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Table of Contents

Advances in Medical, Pharmaceutical and Dental Research Journal (AMPDR)- ISSN 2812-4898
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New Developments in the Optometrist's Assessment and Treatment of Common Headaches

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ABSTRACT:

Headache is pain experienced in the head and neck. It varies in types as it is caused by several reasons but is chiefly of two types, primary and secondary headaches. Primary headaches have no known cause, but secondary headaches have several causes. Tension, cluster and Migraine headaches are examples of primary headaches but hormonal, allergies, caffeine, exertional, hypertension, rebound, post-traumatic and headaches of ocular origin are secondary headaches. Primary and secondary headaches have their respective presentations, signs and symptoms. These headaches also have their peculiar management plans which have been addressed in this article. However, it is expected that the Optometrist will carry out the required eye examination procedures and/or prescribe the ideal medications, if need be, to remedy the headache complaint.

KEYWORDS:

headache, presentation, assessment, treatment & optometrist.

1. Introduction

Headache is pain experienced in the head and neck regions that may be either a disorder in its own right or a symptom of an underlying medical condition.[1] The medical term for headache is "cephalagia".[1] It is estimated that approximately 64–77% of people have a headache at some point in their lives; however, in each year, on average, 46–53% of people have been found to experience a headache.[3] Headache pain is felt when there is a lesion along specialized nerve endings called nociceptors.[4]

Nociceptors are located in the skin, walls of blood vessels, internal organs,[2] cervical spine, the trigeminal, glossopharyngeal, and vagus cranial nerves, the nerves innervating the upper part of the neck, the venous sinuses inside the head, the large arteries at the base of the brain, the large arteries innervating the dura mater, which is the outermost layer of the meninges, and the portion of the dura mater at the base of the skull.[4]

2. Materials and Method

Materials were sourced from the internet using PubMed, Google Scholar, and Science Direct. Recent literature relating to headaches, drugs used for treating headaches, and the eye were used for the review. Relevant papers were also obtained from the university library.

Classification of Headaches

Headaches can be classified into two categories based on cause, namely primary and secondary headaches.[1] Primary headaches are headaches without a known cause; tension, cluster, and migraine headaches are examples. Secondary headaches are caused by diseases, disorders, or accidents; allergies or sinus infections and caffeine. Hormonal, exertional, hypertensive, rebound, and post-traumatic headaches and headaches of ocular origin are examples. Whatever the cause may be, all headaches vary in duration, presentation, and intensity.[1]

Primary Headaches

Tension Headaches

Presentation

Tension headache presents as a reoccurring type of headache pain that lasts from minutes to weeks.[5] The headache is bilateral, pressing with mild to moderate intensity.[5] It is not throbbing in nature, and there is tenderness around the neck, forehead, scalp, and shoulder. Tension

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headaches can occur in anybody, as they are often triggered by stress.[5] Tension headache is of two subtypes. The Episodic and Chronic. The episodic tension headache occurs once in a while and can last from half an hour to several hours, while chronic tension headaches can consistently occur throughout the month. It occurs at 15 or more days per month, lasting hours or days.[8] However, both types of tension headaches share similar clinical features except for frequency.[5]

Cluster Headaches

Presentation

Cluster headaches are characterized by a severe burning and piercing pain.[8] Cluster headaches occur in series, each lasting from 15 minutes to 3 hours, with an average of 45–90 minutes in duration between 6 and 12 weeks.[16,17] It can occur from trauma and affect an eye and one side of the face at the same time. It most often comes with swelling, redness, flushing, sweating, nasal congestion, and tearing that occur on the side that is affected by the headache.[9] Cluster headaches are divided into episodic and chronic types. Episodic cluster headache attacks occur from several days to a year if untreated, and chronic occurs more frequently.[1]

Associated factors

Cluster headaches are associated with some cardiovascular risk factors causing irregularities in blood pressure leading to fluctuating blood pressure levels in the patients.[10] It is found to be associated with acute maxillary sinusitis.[11] Cluster headaches attacks may be triggered by various substances, which include but are not limited to alcohol, fumes from petroleum products, nail varnish, etc.[9,12] The administration of intravenous nitroglycerin can induce cluster headaches.[13] Most studies have shown cluster headaches to be more common in men than in women;[7] men are three times more likely to suffer from this condition than women. However, any age is possible for onset, but the typical age is 30 years.[13] There is a high incidence of cluster headaches in patients with head trauma.[14,15] Other risk factors for cluster headaches include gender, age, history of brain surgery or trauma and family history.[15,16]

Migraine Headaches

Presentation

Migraine is an intense pain experienced deep within the head. It is throbbing and usually one-sided.[18] The pain from migraine can last for days and can significantly limit the individual's ability to carry out daily routine task. Headache comes with nausea, photophobia, phonophobia, rhinorrhea, tearing, and osmophobia.[19] Migraine occurs in four phases.[1] These phases include:

Prodrome: This is the first phase of migraine attacks.[20] Here the migraine headache sufferer experiences symptoms like yawning, mood swings, lethargy, neck symptoms, photophobia, restlessness, craving, sound sensitivity, sweating, excess energy, thirst, and edema.[21]

Aura: Aura is the second phase of migraine. However, it is present in some migraines. Aura can precede a migraine headache or occur simultaneously with it.[22] Symptoms of aura include ear ringing, tingling sensation in the skin, rhythmic movements, vision loss and hearing loss.[1,18,19]

Headache: This is the third phase of migraine attack where the actual headache is felt. This headache phase can last from hours to days. Sufferers may seek relief in dark places, as the pain usually resolves in sleep.[19]

Postdrome: This is the last phase of migraine attack. Common symptoms seen include exhaustion, dizziness, difficult concentration, and euphoria.[20]Associated factors include age, family history and gender. Migraine is common amongst young adults and declines with increase in age.[1,9,18] Migraine is hereditary and is associated with other nervous system conditions.[1,2,10] Migraine also is gender based as women are three times more likely to develop a migraine than men.[9] People with post-traumatic stress disorder have an increased risk of migraine.[18]

Secondary Headaches

Allergy or Sinus Headaches

Presentation

Allergy or sinus headaches are caused by an allergic reaction or infection of any of the paranasal sinuses in the head.[1,11] When the sinuses become infected, inflammation sets in. Symptoms of inflamed sinuses include nasal congestion, running nose, nasal discharge, anosmia, pain when leaning forward and fever.[11] People who have chronic seasonal allergies or sinusitis are susceptible to this kind of headache.

Hormonal Headaches

Presentation

Hormonal headaches are headaches often experienced by women in their reproductive

years. This can take the form of migraines, however it differs from migraines because of its hormonal involvement. Associated factors include menstruation, birth control pills, and pregnancy, which can affect estrogen levels, causing headache.[2,8]

Caffeine Headaches
Presentation

Caffeine consumption can cause headache. However, the relationship between caffeine intake and headache is poorly understood,[2] but what is known is that excess caffeine intake affects the brain and this can cause headache. Also withdrawal from caffeine can trigger headache, too.[8,2,22]

Exertion or Exercise Headaches
Presentation

Prolonged physical exertion can cause these headaches. This form of headache can cause fainting, neck pain, and pain on one or both sides of the head.[11]

Hypertension Headaches
Presentation

This headache occurs when the blood pressure becomes dangerously high. It is bilateral, and it is aggravated by any physical activity. It is pulsating, and there are changes in vision, numbness or tingling in the extremities, nosebleeds, chest pain, or shortness of breath.[8]

Rebound Headaches
Presentation

Rebound headaches (RH), also known as medication overuse headaches or drug-induced headaches, occur in patients with primary headache.[31,32] This results in increased headache frequency, whereby the medication indicated for the treatment of the primary headache becomes the cause of headaches.[1,29] RH is presented like a dull, tension-type headache, or at times like a migraine.[17] Associated factors include regular intake of analgesics such as over-the-counter (OTC) pain relievers like acetaminophen, ibuprofen, aspirin, and naproxen, which will develop RH.[11] Outside OTC medications, there are other risk factors that can cause RH.[33] These include age, gender, anxiety and life style.[33,34,35] RH commonly affect those who are between 30 and 50 years.[1,36] It is more common in males than females.[37] However, metabolic syndromes are common in females with rebound headaches.[34]

Post-traumatic Headaches (PTH)
Presentation

A post-traumatic headache is a type of secondary headache that typically lasts for six to twelve months after a head injury occurs and starts seven days or several months after the shock or injury.[28] There are two types of PTH: acute, which lasts less than a year, and chronic, which lasts for a year or longer. PTH presents bilaterally, with moderate to severe intensity, pressing quality and photophobia [17,35,36] Associated factors linked to PTH include gender, age, greater severity of head injury, anxiety and depression.[37,39,40] Chronic daily headache (CDH) is another subtype of headache that may develop after a head injury.[38] CDH is a headache that occurs more than 15 days per month in a minimum of three months.[14]

Headaches of Ocular Origin
Presentation

Headaches of ocular origin are very common headaches experienced by people having ocular or visual challenges.[1,17] The pain of this type of headache is usually felt on the forehead. It can also be bilateral or localized in one eye or take any form. Symptoms of this type of headache include photophobia, lacrimation, pain, sneezing, inflammation and general ocular discomfort. Associated factors include refractive errors, binocular vision abnormalities, head trauma, family history, birth defects, and eye diseases like glaucoma, uveitis, age related macular degeneration etc.[4,41] Again, spectacles/prismatic prescriptions that are not fully corrective can also cause headaches as a result of a high demand on accommodation. Whatever the cause of these headaches, tackling the primary cause will take away the headache experienced and bring relief to the patient.[51]

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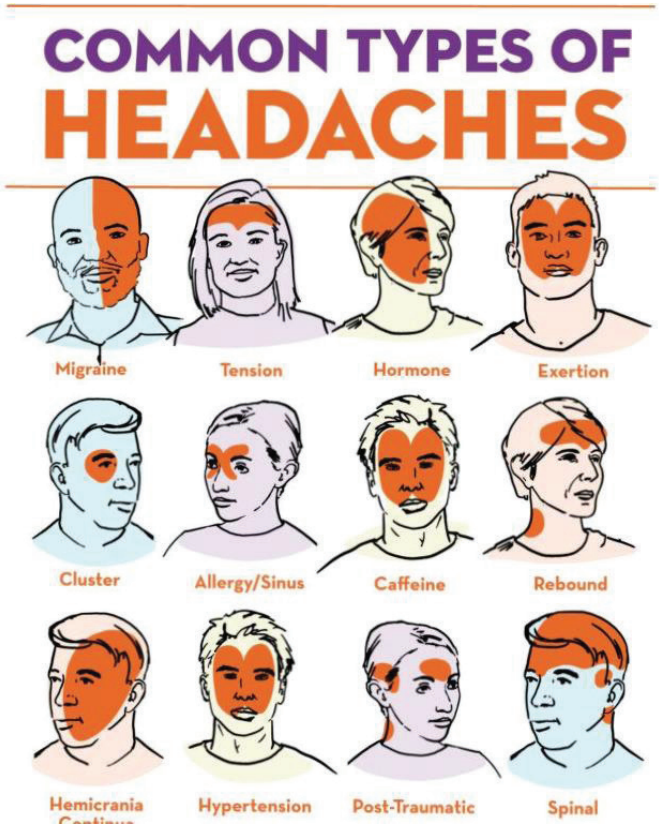

Fig 1: The different types of headaches and their facial presentations (adopted from pinterest.jpy)

3. Assessment

In tackling the primary cause of headaches the optometrist needs to make a differential diagnosis and carry out clinical procedures on the patient. The differential diagnosis and clinical procedures for eye examination for headache patients are:

Case history:

The differential diagnosis of headaches begins with a complete patient history. The history must contain the age, gender, medical, family, social and work life pattern of the patient.[4,16,18,28] The optometrist can make a diagnosis of the type of headache, if the case history is done well. In taking the case history, the following information should be obtained from the patient in a question and answer form below:[54]

Character of the headache: Here the optometrist is to inquire from the patient about the nature of the headache, if throbbing or pulsating, dull, sharp, tight pressing or mild.[1]

Location: the optometrist is to inquire from the patient about the area of the head where the headache occurs.[12,17]

Frequency: Here the optometrist is to find out how often the patient experiences the headache.[30]

Intensity: how debilitating is the headache?[1,5]

Onset: the optometrist is to inquire from the patient about the headache trigger and the time of the day the headache occurs.[11,30]

Duration: Here the timeframe of the headache pain is required from the patient [2,54]

Associated Symptom/Signs: What signs and/or symptoms accompany the headache?[54]

Relieving Mechanism: By what means is the headache easily relieved?[1,54]

In taking case history, it is expected from the optometrist to know that he or she can have patients who fall into any age group, but irrespective of the age of the patient the diagnostic approach remains. However, for very young children who may not be able to verbalize pain well, the optometrist is to observe their body language, as young child is irritated easily when they experience headache.[4]

Visual acuity (VA) testing:

In measuring the VA of a patient with a headache complaint, it is expected that the optometrist observes the reading pattern, the head movement and facial expression of the patient.[4,14,17]

External ocular examination:

The ocular adnexa is to be grossly inspected for inflammation. The face also is to be inspected for possibility of a facial scar from a post-traumatic event. Slit-lamp biomicroscopy should be performed to aid diagnosis.[4,55]

Ophthalmoscopy/Fundoscopy:

During this procedure, the optometrist is to observe the fundus for any abnormal sign, like disc cupping, tear, inflammation, exudates etc.[1,4]

Tonometry:

Tonometry is to be carried out, if glaucoma is suspected following ophthalmoscopy Results from tonometry will determine the likely treatment options to take.[1,12,17]

Refraction: When performing this procedure, the optometrist should be observant and pay close attention to the reaction and facial

expressions of the patient. The prescription that fully provides relief for the patient and a very good and comfortable vision acuity is the best prescription to be given. Again, the optometrist is to find out from the patient, if the headache is relieved as he keeps changing the lens. Most ocular headaches are relieved when the ideal prescription is given. When the prescription does not yield results, it is then obvious that the headache cannot be resolved by spectacles.[55]

Von Graeffe Technique (VGT):

Headaches from uncompensated phoria or tropia can be diagnosed with VGT. In the VGT, the Optometrist is to inquire from the patient of any likely discomfort and or headache and the prism power that relieves the headache.[19,51]

Transcranial magnetic stimulation (TMS) technique:

TMS technique is a noninvasive form of brain stimulation in which a varying magnetic field is used to induce an electric current at a specific area of the brain through electromagnetic induction. In TMS, a stimulator is connected to a magnetic coil that is subsequently connected to the patient's scalp. The stimulator generates an alternating current within the coil which creates a varying magnetic field, inducing a current within a region in the brain itself. TMS is both a diagnostic and therapeutic procedure for headaches. In the therapeutic management of headache, it is done repeatedly.[42]

Functional magnetic resonance imaging (fMRI) scan.

Magnetic resonance imaging, or MRI, is a noninvasive, painless medical scan that produces detailed images of almost every internal structure in the human body. [43] MRI scanners create images of the body using a large magnet, radio waves and a computer. Functional magnetic resonance imaging or functional MRI (fMRI) uses MRI technology to measure brain activity. It does this by measuring blood flow to certain areas of the brain.[53]

4. Treatment

Headache is a common complaint reported by patients visiting the eye clinic. As primary eye care providers, patients with headache complaints will likely meet the optometrist first before other healthcare providers. Optometrists also get referrals from general practitioners and others in the health sector. Whatever the case maybe, the optometrists first line of treatment should be to carryout a detailed case history and eye examination which should culminate in giving the patient the ideal lens or prism prescriptions that will solve the headache problem. Outside of lenses and prisms, the optometrist may add some drugs to the treatment regimen. For tension headache, pain relievers, like Acetaminophen 500mg should be included.[4] If this fails, refer the patient to a general practitioner. The optometrist can also recommend a hot compress for the patient. This is done by placing a hot pad on the patient's neck or the back of the head and massaging for a few minutes the patient's forehead, neck, and temples until the patient is relieved of the headache. Also, for these headaches, amitriptyline and mirtazapine can be prescribed as they have been found to be effective at reducing the frequency and intensity of headaches. Again, both drugs have no associated adverse-effect profile.[7] In treating cluster headaches oxygen therapy, sumatriptan and or lidocaine can be used to provide pain relief.[8] Migraines can be treated or relieved with pain relievers, like oral ergotamine and caffeine since they are efficacious in the treatment of migraine.[9] However, if the pain relievers fail, then triptans such as the sumatriptans should be prescribed. The triptans are available in oral, intranasal powder, liquid nasal spray and subcutaneous injection forms.[4] Rimegepant,[45] a relatively new drug can be used for the treatment of acute migraine and can also be used as a prophylactic treatment for migraines in adult. Pharmacologically, rimegepant is a receptor antagonist to the calcitonin gene related peptide (CGRP).[46] CGRP has been implicated as a cause for migraines.[47] Rimegepant is available in 75mg tab and can be given orally, on or under the tongue once a day. [28]

Another way to treat migraines is by prescribing drugs that prevent its occurrence. These drugs decrease the frequency of migraine attacks and improve the patient's response to acute migraine medications. These drugs also greatly help to improve the quality of life and productivity of people with migraine. Useful preventive medications are propanolol, metoprolol, topimarate, amitriptyline and cold ice.[48] Butterbur,[49] an extract of Petasite has been found to be effective for the prevention of migraine. This natural remedy has no risk of hepatotoxicity.[49,50] This reason makes it more useful for patients who prefer natural remedies. Since migraines come with photophobia, patients and sufferers are to be counseled on the need to use blinds on windows and screens, to be on sunglasses or photochromic lenses when outdoors, to use anti-glare screens when using the computer and to use daylight-spectrum

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fluorescent bulbs in light fixtures. Same time using nasal steroid sprays, phenylephrine, and antihistamines like cetirizine, as these will be useful in relieving the headache.[14, 24] A tentative remediation is by placing a warm cloth on the area that hurts. Hormonal headache treatment is like migraine headache treatment except for the use of alternative remedies that may have a role in decreasing the headache pain. Alternative remedies include relaxation, taking part in yoga and modifying one's diet.[25]

Caffeine headache can be prevented by keeping caffeine intake at a very low level or quitting it entirely.[4,27,28] Exertion headaches usually resolve within a few minutes or several hours. However, if headache continues, analgesics, such as aspirin and ibuprofen should be prescribed to ease the symptoms.[28,29] However, if this fails, then medical attention is required.[23] Hypertension headaches rescind when blood pressure is under control. Medications like Acetazolamide can be prescribed to ease the symptoms.[14,30] The only treatment for rebound headaches is to wean the patient off the medication he/she has been taking to control pain. Patient education and motivation.[31,33] Patient should be educated on the condition and motivated to discontinue the over-used drug.[32] For patients with a high risk of drugs toxicity, it is advised to prescribe an alternative medication that will run for two weeks or less. Post-traumatic headache (PTH) can be treated in several ways. Neutralizing prisms have been found to be a treatment modality for PTH in patients with vertical heterophoria (VH).[51]

Paracetamol and ibuprofen tablets can be prescribed to manage mild to moderate headaches. However, like all analgesics, these drugs should be prescribed with caution especially for young people, as it is shown to cause sterility.[20] But when headache is chronic, Tricyclic antidepressants, or antiepileptic medications such as amitriptyline can also be prescribed. Onabotulinum toxin A given intravenously, has been found to be effective in treating PTH.[52,53] Repetitive transcranial magnetic stimulation (rTMS) is another way for treating PTH. But this is the therapeutic procedure for TMS.[17] Cognitive behavioral therapy, physical therapy, biofeedback and relaxation techniques are psychological approaches that can be used for PTH treatment. Patient counseling and health education should be followed alongside any treatment option the optometrist desires to choose. However, if all this fail, the patient should be referred immediately.

5. Conclusion

Headache is a very common complaint encountered in any healthcare facility as it is a general health burden and because of its association with various health conditions including those that affect the eyes. Thus, making it necessary for optometrists not to overlook and underestimate headache cases irrespective of the type or frequency. Being primary healthcare providers, it is the duty of optometrists to properly diagnose and manage effectively headache complaints that come to them from time to time. Since headache is both a systemic and ocular problem, optometrists should also consider following an interdisciplinary approach.[20] But this should come after the eye examination procedures. The optometrist, whether working single handedly or not, should refer headache cases that are beyond his or her scope of practice and should follow up such cases to ensure complete treatment.

Optometrists are encouraged to know the various examination techniques required in the diagnosis of headache to advise their patients properly on the best treatment to take. Even as more studies are ongoing to further alleviate this global health burden, optometrists are encouraged to be part of these studies by carrying out research that promotes effective patient care in the examination and management of headaches. The management of multidisciplinary and specialist hospitals are to equip their facilities to promote further works on headache and ensure headache patients are given full and complete treatment.

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Advances in Medical, Pharmaceutical and Dental Research Journal (AMPDR)- ISSN 2812-4898
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Piper guineense (Swhan and Thon) Inhibits Lanosterol-14α-demethylase in Multi-Drug Resistant Non-*albicans Candida Species*: In vitro and In silico Studies

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ABSTRACT:

Background:
Candida species are globally recognized for invasive infections with poor prognosis. Burgeoning quest for the discovery of novel therapeutics has increased the scientific scrutiny of several medicinal plants. This study assessed the efficacy of Piper guineense crude extract and fractions against selected multi-drug resistant non- albicans Candida species.

Results
Both the crude and fractionated extracts of P. guineense elicited marked anti-candidal activity. Overall, the crude extract showed better efficacy over the fractions with the highest inhibition zone (25.0mm) recorded against Candida tropicalis; amongst the fractions, n-hexane fraction (F2) produced the highest inhibition zone (23mm) against Candida glabrata. The MIC ranged from 25mg/ml to 50mg/ml, while the MBC ranged between 100 mg/ml to 200 mg/ml. HPLC analysis revealed the presence of 14 compounds in the extract, with prominent members being quercetin, ellagic acid, persin, catechin, p-coumaric acid, and lutein. The binding affinity and free binding energy results reveal that most of these bioactive compounds were better than the standard drug (Fluconazole).

Conclusion:
Conclusively, P. guineense extracts demonstrated impressive anti-candidal properties against the tested multi-drug resistant non-albicans Candida species and could have potential as new drug lead for the treatment of infections resulting from these pathogens.

KEYWORDS:

P. guineense, non-albicans Candida, Lanosterol-14α-demethylase, Antimicrobial resistance

1. Introduction

Each year, more than 6.5 million people contract life-threatening fungal infections; about 1.5 million of these are attributable to invasive candidiasis, with a 63.6% mortality rate [1]. The clinical spectrum of candidiasis, which is caused by yeasts of the genus *Candida,* extends from superficial diseases such as cutaneous, nail, digestive, and genital candidiasis to systemic diseases such as candidemia [2]. *Candida* spp are generally commensal germs that develop in the skin, inside the body, in the mouth, throat, intestines, and vagina, without causing infection [3]. They express their pathogenic power only in the presence of factors favoring the origin of the translation of endogenous commensal to the disease-causing parasite. These factors can be intrinsic or extrinsic to the host, including overweight and prolonged use of broad-spectrum antibiotic therapy and corticosteroids, among others [4]. Immunosuppression remains one of the most prevalent risk factors [5]. The resurgence of diseases weakening the immune system, such as AIDS, and immunosuppressive

treatments, such as heavy chemotherapy, has led to a drastic increase in *Candida* infections, which have become a major cause of morbidity, mortality, and increased treatment expenses in hospitals. The disseminated forms of candidiasis can be life-threatening, with high mortality rates among immunocompromised cancer patients and those exposed to multiple treatments, such as broad-spectrum antibiotics, chemotherapy, immunosuppressive therapy, and antiretroviral therapy [2,6]. On the other hand, inappropriate medical practices such as misdiagnosis and inadequate medication are responsible for the exacerbation, spread, and persistence of the infection. The pathogenicity of *Candida spp.* Emanates from a diversity of factors, including its heightened ability to adapt to stressful conditions [7]. Moreover, virulence attributes such as the expression of surface molecules as adhesins, the ability to change its morphology, biofilm forming capacity, and the secretion of hydrolytic enzymes are essential for establishing infection [4].

Natural products obtained from plants have a vast repertoire of biologically active compounds that represent rich prospects for drug development [8]. *Piper guineense* (African black pepper), belonging to the order Piperales and the Piperaceae family, is one of the most commonly used spices. It is considered as "the king of spices" due to its trade in the international market [9,10]. African black pepper is cultivated in many tropical regions like Brazil, Nigeria, India, Ghana, Indonesia, and Senegal [2]. *P. guineense* adapts well to a broad range of environmental conditions, including vast altitudinal regions [10]. Interestingly, *P. guineense* has been used for several purposes as a natural medicinal agent for the treatment and alleviation of digestive and respiratory disorders [11]. It has also been used in human dietaries and perfumery as a preservative and biocontrol agent [12,13].

Despite therapeutic advances, the incidence of candidiasis continues to aggravate with increasing mortality. Moreover, non-*albicans Candida* species (NACs) are more frequently implicated in the epidemiology of the disease nowadays [6,14,15]. The poor prognosis of candidal infections is partly attributable to the limited antifungal armamentarium, toxicity of antifungal drugs, and the current challenge of antifungal resistance [16,17]. Against this background, it becomes pertinent to develop alternative therapies for treating *Candida* infections. This study, therefore, evaluated the efficacy of *Piper guineense* crude and fractionated leaf extracts against multi-drug resistant NACs.

2. METHODS

Test Organisms and Preparation of Inoculum

The test organisms used in this study were multidrug resistant strains of *Candida krusei, Candida tropicalis,* and *Candida glabrata* obtained from the stock culture of previously identified isolates at the Microbiology Laboratory, AAUA. The test organisms were maintained on Sabauraud dextrose agar (SDA) agar slant and stored in the refrigerator at 4^0C for further studies. McFarland standard (0.5) was prepared by combining 0.05ml of 1% barium chloride Dihydrate ($BaCl_2.2H_2O$) with 9.95ml of 1% Sulfuric acid (H_2SO_4) to yield 1.0%w/v barium sulphate suspension. The accuracy of the density of a prepared McFarland standard was checked by using a spectrophotometer at 625 nm. The McFarland standard was vigorously agitated on a vortex mixer before use. The Inoculum of each test organism was prepared by picking five distinct colonies of approximately 1 mm in diameter from a 24-hour-old culture. Colonies were suspended in 5 mL of sterile 0.85% saline. The resulting suspension was vortexed for 15 seconds, and its turbidity was adjusted to 0.5 McFarland standards. This procedure yielded a yeast stock suspension of 1-5 x 10^6 cells per mL [6].

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Extraction and Fractionation of Plant Material

Fresh leaves of *Piper guineense* (Swhan and Thon) were sourced from AAUA medicinal garden, Akungba-Akoko (Latitude 7.4740 °N and Longitude 5.7379 °E), Ondo state, Nigeria. The plant was authenticated at the Department of Plant Science and Biotechnology Herbarium of our institution. Voucher specimen number PSBH 254 was deposited for the plant.

The maceration method, as described by Oluyele *et al.* [18], was used for the crude-extraction process. Briefly, the leaves of *Piper guineense* were air-dried at room temperature and pulverized. Thereafter, 850g of the powdered leaves were soaked in 2550 ml of 70% ethanol for 7 days with occasional shaking to allow the full extraction of the active ingredients. The mixture was sieved using a muslin cloth and then filtered using Whatman No.1 filter paper. The filtrates were concentrated using a rotary evaporator. The extract obtained was stored in the refrigerator at 4°C for further studies. Fractionation of *P. guineense* crude extract was performed using liquid-liquid extraction according to the method of Pham *et al.* [19]. Briefly, the crude extract was dissolved in distilled water (1:10, w/v), transferred into a separating funnel, and successively fractionated with n-hexane and ethyl acetate to yield different fractions, respectively designated hexane fractions (F1 and F2) and ethyl acetate fraction (F3). The residue was generated, namely aqueous fraction (F4). The resulting fractions (F1, F2, F3, F4) were then concentrated using a rotary evaporator. All fractions were then freeze-dried and stored at 4°C until further experiments.

Antifungal Assay of Piper guineense leaf extract

The agar well diffusion technique, as described by Oluyele *et al.* [18], was used to determine the antifungal activity of the crude extract and fractions. One (1ml) aliquot of each test organism suspension (standardized) was transferred onto the well dried sterile Sabauraud dextrose agar (SDA) plates and was spread evenly using sterile swab sticks. The plates were allowed to dry; a standard sterile cork borer of 6mm diameter was used to cut uniform wells on the SDA plates. Each well was appropriately labeled on the reverse side of the plates. Then, 50 µL of 100 mg/mL of the extract and fractions prepared in 5% dimethyl-sulfoxide (DMSO, Sigma Aldrich, Germany) were filled into the corresponding wells. Fluconazole was used as a control in one of the wells. The plates were allowed to stand for 15 minutes at room temperature to allow proper diffusion of the extract to occur. All the plates were incubated at 35°C for 48 hours, after which the zones of inhibition were measured.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MBC)

The MIC and MFC of the extract were determined using tube-dilution and plating methods respectively [18]. For MIC, different concentrations of the extract were prepared at 200, 100, 50, 25, 12.5, 6.25, and 3.125 mg/ml. This was followed by the addition of 0.1 ml of the standardized test inoculum into each test tube. A set of test tubes containing only sterile Sabauraud dextrose broth (SDB) was used as a negative control, and another set of test tubes containing SDB plus test organisms was used as a positive control. All the test tubes were then incubated at 35°C for 48 hours. Growth in each tube was checked by visible observation and by using a spectrophotometer (Beckman model 35). The concentration that produced no visible turbidity was taken as the MIC. The MFC was determined using the plating method by subculturing from the MIC tube and other tubes that showed no turbidity onto freshly prepared Sabauraud dextrose agar and incubated at 35°C for 48 hours. The concentration that showed no visible growth after incubation was taken as the MFC.

High Performance Liquid Chromatography (HPLC) Analysis of Extract

About 2g of sample was measured into an

amber bottle, followed by the addition of 20mls of (Acetonitrile/methanol) and vigorous agitation for 30 minutes. Thereafter, the aqueous end was run off while the organic solvent end was collected into a 25ml standard flask, made up to the mark, and ready for analysis. The sample was run using gradient elution according to the following chromatographic conditions: reversed phase chromatography (Agilent Technologies 1200 HPLC), mobile phase composition: 0.1% formic acid + acetonitrile, stationary phase: Hypersil BDS C18 (Agilent), column dimension: 250mm x 4.0 mm, injection volume: 20µL, flow rate 0.6 ml/min, detector wavelength: 280 nm. The standard form of analyte profile was first injected into the HPLC, and this generated a chromatograph with a given peak area and peak profile. These were used to create a window in the HPLC in preparation for the test sample analysis. An Aliquot of the extracted test sample was also injected into the HPLC to obtain a corresponding peak area and peak profile in a chromatograph. By keeping track of retention time and analyzing UV spectra, the peaks were identified by comparing them with reference standards [20].

Generation and Preparation of Compound Library

The compounds that were identified from *P. guineense* by HPLC analysis were downloaded from the PubChem (https:// pubchem.ncbi.nlm.nih.gov) repository alongside with the protein standard drug in structure data file (sdf) format. These molecules were exported onto Schrodinger workspace (Schrodinger, 2021v2) and prepared using Ligprep tool for the *in silico* study.

Protein Preparation

The research collaborator for the structural bioinformatics protein databank (RCSB PDB) [www.rcsb.org] website provided the x-ray crystallographic structure of the lanosterol-14α-demethylase complex with ketoconazole having PDB ID of 3LD6. The missing residues and loop in the protein and other side chain anomalies were resolved, followed by energetic optimization with force field OPLS3 using the protein preparation wizard of Schrodinger suit 2021. The receptor grid generator was used to generate a glide grid on the co-ligand (digoxin) attached site with glide coordinates of x = 42.47, y = 4.96, and z = 2.02. The prepared protein crystallographic structure and Ramachandran residues' distribution are shown in Figure 1.

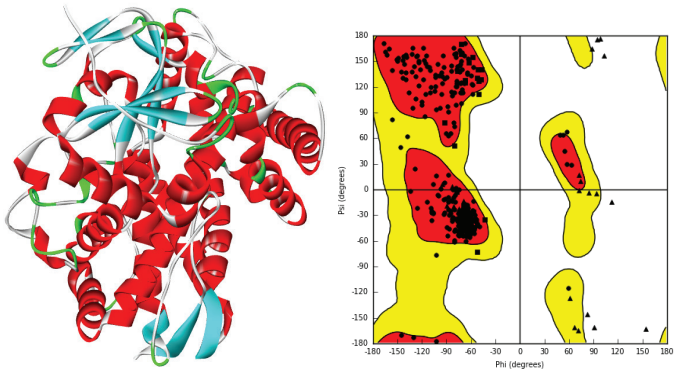

Figure 1: Crystal structure of Lanosterol-14α-demethylase and Ramachandran plot of residues distribution

Structure-based virtual screening

The prepared compounds from *P. guineense* and the standard ligand were screened against lanosterol-14α-demethylase using the extra precision (XP) GLIDE docking filtering procedure in the Maestro Schrodinger suite (v 2021). This scoring function is known for its robustness and discriminating ability, but it requires more time to run [21].

Prime/MM-GBSA calculations

The lanosterol-14α-demethylase-ligand complexes were minimized by using the local optimization feature in Prime; the binding energy (Δ^{bind}) for the complexes was determined using the OPLS3 force field. Molecular mechanics generalized the Born surface area (MM/GBSA) calculation, which was carried out on the docking complexes. The following equation was used to calculate the binding free energy:

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3. RESULTS

Anti-candidal Activity of Piper guineense Extracts

As shown in Tables 1 and 2, the extracts of *P. guineense* showed marked anti-candidal activity in this study. The highest activity was observed against *Candida tropicalis* 25.0mm for the crude extract of *P. guineense*, and against *Candida glabrata* (N-hexane fraction 2) amongst the fractions. The MIC ranged from 25mg/ml to 50mg/ml; while the MBC ranged between 100 mg/ml to 200 mg/ml.

Table 1: Antifungal Potency of Piper guineense Extracts

Organism	CE	F 1	F 2	F 3	F 4	FLC
Candida glabrata	23.0^{b}	21.5^{c}	23.0^{c}	17.5 ± 0.29^{c}	20.5^{b}	26.0^{a}
Candida tropicalis	25.0 ± 0.29^{c}	19.5^{b}	5.5^{a}	4.5^{a}	18.5 ± 0.29^{a}	26.0^{a}
Candida krusei	22.0 ± 0.29^{a}	17.0 ± 0.29^{a}	11.5 ± 0.29^{b}	16.5^{b}	21.5 ± 0.33^{c}	28.0^{a}

Legend: CE- Crude extract, F1- N-hexane fraction 1, F2- N-hexane fraction 2, F3- Ethyl acetate fraction, F4- Aqueous fraction, FLC - Fluconazole. Values with the same superscript across the column are not significantly different.

Table 2: Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal concentration (MFC) of Piper guineense Crude-Extract against Test Organisms

Organism	MIC	MBC
Candida glabrata	50mg/ml	100mg/ml
Candida tropicalis	25mg/ml	100mg/ml
Candida krusei	50mg/ml	200mg/ml

HPLC Identified Compounds of Piper guineense Crude-Extract

As presented in Figure 2 and Table 3, HPLC analysis revealed fourteen compounds in the crude leaf extract of *P. guineense*. Some of the notable constituents include quercetin, ellagic acid, persin, catechin, p-coumaric acid, and lutein.

Table 3: Compounds identified in the Piper guineense Leaf Extract

Compounds	Retention	Area	Height
Ellagic acid	3.70	1781.7085	48.923
Zeaxanthin	5.883	308.7870	8.077
P-Coumaric Acid	7.966	609.2790	13.901
Camphene	9.116	64.5150	3.573
Catechin	10.500	128.7600	3.217
Epicatechin	11.300	108.8825	5.707
Obovaten	12.000	59.3410	4.334
Obovatinal	13.833	75.0890	4.515
Persin	15.500	863.8795	14.749
Quercetin	17.233	2761.1350	37.005
Persenone A	19.166	105.4600	7.437
Lutein	19.950	84.0050	5.832
Scorpoletein	20.500	62.7400	5.332
Afzelin	21.416	66.7880	2.356

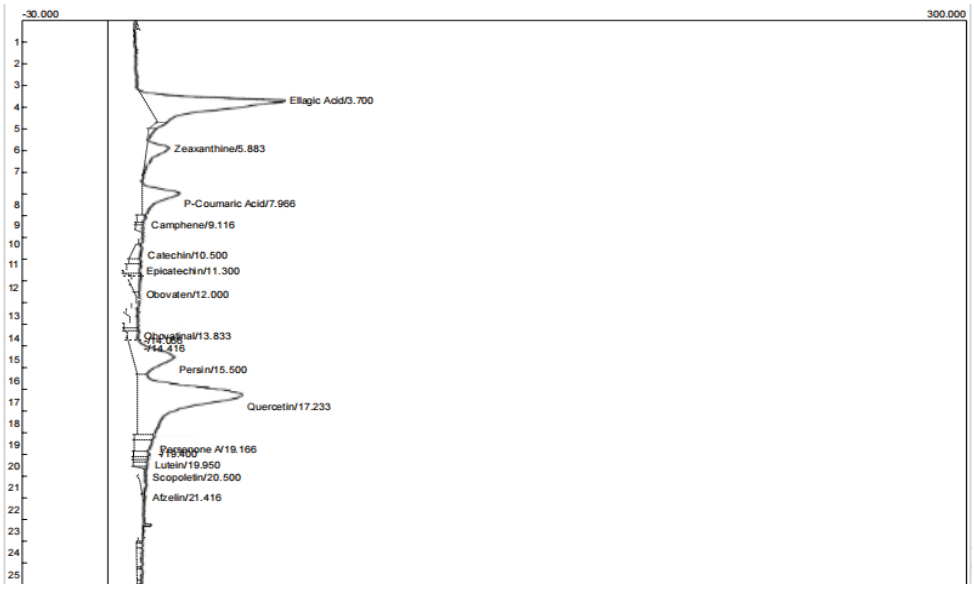


Figure 2: HPLC Chromatograph of Piper guineense

Molecular Docking and MM/GBSA of the bioactive compounds against the target

As shown in Figure 3, the active compounds had varying binding affinities against lanosterol-14α-demethylase ranging from -5.752 to -10.227 kcal/mol, which is comparable with the standard drug (fluconazole = -8.454 kcal/mol). Quercetin, persin, persenone A, afzelin, epicatechin, catechin, and ellagic acid were observed to have better binding affinities (-10.227, -10 027, -9.555, -9.948, -9.081, -9.310 and −9.286 kcal/mol respectively) against lanosterol-14α-demethylase compared with the standard drug. The binding affinities obtained might be attributed to the formation of various interactions between the functional groups of the bioactive compounds and the amino acid residues at the binding site of lanosterol-14α-demethylase. From Figure 4, the active compounds from the plant interacted with various amino acids present at the pocket of the protein through various molecular interactions like van der Waals, alkyl bond hydrogen bonds, and pi-alkyl bonds. Catechin and ellagic acid formed four (4) hydrogen bonds with other hydrophobic interactions while afzelin and obovatin formed three (3) hydrogen bonds with other hydrophobic bonds which is equivalent to the number of hydrogen bonds formed by fluconazole (standard drug) with TYR 131, ILE 397 and MET 378.

The binding free energies of the complex were determined by calculating the molecular mechanics generalized born surface area (MM/GBSA). From Figure 2, all the compounds except p-coumaric acid and camphene has binding free energy more than -40 kcal/mol. Obovaten, ellagic acid, catechin, persin, persenone A and afzelin have better binding free energies than the standard drug (fluconazole = -49.21 kcal/mol).

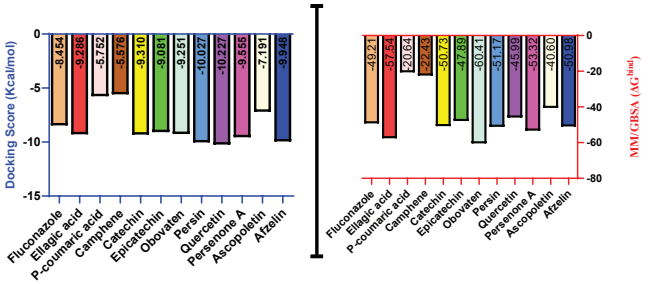
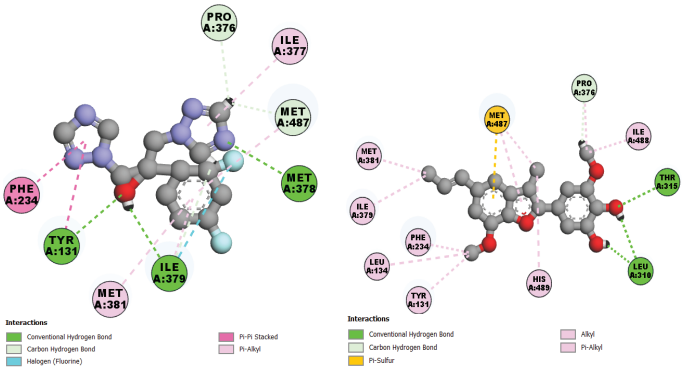


Figure 3: Graphical representation of the binding affinity and binding free energy calculation of bioactive compounds against lanosterol-14α-demethylase



Fluconazole

Obovaten

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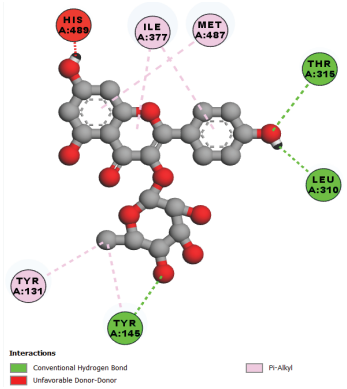
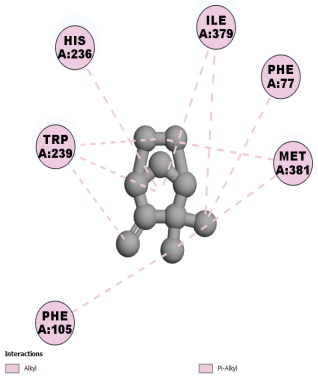
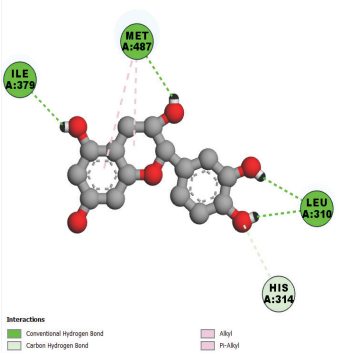


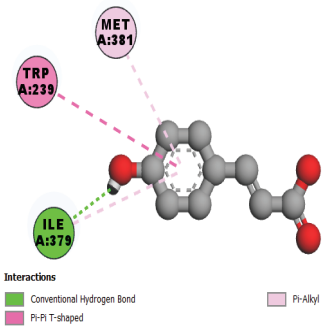
Afzelin



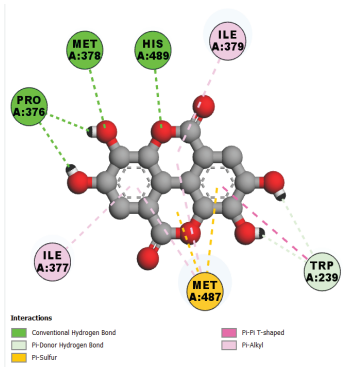
Camphene



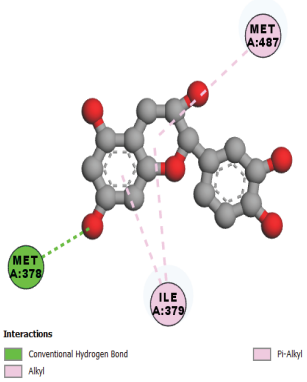
Catechin



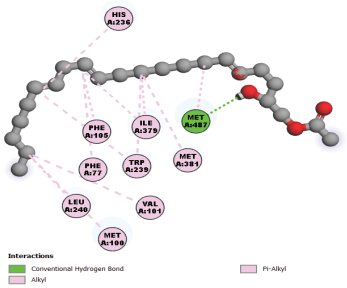
P-coumaric acid



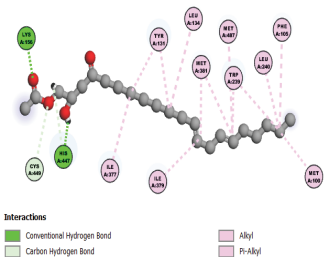
Ellagic acid



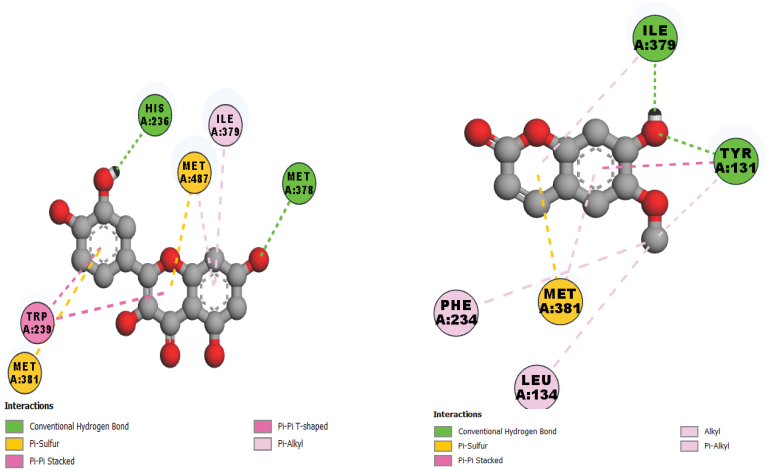
Epicatechin



Persenone A



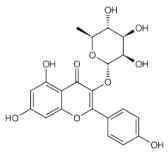
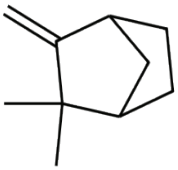
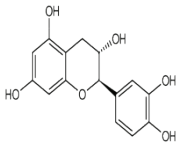
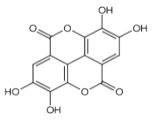
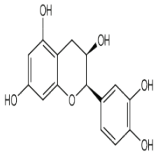
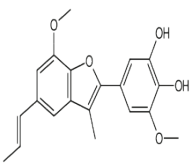
Persin



Quercetin ***Scopoletein***

Figure 4: 2D interaction between the compounds-lanosterol-14α-demethylase complexes

Table 4: Post-docking analysis of Ligand-protein complexes

Name	Structure	Binding Affinity (kcal/mol)	No of H-bonds	Amino Acids
Afzelin		-9.948	3	THR 315; LEU 310; TYR 145
Camphene		-5.576	Nil	Nil
Catechin		-9.310	4	ILE 379; MET 487; LEU 310
Ellagic acid		-9.286	4	PRO 376; MET 378; HIS 489
Epicatechin		-9.061	1	MET 378
Persin		-10.027	2	LYS 156; HIS 467
Obovaten		-9.251	3	THR 315; LEU 318

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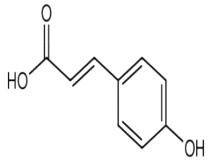
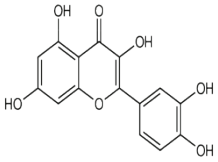
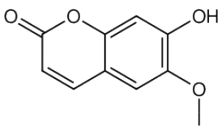
P-coumaric acid		-5.752	1	ILE 378
Quercetin		-10.227	2	HIS 236; MET 378
Ascopoletin		-7.191	2	ILE 379; TYR 131
Persenone A		-9.555	1	MET 487

4. DISCUSSION

Candida spp are globally recognized for invasive infections with poor prognosis [6]. The burgeoning quest for the discovery of novel therapeutics has increased the scientific scrutiny of several medicinal plants. In this study, we evaluated the efficacy of *Piper guineense* crude extract and fractions against some pathogenic multi-drug resistant non-*albicans Candida* species. Both the crude extract and the fractions of *P. guineense* elicited impressive activities against the test pathogens in this study. Overall, the crude extract showed higher efficacy compared to the fractions. This higher activity observed could be attributed to the crude extract containing a larger proportion of the bioactive constituents of the plant, owing to the ability of the extraction solvent to retrieve these constituents. The results obtained showed variations in the inhibition zones (IZs) of the extracts on the test yeast strains. The highest inhibition zones were observed against *Candida tropicalis* for the crude extract, against *Candida glabrata* for F1, F2, and F3, and against *Candida krusei* for F4. Notably, F2 and F3 had minimal effect on *Candida tropicalis,* and the inhibition zones observed were the least overall. The variation seen in the inhibition zones is presumed to be due to different active compounds present in each extract and the susceptibility profile of the *Candida* strains studied.

In tandem with this report, the hexane and ethanolic fruit extracts of *P. guineense* were chronicled to be effective against the growth of *C. albicans* and *C. glabrata* [22]; the hexane leaf extract elicited appreciable activity against *Sarcina* sp., *Staphylococcus aureus,* and *Enterobacter aerogenes*. However, the water extracts were not active against the bacterial strains tested [23]. Moreover, another study revealed that the seed extracts of *P. guineense* showed antimicrobial potency against *Escherichia coli, Klebsiella pneumoniae, Salmonella typhi, Shigella dysenteriae, Aspergillus flavus, Aspergillus niger* and *Candida albicans* [24].

The MIC and MFC of the crude extract were further determined since the IZs were the most prominent of all the extracts. The MIC was 50mg/ml for *Candida glabrata* and *Candida krusei* and 25 mg/ml for *Candida tropicalis,* while the MFC was 100mg/ml for *Candida glabrata* and *Candida tropicalis* and 200mg/ml for *Candida krusei.* The activities of the extracts (especially the crude) compared favorably with the standard drug (Fluconazole) employed in this study, although Fluconazole produced higher inhibition zones. The better activity of the standard drug over the extracts could be connected to the fact that organic extracts are in crude form compared to synthetic antibiotics, which have a high degree of purity; hence, the secondary active metabolites could be present in low concentrations or masked in the extracts [18].

Considering that the crude extract portrayed the overall higher activity in this study, it was subjected to HPLC profiling. Distinctive among the compounds identified include quercetin, ellagic acid, persin, catechin, p-coumaric

acid, and lutein. Notably, these compounds are different from those reported by a previous study [23]. The compounds identified from our analysis are most likely responsible for the observed efficacy of *P. guineense* in our experiment. For instance, quercetin a flavonoid with antioxidant properties and many beneficial effects on health [25], has been reported to show a broad inhibitory effect on bacteria, and its combination with amphotericin-B and fluconazole produced promising synergistic antifungal-activity [26,27]. Quercetin enhanced fluconazole-resistant *Candida albicans*-induced apoptosis by regulating quorum sensing [28]. The presence of ellagic acid in *P. guinesse* studied is of great importance, as it is a bioactive polyphenolic compound naturally occurring as a secondary metabolite in many plant taxa [25]. Varying concentrations of ellagic acid were reported to inhibit the growth of *Candida albicans* [29]. Persin is a fungicidal compound present in avocados [30]. Catechin has been proven to exhibit antimicrobial activity against clinical isolates of methicillin resistant *Staphylococcus aureus* [31]. Lutein from extract of *Helianthus annuus* was found to be active against *Bacillus subtilis, Escherichia coli, Salmonella typhi* and *Staphylococcus aureus* [32].

The virtual screening analysis of the active molecules from the HPLC analysis of the crude extract against the binding site lanosterol-14α-demethylase was carried out. The fungal lanosterol-14α-demethylase is a vital protein in the pathway that synthesizes ergosterol and cholesterol biosynthetic pathways in humans [33]. This cytochrome P450 enzyme remains the target for the azole antifungals used in the treatment of fungal infections in humans [34]. Inhibition of the binding domain of this protein results in the termination of ergosterol biosynthesis and the formation and accumulation of the intermediates that might lead to inhibition of microbial growth [35]. The virtual screening of active molecules through a molecular docking procedure is one of the vital methods of drug design. This method forecasts the interaction between small molecules and the amino acid residues at the binding site of the target [21]. Inhibition of lanosterol-14α-demethylase by bioactive compounds from *Piper guineense* was selected to study the antifungal mechanism of the compounds from the plant and complement the in vitro antifungal activity of the plant observed in this study. The result of the binding affinities obtained in this study, as shown in Fig. 3, suggests that eight out of the eleven active compounds from *Piper guineense* were potent inhibitors of lanosterol-14α-demethylase with better binding affinity compared with fluconazole (standard drug). This result agrees with the findings of Oladimeji *et al*. [33], who observed the inhibition of sterol-14α-demethylase by the active compounds from *E. coccinea* essential oil. Rosam *et al*. [35] have also reported that sterol-14α-demethylase-ligand binding pocket mediated acquired and intrinsic azole resistance in fungal pathogens. The ligand-protein interactions in molecular docking contributed to the binding affinity of small molecules against the target, which has been observed to be a key regulator of their action [36]. Figure 4 showed that afzelin interacted with THR 315, LEU 310, and TYR 145 with hydrogen bonds, catechin interacted with ILE 379, MET 487, and LEU 310 with four hydrogen bonds, ellagic acid interacted with PRO 376, MET 378, HIS 489 with four hydrogen bonds and obovaten interacted with THR 315 and LEU 310 with three hydrogen bonds. These active compounds have the highest hydrogen bond interactions among the compounds identified from *Piper guineense*, which is comparable with the standard drug (fluconazole) interacting with TYR 131, ILE 379, and MET 378 with three hydrogen bonds. Other hydrophobic interactions were also observed in the protein-ligand complex, which might contribute to the docking score. The observed inhibitory activity of these active compounds against fungi lanosterol-14α-demethylase presents the enzyme as the antifungal mechanism of the plant, as observed in the *in vitro* study.

5. Conclusion

Conclusively, *P. guineense* extracts demonstrated impressive anti-candidal efficacy against the test multi-drug resistant non-*albicans Candida* species and could have potential as a new therapeutic agent for the treatment of infections resulting from these pathogens. The efficacy of *P. guineense* recorded in our study could be attributed to the diversity of bioactive compounds identified with the inhibition of lanosterol-14α-demethylase proposed as the mechanism of anti-fungi action. Further, *in vivo* studies on the isolated bioactive compounds are necessary to validate the findings from the *in vitro* and *in silico* analyses in this study for safe therapeutic resolution of Candidal infections.

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Knowledge, Attitude, and Practice towards Voluntary Counseling and Testing Services among University Students in Kigali, Rwanda: A cross-sectional study.

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ABSTRACT:

Background:
Voluntary Counseling and Testing (VCT) is recommended as effective in reducing risks in sexually active young adults, such as college students.

Objective: This study was aimed to assess the level of knowledge, attitude and practice towards VCT service among University students in Kigali City, Rwanda.

Method:
A cross-sectional study was conducted using a stratified sampling method from March to August 2021. A total of 374 participants filled out a structured questionnaire to gather information. A chi-square test was used to determine an association between a number of independent factors and dependent variables.

Result:
A total of 374 participants were interviewed. The majority, 278(74.3%), were in the age bracket of 20-24 years. A total of 223(59.6%) students demonstrated good knowledge of VCT; 219(58.6%) had a positive attitude towards VCT for HIV. Less than half, 160(42.8%), reported that they had VCT for HIV within the past year. Respondents' age (p-value<0.001), religion (p-value<0.001), income category (p-value<0.001), level of education (p-value<0.001), and occupation (p-value <0.001) were among the sociodemographic variables that were significantly associated with practices of HIV prevention and control. The ignorance of the VCT center was reported as the main barrier to VCT uptake.

Conclusion:
The findings show positive views on VCT, but over half didn't get tested due to a lack of knowledge about VCT centers.

Keywords: *knowledge, Attitudes, practices, voluntary counseling, and testing, HIV/AIDS.*

1. Introduction

Every year, a total of 940,000 HIV/AIDS-related deaths occur globally, while 36.9 million people are infected and living with HIV/AIDS.[1] UNAID report indicates that 1.8 million new infections occur every year globally [2]. UNAIDS additionally states that on a global scale, around 5,000 new infections arise each day, with 33% of these cases impacting individuals aged 15 to 24, and among them, 19% are young women.[3] Sub-Saharan Africa is noted as the most severely affected region globally, with approximately 23.5 million people living with HIV infection.[4]

In sub-Saharan Africa, Eastern and Southern Africa are particularly impacted. These regions account for 20.6 million (54%) of the total individuals living with HIV/AIDS worldwide. They also represent 45% of the global HIV infections and 53% of the global population affected by HIV/AIDS.[3] Voluntary testing and counseling (VCT) refers to the procedure whereby an individual receives private counseling to help them make an informed decision about discovering their HIV status and receive guidance on taking suitable steps thereafter.[5] It is a widely embraced method for identifying individuals potentially

exposed to or infected with the HIV virus and serves as a crucial tool in halting the transmission of HIV.[6]976 Ethiopians are currently living with HIV and all of them require antiretroviral treatment (ART Voluntary Counseling and Testing (VCT) has proven to be an effective strategy in encouraging behavioral changes aimed at both preventing HIV transmission and ensuring early access to care and support.[7] VCT has also been shown to play a crucial role in promoting behavioral changes and contributes significantly to the reduction of HIV and other sexually transmitted infections (STIs).[6976 Ethiopians are currently living with HIV and all of them require antiretroviral treatment (ART, 8a person who had no record of contact with the health facility for at least three consecutive months was considered LTFUP. LTFUP incidence rates were computed, and the Fine-Gray's competing risk regression models were used to determine factors associated with time to first LTFUP. Generalized estimating equations (GEEs] To reach the UNAIDS goal of ensuring that 90% of all people living with HIV know their status,[990% of those who are HIV positive treated, and 90% of those treated achieve an undetectable viral load. The latter indicates viral suppression, the goal for clinicians treating people living with HIV (PLWH] targeted HIV testing and counseling (HTC) services need to be implemented across diverse community and facility-based settings. Specifically, adolescent and youth clients aged 15-24 are among the groups prioritized for HIV testing and counseling initiatives. [10respiratory infections, malnutrition, schistosomiasis, malaria, soil-transmitted helminth infections and trachoma from exposure to inadequate drinking-water, sanitation and hygiene behaviours (WASH]

A recent report from 2021 surveys conducted in 12 high-burden sub-Saharan African countries indicates that only 12% of men and 10% of women in the general population have undergone HIV testing and received their results. (11)

In Rwanda, HIV infection remains a critical public health concern, contributing significantly to mortality rates and imposing social and economic burdens that affect both individuals and the nation as a whole. Rwanda has made significant strides in advancing universal access to HIV and AIDS services by implementing comprehensive national multi-sector strategic plans. Top of Form These plans, such as the one spanning from 2005 to 2009 and the subsequent National Strategic Plan from 2013 to 2018, signify Rwanda's commitment to combatting HIV/AIDS comprehensively and effectively. [12]

More than 50% of global HIV cases occur among adolescents aged 10 to 24 years.[13] Voluntary Counseling and Testing (VCT), which remains a crucial gateway for HIV prevention, management, and treatment, VCT services are underutilized by the youths; consequently, many adolescents are unaware of their HIV status.[14]

In Rwanda, there are 256 VCT (Voluntary Counseling and Testing) sites identified by the Treatment and Research on AIDS Centre (TRAC). TRAC was introduced in Rwanda in 1997 with specific objectives focused on AIDS treatment and research. [15]

According to the 2019/2020 Rwanda Demographic and Health Survey, 59% of men and 45% of women aged 15-24 have never undergone HIV testing.[16] Although VCT is a widely embraced method for identifying individuals potentially exposed to or infected with the HIV virus and serves as a key tool in reducing the transmission of the virus,[6]976 Ethiopians are currently living with HIV and all of them require antiretroviral treatment (ART context-based studies on the KAP regarding VCT services in Rwanda are scarce. This study evaluated the knowledge, attitude, and practice of University students towards VCT service in Kigali, Rwanda.

2. Method

Ethical consideration

The study was reviewed by Mount Kenya University's Ethical Review Board. Additional permissions to conduct the research were obtained from the three selected universities involved in the study. Participants were informed that their involvement was voluntary, with the option to withdraw at any point without repercussions. Confidentiality of the provided information was underscored. Before joining the study, participants received comprehensive explanations about its objectives. They were then invited to sign a consent form willingly, without pressure. Measures were implemented to safeguard participants' dignity, respect, and independence throughout the study.

Study area and study setting.

This was a cross sectional study that interviewed students registered in three(3) out of seventeen(17) universities located in Kigali City, Rwanda. Kigali, the capital of Rwanda, is positioned approximately at the country's geographical center, covering an area of 730 square kilometers with a population of 1,745,555.

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[17] The three Universities were Mount Kenya University (MKU), Independent University of Kigali (UK), and University of Rwanda (UR); College of Science and Technology and College of Medicine and Health Sciences. Those higher learning institutions were purposively selected for three main reasons. First, the three are the largest universities in Kigali City in terms of student population. Two, at the time of data collection, all three universities were in session, so the students could be reached for interviews. Third, there is a diversity of students, female and male, as well as national and international students, and there is respect for student culture and policy to abrogate discrimination.

The research undertaken followed a quantitative cross-sectional design and was carried out between March and August 2021 among university students in Kigali, Rwanda. The term "university" in this context encompasses all institutions of higher learning. The study was conducted across three out of the seventeen universities situated in Kigali, Rwanda.

Population, sample size, and sampling techniques

The study population was undergraduate students in the three selected Universities who, at the time of data collection, from March and August 2021, were in session. A sample size of 374 students was determined using the Fischer formula.[18]

$$N = \frac{Z^2\ p\ (1-p)}{d^2}$$

Where,

"d," the preferred margin of error of 5%;

"p," the prevalence (58%) of VCT uptake based on a study conducted in Ethiopia among university students; [19]

"z" is the standard normal deviation (1.96) that matches the 95% confidence level.

Substituting,

$$\frac{(1.96)^2\ 0.58\ (1-0.58)}{(0.05)^2} = 374$$

The study employed a stratified sampling technique to select participants. Strata were defined based on schools, departments, and intake levels. A target population list, including registration numbers, was obtained from each institution's registrar's office. Within each stratum, participants were selected using simple random sampling, achieved through random number generation. Questionnaires were distributed until all selected numbers within each stratum were covered.

Table 1: Distribution of target population

Universities	Target population	Sample size	Sampling technique
Mount Kenya University	2418	Ni/N*n= 2418/8968* 374 = 101	Stratified
Independent University of Kigali	2750	Ni/N*n= 2750/8968*374= 115	Stratified
UR- college of medicine and health sciences	1800	Ni/N*n= 1800/8960*374= 75	Stratified
UR- College of Science and Technology	2000	Ni/N*n= 2000/8968*374= 83	Stratified
Total	8968		
N.B: Ni/N*n; Ni= Total population in the university; N= Grand Total Population; n= Sample size			

The data collection instrument used was a self-administered structured questionnaire. This questionnaire was adapted from previous studies [20,21] and comprised four parts.

Part I focused on sociodemographic characteristics of the students. Part II assessed students' knowledge about Voluntary Counseling and Testing (VCT) for HIV. Part III evaluated students' attitudes towards VCT. Part IV examined their VCT practices.

Knowledge about VCT was assessed using an eleven-item questionnaire that covered aspects such as awareness of VCT centers in Kigali, understanding the voluntary nature of testing, knowledge of locations offering VCT services, and awareness of the importance of VCT in HIV prevention and control.

Attitudes towards VCT were evaluated through an eleven-item questionnaire that explored perceptions related to confidentiality of testing, cost considerations, support from friends during testing, feelings of embarrassment, fairness towards HIV-positive individuals, and perceptions of eligibility for VCT.

The practice of VCT was assessed with a single question: "Have you ever had VCT in the past?" with responses recorded as "Yes" or "No."

The structured questionnaire used in this study was adapted from similar studies conducted in Ethiopia. [20,21]. The questionnaire underwent modifications to align with the context of Rwanda. To guarantee clarity and reliability, a pre-test was conducted with twenty students from another campus of the university, not included in the selection, to test the validity of the tool. The feedback from this pre-test was utilized to refine the phrasing of questions in the questionnaire, ensuring its comprehensibility and effectiveness.

Scoring

For the knowledge about VCT, each question was scored with "Yes" for correct answers and "No" for incorrect answers. The scores of all respondents were summed up, and participants who scored six or more correct answers out of eleven were categorized as good knowledge, while others were categorized as poor knowledge.

Students' attitudes toward VCT service were assessed based on their negative perceptions of HIV-positive individuals in society and among friends, confidentiality during counseling and testing for HIV, among other factors. A positive attitude was indicated by respondents who disagreed with most statements, except for four questions. Participants who scored six or above were classified as having a positive attitude, while those scoring lower were deemed to have a negative attitude.

Practice was evaluated with a single question requiring a "Yes" or "No" response, with those answering "Yes" considered to have undergone VCT services in the past.

Data management and analysis

The collected data were first entered into Excel, where they underwent coding and cleaning procedures. Following this, the data were exported and analyzed using IBM SPSS Statistics for Windows version 25.0. The results were presented in tables, and descriptive statistics were employed to provide a comprehensive overview of background variables such as age, sex, and other parameters outlined in the structured questionnaire.

Frequency distributions of both dependent variables (knowledge, attitude, and practice of VCT) and independent variables (sociodemographic factors) were calculated to understand their distribution within the sample. The association between variables was assessed using the chi-square test, with a significance level set at $p < 0.05$ for all analyses.

This analytical approach aimed to explore relationships and identify significant associations between sociodemographic factors and participants' knowledge, attitudes, and practices regarding VCT.

3. Results

Socio-demographic information

A total number of 374 university students participated in this study. Of these, 193 (51.6%) were male, and 181(48.4%) were. About three-quarters were in the age bracket of 20 to 24; more than half, 202 (54%); Slightly less than half, 177 (47.3%), belonged to category (ubudehe) II; while 161 (44.1%) were in category three. An overwhelming majority, 369 (98.7%), reported that they were not married. The majority, 328 (87.7%) of the respondents were students as occupation and around one-third, 120 (32.1%) of them, were from year one, and 87 (23.3%), 85(22.7%) and 82 (21.9%) were at level two, three and four respectively.

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Table 2: Knowledge of VCT services among university students in Kigali city.

Variables	Frequency (n= 374)	Percent (%)
Ever heard of VCT services		
Yes	319	85.3
No	55	14.7
Where did you hear it from (n = 319)		
Radio	142	38
TV	39	10.4
Friends and family	34	9.1
School	104	27.8
Do you know the place where VCT is provided? (**)		
Government hospital	198	52.9
Private hospital	34	9.1
HIV awareness program	142	38
Do you know that? (**)		
HIV tests are voluntary	188	50.3
HIV test is given with counseling	135	36.1
HIV medication is given at the VCT center	51	13.6
Is VCT important for the prevention and control of HIV/AIDS		
Yes	368	98.4
No	6	1.6
Do you know any VCT centers in Kigali?		
Yes	256	68.4
No	118	31.6
Are prostitutes the only ones visiting VCT?		
Yes	42	11.2
No	332	88.8
Is VCT visit for married only		
Yes	0	0
No	374	100
Is VCT visit for people with multiple partners		
Yes	18	4.8
No	356	95.2
Is VCT visit for people with STD		
Yes	20	5.3
No	354	94.7
To undergo VCT for healthy looking person		
Yes	321	85.8
No	53	14.2
VCT services are for everyone		
Yes	347	92.8
No	27	7.2

(**) means multiple answers

Knowledge on VCT

The majority, 319 (85.3%) of the respondents, had heard about VCT service from some sources. The major sources cited were radio, 142 (38.0%) and, and 104 (27.8%) from schools (see Table 1). A total of 55(14.7%) reported that they had never heard about VCT service in their lifetime. The majority, 368 (98.4%) of respondents, agreed that VCT is important for HIV prevention; 256 (68.4%) knew at least one VCT Centre in Kigali. All the participants, 374 (100%), did not agree that only married had to visit VCT; 332 (88.8%), 356 (95.2%), and 354 (94.7%) don't agree that only prostitutes, people with multiple partners and people with sexually transmitted disease respectively have to visit VCT service. The majority, 347 (92.8%) and 321 (85.8%) of respondents agreed that VCT service is for everyone and healthy-looking persons, respectively. Half, 187(50%) of the respondents, knew that VCT is provided at government hospitals. About 188 (50.3%) of them knew that HIV test is voluntary, 135 (36.1%) knew HIV test is given with counseling, and only 51 (13.6%) of them knew that HIV medication is given at VCT centers.

VCT attitudes of university students in Kigali

Of the study participant, 190 (50.8%) agreed to be embarrassed to go for VCT, and 296 (79.1% agreed their friends should support them if they decided to go for VCT. The majority, 234 (91.1%) of respondents, agreed that VCT is confidential, 319 (85.3%) and 298 (79.7%) agreed to take care of a sick relative with HIV in his house and buy a fresh vegetable from an HIV-positive vendor respectively. About 314 (84%) of respondents disagreed that VCT is expensive for students, 229 (61.2%) and 215 (57.5%) of them disagreed that people could abandon an HIV-positive person and be disclosed to other persons, respectively. The majority, 345 (92.2%) of respondents, disagreed with the fact of not attending VCT service because of no cure for HIV/AIDS, 325 (86.9%) and 198 (52.9%) disagreed that people with HIV should be ashamed of themselves and ashamed of bringing the virus to the community.

Practices towards VCT service among university students.

About 160 (42.8%) of the respondents have had VCT in the past, and 214 (57.2%) of them have never had VCT. Among those who have taken VCT service in the past, 62 (16.6%) of them took their VCT service in the hospital, 54 (14.4%) of them were taken during the HIV campaign, and 130 (34.8%) were taken on campus. The majority, 335 (89.6%) of the respondents, were ready to take the VCT test if available, and 109 (29.1%) of them didn't know the VCT center. The reason for not taking the VCT service, for many, 109 (29.1%), was for not knowing the VCT center, and 51 (13.6%) of them use condoms during sex. The majority, 352 (94.1%) of respondents, agreed to get an HIV test if available and free; only 22 (5.9%) of them were not ready.

The majority, 94.1% (325) of the respondent, decided to take HIV VCT uptake by their own choice, 90.4% (325) of them found it comfortable using HIV VCT service, and 79.9% (299) of them agreed to take HIV VCT services in the campus. Almost half 46% (172) of the respondents admitted that HIV VCT is available on campus, 73.3% (274) of them agreed HIV VCT is affordable, and 78.9% (295) admitted that VCT/HIV service is convenient to them.

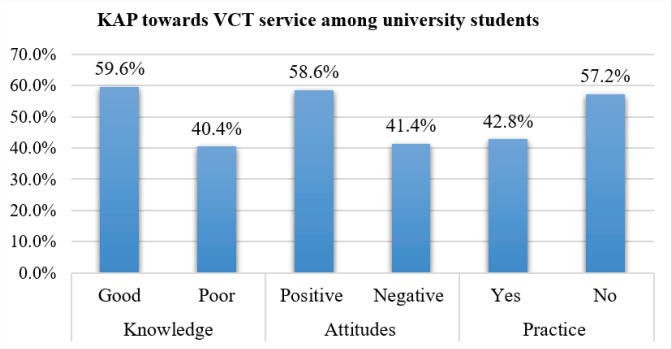

Figure 1: Knowledge, attitude, and practice towards VCT service among University students in Kigali

The finding revealed that 59.6% of the respondent are knowledgeable towards VCT and more than of half 58.6% of students had positive attitudes for VCT. However only 42.8% of the respondent are practiced VCT (Figure 1).

Table 3: Association between sociodemographic variables with the knowledge of respondents about VCT service

Variables	Knowledgeable	Not knowledgeable	χ^2 (P-value)
Gender Male Female	 107 55.4 116 64.1%	 86 44.6% 65 35.9%	 2.902 (0.088)
Age >20 20-24 25-29 30-34	 31 66.0% 152 54.7% 34 79.1% 6 100%	 16 34.0% 126 45.3% 9 20.9% 0 0.0%	 14.428 **(0.02)***

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Marital status Single Married	218 59.1% 5 100%	151 40.9% 0 0.0%	3.432 (0.064)
Year of Study Year 1 Year 2 Year 3 Year 4	69 (57.5%) 53 (60.9%) 53 (62.4%) 48 (58.5%)	51 (42.5%) 34 (39.1%) 32 (37.6%) 34 (41.5%)	0.589 (0.899)
Religion Protestant Catholic Muslim Atheist	33 (35.9%) 142 (70.3%) 36 (81.8%) 12 (33.3%)	59 (64.1%) 60 (29.7%) 8 (18.2%) 24 (40.4%)	50.462 **(<0.001)***
Category (ubudehe) Class1 Class2 Class3	2 (6.3%) 95 (53.7%) 126 (76.4%)	30 (93.8%) 82 (46.3%) 39 (23.6%)	59.678 **(<0.001)***
Parent education No education Primary Secondary University	3 (5.4%) 35 (41.2% 84 (81.8% 104 (77.6%	53 94.6% 50 58.8% 18 18.2% 30 22.4%	118.788 **(<0.001)***
Occupation Student Government employee Businessman/woman	180 (54.9%) 24 (100%) 19 (86.4%)	148 (45.1%) 0 (0.0%) 3 (13.6%)	25.856 **(<0.001)***
Year of Study Year 1 Year 2 Year 3 Year 4	113 (94.2%) 87 (100%) 84 (98.8%) 76 (92.7%)	7 (5.8%) 0 (0.0%) 1 (1.2%) 6 (7.3%)	9.299 **(0.026)***
Religion Protestant Catholic Muslim Atheist	85 (92.4%) 199 (98.5%) 43 (97.7%) 33 (91.7%)	7 (7.6%) 3 (1.5%) 1 (2.3%) 3 (8.3%)	9.043 **(0.029)***
Category (ubudehe) Class1 Class2 Class3	32 (100%) 169 (95.5%) 159 (96.4%)	0 (0.0%) 8 (4.5%) 6 (3.6%)	1.546 (0.462)
Parent education No education Primary Secondary University	53 (94.6%) 79 (92.9%) 98 (99.0%) 130 (97.0)	3 (5.4%) 6 (7.1%) 1 (1.0%) 4 (3.0%)	5.264 (0.153)
Occupation Student Government employee Businessman/woman	314 (95.7%) 24 (100%) 22 (100%)	14 (4.3%) 0 (0.0%) 0 (0.0%)	2.040 (0.361)
Knowledge Knowledgeable Not knowledgeable	216 (96.9%) 144 (95.4%)	7 (3.1%) 7 (4.6%)	0.560 (0.454)

Table 3 presents the factors that were associated with knowledge. Age of the respondents, religion of the respondents, category class (ubudehe) of the respondents, occupation of the respondents and parents education of the respondents (χ^2 = 14.428, P-value < 0.02; χ^2 = 50.462, P-value < 0.001; χ^2 = 59.678, P-value < 0.001, χ^2 = 25.856, P-value < 0.001 and χ^2 = 118.788, P-value < 0.001) respectively were significant associated with the knowledge status on VCT service (table 3)

Table 4: Association between sociodemographic variables and knowledge of VCT with the attitude of respondents towards VCT service

Variables	Positive attitude	Negative attitude	χ^2 (P-value)
Gender Male Female	186 (96.4%) 174 (96.1%)	7 (3.6%) 7 (3.9%)	0.015 (0.903)
Age >20 20-24 25-29 30-34	45 (95.7%) 266 (95.7%) 43 (100%) 6 (100%)	2 (4.3%) 12 (4.3%) 0 (0.0%) 0 (0.0%)	2.193 (0.533)
Marital status Single Married	355 (96.2%) 5 (100%)	14 (3.8%) 0 (0.0%)	0.197 (0.657)

During the survey, it was found that the year of study and the religion of respondents showed a statistically significant association with their attitudes towards VCT services at χ2 (P-value) of 9.299 (<0.026 and 9.043 (<0.029) respectively, as shown in Table 4.

Table 5: Association between sociodemographic variables and knowledge about VCT with practice of VCT service

Variables	Use VCT	Not use VCT	χ^2 (P-value)
Gender Male Female	82 (42.5%) 78 (43.1%)	111 (57.5%) 103 (56.9%)	0.014 (0.906)
Age >20 20-24 25-29 30-34	35 (74.5%) 101 (36.3%) 23 (53.5%) 1 (16.7%)	12 (4.3%) 177 (63.7%) 20 (46.5%) 5 (83.3%)	27.689 **(<0.001)***
Marital status Single Married	159 (43.1%) 1 (20%)	210 (56.9%) 4 (80%)	1.074 (0.3)
Year of Study Year 1 Year 2 Year 3 Year 4	63 (52.5%) 40 (46%) 33 (38.8%) 24 (29.3%)	57 (47.5%) 47 (54%) 52 (61.2%) 58 (70.7%)	11.654 **(0.009)***

Religion Protestant Catholic Muslim Atheist	29 (31.5%) 88 (43.6%) 33 (75%) 10 (27.8%)	63 (68.5%) 114 (56.4%) 11 (25%) 26 (72.2%)	26.784 **(<0.001)***
Category (ubudehe) Class1 Class2 Class3	17 (53.1%) 74 (41.8%) 69 (41.8%)	15 (46.9%) 103 (58.2%) 96 (58.2%)	1.530 (0.465)
Parent education No education Primary Secondary University	29 (51.8%) 32 (37.6%) 38 (38.4%) 61 (45.5%)	27 (48.2%) 53 (62.4%) 61 (61.6%) 73 (54.5%)	3.964 (0.265)
Occupation Student Government employee Business-man/woman	150 (45.7%) 6 (25%) 4 (18.2%)	178 (54.3%) 18 (75%) 18 (81.8%)	9.705 **(0.008)***
Knowledge Knowledge-able Not knowledgeable	108 (48.4%) 52 (34.4%)	115 (51.6%) 99 (65.6%)	7.202 **(0.007)***

Age of the respondent, year of study of respondents, the religion of the respondent, occupation of the respondent, and knowledge of the respondent had a statistically significant association with the attitude towards VCT service at $\chi 2$ (P-value) of 27.689 (<0.001), 11.654 (0.009), 26.784 (<0.001), 9.705 (<0.008) and 7.202 (0.007) respectively (table 5).

4. Discussion

The study aimed to assess the level of knowledge, attitude, and practice toward VCT service among University students in Kigali City, Rwanda. Our findings indicate that the majority, 319 (85.3%), reported that they had never heard about VCT. The finding is lower than the results reported in a similar study conducted among university students in Ethiopia [20], which reported that 93.4% of the participants were knowledgeable about VCT of HIV. This study reports that 59.6% of the students had good knowledge of VCT, which is slightly lower than the findings of a study conducted among university students in Ethiopia, which reported that 66% of the students were knowledgeable about VCT. [22] Other studies in Ethiopia and Nigeria reported much higher knowledge levels, 93.4% and 82.9%, respectively, which is higher compared to the results from this study.[20,23] The majority of the students in Ethiopia with high knowledge are comparatively older than the majority age bracket in Kigali, 20-24 versus 24 and above age groups for Kigali and Ethiopia, respectively. Consequently, they may not be fully informed about available services such as counseling and partner advice, seeing VCT primarily as blood testing. Closing this knowledge gap requires effective communication. Information about VCT services was mainly received from radio (38%) and schools (27.8%). In contrast, a study in Malawi revealed that 42% of students had acquired information about VCT from church or funeral gatherings, while 25% cited friends as their primary source of information [24]. In another study conducted in Nigeria, it was found that mass media and churches were the primary sources of information on VCT. This trend could be attributed to the wide reach of mass media, making it a potent tool for reaching young people, while churches also play a significant role in disseminating information. Schools, on the other hand, are considered a vital avenue for accessing young individuals.[25]

An evaluation of the influence of sociodemographic factors on VCT knowledge revealed significant associations with age, religion, category, parents' education, and occupation of the surveyed respondents. This finding contrasts with a study conducted in Ethiopia, where only the residency status of respondents showed a statistically significant association with VCT knowledge. [22]

As the overall attitude score showed 58.6% of participants have a positive attitude towards VCT, lower results were reported compared with a study from students in North West Ethiopia, in which 73.3% of respondents had favorable attitudes towards VCT services.[21] This is in contrast to the study, which reported 40 % positive attitudes toward VCT. [23] The variation in awareness levels between the study regions and ongoing enhancements in health interventions could account for these differences. Marital status and religious affiliation emerged as significant sociodemographic variables influencing students' attitudes toward VCT. Christian students are often inclined towards abstinence or condom use (if they know their HIV status); if married, they exhibit distinct attitudes. This association with religious beliefs may stem from heightened knowledge among students, thereby influencing their attitudes positively. Conversely, a study in Addis Ababa suggested that students from rural areas, potentially lacking comprehensive information about VCT, might exhibit lower levels of favorable attitudes, albeit not statistically significant. [25which is essential for the management of the disease. This study sought to determine the prevalence

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and factors that influence the utilization of VHCT services among young people. In this study, young people in the Tema Metropolis were cross-sectionally surveyed. The simple random sampling method was used to select the participants. The majority (60%]

The results of this study revealed that 42.8% of respondents demonstrated a positive attitude towards VCT services. In comparison, a study among high school students [26] reported a relatively higher attendance rate of VCT at 65.1%. This finding contrasts with a study conducted at Addis Ababa University [27which is essential for the management of the disease. This study sought to determine the prevalence and factors that influence the utilization of VHCT services among young people. In this study, young people in the Tema Metropolis were cross-sectionally surveyed. The simple random sampling method was used to select the participants. The majority (60%], where only 23% of participants had undergone VCT, with a mere 15.3% of them testing for HIV. [29] In line with a study conducted in Nigeria, the primary reasons cited by individuals who had never undergone VCT in the past were attributed to concerns regarding trust in partners and oneself (18.5%), fear of stigma (21.2%), and apprehension about receiving the test results (18.3%). Despite variations in VCT attendance rates, the primary reasons for not visiting VCT centers appear to be consistent across different study areas, including community-based surveys.[28, 30] Individuals with good knowledge and positive attitudes are more likely to be aware of prevention methods related to HIV/AIDS, suggesting that interventions focusing on improving knowledge could lead to attitude changes and subsequently facilitate the uptake of VCT services. The current study found statistically significant associations between VCT uptake and factors such as age, year of study, religion, occupation, and knowledge level, consistent with findings from previous research. Furthermore, there was a notable association between knowledge about HIV, knowledge about VCT, attitude towards VCT, and VCT practice, indicating their interconnectedness. Therefore, efforts aimed at enhancing knowledge and fostering attitude changes are crucial for increasing the uptake of VCT services.

5. Conclusion

Generally, students' knowledge and attitudes towards VCT services are relatively satisfactory, but their actual practice of utilizing VCT services is relatively unsatisfactory. Information education communication and peer-to-peer discussions are highly valuable in addressing this gap. Factors such as age, religion, socio-economic category (ubudehe), occupation, and parents' education are associated with knowledge about VCT. This allows for targeted interventions toward specific groups when universal approaches are not feasible. Additionally, the year of study and religion are significantly associated with having a positive attitude towards VCT services. Regarding the utilization of VCT services, factors such as age, year of study, religion, occupation of the respondent, and having knowledge about VCT are significantly associated with actually using these services. These findings highlight the importance of targeted interventions and tailored educational efforts to improve VCT uptake among students. As a recommendation, first, it is important to raise awareness within families and higher education institutions to prevent stigma and discrimination among students. Secondly, enhancing knowledge about VCT/HIV among university students can be achieved by expanding VCT facilities that offer services, along with training counselors, which will play a key role in increasing the use of these services.

6. Limitations of the study

The findings reported in this study should be considered within the context of certain limitations. One, as a cross-sectional study, we relied solely on the information provided by respondents, introducing the potential for information bias. Therefore, the results may not be broadly applicable to the wider population of tertiary-level students. Additionally, we did not assess the perceived risk of HIV, which could have strengthened the findings. Despite these limitations, our study offers valuable insights for future research, suggesting the need for multicenter studies involving larger study populations to further validate and expand upon our findings.

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Age And Pattern Of Cervical Smear Cytology In Federal Medical Centre Asaba – A Five Year Review

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ABSTRACT:

Background: *Cancer of the cervix is a leading cause of cancer deaths among women in low and middle-income countries. Screening for premalignant lesions of the cervix using a cervical smear is preventive. The pattern of cytological findings varies with age; this pattern is not well known in our environment.*

Objective: *To assess the pattern of cervical smear cytology in the Federal Medical Centre, Asaba.*

Methods: *This was a retrospective descriptive study of all cervical smears received and processed at the histopathology department of the Federal Medical Centre (FMC), Asaba, between 1st January 2016 and 31st December 2020. Those with inadequate data were excluded from the study. The classification was by the 2014 Bethesda system. Data collected was analyzed using the SPSS (IBM version 20), and the results were presented in frequencies, percentages, and tables.*

Results: *A total of 1184 cervical smear samples were received in the histopathology department of FMC, Asaba, during the period of study. One thousand one hundred and fifty were satisfactory. The mean age was 43+/-10.58 and ranged between 19 and 76 years. The age group with the largest number of cervical smears was 41-50 years (412; 35.8%). About 807(70.2%) were normal smears while 343(29.8%) had abnormal results. Of the abnormal results, 173(15.0%) were negative for intraepithelial cell abnormalities, while 170(14.8%) had intraepithelial cell abnormalities. Of these were ASC-US (13; 1.1%), LSIL (129; 11.2%), HSIL (28; 2.4%). The prevalence of HSIL increased with age; the highest was >60 years (35.7%). The reverse was the case for the lower grade lesions. There was a significant relationship between the ages of the patient and the pattern of cervical smear results (P < 0.01).*

Conclusion: *Population screening programs should be encouraged for the inclusion of younger women in whom lower grade lesions are commoner to reduce the prevalence of cervical cancer in our population.*

Keywords: *Age, screening, cervical smear, cytology, cervical intra epithelial lesion, and cervical cancer.*

1. Introduction

Cancer of the cervix is a potentially preventable disease.[1] Approximately 570 000 cases of cervical cancer and 311000 deaths from the disease occurred in 2018.[2] Cervical cancer was the fourth most common cancer in women, ranking after breast cancer (2·1 million cases), colorectal cancer (0·8 million) and lung cancer (0·7 million). [2] It is the commonest in developing countries, and it progresses slowly in the body. [3] Majority of the morbidities and mortalities from cervical cancer occur mainly in these developing countries; this can be largely as a result of late presentation when only palliative care is available.[4, 5] The goal of cervical cancer screening is to find pre-cancers early so they can be treated before they transform into cancer. [6,

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[7] Although screening is a known cost-effective strategy used in reducing the burden of cervical cancer worldwide, its uptake, particularly in developing countries, is still abysmal. [8, 9]

The Pap smear test results are reported using the 2014 Bethesda nomenclature.[10] According to this classification system, the adequacy of the specimen was properly assessed based on either being satisfactory or unsatisfactory or a general categorization of either being normal or abnormal. The abnormal result could further be divided into negative for intraepithelial lesion/ malignancy, epithelial cell abnormality, which could either be of squamous cell or glandular origin and others.[10] The squamous cell type is further divided into atypical squamous cells (of undetermined significance ASC-US and cannot exclude HSIL – ASC-H), low grade squamous intraepithelial lesion (LSIL), high grade squamous intraepithelial lesion (HSIL) and squamous cell carcinoma.

The glandular cell type is further divided into Atypical glandular cells, endocervical adenocarcinoma in situ, adenocarcinoma, and others.

This study assesses the pattern of Pap smear results in FMC, Asaba, over five years.

2. Methodology

Study Design

This was a retrospective study of all cervical smears received and processed at the Pathology department over 5 years (1st January 2016 - 31st December 2020) in Federal Medical Centre, Asaba.

Setting

Federal Medical Centre Asaba is a tertiary health institution located in Oshimili South LGA of Delta State situated in the South-South Geopolitical zone of Nigeria. It provides primary, secondary and tertiary health services to the Delta populace. It also serves as a referral center for patients from neighbouring states like Anambra and Edo.

The Histopathology department provides services to the entire hospital as the need arises during working hours of weekdays. The department has two Consultants and other support staff that direct activities of the department. The cervical cytology smear testing is one of their services in conjunction with the Obstetrics and Gynaecology department where the samples are collected before being sent for histopathology evaluation.

Study Population

Cervical cytology smears performed at FMC Asaba between January 2016 and December 2020 were studied and analyzed.

Data collection methods

A register of all women who had a cervical cytology smear done at the histopathology department is maintained electronically. This register was used to identify cervical cytology smear results done between 1st January 2016 and 31st December 2020. Relevant data retrieved included the age, cervical cytology smear pattern and the amount of cervical cytology smears done annually. A proforma was designed and used to extract these data.

3. Data Analysis

Data collected was entered into and analyzed using the Statistical Package for Social Sciences (SPSS) version 20.0 (IBM SPSS). The results were presented in statements, frequency distribution tables, and charts. The test of significance was by the chi-square test and Fisher's exact test. The level of significance was considered if the p-value was < 0.05

Ethical Approval

Approval for the study was granted by the Research and Ethics Committee of Federal Medical Centre Asaba.

4. Results

The total number of pap smears received was 1184. Thirty-four (2.9%) were unsatisfactory and, therefore, not included in the study. One thousand one hundred and fifty (1150) smears were analyzed. The age range of the women screened was between 19 and 76 years. The largest number of smears was between ages 41-50years (412 = 35.8%) and 31-40years (366 = 31.8%) while (177 = 15.4%) was for age bracket 51-60years, (119 = 10.3%) for 21-30years and (75 = 6.5%) for ages above 61years. Only 0.1% was found in the 20-year age group below. (Table 1)

The year 2019 had the highest amount of cervical cytology tests done, with 299(26.0%) smear results. This was followed by 275(23.9%) for 2017, 232(20.2%) for 2016 and 216(18.8%) for 2018. The year 2020(128=11%) witnessed the lowest amount of Pap smears done in the last

five years, as shown in Figure 1.

Majority of the smears 807(70.2%) were in the category of normal while 343(29.8%) had an abnormal Pap smear result. Of the abnormal results, 173(15.0%) were negative for intraepithelial lesion whereas 170(14.8%) were positive. These positive smear results were made of ASC-US 13(1.1%), LSIL 129(11.2%) and HSIL 28(2.4%). There was no invasive carcinoma found as shown in Figure 2.

Smears that revealed an HSIL occurred more in the 51-60 years age group and were closely followed by the 41- 50 years age group, while smears that revealed an LSIL occurred more in the 41-50 years age group and were followed by the 31-40 years age group. Cervical smears with ASCUS also occurred more in the 41-50 years age group. There appeared to be a significant relationship between the age of the patients examined under the period and their cervical cytology results, as the P-value was < 0.05. See Table 4

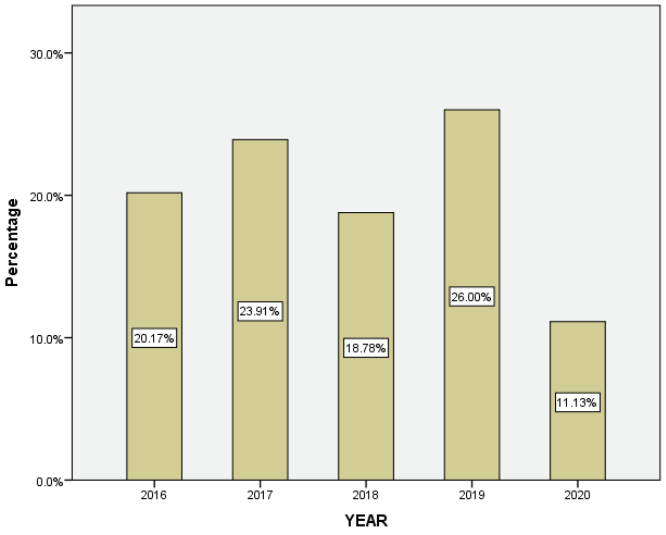

Figure 1. The yearly trend of uptake of cervical cytology smears.

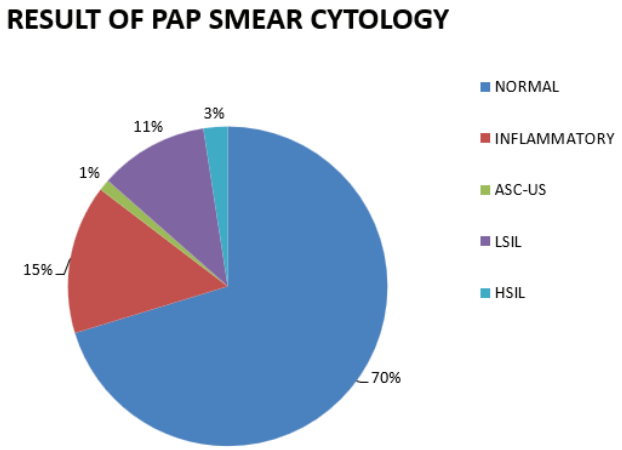

Figure 2 Pie Chart showing the pattern of distribution of the cervical smear

Table 1. Age distribution of the cervical smears

Age	Frequency	Percentage(%)
<20	1	.1
21-30	119	10.3
31-40	366	31.8
41-50	412	35.8
51-60	177	15.4
>61	75	6.5
Total	1150	100.0

Using the chi-square test and Fisher's exact test for correction, as more than 20% of the cells have an expected count of less than 5.

Table 4. Relationship between the age of the patient and their cervical cytology result.

NORMAL		RESULT					Total	X^2	P value
		INFLAMMATORY	ASC-US	LSIL	HSIL				
AGE	<20	1	0	0	0	0	1	79.854	0.000
	21-30	92	17	2	8	0	119		
	31-40	268	62	1	32	3	366		
	41-50	301	53	5	46	7	412		
	51-60	111	28	4	26	8	177		
	>61	34	13	1	17	10	75		
Total		807	173	13	129	28	1150		

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From the table above, there is a significant relationship between age and cervical cytology results of patients. The p value is <0.05.

5. Discussion

Cervical cytology smears are usually taken for screening of cervical cancers as this disease may be prevented by early detection alongside appropriate management of precursor lesions.[2, 11] The low rates of cancer of the cervix in more developed climes are probably the result of successful cytological screening. This is possible because of the routine against opportunistic and institution-based screening in low- and middle-income countries; the latter is erratic and does not cover all the relevant groups. These screening programs have counteracted the increased exposure to risk factors among generations born after 1945, as established from age-period-cohort analyses.[2]

Of the 1150 smears analyzed, 807(70.2%) were normal smears, while 343(29.8%) had abnormal Pap smear results. The abnormal Pap smear result obtained in this study was similar to the results in Imo[12] and Zaria[13] but was higher than the results obtained in Calabar,[14] Enugu[15], and Saudi Arabia.[16] It was, however, lower than the results obtained in Osun,[17] Gombe[7], and India.[18] Of the abnormal results, 173(15.0%) were negative for intraepithelial lesions whereas 170(14.8%) were positive for intraepithelial lesions. Inflammatory lesions (15%), such as acute and chronic cervicitis, accounted for the smears that were negative for intraepithelial lesions. This was similar to the 14.6% published by Obaseki et al. in Benin[19] and 13% in Zaria,[13] but lower than 52.5% in Gombe,[7] 29.9% in Ile-Ife[17], and 50.2% in India.[18] Inflammatory lesions obtained in our study were higher than the 5.2% obtained in Saudi Arabia.[16]

The LSIL 129(11.2%) was the commonest intraepithelial lesion seen in this study and was similar to the findings in Zaria,[13] Ile-Ife[17], and Benin.[19] This was followed by HSIL 28(2.4%) and ASC-US 13(1.1%). However, Duru et al. in Imo reported HSIL(36.8%) to be the commonest intraepithelial lesion, followed by squamous cell carcinoma(35.1%) and LSIL(14.0%)[12] while Samar et al. [20] and Elhakeem et al. [21] reported ASCUS to be the commonest intraepithelial lesion in Saudi Arabia. The varying pattern could be due to the differences in the population and method of screening.

The year 2019 had the highest number of cervical smears done over the five-year review of our study, while 2020(11.1%) had the lowest amount of Pap smears done. This was probably due to the effect of the Covid-19 pandemic on the reproductive health of women.

The oldest patient with a positive smear result in this series was 76 years old, supporting the facts by some authors that there should be no upper age limit for the first smear.[15] The majority of the women who had a pap smear were between the 41- 50 years age group 412(35.8%). This was followed by the 31-40 years age group, 366(31.8%). This was comparable with similar studies in Imo,[12] Zaria,[13] Calabar,[14] Ile-Ife,[17] Benin,[19] India[18] and Saudi Arabia.[21] The majority of the ASC-US and LSIL cases also occurred in this 41-50-year-old age group, while a higher proportion of pap smear results with HSIL occurred in the 51-60-year-old age group. This was in contrast with findings in Benin[19] and Saudi Arabia[20], where LSIL occurred more in the 50-59-year-old group while HSIL occurred more in the 40-49-year-old age group.[20]

This study showed a significant relationship between age and the pattern of cervical smear results, as the p-value was < 0.05. It also showed that the number of abnormal lesions in the different age brackets increased with age. This emphasizes the fact that there are more women affected by increasing age, hence the importance of organized screening.

6. Conclusion

Screening for cancer of the cervix has been proven to be an effective tool in the detection of pre-invasive stages of cervical cancer. Population based screening should be encouraged for adequate inclusion of all relevant age groups, especially the younger ones in whom the lower grade lesions are commoner. Hence, all tiers of government and the organized private sector should devise a strategy for effective cervical smear uptake with follow-up and referrals to specialist clinics for treatment when abnormal smears are found.

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Context-Dependent Autophagy in Cancer: Deciphering Cytoprotective vs. Cytotoxic Roles and Therapeutic Modulation Strategies

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ABSTRACT:

Autophagy is a highly conserved cellular process activated when cells are exposed to stress. It is responsible for maintaining cellular homeostasis by degrading and recycling the exhausted cell components. However, autophagy is considered a cell survival mechanism; recent studies revealed its dual role in cancer. Autophagy can act as a cytoprotective in early tumor stages or cytotoxic in advanced malignancies. This review explores the underlying molecular mechanisms and key regulatory pathways of autophagy, such as mTOR, AMPK, and p53, and their roles in tumorigenesis. The contradictory nature of autophagy in cancer varies according to the cellular context and depends on genomic stability, metabolic adaptation, immune evasion, and therapy resistance. Additionally, this review discusses different therapeutic strategies targeting autophagy, including inhibitors such as hydroxychloroquine and inducers like rapamycin, which have shown promise in modulating autophagy for improved cancer treatment outcomes. The review also examines the role of autophagy in cancer stem cells, metastasis, and therapy resistance, providing insights into how autophagy modulation can enhance chemotherapy, radiotherapy, and immunotherapy. Given its context-dependent functions, a deeper understanding of autophagy's intricate regulation is crucial for developing precision medicine approaches to effectively integrate autophagy-targeting strategies in cancer treatment.

Keywords: *autophagy, cytoprotective, cytotoxic, cellular context, cancer .*

1. Introduction

Autophagy is a cellular process that is responsible for maintaining cellular homeostasis by degrading and recycling the intracellular components. It has been preserved throughout evolution [1]. The term autophagy is derived from two Greek words, "auto" (self) and "phage" (eating), which allows cells to remove damaged organelles, misfolded proteins, and infections to protect their cellular integrity and survival [2]. The process begins with the formation of the phagophore, which is a double membrane vesicle that engulfs the cytoplasmic debris until maturing into an autophagosome. Subsequently, the autophagosome fuses with hydrolytic lysosome enzymes that break down the engulfed content and release macromolecule building blocks to be reused by the cell. Control of autophagic activity in response to food availability and cellular stress comes from autophagy-related (ATG) genes and signaling pathways, including mTOR and AMP-activated protein kinase (AMPK) [3]. The beginning of the study of autophagy stretches back to the 1950s and 1960s when Christian de Duve discovered that intracellular breakdown is done by the lysosomes. Hereafter, the word "autophagy" was created in 1963, but its importance in cellular homeostasis was not completely understood until the 1990s when Yoshinori Ohsumi discovered ATG genes in yeast, which earned him the 2016 Nobel Prize in Physiology or Medicine [4]. Recently, autophagy has been associated with various physiological and pathological processes, including immunological modulation, neurodegeneration, and, most importantly, cancer. Recent findings

have increased our knowledge of autophagy's involvement in cellular metabolism, aging, and disease development, emphasizing its potential as a therapeutic target [1, 5]. Autophagy is well known for its controversial role in cancer, as it acts by dual function, and it may operate as a tumor suppressor or promoter depending on the cellular context. Autophagy protects normal cells by eliminating damaged organelles and reducing genetic instability, which inhibits tumorigenesis. However, in some tumors, cancer cells may adapt cytoprotective autophagy to evade apoptosis, increase cell survival under metabolic stress, and develop drug resistance [6]. The dual function of autophagy complicates therapy attempts as, depending on the kind and stage of cancer, both repression and activation have benefits. Designing effective cancer treatments depends on understanding the processes behind cytoprotective rather than cytotoxic autophagy [7]. Autophagy has a significant impact on cancer treatment and drug resistance. Many cancers use autophagy to adapt to harsh microenvironmental circumstances, including hypoxia and food deprivation, which help cell growth and survival. Moreover, autophagy helps chemoresistance by boosting cytotoxic agent breakdown and inhibiting drug-induced apoptosis [8]. This has led to using autophagy inhibitors in clinical trials, such as hydroxychloroquine, as adjuvant chemotherapy to enhance the effect of the treatment [9]. In contrast, in malignancies, when autophagy is reduced, reactivating autophagy may restore normal cellular functions and trigger tumor cell death [10]. Small-molecule autophagy activators, such as rapamycin and metformin, have been shown in certain studies to have anti-cancer characteristics by increasing autophagy-mediated cell death and decreasing resistance to treatment. Given the context-dependent nature of autophagy in cancer, targeting autophagic pathways offers a viable route for precision medicine techniques [7]. This review attempts to clarify the molecular mechanisms controlling autophagy by means of key regulatory pathways and signaling networks, differentiate between the conditions under which autophagy displays cytoprotective rather than cytotoxic activities in cancer, and investigate therapeutic strategies targeting autophagy, including both inhibitors and activators, to enhance the outcome of cancer treatment.

1. Types of Autophagy

Autophagy has three forms: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA), all of which depend on lysosomal degradation [11]. Macroautophagy is the most studied form that involves the formation of autophagosomes to sequester damaged organelles, misfolded proteins, and pathogens. Then, the autophagosome is fused with the lysosome for content degradation. This form of autophagy is regulated by mTOR and AMPK signaling and is important for cancer suppression, neuroprotection, and immune function. Any dysregulation with macroautophagy is always associated with neurodegenerative diseases and cancer [1]. The microautophagy directly engulfs the small cytoplasmic portions with the aid of lysosomes without autophagosome formation. It is responsible for organelle turnover, lipid metabolism, and protein homeostasis, with roles in aging, metabolic disorders, and neurodegeneration [12]. On the other hand, chaperone-mediated autophagy is a specific mode of lipophagy that begins with the recognition of a conserved KFERQ peptide sequence in the target proteins, LD coat proteins, including PLIN2 and PLIN3, which are demonstrated to be substrates of this pathway. Specifically, cargos to be degraded are recognized by a chaperone, Hsc70. Hsc70 thereby links these cargo molecules to a lysosomal structural protein called LAMP2A. It is vital for protein quality control, oxidative stress response, and metabolic regulation, with implications in aging, cancer, and neurodegenerative diseases [13]. Although different, these autophagy mechanisms are constantly controlled depending on cellular demands and are linked, so their regulation has immense potential as a therapeutic approach for disorders like cancer and neurodegeneration. Therefore, their crosstalk is an important focus for further studies.

2. Molecular mechanism of autophagy

Autophagy's molecular mechanism is a process controlled by many proteins and signaling pathways that guarantee exact execution and control by means of several consecutive phases. The induction phase of autophagy is the one in which cellular stress or nutrient deprivation inhibits the mTOR complex 1 (mTORC1) [14]. The inhibition of mTOR activates Unc-51-like autophagy, which further activates the kinase 1 (ULK1) complex, which includes ULK1, ATG13, FIP200, and ATG101. After activation of the ULK1 complex, the nucleation phase is initiated, recruiting ATG proteins to form the pre-autophagosomal structure (PAS) [15]. Nucleation is facilitated by the class III PI3K complex, consisting of VPS34, Beclin-1, VPS15, and ATG14L. These complexes form phosphatidylinositol 3-phosphate (PI3P) at the PAS, which leads to

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the accumulation of ATG proteins necessary for the elongation of the autophagosome membrane [16]. Membrane elongation and expansion depend critically on the ATG12-ATG5-ATG16L1 complex and microtubule-associated protein one light chain 3 (LC3). LC3-II is formed by conjugating phosphatidylethanolamine with LC3-II, which hooks the autophagosomal membrane to permit cytoplasmic cargo to be engulfed [17]. Some adaptor proteins like p62/SQSTM1, NBR1, and optineurin will bind to ubiquitinated cargo and LC3 to guarantee the selective destruction of certain cellular components [18]. Linking ubiquitinated proteins to the autophagic machinery for destruction and acting in the nuclear factor-kappa B (NF-κB) pathway, P62 functions both in autophagy and signaling pathways [19].

Autophagosome closure involves additional ATG proteins and membrane fusion events, leading to a mature autophagosome formation. Then, the lysosomes are fused with the autophagosome; this fusion process is regulated by SNARE proteins such as syntaxin 17, vesicle-associated membrane protein 8, and synaptosomal-associated protein 29. Consequently, the autolysosome is formed after the fusion, where lysosomal hydrolases degrade the autophagic cargo [20]. Autophagy is regulated by miRNA-34 by targeting ATG, which induces the expression and function of proteins involved in autophagic pathways [21]. P2X7 receptor also stimulates autophagy by increasing calcium influx and thereby activating downstream signaling pathways [22]. Interacting with Beclin-1, Bcl-2 is an anti-apoptotic protein that reduces autophagy under normal settings. BH3-only proteins may replace Beclin-1 from Bcl-2 under stress, thereby enabling autophagy [23]. Additionally, autophagy is activated when the AMPK pathway inhibits mTORC1 and directly phosphorylates ULK1 [24]. The JNK pathway can also regulate autophagy by phosphorylating Bcl-2, disrupting its interaction with Beclin-1 [25].

Overall, autophagy regulation is a complex process involving interactions between molecules and interplay of many signaling pathways. Understanding these mechanisms, including the roles of miRNA-34, P2X7, p62, Bcl-2, AMPK, and JNK, is important to develop new therapeutic strategies to modulate autophagy in diseases such as cancer, where autophagy can play both protective and detrimental roles depending on the context.

3. *Functional Roles of Autophagy: Cellular Homeostasis, Organelle Turnover, and Response to Stress*

Autophagy maintains cellular homeostasis through the degradation and recycling of damaged organelles, misfolded proteins, and any toxic content. This process is crucial to preserve cellular integrity and prevent dysfunction, especially in metabolically active tissues like neurons, muscles, and the liver [26]. However, autophagy is responsible for removing aged proteins and defective cytoplasmic content; its malfunction leads to aging and cancer progression, making it a promising therapeutic target [6].

One of the major functions of autophagy is organelle turnover to ensure the removal of dysfunctional organelles such as mitochondria, peroxisomes, and the endoplasmic reticulum (ER) [1]. For example, mitophagy is the selective clearance of damaged mitochondria, which further prevents oxidative stress and inflammation and preserves redox homeostasis to maintain metabolic balance [27]. Similarly, reticulophagy is responsible for removing damaged ER segments and preventing ER stress, which is a key factor in Alzheimer's and Parkinson's [28].

Furthermore, autophagy acts as a first-line defense when the cell is exposed to nutrient deprivation, hypoxia, infection, and oxidative stress. Under these conditions, it provides an alternative energy source by repurposing the intracellular macromolecules for metabolism. It also gets rid of oxidized proteins and damaged mitochondria to prevent excessive ROS accumulation and reduce age-related disorders [29]. However, excessive autophagy for a prolonged duration of stress may result in autophagy-dependent cell death (ADCD) and is known as cytotoxic autophagy, which highlights its dual role in cell survival and disease [30].

The balance between cytoprotective and cytotoxic autophagy relies on cellular context, activation intensity, and environmental conditions [31]. Understanding its molecular mechanisms will allow for precise therapeutic modulation and offer potential treatments for neurodegeneration, cancer, metabolic disorders, and aging-related diseases.

4. Role of autophagy in tumorigenesis

4.1. Tumor-Suppressive Role of Autophagy in Early Tumorigenesis

In the early stages of tumorigenesis, autophagy acts as a tumor suppressor by maintaining cellular homeostasis and genomic integrity by removal of damaged organelles and misfolded proteins to prevent the release of ROS and genomic instability, which may lead to carcinogenesis [32]. Previous studies highlight the role of autophagy as a preventive factor of tumorigenesis by the elimination of precancerous cells and inhibiting oncogenic mutations. For example, in mouse models, deletion of ATGs, including ATG5, ATG7, and Beclin-1, has been found to speed carcinogenesis, especially in liver and breast cancer models [33], thereby highlighting the protective function of autophagy in early cancer suppression.

Moreover, as discussed, autophagy uses a selective mechanism called mitophagy to break down defective mitochondria, hence promoting metabolic balance. Early tumor formation is characterized by mitochondrial dysfunction as it causes metabolic reprogramming and increases oncogenic signaling. By preventing mitochondrial dysfunction, autophagy inhibits cancer progression [27]. Furthermore, autophagy is known to regulate oncogene-induced senescence (OIS), which is a tumor-suppressive mechanism that decreases the proliferation of cells carrying oncogenic mutations. This results in inhibiting the escape of precancerous cells for senescence and reducing malignant transformation [34].

Autophagy also has an immune function that is involved in tumor suppression through intracellular pathogen degradation and antigen presentation. Autophagy also enhances the ability of various immune cells, such as natural killer cells and cytotoxic T cells, to recognize and eliminate early tumor cells. The failure of autophagy-mediated immune surveillance allows pre-malignant cells to evade detection and progress to more aggressive tumor phenotypes [35]. This highlights the importance of autophagy in preventing tumor initiation by integrating metabolic regulation, immune response, and genomic stability.

4.2. Tumor-Promoting Role of Autophagy in Established Cancers

Autophagy, in some cases, shifts from a cytoprotective form into a cytotoxic one as the tumor progresses, which promotes the transformation of normal cells to cancerous ones and increases therapy resistance. When cancer cells are exposed to a hypoxic and nutrition–deprived environment, autophagy will degrade the cellular components to sustain the tumor growth and survival, as seen in pancreatic, lung, and colorectal cancers [36]. Furthermore, the poor prognosis of cancer and treatment resistance is proved by high autophagic flux.

Autophagy provides the cancer cell with an alternative energy source through the AMPK/mTOR pathway to ensure tumor cell progression and proliferation [37]. Autophagy also helps cancer cells to evade immune cells by degrading damage-associated molecular patterns (DAMPs) and inhibiting type I interferon signaling, subsequently preventing effective immune responses [38].

Furthermore, autophagy is widely known nowadays as a tumor protector because it increases chemotherapy resistance and protects tumor cells from radiation and targeted therapy through its cleaning machinery process. Previous studies in glioblastoma and breast cancer have shown that chemotherapy-induced autophagy prevents apoptosis and reduces treatment efficacy [39, 40]. Some autophagy inhibitors like chloroquine (CQ) and hydroxychloroquine (HCQ) are used to increase cancer sensitivity to therapy, though their effectiveness varies by tumor type and stage [41].

5. Molecular Pathways Involved in Cancer-Associated Autophagy: PI3K/AKT/mTOR, p53, and Hypoxia-Inducible Factors

Autophagy in cancer is regulated by various signaling pathways such as PI3K/AKT/mTOR, p53, and HIF-1α pathways that play a crucial role in tumor progression, cell survival, and drug resistance [7]. Recent studies demonstrated that these pathways are promising targets for cancer therapy.

The activation PI3K/AKT/mTOR pathway upon decreasing cell nutrients, inhibits autophagy by phosphorylating ULK1/2 and further prevents autophagosome formation [42]. This pathway is highly activated in breast [43], prostate [44], and lung cancers [45], which inhibits autophagy and supports tumor growth and therapy resistance.

However, mTOR inhibitors like rapamycin, everolimus induce autophagy, which can either increase cancer cells sensitivity to chemotherapy or enhance their survival by providing metabolic flexibility [46]. Combining mTOR inhibitors with

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autophagy inhibitors like CQ is now considered an inducer of cancer cell death [47].

P53 has dual roles in autophagy. Nuclear p53 acts as an autophagy inducer through activation of DRAM1 and SESN2, suppressing mTOR and preventing genomic instability, which will suppress tumor growth. Conversely, cytoplasmic p53 acts as an autophagy inhibitor and promotes tumor growth and survival, furthermore increasing therapy resistance as seen in glioblastoma and pancreatic cancers [48].

More than 50% of cancers develop p53 mutations that lead to dysregulation in autophagy and increasing tumor growth [49]. Therefore, therapies like PRIMA-1 and APR-246 aim to restore p53-dependent autophagy, promoting tumor cell death [50].

In solid tumor, hypoxia is known to stabilize HIF-1α, which will induce autophagy via BNIP3/BNIP3L and further inhibit mTOR and enable tumor adaptation. HIF-1α-driven autophagy enhances metabolic reprogramming (Warburg effect) and therapy resistance, as demonstrated in glioblastomas and pancreatic and liver cancers [51].

Digoxin, acriflavine, and PX-478 are HIF-1α inhibitors and are used to block hypoxia-induced autophagy and enhance chemotherapy and radiation sensitivity [52]. Combining HIF-1α and autophagy inhibitors represents a promising anti-cancer strategy.

6. *Autophagy and Cancer Metabolism: Adaptation to Metabolic Stress*

Autophagy is one of the cell machinery processes that help cancer cells to adapt to nutrient deprivation, hypoxia, oxidative stress, and chemotherapy by recycling the intracellular components to sustain metabolism. Through playing a dual role, autophagy supports tumor survival in stressed microenvironments while also acting as a potential mechanism of cell death in specific contexts [7].

Autophagy can recycle nutrients under high metabolic stress through the degradation of damaged organelles and lipid droplets to generate energy. One such mechanism is lipophagy, which releases free fatty acids and induces tumor growth in cancers like pancreatic ductal adenocarcinoma (PDAC) and glioblastoma. High autophagic flux in tumors also enhances amino acid homeostasis, which maintains tumor growth even under systemic nutrient depletion [53].

HIF-1α induces BNIP3/BNIP3L-mediated autophagy, known as mitophagy, in case of hypoxia, which prevents the release of ROS and shifts the cell metabolism towards anaerobic glycolysis (Warburg effect) [51]. This adaptation has been shown in lung, breast, and colorectal cancers, and this is believed to support tumor survival and therapy resistance [36].

Finally, autophagy has a significant role in helping chemotherapy-resistant tumors to survive the metabolic stress induced by therapy through maintaining glutamine metabolism and redox balance. It acquires glutaminolysis to sustain the TCA cycle and relieves oxidative damage by destroying dysfunctional mitochondria [54]. The observed active autophagic flux seen in multiple myeloma and ovarian cancer is always associated with drug resistance [55].

7. *Autophagy in Cancer Stem Cells (CSC) and Metastasis*

Autophagy is one of the cell signaling pathways that is essential for CSC survival, therapy resistance, and metastasis, which enables tumors to adapt to stress, escape immune cells, and sustain growth [56]. CSCs use autophagy to survive hypoxic and nutrient-poor environments, which further prevents apoptosis and maintains stemness factors such as SOX2, OCT4, and NANOG. This directs the cell to self-renewal and resistance to chemotherapy and radiotherapy [57]. Previous studies in glioblastoma, colorectal, and pancreatic cancers have shown that targeting ATG5, ATG7, and Beclin-1 could reduce CSC tumor-initiating capacity and enhance chemotherapy sensitivity [58].

Autophagy aids the metastatic cancer cells in surviving harsh oxidative and metabolic stress. Epithelial-to-mesenchymal transition (EMT) is one of the crucial processes for metastasis; it is widely known to be regulated by some transcription factors derived from autophagy, such as ZEB1, SNAIL, and TWIST [59]. Studies indicate that inhibiting autophagy suppresses EMT and reduces metastasis in lung and breast cancer [60].

Autophagy can also adapt to mitochondrial dysfunction by acquiring lipophagy that provides metastatic growth by converting the lipid content into free fatty acids to sustain oxidative phosphorylation in distant organs like the liver and lungs [53].

Furthermore, autophagy degrades MHC

proteins to help circulating tumor cells evade immune destruction, which results in preventing recognition by NK cells and cytotoxic T cells. It also promotes metastatic potential by allowing disseminated tumor cells (DTCs) to survive in a dormant state before transforming into secondary tumors [61]. Therefore, targeting autophagy in latent cells is considered a promising approach to prevent metastasis.

Recently, clinical trials are investigating the role of the combination of autophagy inhibitors such as CQ and HCQ with chemotherapy, radiotherapy, and immunotherapy to inhibit CSC survival and metastasis [62]. Some metabolic strategies using glutaminase and glycolysis inhibitors can target lipophagy and oxidative phosphorylation, which could deprive metastatic cells of essential nutrients [63]. Moreover, autophagy-targeting immunotherapies seek to boost antigen presentation to restore anti-tumor immune responses [61].

8. Determinants of Cytoprotective Versus Cytotoxic Autophagy

8.1. Determinants of Cytoprotective Versus Cytotoxic Autophagy: Intracellular Determinants

P53, Beclin-1, PI3K/AKT/mTOR, AMPK, and stress-responsive pathways are key regulators, which determine whether autophagy will induce tumor suppression or trigger tumor growth [64, 65]. More research is done to investigate those molecular determinants to help in developing promising therapies against autophagy.

8.1.1. p53 and Beclin-1: Key Regulators of Autophagy Fate

Wild-type p53 normally inhibits mTOR and activates DRAM1 and SESN1/2, which in turn enhances cytoprotective autophagy and promotes the degradation of dysfunctional organelles. Consequently, the cell preserves its genomic stability and prevents tumorigenesis. However, mutant p53 inhibits autophagy, promotes tumor survival, and increases drug resistance. At the same time, cytoplasmic p53 prevents the initiation of through interaction with ATG7 and Beclin-1, which further halts autophagosome formation [65]. On the other hand, tumors with p53 deficiency will exhibit a highly active autophagic flux that allows the cancer cells to escape immune cells and develop resistance to chemotherapy [66]. That is why targeting autophagy-mediated cell death in p53-mutant cancers is a promising therapeutic strategy.

Beclin-1 is a key component of the PI3KC3 complex, and it is important for autophagy to initiate and maintain the integrity of the phagophore membrane [67]. In some cancers, Beclin-1 is lost, such as breast, ovarian, and prostate cancers, which will impair autophagy and lead to genomic instability and tumor metastasis [68]. Bcl-2 and Bcl-XL usually inhibit Beclin-1 as they bind and inhibit it, further preventing excessive autophagy. Therefore, recent studies aim to disrupt Bcl-2/Beclin-1 interactions to enhance cytotoxic autophagy, making it a potential therapeutic target [69].

Autophagy-related biomarkers have significant potential in cancer diagnosis, prognosis, and treatment stratification. Among these, LC3-II, p62/SQSTM1, and Beclin-1 are widely studied for their roles in autophagy regulation and their expression patterns in various malignancies. Their levels can reflect autophagy activity and correlate with therapeutic response and clinical outcomes [70]. T**able 1** below summarizes the key autophagy biomarkers, their biological functions, associated tumor types, and their clinical significance in guiding biomarker-driven therapies.

Table 1: Autophagy Biomarkers in Cancer

Biomarker	Biological Function	Associated Cancers	Clinical Relevance
LC3-II	The marker of autophagosome formation reflects autophagy flux	Pancreatic, Breast, Colorectal	High levels indicate active autophagy, linked to poor prognosis in pancreatic cancer, a potential predictor of response to autophagy inhibitors. [71]
p62/SQSTM1	Selective autophagy substrate accumulates when autophagy is impaired	Hepatocellular, Colorectal, Lung	Elevated levels correlate with therapy resistance and reduced survival, potential prognostic and predictive biomarkers. [72]
Beclin-1	Initiates autophagosome formation; regulates autophagy initiation	Breast, Glioblastoma, Ovarian	Reduced expression in breast cancer associated with tumor progression; overexpression in glioblastoma linked to chemoresistance. [73]

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The clinical utility of autophagy biomarkers extends beyond their prognostic value, offering significant potential for guiding biomarker-driven cancer therapies. These biomarkers can facilitate patient stratification based on autophagy dependency, predict therapeutic response, and refine personalized treatment approaches. Their integration with genomic and transcriptomic profiling enables a precision oncology framework, supporting the development of tailored autophagy-targeted interventions [74]. The following **table 2** outlines key clinical applications of autophagy biomarkers and examples of their relevance in current therapeutic strategies.

Table 2: Clinical Applications of Autophagy Biomarkers in Biomarker-Driven Cancer Therapy

Clinical Application	Biomarker(s) Involved	Example/Context	Implications
Patient Stratification Based on Autophagy Dependency	LC3-II, p62/SQSTM1	High LC3-II or p62 levels may identify patients suitable for autophagy inhibition therapy.	Enhances treatment personalization; avoids ineffective therapies in autophagy-independent tumors [75]
Predicting Therapeutic Response to Autophagy Inhibitors	LC3-II, p62/SQSTM1	Trials using hydroxychloroquine + chemotherapy stratify patients by autophagy biomarker levels.	Improves therapeutic efficacy; reduces adverse effects from unnecessary drug exposure [76]
Integration with Omics Data for Precision Medicine	LC3-II, Beclin-1, p62 + Genomic Profiling	TCGA data integration correlates biomarker levels with tumor genotype and transcriptome	Enables development of personalized, genotype-specific autophagy-targeted therapies [77]

8.1.2. Signaling Pathways Controlling Cytoprotective vs. Cytotoxic Autophagy

The PI3K/AKT/mTOR pathway is known to inhibit ULK1/2, which inhibits autophagy and promotes cancer cell survival. Hyperactivation of PI3K/AKT in cancer cells shifts autophagy to a cytoprotective form, which helps tumors withstand harsh metabolic stress by recycling intracellular components. However, mTOR inhibitors such as rapamycin, everolimus, or temsirolimus induce cytotoxic autophagy and lead to apoptosis. Therefore, recent studies investigate the combination effect of mTOR inhibitors with autophagy modulators, which may enhance cancer therapy [44].

AMPK is another signaling pathway known as a metabolic stress sensor that activates autophagy by inhibiting mTOR and activating ULK1. Unlike PI3K/AKT, AMPK is considered a cytotoxic autophagic factor inducer, particularly in tumors that depend on glycolysis, as AMPK activation results in the induction of mitophagy, which further decreases ATP production and induces apoptosis in cancer cells [42]. One example of an AMPK activator is metformin, which is combined with autophagy inducers, and recently, this combination has shown a promising therapeutic option to induce autophagy-dependent cancer cell death, particularly in therapy-resistant tumors [78].

Under oxidative stress and DNA damage, JNK and MAPK pathways control autophagy. JNK activation phosphorylates Bcl-2, therefore disturbing its regulation of Beclin-1 and triggering autophagy-mediated cell death [79]. Furthermore, autophagy driven by ER stress via the PERK-eIF2α pathway may cause death in aggressive cancers. A proteasome inhibitor, bortezomib, causes ER stress and excessive autophagy, which results in cell death in many myelomas [80].

8.2. Determinants of Cytoprotective Versus Cytotoxic Autophagy: Extracellular Factors

Autophagy is highly affected by tumor microenvironment conditions such as availability of nutrients, hypoxic conditions, and immune signaling. Studies confirm that tumors acquire autophagy to adapt to high-stress conditions. However, excessive autophagy can shift into a cytotoxic form, which induces cancer cell death under certain conditions [81].

The TME is a composition of cancer cells, stromal fibroblasts, immune cells, and ECM components, which are the determinants of whether the cell will acquire cytoprotective or cytotoxic autophagy. In response to harsh TME conditions, autophagy helps cancer cells maintain cell integrity, genomic stability, metabolic balance, and resist therapy [82]. Cytokines and growth factors such as TGF-β, IL-6, TNF-α, and IL-1β are secreted by stromal cells and tumor-associated macrophages and will upregulate pro-survival autophagy under metabolic stress. However, tumors in the early stages will adapt

autophagy to remove the damaged organelles and maintain the metabolic balance to prevent genetic mutation function as tumor suppressors. In advanced cancer, tumors hijack autophagy for self-survival and therapy resistance; that's why it is considered an attractive therapeutic target [83].

As tumors proliferate, the level of glucose, amino acids, and lipids is decreased, which inhibits mTOR through AMPK activation that results in activation of autophagy and sustained energy homeostasis [84]. Lipophagy is an activated specialized form of autophagy that converts the lipid droplets to free fatty acids to fuel oxidative phosphorylation and support tumor survival and growth under metabolic stress. This is demonstrated in several types of cancer, such as pancreatic and colorectal cancers, which depend mainly on lipid metabolism. However, nutrient deprivation for prolonged durations may lead to excessive autophagy that results in ADCD, further degradation of mitochondria and ER, and the release of ROS and apoptosis [53].

A common characteristic of solid tumors is hypoxia that stabilizes HIF-1α and further activates ATGs (BNIP3, BNIP3L) to induce mitophagy, hence avoiding ROS buildup and facilitating tumor survival under low oxygen conditions. In some types of cancer, such as glioblastoma, hepatocellular carcinoma, and breast cancer, autophagy induced by hypoxia is considered a cytoprotective one and correlates with increased tumor aggression and therapy resistance [36]. However, therapies like radiation therapy can exacerbate hypoxia and shift autophagy to a cytotoxic one, which is favorable in cancer treatment. Targeting HIF-1α or decreasing autophagy induced by hypoxia could enhance radiation therapy efficacy in hypoxia-adaptive tumors [85].

8.3. Determinants of Cytoprotective vs. Cytotoxic Autophagy: Role of Cellular Context and Stressors

Autophagy in cancer is influenced by cellular context as well external stress factors such as chemotherapy, radiotherapy, and oxidative stress. Although tumors often use autophagy as a defense mechanism against therapy and metabolic changes, excessive autophagy activation may result in ADCD. This balance relies on stressor type, duration of autophagy, and tumor genetics [86].

8.3.1. Autophagy in Chemotherapy: Survival vs. Cell Death

Chemotherapy is known to damage DNA and increase the release of ROS, which leads to cell apoptosis. However, cancer cells induce autophagy as a protective mechanism to counteract these effects. Some chemotherapies, such as cisplatin, doxorubicin, and temozolomide, activate p53 and inhibit mTOR, which consequently activate cytoprotective autophagy and develop chemoresistance [87]. For example, ovarian cancer adapts autophagy to resist chemotherapy and promote tumor recurrence, which is demonstrated by increased autophagic flux biomarkers such as Beclin-1 [88]. Cytoprotective autophagy in leukemia, glioblastoma, and pancreatic cancer decreases ATP, which further increases ROS [89]. Recent studies support the use of autophagy inhibitors such as CQ and HCQ to suppress cytoprotective autophagy and improve the effect of chemotherapy [62].

8.3.2. Autophagy in Radiotherapy: A Double-Edged Sword

Radiotherapy is widely used nowadays in early-stage cancer due to its reduced side effects, and it kills tumor cells by inducing DNA damage and oxidative stress induction, but tumors subjected to radiotherapy activate autophagy as a survival mechanism [90]. In glioblastoma, prostate cancer, and hepatocellular carcinoma, the cancer cells induce mitophagy, which helps radio-resistance by removing damaged mitochondria and reducing ROS released from the tumor [91]. Additionally, radiation-induced autophagy promotes CSC survival, increasing therapy resistance. However, excessive autophagic flux can push cells into autophagy-mediated apoptosis [57]. Autophagy inducers (rapamycin, resveratrol) have shown potential in sensitizing tumors to radiation [92]. Recent clinical trials have used autophagy inhibitors as lysosomal inhibitors to prevent DNA repair induced by autophagy and, hence, enhance radiotherapy outcomes [93].

8.3.3. Oxidative Stress and Autophagy: A Critical Balance

ROS affects responsiveness to treatment and tumor development. Moderate ROS levels enable autophagy for survival, therefore enabling tumors to adapt to hypoxia and inflammation [89]. In colorectal and breast cancer, KEAP1-NRF2 is activated to maintain redox homeostasis by autophagy [94]. However, excessive ROS shifts autophagy to a cytotoxic one that is irresistible to antioxidant defenses and degrades mitochondria. Lung, ovarian, and pancreatic tumors, where oxidative stress-

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induced autophagy drives death, have also shown this impact [86]. Recently, some pro-oxidant therapies, such as arsenic trioxide and photodynamic therapy, utilize ROS overload to induce cytotoxic autophagy. The combination of an autophagy inducer and oxidative stress shows a profound effect as an anti-cancer strategy [95].

8.4. Biomarkers for Predicting Autophagic Response: Determinants of Cytoprotective Versus Cytotoxic Autophagy

Biomarkers play a vital role in predicting the fate of autophagy; therefore, it is crucial to identify them for targeted cancer therapy. These biomarkers include ATGs, transcription factors, metabolic markers, and lysosomal activity, which serve as key determinants of autophagic responses [6].

8.4.1. Autophagy-Related Gene (ATG) Expression as Biomarkers

ATGs such as ATG5 and ATG12 are considered autophagy key markers as they are known to regulate autophagosome formation and autophagic flux [96]. In breast and lung cancer, the presence of high levels of ATG12 is often associated with therapy resistance due to cytoprotective adaptations by these cancers [97]. Conversely, ATG5 induces cytotoxic autophagy and is considered a target to increase therapy response. LC3 is a protein that maintains the integrity of the autophagosome membrane, and it is widely used as an autophagic flux marker. Its accumulation is an indicator of enhanced autophagic activity, which is often associated with therapy resistance [98]. However, in the presence of lysosomal inhibitors such as CQ, the increased level of LC3-II may be an indicator of impaired degradation, which further leads to cytotoxic autophagy [99].

8.4.2. Beclin-1 and PI3K/AKT/mTOR Pathway Components

Another biomarker is Beclin-1, which is a key autophagy initiator. Recent studies in breast and ovarian cancers demonstrated a downregulation in Beclin-1 and promoted tumor progression [100]. However, in some cancers, such as glioblastoma and colorectal cancer, the increased level of Beclin-1 is associated with tumor suppression and is considered a biomarker for cytotoxic autophagy [101]. The PI3K/AKT/mTOR pathway is an important one that inhibits autophagy. The elevated levels of phosphorylated AKT (p-AKT) and activation of mTOR are always accompanied by decreased autophagy processes that indicate the tumors suppress autophagy for cytoprotective functions. Low mTOR activity, on the other hand, seen in metabolically stressed tumors, are indicators of enhanced autophagic flux, which depends on context and may either contribute to cancer cell survival or death [44].

8.4.3. Lysosomal Markers and Autophagic Degradation Biomarkers

LAMP1 (Lysosomal Associated Membrane Protein 1) is one of the lysosomal activity's key determinants of autophagic function and is used to assess autophagic degradation. A high level of LAMP1 is associated with an efficient lysosomal function that enhances tumor growth even under stress conditions [102]. However, HCQ, one of the lysosomal inhibitors, can block LAMP1-mediated autophagy, which shifts autophagy to a cytotoxic one [103].

p62 (SQSTM1) is another cargo protein that is decreased by enhanced autophagic processes while its level is increased upon autophagy impairment. The elevated expression level of p62 is an indicator of poor response to autophagy-targeting therapies, while reduced levels of p62 are linked to cancers, which adapt cytotoxic autophagy [104].

8.4.4. Metabolic and Redox Biomarkers for Autophagic Response

Autophagy is intricately linked to tumor metabolism. Glutamine metabolism under control by glutaminase (GLS1) predicts survival driven by autophagy in pancreatic cancer. While GLS1 inhibition with autophagy activation pushes cells toward autophagic death, high GLS1 expression shows autophagy promotes metabolism [105]. Oxidative stress markers such as ROS levels and Nrf2 activation are used as detriments of autophagic response. Increased activation of Nrf2 enhances cancer cells to acquire cytoprotective autophagy; conversely, excessive ROS accumulation promotes autophagy-mediated cell death. Reducing Nrf2 activation in tumors associated with high autophagic activity could shift autophagy toward cytotoxicity and enhance therapy [106].

8.4.5. Autophagy-related miRNAs and Predictive Genetic Markers

Different families of microRNAs (miRNAs) regulate autophagy. For example, the downregulation of miR-148a-3p could enhance autophagic flux and promote chemotherapy resistance in gastric cancer [107]. On the other

hand, some miRNAs, such as miR-101, suppress Beclin-1-mediated autophagy and push tumors toward cytotoxic autophagy [108].

Autophagic fate in ATG genes is influenced by genetic variants. In colorectal cancer, ATG16L1 mutations reduce autophagy, hence increasing sensitivity to treatments that induce autophagy. Also, mutations in ULK1 may disrupt autophagy initiation and potentially make tumors more sensitive to autophagy activators [109].

9. Therapeutic Implications: Autophagy Modulators in Cancer Therapy

Autophagy modulators could be autophagy inhibitors such as CQ and HCQ or autophagy inducers such as rapamycin and everolimus. Both autophagy inhibitors and inducers have gained attention as potential co-adjuvant therapies to traditional cancer therapies. The ability to either prevent or promote autophagy offers a special opportunity to sensitize tumors to chemotherapy, radiation, and immunotherapy, thereby improving the effectiveness of cancer treatment [110].

9.1. Autophagy Inhibitors in Cancer Therapy

CQ and HCQ are used as autophagy inhibitors that prevent lysosomal acidification, which disrupts the last step of autophagic degradation. It works by preventing the fusion of autophagosomes with lysosomes and the formation of autolysosomes, which further leads to this accumulation of defective proteins and organelles and finally shifts the cancer cells to apoptosis [103]. Recent studies in pancreatic cancer, glioblastoma, and non-small cell lung cancer (NSCLC) have demonstrated that when CQ and HCQ are used in combination with chemotherapy, the effect of the treatment increases compared to chemotherapy alone [111]. One such known combination is HCQ and gemcitabine, doxorubicin, and temozolomide, which is used in pancreatic cancer, and this combination has the effect of inhibiting tumor growth by inhibiting the cytoprotective autophagy [112]. Similarly, in GBM, the combination treatment of CQ and temozolomide has been reported to enhance the cytotoxic effects and prevent chemoresistance induced by autophagy [113]. Also, their ability to inhibit autophagy to overcome therapy-induced resistance is well-studied in CSCs. Recent research investigated the effect of CQ to reduce the stemness properties of CSCs, which prevents tumor relapse and metastasis [114]. Furthermore, clinical trials are actively examining CQ and HCQ as adjuvants in combination with chemotherapy and immunotherapy for therapy-resistant cancers. However, CQ and HCQ have some limitations, such as potential toxicity and off-target effects, especially for prolonged treatment duration, including retinopathy and cardiotoxicity [115]. Therefore, more studies are being conducted to develop next-generation autophagy inhibitors with greater specificity, such as Lys05 and dimeric CQ derivatives, which exhibit enhanced potency with reduced toxicity [116].

9.2. Autophagy Inducers in Cancer Therapy

Rapamycin is an mTOR inhibitor. It is one of the most widely studied autophagy inducers in cancer therapy. When mTOR is inhibited by rapamycin, autophagy is enhanced, which may lead to cancer suppression and increase the treatment response when used in combination with antitumor therapies [46]. Recent studies done on breast cancer, renal cell carcinoma, and glioblastoma have investigated the effect of other mTOR inhibitors, such as everolimus, temsirolimus, and sirolimus, to determine their anti-tumor effect [117]. Rapamycin is known to suppress tumor growth through the induction of cytotoxic autophagy, especially in cancer with hyperactive PI3K/AKT/mTOR signaling. Research shows that rapamycin restores autophagic flux in PTEN-deficient cancers, which show constitutive mTOR activity, therefore suppressing the tumors [118]. Triple-negative breast cancer (TNBC) has very clearly shown this impact where PI3K/mTOR pathway dysregulation is prevalent [119]. Another novel strategy is to combine autophagy inducers with apoptosis inducers to promote cancer cells to adapt to cytotoxic autophagy. For example, in leukemia and lymphoma, studies have demonstrated that rapamycin has a synergistic effect with Bcl-2 inhibitors, such as venetoclax, to enhance cell death autophagy. This strategy is justified by the fact that autophagy stimulation may provide metabolic stress, which drives cancer cells into irreversible cell death pathways [120].

However, using rapamycin and its analogs as autophagy inducers is their context-dependent effects is still a major challenge because in some cancers, such as glioblastoma, the prolonged use of rapamycin could induce adaptive resistance mechanisms, where tumors use autophagy to prevent themselves from dying [121].

9.3. Therapeutic Implications: Combination Therapies Targeting Autophagy

Recently, studies demonstrated that combination of different classes of autophagy modulator could enhance the therapeutic

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outcome, which has been reported in different types of cancer as shown in table 3.

Table 3: Combination Strategies Targeting Autophagy to Enhance Cancer Therapy

Combination Therapy	Strategy	Key Findings	Cancer Types
Autophagy Inhibition + Chemotherapy	Blocking autophagic degradation to enhance chemosensitivity	- CQ and HCQ block lysosomal degradation, sensitizing tumors to chemotherapy [122]. - HCQ + gemcitabine/nab-paclitaxel improves survival in pancreatic cancer [123]. - CQ + temozolomide enhances chemotherapy response in glioblastoma [124]. - CQ reduces CSC survival in breast and colorectal cancers, preventing relapse [114].	Breast cancer [114, 122], Pancreatic cancer [123], Glioblastoma [124], Colorectal cancer [114]
Autophagy Induction + Chemotherapy	Inducing autophagic apoptosis to increase DNA-damaging agent efficacy	- mTOR inhibitors (rapamycin, everolimus) shift cells into autophagic apoptosis [125]. - In PTEN-deficient prostate and breast cancers, rapamycin + etoposide/cisplatin enhances tumor suppression [126, 127].	PTEN-deficient prostate cancer [125, 126], Breast cancer [127]
Autophagy Inhibition + Immunotherapy	Inhibiting autophagy to improve immune checkpoint blockade (ICB) response	- CQ + anti-PD-1 therapy increases CD8+ T-cell infiltration in TNBC, improving immune response [128]. - CQ and HCQ stimulate cytotoxic T cells to recognize tumors in lung cancer and melanoma [41]. - Autophagy inhibition in Tregs and MDSCs reduces tumor immune suppression, enhancing PD-1 and CTLA-4 blockade responses [129].	TNBC [128], Melanoma [41, 129], Lung cancer [129]

10. Challenges and Future Directions in Autophagy-Targeting Cancer Therapy

Recently, autophagy has become an essential target in cancer therapy, which can help in tumor suppression and decreasing treatment resistance. Although there are many promising preclinical and clinical findings, several major challenges remain in translating autophagy-targeting strategies into effective personalized cancer treatments. The most common challenge is overcoming autophagy-induced resistance and developing individualized therapy tailored to the genetic profile of the specific tumor. Therefore, recent studies highlight the need for developing novel strategies to modulate autophagy while addressing therapy resistance and inter-patient variability [130].

10.1. Overcoming Resistance to Autophagy-Targeting Therapies

10.1.1. Adaptive Resistance to Autophagy Inhibition

Tumors always use the possibility of switching in the autophagy pathway to switch between cytoprotective and cytotoxic autophagy. This phenomenon has been investigated in tumors that are subjected to prolonged durations of autophagic inhibition, leading to exhibiting a compensatory metabolic reprogramming, which allows cancer cells to survive by stimulating other nutrient scavenging pathways such as macropinocytosis and mitochondrial biogenesis [1, 3, 6].

For example, in PDAC, where autophagy is a vital survival strategy, extended suppression with either HCQ or CQ has been demonstrated to induce metabolic changes that maintain tumor development despite autophagy inhibition [131]. Likewise, chronic autophagy suppression increases mitochondrial oxidative phosphorylation in GBM, therefore enabling tumor cells to survive in nutrient-deprived environments [121].

To overcome this adaptive resistance, some recent researchers investigated the dual-targeting strategies that combine autophagy inhibition with metabolic inhibitors, such as the combination of autophagy inhibitors like CQ or HCQ with glycolysis inhibitors (2-deoxyglucose). This combination has been shown to have a better effect against tumors more effectively than monotherapy [132]. Another combination that targets both autophagy and mitochondrial function, such as metformin + HCQ, has been proposed as a strategy to prevent metabolic compensation [133].

10.1.2. Overcoming Resistance to Autophagy Induction Therapies

While autophagy induction has been investigated as a means of inducing tumor cell death, certain cancer cells acquire resistance by altering important survival pathways. One of the main obstacles is that tumors with intact PI3K/AKT/

mTOR signaling generally restrict the efficacy of autophagy inducers such as rapamycin and everolimus by suppressing autophagy induction [43]. Recent research suggests that tumors that develop mTOR-resistant need additional combination approaches, such as combining autophagy inducers with proteasome inhibitors (rapamycin + bortezomib), which could switch the autophagy pathway to a cytotoxic one as seen in hematological malignancies [134]. Another strategy is combining autophagy inducers with checkpoint inhibitors that may enhance T-cell activation and tumor cell recognition [128]. Additionally, heterogeneity plays a key role in therapy resistance. For example, different subpopulations within a tumor may show various levels of autophagic activity. For instance, single-cell RNA sequencing studies have demonstrated that some cells within TNBC depend mainly on autophagy for survival and growth, while other cells demonstrate autophagy suppression [135]. This highlights the need for individualized treatment approaches that consider tumor heterogeneity when designing targeted therapies against autophagy.

10.2. Developing Patient-Specific Autophagy-Targeting Therapies

10.2.1. Biomarker-Driven Personalized Treatment Approaches

The development of biomarker-driven, patient-specific treatment plans is one of the main potential paths in autophagy-targeting therapy. Given the double function of autophagy in cancer, it is essential to predict whether a patient's tumor will react to autophagy suppression or autophagy stimulation. Insight into autophagic flow and tumor dependence on autophagy may come from biomarkers such as LC3-II/LC3-I ratios, p62/SQSTM1 levels, Beclin-1 expression, and lysosomal activity indicators. Since cancers with high LC3-II and low p62 expression are often autophagy-dependent, indicating that autophagy suppression might be a more effective treatment strategy [136]. Conversely, cancers with defective autophagy with low Beclin-1 expression may benefit from autophagy-inducing treatments such as caloric restriction mimics or mTOR inhibitors [137].

Future studies should concentrate on creating real-time imaging tools to evaluate tumor autophagy levels, therefore enabling tailored, real-time changes to treatment plans.

10.2.2. Integrating Artificial Intelligence (AI) and Precision Medicine in Autophagy Therapy

Recently, with the evolution of precision oncology, machine learning, and AI-driven approaches are being evaluated to predict autophagy dependence in tumors and optimize treatment regimens. AI-driven models can integrate multi-omics data such as genomics, proteomics, and metabolomics, which help in classifying tumors based on autophagy signatures, which further predict whether inhibition or induction of autophagy would have a better effect [138]. Hence, identifying novel drug combinations tailored to specific autophagic responses that reduce trial-and-error in clinical settings. For example, an AI-driven model published recently is used to analyze more than 10,000 tumor samples to predict which tumors are most likely to develop resistance to autophagy inhibitors [139]. This approach further guides clinical trial design and patient selection.

11. Future Directions: Expanding Autophagy-Targeting Approaches in Clinical Trials

Currently, several autophagy-targeting therapies are being evaluated in clinical trials; for instance, phase II trials use CQ and HCQ as adjuvant therapy in pancreatic cancer, glioblastoma, and NSCLC [115] and determine the effect of combinations of rapamycin with chemotherapy and immunotherapy in triple-negative breast cancer and melanoma [41, 128]. Several novel agents, such as HCQ derivatives (e.g., Lys05) and specific VPS34 inhibitors (e.g., SAR405), are under investigation in preclinical and early-phase clinical trials, aiming to selectively inhibit autophagy in tumor cells while minimizing systemic toxicity [140]. Many areas still need further research despite these developments. Optimizing The ideal timing and dose techniques for combining autophagy modulators with chemotherapy, radiation, or immunotherapy must be found via further study. Also, reducing off-target effects requires the development of new autophagy modulators with enhanced selectivity and less toxicity. Therapies should be targeted to tumor microenvironmental circumstances, as hypoxia, immune cell penetration, and metabolic stress affect autophagy. Finally, future clinical studies should stratify patients depending on autophagy-related indicators so that treatments are administered only in malignancies where autophagy targeting is probably advantageous.

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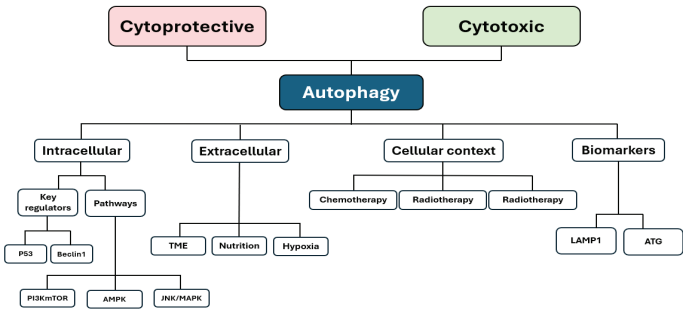

Graphical abstract

Data Availability

This study did not perform any data

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Modeling Schizophrenia Endophenotypes In Drosophila Melanogaster: Effects Of Ketamine On Anxiety, Aggression, Locomotion And Inflammatory Responses

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ABSTRACT:

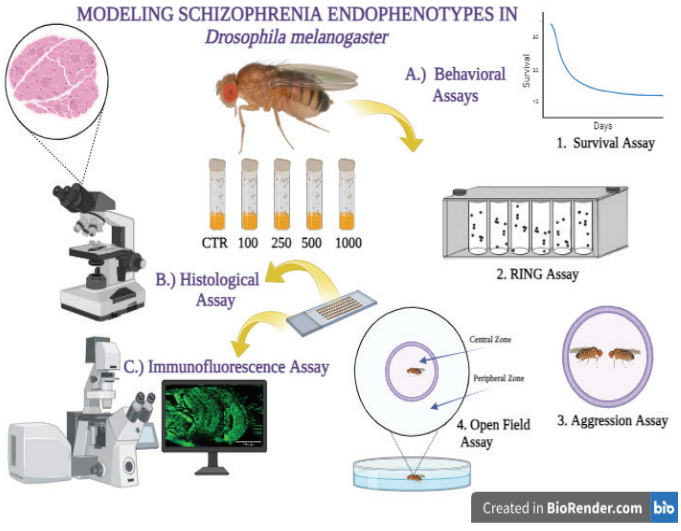
Schizophrenia presents a significant challenge in mental health, characterized by a profound distortion of reality, often accompanied by hallucinations, delusions, cognitive deficits, and neuroinflammatory processes. Ketamine has been widely used as a pharmacological agent to model schizophrenia symptoms in both human and animal studies. However, the potential of ketamine to induce schizophrenia-like phenotypes in Drosophila melanogaster remains under investigated. This study, therefore, investigated the effects of ketamine on anxiety, aggression, locomotor activities, and inflammatory response in Drosophila melanogaster as a preliminary step toward developing a pharmacological model of schizophrenia in this organism.

Virgin male and female Oregon-R flies were collected after eclosion and exposed to four different concentrations of ketamine (100, 250, 500, 1000 µg/mL) for 1 week under standard laboratory conditions (22-25°C, 50-60% humidity). Experimental groups consisted of 10 vials, each containing 10 flies. Anxiety, aggression, and locomotory functions were assessed behaviorally through the open field, aggression, and rapid iterative negative geotaxis (RING) assays. Pro-inflammatory and astrogliotic responses were measured immunohistochemically using Tumor necrosis factor-alpha (TNF-α) and Glial fibrillary acidic protein (GFAP) antibodies. The general neuronal architecture was evaluated using the H&E histological staining techniques.

The results showed a dose-dependent induction of aggressive behavior. Motor function assays demonstrated that ketamine impaired these functions in a dose-dependent manner. Survival assays indicated that higher doses of ketamine reduced survival rates. Immunohistochemical analysis revealed a dose-dependent increase in TNF-α and GFAP mean fluorescence intensity across the treatment groups, indicating upregulation of TNF-α and GFAP expressions. This suggests a robust pro-inflammatory and astrogliotic response to ketamine administration, aligning with the emerging neuroinflammatory endophenotype theory of schizophrenia aetiology and its experimental modeling. Histological analysis displayed significant dose-dependent histopathological changes, including increased cell loss and vacuolization at higher ketamine concentrations.

In conclusion, the findings suggest that ketamine has potential as a pharmacological model of schizophrenia in Drosophila. Overall, these results contribute to the understanding of how ketamine influences key behavioral and neurobiological parameters, offering insights into their potential roles in inducing schizophrenia-like phenotypes like altered behavior and histopathology.

Keywords: *Ketamine, Drosophila melanogaster, Neuroinflammation, TNF-α, Schizophrenia, GFAP.*



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1. Introduction

Schizophrenia is a severe mental disorder affecting approximately 1% of the global population, significantly impacting individuals, families, and healthcare systems. It is characterized by positive symptoms (hallucinations, delusions), negative symptoms (social withdrawal, avolition), and cognitive impairments, leading to substantial functional disability and reduced quality of life (1). Current treatments often fail to address the full spectrum of symptoms, especially cognitive and negative symptoms, highlighting the need for novel therapeutic strategies (2).

Ketamine, an NMDA receptor antagonist initially developed as an anesthetic, has gained attention for its psychotomimetic effects and rapid antidepressant properties (3). Its use as a schizophrenia model is due to its ability to induce psychotic-like symptoms and cognitive impairments in healthy individuals, closely resembling those seen in schizophrenia patients (4). Acute ketamine administration produces positive symptoms (perceptual alterations, thought disorder), negative symptoms (blunted affect, social withdrawal), and cognitive deficits in attention, working memory, and executive function. These effects are linked to ketamine's disruption of glutamatergic neurotransmission, aligning with the glutamate hypothesis of schizophrenia (5).

Neuroinflammation has recently emerged as a critical factor in schizophrenia pathophysiology, providing new insights into its etiology and treatment (6). It involves an immune response within the central nervous system, marked by the activation of microglia and astrocytes and the release of pro-inflammatory cytokines (7). Studies have shown elevated inflammatory markers in the blood and cerebrospinal fluid of schizophrenia patients (8), and neuroimaging reveals increased microglial activation in several brain regions (9). GFAP, a key marker for astrocytes, provides insight into neuroinflammatory responses and potential neuropathological states (10). TNF is a critical mediator of neuroinflammation, often exacerbating neurodegeneration, synaptic dysfunction, and cognitive impairments (11).

Drosophila melanogaster is a valuable model organism for studying complex neurological disorders. Despite its simplicity, it shares many molecular and cellular mechanisms with humans, making it a powerful tool for neuroscience research (12). Its advantages include a short lifespan, rapid generation time, and advanced genetic tools. Drosophila also exhibits complex behaviors, such as learning, memory, circadian rhythms, and social interactions, which can be used to model neuropsychiatric disorders and screen for therapeutic compounds (13)autism spectrum disorders and Down syndrome, among others. They are characterized by limitations in adaptive and social behaviors, as well as intellectual disability (ID. While ketamine's effects are well-studied in mammalian models, its impact on Drosophila's cognitive function and neuroinflammatory responses remains underexplored, limiting the fly's utility in schizophrenia research. This study thus investigated the effects of ketamine on anxiety, aggression, and locomotory functions through the open field, aggression chamber, and rapid iterative negative geotaxis (RING) assays, lifespan, neuroinflammatory responses, and neuroarchitecture in Drosophila melanogaster. These findings contribute to our understanding of ketamine's effects on Drosophila and support its use as a model for schizophrenia research.

2. METHODS

Acquisition and Breeding of Flies

Wild-type *Drosophila melanogaster* (Oregon-R strains) were obtained from the Group for Biopsychiatry Research and Innovative Neuroscience at the Department of Anatomy, Olabisi Onabanjo University, Ogun State. The culture and feeding medium, primarily corn-meal-based, was hygienically prepared according to the protocols adopted by (14). The flies were maintained under standard laboratory conditions (22-25°C, 50-60% humidity) with natural day/night cycles in the animal holding facility of the Department of Anatomy at Olabisi Onabanjo University, Sagamu, Nigeria. Ketamine HCl injection (50 mg/ml) was sourced from Jawa® Group in Lagos, Nigeria.

Experimental Design and Dosing

The experimental design for the research included the following groups: a control group, which was maintained on standard fly food ad libitum, and a ketamine-administered group, which received varying concentrations of ketamine hydrochloride (100, 250, 500, and 1000 µg/mL) mixed into the fly food and allowed to feed ad libitum. The dosage of ketamine used in this study was informed by Koksal and Gürbüzel's (2020)(15) study, which provided a foundational understanding of ketamine's effects on *Drosophila*, identifying dosage ranges that elicit significant biological responses

without causing immediate toxicity.

Each vial contained 5 mL of the prepared feed dispensed into standard vials, with the appropriate volumes of ketamine added shortly after cooking the feed, prior to dispensing into the treatment vials. Each experimental group was replicated in ten vials, with each vial containing ten flies. All treatments were administered continuously for one week, and the flies were monitored daily.

Table: Experimental Design

Groups	Regimen	No flies/ vial
Control	Standard fly food	10
KT1	100 µg/mL Ketamine in fly food	10
KT2	250 µg/mL Ketamine in fly food	10
KT3	500 µg/mL Ketamine in fly food	10
KT4	1000 µg/mL Ketamine in fly food	10

Behavioural Assays

1. Survival Assay

Newly eclosed flies were transferred into culture vials containing different concentrations of ketamine mixed into the diet and vials without ketamine for the control with ten flies per vial. The number of dead flies was recorded at regular intervals of three days (16)leading to reduced physical performance and increased risk of disease. Individual aging is manifest at the population level as an increase in age-dependent mortality, which is often measured in the laboratory by observing lifespan in large cohorts of age-matched individuals. Experiments that seek to quantify the extent to which genetic or environmental manipulations impact lifespan in simple model organisms have been remarkably successful for understanding the aspects of aging that are conserved across taxa and for inspiring new strategies for extending lifespan and preventing age-associated disease in mammals. The vinegar fly, Drosophila melanogaster, is an attractive model organism for studying the mechanisms of aging due to its relatively short lifespan, convenient husbandry, and facile genetics. However, demographic measures of aging, including age-specific survival and mortality, are extraordinarily susceptible to even minor variations in experimental design and environment, and the maintenance of strict laboratory practices for the duration of aging experiments is required. These considerations, together with the need to practice careful control of genetic background, are essential for generating robust measurements. Indeed, there are many notable controversies surrounding inference from longevity experiments in yeast, worms, flies and mice that have been traced to environmental or genetic artifacts(1-4. The study examined ketamine's effects on the survival of *Drosophila melanogaster*, with daily monitoring of treatment groups consisting of 50 flies per group, separated by sex.

2. Rapid Iterative Negative Geotaxis (RING) Assay

Ten flies from each treatment vial were introduced into a climbing apparatus consisting of vertical columns made from high-density polyethylene (15 cm in length and 2 cm in diameter) on the final day of administration. After being anesthetized with mild ice, the flies were allowed to recover for one hour. A white background with horizontal lines spaced 1 cm apart was positioned behind the vials. The flies were gently tapped to the bottom of the column by lightly banging it on the assay platform. The number of flies that reached the 8 cm mark within ten seconds was recorded. Each assay was conducted five times for the same group, with a one-minute rest period between trials. The score for each trial was calculated as the average of five measurements (17).

Figure 1: RING Assay Apparatus

Schematic representation of the Rapid Iterative Negative Geotaxis (RING) assay used to assess locomotor function in Drosophila. The setup consists of vertical columns (15 cm in length, 2 cm in diameter) with a white background marked with horizontal lines at 1 cm intervals. Flies are tapped to the bottom, and their climbing ability is measured by recording the number of flies reaching the 8 cm mark within 10 seconds.

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3. Open Field Assay set up

The open field assay provides valuable insights into anxiety-like behaviors and exploratory drive across treatment groups. The open field arena was set up by placing a 10 cm diameter Petri dish on a circular arena drawn on paper, divided into a central zone (50% of the total area) and a peripheral zone (50% of the total area), with a grid overlay for distance measurement and further divided into four quadrants. A video camera was positioned directly above the arena for full visibility.

Flies were acclimatized to the testing room conditions for at least one hour before the assay. A single fly was gently aspirated into the center of the arena without anesthesia to avoid altering its behavior. Video recording began immediately, allowing the fly to explore the arena for ten minutes. After each trial, the fly was removed, and the arena was cleaned with 70% ethanol to eliminate olfactory cues. This process was repeated for all experimental groups, alternating between control and treatment groups to minimize time-of-day effects. The recorded videos were analyzed for parameters including time spent in the central and peripheral zones and frequency of zone crossings (18).

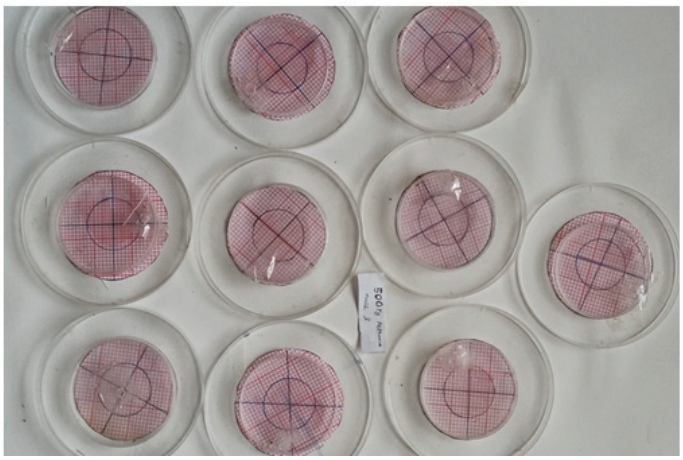

Figure 2: Open Field Assay Setup

Diagram of the open field assay setup used to analyze anxiety-like behavior and exploratory activity in Drosophila. The arena consists of a 10 cm diameter Petri dish placed on a circular zone drawn on paper, divided into a central and peripheral zone. A video camera positioned above records movement patterns, including time spent in each zone and frequency of zone crossings.

4. Aggression Assay

The aggression assay was conducted to quantify aggressive behaviors in male and female *Drosophila melanogaster* after various treatments. The assay used an arena chamber made from a 96-well plate, a video camera, and a timer. Pairs of male and female flies were gently placed into each cell of the arena chamber using an aspirator. The chamber was then positioned under the video camera to ensure unobstructed recording. After loading the flies, they were given 5 minutes to acclimate to reduce handling stress and encourage natural behavior. Once the acclimation period ended, a 10-minute recording session began to capture the flies' behavior. Aggressive behaviors were observed and scored based on fighting frequency and latency to fighting (19).

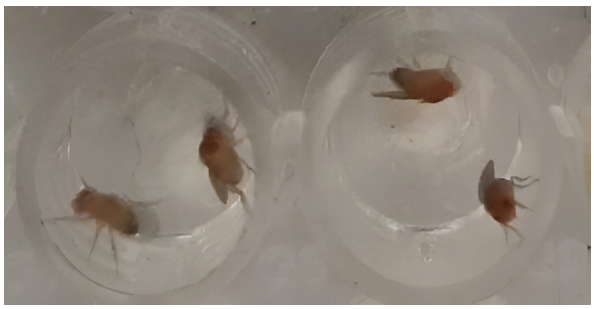

Figure 3: Aggression Assay Chamber

Illustration of the aggression assay setup used to assess aggressive interactions in Drosophila. The assay utilizes a 96-well plate as an arena chamber, with pairs of flies placed in individual wells. A video camera records interactions, scoring fighting frequency and latency to aggression.

Histology and Immunofluorescence Analysis

Following administration, the Drosophila were anesthetized on ice and transferred to a dissection dish containing ice-cold phosphate-buffered saline (PBS, pH 7.4). Under a stereomicroscope, the heads were carefully separated from the bodies using fine forceps. Following the protocol of Karmakar & Mishra, 2020, the heads were then immediately placed into a microcentrifuge tube containing 4% paraformaldehyde (PFA) in PBS for fixation, which was carried out for 20 minutes at room temperature with gentle agitation. Following fixation, the samples were washed three times with PBS to remove excess fixative. After anesthetizing the flies on ice, their heads were carefully removed and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 2 hours at room temperature. Following fixation, the samples were washed in PBS and dehydrated through a graded ethanol series (30%, 50%, 70%, 90%, 100%) for 5 minutes each. The dehydrated heads were cleared in xylene

and embedded in paraffin. Sections were cut at a thickness of 5 μm using a rotary microtome and mounted on gelatin-coated slides.

To assess neuroinflammation and neuronal integrity, two primary antibodies were used: rabbit monoclonal anti-TNF-alpha (1:200, medchem express) to detect neuroinflammatory responses and anti-GFAP (1:500, Sigma-Aldrich) to assess glial reactivity. After fixation and washing, the samples were permeabilized with 0.1% Triton X-100 in PBS for 20 minutes at room temperature, followed by blocking with 5% normal goat serum in PBS for 1 hour to reduce non-specific binding.

Primary antibodies diluted in 0.1% Triton X-100 in PBS were applied to the samples and incubated overnight at 4°C. The next day, samples were washed three times with PBS to remove unbound primary antibodies. Fluorophore-conjugated secondary antibodies (anti-rabbit donkey CF647, 1:500, Sigma-Aldrich) were then applied to the samples for 2 hours at room temperature in the dark, followed by washing three times with PBS. DAPI (1:500 dilution in 0.1% Triton X-100 in PBS) was applied for 10 minutes to counterstain nuclei. After a final PBS wash, the samples were mounted on glass slides using Vectashield anti-fade mounting media and cover-slipped. Image acquisition was conducted using a Zeiss LSM-700 confocal microscope with standardized settings to ensure consistency. The images were processed using ImageJ/Fiji software, where they were converted to 8-bit grayscale for intensity analysis.

Hematoxylin and Eosin (H&E) staining was performed following standard protocols (20). The sections were deparaffinized in xylene, rehydrated through a descending ethanol series, and stained with Harris hematoxylin for 5 minutes. After rinsing in running tap water, the slides were counterstained with eosin for 2 minutes. The sections were then dehydrated through an ascending ethanol series, cleared in xylene, and mounted with DPX mounting medium under coverslips. Images of the H&E-stained sections were captured using an Olympus BX53 bright-field microscope equipped with a digital camera, utilizing 20x and 40x objectives for detailed visualization.

3. Statistical Analysis

Image analysis was performed using ImageJ software (FIJI) to measure fluorescence intensity.

Statistical analyses were conducted using Microsoft Excel. For each quantitative measure, the mean, standard deviation, and standard error of the mean were calculated.

4. RESULTS AND DISCUSSION

Survival Assay

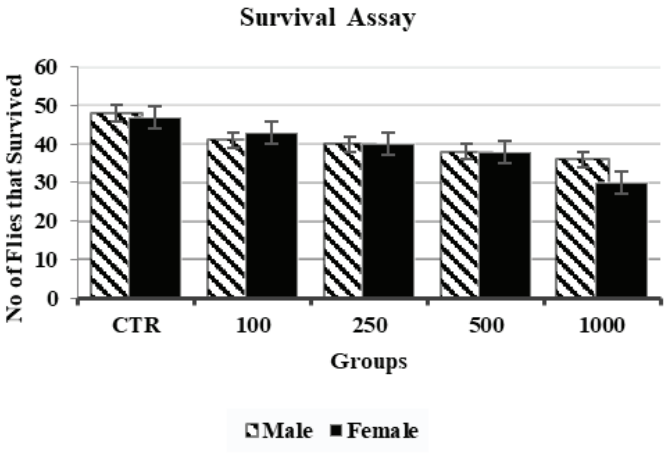

Figure 4: Graph of Survival Assay

Results indicated a clear dose-dependent decrease in survival $p \approx 0.018$ (< 0.05) for males and $p \approx 0.028$ (< 0.05) for females, with the control group exhibiting high survival rates (48 males, 47 females), while the highest ketamine dose (1000 μg/mL) led to significant reductions (36 males, 37 females surviving). These findings are consistent with previous studies, such as Zou et al. (2009) (21), which reported dose dependent neurotoxicity in rat models.

In vivo, reduced survival rates imply significant physiological stress and potential neurotoxicity at higher ketamine doses. Interestingly, our results showed similar survival trends between males and females across all treatment groups, suggesting that ketamine's effects on Drosophila survival may not be strongly sex-dependent. This observation contrasts with some mammalian studies, such as (Franceschelli et al., 2015) (22), who reported sex-specific behavioral responses to ketamine in mice.

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Open Field Assay

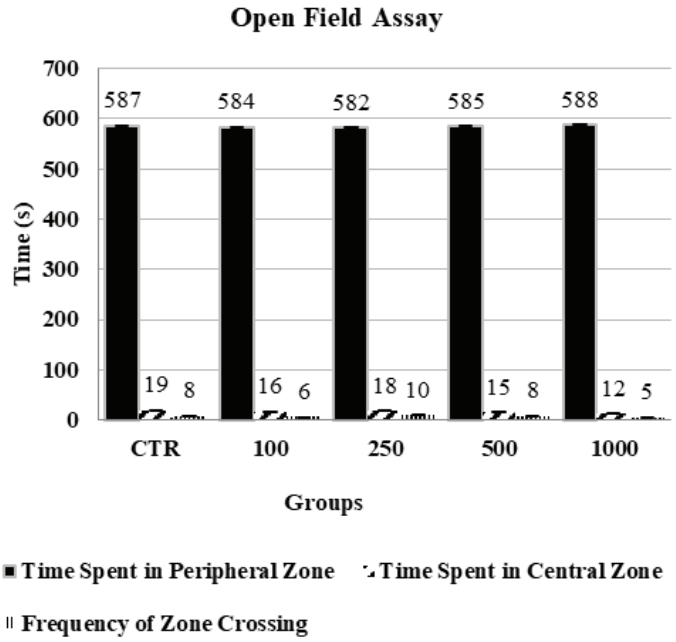

Figure 5: Graph of Open field Assay

The control group established baseline exploratory behavior, spending 19 seconds in the central zone and 587 seconds in the peripheral zone, with an average of 8 zone crossings.

Ketamine treatment showed no statistically significant differences despite appearing notable. For time spent in the central zone, p-value ≈ 0.089; for Time in the Peripheral Zone:

p ≈ 0.94 while For Zone Crossings: p ≈ 0.132 (> 0.05).

Although not significant, the trend suggests a possible dose-dependent decrease with higher doses leading to increased thigmotaxis (preference for the peripheral zone), which is often interpreted as increased anxiety-like behavior in rodent models Simon et al., (1994) (23). This suggests that while ketamine may influence anxiety-like behaviors, its effects in Drosophila may be more subtle than those observed in mammalian models.

Aggression Assay

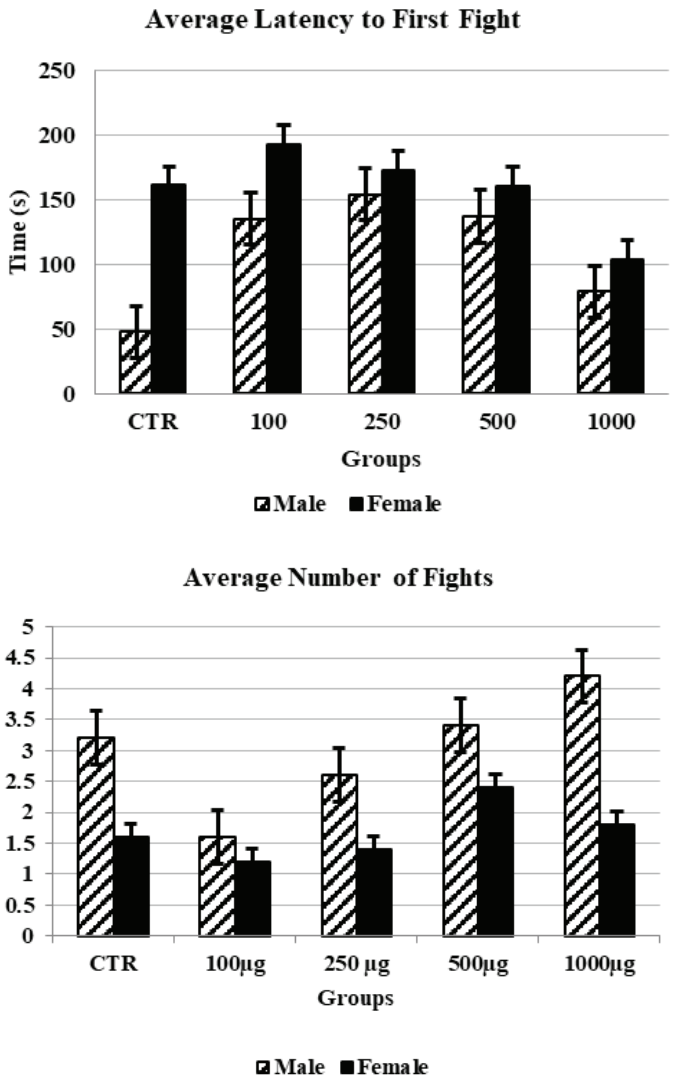

Figure 6: Graph of Aggression Assay

In the control group, males exhibited a 50-second latency and an average of 3.2 fights, while females showed a 160-second latency and an average of 1.6 fights. Ketamine treatment resulted in a significant dose-dependent increase in number of fights, p ≈ 0.031 in male although there was an initial suppression of aggression at lower doses and p ≈ 0.047 in female with moderate reduction at lower doses and peak increase at 500 µg/mL.

Lower doses (100 µL) lead to mild aggression, longer latencies (140 seconds for males, 190 seconds for females), and fewer fights (1.6 and 1.2 fights for males and females, respectively). As doses increased (500 µL, 1000 µL), latencies decreased, and fighting frequency rose, with the highest aggression observed at 1000 µL (65 seconds for males, 102 seconds for females; 4.2 and 1.8 fights). These findings align with studies like Ye et al. (2019) (24)6R, linking ketamine metabolites to altered aggression via its effects on glutamatergic neurotransmission.

Negative Geotaxis Assay

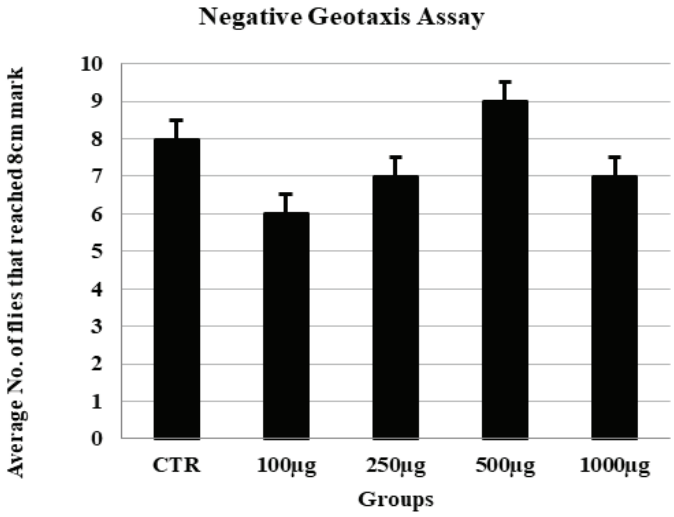

Figure 7: Graph of Negative Geotaxis Assay

The control group established a baseline climbing performance, while ketamine-treated groups showed a dose-dependent increase, most notably in the 500 µL group, where climbing ability was markedly increased. Although the differences between the groups are not statistically significant at p ≈ 0.086

This dose-dependent effect of ketamine on motor function aligns with findings from Imre et al. (2006) (25) and Chen et al. (2023) (26), who reported dose-dependent effects of ketamine on locomotor activity in rats. A dose-dependent enhanced negative geotaxis in Drosophila has previously been reported using Dizocilpine, an NMDA receptor agonist widely used to model schizophrenia-like-phenotypes (27).

However, the 1000 µL group showed a reduction in motor function, suggesting that high doses of ketamine could impair locomotion.

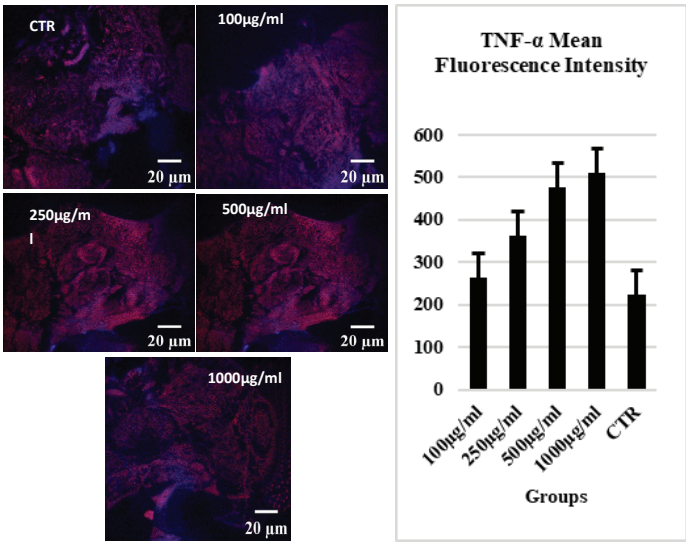

Figure 8: Section of Drosophila brain showing the distribution and localization of TNF-alpha (red).

Immunofluorescence and Histological Analysis

Figure 8: Section of Drosophila brain showing the distribution and localization of TNF-alpha (red). DAPI (Blue) is used as a nuclear stain to visualize the whole brain morphology, and the graph shows a comparison of the mean fluorescence intensity of TNF-alpha across different experimental groups.

TNF-α expression analysis revealed highly significant differences between groups ($p < 0.0092$), characterized by a strong dose-dependent increase. The highest ketamine concentration resulted in more than doubled TNF-α expression compared to control levels, providing clear evidence of an inflammatory response. This pattern aligns with prior studies that link increased doses of pro-inflammatory agents to heightened TNF levels, reinforcing the inflammatory cascade's sensitivity to modulation (28)

The pronounced increase in TNF-α expression indicates robust neuroinflammatory activation at higher ketamine doses. Similar findings were reported by Wang et al. (2015) (29), who observed that chronic ketamine administration led to elevated pro-inflammatory cytokine levels, including TNF-α, in the hippocampus of rodents. Additionally, Li et al. (2017) (30) found that Single administration of ketamine increased the level of TNF-α, whereas multiple and long-term administration decreased it significantly. In Drosophila, elevated TNF levels likely mirror mammalian inflammatory processes, given the conservation of TNF pathways across species (31).

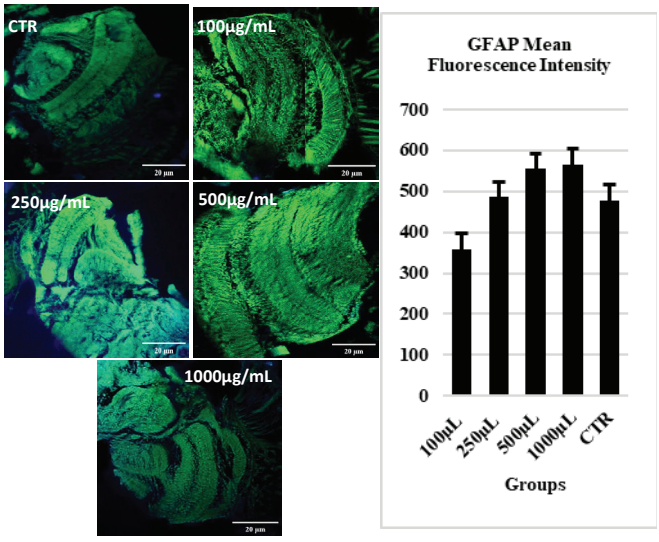

Figure 9: Section of Drosophila brain showing the distribution and localization of GFAP (green).

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DAPI (Blue) is used as a nuclear stain to visualize the whole brain morphology, and Graphs show a comparison of the mean fluorescence intensity of GFAP across different experimental groups.

The analysis of GFAP expression revealed significant differences between treatment groups ($p < 0.038$), demonstrating a complex dose-dependent response to ketamine exposure. At the lowest concentration (100 µg/mL), GFAP expression showed an initial decrease compared to control levels, followed by a return to baseline at 250 µg/mL. Higher concentrations (500-1000 µg/mL) induced a progressive increase in GFAP expression, with peak levels observed at 1000 µg/mL, suggesting dose-dependent astrogliosis at higher concentrations.

The biphasic response of GFAP expression is particularly noteworthy. The initial decrease at low doses followed by elevation at higher concentrations suggests that ketamine's effects on astrocyte activation are dose-threshold dependent. This pattern aligns with findings from Keilhoff et al. (2004) (32), who demonstrated that repeated ketamine exposure in rats led to reactive gliosis and increased GFAP immunoreactivity.

This dose-dependent trend is consistent with previous studies showing increased astrocyte activation at higher ketamine doses (33). Lower GFAP expression at 100µL may reflect neuroprotective effects, whereas higher doses likely indicate pro-inflammatory or neurotoxic effects (34).

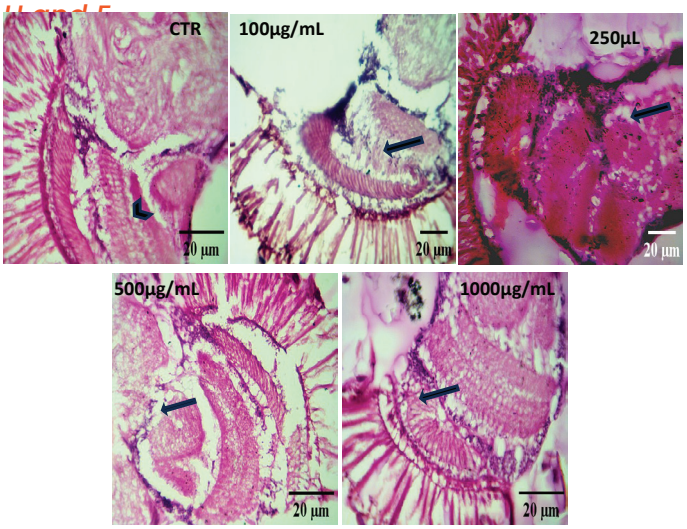

Figure 10: Histological Analysis of Drosophila Brain Sections

Hematoxylin and eosin (H&E)-stained brain sections of Drosophila showing structural changes across treatment groups.

Brains from control flies exhibited normal histological features with intact neuropil (Arrowhead) and no significant vacuolation across all brain regions. 100 µL Ketamine groups displayed mild degenerative changes as scattered vacuoles were observed (arrow). Moderate vacuolation was observed in the 250 µL Ketamine groups with increased disorganization of neuropil fibers (Figure 10). Marked degeneration was evident in the 500 µL Ketamine with prominent vacuolar lesions (arrow) disrupting the neuropil and necrotic cell bodies. Severe neurodegeneration was apparent, with extensive vacuolation and fragmentation in the 1000 µL Ketamine group. These findings are consistent with previous studies on ketamine's neurotoxic effects in both invertebrate and vertebrate models. For instance, Liu et al., (2013) (35) reported that ketamine induced widespread neuroapoptosis in the developing rat brain, with particular vulnerability in certain brain regions.

5. CONCLUSION

In conclusion, our study demonstrates the potential utility of Drosophila as a model organism for investigating complex neuropsychiatric phenomena. The observed effects of ketamine, particularly its ability to induce some behavioral and histopathological changes, suggest potential avenues for its use as a pharmacological model of schizophrenia. However, future research should focus on elucidating the molecular mechanisms underlying these effects and exploring their relevance in mammalian models. The complex effects underscore the need for careful, nuanced approaches in translating these findings to clinical applications.

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Bispecific T-Cell Engagers and Related Immunotherapies: Advances and Challenges

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ABSTRACT:

Bispecific T-cell engagers (BiTEs) have revolutionized cancer immunotherapy, especially in hematological malignancies. These molecules simultaneously target tumor antigens and engage T-cells, which demonstrating potent antitumor activity in various cancers. However, challenges such as rapid drug clearance, off-target effects, and cytokine release syndrome limit their broader use. Recent advances in BiTE design aim to address these obstacles, expanding their therapeutic potential. This review discusses the latest progress in BiTEs and related immunotherapies, as well as strategies to overcome current challenges.

Keywords: *Bispecific, immunotherapy, cancer, cytokine, antibodies.*

1. Introduction

Before bispecific antibodies (BsAbs) were introduced, traditional cancer immunotherapy relied on monoclonal antibodies (MoAbs) molecules targeting a single tumor antigen **[1]**. However, the complex nature of some cancers, with their ability to switch signaling pathways and evade immune responses, posed challenges for this approach **[2]**. A prime example is the interaction between Programmed Cell Death Protein 1 (PD-1) and Programmed Cell Death Ligand 1 (PD-L1), where tumor cells exploit this interaction to attenuate the immune response **[3]**. This manipulation involves inducing apoptosis in antigen-specific T cells and inhibiting the apoptosis of regulatory T cells, affecting the efficacy of single antibody-targeted immunotherapy **[3]**. The arrival of bispecific antibodies marks a significant shift in addressing these challenges, offering a promising avenue for more effective cancer treatment.

BsAbs are a promising type of therapy that can target two different tumor antigens simultaneously **[4]**. These antibodies typically consist of two single-chain variable fragment (scFv) antigen-binding parts linked by a flexible amino acid linker, offering a more refined approach against cancer cells **[5]**. In the case of the PD-1/PD-L1 axis, bispecific antibodies can be designed to bind both PD-1 and a tumor-specific antigen at the same time. This disrupts immune evasion mechanisms and strengthens the immune response **[6]**. Over 100 bispecific antibodies have been evaluated across various cancer types, with many receiving marketing approvals **(Table 1) [7, 8]**. A significant achievement occurred in 2022 when the FDA approved a Bispecific T cell Engager (BiTE) product targeting CD3/BCMA for treating relapsed or refractory multiple myeloma **[9]**. Subsequently, talquetamab and elranatamab, both CD3 T-cell engagers, received FDA approval in 2023 for multiple myeloma treatment **(Table 1) [10, 11]**. These approvals mark substantial progress in treating adult patients with relapsed or refractory multiple myeloma.

While most bispecific antibodies focus on cancer treatment, some are directed at chronic inflammatory, autoimmune, and neurodegenerative diseases and infections. Examples include emacizumab and faricimab, both developed for hemophilia A and retinal vascular disease treatment, respectively

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[12, 13]. These diverse applications highlight the expanding role of bispecific antibodies in transformative therapeutic interventions. Although BsAbs have been effective in cancer treatment, they still face challenges like a short in vivo half-life, on-target off-tumor effects, cytokine release syndrome, and issues in manufacturing **[14-16]**. These challenges have hindered their broader application, indicating the need for advanced formats. Recent advances have led to innovative approaches addressing these challenges, paving the way for improved clinical practices.

In this review, we shed light on the evolving field of bispecific antibodies, providing insights into their present status in clinical development. Additionally, we delve into the challenges associated with bispecific antibodies and explore recent modifications aimed at enhancing their therapeutic efficacy.

2. Bispecific T cell Engager

The concept of bispecific antibodies (BsAbs) has evolved significantly since their initial description by Nisonoff in 1960, resulting in the development of several hundred formats categorized into six diverse mechanisms of action: (1) bridging cells, (2) receptor inhibition, (3) receptor activation, (4) co-factor mimetic, (5) piggybacking I, and (6) piggybacking II **[8, 17]**. These diverse BsAb formats have been engineered to target various components such as tumor signaling pathways, immune checkpoint inhibitors (ICIs), inflammatory cytokines, and more **[18-20]**. Among these formats, the bridging cell or Bispecific T-cell Engager stands out as the most common BsAb employed for the treatment of both liquid and solid tumors **[21]**. A crucial aspect of BiTEs is their ability to redirect naïve T cells to target tumor cells, leading to T-cell activation, clonal expansion, and subsequent tumor cytotoxicity **Figure 2** **[21, 22]**. First-generation BiTE constructs were typically designed with two monoclonal antibodies (mAb) moieties tandemly fused, with one moiety targeting a specific tumor antigen and the other binding to CD3 antigen on T-cell surfaces. This design ensures that T cells engaged by BiTE molecules become activated and effectively eliminate malignant cells **[23]**. More than six decades, seven BiTEs have been approved for cancer treatment (**Table 1**), and several more are undergoing clinical testing **[24]**. Despite their efficacy, the use of BITE has faced challenges associated with 'on-target, off-tumor' toxicities **[25, 26]**. BiTE therapy primarily involves identifying suitable tumor-associated antigens (TAAs) on target cells that differ from those on normal cells, aiming to prevent on-target/off-tumor toxicity **[25]**. However, the identification of antigenic targets exclusive to tumor cells presents challenges, as many target antigens are expressed on both normal and tumor cells **[27]**. Even minimal antigen expression on normal cells can result in adverse on-target off-tumor toxicities, leading to cytokine release syndrome (CRS). CRS, characterized by an excessive immune response leading to the release of proinflammatory cytokines, can potentially result in organ failure and, in severe cases, death **[28]**. Currently, the primary clinical interventions to manage CRS in T cell-engaging bispecific antibody (TCE) therapies involve dose reduction or the administration of anti-interleukin antibodies and corticosteroids **[28]**. While these interventions have proven effective in certain scenarios, they do not provide a complete prevention of CRS. Accordingly, increasing reports have highlighted the occurrences of off-target, on-target toxicity associated with bispecific antibody molecules, especially BiTE therapeutics **[23, 29, 30]**.

To overcome the significant challenge of on-target, off-tumor adverse effects, including CRS, and enhance the therapeutic index of BsAbs, particularly in the context of solid malignancies, researchers have been exploring several modification strategies. One such strategy focuses on employing avidity-mediated specificity or the 2 + 1 architecture **[31, 32]**. In this novel approach, a bivalent antibody with low affinity for the tumor antigen is combined with a monovalent anti-CD3 molecule **[32]**. This unique design enables the BiTE to selectively bind to tumor cells that overexpress the target tumor-associated antigen (TAA), facilitating the specific killing of tumor cells while sparing normal cells expressing the target antigen at lower densities.

A study conducted by Bacac et al. exemplifies this approach, utilizing a bivalent anti-CEA scFv domain linked with a monovalent anti-CD3 domain for the treatment of solid tumors expressing carcinoembryonic antigen (CEA) **[33]**. CEA, also known as carcinoembryonic antigen-related cell adhesion molecule 5 (CEACAM5), is associated with glycosylphosphatidylinositol and is overexpressed in various cancers, playing a role in adhesion and invasion **[34]**. The resulting CEA T cell bispecific (TCB) demonstrated sustained antitumor activity in a preclinical model, exhibiting a notable increase in T-cell longevity **[33]**. Moreover, the CEA+CD3 TCB transformed PD-L1-negative tumors into PD-L1-positive, creating a highly inflamed tumor

microenvironment. This promising development has advanced to phase 1 clinical investigation (NCT02324257), showcasing pronounced efficacy and manageable safety profiles **[33]**.

In line with these advancements, another group used an anti-HER2/CD3 T cell-dependent bispecific (TDB) antibody to redirect T cells to eliminate HER2-overexpressing cells, demonstrating potent antitumor activity **[31]**. This suggests that avidity-mediated selection holds promise for treating solid tumors, as it potentially addresses one of the major challenges associated with TCE therapies, offering a more targeted and controlled immune response. However, since high expression levels of the TAA are crucial for avidity specificity and bispecific antibody-mediated tumor lysis, this strategy applies primarily to cancer cells expressing very high levels of the target antigen. The challenge arises when dealing with solid tumors expressing variable densities of the target antigen. To address this challenge and enhance the versatility of the approach, future studies are needed to develop a dual bispecific antibody with a 2+1+1 architecture, where one target incorporates avidity-mediated specificity and the other features high-affinity binding. This approach would offer a comprehensive solution to rapidly target and eliminate solid tumors expressing differential levels of the target antigen.

Generally, there is currently no FDA-approved BiTE molecule for treating solid malignancies. However, catumaxomab, the first bispecific T-cell engager approved by the EMA in 2009 to treat malignant ascites of epithelial cancers, was later withdrawn from the market due to severe adverse events, including CRS and dose-dependent liver toxicity **[35]**. Ongoing research and development aim to address these challenges and further enhance the clinical applicability of BiTEs, emphasizing their significance in advancing cancer immunotherapy.

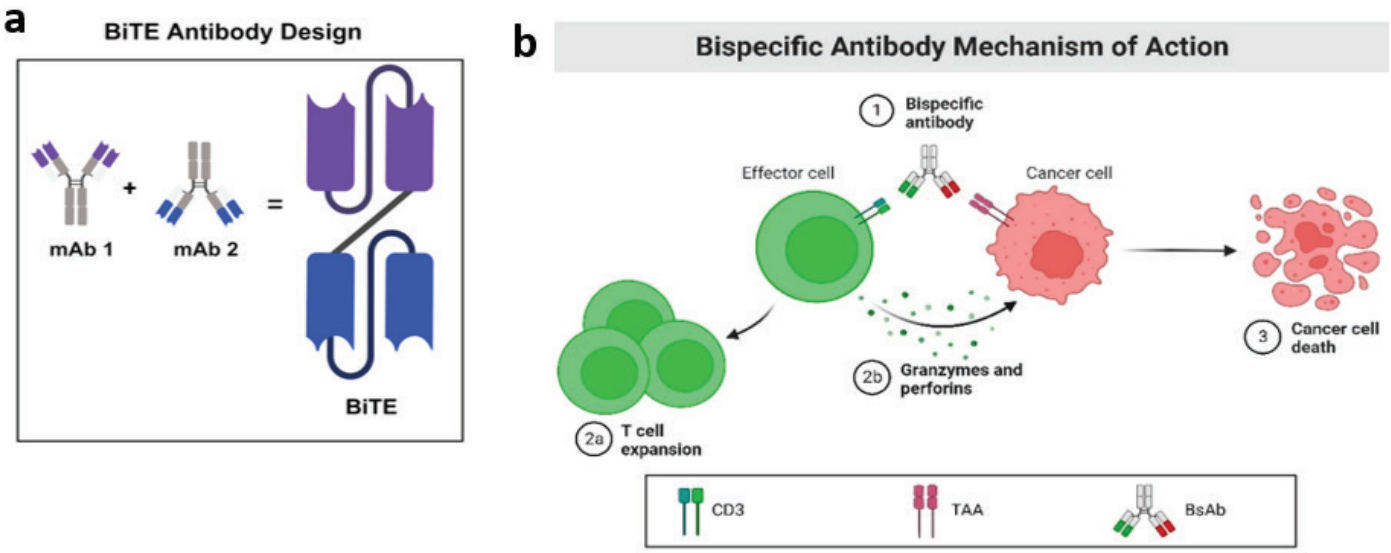

Figure 1: BiTE and its mechanism of action.

a. BiTE antibody construct comprises two single-chain variable fragments of monoclonal antibodies linked together through a flexible linker. b. One arm of the BiTE molecule is designed to bind to CD3, an antigen located on the surface of T cells. Simultaneously, the other arm is engineered to bind to a tumor-associated antigen (TAA). Upon successful binding of both arms to their specific targets, a synapse is formed between the T cell and the cancer cell. Subsequently, the T cells undergo expansion and release perforin, creating a pore in the cancer cell's membrane. This pore allows toxic molecules called granzymes to flow through, ultimately inducing the death of the cancer cell.

While BiTEs encounter challenges in battling solid tumors, a promising alternative, immune-mobilizing monoclonal T-cell receptors against cancer (ImmTACs), has emerged **[36]**. Like BiTEs, ImmTACs facilitate the interaction between cancer cells and T cells by simultaneously engaging their proteins. However, ImmTACs take a different approach by employing a T-cell receptor instead of an antibody fragment to recognize proteins in cancer cells [36]. This unique strategy allows ImmTACs to bind to intracellular proteins processed and presented externally, expanding their target range beyond cell surface proteins. This characteristic makes ImmTACs more effective in addressing

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solid tumors, where many cancer-specific proteins are primarily expressed inside the cell. Tebentafusp (Kimmtrak), an ImmTAC therapeutic, has already gained approval for treating uveal melanoma **[37]**. Considering the risks associated with BiTEs in solid tumors, especially CRS, ImmTACs emerge as a promising class of therapeutics, offering cancer-fighting immune cells a distinct advantage.

Blinatumomab, the first FDA-approved BiTE construct

Blinatumomab stands out as a significant success in BiTE therapy, marking the first FDA-approved BiTE molecule to treat B-cell acute lymphoblastic leukemia (ALL), **Figure 2 [38]**. This therapy combines anti-CD19 and anti-CD3 scFv, demonstrating notable clinical efficacy. Many patients experienced complete tumor regression, contributing to improved overall survival rates **[39]**. In a study with 54 relapsed or refractory (R/R) patients, 91% (49/54) achieved a complete response with blinatumomab treatment, highlighting its clinical effectiveness in challenging R/R settings **[40]**. These outcomes emphasize blinatumomab's therapeutic potential and its crucial role in advancing treatment options for B-cell ALL patients. Importantly, blinatumomab's activity is independent of major histocompatibility complex activation, ensuring rapid activation of T cells and the destruction of tumor cells **[41]**.

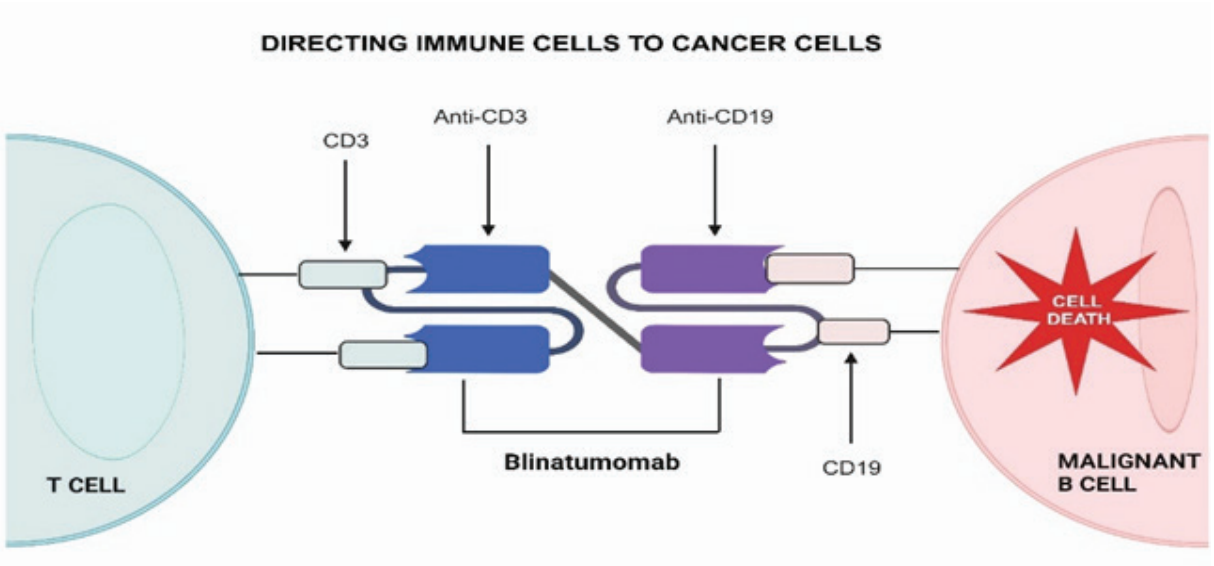

Figure 2 The mechanism of action for blinatumomab, the first-in-class BiTE, involves one arm binding to CD3 and the other to CD19. This interaction activates unstimulated T cells, initiating their attack on CD19+ cells.

Although blinatumomab has demonstrated significant success, crucial challenges persist. Factors such as rapid drug clearance, on-target/off-tumor adverse effects, cytokine release syndrome, and activation of peripheral immune cells may potentially limit therapeutic efficacy in both hematological malignancies **[42]**. Recent reports indicate instances of relapse among patients following blinatumomab treatment, with the phenomenon associated not only with the loss of CD19 but also CD58, as proposed by Jabbour et al. **[43]**. Previous research has explored mechanisms contributing to CD19 escape, including CD19 mutations, CD19-mutant allele-specific expression, low CD19 RNA expression, and mutations in CD19 signaling member CD81 **[44]**. However, limited attention has been given to CD58 loss and its mechanism in the context of Blinatumomab treatment.

A recent study by Yizhen et al. has identified a crucial intrinsic factor, PAX5 mutation, significantly downregulating CD58. This downregulation has been linked to a reduction in blinatumomab activity, particularly observed in patients with ALL **[45]**. Further research is needed to address the PAX5 mutation in ALL models under Blinatumomab treatment, providing a more comprehensive understanding of the role of PAX5 in CD58 loss. Moreover, additional studies have suggested regulatory T cells (Tregs) as potential regulators in the resistance process against blinatumomab, indicating that multiple factors may contribute to resistance and a reduced response rate to this therapeutic approach **[44]**. These findings suggest the complexity of the mechanisms behind resistance to Blinatumomab, emphasizing the necessity for ongoing research to unravel these intricacies

and ultimately pave the way for more effective and personalized treatment strategies.

Furthermore, the phenomenon of lineage switch represents a significant challenge associated with blinatumomab treatment, wherein refractory B lymphoblastic leukemia (B-ALL) can undergo a transition to acute myeloid leukemia (AML) **[46-48]**. This shift in lineage was initially documented by Stass and colleagues following standard chemotherapy for acute leukemia **[49]**. The occurrence of lineage switching has been observed not only in blinatumomab therapy but also in other immunotherapies, including CD19-specific chimeric antigen receptor (CAR) T cells **[50]**. It is particularly noteworthy that this switch occurs when CD19 B-cells acquire a distinct phenotype after the loss of CD19 **[51-53]**. While several other theories have been proposed to explain the mechanisms leading to lineage switch **[54, 55]**, the prevailing view suggests that the selective pressure resulting from CD19-directed therapy plays a crucial role in this phenotypic transition **[56-58]**. Studies on lineage switching highlight various rearrangements of the gene encoding histone–lysine N-methyl-transferase 2A (KMT2A, also known as mixed-lineage leukemia, MLL) as a key regulator of this switch **[59-61]**. The development of this immunophenotype is recognized as a critical factor contributing to relapses and resistance to several antibody-targeted therapies.

In the case of blinatumomab, five chromosomal rearrangements linked to lineage switch have been identified: KMT2A-AFF1 **[62, 63]**, KMT2A/AFF4 **[58]**, BCR-ABL1 **[64]**, hyperdiploidy **[65]**, and KMT2A/EPS15 **[66]**. The t(4;11) (q21;q23) rearrangement with the KMT2A/AFF1 fusion protein is particularly common, especially in infants with ALL **[67-69]**. Lineage conversion has been observed in pediatric patients with ALL, impacting blinatumomab treatment monitoring. A switch from CD19-positive B-precursor ALL to CD19-negative AML has been documented following blinatumomab therapy **[47]**. Efforts to overcome this challenge include incorporating blinatumomab into the Interfant-06 backbone regimen. In an analysis of 30 infants with acute leukemia treated with standard chemotherapy and post-induction blinatumomab, no lineage switches were observed **[70]**. Similarly, promising outcomes have been reported in infants with KMT2A-rearranged ALL, where the addition of blinatumomab to the Interfant-06 chemotherapy trial significantly improved the 2-year overall survival compared to the Interfant-06 alone **[71]**. It is essential to note that the follow-up time in these studies was relatively short, and longer-term monitoring is required to comprehensively evaluate the safety and efficacy of this combined therapy. Furthermore, blinatumomab has shown promise as an effective salvage therapy following anti-CD19-CAR-T failure, surpassing chemotherapy options. In R/R B-ALL patients, blinatumomab showed an improved complete remission rate, even in those expressing low CD19 levels **[72]**. However, inconsistent findings warrant further comparable studies to validate its potency as a rescue or pretreatment therapy, as some reports suggest prior blinatumomab treatment can maintain anti-CD19-CAR-T efficacy **[73].**

Table 1: Summary of BsAbs approved for the market worldwide for clinical use as of 2014

Drug (Company)	Trade name	Target antigen	Approved Countries	Year Approved	Approved indications
Blinatumomab (Amgen)	Blincyto	CD3/CD19	FDA	2014	Adults and children with B-cell precursor ALL in first or second complete remission with minimal residual disease (MRD) greater than or equal to 0.1%. **[74]**
Emacizumab-kxwh (Genentech)	Hemlibra	FIXa/ FX	FDA	2017	The treatment is recommended for adult and pediatric patients, including newborns, with hemophilia A. This includes individuals with congenital factor VIII deficiency, whether or not they have developed factor VIII (FVIII) inhibitors **[75]**

Advances in Medical, Pharmaceutical and Dental Research Journal (AMPDR)- ISSN 2812-4898
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Amivantamab-vmjw(-Janssen Biotech)	Rybrevant	EGFR/c-Met	FDA/EMA	2021	Adult patients with locally advanced or metastatic non-small cell lung cancer who have EGFR exon 20 insertion mutations and have previously received platinum-based chemotherapy **[76]**
Tebentafusp-tebn (Immunocore)	Kim-mtrak*	CD3/ gp100	FDA	2022	For the treatment of adult patients with unresectable or metastatic uveal melanoma who are HLA-A*02:01-positive. **[77]**
Faricimab-svoa (Roche)	Vabysmo	VEGF-A/Ang-2	FDA	2022	To treat neovascular (wet) age-related macular degeneration and diabetic macular edema **[78]**
Mosunetuzumab-axgb (Genentech)	Lunsumio	CD3/CD20	EMA/FDA	2022	Patients with advanced non-small cell lung cancer (NSCLC) harboring EGFR exon 20 insertion mutations face disease progression after platinum-based chemotherapy **[79]**
Cadonilimab (Akeso)	Kaitanni	PD-1/CTLA-4	CFDA	2022	For patients with relapsed or metastatic cervical cancer (r/mCC) who have experienced disease progression following platinum-based chemotherapy **[80]**
Teclistamab-cqyv (Janssen Biotech)	Tecvavli	CD3/BCMA	EMA/FDA	2022	Adult patients with relapsed or refractory multiple myeloma who have received at least four prior lines of therapy, including a proteasome inhibitor, an immunomodulatory agent, and an anti-CD38 monoclonal antibody **[81]**
Epcoritamab-bysp (Genmab)	Epkinly	CD3/CD20	FDA/EMA	2023	Adults with relapsed or refractory diffuse large B-cell lymphoma (DLBCL), including cases arising from indolent lymphoma and high-grade B-cell lymphoma after two or more lines of systemic therapy **[82]**
Glofitamab-gxbm (Genentech)	Columvi	CD3/CD20	FDA	2023	For adults with relapsed or refractory diffuse large B-cell lymphoma (DLBCL, NOS) or large B-cell lymphoma (LBCL) arising from follicular lymphoma after two or more lines of systemic therapy **[83]**.
Talquetamab-tgvs (Janssen Biotech)	Talvey	GPRC5D/ CD3	EMA/FDA	2023	Adults with relapsed or refractory multiple myeloma who have undergone at least four prior lines of therapy, including a proteasome inhibitor, an immunomodulatory agent, and an anti-CD38 monoclonal antibody. **[84]**

Elranatamab (Pfizer)	Elrexfio	BCMA/CD3	FDA/EMA	2023	For adults with relapsed or refractory multiple myeloma who have received at least four prior lines of therapy, including a proteasome inhibitor, an immunomodulatory agent, and an anti-CD38 monoclonal antibody **[85]**
Odronextamab (Ordspono)	Regeneron	CD20/CD3	FDA	2024	Adult patients with relapsed/refractory (R/R) follicular lymphoma (FL) or R/R DLBCL who have progressed after at least two prior systemic therapies **[86]**

**Kimmtrak* *is technically a bispecific molecule, not a bispecific antibody. Like some of the other bispecific antibodies used to treat some cancers, Kimmtrak has one arm using an antibody fragment to bring killer T cells to the tumor. Kimmtrak's other arm is an analogous structure found on T cells, the T cell receptor, instead of an antibody fragment to target a tumor antigen.*

Immune checkpoint bispecific antibodies

In cancer immunotherapy, the use of immune checkpoint inhibitors (ICIs) has been a breakthrough, particularly when used as monotherapies **[87, 88]**. These inhibitors tap into the potential of natural T cells that infiltrate tumors. Cancer cells often exploit immune checkpoints to avoid immune responses, and ICIs counteract this by blocking specific checkpoints **[89, 90]**. Approvals of drugs like ipilimumab, pembrolizumab, and nivolumab signify significant strides in ICI development (**Table 2**) **[87]**. However, the effectiveness of single antibody targets against immune checkpoints and their ligands has shown limited impact, especially in treating "cold tumors" – tumors that hinder immune responses by preventing the infiltration of immune cells into the tumor **[91, 92]**. Consequently, only a minimal fraction of the patient population has experienced significant benefits from ICI monotherapies.

Recent advancements in bispecific antibodies have addressed this limitation by focusing on the dual targeting of immune checkpoints, encompassing both receptors and ligands **[93]**. Notably, programmed death protein 1 (PD-1) and programmed cell death ligand 1 (PD-L1) checkpoint inhibitors have gained attention for their ability to restore T cells exhausted due to tumor-induced suppression [79]. PD-L1 and PD-L2, widely expressed ligands across various cancer types, have been a focus of study. PD-L2, known to bind PD-1 more strongly than PD-L1, presents an opportunity for more impactful outcomes when targeted **[94, 95]**. In contrast to monospecific PD-1 and PD-L1 antibodies, bispecific antibodies targeting both PD-1 and PD-L1 have demonstrated powerful antitumor responses. LY3434172, a bispecific antibody co-targeting PD-1 and PD-L1, exhibited significant in vivo antitumor potency even at lower doses in preclinical studies, suggesting a synergistic effect and a distinctive pathway interaction in modulating immune responses **[96]**.

Approximately 60% of cancers express both PD-L1 and PD-L2, while around 30% express either PD-L1 or PD-L2, expanding the binding effect and reducing off-target toxicities of bispecific antibody constructs **[97]**. Ongoing studies are exploring dual-specific antibodies to co-target stromal cells, Tregs, and myofibroblasts in the tumor microenvironment, facilitating the influx of T cells into poorly infiltrated tumors **[98]**. Emerging strategies aim to target specific surface proteins, including PD-L1/PD-L2, CD25/CTLA-4, PD-L1/ICOS, PD-1/CD47, and PD-L1/T cell immunoreceptor with Ig and ITIM domains (TIGIT) (**Table 1**) **[99, 100]**. For instance, dual-specific monoclonal antibodies designed to bind PD-L1 and PD-L2 have demonstrated enhanced immune-driven anti-tumor activity **[101]**. In the context of treating HER2-positive solid tumors, a bispecific combination of PD1 and HER2 exhibited high effectiveness in killing HER2-positive tumor cells through antibody-dependent cellular cytotoxicity **[102]**.

Undoubtedly, bispecific antibodies tailored against PD-L1 and PD-L2 play a pivotal role in facilitating the migration of host immune responses to tumor cells, thereby enhancing antitumor responses. The targeting of PD-L1 in dual antibody regimens has demonstrated effectiveness in various settings of human tumors, as evidenced by the numerous ongoing clinical trials exploring PD1/PDL1 combination regimens **[103]**

Advances in Medical, Pharmaceutical and Dental Research Journal (AMPDR)- ISSN 2812-4898
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Table 2: Studies investigating the efficacy of PD1/PDL1 combination regimens in patients with advanced solid tumors (Clinical trials are registered at clinicaltrials.gov)

Target	Name	Condition	Status	Phase	NCT ID
PD-L1 and TGF-β	SHR-1701	Advanced solid tumors	Unknown	Phase I	NCT03710265
CTLA-4×PD-L1	KN064	Advanced Solid Tumors	Completed	Phase 1	NCT03733951
PD-1 and CTLA-4	MEDI5752	Advanced solid tumors	Recruiting	Phase I	NCT03530397
	MGD019	Advanced solid tumors	Active, not recruiting	Phase 1	NCT03761017
	AK104	Hepatocellular carcinoma	Recruiting	Phase I/II	NCT04444167
	COMPASSION-03	Advanced solid tumors	Active, not recruiting	Phase I/II	NCT03852251
LAG-3 × PD-L1	ABL501	Advanced solid tumors	Recruiting	Phase I	NCT05101109
	FS118	Advanced solid tumors	Active, not recruiting	Phase I/II	NCT03440437
	AK104	NSCLC	Active, not recruiting	Phase I/II	NCT04646330
LAG-3 × PD-1	MGD013	Advanced liver cancer	Terminated	Phase I/II	NCT04212221
	RG6139	Advanced solid tumors	Recruiting	Phase I/II	NCT04140500
	Not Given	Advanced solid tumors	Recruiting	Phase I	NCT05577182
TIM-3 × PD-L1	LY3415244	Advanced solid tumors	Terminated	Phase I	NCT03752177
	ABL501	Advanced solid tumors	Recruiting	Phase I	NCT05101109
TIGIT×PD-L1	HLX301	Advanced solid tumors	Recruiting	Phase I/II	NCT05102214
TIGIT×PD-1	ARTEMIDE-01	Advanced NSCLC	Recruiting	Phase I/II	NCT04995523
	LB1410	Advanced Solid Tumor	Recruiting	Phase I	NCT05357651

TIM-3 × PD-1	AZD7789	Lymphoma	Recruiting	Phase I/II	NCT04931654
	RG7769	Advanced Solid Cancer	Recruiting	Phase I	NCT03708328
	Lomvas-tomig	Advanced Solid Cancer	Active, not recruiting	Phase II	NCT04785820
	Tobem-stomig	Non-small Cell Lung Cancer	Recruiting	Phase II	NCT05775289
4-1BB×PD-L1	ABL503	Advanced Solid Cancer	Recruiting	Phase I	NCT04762641
	PRS-344	Advanced Solid Cancer	Recruiting	Phase I/II	NCT05159388
	GEN1046	Advanced Solid Cancer	Recruiting	Phase I/II	NCT03917381
CD27×PD-L1	CDX-527	Advanced Solid Cancer	Completed	Phase I	NCT04440943
PD-L1 and CD137	MCLA-145	Advanced Solid Cancer	Recruiting	Phase I	NCT03922204
	AP203	Advanced Solid Cancer	Not yet recruiting	Phase I/II	NCT05473156
	FS222	Advanced Solid Cancer	Recruiting	Phase I	NCT04740424
PD-L1 and VEGF	PM8002	Advanced Solid Cancer	Recruiting	Phase II	NCT05879055
	HB0025	Advanced Solid Cancer	Recruiting	Phase I	NCT04678908
	IMM2510	Advanced Solid Cancer	Recruiting	Phase I	NCT05972460
PD-1/ VEGF	AK112	NSCLC	Recruiting	Phase II	NCT04736823

3. Future directions

Looking ahead, the success of BsAbs in effectively treating hematological malignancies is evident with FDA approvals. However, it's noteworthy that there is currently no FDA-approved BiTE molecule for addressing solid malignancies. Ongoing initiatives are exploring innovative approaches, such as incorporating masks linked through protease-cleavable linkers into first-generation TCEs, including Conditional Bispecific Redirected Activation, Probody TCB, and precision-activated TCEs. These attempts aim to enhance the therapeutic efficacy of bispecific T-cell engagers in treating solid tumors.

In addressing complications like cytokine release syndrome CRS associated with BsAbs therapy, future research is focused on optimizing the design to trigger immunological responses exclusively towards tumors. Unlike previous designs involving a single BsAb agent, emerging strategies adopt a unique approach by employing two Bispecific Antibodies BsAb components. Each component features a split anti-CD3 paratope and a binding moiety for a tumor antigen. These advancements signify a promising direction in the evolution of BiTE for more effective and targeted treatments of solid tumors.

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4. Conclusion

The field of bispecific antibodies BsAbs, particularly exemplified by BITE therapies, has witnessed remarkable strides in cancer immunotherapy and appears superior to conventional chemotherapy in at least hematological malignancy settings. The clinical success of over 100 evaluated bsAbs, with seven BiTE approved for market use, highlights their remarkable achievements. However, challenges such as rapid drug clearance, off-target effects, and cytokine release syndrome persist, limiting their widespread application. Despite this, innovative modifications, including avidity-mediated specificity, paratope masking, and the two BsAbs system, hold promise in addressing on-target/off-tumor adverse effects. Moreover, immune checkpoint bispecific antibodies, co-targeting receptors, and ligands like PD-1 and PD-L1 present a paradigm shift in cancer immunotherapy, offering enhanced antitumor responses. The evolving landscape of bispecific immunotherapeutics holds great potential in advancing personalized and effective cancer treatments, emphasizing the need for ongoing research and development to overcome existing challenges and broaden therapeutic applications.

Data Availability

This study did not perform any data

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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BiTEs encounter challenges in battling solid tu

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Unveiling the Multi-Targeted Therapeutic Potentials of Vernonia amygdalina: A Comprehensive Review of Bioactive Compounds, Molecular Mechanisms, and Clinical Opportunities

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ABSTRACT:

Vernonia amygdalina (bitter leaf) has been used as traditional medicine across Africa and beyond because of its diverse pharmacological activities. While previous studies have validated its therapeutic potential, a comprehensive understanding of the underlying molecular mechanisms driving these effects remains incomplete. This review critically examines the bioactive compounds present in V. amygdalina, their documented pharmacological effects, and the potential therapeutic applications of this plant, focusing specifically on elucidating the key molecular pathways modulated by its constituents. A systematic literature search was conducted using Web of Science, Scopus, PubMed, and ScienceDirect. Identification and analyses of a wide array of bioactive compounds, including sesquiterpene lactones (vernodalin, vernolepin), flavonoids (luteolin, apigenin), and saponins were carried out. Critical analyses revealed that these compounds exert their effects through multiple, interconnected pathways, including but not limited to the method of extraction, their robust antioxidant and cytoprotective effects, and the direct cytotoxic effects on cancer cells through induction of apoptosis and cell cycle arrest. Bitter leaf is used in the traditional treatment of malaria, diabetes, and gastrointestinaldisorders.Inlightoftheseestablished mechanisms, its antimicrobial properties and its effectiveness in managing metabolic syndromes were discussed. However, significant challenges remain in translating V. amygdalina's therapeutic benefits into standardized and safe clinical applications, including variability in bioactive compound concentrations due to environmental factors and preparation methods, limited data on human bioavailability and pharmacokinetics and a lack of rigorous clinical trials demonstrating efficacy and safety. Addressing these challenges through targeted research into optimized extraction techniques, formulation development, and well-designed clinical studies are crucial. Bitter leaf represents a promising source of novel drug leads, particularly for the development of multi-targeted therapies for complex diseases. Future research should prioritize the identification and validation of specific bioactive compounds responsible for the observed effects and the elucidation of their detailed molecular mechanisms of action.

Keywords: *Vernonia amygdalina, bioactive compounds, therapeutic applications, pharmaceutical potentials, ethnomedicine.*

1. Introduction

Natural products have been critical in drug discovery/development for centuries for several therapeutic purposes [1, 2]. The potential therapeutic benefits of medicinal plant-derived compounds, including enhanced efficacy, minimized toxicity, and increased bioavailability, have revitalized research interest in plants [3, 4]. *Vernonia amygdalina*(bitter leaf) is an angiosperm that belongs to the plant family known as Asteraceae and the genus Vernonia. Of over 1000 species of this genus available, 300 species are common in Africa. Among the four species whose leaves are used as vegetable including, bitter leaf (*Vernonia amygdalina*), sweet bitter leaf (*V. hymenolepis*), star-flowered bitter leaf (*V. colorata*) and country bitter leaf

(*V. thomsoniana*); *Vernonia amygdalina* is most common because of its versatility. It thrives well in tropical Africa and Asia and grows to about 2-5 m high with oval-shaped leaves of about 20 cm [5]. The leaf extracts stems, and barks are of immense significance due to their usefulness for culinary, medical, and curative reasons in many parts of Africa and beyond. In fact, traditionally, it has been effective in the treatment of some common ailments such as fever, stomach aches, malaria, wounds, and so on. [6]. Bitter Leaf's long time track records of medicinal and curative values have earned it recognition in the pharmaceutical space. Notably, the acceptance and usefulness of bitter leaf as an alternative source of medicine is gaining recognition across different countries, including Nigeria [5, 6, 7]. This review is designed to unveil the pharmaceutical worth of *V. amygdalina* and its bioactive compounds, highlighting their potential applications in contemporary medicine.

2. Methodology

A comprehensive literature search was conducted using prominent databases, including Web of Science, Scopus, PubMed, Springer, ScienceDirect, and Google Scholar. English-language publications were exclusively utilized for this review. Relevant studies were identified by employing strategic keyword combinations and concatenation, such as "*Vernonia amygdalina* bioactive compounds," "ethnopharmacology of bitter leaf," and "*Vernonia amygdalina* pharmacology." The search yielded a range of studies examining the phytochemical, ethnobotanical, and pharmacological properties of *Vernonia amygdalina*. The retrieved articles were meticulously reviewed, with particular attention paid to their titles, abstracts, and results.


A


B

Plate 1: Vernonia amygdalina: (A) Life V. amygdalina in a garden located at Ishiagu, Ebonyi State, Nigeria (B) V. amygdalina after its leaf extraction

Chemical composition of bitter leaf

The actual chemical composition, particularly bioactive chemical compounds of bitter leaf, has yet to be fully explored. Notably, substantial scientific evidence exists concerning bitter leaf's increasing usefulness attributable to its vast bioactive compounds (3, 4). The nutritive constituents of the bitter leaf include protein, sugars, iodine, lipids, copper, iron, thiamine, vitamins A and E, etc. [7]. Aside from these nutrient components, it also harbors many secondary metabolites termed bioactive compounds, which are referred to as phytochemicals. These include saponins, coumarins, flavonoids, lignans, alkaloids, xanthones, etc. (Figure 2).

According to a study by Edo *et al.* [7], about 20 different alkaloids of bioactive significance have been detected in bitter leaves. The vast therapeutic applications or effects of the bitter leaf have been attributed to biologically important chemical compounds harbored in its leaf, stem as well as in root. Bioactive members of sesquiterpenes contained in bitter leaf are numerous; they include vernolide, vernolepin, vernodalinol, hydroxyvernolide, vernolic, vernodalin, vernomenin, and vernomydin [7, 8]. Compounds such as Lupeol and α-amyrin are examples of terpenes found in bitter leaves [7, 8, 9]. Studies have also revealed isorhamnetin, rhamnetin, luteolin 7-O-b-gluconide, and luteolin 7-O-b-glucoronoside as important examples of flavonoids in bitter leaves [2, 7, 10]. Ugbogu *et al.* [2] highlighted the vigorous

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antioxidant activities of three flavones, especially luteolin (3´,4´,5,7 tetrahydroxyflavone) present in bitter leaf extract.

According to studies, many bioactive compounds were unraveled in the classes of alkaloids, terpenoids, flavonoids, saponins, and tannins (Figure 2) [2, 7, 11-12]. Based on the studies, the solvents employed for extraction were mainly ethanol and methanol. It is imperative to note that the specific bioactive compounds present in the bitter leaf may vary depending on some factors, which include: the plant's geographic location, growing conditions, processing, and extraction methods.

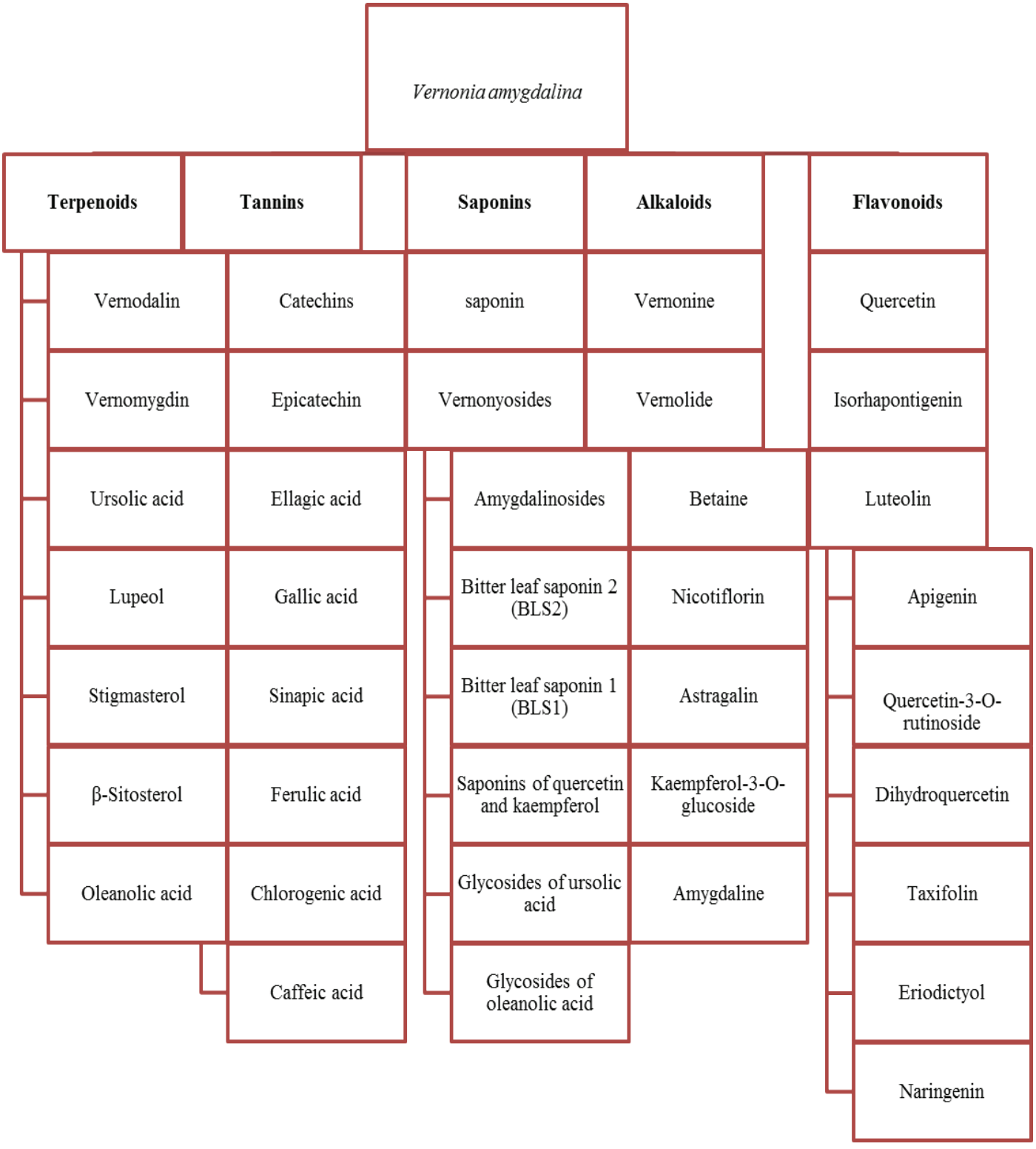

Figure 1: Bioactive chemical composition of bitter leaf

Pharmacological properties of bitter leaf and its bioactive compounds underlying principles of action

Bitter leaf contains interesting bioactive compounds with potential therapeutic applications (Figure 2). Various studies have shown that bitter leaf has significant antimicrobial activity against many bacterial, fungal (yeast inclusive), and viral pathogens: *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans*, and *Salmonella typhi*. Using chloroform and methanol extraction methods, among others, bitter leaf extracts have been reported to have antimicrobial/antifungal effects against *Staphylococcus aureus, Salmonella enterica, Bacillus subtilis, Klebsiella pneumonia*, *Toxoplasma gondii*, *Pseudomonas aeruginosa, Botrytis cinereal*, *H. contortus*. [2, 7, 9, 13-16]. The alkaloid and flavonoid constituents of the bitter leaf have been reported to be responsible for its antimicrobial activities. Edo *et al*. [7] further reported that bitter leaf generates superoxide radicals, which possess antimicrobial activity by inhibiting the growth and proliferation of human pathogens.

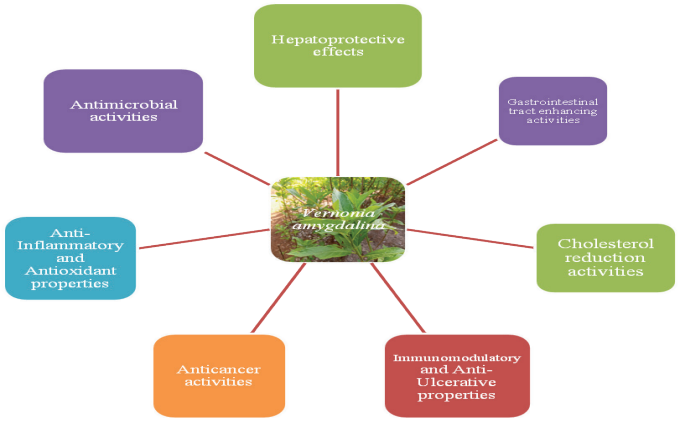

Figure 2: Pharmacological properties of bitter leaf

Many researchers have reported the antioxidant potentials of bitter leaf: in an *in vivo* investigation using mice as a model organism, the extract from the stem-bark of bitter leaf demonstrated free-radical scavenging activities against toxicity caused by acetaminophen, thus depleting the oxidative stress. A significant reduction of brain tissue oxidative stress was also reported on exposure to bitter leaf extracts. Bitter leaf extracts demonstrated antioxidant activity when administrated to a model rat exposed to arsenic-induced free radicals. However, the efficacy of the extract depends on the extraction method used; the leaf extract obtained from the methanol extraction method proved to have the most antioxidant potency when compared with acetone and water extraction methods [2, 12, 17]. It is therefore recommended that the methanol extraction method be utilized for optimal antioxidant benefits of bitter leaf extract.

Several studies have supported the anti-inflammatory potential of bitter leaves. Many bioactive compounds like cynaroside, vernonioside, and zinc oxide isolated from bitter leaf showed anti-inflammatory effects in mice. Bitter leaf extracts exhibited significant anti-inflammatory activities against inflammation, fever, and pain artificially generated in rats using carrageenan when compared to standard drugs (diclofenac) [2, 18, 19]. Bitter leaf contains bioactive compounds like tannins, quercetin, and kaempfero; they have been attributed to its antioxidants' capabilities, which scavenge free radicals, obstruct appropriate pyrogens, and shield vital body components against oxidative stress [9, 7, 20].

Researchers have reported anti-cancerous properties of bitter leaf extracts: their mechanism of action includes the cause apoptosis of infected cells, elevated cell inactivity during the mitotic phase, and obstructed cellular communication networks; the compounds in the extracts assist in suppressing the proliferation of cells. Ugbogu *et al.* [2] reported that the extract of bitter leaf stimulated the production of phosphatidylinositol-3-kinase, which mechanistically triggered the appearance of rapamycin among murine mammary carcinoma cells. Myasita *et al.* [11] further reported the inhibitory activities of bitter leaf extracts against the multiplication of human cancer cells. In the same vein, Yedjou *et al*. [21] reported the potential of bitter leaf extract to obstruct the progression of breast carcinomas. Additionally, bitter leaf extracts have been reported to cause significant depletion in the volume of MDA-MB-468 tumor cells and obstructed MCF-7 and MDA-MB-231 growth [22-23]. The anti-cancer potential of bitter leaf extract is attributed to its sesquiterpene lactones content, notably vernolide and vernodalin, which have been demonstrated to exhibit cytotoxic and ant proliferative abilities [7, 8].

The pharmacodynamics of bitter leaf extracts have also been reported in *vivo* experimentations with rats: Ugbogu *et al*. [2], while evaluating the phytochemistry, ethnobotanical, and pharmacological potentials of *V. amygdalina*, reported the anti-hepatoprotective effects of

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bitter leaf extracts on experimental mice with liver disorder induced by acetaminophen. The extract was also reported to have enhanced the antioxidant potency of certain enzymes against carbon tetrachloride (CCl4) given to mice. Bitter leaf extracts exhibited hepatoprotective effects synergistically with certain drugs, including isoniazid and rifampicin, in the mice. Bitter leaf extract generally demonstrated its ability to protect the liver and kidney against the deleterious effects of heavy metals and liver enzyme depletion, such as aspartate aminotransferase, alanine aminotransferase, and gamma-glutamyltransferase, etc. A combination of bitter leaf and red sorrel extracts with ascorbic acid showed hepatoprotective activities against rat disease conditions caused by gamma radiation (4 Gy) [2]. A combination of vitamin C and bitter leaf extracts was reported to deplete the volume of alkaline phosphatase and lipid peroxidation (LPO) [24]. These activities have been reported to be powered by the flavonoid and saponin constituents present in bitter leaf extracts [7, 20].

The therapeutic efficacy of bitter leaf has also been widely demonstrated by several studies, which have evaluated its potential medicinal applications. In the work of Yunusa et al. [25] on the cytotoxic and the genotoxicity evaluation of *V. amygdalina,* the presence of alkaloids and flavonoids in the extract of bitter leaf reportedly had immunomodulatory properties which elevated immune responses and formed a shield against gastric ulcers. Many researchers like Edo *et al*. [7] and Egharevba [26] reported strong anti-parasitic activities of bitter leaf extracts which have the ability to fight against certain protozoan diseases such as leishmaniasis, malaria, and trypanosomiasis; they stressed that the presence of terpenoids in bitter leaf extract slowed down the metabolic processes of the cell membrane, proteins and the mitochondria of the parasitic organisms. A notable decrease in parasite density of *Plasmodium berghei* was further reported in malaria-infected mice after administration of bitter leaf extract, hence highlighting the anti-malarial abilities of the aqueous and acetone-water extracts of bitter leaf leaves and roots [2]. The presence of saponin compounds, dioscin, gracillin, and pseudo-protodioscin in bitter leaf extract has been reported to have anti-rheumatic properties [27].

Bitter leaf extract has demonstrated its ability to decrease lipid serum (triacylglycerol) levels, lower blood pressure, increase blood clotting abilities as well and control immune response; these abilities are attributed to the tannins contents of the bitter leaf extract [7, 28]. Bitter leaf extract also demonstrated its ability to reduce obesity in rats treated with obesity-generating substances. Ugbogu *et al* [2] reported a significant decrease in body weight and cholesterol in the brain, as well as a reduction in insulin and leptin after the administration of bitter leaf extract to the model organism.

The anti-diabetic properties of the bitter leaf have been extensively investigated, with numerous studies revealing its potential in managing both type 1 and type 2 diabetes [2, 12, 29]. Bitter leaf extracts have been shown to significantly reduce diabetes in mice induced with streptozotocin (STZ) [12]. Furthermore, phytochemical compounds isolated from bitter leaf stem-bark, namely 6β,10β, 14β-trimethylheptadecan-15α-oyl-15-O-β-Dglucopyranosyl-1,5β-olide, have been found to lower glucose levels in rats with STZ-induced hyperglycemia [29]. In the same vein, hot water extracts of bitter leaf have been shown to inhibit α-glucosidase activity and enhance insulin secretion by stimulating β-cell activity [2, 12, 29]. The synergistic anti-diabetic effects of bitter leaf extract have also been demonstrated in combination with metformin, resulting in improved glucose control in mice with alloxan-induced hyperglycemia [12]. Moreover, a decoction of African basil and bitter leaf has been found to reduce blood glucose levels in diabetic rats [2, 12, 29].

Some researchers have also reported the purgative potentials of bitter leaf extract. Ugbogu *et al*. [2] reported the cathartic activity of bitter leaf extracts, while mice fed with charcoal meal experienced constipation, but on the administration of bitter leaf extract, there was a speed-up of catabolic process within the mice, which caused it to have a bowel movement; thus, releasing feces. Nitrobenzene was reportedly used to cause neurological disease in rats and was treated with bitter leaf extracts in methanol afterward; the results revealed its ability to enhance neuronal growth and health [30]. Studies have also uncovered bitter leaf extract's anti-diarrheal properties; diarrhea was induced in mice by exposing it to castor oil, *Vibrio cholera,* but after the administration of bitter leaf extract, there was a significant improvement in the stoppage of the diarrhea [2, 31].

Interestingly, a study conducted by Ugogbu

et al. [2] revealed that the administration of bitter leaf extracts to HIV-positive patients undergoing anti-retroviral therapy resulted in a significant increase in lymphocyte levels. Notably, the lymphocyte count was found to be dose-dependent, with higher concentrations of bitter leaf extracts yielding greater increases in lymphocyte levels. Again, the study also indicated an enhancement in immune cell levels, suggesting a potential immunomodulatory effect of bitter leaf extracts in HIV-positive individuals.

Based on scientific evidence, bitter leaf extract's vast pharmacological properties have been attributed to its numerous phytochemicals and bioactive compound contents; it is also worth noting that the method of extraction contributes to the efficacy of the extracts.

Vernonia amygdalina extracts have been shown to be therapeutic in female health: it has been reported to have uterine contraction abilities in both humans and model organisms [38, 39], as well as used in the treatment of polycystic ovarian syndrome (PCOS) in female Wistar rats [40]. Attah *et al.* [38] reported that freshly squeezed bitter leaf extract administered to women during labor aided easy labor progression, strengthened or toned the uterus muscle, and prevented complications such as pain and bleeding. Among all other plant extracts used, bitter leaf extracts yielded the highest uterine contractility of 28.3 % at 150 minutes. The contractile effect was reported to last for 2.5 to 3.5 hours, suggesting that bitter leaf extract exhibits uterotonic activity, which may facilitate easy and uncomplicated labor in humans by enhancing uterine contractions. In the work of Ijeh *et al.* [39] on the effect of leaf extracts of *Vernonia amygdalina* on the contraction of the mammary gland and uterus of guinea pigs, they reported that the extracts of bitter leaf contain a potent uterotonic agent that induced uterine contraction amplitudes in guinea pig at 100 mg/ml. They also reported that the guinea pigs exposed to varying concentrations of bitter leaf extracts produced more milk than the control groups of guinea pigs.

These assertions authenticate the reasons why traditional healers and midwives in maternity clinics and rural communities use herbal remedies to speed up contractions and childbirths.

In another study with Wistar rats, Adedoun *et al.* [40] reported that after administering extracts of bitter leaf for 14 days to female Wistar rats with PCOS, there was a significant reduction of insulin and luteinizing hormone levels, increased follicle-stimulating hormone and progesterone levels when compared with the PCOS untreated group. They also reported the reduction of Serum IL-6, CRP, and TNF-α levels in the treated group when compared with the untreated group. A significant reduction in malondialdehyde level was also reported, as well as a significant reduction in triglycerides and LDL-C levels in the bitter leaf extract-treated rats.

Therapeutic Applications

The pharmaceutical potentials of bitter leaf extracts have been extensively investigated and substantially documented in various therapeutic applications (traditional, modern, and nutraceutical); these are attributed to their unique phytochemical profile and pharmacological properties. Bitter leaf has been deployed to cure several diseases such as fever, malaria, gastrointestinal conditions, respiratory ailments, skin infections, wounds, and so on, especially in Africa and Asia, for many decades as traditional medicine. Various formulations used were infusions, decoctions, and topical [5, 9, 13]. Notably, bitter leaf has gained positive recognition as a *novel* pharmaceutical lead, showing a broad spectrum of biological activities, including antimicrobial, anti-inflammatory, anticancer, hepatoprotective, and immunomodulatory abilities [9, 13, 20, 22, 25]. Thus, according to Li *et al.* [4], the bitter leaf has been incorporated into food in the form of dietary supplements, functional foods, herbal teas, capsules, or tablets, providing certain nutraceutical advantages such as antioxidant effects [36], anti-inflammatory impacts, immune system assistance, and digestive enhancement.

Additionally, bitter leaf extracts has demonstrated strong effects against hypertension when evaluated using laboratory mice, even producing high level of synergistic activity in combination with *Ocimum gratissimun* (African basil) [7]. Some studies have affirmed bitter leaf's ability to reduce surplus calories and fat [2, 7]. Bitter leaf's bioactive compound (elotides), a hormone balancing compound enables it boost the fertility of women [2].

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The Bitter Taste of Bitter Leaf and Chloroquine: Pharmaceutical Implication

The bitter taste shared by bitter leaf and chloroquine is a result of the similar chemical structures they possess; alkaloids are partly responsible for the bitter taste. Alkaloids in bitter leaf and chloroquine possess antimicrobial properties [9, 13, 20]. Both have played critical roles in the treatment of human ailments for a long time. Chloroquine is a well-known anti-malarial drug, while bitter leaf has shown potential for anti-malarial properties [5, 25]. Regarding their taste, both activate bitter taste receptors in the tongue, thereby triggering a bitter sensation [32]. Structurally, chloroquine is C18H26ClN3 [33], while bitter leaf alkaloids (vernoline) are C21H25NO4 [9]. Chloroquine's structure consists of a quinoline ring, a chlorine atom, and a side chain containing two nitrogen atoms [33]. This quinoline ring plays a vital role in its anti-malarial properties [34]. Chlorine atom contributes to its pharmacological properties, including antimicrobial and anti-inflammatory effects [35]. On the other hand, bitter leaf alkaloid is (e.g., vernoline, C21H25NO4); the structure of vernoline comprises a sesquiterpene lactone ring, a nitrogen atom, and four oxygen atoms. According to a study, the antimicrobial and anti-inflammatory properties exhibited by vernoline are thought to be due to the presence of a sesquiterpene lactone ring [9]. Yunusa et al. [25], in their findings, suggested that the presence of four oxygen atoms in vernoline may play a role in its antioxidant effects. Consequently, the interesting pharmaceutical implications are significant. It impacts its anti-malarial property, attributing it to the quinoline ring component of chloroquine, suggesting that bitter leaf alkaloids may demonstrate similar anti-malarial effects driven by the presence of their sesquiterpene lactone ring [34].

Secondly, the antimicrobial and anti-inflammatory activities reveal that chloroquine and bitter leaf alkaloids display antimicrobial and anti-inflammatory activities because of their nitrogen-containing rings [9, 35]. Furthermore, the antioxidant abilities maintained by the bitter leaf's structure of alkaloids are probably due to being rich in oxygen, which might play a vital role in their antioxidant effects, making them different from chloroquine [25]. Regarding drug design and development, novel anti-malarial and antimicrobial agents may be possible by rigorously investigating the structural analogies and differences between chloroquine and bitter leaf alkaloids. It was noted in some studies that well-defined chemical structures may affect the pharmacokinetics and pharmacodynamics of chloroquine and bitter leaf alkaloids, influencing their bioavailability. [9, 20].

Summary of a critical analysis of the studies cited in this review article

The researchers seem to have done rigorous studies over the years covering the various aspects of bitter leaf's pharmaceutical prospects; however, there appear to be gray areas that need some attention; they are as listed below:

a. Methodological limitations

i. Lack of standardization: Many studies used different extraction methods, solvents, and concentrations, making it challenging to compare results.

ii. Limited sample sizes: Many of the studies had small sample sizes, reducing the precision of the results while broadening the margin of error in their studies.

iii. *In vitro* and *in vivo* studies: While *in vitro* studies provide valuable insights, more *in vivo* studies are necessary to confirm the efficacy and safety of bitter leaf extracts in humans.

b. Pharmacological properties

i. Antimicrobial activity: The antimicrobial activities of bitter leaf extracts against various micro-organisms are well documented. However, the mechanisms of action and the specific compounds responsible for this activity require further investigation.

ii. Anti-inflammatory activity: Bitter leaf extracts have shown anti-inflammatory activities in various studies. However, the specific compounds responsible for this activity and the underlying mechanisms require more investigation.

iii. Anti-cancer activity: The anti-cancer activities of bitter leaf extracts have been reported in several studies, although the specific compounds responsible for these activities have not been elucidated; thus, further investigations are needed to pinpoint the proteins and bioactive compounds of interest so that they can be isolated and used for pharmaceutical drug preparation at scale.

c. Therapeutic applications

i. Traditional medicine: Bitter leaf has been used in traditional medicine for various purposes. However, the efficacy, adequate dosage and safety of these uses require further investigations.

ii. Modern medicine: Bitter leaf extracts have shown potential in modern medicine, particularly in the treatment of infectious diseases, cancer, and inflammatory disorders. However, further research is necessary to confirm the efficacy and safety levels of their uses.

iii. Nutraceuticals: Bitter leaf extracts have been incorporated into food products and dietary supplements. However, the safety, dosage, and efficacy of these products require further investigation.

Future research directions on bitter leaf's pharmaceutical potential

In order to fully exploit the pharmaceutical potential of bitter leaf and unlock its broad therapeutic applications, future research should focus on the isolation and characterization of its bioactive compounds, pharmacokinetics and pharmacodynamics studies of bitter leaf-based products, clinical trials for the efficacy and safety dosage of bitter leaf based-products, toxicity and biosafety of bitter leaf based-products, and Regulatory guidelines (Figure 3).

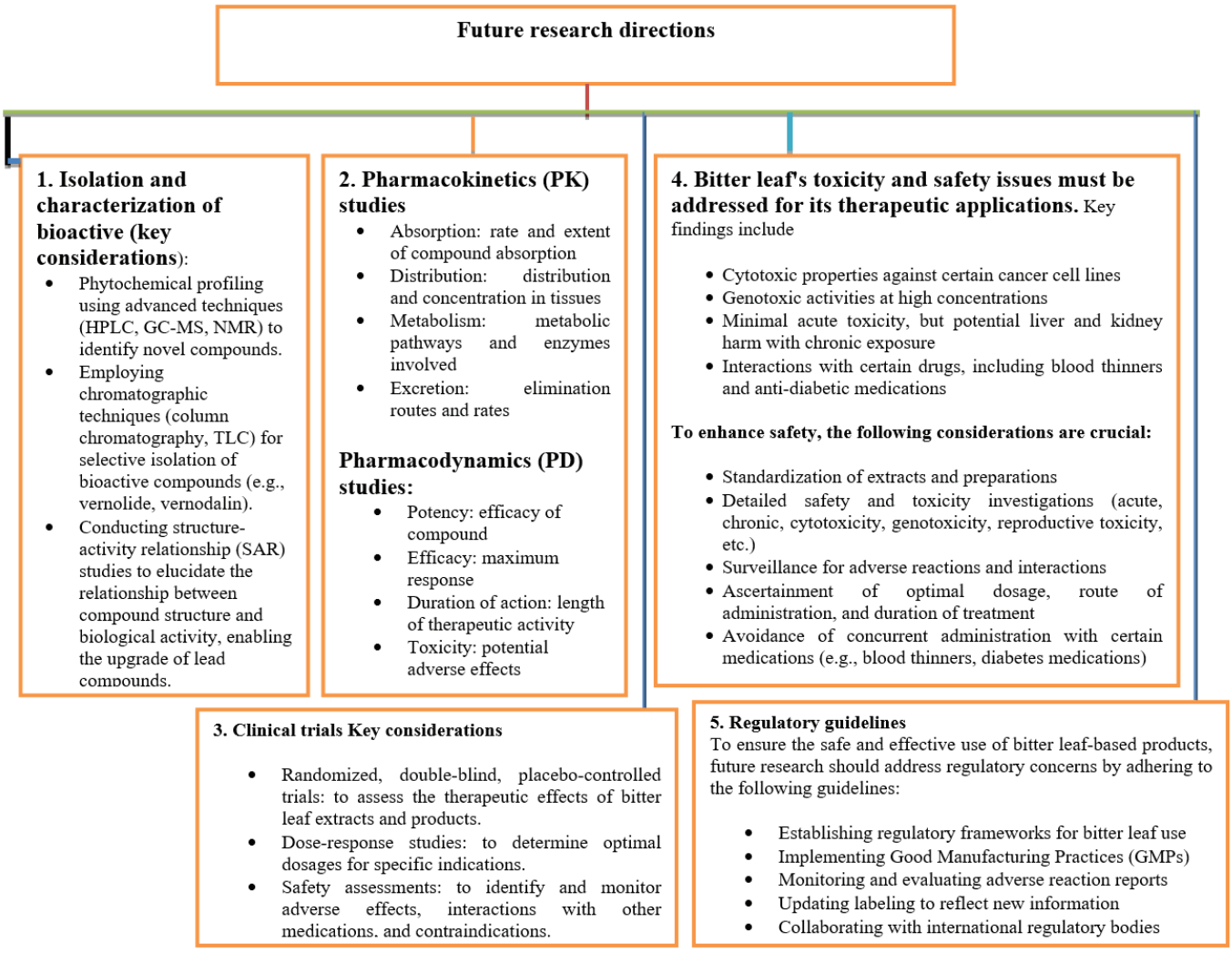

Figure 3: Future research focuses on the pharmaceutical potentials of bitter leaf

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Challenges and Opportunities

The major obstacles and prospects in tapping bitter leaf's full pharmaceutical potential are scalability and sustainability (scalability and sustainability entail producing bitter leaf-based products in large quantities without reducing the product's quality or environmental sustainability), regulatory frameworks (policy formulation and implementation for the regulation and standardization of bitter leaf-based products), and patent and intellectual property (protecting *novel*/native knowledge and inventions).

3. Conclusion

Vernonia amygdalina, generally known as a bitter leaf, has drawn notable recognition for its tremendous pharmaceutical potential. This review has comprehensively examined several bioactive compounds and pharmacological effects of bitter leaf, highlighting its potential as a therapeutic agent. Bitter leaf contains many bioactive compounds, including alkaloids, flavonoids, terpenes, and sesquiterpene lactones. However, further research is necessary to confirm the efficacy and safety of bitter leaf extracts in humans. Standardization of extraction methods, solvents, and concentrations is essential to ensure consistency in results. Additionally, *in vivo* studies and clinical trials are necessary to confirm the therapeutic potential of bitter leaf extracts.

Ethics approval
Ethics approval is not applicable; a review article.

Consent to participate
It does not apply to a review article.

Consent for publication
The authors have given their consent for the article to be published.

Availability of data and materials
As synthesized from databases.

Competing interests
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Both authors contributed equally to the production of this manuscript.

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Pancytopenia in Epidemic Dropsy: A Case Report

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ABSTRACT:

Epidemic dropsy is caused by the consumption of mustard oil contaminated with argemone oil, characterized by pitting edema of the extremities, especially lower limbs; cutaneous erythema and local tenderness; Glaucoma and other visual field defects, leading to blindness; cardiac and respiratory problems leading to death are among the most severe manifestations. A family consuming self-cultivated contaminated mustard oil suffered from dyspnoea and pedal edema; 5 of them approached the tertiary center after the demise of two members, an elderly and a kid, at home. Out of those five, three were hospitalized in the medicine department, while 2 were admitted to the pediatric department. So here we report a case series of 3 patients, residents of Prayagraj Uttar Pradesh, India, who presented in March 2023 with all the classical features of epidemic dropsy (ED), all belonging to the same family admitted in the medicine department. They had variable presentations, but surprisingly, all of them had pancytopenia, all had AKI(acute kidney injury), all had a reticular hyperpigmented rash, all landed up in noncardiogenic edema but the one with underlying Perimembraneous VSD(ventricular septal defect) had increased severity, two had renal concretions, one had visual defect and the one using it topically too, had diffuse hyperpigmented skin and hair loss as well. The adulteration of oil with argemone was confirmed by nitric acid test and ferric chloride test. Patients felt improvement in conservative management. AKI improved in 45 days on average. This is the first case report having pancytopenia in all cases never found in any single family outbreak or multiple family outbreak of epidemic dropsy.

Keywords: *Epidemic Dropsy, Mustard oil, Argemone maxicana, AKI, Pancytopenia.*

1. Introduction

Epidemic dropsy is caused by the consumption of mustard oil contaminated with argemone oil. The seeds of the weed plant Argemone Mexicana mimic mustard seeds, and the season of blooming is the same. Besides, the flowers are the same color. Thus making the removal of weed cumbersome. Moreover, mustard oil is completely miscible with argemone oil.[1-4] Epidemic dropsy has been prevalent in the community for ages now. Evidence of this dates back to the Mughal armies being affected by several diseases, including dropsy. [5]. Traditionally, the use of this plant, *Argemone mexicana*, has been studied extensively. The seeds of this plant have been used as antidotes in snake poisoning. Other uses of the plant include dermatological use, cold sores, ophthalmic problems, controlling blood sugar levels, scorpion bites, and malaria fever. The use of this plant is not limited to its seeds, but literature shows data regarding several uses of the plant oil, leaves, and yellow juice.[6]

Epidemic dropsy is characterized by pitting edema of the extremities, especially of the lower limbs, cutaneous erythema, and local tenderness. Sanguinarine and dihydrosanguinarine are the toxins in Argemone (*katkar*) oil that are responsible for the pathogenesis causing enhanced permeability and leaking of proteinaceous plasma in interstitium and dilatation of capillaries resulting in anasarca, specifically oedematous feet. [7]

2. Case report

Out of a family of 7 members from Prayagraj, five presented to our hospital with bilateral pedal edema, hyperpigmented rash, and dyspnoea for two weeks; two of them were children. They

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all were receiving treatment from the local hospital for similar complaints, but two of the family members expired during the treatment before coming to our tertiary care center. Out of the surviving five, three were hospitalized in the medicine department, while two were admitted to the pediatric department. So here we report a case series of 3 patients belonging to the same family and residents of Prayagraj Uttar Pradesh, India, who presented in March 2023 with all the classical features of epidemic dropsy (ED). The family was primarily involved in agriculture and belonged to the lower middle class. The patient's age varied from 5 to 45 years. All the members of the family were consuming oil of mustard seeds cultivated in their own land for the first time in their life from last 1 month. The clinical details of all three cases are shown in Table 1.

Table 1: shows the clinical manifestations of all the patients

CASE	1	2	3
Age/Gender	35/male	30/female	25/male
Symptoms from the last 2 weeks	B/L lower limb swelling, chest pain &dyspnoea, diminution of Right eye vision	B/L lower limb swelling and dyspnoea on exertion	B/L lower limb swelling, dyspnoea on exertion
General examination	Pallor, B/L pitting pedal edema, and hyperpigmented rash over both legs, chest, and trunk were present.	Pallor, B/L pitting pedal edema, and hyperpigmented rash all over the body were present.	Pallor, B/L pitting pedal edema, hyperpigmented rash all over the body
Cardiac examination	pan systolic murmur present	WNL	WNL
Respiratory examination	Right sided pleural effusion	WNL	WNL
Refraction Right eye Left eye-	 6/24 6/9	 6/6 6/6	 6/6 6/6
Fundus examination Right Eye- Left Eye-	premacaluar hemorrhages with peripapillary superficial retinal hemorrhages superficial peripapillary hemorrhages with few para macular hemorrhages.	No Abnormality Detected in both the eyes	No Abnormality Detected in both the eyes

B/L bilateral

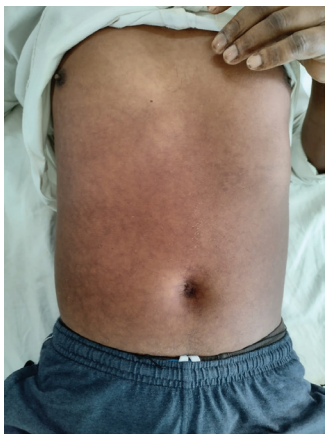

Fig 1a

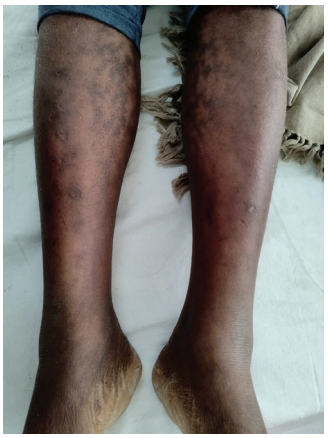

Fig 1b

Fig 2

Fig1 & 2 show the skin manifestations of the cases.

Fig 1 (a) Case 1 hyperpigmented reticular Rash over chest and abdomen
Fig 1 (b) Case 1 showing B/L pedal edema with hyperpigmented reticular rash.
Fig 2 Case 3- showing hyperpigmented reticular rash over chest and abdomen and hyperpigmentation of hands and face due to topical application of contaminated mustard oil

Patients were thoroughly investigated, and reports are shown in Table 2.

Table 2: Showing Investigations of all the cases

Investigation (Reference range)	Case 1	Case 2	Case 3
Blood tests Hb(12-16 g/dl) TLC(4000-11000cells/mm³) Platelets(1.5-4.5lac cells /mm³) MCV(84-100fl) GBP Bone Marrow Aspiration Vitamin B12(normal Range= 189-889ng/dl/) Ferritin (30-333ng/ml) Folic acid(2.5-20ng/ml)	 6.1 3600 0.55 88.6fl Normocytic normochromic anemia Hypercellular 180 45 12	 5.2 2200 0.40 78.6fl Normocytic normochromic anemia Normocellular 320 289 23	 7.9 3200 0.80 72.6fl Normocytic normochromic anemia Normocellular 280 302 16
KFT Creatinine(<1mg/dl)	 2.35	 1.78	 1.92
Urine R/M 24 hour urinary protein	Proteinuria + 956mg in 24 hours	WNL 250mg in 24 hours	WNL 390 mg in 24 hours
USG Abdomen	WNL	WNL	WNL
2D ECHO	Global hypokinesis, Severe MR, TR with Perimemberaneous VSD Left to Right shunt	WNL	WNL
Xray chest PA view	Right sided pleural effusion	WNL	WNL
Nitric acid test Ferric chloride test	Mustard Oil from home tested positive for argemone oil contamination by Nitric acid test and ferric chloride test.		

B/L bilateral, WNL-with in the normal limit, Hb-hemoglobin, TLC-total leucocyte count, MCV-mean corpuscular volume, GBP-general blood picture, Urine R/M- routine and microscopic, KFT-kidney function test, USG-ultrasonography, 2D ECHO-2-dimensional echocardiography, MR-mitral regurgitation, TR-tricuspid regurgitation, VSD-ventricular septal defect, +present, PA-posterior-anterior view.

3. Differential Diagnosis:

Food poisoning was the foremost differential, as all the members of a single family were involved. Water contamination was ruled out as other families residing around were using water from the same water supply, and none suffered. Hypoalbuminemia, congestive heart failure, wet beriberi, and nephrotic syndrome have similar clinical presentations as dyspnoea and pedal edema, but none of the above investigating reports favored. Diagnosis of noncardiogenic edema, pancytopenia, and AKI(acute kidney injury) was made in all three cases, besides a component of CHF(congestive heart failure) was seen too and more evident in case 1 as case 1 had Ventricular Septal Defect and retinal hemorrhage too was seen in Case 1.

Nitric acid test was done in our own biochemistry lab by us in which 5ml of adulterated oil was mixed with equal amount of nitric acid and was shaken. On standing acid layer turned orange. (As shown in Figure 3).

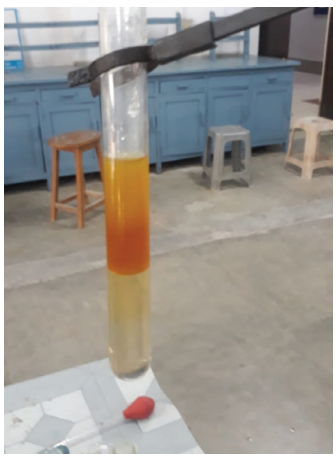

Figure 3: showing Nitric Acid test positive –orange, suggesting the presence of argemone oil

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Ferric chloride test-2ml oil and 2ml of concenterated HCL(hydrochloric acid) and heated in water bath at 33.5-35C for 2minutes, then 8 ml of ethyl alcohol added and ferric chloride was added and again heated for 10 minutes. Orange red precipitate was formed.

Thus, both tests suggested adulteration of the mustard oil with argemone Mexicana.

Levels of toxins in blood could not be tested due to nonavailability of tests.

4. Treatment

Cases were notified to the District Chief Medical Officer(CMO), and they visited the patients to interview, and surveillance was done at the hospital bedside beside their village residence area.

Case 1: was managed on IV antibiotics and Inj furosemide 40mg IV 8hrly, Tab Metoprolol 25 BD. The patient also required moist O2 inhalation at 2l through a Flexi mask for the first 3 days; then, he was off oxygen. A total of four units of PRBC were transfused. Injection of Vitamin B12 was also given. The hospital stay was for 40 days, and he was discharged on Tab Metoprolol 25 BD, Tab (Torsemide+spironolactone) 10/50 OD.

Case 2: A total of 2 units of PRBC were transfused and were managed on IV antibiotics and Inj Furosemide for 7 days only. The patient got symptomatically better after a blood transfusion. The hospital stay was 15 days. The case was discharged on Hematinics.

Case 3: No PRBC transfusion was required in case 3, and it was managed with diuretics for 7 days only. The hospital stay was 10 days.

All the three patients were given Tab Vitamin C 500 BD, Tab Zinc 50 OD, Tab B Complex BD and Tab calcium 500 BD.

5. Discussion

Dropsy is a disease that usually occurs in epidemics; isolated cases are seldom seen and reported [8]. Cases of epidemic drops are seen in northern regions of India, such as Uttar Pradesh, as in southern regions, coconut oil is consumed [9,10].

Epidemic Dropsy is seen in poor socioeconomic people as they cannot afford high-quality edible oils that are commercially tested prior with AGMARK(Agriculture Mark- Agriculture and Marketing Advisory To the Government of India, is a third-party guarantee for agricultural product) mark approving to be accordance to Indian standards. Confirmation of the adulterated oil is cumbersome, and detection of edible mustard oil being consumed necessitates laboratory investigation. Adulteration with even 1% of Argemone oil can produce manifestation. [11]

The reasons for this outbreak being restricted to a particular family can be explained as the contamination of mustard seeds with argemone seeds that occurred at the household level, where the mustard oil was extracted from the seeds of the plants cultivated on their own land. The family's low socioeconomic status and lack of knowledge prevented them from availing good quality edibles, and poor literacy was responsible for their ignorance regarding adulteration in mustard seeds being contaminated with argemone seeds, which was the source of the current outbreak. Target organs for Argemone oil poisoning include the gastrointestinal tract, heart, liver, lungs, kidney, and serum, as sanguinarine can stay in these sites for longer than 4days.[7]Dropsy patients have low levels of antioxidants, especially vitamins E and A.[12]

Sanguinarine causes inactivity of cytochrome P-450, and accumulation of sanguinarine further leads to its cumulative toxicity.[13,14] Additionally, sanguinarine acts on the Na+-K+-ATPase pump in intestinal cells, resulting in glycogenolysis and causing an accumulation of pyruvate in the blood. A similar effect is seen in the heart, which may provoke cardiac failure.[15] The commonest presentation of Argemone oil poisoning is an acute, bilateral lower limb swelling, which was found in all of our cases. Nausea, vomiting, diarrhea, mild liver dysfunction, and hepatomegaly are a frequent feature found in almost all patients, but none was seen in any of the members of this family in our case study. In extreme cases, glaucoma and even death due to cardiac arrest have been encountered.

The majority of cases reported are from groups of families, and outbreaks in single families are less in the medical literature.

Most of the cases reported had diarrhea at the onset. Surprisingly, none of our patients had diarrhea all through the course. Sunil Kumar Rao et al. in 2019[16] also reported epidemic

dropsy in a single joint family with none of the patients having diarrhea. All the patients presented with dyspnoea at the onset of pedal edema and the probable mechanism was increased permeability in capillaries due to sanguinarine. Pleural effusion was seen in case 1, who had additional congenital heart disease, i.e. Perimemberaneous VSD, and was diagnosed incidentally on Echo being indicated because of an alarming murmur he had on clinical physical examination and as a workup for dyspnoea and pedal edema even though the probable mechanism is noncardiogenic edema [17].

There is single case report in Epidemic dropsy having manifestation of AKI at the onset[18] and that too without any prior dehydrating illness like diarrhoea and vomiting in medical literature similar to that which is seen in our study and our all patient suffered from it which was reversible and improved on an average of 45-60 days and none had any dehydrating manifestation like vomiting or diarrhoea prior to it.

Regarding vision diminution, case 1 had macular hemorrhages with peripapillary superficial retinal hemorrhages in the right eye on fundoscopy with 6/24 refractive error and superficial peripapillary hemorrhages with few para macular hemorrhages in the left eye on fundoscopy with 6/9 refractive error. Kamal Singh et al. reported retinal abnormality in their case study as superficial retinal hemorrhages were present in both eyes in a boy of 12 yr and soft exudates in the left eye in a 55 yr old male in an outbreak in a single family[17]. Sunil Rao reported subconjunctival hemorrhage in one of the members of his case report.[16] Raised intraocular pressure is noticed in 0–12% of cases, and retinal hemorrhage, if present, requires urgent surgical intervention to prevent blindness [19, 20]

Our all the members of the family had pancytopenia which has never been seen in any case reports. Other etiologies of pancytopenia, was searched. Our cases didn't have hepatosplenomegaly; nutritional cause was ruled out as just only one case had borderline low Vitamin B12, rest all folate and ferritin were normal. MCV was normal in all the patients .Bone marrow disclosed no clue in any.

In our case, case 3 also had hyperpigmentation of the skin and increased hair loss as he was applying the oil to his face and hair. This darkening might be attributed to the topical application of oil to the face, leading to transcutaneous absorption of argemone oil and sanguinarine toxicity. In some of the case reports, red macules and telangiectasia have been documented, but patchy reticular hyperpigmentation in all the members of the family and diffuse hyperpigmentation because of the effect of topical application is the first time seen and reported here only.

Organs of predilection for Argemone oil include the gut, cardiac, hepatic, pulmonary, kidneys, and serum because sanguinarin persists in these sites for longer than 4days.[7].Dropsy patients have low antioxidants, especially vitamins E and A.[12].

Recovery time is about 3 months.[17]. Our patients had a history prior to admission of 15 days and remained hospitalized for one and a half months till they became symptomatically relieved, so the total duration of hospitalization and significant improvement was 2 months. The patients came in for follow-up, and total improvement took nearly 3 months.

The differential diagnosis to be considered includes nephrotic syndrome, congestive cardiac failure, myxoedema, and anemia, and all of these are usually not seen in all the family members simultaneously, except one more differential beriberi, which was ruled out as no alteration in the usual routine diet was seen. The only change in lifestyle was a change in oil consumption, and the symptoms appeared after around 15 days of consumption. In a similar case report, symptoms started after 15 days of consumption.[17]Toxins in water were not considered to be attributable as they were not seen in members of the localities who all consumed water from the same water supply. A correct diagnosis requires a high index of suspicion, as the disease is rarely seen in regular clinical practice.

As anemia was normocytic normochromic and was found in all the cases, the probable cause might be bleeding in

the gut, inactive bone marrow, and decreased red blood cell survival.[21]

Nitric acid testing and ferric chloride are the two easily done lab tests[22]

Nitric acid Tests can detect even if the concentration of argemone is as low as 0.25%. Nitric acid and Ferric Chloride Tests were done in the oil brought from their home. If argemone oil

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is present, an orange-red precipitate is formed, as in our study.

Paper Chromatographic Method – This is supersensitive, detecting as low as 0.0001% Argemone oil contamination.

The foremost need is to withdraw the culprit oil. Avoidance of exertion and leg dangling must be advised. Judicious and cautious use of diuretics with vigilance of hypovolemia and electrolyte imbalance is needed. The pleural effusion of case 1 improved with diuretics. Replenishment of antioxidants (vitamins A, C, E), Vitamin B1, Protein, and calcium must be done. AKI(acute kidney injury), in our cases, didn't need dialysis. Patients were discharged on significant improvement, and case 1 was referred to an eye surgeon for his retinal hemorrhage. Poor prognostic factors like ARDS (adult respiratory distress syndrome), heart failure, and AKI were which were seen in our cases, and besides pneumonia, can be complicated with medical literature showing a 5% mortality rate.

Suspiciousness and vigilance in the right direction is must for detection of argemone oil in mustard oil and ruling out differentials is must to reach the final diagnosis of epidemic dropsy.Timely Blood transfusions in our cases of pancytopenia was very much warranted.

Our patients were having a multitude of life-threatening manifestations such as ARDS, CHF, AKI, and debilitating vision diminution to the extent of blindness and pancytopenia.

6. Conclusion

Strong suspicion and sharp vigilance in a group of people belonging to a single family presenting with dyspnea and pedal edema unmasked the diagnosis of Epidemic dropsy with grave complications of ARDS, AKI, CHF, pancytopenia and retinal hemorrhages, and this life-threatening situation could be salvaged. This case report further highlights the need to educate farmers regarding food adulteration.

Public and Patient Involvement

The diagnosis of epidemic dropsy should be strongly suspected if there is a simultaneous complaint of dyspnea and bilateral pedal edema within the group of people sharing the same kitchen in a single family, especially where the preferred edible oil is mustard oil. Patients and the Public, especially farmers, must be educated about the Food Adulteration Act, argemone weed growing rampantly in the same season amongst mustard plants and mimicking in all aspects in appearance and smell, and the need to prevent dropsy.

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A Primer on Fabricating Bioactive Regenerative Scaffolds – From Materials and Surface Properties to Microenvironment

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ABSTRACT:

Scaffold-based regenerative medicine is based on the creation of biomaterial constructs that effectively mimic the native tissue extracellular matrix (ECM), guiding cell behavior and enhancing functional tissue regeneration. Achieving this requires a multifaceted approach that includes careful material selection, control over scaffold architecture, enhancement of bioactivity, and the application of appropriate fabrication techniques. This review offers a comprehensive exploration of these core design principles, equipping researchers with the knowledge to engineer successful scaffolds for a range of regenerative applications. The review examines a spectrum of biocompatible materials and their surface characteristics like roughness, topography, and wettability, carefully weighing their strengths and limitations with respect to mechanical properties, degradation kinetics, potential immunogenicity, and bioactivity. Furthermore, scaffold architecture—encompassing pore size, interconnectivity, and fiber alignment—that plays a crucial role in mediating cell infiltration, nutrient transport, and tissue organization will be discussed. The review also covers the different aspects of increasing scaffold bioactivity, like functionalization with cell adhesion motifs, incorporation of encapsulated growth factors, phytoconstituents, and immunomodulation to create a pro-regenerative microenvironment. Finally, the review discusses the application of various techniques like 3D printing and electrospinning, among others, in scaffold fabrication. By effectively integrating these elements, researchers can design scaffolds that not only provide structural support but also actively orchestrate the regenerative process for better treatment outcomes.

Keywords: *scaffold, biopolymer, regenerative medicine, drug loading, mechanical properties, porosity, immunomodulation, degradation, surface topography, bioactivity.*

1. Introduction

Regenerative medicine, once considered "a dream," is now a fast-developing scientific area aiming to restore, maintain, or improve damaged tissues and organs affected by disease, injury, or congenital conditions [1]. It combines various knowledge disciplines, including cell biology, materials science, and engineering principles, to offer treatment options that traditional methods lack [2]. Within regenerative medicine, tissue engineering is a strategy that makes use of scaffolds as temporary 3D frameworks similar to native tissue extracellular matrices (ECMs) that facilitate cell attachment, proliferation, differentiation, and the formation of functional tissue [3]. In the last 20 years, an increase in the number of relevant publications on the Scopus Database (Figure 1) has been noted, which indicates its importance.

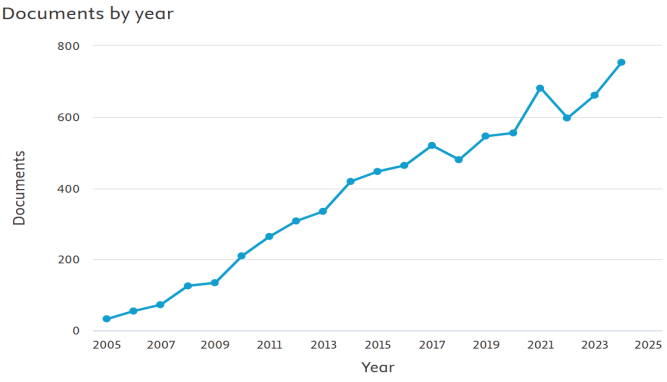

Figure 1: Number of publications over the past 20 years related to the terms "Regenerative medicine" and "Tissue engineering" and "Scaffold" on the Scopus Database.

Engineering of a successful regenerative scaffold is complex and relies on the scaffold's ability to mimic the native tissue extracellular matrix (ECM) microenvironment. This necessitates careful consideration of key design principles (like material selection, scaffold design, bioactivity, and suitable fabrication technique)

that influence cellular responses and tissue development [1, 4]. Failure to properly balance these aspects can ultimately lead to scaffold failure.

This review aims to help junior researchers explore the core design principles that govern scaffold performance in regenerative medicine. By analyzing the interplay of materials, structure, and biological activity, the review underscores the need for an integrated, interdisciplinary approach to engineer a successful scaffold. This review further explores the latest trends in material research and fabrication, aligning them with the evolving needs of regenerative strategies to address the development of scalable and cost-effective manufacturing techniques for complex scaffold designs, an area requiring further attention. Ultimately, this understanding will empower researchers to effectively develop more clinically relevant scaffolds to address tissue and organ damage.

2. Key Design Considerations

A. Material Properties:

1. Material Biocompatibility

Materials selection for scaffolds depends on the application and desired tissue properties [5]. The chosen materials dictate mechanical properties, degradation kinetics, and, critically, the host scaffold interactions through cell adhesion, and immune response [6]. Inappropriate materials selection can trigger inflammatory immune responses, that ultimately leads to scaffold rejection and treatment failure.

Materials used in any scaffold fabrication can be generally grouped into polymers (natural and synthetic), bio-ceramics, and biodegradable metals, [7] each with unique biocompatibility profiles and application suitability. Using combinations of these biomaterials in composites can open an avenue for properly tailoring scaffold properties to intended applications [8]. A summary of the popular materials used in scaffold fabrication is presented in Table 1.

a. Polymers

Polymeric biomaterials can be derived from both natural and synthetic sources. Natural polymers used in scaffolds encompass carbohydrates like alginate and hyaluronic acid alongside proteins like collagen and elastin alone or in combinations [9]. These biopolymers are widely available, generally non-toxic, and highly biocompatible due to their close resemblance to ECM [10] and provide excellent support for cell adhesion, proliferation, and differentiation [11]. However, natural polymers often suffer from sensitivity and degradation at elevated temperatures, necessitating purification to prevent immunological responses after implantation, and poor mechanical properties. Crosslinking, both chemical and physical, remains a primary method to address the mechanical limitations of natural polymers in scaffold fabrication. Chemical crosslinking, using agents like genipin (favored for its lower cytotoxicity compared to glutaraldehyde) or enzymatic methods, increases polymer network density and enhances mechanical properties. Physical crosslinking, methods like dehydrothermal treatment (DHT) or ionic crosslinking with multivalent ions, offers improvements while often maintaining better biocompatibility [12, 13].

In contrast to natural polymers, synthetic polymers provide enhanced control over scaffold properties such as mechanical properties, degradation rates, and minimized immunogenicity [14, 15]. However, they often lack inherent bioactivity, requiring surface modification or mixing with other natural polymers to facilitate cell attachment [15]. Common synthetic polymers used in tissue engineering and scaffold fabrication include PCL [16] and PLA [17], as well as PLGA [18], PEG [19], and emerging materials like poly(hydroxyalkanoates) (PHAs) [20].

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Table 1: A summary of the popular materials used in scaffold fabrication and their properties.

Material Category	Material	Advantages	Disadvantages	Applications	Ref.
Natural Polymers	Collagen	Excellent biocompatibility, cell adhesion, and biodegradability.	Batch-to-batch variability, limited mechanical strength, potential immunogenicity.	Skin, bone, cartilage, and vascular tissue engineering.	[21-25]
	Gelatin	Similar to collagen, but often more processable and readily available.	Lower mechanical strength than collagen, potential immunogenicity.	Wound healing, drug delivery, cell encapsulation.	[26-28]
	Hyaluronic Acid (HA)	Excellent biocompatibility, inherent bioactivity (CD44 receptor binding), regulates inflammation.	Rapid degradation and limited mechanical strength.	Cartilage, skin, and wound healing applications.	[28]
	Alginate	Easy gelation, biocompatible, relatively inexpensive.	Limited cell adhesion, rapid degradation in vivo.	Cell encapsulation, drug delivery, wound dressings.	[29, 30]
	Chitosan	Antimicrobial properties promote wound healing.	Variable purity, limited mechanical strength.	Wound healing, bone regeneration, drug delivery.	[31]
	Silk Fibroin	High mechanical strength, biocompatibility, slow degradation.	It can be challenging to process, potential for immunogenicity.	Bone, cartilage, and tendon/ligament regeneration.	[32, 33]
Synthetic Polymers	Poly(lactic-co-glycolic acid) (PLGA)	Biodegradable, biocompatible, tunable degradation rate.	Acidic degradation products, lack cell adhesion sites.	Drug delivery, bone regeneration, suture material.	[18, 34]
	Polycaprolactone (PCL)	Biodegradable, slow degradation rate, good mechanical properties.	Hydrophobic, slow degradation, lack of cell adhesion sites.	Bone regeneration, vascular grafts, long-term implants.	[16, 35]
	Poly(lactic acid) (PLA)	Biodegradable, biocompatible, good mechanical properties.	Brittle, slow degradation, acidic degradation products.	Bone screws, suture material, drug delivery.	[17, 35, 36]
Bioceramics	Hydroxyapatite (HAp)	Osteoconductive, biocompatible, promotes bone ingrowth.	Brittle, low tensile strength.	Bone regeneration, dental implants, drug delivery.	[37, 38]
	Tricalcium Phosphate (TCP)	Osteoconductive, biocompatible, bioresorbable.	With lower mechanical strength than HAp, the degradation rate can be difficult to control.	Bone regeneration, drug delivery.	[39, 40]
	Bioactive Glasses (BG)	Osteoconductive and bioactive promote angiogenesis.	Brittle can be challenging to process.	Bone regeneration, wound healing.	[41, 42]
Biodegradable Metals	Magnesium (Mg) and its alloys	Biodegradable, good mechanical properties, promotes bone formation.	Rapid degradation and hydrogen gas evolution can cause local alkalization.	Bone fixation screws, cardiovascular stents.	[43-45]
	Zinc (Zn) and its alloys	Biodegradable, essential trace element, antibacterial properties.	Limited mechanical strength can be brittle.	Small bone implants, wound healing.	[44, 45]
	Iron (Fe) and its alloys	Biodegradable, good mechanical strength.	Slower degradation than Mg, the potential for iron overload.	Cardiovascular stents, bone fixation devices.	[46, 47]

Composite Materials	Hydroxyapatite (HAp) / Polymer Composite	Combines osteoconductivity of HAp with tunable degradation and mechanical properties of the polymer.	It can be challenging to achieve uniform distribution of HAp, which has the potential for delamination.	Bone and dental regeneration.	[21, 26, 37, 48, 49]
	Collagen and polymer Composite	Enhanced biocompatibility promotes cell adhesion. Antimicrobial properties (chitosan), tunable mechanical properties (synthetic polymers)	Chitosan variability, limited mechanical strength, potential immunogenicity, degradation control challenges.	Wound healing, bone/ cartilage regeneration, nerve conduits.	[50-52]
	Decellularized ECM / Synthetic Polymer Composite	Provides natural ECM signals with enhanced mechanical properties and processability from synthetic components	Decellularization can change native architecture, and it can be challenging to maintain ECM bioactivity during the processing.	Vascular grafts, soft tissue, and nerve repair	[53-55]

b. Bioceramics

Bioceramics like Hydroxyapatite (HAp) and tricalcium phosphate (TCP) are widely used in dental and bone tissue engineering due to their biocompatibility, osteoconductivity, and ability to promote bone regeneration [21, 26]. While offering advantages like non-toxicity and inherent bioactivity, they are brittle and have low elasticity, promoting their use in composites for weight-bearing applications [26].

c. Metal implants

Traditional metal implants, made of stainless steel [56] and titanium alloys [57], are characterized by their strength, corrosion resistance, and cost-effectiveness [58], but their non-biodegradable nature has inspired the development of biodegradable porous metal implants, such as magnesium [43], iron [46] and zinc [44] based implants, which offer controlled corrosion properties. While these biodegradable metals offer biocompatibility and bone-like mechanical properties, they face challenges, including slow degradation and MRI incompatibility [59].

d. Composite Materials

Combining various materials in scaffold design can provide tunable properties to simulate target tissue properties. Introducing synthetic polymers, such as PLGA or PCL, often enhances the mechanical strength, degradation control, and processability of scaffolds derived from naturally sourced materials like collagen, chitosan, or alginate. However, the inherent biocompatibility of natural polymers can be compromised. Synthetic polymers may elicit an inflammatory response or generate acidic degradation products that negatively affect cell viability and tissue regeneration. Ultimately, designing successful synthetic-natural polymer blends necessitates a delicate balance to optimize mechanical properties while minimizing adverse biological responses and promoting effective tissue integration. Collagen-PLGA composites offer enhanced cell adhesion and controlled degradation [59]. Incorporating bioactive ceramics like hydroxyapatite into polymer matrices enhances osteoconductivity for bone regeneration [38]. Silk fibroin-based composites are also gaining attention due to their excellent mechanical properties and biocompatibility in cartilage regeneration [32].

It is worth noting that standardized testing and validation for demonstrating scaffold biocompatibility is essential for the successful translation of scaffold-based therapies. Available in vitro and in vivo techniques to evaluate the scaffold's biocompatibility include cell viability assays, cytokine release assays and histological analysis [60].

2. Scaffold degradation

The scaffold degradation process (including both biodegradation and biosorption) is a critical design parameter to achieve optimal outcomes in regenerative scaffolds. Ideally, a scaffold should degrade at a rate proportional to new tissue formation to ensure that the mechanical support provided by the scaffold gradually diminishes as the newly formed tissue gains its own structural integrity. Disproportionality in these rates can lead to a variety of complications where premature degradation causes scaffold collapse, while slow degradation hinders tissue remodeling.

Several factors influence the degradation rate of a scaffold, including the material composition, crosslinking density, porosity, and the presence of enzymes [36]. Synthetic polymers like

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polycaprolactone (PCL) and poly(lactic acid) (PLA) are widely used due to their controllable degradation profiles [35]. Also, it has been reported that incorporating bioceramic materials such as hydroxyapatite (HA) into polymer scaffolds in high concentrations can often lead to slower scaffold degradation rates [37].

In some cases, scaffold degradation products can promote tissue regeneration. The release of Zn^{2+} ions from degrading zinc alloys can promote osteogenesis and angiogenesis [45]. Recent studies have explored developing "smart" scaffolds that respond to microenvironmental stimuli and degrade in a controlled manner [61].

Scaffold degradation mediated by enzymes is a key contributing factor to the success of regenerative scaffolds. Collagenases, specifically matrix metalloproteinases (MMP) secreted by fibroblasts and immune cells, metabolize collagen in scaffolds such as collagen-based sponges or decellularized ECM, thereby affecting the rate of tissue integration and vascularization [62]. In addition, Esterases hydrolyze ester bonds prevalent in synthetic biodegradable polymers like poly(lactic acid) (PLA) and poly(ε-caprolactone) (PCL) [63].

Recent studies have shown that a variety of techniques can be used to monitor in vivo degradation, such as histological evaluation, imaging modalities, and biochemical assays [64]. Further research into the mechanisms of in-vivo scaffold degradation and the effects of degradation products on the host response will pave the way for the development of more effective and biocompatible regenerative therapies.

3. Mechanical Properties

In designing scaffolds, understanding how mechanical properties influence cell behavior and tissue regeneration is critical due to the mechanosensitive nature of cells. Scaffold's mechanical characteristics, such as stiffness (Young's modulus), tensile strength, elasticity, viscoelasticity, and compressive strength, may govern cell fate, ECM production, and, ultimately, the functional integrity of engineered tissues [24]. Consequently, studies to balance these mechanical attributes are essential to effectively mimic the native tissue microenvironment in engineered scaffolds. A summary of scaffold mechanical properties in relation to engineered tissue is presented in Table 2.

Scaffold stiffness plays a crucial role in directing cell behavior and tissue regeneration. Cells, being mechanosensitive, will migrate towards regions of optimal stiffness for their specific phenotype. Mesenchymal stem cells (MSCs), for instance, exhibit differential differentiation based on substrate stiffness, with softer substrates promoting neurogenic lineages and stiffer substrates favoring osteogenic differentiation. This mechanotransduction mechanism highlights the need to carefully consider the target tissue's inherent stiffness during scaffold design [65].

Furthermore, the presence of stiffness gradients within a scaffold is a feature that directs regeneration events toward mimicking the mechanical properties of native tissues. Native tissues, such as bone or cartilage, rarely exhibit uniform stiffness; instead, they display gradual transitions in mechanical properties across different regions and interfaces, providing cells with positional information and guiding their migration and differentiation.

Table 2: A summary of scaffold mechanical properties in relation to engineered tissue.

Tissue	Key Mechanical Property	Desired Range	Typical Scaffold Materials	Ref.
Bone	Compressive Strength	2-200 MPa (Cancellous) 100-200 MPa (Cortical)	Hydroxyapatite (HAp), Tricalcium Phosphate (TCP), PLGA/HAp Composites, PCL, Metals (Ti, Mg)	[66]
	Elastic Modulus	0.02-20 GPa (Cancellous) 10-30 GPa (Cortical)	HAp, TCP, PLGA/HAp Composites, PCL	
	Viscoelasticity	energy absorption during impact.	HAp/Polymer Composites, Collagen-mineral composites	
Cartilage	Compressive Modulus	0.1-10 MPa	Collagen, Hyaluronic Acid (HA), Agarose, Alginate, PCL/Collagen Composites	[67]
	Tensile Strength	1-10 MPa	Collagen, Silk Fibroin, HA	
	Viscoelasticity	load distribution and shock absorption.	HA, Agarose, Alginate, crosslinked Collagen hydrogels with specific crosslinking.	

Tendon/ Ligament	Tensile Strength	50-100 MPa	Collagen, Silk Fibroin, Electrospun Polymers (PCL, PLA), Collagen/Polymer Composites	[68]
	Elastic Modulus	100-1000 MPa	Collagen, Silk Fibroin, Electrospun Polymers (PCL, PLA)	
	Viscoelasticity	energy dissipation under dynamic loading.	Aligned Collagen fibers, PCL, and composite materials.	
Skeletal Muscle	Elastic Modulus	1-100 kPa	Hydrogels (Fibrin, GelMA, PEG), Electrospun Polymers	[68]
	Tensile Strength	0.1-1 MPa	Hydrogels (Fibrin, GelMA, PEG)	
	Viscoelasticity	muscle's ability to withstand contractions.	Fibrin, GelMA, composites with tunable degradation.	
Vascular Tissue	Tensile Strength	1-3 MPa (Artery)	Elastin, Collagen, PCL, Decellularized Vessels, Elastin-like Polypeptides (ELPs)	[69]
	Elastic Modulus	0.1-10 MPa (Artery)	Elastin, Collagen, PCL, Decellularized Vessels, ELPs	
	Viscoelasticity	damping pulsatile blood flow and preventing aneurysms.	Elastin-rich materials, dynamically crosslinked polymers.	
Neural Tissue	Elastic Modulus	0.1-10 kPa	Hydrogels (Agarose, Hyaluronic Acid, PEG), Self-Assembling Peptides	[70]
	Compressive Modulus	Similar to Elastic Modulus	Hydrogels (Agarose, Hyaluronic Acid, PEG), Self-Assembling Peptides	
	Viscoelasticity	Plays a role in neuronal signaling and axonal guidance.	Self-assembling peptides, very soft hydrogels	

Processes. In this context, scaffolds engineered with stiffness gradients create a more biomimetic environment that can trigger different intracellular signaling pathways through varying mechanical forces promoting controlled tissue regeneration [71].

Tensile strength, the capacity of a scaffold to resist breaking under tension, is also a fundamental determinant of scaffold performance. A scaffold with low tensile strength will prematurely fail under physiological loads, compromising its structural integrity and impeding tissue regeneration. A scaffold's tensile strength requirements vary considerably depending on the target tissue, reflecting the diverse mechanical demands of different organs [72]. For highly tensile tissues like tendons and ligaments, scaffold tensile strength is paramount to withstand physiological loads during movement [73]. In vascular tissue engineering, scaffolds must maintain structural integrity while preventing aneurysmal dilation or rupture of engineered blood vessels under pulsatile blood pressure [74]. In bone regeneration, higher tensile strength correlates with slower degradation, which is important where the scaffold needs to provide long-term support for bone ingrowth and mineralization [75]. For skin regeneration, adequate tensile strength in dermal scaffolds provides a stable platform for fibroblast infiltration, growth, and production of ECM, preventing wound contraction and promoting a natural skin architecture [76].

Elasticity, defined as a material's ability to return to its original shape after deformation, directly impacts the performance of scaffolds in tissue engineering applications. For highly elastic tissues such as arteries and lung alveoli, the scaffold must exhibit sufficient elasticity to withstand repetitive cycles of expansion and contraction without permanent deformation, ensuring long-term structural integrity and functionality [75]. Furthermore, scaffold elasticity directly influences cellular mechanotransduction. Cells adhere to the scaffold via integrins, forming focal adhesions that link the scaffold matrix to the intracellular cytoskeleton. The scaffold's elastic behavior then creates a biomimetic microenvironment that influences intracellular signaling pathways that regulate cell proliferation and differentiation [77] to promote successful tissue regeneration.

Viscoelasticity, which describes a material's time-dependent response to applied stress, affects scaffold performance by influencing and modulating cell-matrix interactions within the scaffold [78]. Viscoelastic scaffolds exhibit energy dissipation, with a portion of the energy being dissipated as heat due to internal friction. The significance of viscoelasticity stems from its ability to more closely mimic the mechanical behavior of many native tissues, particularly those subjected to complex, dynamic loading [79]. Cartilage, a highly viscoelastic tissue, exhibits a time-dependent response to compressive forces, allowing it to efficiently

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dissipate energy during joint loading. Scaffolds that mimic this viscoelastic behavior can better protect chondrocytes from excessive mechanical stress, promoting matrix synthesis and preventing tissue degradation [79].

At the cellular level, the viscoelastic properties of a scaffold can directly modulate the dynamics of cell-matrix interactions. Cells respond not only to the magnitude of the applied force but also to the rate at which the force is applied, a feature that is inherently encoded in viscoelastic materials [80]. For example, studies have shown that viscoelastic substrates can promote enhanced integrin clustering and focal adhesion formation, leading to increased cell adhesion and spreading [81], and can provide dynamic mechanical guides that promote stem cell differentiation along specific lineages, such as osteogenesis or chondrogenesis [30].

Compressive strength, defined as a material's ability to withstand axial compressive loads before failure, is a critical mechanical property in scaffold design. A scaffold with insufficient compressive strength will deform or collapse under physiological loading conditions, compromising its structural integrity and hindering tissue regeneration. Bone scaffolds, for example, must possess adequate compressive strength to support weight-bearing and promote bone ingrowth [82]. Similarly, cartilage scaffolds must withstand compressive forces within joints, maintaining joint space and facilitating shock absorption [31]. In addition, compressive strength also influences cellular behavior within the scaffold. For instance, compressive forces can enhance chondrocyte differentiation and promote the synthesis of cartilage-specific extracellular matrix components [83]. Therefore, matching the compressive strength of the scaffold to the target tissue's mechanical demands is crucial for successful integration and long-term functionality.

4. Surface Properties

The design of regenerative scaffolds depends not only on bulk properties but on scaffold surface characteristics as well, where the scaffold-cell interactions govern the overall success of the regeneration process. Key factors that influence cell fate on scaffolds include surface topography (particularly roughness and surface pits/grooves) and surface chemistry (particularly wettability and functional groups). A summary of scaffold surface properties in relation to cell adhesion and differentiation is presented in Table 3.

Studies have demonstrated the significant influence of scaffold topography on cell behavior [71]. Implants with nano grooves/pits exhibit enhanced bone ingrowth compared to smooth surfaces. Furthermore, the size and distribution of surface features can influence cell adhesion, with certain configurations promoting enhanced osteoblast attachment. Surface roughness also differentially affects cell types, with osteoblast proliferation increasing with roughness while fibroblast proliferation decreases [84].

Scaffold surface chemistry also plays a crucial role in cell adhesion. Generally, an increase in scaffold hydrophilicity promotes osteoblast adhesion, while fibroblast adhesion peaks at intermediate wettability. Conversely, hydrophobic surfaces may hinder cell adhesion and can even promote microbial biofilm formation. In addition, hydrophilic surfaces also facilitate protein adsorption and cell spreading, fostering a more favorable environment for cell growth. An excellent review by Idaszek J. et al. discusses the effect of surface properties on scaffold performance [85].

Furthermore, scaffold surface modification can improve biocompatibility and enhance regenerative outcomes. By strategically modifying the scaffold surface, researchers can create a microenvironment that provides control over cell adhesion, spreading, migration, differentiation, and the production of extracellular matrix. Surface modification strategies can be generally classified into physical, chemical, and biological modifications [86].

Table 3: A summary of scaffold surface properties in relation to cell adhesion and differentiation.

Surface Property	Impact on Cell Attachment	Impact on Cell Differentiation	Modification Techniques	Ref.
Surface Roughness (Ra, Sq)	Increased roughness generally enhances cell adhesion by providing more surface area for cell attachment and promoting integrin clustering. Nanoroughness can be particularly effective.	It can direct cell fate. Certain lineages (e.g., osteogenic) are often enhanced on rougher surfaces.	Sandblasting, acid etching, plasma etching, micro/nanofabrication, self-assembling nanostructures.	[87, 88]
Surface Topography (Grooves, Pits, Nanopatterns)	Aligned features (e.g., grooves, nanofibers) provide contact guidance cues, directing cell alignment and elongation.	Can influence cell lineage commitment and function. Aligned features can promote differentiation along specific lineages (e.g., tenogenic, myogenic).	Micro/nanofabrication (e.g., lithography, etching), electrospinning, microcontact printing.	[39, 84]
Wettability (Hydrophilicity/ Hydrophobicity)	Hydrophilic surfaces generally promote protein adsorption, which is crucial for initial cell attachment.	It can influence the lineage commitment of stem cells. Some lineages (e.g., fibroblast) prefer moderately hydrophilic surfaces.	Plasma treatment, surface grafting of hydrophilic polymers (e.g., PEG), self-assembled monolayers (SAMs) with hydrophilic terminal groups.	[89, 90]
Surface Charge (Positive/Negative)	Influences protein adsorption (charge-charge interactions). Positive charges can enhance cell attachment in some cases.	It can affect signaling pathways involved in cell differentiation.	Plasma treatment, surface grafting of charged polymers (e.g., poly(acrylic acid)), chemical modification with charged functional groups.	[91]
Growth Factor Immobilization	Enhances cell recruitment and differentiation by providing localized growth factor signaling.	Directly promotes differentiation by activating specific growth factor receptors and downstream signaling pathways.	Physical adsorption, covalent grafting, encapsulation within micro/nanoparticles.	[92, 93]

Physical methods, such as plasma treatment, surface roughening, and micro/nanofabrication, alter the scaffold's topography and roughness to influence cell adhesion and alignment. It was found [94] that increasing the roughness of a titanium implant surface can promote osteoblast adhesion and the subsequent growth of bone tissue while creating aligned microgrooves on a polymer scaffold can guide cell orientation and the deposition of extracellular matrix in anisotropic tissues like muscle or nerve [95].

Chemical methods involve introducing or modifying functional groups on the scaffold surface. This affects properties like wettability, which can improve protein adsorption and cell adhesion. In addition, a surface charge can influence the recruitment of specific proteins or cells. Techniques like chemical self-assembled monolayers and surface grafting can be used to create surfaces with specific chemical functionalities [86].

Finally, biological methods involve immobilizing bioactive molecules such as cell adhesion peptides directly onto the scaffold surface [87]. This technique allows control over cell signaling to promote very specific cellular responses. The optimal surface modification strategy will depend on the specific needs of the tissue that is being engineered and the precise cellular behaviors that need to be encouraged.

B. Scaffold Architecture:

While material selection and bioactivity are crucial for proper scaffold design, it is the architecture of the scaffold that exerts a dominant influence on its clinical success. Scaffold architecture features like porosity and fiber alignment will be discussed.

1. Porosity

Porosity exerts a dominant influence on the scaffold microenvironment and, consequently, its performance. The pore's size and interconnectivity control nutrient and waste transport through the scaffold and cell infiltration.

a. Pore size

The importance of pore size in facilitating cell migration, nutrient diffusion, and vascularization

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within the scaffold for successful tissue regeneration is well recognized. Macropores create pathways for cells to migrate into the interior of the scaffold, access nutrients, and get rid of metabolic waste products. It is noteworthy that the optimal pore size can vary depending on the specific tissue being engineered. For bone tissue, studies suggest that a combination of smaller pores (50-100 μm) to promote initial cell attachment and larger pores (200-400 μm) to enhance nutrient diffusion and angiogenesis is most effective. For other tissues, such as skin, smaller pores (1-12 μm) have demonstrated the greatest support for cell attachment [96, 97].

b. Interconnectivity

Similar to pore size, the interconnectivity of scaffold pores is also crucial. While pore size dictates cellular accessibility, interconnected pores establish continuous pathways for mass transport throughout the scaffold 3D structure, enhancing diffusion and transport of essential nutrients to cells deep within and facilitating the efficient removal of metabolic waste products, preventing accumulations that can inhibit cellular function, leading to apoptosis or necrosis, and compromise extracellular matrix (ECM) synthesis. In this context, pores interconnectivity is essential for maintaining cellular viability and promoting proliferation [98].

In large, complex scaffolds with increased diffusion distances, pore interconnectivity becomes more significant in preventing nutrient depletion and waste accumulation in the scaffold core by providing efficient mass transport pathways across the scaffold volume. This transport system helps keeping a proper microenvironment across the scaffold, promotes uniform cellular distribution, robust tissue formation, and long-term functional integration [99]. However, scaffold porosity can compromise its mechanical properties. This creates a necessity to balance scaffold requirements for the design of effective scaffolds.

2. Fiber Alignment

Many native tissues, including nerves, muscles, tendons, and blood vessels, exhibit highly organized microstructures in which cells and ECM components are aligned in a specific direction (anisotropic). This precise alignment is vital for imparting the required functional mechanical characteristics, facilitating cell communication, and, ultimately, ensuring proper tissue function. In this context, fiber alignment within regenerative scaffolds (mimicking the anisotropic architectures of native tissues) exerts a significant influence on cellular organization and function. Scaffolds with controlled fiber alignment guide cell interactions within the scaffold by providing topographical signals that influence extracellular matrix (ECM) deposition, induce cell directional orientation, promote cell-cell communication along the longitudinal axis, and mirror the native tissue structure [100].

The effects of fiber alignment are particularly relevant in musculoskeletal system regeneration. In tendon and ligament engineering, it was reported [101] that aligned electrospun nanofibers were able to guide stem cell differentiation toward the tenocyte lineage, fostering the development of longitudinal organization of collagen fibrils with enhanced mechanical properties and the upregulation of tenogenic markers in seeded cells. Furthermore, in neural tissue engineering, aligned nanofibers were found to create guidance templates for axonal extension, promoting directional nerve regeneration [102].

C. Bioactivity

Regenerative scaffolds provide an artificial ECM for cells to adhere, proliferate, and differentiate. The ability of cells to interact effectively with the microenvironment within the regenerative scaffold (bioactivity) directs the formation of new tissue. Consequently, increasing scaffold bioactivity can help achieve scaffold functional success. Various strategies are adopted to increase scaffold bioactivity, including the incorporation of cell adhesion motifs, growth factors, immunomodulators, and the addition of encapsulated phytoconstituents. A summary of scaffold bioactivity strategies is presented in Table 4.

Table 4: A summary of the scaffold bioactivity strategies.

Bioactivity Strategy	Mechanism of Action	Examples	Benefits	Limitations	Representative Tissues Studied	Ref.
Incorporation of Cell Adhesion Motifs	Promotes cell attachment and spreading by providing specific binding sites for integrins	-RGD (Arginine-Glycine-Aspartic acid) peptides -YIGSR (Tyrosine-Isoleucine-Glycine-Serine-Arginine) peptides	Enhances cell adhesion, migration, and ECM production; improves cell-scaffold integration; enables selective cell binding through specific motif choice.	It may not be sufficient for all cell types or tissue types, requiring combinations of motifs; it can be costly to synthesize peptides; motif presentation and accessibility can be challenging; and susceptibility to enzymatic degradation.	Bone, Cartilage, Skin, Neural Tissue	[103-105]
Growth Factor Delivery (Controlled Release)	It provides tissue-specific localized and sustained stimulation of cell proliferation, differentiation, and angiogenesis.	- BMP-2 (Bone Morphogenetic Protein-2) - VEGF (Vascular Endothelial Growth Factor) - TGF-β (Transforming Growth Factor-β) - NGF (Nerve Growth Factor)	Promotes targeted cell responses, enhances tissue formation, accelerates healing; allows for precise control over GF dose and release kinetics.	Requires careful optimization of release kinetics and dose to avoid supra-physiological levels; potential for off-target effects if GF diffuses away from the intended site; GFs can be unstable and prone to degradation.	Bone, Cartilage, Vascular Tissue, Neural Tissue, Wound Healing	[106-109]
Immunomodulation	Twists macrophage polarization from pro-inflammatory (M1) to pro-regenerative (M2); reduces inflammation and promotes tissue repair.	- Incorporation of M2-polarizing cytokines: (IL-4, IL-10) - Use immunomodulary biomaterials (Decellularized ECM, hyaluronic acid, and chitosan).	Enhanced tissue regeneration; reduced fibrosis and scar formation; improved cell survival; modulated host-graft response.	Potential for off-target effects; requires careful control of cytokine dosage and release kinetics; dECM can be immunogenic if not properly processed; long-term effects on immune system not fully understood.	Bone, Cartilage, Wound Healing, Spinal Cord Injury, Vascular Grafts	[110-112]
Incorporation of Phytoconstituents	Modulate cell behavior through reduced oxidative stress and promote wound healing through natural bioactive compounds.	- flavonoids and essential oils (antioxidant, anti-inflammatory) - Aloe vera (wound healing)	Enhanced cell proliferation, differentiation, and migration; reduced inflammation and oxidative stress; improved angiogenesis and ECM deposition; can have antimicrobial properties.	Bioavailability limitations; potential for cytotoxicity at high concentrations; stability issues; batch-to-batch variability; some have limited aqueous solubility.	Skin, Wound Healing, Bone Regeneration, Nerve Regeneration	[113-115]

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1. Cell Adhesion Motifs

Cell adhesion motifs are short peptide sequences derived from ECM proteins, that mimic the natural signals that cells utilize to attach to and interact with their surroundings, thereby, influencing cell morphology, migration, gene expression, and ultimately, tissue organization.

Integrins, a family of transmembrane receptors, are the primary mediators of cell adhesion to the ECM. Integrins recognize and bind to specific amino acid sequences within ECM proteins, triggering intracellular signaling pathways that regulate cell behavior. As such, incorporating integrin-binding motifs such as arginine-glycine-aspartic acid (RGD) sequence (found in a variety of ECM proteins, including fibronectin, vitronectin, and laminin) can effectively promote cell adhesion, spreading, and migration on a wide range of scaffold biomaterials. This sequence is one of the most widely studied cell-adhesive motifs and is recognized by several different integrins. RGD functionalization has also proven valuable in stimulating the differentiation of mesenchymal stem cells [33].

The method of presenting cell adhesion motifs is also an important consideration. Simply incorporating adhesion ligands into the scaffold bulk may not be sufficient to promote cell adhesion, as they may be buried within the material core and inaccessible to cells. Surface modification methods like plasma treatment, chemical grafting, and layer-by-layer assembly can be used to ensure proper surface presentation [116]. Continued research into novel ECM-derived motifs, delivery strategies, and the synergistic effects of multiple bioactive signals will pave the way for the development of even more effective and clinically relevant regenerative therapies.

2. Growth Factor Delivery

Growth factors (GFs) are naturally occurring signaling proteins that play an essential role in almost all aspects of scaffold biological performance, regulating cell proliferation, differentiation, migration, and ECM synthesis. Although integrating growth factors within scaffolds can be challenging due to the inherent problems of bioavailability, short half-life, and potential side effects, their delivery from biomaterial scaffolds is swiftly becoming a vital research topic for promoting tissue regeneration. The choice of growth factor and its delivery strategy are highly tissue-specific. Scaffolds serve as vehicles for delivering GFs to the site of tissue regeneration. The controlled release of GFs from scaffolds is crucial, as the timing and dose can profoundly affect cell response, including cell recruitment, tissue-specific differentiation, and tissue development [117].

Delivery of GFs ranges from simple GF incorporation within the scaffold matrix to sophisticated micro- or nano-encapsulation that provides sophisticated control over the GFs release within scaffolds, enhancing their regenerative potential [118]. Microparticles and nanoparticles can encapsulate GFs within a variety of materials, including polymers, lipids, and inorganic materials, protecting the GFs from degradation and enabling sustained release profiles. These systems are integrated into the scaffold structure, allowing for localized GF delivery [119]. PLGA microspheres loaded with bone morphogenetic protein-2 (BMP-2) were found to be successful in bone regeneration, providing a controlled release of BMP-2 that promotes osteoblast differentiation and bone formation [106]. Transforming growth factor-β (TGF-β) has been incorporated into hydrogels for cartilage regeneration, promoting chondrogenesis and cartilage matrix synthesis [120]. In addition, the liposomal hydrogel was found to deliver vascular endothelial growth factor (VEGF) in a sustained manner in order to enhance the osteogenesis of MG-63 cells [121]. Overall, growth factors have become important means of promoting certain cellular behaviors by directing cells toward desired outcomes.

3. Immunomodulatory signals.

The host's immune response to the implanted scaffold and/or its degradation products represents one of the primary concerns for developing a successful regenerative scaffold [22]. Scaffolds can trigger immune responses, directing macrophage polarization towards either pro-inflammatory M1 or pro-regenerative M2 phenotypes. This property is a key factor that governs the overall healing outcomes. M1 macrophages release pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6, promoting chronic inflammation and scaffold failure, while M2 macrophages secrete anti-inflammatory cytokines such as IL-10 and TGF-β that promote tissue repair, angiogenesis, ECM remodeling, and resolution of inflammation, making them highly desirable in regenerative settings. Consequently, the modulation of macrophage polarization towards the M2 phenotype is an increasingly desirable strategy in scaffold design.

Several approaches are being explored to achieve this immunomodulation. Certain biomaterial

hydrogels, such as hyaluronic acid and chitosan, possess inherent immunomodulatory properties, capable of steering the immune response toward a pro-regenerative state [122]. The use of decellularized ECM (dECM) holds significant promise, as it inherently contains tissue-specific growth factors and ECM proteins that can influence macrophage behavior [123].

4. Phytoconstituents

Incorporating phytoconstituents such as flavonoids and polyphenolic compounds in wound healing scaffolds significantly enhances their regenerative potential. They act as antioxidants, reduce inflammation, and promote a wound healing microenvironment. They offer distinct advantages of biocompatibility and complex mechanisms of action. Optimized phytoconstituents loading, release kinetics, and loaded scaffold degradation profiles are all responsible for attained therapeutic efficacy [124, 125].

In addition, encapsulating essential oils (EOs) within scaffolds also represents a promising strategy for enhancing their performance in a multifaceted approach. First, many EOs exert antimicrobial activity (disrupt bacterial cell membranes and inhibit biofilm formation), creating a cleaner environment that promotes cell survival and proliferation within the scaffold, reducing the risk of scaffold-associated infections, a significant barrier to successful tissue integration. In addition, many EOs possess anti-inflammatory properties, reducing the expression of pro-inflammatory cytokines and promoting an immunomodulatory shift towards a pro-regenerative immune response and preventing chronic inflammation, which can hinder tissue repair and lead to fibrosis. Also, certain EOs promote the release of vascular endothelial growth factor (VEGF), stimulating angiogenesis essential for nutrient supply and waste removal within the scaffold [126].

Encapsulation of EOs from scaffolds not only protects EOs from degradation but also allows for sustained and localized delivery, maximizing their therapeutic effect while minimizing potential cytotoxicity associated with high concentrations. Various encapsulation methods, including microencapsulation, nanoencapsulation, and complexation with cyclodextrins, are employed to create EO-loaded delivery systems suitable for incorporation into a variety of scaffold materials [127]. This multifaceted modulation of the scaffold microenvironment by EOs helps increase their bioactivity for successful tissue regeneration and functional integration.

4. Fabrication Techniques

The proper selection of a suitable fabrication technique directly influences the scaffold's porosity, mechanical properties, and drug delivery capabilities, all of which are vital to the proper direction of cell behavior and tissue regeneration. In this context, the ideal fabrication method must be carefully considered with respect to the specific requirements of the target tissue and the desired scaffold characteristics, in addition to the scalability and cost-effectiveness of the technique for eventual clinical translation [128]. A summary of common scaffold Fabrication Techniques is presented in Table 5.

Traditional fabrication methods are simpler and more accessible and still present potential value. Solvent casting and particulate leaching are foundational for creating porous scaffolds with controlled pore size, are useful in bone regeneration, and provide a basic template for osteoblast attachment. Gas foaming, known for avoiding organic solvents, is suitable for enhancing biocompatibility in scaffolds designed for soft tissue applications. Freeze-drying, or lyophilization, creates highly porous structures, mimicking the architecture of ECM and serving as a versatile starting point for deposition and cell infiltration in bone and skin regeneration. Each technique, though established, continues to be refined and adapted for novel applications [128, 129]. Advanced fabrication technologies, on the other hand, provide broader outcomes and versatility. Electrospinning creates nanofiber scaffolds with control over fiber alignment, which is essential for directional cell organization in anisotropic tissues like tendons, ligaments, and nerves. 3D printing, encompassing techniques like fused deposition modeling, stereolithography, and bioprinting, enables the creation of complex, customized scaffolds tailored to the patient's anatomy.

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Table 5: Summary of common scaffold Fabrication Techniques.

Fabrication technique	Process description	Advantages	Limitations	Examples	Ref.
Solvent casting/particulate leaching	The polymer solution is cast into a mold with porogen (salt, sugar); the solvent evaporates, and the porogen leaches out.	Simple, cost-effective, controlled pore size and porosity.	Poor pore interconnectivity hinders nutrient transport in larger constructs, as well as difficult, complex geometries and potential solvent contamination.	PLGA scaffolds for non-load-bearing bone regeneration.	[129-131]
Gas foaming	Polymer saturated with gas (CO_2) under pressure; pressure release creates pores.	Avoids organic solvents, potentially improving biocompatibility.	Difficult to control pore size and interconnectivity; often, closed-pore structures restrict cell infiltration and nutrient diffusion.	PCL scaffolds for soft tissue regeneration.	[129, 132]
Freeze-drying (lyophilization)	The polymer solution was frozen, and the solvent was sublimated under a vacuum.	Simple, versatile; high porosity promotes cell infiltration.	Limited control pore size and interconnectivity; anisotropic pore structures restrict cell migration and ECM deposition.	Collagen scaffolds and skin regeneration.	[129, 133, 134]
Temperature-induced phase separation (TIPS)	Polymer solution undergoes liquid-liquid or solid-liquid phase separation upon cooling, followed by solvent extraction.	Good control over pore size and morphology; highly interconnected porous structures can be achieved, which promotes cell infiltration and nutrient transport.	It can be challenging to remove all solvents; mechanical properties can be limited.	PCL, PLGA, and collagen scaffolds for various tissue engineering applications.	[129, 135, 136]
Electrospinning	The polymer solution is ejected through a spinneret under high voltage, forming nanofibers.	High surface area promotes cell adhesion, and controlled fiber alignment guides cell orientation.	Limited control of pore size and porosity hinders cell infiltration and transport.	PU scaffolds for vascular grafts.	[129, 137]
3D printing (additive manufacturing)	Digital design builds scaffolds layer by layer (FDM, SLS, SLA, bioprinting).	Precise control geometry, pore size/interconnectivity, promotes optimal cell infiltration, nutrient transport & material composition; customized scaffolds provides control over mechanical properties.	Time-consuming, expensive, specialized equipment, material limitations.	PCL bone scaffolds, hydrogel cartilage scaffolds.	[129, 137, 138]
Decellularization	Cells removed from native tissues/organs, leaving ECM scaffold.	Retains natural ECM architecture, composition, and biomechanical properties, which provide a natural microenvironment.	Difficult complete decellularization, potential immune response, limited control geometry and properties.	wound healing, heart valves for valve replacement.	[123, 139, 140]

Combining different scaffold fabrication techniques is quickly gaining interest in overcoming the limitations of individual methods and creating scaffolds with tailored properties for complex applications. Examples of commonly combined fabrication techniques are listed in Table 6. By synergistically integrating multiple approaches, researchers can achieve greater control over scaffold architecture, mechanical properties, and bioactivity. These hybrid approaches leverage the strengths of each technique, resulting in scaffolds that better mimic the complex microenvironment of native tissues and promote enhanced tissue regeneration.

As the field advances, further research is needed to develop novel fabrication techniques, optimize existing techniques, and translate these promising technologies into clinical applications.

5. Conclusion

Successful scaffold design for regenerative medicine requires a meticulous balance of material properties, geometry, bioactivity, and selected fabrication technique. To optimize material properties, continued research into biocompatible materials is crucial for minimizing adverse host responses, while controlled degradation mechanisms are necessary to ensure scaffold breakdown aligns with new tissue formation. Matching scaffold mechanical properties to native tissue properties is vital for creating biomimetic environments that promote appropriate cell responses. Regarding geometry, advanced fabrication methods are critical for achieving precise control over architecture, porosity, and pore interconnectivity, facilitating optimal cell infiltration and nutrient transport. Simultaneously, ongoing investigations into microfabrication techniques allow for the creation of surface features that enhance cell adhesion and direct cell behavior. Ultimately, optimizing scaffold bioactivity depends on research into novel ECM-derived motifs for cell signaling, innovative delivery strategies for growth factors and therapeutic agents, and a deeper understanding of the synergistic effects of multiple bioactive signals. A careful selection and implementation of suitable fabrication techniques are critical for improving tissue regeneration outcomes and successful clinical translation.

Table 6: Examples of Combined Scaffold Fabrication Techniques.

Combination of Techniques	Key Benefits Achieved	Target Tissue	Ref.
3D Printing + Electrospinning	Precise macropore architecture (3D printing) combined with aligned nanofiber guidance (electrospinning) for enhanced osteoblast differentiation and bone ingrowth.	Bone	[137, 141, 142]
Freeze-Drying + Gas Foaming	High porosity from freeze-drying, combined with interconnected pores, is achieved through gas foaming.	Bone	[143, 144]
Electrospinning + Solvent Casting/ Particulate Leaching	Aligned nanofibers for chondrocyte alignment, combined with macroporous structure for nutrient transport.	Cartilage	[145]
Electrospinning + Decellularization	Aligned nanofibers for chondrocyte alignment and decellularized ECM for enhanced biocompatibility and cell-specific cues	Cartilage	[146]
3D Printing + Decellularization	3D printed support structure providing mechanical strength, combined with decellularized ECM for enhanced biocompatibility and cell-specific signals.	Vascular Graft	[147, 148]
Electrospinning + Bioprinting	The electrospun layer provides dermal support, combined with a printed epidermal layer for enhanced skin regeneration.	Skin	[149, 150]

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Dose-dependent response of metformin in enhancing motor performance and dopamine release in C57BL/6 mice afflicted by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)

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ABSTRACT:

Introduction: *Clinically, metformin has been used as a cornerstone medicine in blood sugar homeostasis for further than 40 times It was obviously the first line treatment among type 2 diabetes mellitus (T2DM) cases. Recently, fresh places of metformin in cancer & neurodegenerative conditions came apparent. Then, we delved the capabilities of this magic medicine in enhancing motor performance, dopamine (DA) release and TH- protein expression.*

Methods: *C57BL/6 mice were grouped into 4 via; Group1 (Saline), Group2 (MPTP), Group3 (MPTP + Met200), Group4 (MPTP + Met400). After acute administration of MPTP (25mg/kg for 5- successive days) and attendant follow-up by metformin (200 & 400 mg/kg), mice were exposed to several behavioral tests and later sacrificed for amperometric DA release measures.*

Results: *MPTP mice showed a significant drop in motor functions and amperometric amplitude ($P<0.05$), as well as the vesicle recycling as measured by pair-pulse ratio. Interestingly, metformin proves decisive in mollifying the motor dysfunctions caused by MPTP, with Met400 being more potent. It inversely improves the DA release as well the expression of its biomarker (Tyrosine Hydroxylase) in both striatum and Substantia Nigra pars compacta. This, in substance, has always indicated a functional part of metformin in employing the motor functions and DA release in the Parkinson's disease (PD) mode.*

Conclusion: *Our study demonstrated that metformin enhances motor function, DA release, and DA expression in C57BL/6 exposed to acute MPTP-induced neurotoxicity, possibly through vesicle recycling. These findings may facilitate the clinical application of metformin in the treatment of motor and even non-motor symptoms of PD.*

Keywords: *Metformin; Parkinson's Disease; Dopamine Release; MPTP; Motor Performance.*

1. Introduction

Metformin is conventionally used as a foundation medicine in the treatment of type II diabetes mellitus (T2DM) and other metabolic syndromes (Patil et al. 2014). A long history of efficacy, energy, and safety has made metformin one of the most generally specified medications globally (Rena et al., 2013). Lately, it was set up to have profound eventuality in reducing the pitfalls of Parkinson's disease (PD) and other age-related central nervous system (CNS) diseases (Adedeji et al. 2014). The versatility of this magic medicine in upgrading the ruinous goods of both metabolic dislocations (e.g., T2DM) and neurodegenerative (e.g., PD) diseases is obviously due to the

participated dysregulated pathways (Santiago and Potashkin 2014). It's vehemently believed that exposure to environmental factors and inheritable vulnerability is markedly associated with the etiology and progression of both conditions (Bayliss et al. 2016). Also, a compelling number of attestations from epidemiological studies suggested that T2DM is a threat factor for PD, although an implicit link between PD and T2DM remains controversial (Chen and Tsai 2010). Metformin also promotes neurogenesis and enhances spatial memory conformation through the activation of atypical PKC-CBP pathway (Wang et al. 2012). Metformin, besides it is currently trending role in neurodegenerative diseases (Alzheimer's disease, AD, and PD), was also found to have a profound role in mitigating the pathogenesis associated with cancer, non-alcoholic fatty liver disease (NAFLD), inflammation, heart attack and polycystic ovarian syndrome (PCOS) (Mahmood et al. 2013). Also, it's an important seeker in suppressing appetite and promoting weight loss (Day et al. 2019).

Levodopa, the current gold-standard medicine in the treatment of motor symptoms associated with PD, has been under pitfalls due to its incapability to upgrade the ruinous non-motor symptoms. Still, under a long-term scale, it causes serious dyskinesia (involuntary muscle movements) called levodopa-induced dyskinesia (LID), and this, thus, corroborated the critical need for a more presumptive and potent medicine devoid of any long-term complications. Strong substantiation suggested the positivity associated with metformin in mitigating motor symptoms of PD and perfecting mitochondrial integrity by enhancing the upregulation of mitochondrial marker proteins such as heat shock protein 60 (HSP60) (Kang et al. 2017; Katila et al. 2017). Additionally, current progress is that mitochondrial dysfunction, oxidative stress (substantially caused by occupational exposures to environmental poisons), and protein mishandling have a crucial part in PD pathogenesis (Martin et al. 2011). The neuropathological hallmark of PD is the presence of alpha-synuclein eliminations called Lewy- bodies in the midbrain, associated with progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNc) (Kakish et al. 2015). However, the precise medium underpinning the part of Lewy- bodies in PD cases isn't well established. Whereas Lewy bodies may represent an original attempt to sequester misfolded proteins, they may, at some point, come as a trigger for a seditious response, which, in turn, damages DA neurons (Miller et al. 2019). In addition to Lewy bodies, activated microglia are also present in the brains of people with PD and in laboratory animals like mice and rodents. Inheritable factors are also attributed immensely to the etiology of PD, and until now, no available data has suggested the neuroprotective prowess of metformin on genetically caused PD. Numerous genes mutations similar to PINK1 (PARK6), LRRK-2, PARK2 (PRKN), MC-1, and UCHL-1 contributed putatively to the domestic PD (Lauretti et al. 2016; Pickrell et al. 2015). More so, gene mutations of PARK2 and PARK6 were reported to give rise to autosomal recessive youthful-onset Parkinsonism, a primary mitochondrial cytopathy (Goedert and Compston, 2018). However, it's imperative to understand that no available substantiation suggests that PD can be caused by inheritable factors single-handedly. However, a significant number of studies reported that a positive family history has been associated with a high risk of PD (Rotermund et al., 2018). Shen et al. (2018) reported that inheritable omission of vesicular glutamate transporter in DA neurons increases vulnerability to MPTP-induced neurotoxicity in mice.

In this study, we conducted four of the most constantly used behavioral assays (Open Field tests, Elevated Plus Maze, Rotarod, and Footprints), aimed primarily at the evaluation of motor activity, anxiety-related actions, and emotionality characteristics of C57BL/6 mice exposed to colorful treatments. We used an acute protocol of MPTP administration, which consists of administering 25mg/kg MPTP intraperitoneally for 5-successive days. As recently reported, metformin prevents dopaminergic neuron death in MPTP-induced mice models via autophagy and mitochondrial ROS concurrence (Patil et al. 2014; Martin et al. 2011). We recently found that metformin enhances DA release and, therefore, vesicle recycling in dopaminergic neurons. Although its role in PD has been reported, no previous study has delved into its dose-dependent role in perfecting DA release on MPTP mice models. Most of the studies conducted so far concentrated on assessing its part in enhancing the integrity of mitochondrial proteins and posterior scavenging of reactive oxygen species (ROS) (Kang et al. 2017). More so, the series of behavioral assays we conducted enabled proper evaluation of its role in motor performance and inescapably proved its eventuality in the treatment of motor symptoms.

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Positive results attained from open field test and elevated plus maze test further indicated its propensity in treating non-motor symptoms that are similar to anxiety, a hallmark that wasn't hypothecated ahead. Eventually, our study suggests that metformin is a neuroprotective agent that mediates motor function, DA release, and vesicle recycling, a role that might be of remedial value for PD cases. Metformin might also be a rising pharmacological seeker against neurological diseases and, particularly, as a future replacement for Levodopa.

2. Materials and Methods

2.1. Animals and MPTP & Metformin Treatments

Twenty-five male C57BL/6 mice weighing 25–30 grams have been used in this study. All the experiments were intuitively conducted in the central laboratory and animal center of the School of Life Science and Technology of Xi'an Jiaotong University (XJTU), China, under the guidelines and directives of the Ethical Committee for Animal Care Protocol and Use. Adequate measures were put in place in order to minimize animal death, pain, and/or discomfort. Mice were equally exposed to the trial/training phase prior to the behavioral tests for habituation and acclimatization.

The MPTP administration protocol was developed by Jackson-Lewis and Przedborski (2007) (with little modification). The acute protocol (25 mg/kg for five consecutive days intraperitoneally) was designed to produce desirable motor dysfunctions and dopaminergic loss. Since dopaminergic loss is a hallmark of PD, we analyzed whether acute MPTP administration can cause a loss of these DA neurons by investigating the level of tyrosine hydroxylase (being a major biomarker of PD) expression in both substantia nigra pars compacta (SNc) and striatum. Metformin (Sigma-Aldrich, ID# DST200524-178, CAS: 1115-70-4), on the other hand, was administered in two doses via mid-dose of 200 mg/kg and high-dose of 400 mg/kg. The potency and efficacy of each dose were analyzed accordingly.

Mice were kept for a couple of weeks in an animal center (five per cage) at light-dark cycles of 12 hours with free access to food and water. Prior to the treatments, mice were divided into four groups of 5-mice each and treated as follows: Group 1 was treated with saline and served as control, Group 2 was treated with MPTP (cumulative dose, 100 mg/kg of free MPTP), Group 3 was treated with MPTP and 200 mg/kg metformin (Met200) & Group 4 was treated with MPTP and 400 mg/kg metformin (Met400). After these treatments, mice were kept for a few days under the same environmental conditions and later sacrificed for further analysis using deep isoflurane anesthesia.

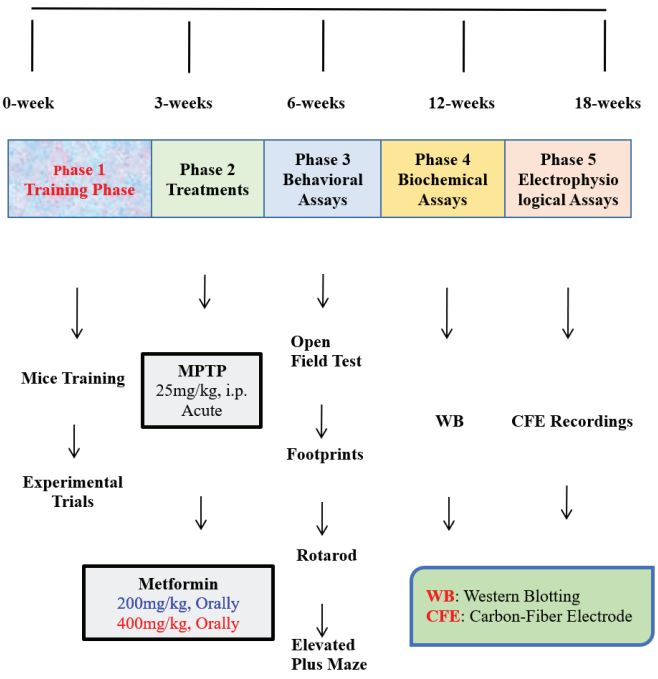

Figure 1 | Research Plan

The whole experiment was conducted in 3-4 months. 3-weeks are dedicated to mice's habituation and experimental trials. The next 3-weeks were dedicated to the administration of MPTP and Metformin. Four behavioral assays (EPM, OFT, Rotarod, and Footprints) were conducted between weeks 6th and 12th, including training. Mice were sacrificed, and biochemical and electrophysiological tests continued through week 18th.

2.2. Study Design

Thirty C57BL/6 male mice were randomly grouped into four as follows: Group 1 was treated with Saline and served as control. Group 2 was treated with MPTP (25 mg/kg intraperitoneally) for five consecutive days, and Group 3 and 4 were treated with 200 mg/kg and 400 mg/kg (met200 & met400) metformin, respectively. As an experiment that involved highly toxic

agents, five extra mice were added to each group as a backup. The mice were serially trained for 14 consecutive days (2 weeks) to acclimatize and adopt the different motor tests (**Figure 1**). Metformin (dissolved in Saline) was administered orally using a plastic enteral feeding tube. Behavioral assessment was carried out on the 4th day following the administration of MPTP and Metformin. Later, the mice were sacrificed, and brain slides were systematically cut for DA release measurement using carbon-fiber electrodes (CFE recordings).

2.3. Immunoblotting

Cells were isolated in lysis buffer containing 50mM HEPES, 150mM NaCl, 100mM NaF, 10mM sodium pyrophosphate, 5mM EDTA, 250mM sucrose, 1mM dithiothreitol and 1mM sodium orthovanadate with 1 % Triton-X and one tablet of complete Protease Inhibitor Cocktail (Roche, 11697498001) per 50mL, then stored at 80% until analysis. Protein concentration was determined with the Pierce BCA Protein Assay kit (Thermo Fisher). Lysates were then diluted with sample buffer and run on a polyacrylamide gel to separate proteins based on size. Next, samples were transferred to a polyvinylidene difluoride membrane and blocked in a 5% BSA for 1 hour at room temperature (20-25^{0}C). Membranes were incubated with primary antibody (1:1000 except α-actin 1:1,500) overnight at 4^{0}C. Appropriate secondary antibodies were used at a concentration of 1:10,000. Bound antibodies were detected using Clarity Western ECL Substrate (BioRad) (Vivacqua et al. 2020).

2.4. Open Field Test

In our laboratory settings, the Open Field Test (OFT) apparatus consists of a rectangular plastic arena measured (40 X 40 X 50) cm^3. The area is well-tight to prevent fluid absorption. The whole arrangement is connected with a video tracking system in which the area is divided into small squares of 5cm by 5cm. The trajectory path of the mice was traced and recorded within the center and corner zones, respectively, using an automatic video tracking system mounted above the maze. The open field maze was cleaned thoroughly after each experiment with 70% ethyl alcohol to get rid of the odor signal and allowed to dry completely prior to testing other mice. The mice were taken from their respective cages to the behavior room directly and tested once at a time for 30 minutes. Occasionally, a spherical beaker (or any other object) is placed at the center to measure the number of times the mice visited the region (center), and that will incontrovertibly mandate their social novelty, which, in substance, gives an evaluation of the position of depression and anxiety (Hwang et al. 2019).

2.5. Footprints

Footprint gaits were analyzed as preliminarily described by Wang et al. (2018). The hind paws and forepaws were coated with Red and Blue non-toxic ink. The mice were trained to walk along a 100cm-150cm long and 10cm wide open-top runaway (with 10cm high walls) with three runs per day for three successive days. A fresh sheet of white paper was placed on the bottom of the runway for each run. The footprint pattern was assessed quantitatively by stride length and front/hind footprint overlap or imbrication.

2.6. Rotarod

This test is used to evaluate mice's forelimb and hind-limb motor balance and coordination. In the 3rd week, mice were placed in a separate compartment on the rod and tested at an initial speed of 5 rpm until it reached 40 rpm. The latency to fall time (time on the rod) was recorded accordingly. Adapted from Ishaq et al. (2020) with little variations.

2.7. Elevated Plus Maze

The Elevated Plus Maze (EPM) test is used to assess anxiety-related behavior in mice models of Parkinson's disease and other Central Nervous System (CNS) disorders. The EPM set-up consists of a plus (+) – shaped maze elevated at roughly 100cm above the floor with two oppositely positioned closed arms, two oppositely positioned open arms, and a central area. As mice freely explore the maze, their behavior is recorded by means of a videotape camera placed above the maze and analyzed using a video tracking system. The preference for being in open arms over closed arms is calculated to measure anxiety-related behaviors. In order to detect zone entries and exits with perfection, the video was viewed in ANY-maze (Ojo et al. 2016).

2.8. Electrophysiological Recordings (DA

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Release Measurement)

Amperometric recordings in dorsal striatum slices were made using carbon fiber Electrodes (CFEs). Mice were anesthetized with isoflurane (1.5 g/kg, intraperitoneally) and transcardially perfused with approximately 50 ml ice-cold artificial cerebrospinal fluid-A (**Sectioning CSF**) (110 C5H14NClO, 2.5 KCl, 0.5 CaCl2, 7 MgCl2, 1.3 NaH2PO4, 25 NaCO3, 25 glucose mM, saturated with 95% Oxygen and 5% Carbon dioxide). Next, the brain was quickly removed and cut into 300-micrometer horizontal slices on a vibratome (Leica VT 1000; Nussloch, Germany). Slices containing the striatum were collected at +0.0 to 1.2mm from bregma. Slices were allowed to recover for 30 min in another artificial cerebrospinal Fluid-B (**recording CSF**) (125 NaCl, 2.5 KCl, 2 CaCl2, 1.3 MgCl2, 1.3 NaH2PO4, 25 NaCO3, 10 glucose mM, saturated with 95% oxygen and 5% Carbon dioxide at 37^{0}C, and then kept at room temperature for recording. CFEs 7 micrometers in diameter with an approximate 200-micrometer sensor tip were used to measure DA release in the striatum. The exposed CFE tip was fully fitted into the sub-surface of the striatal slice at an angle close to 30^0. A holding potential of 780 mV was applied to the electrode by an EPC9/2 amplifier and controlled by pulse software (HEKA Electronic, Lambrecht/Pfalz Germany). Single electrical field stimulation (Estim) pulses (0.2 ms, 0.6 mA) or trains (10 pulses at 20 Hz) were delivered through a bipolar platinum electrode (150-micrometer in diameter) and generated by a Grass S88K stimulator (Astro-Med). The amperometric current (I_{amp}) was low-pass filtered at 100 Hz and digitized at 3.13 kHz. The amplitude of amperometric current I_{amp} is proportional to the local DA overflow concentration [DA] with a calibration factor of 1 pA for nearly 6 nM. Off-line analysis was performed using Igor software (WaveMetrix). This protocol was adopted by Hwang et al. (2019) with little revision.

2.9. Statistical Analysis

Results are presented as the mean ± standard error of the mean (SEM). Statistical significance was assessed either via an unpaired 2-tailed student's t-test for two group comparisons or an ANOVA test with Turkey's HSD post hoc analysis for comparison of more than three groups. Statistical difference was considered significant at the level of $P < 0.05$ (5 % alpha). The results were analyzed using IgorPro, OriginPro2018, and SPSS 13.0 (statistical Packages for Social Sciences). Trajectory path/tract of the open field and elevated plus maze were obtained directly from the software (ANY-maze) and Bandicam independently with a videotape system mounted above the maze.

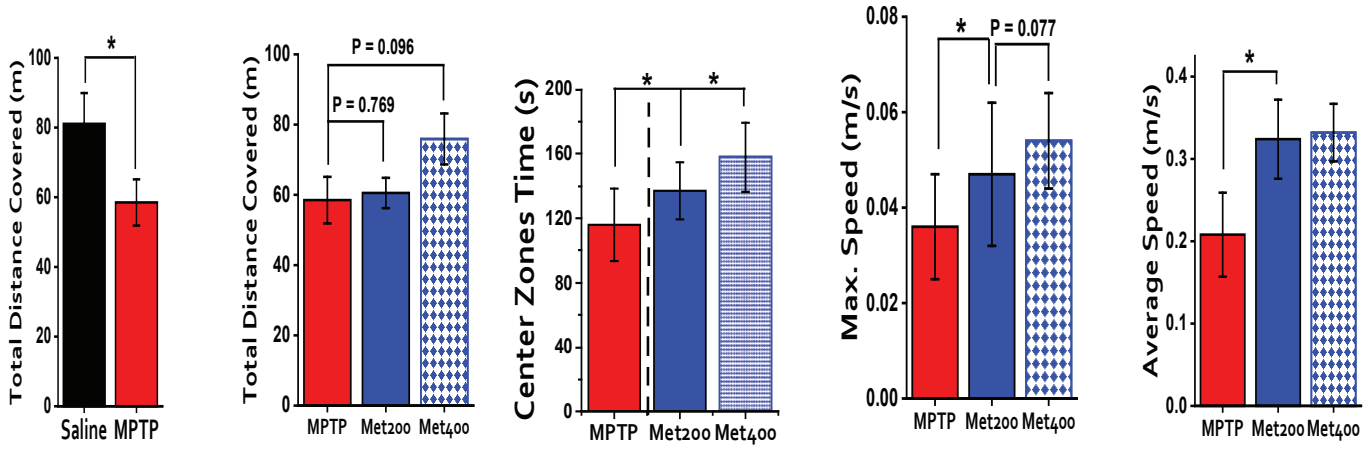
3. Results and Discussion

3.1. Metformin Improves locomotion in C57BL/6 mice

In this study, we conducted a series of behavioral tests using intact mice in order to evaluate the role of metformin in improving motor performance (especially locomotion). Open field test, being an important sensorimotor test used to determine gross locomotor activity and exploration habits in mice models exhibiting PD symptoms, was conducted in an open field maze with the mice allowed to move freely for 30 minutes while being recorded by an overhead camera. Total distance covered, time spent in pre-defined zones (center and corners), and average and maximum speed were all recorded and analyzed accordingly (**Fig. 2A-E**). The PD models (mice treated with MPTP) tend to cover a shorter distance and spent little time at the center of the maze when compared to the control (**Fig. 2A**). This, in essence, was attributed to the lack of balance and cognitive function following the predisposed DA neuronal loss in the striatum caused by MPTP. The cognitive dysfunctions (mostly anxiety and depression), however, are a result of precise damage of the DA neuron terminals in the ventral tegmental area (VTA) (Vivacqua et al. 2020). Another possible explanation for the decreased movement might be the increase in the actions of the direct pathway within the basal ganglia. It is obvious that deficiency of DA in the brain may lead to delayed and awkward movement (**Fig. F {PD models}**), and likewise, it is excess causes the body to make unnecessary movements such as repetitive tics or repetitious singularities (Ojo et al. 2016).

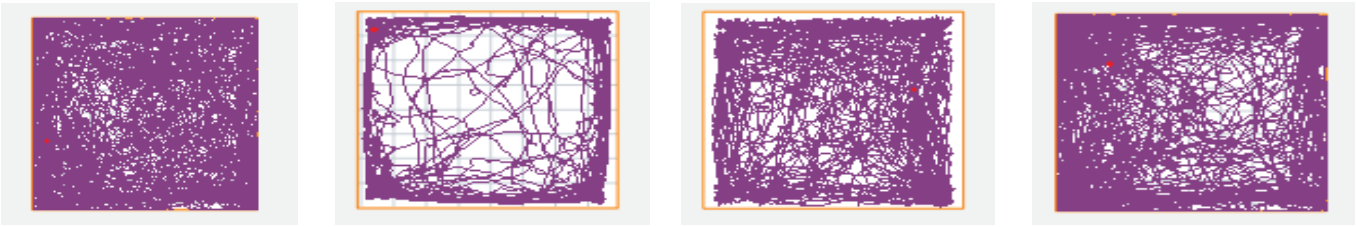
Metformin (especially Met400) showed some promising neuroprotective prowess with respect to locomotion as well as anxiety-related behaviors. The total distance covered by the MPTP group was improved handsomely following the administration of Met200 and Met400, with the latter being more potent and efficient (**Fig. 2A**). More so, the time spent in the inner/center zones in the MPTP group was improved significantly ($P < 0.05$) following the administration of

metformin. This indirectly measures thigmotaxis or wall-hugging behavior, and it is invariably indicative of anxiety-related behavior (**Fig. 2F**). Maximum and average speed further explained the redundancy of the mice to explore more areas of the maze, and this might be attributed to the depression they suffered following the MPTP damage (**Fig. 2D/E**). The overall result, in essence, proves beyond doubt that metformin has potential with respect to neuroprotection.



(A) **(B)** **(C)** **(D)** **(E)**

(F) Trajectory Paths as Recorded by Video Assistant System, VAS



Control **PD Model** **Met200** **Met400**

Figure 2 | Open Field Test

Saline, MPTP, Met200 & Met400 (n =5, each) mice were subjected to the OFT, and Total distance covered, time spent in center zones, and average and maximum speed were statistically analyzed. **Fig. 2A.** Bar chart representation of the total distance covered by saline/control and PD model/MPTP. **Fig. 2B**. Bar chart showing the total distance covered by MPTP and Metformin groups. Met200 covered almost the same distance as the MPTP group, whereas Met200 was higher but still statistically insignificant (P = 0.096). This showed some promising neuroprotective role of metformin in improving motor performance. **Fig. 2C**. Bar chart representation showing the time spent in the inner/center zones. Time spent in the center zones is very limited in the MPTP group when compared with metformin groups (P < 0.05). **Fig. 2D/E**. Bar charts represent the maximum and average as indicative of locomotion and redundancy. **Fig. 2F**. Trajectory paths as recorded by Video Assistant System, VAS: The mice were allowed to move freely in an open field rectangular space, and the corresponding movement (locomotion) was recorded by ANYMAZE software and video system. Center zones and corner zones were selected accordingly. MPTP-treated mice move more predominantly within the corner zones in contrast to the saline (control), which moves more evenly. The results for the data were expressed as mean ± SEM. Statistical analysis (*t-test*) was performed using SPSS and Microsoft Excel 2010. Differences were considered significant at $P < 0.05$.

3.2. Metformin Plays a Key Role in Anxiety-Related Disorders

Following the establishment of some non-motor function of metformin in C57BL/6 mice

Advances in Medical, Pharmaceutical and Dental Research Journal (AMPDR)- ISSN 2812-4898
http://dx.doi.org/10.21622/AMPDR.2025.05.1.1248

afflicted by MPTP, we investigated further using Elevated Plus Maze (EPM). EPM is used to assess anxiety-related behavior in rodent models of CNS disorders. As an anxiety test, we performed the test 3 days after the open field, Rotarod, and footprints in order to ensure full coordination and cognition. We analyzed their propensity to explore the open and closed arms of the maze, and from there, we evaluated the function of each treatment. In this study, we stationed open field tests together with EPM to evaluate spontaneous locomotors and exploratory conditioning. The number of arm entries (open and closed), as well as the duration spent in each, was the index we used to predict the anxiety-related behavior of each group. Interestingly, the time spent in the open and closed arms is significantly higher than that of the PD model (MPTP group) ($P < 0.05$). Metformin (Met400), on the other hand, increases the duration of the MPTP mice in both arms. The restoration conferred by Met400 was neither total nor absolute but proved decisive and promising. The explanation, then, is that when the mouse feels anxious, it tends to remain in the open arm, and that is what we discovered in our findings. The mice induced with MPTP (presumably, PD models) spent more time in the closed arms, and this clearly indicated an increase in anxiety as associated with PD subjects (**Fig. 3B**). We discovered that PD models have a limited number of arm entries and this indicated that, besides their motor impairment, there is a massive reduction in their cognitive function with respect to anxiety and depression (**Fig. 3C**). It appeared to us that the PD models feels very anxious to explore the various arms and slowly leads to slowness in decision and redundancy (**Fig. 3E**). We presumably postulated this theory based on the longest time they spent in the center when compared to other groups. Conclusively, motor impairment and cognitive function (specifically anxiety) are inversely proportional to the frequencies of arm entries and time spent in open arms. Furthermore, the impairment was to be directly proportional to the time spent in closed arms.

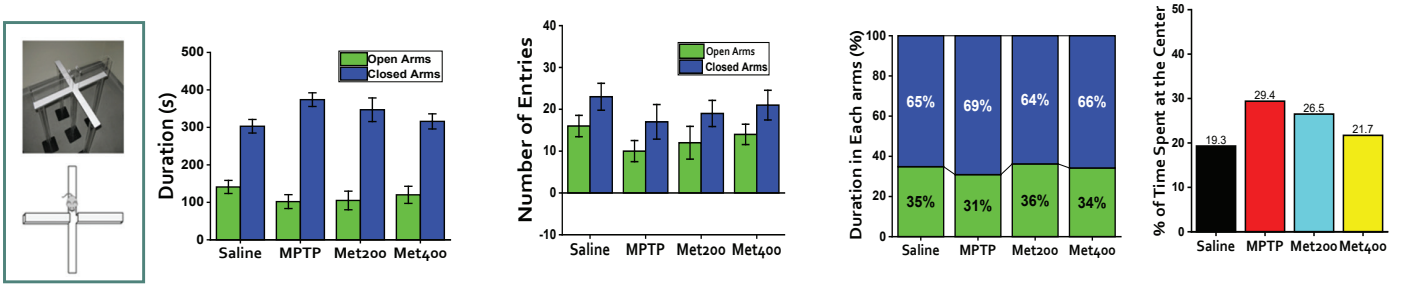


Figure 3 | Elevated Plus Maze Test

Fig. 3A. EPM set-up: The maze is settled with a height of about half-meter above the floor, with pillars standing from the ground floor. The maze is made up of two arms (2-open & 2-closed) crossing each other perpendicularly. The two paths facing each other have halls (hence called closed arms), and the other two have no walls (hence called open arms). **Fig. 3B.** Category Plot Bar chart representation of the time spent in the open and closed arms of the 4-groups. A very sharp and significant difference was observed between the two arms of each group ($P < 0.05$) and, obviously, higher time spent in closed arms. **Fig. 3C.** Bar chart representation showing the frequencies of arm entries. The frequency of arm entries decreases with an increase in motor impairment, and obviously, the MPTP group has the lowest. **Fig. 3D.** 100 % stacked column chart indicating the relative percentage of the time spent in each arm. **Fig. 3D.** A simple bar chart representation shows the percentage of time spent in neither of the arms (center) of the respective groups. MPTP group has the highest.

3.3. Metformin Enhances Balance and Motor Coordination

Motor coordination and balance were intimately evaluated and quantitatively assessed using the Rotarod test. The C57BL/6 mice were exposed to a series of experimental trials and 3-week training prior to taking readings/data to ensure full adaptation and learning the task to the same degree. Data were collected in batches throughout the 9th and 12th weeks (**Fig. E-G**). And at the end of week 12th (**Fig. B-D**). Testing consists of three sessions every week. Then, mice were tested on the accelerating rotation protocol in which they were placed on a rod that accelerates initially from 0-5

rpm and then gradually from 5 to 40 rpm until they collapsed. We observed carefully that the control and Met400 mice were able to withstand the rotating rod at the maximum speed (40 rpm) until they reached RAMP at 300 seconds (**Fig. 4B/D**). Meanwhile, the MPTP and Met200 mice withstood the rotation only hard and fell at a fairly lower time (**Fig. 4C**). As time progressed, we observed a massive improvement in the endurance, and metformin proved decisive over a period of 4 weeks. Initially (in the first 2 weeks), there was no statistical difference between the MPTP and metformin groups (Met200 & Met400). At week 3, we recorded a significant change in the endurance on the rod between the MPTP and Met400 (**Fig. 4G**), indicating positivity surrounding the neuroprotective training of metformin in improving motor performance; meanwhile, no progress was observed with respect to Met200. Unexpectedly enough, the loftiest latency to fall time was observed in the 3rd week, not 4th and the only conclusion we can decide then may be muscle fatigue, whereas the impotency in the first 2-weeks might be associated with motor learning skills/behaviors. Importantly, metformin showed some promising role in ameliorating the adverse effects of MPTP by nearly restoring balance and maybe social

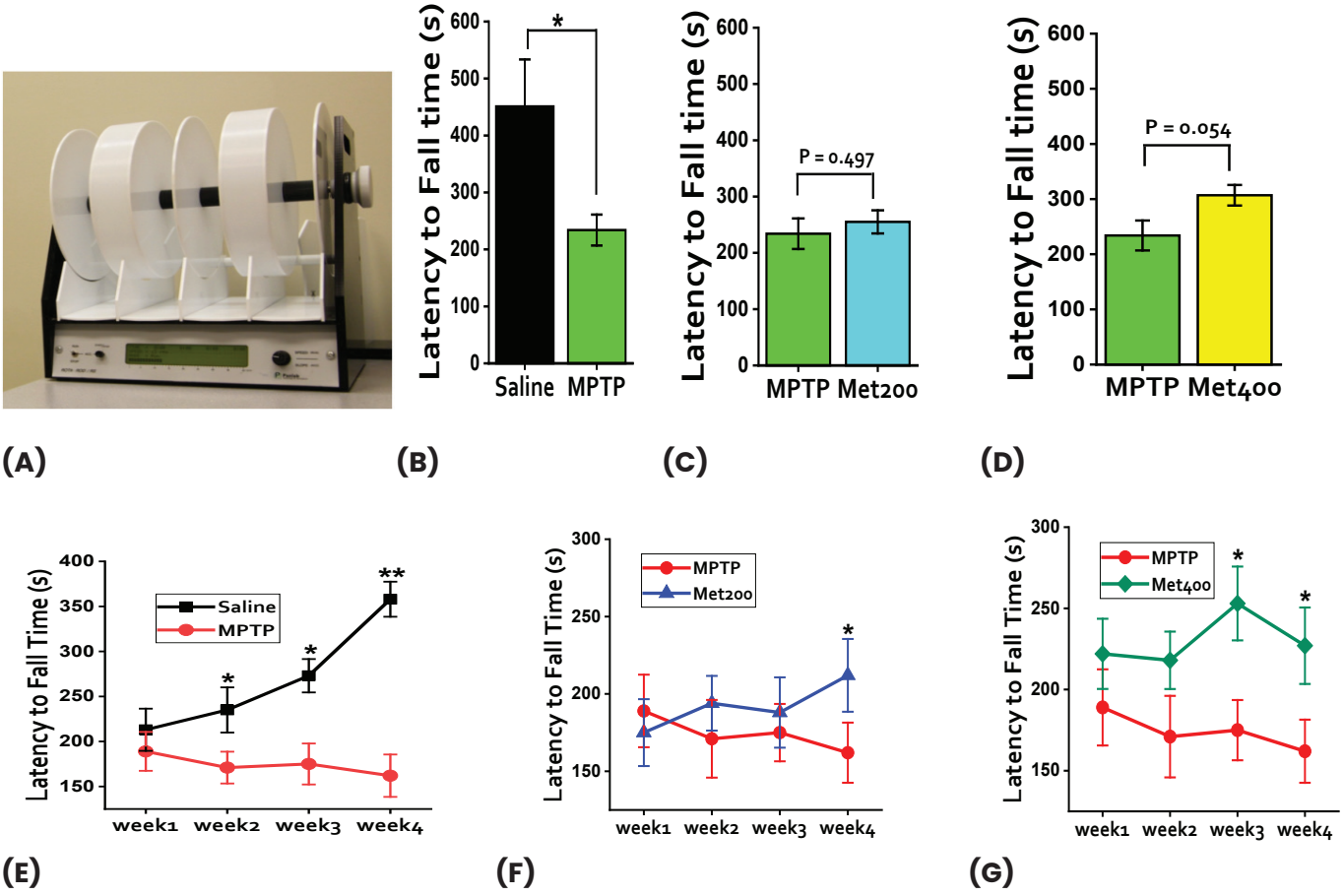


Figure 4 | Rotarod Test

Fig. 4A. Mouse Rotarod Set-up: The apparatus consists of a circular rod turning at increased speed (Initial of 5 rpm and Max. of 40 rpm). Mice were placed on the rotating rod to maintain balance and coordination. It is well automated, and we were able to test 5-mice at a time. Vertical metallic plates served as barriers to separate each. The latency to fall time was recorded and analyzed accordingly. **Fig 4B: Rotarod (Saline Vs. MPTP):** Expectedly, the Latency to falling time in the Saline is significantly longer than that of MPTP ($P < 0.05$). **Fig 4C. Rotarod (MPTP Vs. Met200):** Met200 failed **Fig. 4D. (MPTP Vs. Met400):** Increased time was sustained on the rod in the same group of mice treated with MPTP. Surprisingly, during the first 2-weeks, there was not much difference with the MPTP group. **Fig. E-G.** Analyze the endurance level in 4 weeks. Motor impairment increases with time as MPTP consistently causes damage, and subsequently, the gap widens (**E**). Met200 appeared to be less potent here, and the mice fell almost at the

Advances in Medical, Pharmaceutical and Dental Research Journal (AMPDR)- ISSN 2812-4898
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same time as that of the MPTP group (**F**). Values are expressed as mean ± SEM of the triplicate readings and considered significant at ** P < 0.05 and ** P < 0.01.*

3.4. Metformin Treatment maintained stride length in Footprint Gait analysis

It has been established profoundly that MPTP causes motor deficits such as involuntary movements (Di Biase et al. 2020). Objective evaluation and analysis of the gait cycle is, therefore, pivotal in understanding the severity and inflexibility of motor impairment. Gait impairment is an evolving condition, and a series of gait disturbances are evident as the disease progresses. Here, we assess the progression and severity of motor abnormalities induced by MPTP. Footprint gait analysis is used as an evaluation index. Metformin improves the unbalanced gait induced by MPTP (**Fig. 5C/E**). The stride length of saline, when compared to MPTP, was found to be significantly different at *P < 0.05*. They showed greater variation with the MPTP group in both the SD and range of stride length (**Fig. B/D**). Our findings proved that metformin can ameliorate the unsupportable deterioration caused by MPTP in the performance of motor tasks. Both Met200 and Met400 exert a very amicable impact in subsidizing deficits within cognitive and behavioral domains. However, in order to ascertain the impact, only 5-6 footprints of each group were sufficiently clear to be analyzed. Collectively, these findings indicate that metformin at both doses is sufficient to ameliorate locomotor deficits associated with gait abnormality, among others.

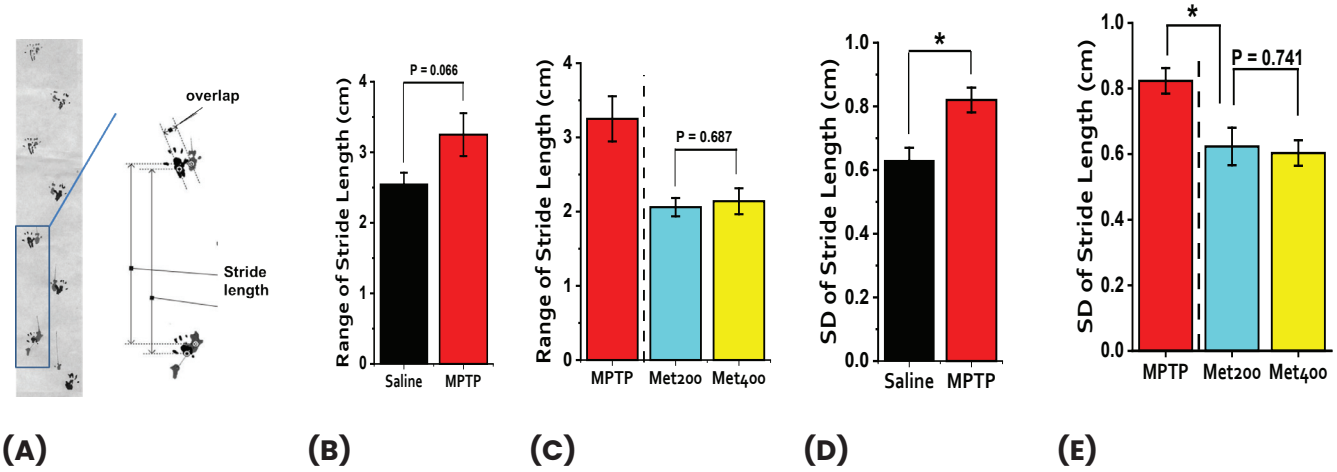

Figure 5 | Footprint Gait analysis

Fig. 5A. Footprints: footprints showing the stride length and overlap. **Fig. 5B.** Footprints of the MPTP and Saline. Concentrated stride length in the MPTP models indicates lower numbers of overlap as well as irregularity in their movement (gait abnormality). **Fig. 5C/E.** Footprints (MPTP VS Metformin). The number of overlaps indicates the regularity and order of movement. Expectedly, MPTP models showed a significantly limited number of overlaps (P < 0.001). Metformin reverses some of the motor deficits caused by MPTP (P < 0.01).

3.5. Metformin Improves DA Release and Vesicle Replenishment in both Striatum and SNc

Grounded on the compelling number of former research, we heavily believed and established the fact that MPTP causes a series of neurotoxic insults by inhibiting complex I protein of the electron transport chain (ETC) of the mitochondria (Hwang et al. 2019). This inhibition will integrate DA transporters (DAT) and block mitochondrial oxidative respiration, which will later result in the dysfunction of some mitochondrial proteins as well as the overall drop in mitochondrial energy and integrity (Mugikura et al. 2016). Accordingly, this will result in a lower release of neurotransmitters (specifically, DA) in the regions affected (Striatum and SNc). Then, we made amperometric recordings with electrochemical carbon-fiber electrodes (CFEs) in striatal and SNc slices to determine whether metformin can ameliorate the impaired DA release from the nigrostriatal terminals in mice induced by MPTP **(Fig. 6B)**. When a local original electrical stimulus (**Fig. 6A**) was applied to the brain slice (striatal slice in particular), there was a transient increase in amperometric current with a subsequent decay to baseline. This laterally represents a transient increase in extracellular DA concentration. Our findings

showed that metformin (especially Met400) can reverse some of the effects caused by MPTP (**Fig. 6C/G**). No former exploration or research delved into the neuroprotective role of different doses of metformin on MPT-induced PD models. Kang et al. (2017) conducted their evaluation on 6-OHDA PD models with no account of DA release. Next, we evaluated the role of metformin on vesicle recycling to determine whether it favors exocytosis and/or endocytosis by assaying the pair-pulse ratios at different stimulus intervals (10s, 20s, 30s, 40s) (**Fig. 6D/E/H/I**).

Expectedly, we discovered a massive reduction in the DA release and vesicle recycling in the MPTP mice as compared to the control. Both the amplitude as well as the pair-pulse ratios are reduced significantly ($P < 0.05$) in both the striatum and the SNc regions. And has inevitably indicated that vesicle replenishment was vehemently inhibited. However, burst stimulation using 10 a train of 10 pulses at a frequency of 20Hz revealed a reduced releasable vesicle pool in the striatal DA terminals only. Furthermore, we assessed the role of metformin in vesicle recycling by stimulating the regions at different times. Originally (at 10s and 20s), the DA release was veritably harmonious and steady but nearly the same as the baseline, and there was no significant difference between the groups. This, in essence, indicates that transient stimulation is sufficient to initiate release while potency/energy increases only with time (**Fig. 6D/H**). From the lower amplitude obtained in Met200, we stimulated only the Met400 strategically over four separate periods. Interestingly, we found that Met400 is potent enough to improve the DA release and vesicle recycling as time progresses (**Fig. 6E/I**).

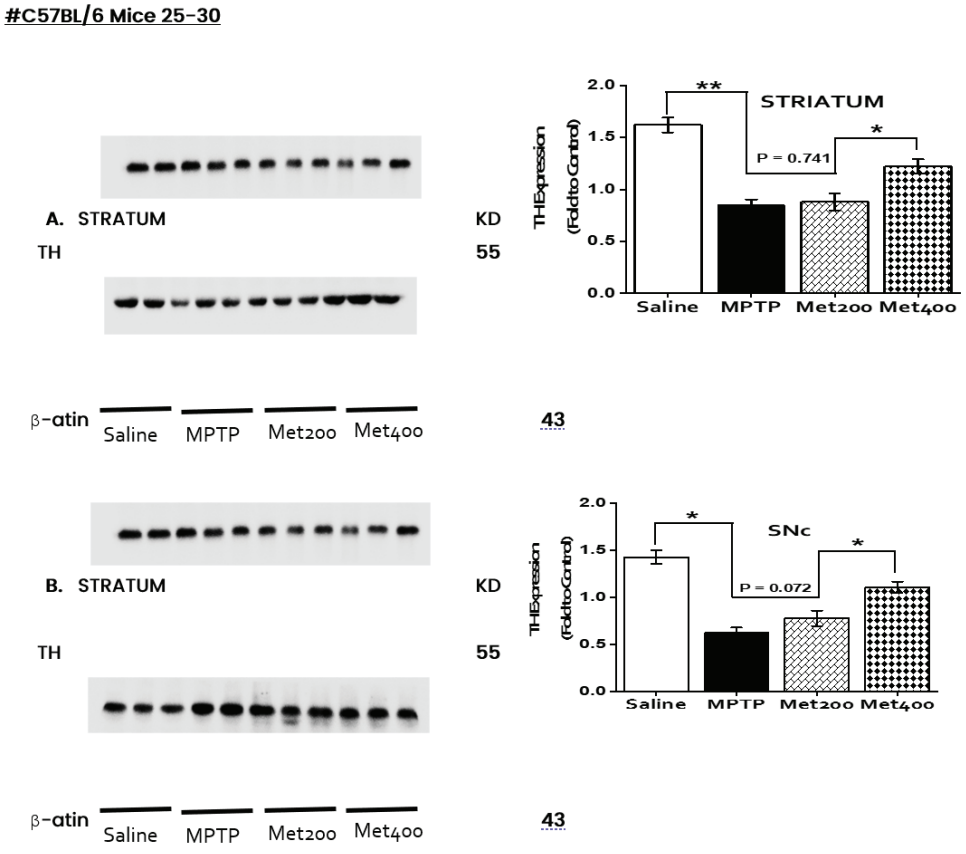

Figure 6A | DA release in Striatum and SNc

Fig. 6A: Carbon-Fiber Electrode Recording Set-up: Showing the arrangement for measuring the DA release in the striatum and substantia nigra. Representative amperometric currents in *Pico* ampere and statistics showing the intact DA release from dopaminergic terminals in the striatum and corresponding MPTP-damaged region. Carbon fiber is used to detect the release of DA. Estim serves as an electrophysiological stimulator that triggers the release of DA at different frequencies. **Fig. 6B/F: DA release of MPTP-mice:** striatal and SNc slices of the brain treated with MPTP show a massive reduction in the DA release when compared to the saline (P

Advances in Medical, Pharmaceutical and Dental Research Journal (AMPDR)- ISSN 2812-4898
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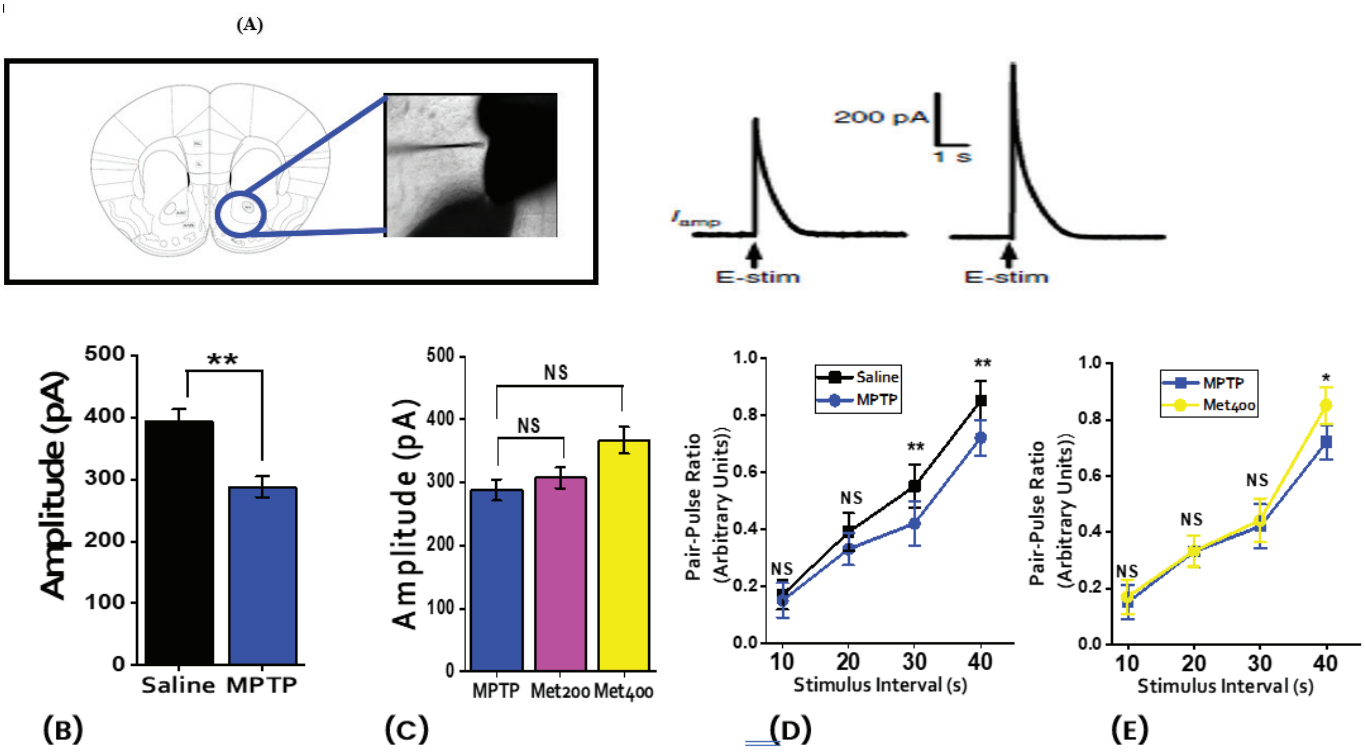
< 0.01) in both regions. This inarguably confirms the successful administration of MPTP to the target area and paves the way for further tests. **Fig. 6C/G: protective effects of metformin at different concentrations**: Both Met200 and Met400 slightly improve the release of DA in the striatum and SNc. Expectedly, the highest amplitude was detected at 400mg/kg in both regions. Metformin displayed a certain level of DA replenishment against the effect induced by MPTP significantly in the SNc (P < 0.01) and statistically insignificant in the striatum (NS, P = 0.939). **Fig. 6D/H: Vesicle Recycling (Saline vs. MPTP) in the striatum and SNc:** MPTP inhibits vesicle pool replenishment in dopaminergic neurons in both regions at the 30s and 40s stimulus intervals. **Fig. 6E/I: Vesicle Recycling (MPTP vs. Metformin) in the striatum and SNc:** Both Met200 and Met400 enhance the vesicle recycling in a stimulus-dependent pattern in the two brain regions. At 10s and 20s, the DA release is not significant enough as compared to baseline, and there is no improvement in the vesicle recycling, whereas, at 30s and 40s, there is enough recycling of dopaminergic neurons from their vesicles (P < 0.01) (P < 0.05) respectively. Pair-pulse ratios of DA release with different stimulus intervals (10s, 20s, 30s, 40s) were recorded accordingly. Data were collected from three different experiments and were expressed as mean ± SEM and unpaired student *t*-test.

3.6. Metformin enhances the expression of DA Biomarker (Tyrosine Hydroxylase) in both Striatum and SNc

Western blot analysis of the striatum and SNc of the MPTP mouse models showed that metformin (both Met200 and Met400) increased the levels of TH expression, and this may potentially lead to the defensive effects of metformin in rescuing dopaminergic neurons (**Fig. 7A/B**). Subsequent MPTP intoxication led to relative suppression of the catecholaminergic protein, TH, in both regions of the brain, and hence, metformin can only reverse the degeneration slightly. Based on our data, we found that the intoxication caused by MPTP was not unrecoverable, and the architecture of the affected regions can get back to normalcy with appropriate interventions. Interestingly, the striatum and the SNc of the group treated with metformin (especially Met400) demonstrated maintenance of TH expression when compared to the treated group (control). In conclusion, our results from this analysis suggested that metformin plays a crucial part in the survival of dopaminergic neurons in the midst of intoxication and subsequent oxidative stress. More so, a compelling number of studies have reported unequaled neuroprotective role(s) of metformin in MPTP-induced PD models. However, Curry et al. (2018) claimed that metformin is ineffective in 6-hydroxydopamine (6-OHDA)-induced PD models. Presently, no available data shows the neuroprotective prowess of metformin on rotenone-induced models.



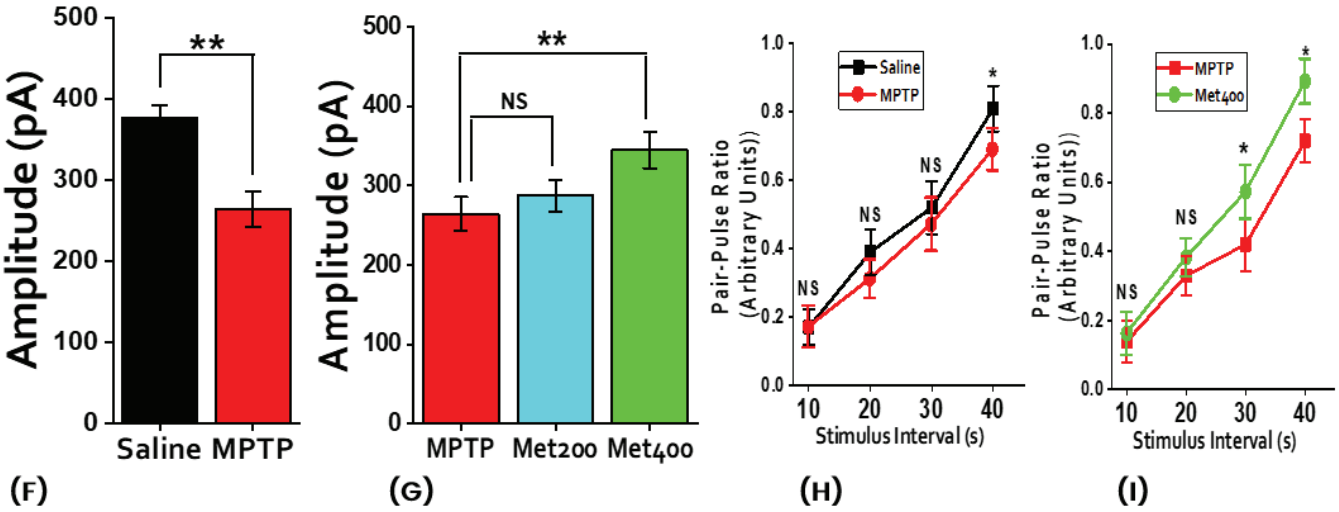


Figure 7 | TH expression in Striatum and SNc/Densitometric Analysis

Acute administration of MPTP decreases TH protein level. **Fig. 7A. Striatum:** Representative western blots showing the expression of Tyrosine Hydroxylase (TH) in the SNc and striatum of C57BL/6 mice treated with MPTP or metformin or both as determined by western blot with the indicated antibody. Western blots were performed 4 weeks after the last MPTP injection. **Fig. 7B.** Bar chart representation showing the optical density of TH protein expression in the striatum region of the brain. The expression in the MPTP-treated mice is significantly reduced when compared to control/saline ($P < 0.01$) and almost significant when compared to a lower dose of metformin (Met200) ($P = 0.741$). However, Met400 enhances the clarity and, hence, the expression of the TH-protein in the MPTP-treated mice significantly ($P < 0.05$). **Fig. 7C. SNc:** Representative western blots showing the expression of TH in SNc of the midbrain performed 4 weeks after the last MPTP injection. **Fig. 7D.** Bar chart showing optical density fibers in the SNc. A significant reduction in expression of the TH in the MPTP group was observed when compared to the control ($P < 0.05$), whereas Met400 rescued some dopaminergic neurons in the region ($P < 0.05$) as Met200 showed no difference. Values were expressed as fold to control and analyzed using Image J and OriginPro18 accordingly. * indicated a significant difference at 5% confidence interval (CI), ** at 1% confidence interval (CI).

3. Discussion

Metformin was well known for its role in metabolic syndromes and established a reputable status as a cornerstone remedial and therapeutic option for T2DM almost 5 decades ago. Recently, besides the aforementioned role, metformin was found to be effective in neurodegenerative diseases (e.g., PD) and cancer (Rotermund et al. 2018). Mechanistically, it affects several tissues that are central to metabolic homeostasis, including the pancreas, liver, brain, and skeletal muscle. In view of it is different physiological functions, it is not even surprising that metformin has an eventuality of improving motor deficits and DA release (Tayara et al. 2018; Lee et al. 2020)

In recent years, metformin was set up to be effective in perfecting motor symptoms of PD in experimental animals and also eased the L-DOPA-induced motor complications (Ryu et al. 2018). Motor dysfunctions similar to resting tremors, rigidity, and bradykinesia were the most common and indeed the most visible symptoms of PD and have a negative impact on the quality of life of cases with the complaint (Murata et al. 2016; Ishaq et al. 2020). So far, the open Field Test (OFT) is the most effective canonical assay for the relative assessment of changes in locomotor function (Samson et al. 2015). Regardless of the dose, metformin-treated mice showed increased entries of the center zone in the open field maze, which always indicated an increased curiosity and lower situations of anxiety (El-Sisi et al. 2015). This, thus, verified the role of metformin in ameliorating motor deficits. OFT, in combination with the Elevated Plus Maze test (EPM), is typically used to probe the behavioral function of certain compounds (e.g., anxiolytic agents) in preclinical settings involving PD models (Ojo et al. 2016; Ramos et al.

Advances in Medical, Pharmaceutical and Dental Research Journal (AMPDR)- ISSN 2812-4898
http://dx.doi.org/10.21622/AMPDR.2025.05.1.1248

2008; Sestakova et al. 2013). Here, we combined OFT and EPM results as indices of locomotion, anxiety, and exploration to predict the possible pharmacological role of metformin (Met400 in particular) in alleviating motor deficits caused by MPTP. As these two tests (OFT and EPM) all rely on the unconditioned avoidance of threatening situations and free moving within the maze, it could be hypothesized that metformin is not only effective in motor functions but also enhances non-motor deficits such as anxiety. In essence, Met400 improves locomotor function and mediates anxiety-related behaviors and gross motor functions in C57BL/6 mice induced by MPTP (**Fig. 2B-F & 3B-D**).

Having established the dose-dependent response of metformin on MPTP-induced PD models in locomotion and anxiety, we also set to explore the part of metformin in the conservation of balance and coordination. Postural balance, gait normality, and muscle strength were analyzed sequentially using Rotarod tests and footprints gait analysis (Hu et al. 2018). Here, we observed that mice treated with saline and metformin showed better balance and muscle coordination in the rotarod and footprints tests than the MPTP group (presumably PD). Groups treated with Met200 and Met400 may protect the dopaminergic neurons within the striatum from degeneration, leading to enhanced motor coordination, balance, and muscle strength. Also, as a hallmark of PD-like pathology in experimental animals, we measured the level of DA release using an electrophysiological carbon-fiber electrode. The amperometric amplitude (PA) was assessed, as well as the pair-pulse ratios at different stimulation intervals (Mosharov et al. 2005; Lahiri and Bevan 2020; Ivanova et al. 2020). Our findings, for the first time, reveal that metformin enhances DA release in a dose-dependent manner. Met400 was found to favorably increase the DA release magnitude compared to the MPTP group (**Fig. 6**).

Lower level and/or expression of TH- protein was one of the hallmarks attributed to PD pathogenesis. The expression of this biomarker is constitutively associated with catecholamines such as DA. Formerly, metformin was proved decisive in improving the number of TH-positive neurons in the striatum and SNc as evidenced by immunohistochemical assays/images (Patil et al. 2014; Bayliss et al. 2016; Kang et al. 2017; Vivacqua et al. 2020; Lee et al. 2020). Here, we observed that metformin has the ability to stimulate TH-protein expressions in both striatum and SNc. Met200 did not show any appreciable change compared to the control (**Fig 7**), and this demonstrated that only a high dose (Met400) of metformin is capable of inducing TH expression in both regions. A novel observation from our study is that the expression of TH in both striatum and SNc is fairly the same despite the unequal distribution of proteins.

A limitation of our study was that we could not give long-term experimental follow-up of metformin and the effects of metabolic phenotypes of C57BL/6 mice on the dose-dependent neuroprotective response of metformin and consequent deceleration of body weight in MPTP-treated groups. Therefore, further studies should concentrate on histological morphologies, body weight variation, long-term effects of metformin (say 3-4 months), and proteomic changes associated with the major mitochondrial protein biomarkers. This is in view of the fact that MPTP specifically inhibits complex I of the electron transport chain (Kang et al. 2017; Jackson-Lewis and Przedborki 2007; Lee et al. 2020; Williams-Langson et al. 1985) and inevitably disrupts the integrity of Mitochondria as a whole. The consequences of this might lead to metabolic changes, including weight loss.

4. Conclusion

Our study demonstrated that metformin enhances motor function, DA release, and DA expression in C57BL/6 exposed to acute MPTP-induced neurotoxicity, possibly through vesicle recycling. Our findings may facilitate the clinical application of metformin in the treatment of motor and even non-motor symptoms of PD. We greatly believed that metformin might be the future clinical substitute for Levodopa.

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Author Contributions

Daha Umar Ishaq: Conceptualization, Methodology, Software. **Daha Umar Ishaq.:** Data curation, Writing-Original draft preparation. **Daha Umar Ishaq**: Visualization, Investigation. **Daha Umar Ishaq**: Software, Validation: **Binta Garba Kurfi** and **Solomon Ojodemi Oguche:** Writing-Reviewing and Editing. **Daha Umar Ishaq** and **Solomon Ojodemi Oguche**.: Formal Analysis & Project Administration.

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