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Chemical Constituents Analysis of Blue Vitriol, Brimstone and Black Stone Using X-ray Fluorescence Technology and GC-MS: A Quest for Novel Antifungals

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ABSTRACT:

New drugs especially antifungals are continually required by healthcare systems to address serious public health challenges such as resistance, toxicity, cost and scarcity. This research aimed at analyzing the chemical constituents of blue vitriol, brimstone and black stone using x-ray fluorescence technology and gas chromatography-mass spectroscopy (GC-MS), a quest for novel antifungal agents. The test isolates were obtained from high vaginal swab specimens of patients attending a University Teaching Hospital in Anambra State, Nigeria. The isolates were identified based on their morphological, physiological and molecular characteristics. The minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) were determined using the broth dilution method. The inorganic and organic constituents of the natural compounds were determined using x-ray fluorescence technology and GC-MS, respectively. The isolates include Candida albicans, C. tropicalis, C. glabrata and C. parapsilosis. The MIC and MFC of blue vitriol against the isolates ranges from 12.50mg/mL – 100mg/mL, brimstone (50mg/mL – 200mg/mL), black stone (200mg/mL) and ketoconazole (positive control):12.50mg/mL - 50mg/mL. The inorganic components found in blue vitriol include oxides of copper (43.7%), sulphur (29.4%), aluminium (1.2%); while the heavy metals are arsenic, lead and nickel (all <0.009%). Linoelaidic acid (24.92%) and oleic acid (12.97%) make up the organic components. Brimstone contains oleic acid (10.26%), decanoic acid (28.37%), sulphur (94.72%), aluminum (3.46%), while lead, arsenic and nickel all accounted for <0.0007%. Black stones comprise hexanoic acid (1.62%), oxides of iron (1.9%), sulphur (1.64%), potassium (2%), copper (0.2%), chlorine (0.98%); while nickel, lead and arsenic all accounted for <0.002%. The study has revealed that blue vitriol, brimstone and black stones are good alternatives to conventional antifungal drugs.

Keywords: *Antifungals, analysis, blue vitriol, brimstone and black stone.*

1. INTRODUCTION

Mycosis, also known as fungal infection, refers to a group of diseases caused by various species of fungi. While many fungal infections are mild and easily treatable, some can be severe and pose significant health risks, particularly in individuals with weakened immune systems (1).

Antifungal agents otherwise known as antimycotic agents are drugs that selectively eliminate fungal pathogens with minimal toxicity to the host. The four main types of antifungal drugs are polyenes, azoles, allylamines and echinocandins. Depending on the nature of the infection, a topical or systemic agent may be used. The development of antifungal agents has lagged behind antibacterial agents' development. This is a predictable consequence

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of the cellular structure of the organisms involved. Bacteria are prokaryotic and hence offer numerous structural and metabolic targets that differ from those of the human host. Fungi, in contrast, are eukaryotes, and consequently most agents toxic to fungi are also toxic to the host. Furthermore, because fungi generally grow slowly and often in multicellular forms, they are more difficult to quantify than bacteria. Despite these limitations, numerous advances have been made in developing new antifungal agents (2, 3).

Fungi can develop resistance to antifungal drugs the same way bacteria can develop resistance to antibiotics. Resistance happens when germs develop the ability to defeat the drugs designed to kill them. Currently, only a small number of antifungal drugs exist, so resistance can severely limit treatment options (4).

To avoid the limitations of the clinically available antifungals, several investigational agents are currently under evaluations that have novel mechanisms of action against targets. These include those that also target either ergosterol or 1, 3-β-D-glucan, but have advantages over currently available drugs. Among these are the tetrazoles VT-1129, VT-1161, and VT-1598 that are more specific for fungal Cyp51 and less so for mammalian CYP 450 enzymes. Also, a series of molecules with antifungal activity against different strains of fungus have been found in plants, which are of great importance to humans. These molecules may be used directly or considered as a precursor for developing better molecules (5, 6).

In addition, several agents with novel mechanisms of action such as blue vitriol, brimstone and black stone, are also under investigation. Blue vitriol otherwise called blue stone or Roman vitriol is a pentahydrate bright blue crystal with the molecular formular $CuSO_4 \cdot 5H_2O$. It exothermically dissolves in water to give the aquo complex $[Cu(H_2O)_6]^{2+}$, which has octahedral molecular geometry. The structure of the solid pentahydrate reveals a polymeric structure wherein copper is again octahedral but bound to four water ligands. The $Cu(II)(H_2O)_4$ centers are interconnected by sulfate anions to form chains. Copper sulfate has been used for control of algae in lakes and related fresh waters subject to eutrophication. It remains the most effective algicidal treatment. Bordeaux mixture, a suspension of copper(II) sulfate ($CuSO_4$) and calcium hydroxide ($Ca(OH)_2$), is used to control fungus on grapes, melons, and other berries (7, 8, 9, 10).

Brimstone is derived from the Old English *brynstan* and a root meaning "to burn." It is an alternative name for sulfur. It is an element that also forms a yellow crystal. Sulfur (or sulphur) is an abundant mineral and Earth's fifth most common element. It can be considered a semi-precious gemstone. Industrial sulfur applications include rubber vulcanization, matches production, insecticide and fungicide. Sulfur (specifically octasulfur, S_8) is used in pharmaceutical skin preparations for the treatment of acne and other conditions. It acts as a keratolytic agent and also kills bacteria, fungi, scabies mites and other parasites. Precipitated sulfur and colloidal sulfur are used, in form of lotions, creams, powders, soaps, and bath additives, for the treatment of acne vulgaris, acne rosacea, and seborrhoeic dermatitis. Most β-lactam antibiotics, including the penicillins, cephalosporins and monobactams contain sulfur (11, 12, 13).

Black stone otherwise called serpent stone or snake stone has been used since antiquity to treat snake bites and local infections. Black stone is a rocklike substance that extracts poison from the body when placed on the wound inflicted by poisonous creatures like snakes and scorpions. It relieves one from boils or other bodily blisters caused by germs (14, 15).

This research aimed at analyzing the chemical constituents of blue vitriol, brimstone and black stone using x-ray fluorescence technology and GC-MS, a quest for novel antifungal agents.

2. Materials and Methods

Study area

The study was carried out at the Laboratory Unit of Department of Applied Microbiology and Brewing, Nnamdi Azikiwe University, Awka, Anambra State, South-East geopolitical zone of Nigeria.

Samples Collection

Blue vitriol, brimstone and black stone were hygienically selected and purchased at the Eke-Awka market in Awka South Local Government Area of Anambra State, Nigeria. They were transferred into sterile containers and transported to the laboratory for analysis after proper identification at the Laboratory unit of Department of Geological Sciences, Nnamdi Azikiwe University, Awka, Anambra State, Nigeria. The method described by Kamka-Evans *et al.* (16) was adopted.

Isolation and Identification of the fungal organisms

The isolates were obtained from high vaginal swab samples and identified based on their morphological, physiological and molecular characteristics. The identification tests include

sugar fermentation test [18], growth on cornmeal agar [17], germ tube test [17], growth on Chromogenic *Candida* agar [18], and nucleic acid sequence analysis [26].

MIC and MFC Determination using the Broth Dilution Method

From the stock concentration of 200 mg/ml of the test agents, various concentrations of the test agents were made in Sabouraud dextrose broth (10mL: 10mL ratio) by double fold serial dilution to obtain 100 mg/mL, 50 mg/mL, 25 mg/mL, 12.25 mg/mL, 6.325 mg/mL, 3.125 mg/mL and 1.5625 mg/mL. Each dilution in a test-tube was inoculated with 0.2 mL of the broth culture of test isolates (0.5 McFarland standards). All the tubes were incubated at 25°C for 24 hours. The lowest concentration showing no visible growth (as compared with a negative control) was recorded as the minimum inhibitory concentration (MIC) for each organism (18).

The MFC was determined by transferring 0.2mL from each negative tube in MIC assay, onto the surface of freshly prepared Sabouraud dextrose agar plates using the spread plate method. This was incubated at 25°C for 48 hrs. The lowest concentration showing no visible growth on SDA was recorded as minimum fungicidal concentration (MFC) for each organism (18).

Gas Chromatography-Mass Spectroscopy (GC-MS) for organic constituents' analysis

The GC–MS analysis of the solutions was done using Agilent Technologies GC systems with GC-7890A/MS-5975C model (Agilent Technologies, Santa Clara, CA, USA) equipped with HP-5MS column (30 m in length × 250 μm in diameter × 0.25 μm in thickness of film). Spectroscopic detection by GC–MS involved an electron ionization system which utilized high energy electrons (70 eV). Pure helium gas (99.995%) was used as the carrier gas with flow rate of 1 mL/min. The initial temperature was set at 50 –150°C with an increasing rate of 3°C/min and holding time of about 10 min. Finally, the temperature was increased to 300°C at 10°C/min. One microliter of the prepared 1% of the extracts diluted with respective solvents was injected in ansplitless mode. Relative quantity of the chemical compounds present in each of the samples was expressed as percentage based on peak area produced in the chromatogram. Bioactive compounds in the various solutions were identified based on GC retention time on HP-5MS column and matching of the spectra with computer software data of standards (Replib and Mainlab data of GC–MS systems) as described by Buss and Butler (19).

X-Ray fluorescence spectroscopy for inorganic constituents' analysis

The samples size was first reduced to meet a ~10-μm particle size fraction. Once in powdered form, the sample was packed into a sample tray and analyzed by XRF. The XRF analysis provides quantitative data for a suite of minerals. Identification of minerals is based on the location and intensity of peaks on the 2θ scale. Samples prepared were scanned with a Scintag® XFS2000 X-ray fluorescence using CuKα radiation at 40 kV and 30 mA. The majority of the scans were performed using a continuous scan mode from 2 to 34° 2θ with a 0.05 step size at 2 degrees per minute. The collected data were then analyzed using Jade 9+® software. To conclude the process, the results were assembled into easy-to-read spreadsheets, and the XRF trace for each sample was put into the form of a jpg image as described by Maddix *et al.* (20).

3. RESULTS

Table 1: MIC of the test agents against Candida albicans using broth dilution method

Concentration (mg/mL)	Blue Vitriol	Brimstone	Black stone	Ketoconazole (Control)
1.56	+	+	+	+
3.13	+	+	+	+
6.25	+	+	+	+
12.50	+	+	+	+
25	+	+	+	+
50	-	+	+	-
100	-	+	+	-
200	-	-	-	-

\+ Presence of growth
\- No visible growth

Table 2: MIC of the test agents against Candida tropicalis using broth dilution method

Concentration (mg/mL)	Blue stone	Sulphur stone	Black stone	Ketoconazole (Control)
1.56	+	+	+	+
3.13	+	+	+	+
6.25	+	+	+	+
12.50	+	+	+	+
25	-	+	+	-
50	-	-	+	-
100	-	-	+	-
200	-	-	-	-

\+ Presence of growth
\- No visible growth

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Table 3: MIC of the test agents against Candida glabrata using broth dilution method

Concentration (mg/mL)	Blue Vitriol	Brimstone	Black stone	Ketoconazole (Control)
1.56	+	+	+	+
3.13	+	+	+	+
6.25	+	+	+	+
12.50	-	+	+	+
25	-	+	+	+
50	-	-	+	-
100	-	-	+	-
200	-	-	-	-

\+ Presence of growth
\- No visible growth

Table 4: MIC of the test agents against Candida parapsilosis using broth dilution method

Concentration (mg/mL)	Blue Vitriol	Brimstone	Black stone	Ketoconazole (Control)
1.56	+	+	+	+
3.13	+	+	+	+
6.25	+	+	+	+
12.50	-	+	+	-
25	-	+	+	-
50	-	+	+	-
100	-	-	+	-
200	-	-	-	-

\+ Presence of growth
\- No visible growth

Table 5: MFC of the test agents against Candida albicans using broth dilution method

Concentration (mg/mL)	Blue vitriol	Brimstone	Black stone	Ketoconazole (Control)
1.56	+	+	+	+
3.13	+	+	+	+
6.25	+	+	+	+
12.50	+	+	+	+
25	+	+	+	+
50	+	+	+	-
100	-	+	+	-
200	-	-	-	-

\+ Presence of growth
\- No visible growth

Table 6: MFC of the test agents against Candida tropicalis using broth dilution method

Concentration (mg/mL)	Blue vitriol	Brimstone	Black stone	Ketoconazole (Control)
1.56	+	+	+	+
3.13	+	+	+	+
6.25	+	+	+	+
12.50	+	+	+	+
25	+	+	+	-
50	-	+	+	-
100	-	-	+	-
200	-	-	-	-

\+ Presence of growth
\- No visible growth

Table 7: MFC of the test agents against Candida glabrata using broth dilution method

Concentration (mg/mL)	Blue vitriol	Brimstone	Black stone	Ketoconazole (Control)
1.56	+	+	+	+
3.13	+	+	+	+
6.25	+	+	+	+
12.50	+	+	+	+
25	-	+	+	+
50	-	-	+	-
100	-	-	+	-
200	-	-	-	-

\+ Presence of growth
\- No visible growth

Table 8: MFC of the test agents against Candida parapsilosis using broth dilution method

Concentration (mg/mL)	Blue vitriol	Brimstone	Black stone	Ketoconazole (Control)
1.56	+	+	+	+
3.13	+	+	+	+
6.25	+	+	+	+
12.50	+	+	+	+
25	-	+	+	-
50	-	+	+	-
100	-	-	+	-
200	-	-	-	-

\+ Presence of growth
\- No visible growth

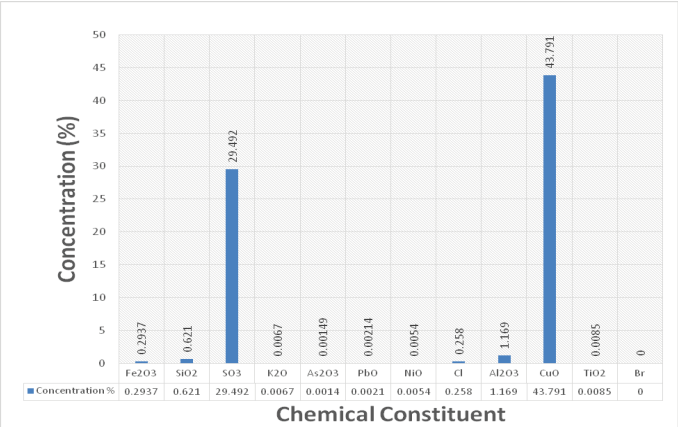

Figure 1: Inorganic chemical constituents of blue vitriol using X-Ray fluorescence spectroscopy

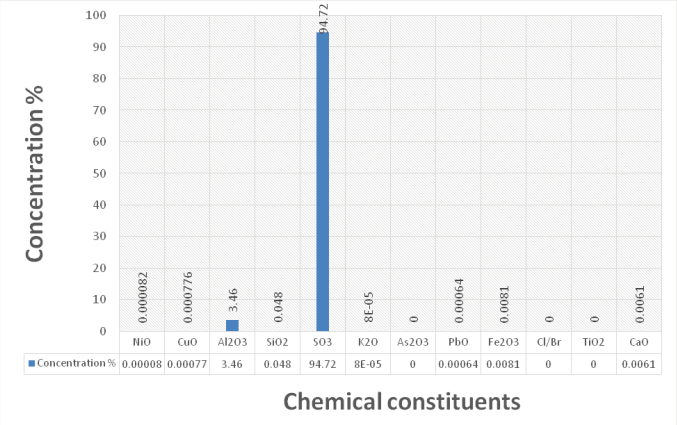

Figure 2: Inorganic chemical constituents of brimstone using X-Ray fluorescence spectroscopy

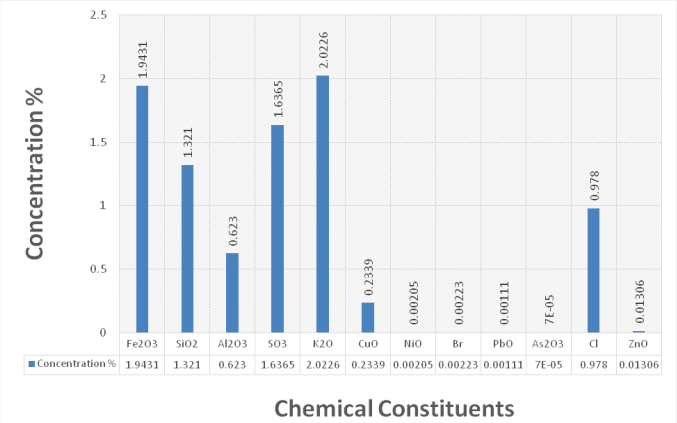

Figure 3: Inorganic chemical constituents of black stone using X-Ray fluorescence spectroscopy

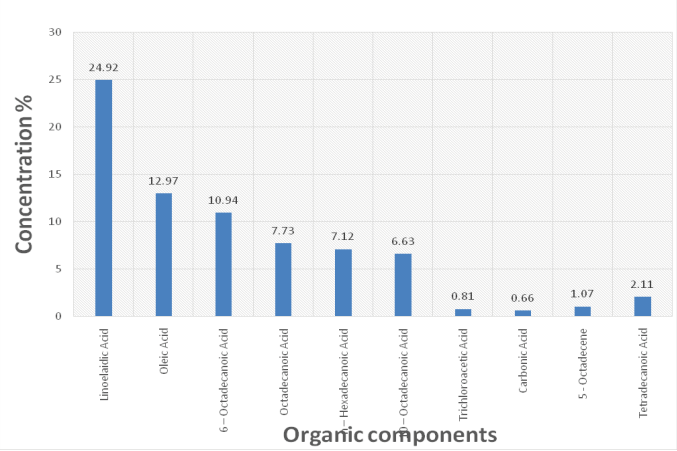

Figure 4: Organic chemical constituents of blue vitriol using gas chromatography-mass spectroscopy

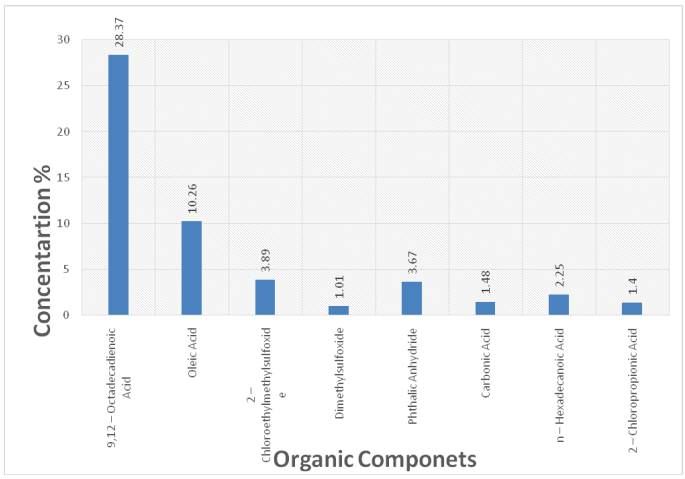

Figure 5: Organic chemical constituents of brimstone using gas chromatography-mass spectroscopy

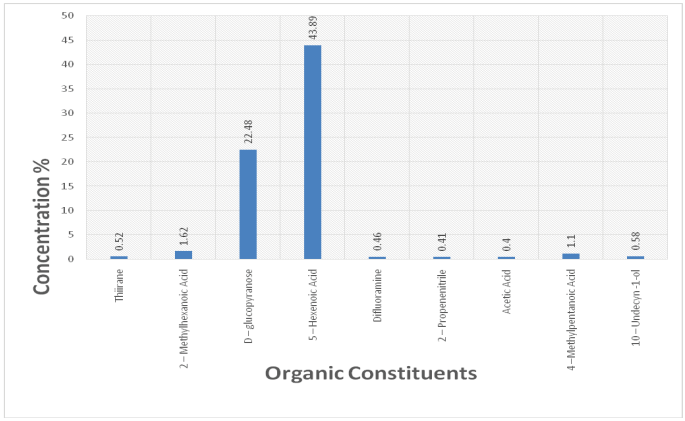

Figure 6: Organic chemical constituents of black stone using gas chromatography-mass spectroscopy

4. DISCUSSION

The increased risk of fungal diseases particularly in immune compromised patients, emerging fungal pathogens, limited repertoire of antifungal drugs, toxicity and the development of resistance to the available antifungal drugs, have increased the demand for the development of new and effective antifungal agents. These have resulted in intensified efforts on antifungal drug search to develop clinically effective and safer antifungal agents. Antifungal research is particularly challenging, with little achievements recently.

The natural compounds studied (blue vitriol, black stones and brimstone) are good antimicrobial agents showing a varying degree of growth inhibition. Their antimicrobial activities increased with concentration. Blue vitriol showed remarkable growth inhibition as observed in its MIC and MFC values in tables 1 to 8. This is probably due to its chemical constituents such as copper, sulphur, oleic acid, linoelaidic acid and octadecanoic acid (figures 1 and 4). The findings are in line with

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Essa and Khallaf (21) who reported the antifungal activity of different solutions of copper sulphate in deionized distilled water against *Aspergillus flavus, A. niger, Penicillium chrysogenum, Fusarium solani* and *Alternaria solani.* Tables 1 – 8 show the MIC values of blue vitriol against the isolates: *Candida albicans* (50mg/mL), *C. tropicalis* (25mg/mL), *C. glabrata* (12.50mg/mL), and *C. parapsilosis* (12.50mg/mL), while the MFC values are *C. albicans* (100mg/mL), *C. tropicalis* (50mg/mL), *C. glabrata* (50mg/mL) and *C. parapsilosis* (25mg/mL). Figure 1 shows the inorganic components found in blue vitriol include oxides of copper (43.7%), sulphur (29.4%), and aluminium (1.2%). The heavy metals which are present in very negligible amount include arsenic, lead and nickel (all <0.009%) thus making it safe especially for topical applications. Even though Copper Sulphate is considered toxic for parenteral use, it is still listed as an antidote (an emetic) in the WHO Anatomical Therapeutic Chemical Classification system (22). Figure 4 shows the organic components which are basically natural fatty acids such as linoelaidic acid and oleic acid. Sulphur has been applied in the treatment of various skin diseases owing to its safety in topical applications (23). Based on the findings (figures 2 and 5), brimstone was observed to be quite effective against the test isolates, probably due to the elemental sulphur, octadecanoic acid and oleic acid. Tables 1 to 8 show the MIC values of brimstone against the isolates: *Candida albicans* (200mg/mL), 50mg/mL, *C. tropicalis* (50mg/mL), *C. glabrata* (50mg/mL), and *C. parapsilosis* (100mg/mL); while the MFC values are *C. albicans* (200mg/mL), *C. tropicalis* (100mg/mL), *C. glabrata* (50mg/mL) and *C. parapsilosis* (100mg/mL). Ismail *et al.* (24) reported that medical drugs, based in elemental sulphur, are well demanded nowadays due to their high efficacy and low cost. The major components of brimstone are elemental sulphur (94.72%), and aluminum (3.46%) (figure 2). In figure 5, lead, arsenic and nickel are less than 0.0007%. Oleic acid, decanoic acid and other organic acids make up the organic components. They probably also contribute to the antimicrobial activity of brimstone.

Black stones traditionally known as snake stone in some African countries have been used since antiquity to treat snake bites and local infections, though there was no evidence of its antimicrobial property either (25). Black stones comprise oxides of iron (1.9%), sulphur (1.64%), potassium (2%), copper (0.2%), chlorine (0.98%), nickel, lead and arsenic (all <0.002%) as shown in figure 3. Hexanoic and Pentanoic acid make up the organic components (figure 6). The above listed components must have contributed to the mild *in vitro* antimicrobial effect of black stone on the isolates. The MIC value of black stone against all the isolates was 200mg/mL (tables 1 to 4). Table 1 shows the MIC of ketoconazole, the positive control ranging from 12.50mg/mL to 50mg/mL. From the above findings, blue vitriol, brimstone and black stone are good antimicrobial agents owing primarily to their chemical constituents.

5. Conclusion

Though several antifungal agents are available in the marketplace, their therapeutic outcome is less than optimal. This is probably due to some limitations such as efficacy, resistance and toxicity. The findings have revealed that blue vitriol, black stone and brimstone are potentially safe and effective in the treatment of candidiasis; therefore, could serve as substitutes to conventional antifungal antibiotics.

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