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Combined activity of blue vitriol, brimstone and black stone on clinical *Candida species* isolates using fractional inhibitory concentration Index

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Abstract

Combination antimicrobial therapy (CAT) involves intentional addition of two or more antimicrobial agents for better therapeutic outcome. CAT is normally applied for treatment of polymicrobial infections, life-threatening infections and prevention of the emergence of resistance. This research aimed at determining the combined activity of blue vitriol, brimstone and black stone on clinical *Candida species* isolates using Fractional Inhibitory Concentration Index. The brimstone, blue vitriol and black stone samples were purchased from a community market in southeastern Nigeria. The isolates were obtained from high vaginal swab samples of patients attending a Teaching hospital in Nigeria and identified based on their morphological, physiological and molecular characteristics. The minimal inhibitory concentrations (MIC) of the samples were determined using the broth dilution method. The fractional inhibitory concentration indexes (FICI) of the samples against the isolates were obtained from their individual FIC values. FICI of <1 indicates synergism; 1 to 2 (Indifference); while >2 (antagonism). All the test agents were synergistic against *C. albicans* (FICI < 1). All the test agents were indifferent against *C. tropicalis* (FICI 1 - 2). Brimstone + black stone combination was indifferent (FICI 1 - 2) against *C. glabrata;* while all the test agents were antagonistic against the isolate (FICI > 2). Blue vitriol + Black stone combination was antagonistic (FICI > 2) against *C. parapsilosis*; Blue vitriol + brimstone was indifferent (FICI 1 - 2); while brimstone + black stone was synergistic (FICI < 1). The findings have revealed that antimicrobial combination outcome could be synergistic, antagonistic or indifferent; thus the need for proper *in vitro* screening before combination therapy.

Keywords: Combination; Antimicrobial; Therapy; Antagonism; Synergism and Indifferent

1. Introduction

Fungi are eukaryotic organisms found in the form of yeasts, molds, or dimorphic fungi. *Candida* is a form of yeast. Candidiasis or thrush is an opportunistic fungal infection caused by any of the species from the genera *Candida*, amongst which *C. albicans* is the most common causative species. The infection is technically referred to as candidosis, moniliasis as well as oidiomycosis. These infections are broad spectrum, ranging from superficially oral thrush and vaginitis to deep cited systemic, which are mostly life threatening (Raesi *et al*., 2019). Predisposing factors of candidiasis include prolonged antibiotic use, diabetes mellitus, tuberculosis, myxedema, oral contraceptive use, hypoparathyroidism, Addison's disease, nutritional deficiency (vitamin A, B6, Iron), xerostomia, smoking, poorly maintained dentures, intraveinous tubes, catheters, heart valves, old age, infancy, and pregnancy (Bertolini and

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Dongari-Bagtzoglou, 2019). *Candida* infections are treated with antifungal medications such as nystatin, clotrimazole, amphotericin B and miconazole. Mild or moderate genital *Candida* infections can be treated using antifungal vaginal creams (Fang *et al*., 2021). The incidence of invasive and disseminative candidiasis has been on the rise globally, and people with an impaired immune system are the most vulnerable (Dubey and Singla, 2019).

The discovery of new antibiotic classes in the past three decades has been slow. The reasons include limited investment in drug discovery endeavors by major pharmaceutical companies, depletion of low-hanging fruits from the previous decade, and exhaustion of new drug candidates because of the use of the same drug compound libraries (McKenna, 2020). New drugs are continually required by the healthcare systems to address unmet medical needs across diverse therapeutic areas, and pharmaceutical industries primarily strive to deliver new drugs to the market through the complex activities of drug discovery and development (*Newman and Cragg, 2016).* Natural products may be useful as a source of novel chemical structures for modern techniques of development of antimicrobial therapies *(Harvey et al., 2015; Newman and Cragg, 2016; Ahn, 2017; Torre and Albericio, 2017).*

Brimstone, blue vitriol and black stone have been used traditionally for many years. *Brimstone* is derived from the Old English *brynstan* and a root meaning "to burn." Historically and in literature, sulfur is also called brimstone, which means "burning stone". Sulfur is the tenth most abundant element by mass in the universe and the fifth most abundant on Earth. Though sometimes found in pure native form, sulfur on Earth usually occurs as sulfide and sulfate minerals. Being abundant in native form, sulfur was known in ancient times, being mentioned for its uses in ancient India, ancient Greece, China, and ancient Egypt (*Greenwood and Earnshaw,1996; Chisholm, 1911).* Elemental sulfur is one of the oldest fungicides and pesticides. "Dusting sulfur", elemental sulfur in powdered form, is a common fungicide for grapes, strawberry, many vegetables and several other crops. It has a good efficacy against a wide range of powdery mildew diseases as well as black spot (*Hyndman et al., 1982).*

Vitriol is the general chemical name encompassing a class of chemical compounds comprising sulfates of certain metals – originally, iron or copper. Those mineral substances were distinguished by their color, such as green vitriol for hydrated iron (II) sulfate and blue vitriol for hydrated copper (II) sulfate. These materials were found originally as crystals formed by evaporation of groundwater that percolated through sulfide minerals and collected in pools on the floors of old mines. The word *vitriol* comes from the Latin word *vitriolus*, meaning "small glass", as those crystals resembled small pieces of colored glass (*Karpenko and Norris, 2002).* Copper sulfate is highly soluble in water. The largest health benefit of copper sulfate is that it is used to control bacteria and fungus growth on fruits, vegetables, and other crops (Zumdahl and Decoste, 2013).

Black stone also known as snakestone or serpent stone has been used since antiquity to treat snake bites and local infections; though its efficacy is debated (Szweda *et al.*, 2015).

A combinationantibiotic is one in which two ingredients are added together for additional therapeutic effect. One or both ingredients may be antibiotics (*Bassetti and Righi, 2015).* Combination therapy comprises treatment regimens that include multiple antifungals from different classes and antifungal agents combined with non-antifungal agents. Non-antifungal drug targets include heat shock proteins, calcineurin, lysine acetyltransferase, lysine deacetylase, protein kinase C, and fungal sphingolipids (Spitzer *et al*., 2017). Combining drugs has the potential to confer enhanced efficacy and specificity compared to individual drug treatments and can slow the evolution of resistance. Further, by carefully selecting specific drug combinations, microbial drug resistance may not only be neutralized but also reversed through a process called selection inversion. Combination therapy is already the treatment of choice for many infectious diseases including HIV, tuberculosis and malaria. Consequently, the use of drug combinations to treat fungal pathogens has garnered considerable interest over the past several years (Zimmermann *et al.*, 2007; Hill and Cowen, 2015; Baym *et al*., 2016; Zumla *et al*., 2012).

Many approaches have been developed to deal with the complex nature of compound interactions that are based on the additive or multiplicative models just described. Quantification of compound interactions in the laboratory is traditionally done by calculation of fractional inhibitory concentration (FIC) index. Other methods to assess compound interactions include E-test, time-kill and disk diffusion assays (Greco *et al*., 1995; Eliopoulos and Moellering, 1996; Rex *et al*., 2001; Canton *et al*., 2014).

This research was aimed at accessing the combined activity of blue vitriol, brimstone and black stone on clinical *Candidaspecies* isolates using Fractional Inhibitory Concentration Index (FICI).

2. Materials and Methods

2.1. Study Area

This study was carried out at the Laboratory Unit of Department of Applied Microbiology and Brewing, Nnamdi Azikiwe University, Awka, Anambra State, Nigeria.

2.2. Samples Collection and Processing

The brimstone, vitriol and black stone samples were hygienically selected after purchase from the Eke-Awka market in Awka South Local Government Area of Anambra State, Nigeria. The samples were transferred into sterile containers and transported to the laboratory for processing and analysis as described by Kamka-Evans *et al*. (2013).

Obvious impurities were gently and carefully removed manually, from the stones after which known weights of the natural compounds were soaked in water at room temperature and placed in the shaker at 60rpm at 40 ^{o}C. The samples dissolved completely within two hours; except sulphur stone that dissolved within four hours (Udemezue and Oyeka, 2021).

2.3. Identification of yeast isolates

The yeast isolates were obtained from high vaginal swab samples aseptically and properly collected from patients suspected of suffering from vulvo-vaginal candidiasis (Udemezue and Oyeka, 2021). They were identified based on their morphological, physiological and molecular characteristics which included sugar fermentation test (Pincus *et al*., 2007), growth on cornmeal agar (Pincus *et al*., 2007), germ tube test (Moya-Salazar and Rojasa, 2018) growth on Chromogenic *Candida* agar (Cheesbrough, 2018) and nucleic acid sequence analysis (Morey *et al*., 2013; Todd, 2012).

2.4. Combination Study: Determination of Minimal Inhibitory Concentration (MIC) and Fractional Inhibitory concentration index (FICI) using 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 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 (Cheesbrough, 2018; (Udemezue and Oyeka, 2021).

Interaction study techniques as employed by Afunwa *et al.,* 2011, were used in this study with a little modification. Stock solutions of 200mg/ml were prepared for Blue Vitriol (A), Brimstone (B) and Black stone (C). The stock solutions were combined (Blue vitriol + Brimstone, Blue vitriol + Black stone and Brimstone + Black stone), in a ratio of 1:1. A two-fold serial dilution was performed on the combination ratio using Mueller-Hinton broth as the diluent. Each dilution in a test-tube was inoculated with 0.2 ml of the broth culture of test isolates diluted to 0.5 McFarland standards. All the tubes were incubated at 25 °C for 24 hrs. The lowest concentration of the combined compounds in the test tubes with no observable growth was taken as the minimum inhibitory concentration (MIC) (Ofokansi *et al.,* 2013). Interaction between the compounds for each isolate, in a 1:1 ratio was determined by calculating their Fractional inhibitory concentration Index (FIC index) using the Equation below:

- MIC A: Minimum inhibitory concentration of first agent in combination ratio
- MIC B: Minimum inhibitory concentration of second agent in combination ratio
- MIC C: Minimum inhibitory concentration of third agent in combination ratio
- FIC: Fractional inhibitory concentration

$$\text{FIC index} = \text{FIC A} + \text{FIC B} + \text{FIC C}$$

$$\text{Where: FIC A} = \frac{Mic\ of\ Drug\ A\ in\ combination\ with\ Drug\ B}{Mic\ of\ Drug\ A\ alone}$$

$$\text{FIC B} = \frac{Mic\ of\ Drug\ B\ in\ combination\ with\ Drug\ A}{Mic\ of\ Drug\ B\ alone}$$

$$\text{FIC C} = \frac{Mic\ of\ Drug\ C\ in\ combination\ with\ Drug\ B}{Mic\ of\ Drug\ C\ alone}$$

Where A, B and C are the antimicrobial agents being combined; A = Blue vitriol, B = Brimstone, C = black stone

The drug interaction is interpreted as synergism if FICI < 1.0; additive (FICI = 1.0) indifference (FICI = 1 – 2), antagonism (FICI > 2.0) and no activity (FICI = 0).

3. Results

Table 1 Combined activity of brimstone, blue vitriol and black stone against *Candida albicans*

Agent	MIC A (mg/ml)	MIC B (mg/ml)	MIC C (mg/ml)	Combined MIC	FIC A	FIC B	FIC C	FIC index	INFERENCE
Blue vitriol + Brimstone	50	200	-	20.63	0.4126	0.10315	-	0.51575	Synergism
Blue vitriol + Black stone	50	-	200	35.7	0.714	-	0.1785	0.8925	Synergism
Brimstone + Black stone	-	200	200	35.1	-	0.1755	0.1755	0.351	Synergism

Table 2 Combined activity of brimstone, blue vitriol and black stone against *Candida tropicalis*

Agent	MIC A (mg/ml)	MIC B (mg/ml)	MIC C (mg/ml)	Combined MIC	FIC A	FIC B	FIC C	FIC index	INFERENCE
Blue vitriol + Brimstone	25	50	-	20.76	0.8304	0.4152	-	1.2456	Indifference
Blue vitriol + Black stone	25	-	200	33.77	1.3508	-	0.16885	1.51965	Indifference
Brimstone + Black stone	-	50	200	56.14	-	1.1228	0.2807	1.4035	Indifference

Table 3 Combined activity of brimstone, blue vitriol and black stone against *Candida glabrata*

Agent	MIC A (mg/ml)	MIC B (mg/ml)	MIC C (mg/ml)	Combined MIC	FIC A	FIC B	FIC C	FIC index	INFERENCE
Blue vitriol + Brimstone	12.5	50	-	20.76	1.6608	0.4152	-	2.076	Antagonism
Blue vitriol + Black stone	12.5	-	200	33.77	2.7016	-	0.16885	2.87045	Antagonism
Brimstone + Black stone	-	50	200	56.14	-	1.1228	0.2807	1.4035	Indifference

Table 4 Combined activity of brimstone, blue vitriol and black stone against *Candida parapsilosis*

Agent	MIC A (mg/ml)	MIC B (mg/ml)	MIC C (mg/ml)	Combined MIC	FIC A	FIC B	FIC C	FIC index	INFERENCE
Blue vitriol + Brimstone	12.5	100	-	20.76	1.6608	0.2076	-	1.8684	Indifference
Blue vitriol + Black stone	-	12.5	200	33.77	-	2.7016	0.16885	2.87045	Antagonism
Brimstone + Black stone	-	100	200	56.14	-	0.5614	0.2807	0.8421	Synergism

4. Discussion

The discovery of novel antimicrobials in the past three decades especially antifungals, has been very slow. Thus, there is urgent need to develop new, readily available, more potent and safe antimicrobials. Natural compounds such as brimstone, blue vitriol and black stone could serve as excellent alternatives to conventional antifungal agents.

Combination antimicrobial therapy has been used over the years for many reasons including empirical treatment of life-threatening infections; treatment of polymicrobial infections; prevention of the emergence of resistance; and for synergism. Winzeler *et al*., (1999) and Costanzo *et al*., (2010) noted that an additional advantage of combination therapy is that it has the potential to unveil a plethora of additional antifungal targets given that eukaryotic genomes are proven to be highly interconnected and functionally redundant.

The fractional inhibitory concentration index (FICI) showed the outcome of the combination. If the FIC index is less than one, the combined activity is synergism, between 1 and 2 (Indifference) while above 2 is antagonism. In Table 1, all test agents were synergistic against *C. albicans* (FICI < 1). This therapeutic outcome has numerous advantages over monotherapy; thus supporting the findings by Zimmermann *et al*. (2007) and Hill *et al*., (2015) who stated that "combining drugs has the potential to confer enhanced efficacy and specificity compared to individual drug treatments and can slow the evolution of resistance". Onyewu *et al.* (2003) also observed that compound combinations might improve fungicidal efficacy through synergy and result in greater therapeutic effect and broader activity than can be achieved with either drug alone.

In Table 2, all the test agents were indifferent against *C. tropicalis* (FICI between 1 and 2). All the test agents were antagonistic against *C. glabrata* (FICI > 2) while brimstone + black stone combination was indifferent (FICI between 1and 2) against the isolate as shown in Table 3.

The combined activity against *C. parapsilosis* varied among the test agents as shown in Table 4. Blue vitriol + Black stone combination was antagonistic (FICI > 2); Blue vitriol + brimstone (indifferent with FICI between 1 and 2); while brimstone + black stone (Synergistic with FICI < 1).

Combination therapy is already the treatment of choice for many infectious diseases including HIV (Bock and Lengauer, 2012), tuberculosis (Zumla *et al.*, 2012) and malaria (Eastman and Fidock, 2009). Consequently, the use of drug combinations to treat fungal pathogens has garnered considerable interest over the past several years. This research has revealed that quantification of antimicrobial interactions can be done *in vitro* by calculation of fractional inhibitory concentration (FIC) index (FICI) of the various drugs combinations.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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