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Original Research Article

The Effects of Blue Vitriol and Brimstone on Etiological Agents of Bacterial Vulvovaginitis

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ABSTRACT

Vulvovaginitis is an inflammation of the vulva and/or the vagina which may be symptomatic or asymptomatic. It is a common public health problem that can affect women and girls of all ages. The study aimed at evaluating the effects of blue vitriol and brimstone on etiological agents of bacterial vulvovaginitis. One hundred high vaginal swab specimens were aseptically and properly collected from suspected cases of vulvovaginitis. Plates of nutrient agar and blood agar were inoculated and incubated aerobically at 25 ^{o}C for 24 hours. The isolates were identified based on their morphological, physiological, biochemical and molecular characteristics. The antimicrobial activity of blue vitriol and brimstone was determined using the agar-well diffusion method. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of the test samples were determined using the broth dilution method. The isolates include *Staphylococcus epidermidis*, *Escherichia coli*, *Lactobacillus spp. and Staphylococcus aureus.* The inhibition zone diameter (IZD) of blue vitriol against the isolates ranges from 30.00±0.00mm to 31.50±2.12mm at concentrations of 50mg/ml to 200mg/ml. The IZD of brimstone against the isolates ranges from 29.00±1.41mm to 11.00±0.00mm at concentrations of 50mg to 200mg/ml. The positive control, ciprofloxacin gave IZD of 17.50±2.12mm to 21.50±2.12mm. The MIC of blue vitriol against all the isolates was 12.50mg/ml; while the MBC ranges between 25mg/ml to 50mg/ml. The MIC of brimstone was 25mg/ml to 200mg/ml; while the MBC was 50mg/ml to 200mg/ml. Ciprofloxacin gave MIC and MBC ranging from 25mg/ml to 100mg/ml against all the isolates. The study has shown that natural compounds such as blue vitriol and brimstone are good antimicrobials; and could serve as substitutes to conventional antimicrobial agents in the treatment of bacterial vulvovaginitis.

Keywords: Vagina, Vulva, Vulvovaginitis, Blue vitriol, Brimstone, Antimicrobials.

INTRODUCTION

Vulvovaginitis also called vaginitis is an inflammation or infection of the vagina. It can also affect the vulva, which is the external part of a woman's genitals. It usually happens when there is a change in the balance of bacteria or yeast that is normally found in the vagina. Vaginitis is the most common vaginal infection in women within the ages of 15 - 44. Although vulvovaginitis occurs in 75% of healthy women over the course of their lifetimes, certain conditions are associated with increased risk, including age, phase of the menstrual cycle, HIV infection, pregnancy, diabetes mellitus, exposure to broad-spectrum antibiotics, douching, use of intrauterine device (IUD), having unprotected sex with a new partner and having multiple sexual partners (1).

Trichomoniasis (TV), bacterial vaginosis (BV) and vulvovaginal candidiasis (VVC) are the most common etiologies of vaginitis worldwide (2, 3). The global prevalence of TV and BV is estimated to be 5.3% and 26%, respectively, with variation among populations with different background characteristics and geographic locations (4, 5). The bacterial microbiota in the vagina of a healthy woman of reproductive age is dominated by lactobacilli (6). Lactobacilli produce lactic acid as a result of the fermentation of carbohydrates, mainly glycogen, present in the vaginal epithelium of women at menarche. This acidic environment provides protection against infectious diseases by preventing the vaginal colonization of potential pathogens. Vaginitis may be symptomatic or asymptomatic. In general, vaginitis symptoms include: a change in the color, odor, or volume of vaginal discharge, abdominal pain, discolored or swollen vulva, discomfort during sex, itching, burning, or pain in the vagina or vulva, light vaginal bleeding or spotting, and painful urination (7).

Treatment of asymptomatic bacterial vaginosis in pregnancy has not been found to alter pregnancy outcomes. Treatment may be oral or intra-vaginal. Studies comparing oral versus topical therapy suggest higher cure rates with seven days of oral metronidazole or clindamycin; however this must be balanced against its higher rate of adverse effects. For pregnant women, clindamycin is category A. Metronidazole is in category B2 for pregnancy, but has not been proven to be harmful (8).

Vaginitis is one of the most common gynecological problems in reproductive age. Because of the limitations of the conventional drugs such as cost, resistance and poor efficacy, identification of new pharmacological interventions for this disease seems to be necessary (9, 10).

Blue vitriol, or copper sulfate, is a water soluble, blue crystalline powder typically sold as a chemical reagent in solid form. It is hydrated copper sulfate (pentahydrate) and its chemical formula is $CuSO_4.5H_2O$. It forms as large, bright blue crystals containing five molecules of water ($CuSO_4 \cdot 5H_2O$). The anhydrous salt is produced by heating the hydrate to 150 °C (300 °F). Cupric sulfate is utilized chiefly for agricultural purposes, as a pesticide, germicide, feed additive, and soil additive. Among its minor uses are as a raw material in the preparation of other copper compounds; as a reagent in analytic chemistry; as an electrolyte for batteries and electroplating baths; and in medicine as a locally applied fungicide, bactericide, and astringent. Copper sulfate was once used to kill bromeliads, which serve as mosquito breeding sites. Copper sulfate is used as a molluscicide to treat bilharzia in tropical countries. Older names for the pentahydrate include blue vitriol, bluestone, vitriol of copper, and Roman vitriol (11, 12, 13, 14, 15, 16).

Brimstone is derived from the Old English *brynstan* and a root meaning "to burn." These days, few people refer to the non-metallic chemical element sulfur this way, but are more likely to use the word in the Biblical phrase "fire and brimstone." Sulfur is a chemical element that is present in all living tissues. After calcium and phosphorus, it is the third most abundant mineral in the human body. Sulfur is also found in garlic, onions and broccoli. Sulfur seems to have antibacterial effects against the bacteria that cause acne. It is also applied to the skin for acne and skin redness (rosacea) and taken orally for many other conditions, but there is limited scientific evidence to support these uses (17, 18, 19, 20, 21).

The research work aimed at evaluating the effects of Blue Vitriol and Brimstone on etiological agents of bacterial vulvovaginitis.

MATERIALS AND METHODS

Study area

This 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.

Ethical Considerations

Ethical Clearance was obtained from the Ethical Committee of Chukwuemeka Odumegwu Ojukwu University Teaching Hospital, Awka, Anambra State, Nigeria.

Specimens Collection

One hundred high vaginal swab specimens were aseptically and properly collected from suspected cases of vulvovaginitis at Chukwuemeka Odumegwu Ojukwu University Teaching Hospital.

Isolation and Identification of Isolates

Plates of blood agar and nutrient agar supplemented with of nystatin (100 U/mL) were inoculated and incubated aerobically at 25 ^{o}C for 24 hours (22). The isolates were identified based on their morphological, physiological, biochemical and molecular characteristics. The identification tests include Gram staining, indole test, Voges-Proskauer (VP), citrate utilization, nutrient utilization (carbohydrate utilization, amino acid degradation, lipid degradation), enzyme production (oxidase, catalase, coagulase, hemolysins) (23). The molecular identification procedures include Genomic DNA isolation of the microbial isolates, polymerase chain reaction test and nucleic acid sequencing analysis (10).

Evaluation of Antimicrobial Activity of Blue vitriol and Brimstone Using the Agar-Well Diffusion Method

Stock solutions of blue vitriol and brimstone were prepared by dissolving 2g each of the test agents in 10ml of sterile water to obtain 200 mg/mL stock solutions. Double-fold serial dilution of the stock solutions was performed to obtain 100 mg/mL, 50 mg/mL, 25 mg/mL, 12.5 mg/mL, 6.25 mg/mL and 3.125 mg/mL concentrations. Using 6mm cork-borer, wells were bored on plates of Muller Hinton Agar. The agar plates were seeded with 0.1 mL (equivalent of 0.5McFarland standard: 2 x 10^{5}cfu/ml) of the bacterial isolates using spread plate method. The test solutions (0.5 mL) were added into the wells using 1ml sterile glass pipette. The positive and negative controls were ciprofloxacin and distilled water respectively. The experimental setup was incubated aerobically at 25 ^{o}C for 24 hours. All the experiments were performed in triplicate and the results reported as average of 3 experiments. The inhibition zone diameter (IZD in mm) produced was reported as Mean ± Standard deviation (10, 24).

MIC and MBC determination using broth dilution method.

From the stock concentration of 200 mg/mL, various concentrations of the test agents were made in nutrient broth using double-fold serial dilution to obtain, 100 mg/mL, 50 mg/mL, 25 mg/mL, 12.25 mg/mL, 6.325 mg/mL and 3.125 mg/mL. Each dilution in a test tube was inoculated with 0.2mL of broth culture of the test isolates diluted to 0.5 McFarland standards. All the tubes were incubated aerobically at 25 ^{o}C for 24 hours. The lowest concentration showing no visible growth was recorded as the minimum inhibitory concentration (MIC) for each organism. From each negative tube in MIC assay, 0.2 mL was transferred onto freshly prepared nutrient agar plates using the spread plate method and incubated at 25 °C for 24 hours. The lowest concentration showing no visible growth on nutrient agar was recorded as minimum bactericidal concentration (MBC) (10, 25).

RESULTS

Table 1: Inhibition zone diameter of blue vitriol and brimstone against *Staphylococcus epidermidis* using agar-well diffusion method

Concentration (mg/ml)	Blue vitriol (mm)	Brimstone (mm)	Ciprofloxacin
200	30.00±0.00	20.50±2.12	19.50±0.71
100	20.50±0.71	15.00±1.41	-
50	19.00±1.41	-	-
25	15.50±0.71	-	-
12.5	-	-	-

Table 2: Inhibition zone diameter of blue vitriol and brimstone against *Escherichia coli* using agar-well diffusion method

Concentration (mg/ml)	Blue vitriol (mm)	Brimstone (mm)	Ciprofloxacin
200	30.00±0.00	19.00±1.41	27.50±0.71
100	21.50±2.12	11.50±2.12	21.00±2.12
50	19.00±1.41	-	16.00±1.41
25	15.50±0.71	-	-
12.5	-	-	-

Table 3: Inhibition zone diameter of blue vitriol and brimstone against *Lactobacillus spp* using agar-well diffusion method

Concentration (mg/ml)	Blue vitriol (mm)	Brimstone (mm)	Ciprofloxacin
20	29.00±1.41	16.50±2.12	29.00±1.41
100	21.50±2.12	10.00±0.00	23.00±1.41
50	19.00±1.41	-	18.50±2.12
25	12.50±0.71	-	-
12.5	-	-	-

Table 4: Inhibition zone diameter of blue vitriol and brimstone against *Staphylococcus aureus* using agar-well diffusion method

Concentration (mg/ml)	Blue vitriol (mm)	Brimstone (mm)	Ciprofloxacin
200	31.50±2.12	11.50±0.00	17.50±2.12
100	25.00±1.41	-	12.50±0.71
50	15.50±0.71	-	-
25	-	-	-
12.5	-	-	-

Table 5: MIC of blue vitriol and brimstone against *Staphylococcus epidermidis* using broth dilution method.

Concentration (mg/ml)	Blue vitriol	Brimstone	Ciprofloxacin (control)
200	-	-	-
100	-	-	-
50	-	+	-
25	-	+	+
12.5	-	+	+
6.25	+	+	+
3.125	+	+	+

+ Presence of growth
- No visible growth

Table 6: MIC of blue vitriol and brimstone against *Escherichia coli* using broth dilution method.

Concentration (mg/ml)	Blue vitriol	Brimstone	Ciprofloxacin (control)
200	-	-	-
100	-	-	-
50	-	-	-
25	-	-	-
12.5	-	+	+
6.25	+	+	+
3.125	+	+	+

+ Presence of growth
- No visible growth

Table 7: MIC of blue vitriol and brimstone against *Lactobacillus spp* using broth dilution method.

Concentration (mg/ml)	Blue vitriol	Brimstone	Ciprofloxacin (control)
200	-	-	-
100	-	-	-
50	-	+	-
25	-	+	-
12.5	-	+	+
6.25	+	+	+
3.125	+	+	+

+ Presence of growth
- No visible growth

Table 8: MIC of blue vitriol and brimstone against *Staphylococcus aureus* using broth dilution method.

Concentration (mg/ml)	Blue vitriol	Brimstone	Ciprofloxacin (control)
200	-	-	-
100	-	+	-
50	-	+	+
25	-	+	+
12.5	-	+	+
6.25	+	+	+
3.125	+	+	+

+ Presence of growth
- No visible growth

Table 9: MBC of blue vitriol and brimstone against *Staphylococcus epidermidis* using broth dilution method.

Concentration (mg/ml)	Blue vitriol	Brimstone	Ciprofloxacin (control)
200	-	-	-
100	-	+	-
50	-	+	-
25	-	+	+
12.5	+	+	+
6.25	+	+	+
3.125	+	+	+

+ Presence of growth
- No visible growth

Table 10: MBC of blue vitriol and brimstone against *Escherichia coli* using broth dilution method.

Concentration (mg/ml)	Blue vitriol	Brimstone	Ciprofloxacin (control)
200	-	-	-
100	-	-	-
50	-	-	-
25	-	+	-

12.5	+	+	+
6.25	+	+	+
3.125	+	+	+

+ Presence of growth
- No visible growth

Table 11: MBC of blue vitriol and brimstone against *Lactobacillus spp* using broth dilution method.

Concentration (mg/ml)	Blue vitriol	Brimstone	Ciprofloxacin (control)
200	-	-	-
100	-	-	-
50	-	+	-
25	-	+	-
12.5	+	+	+
6.25	+	+	+
3.125	+	+	+

+ Presence of growth
- No visible growth

Table 12: MBC of blue vitriol and brimstone against *Staphylococcus aureus* using broth dilution method.

Concentration (mg/ml)	Blue vitriol	Brimstone	Ciprofloxacin(control)
200	-	-	-
100	-	+	-
50	-	+	+
25	+	+	+
12.5	+	+	+
6.25	+	+	+
3.125	+	+	+

+ Presence of growth
- No visible growth

DISCUSSION

Vulvovaginitis is a highly burdensome, long-lasting medical condition that heavily compromises the activities of women and their quality of life. Current antifungal interventions have experienced a lot of setbacks, partly because the spectrum of the activity of conventional systemic antifungal drugs is narrow while the development of new antifungal drugs has become stagnant, thus the need to develop new and effective antifungal agents.

The bacteria isolates obtained from the high vaginal swab specimens include *Staphylococcus epidermidis, Escherichia coli, Lactobacillus spp. and Staphylococcus aureus.* The presence of *Lactobacillus spp* in the specimens is in line with the findings by Ventolini (26) who noted that the healthy vaginal tract of reproductive-aged women is colonized by normal microbiota dominated by lactobacilli, which protect against pathogenic bacteria species when present in sufficient numbers. Exposure to estrogen during puberty causes the vaginal mucosal epithelium to develop intermediate and superficial layers that become stratified, thicker, and appear light pink. These changes promote a vaginal micro biome that is dominated by *Lactobacillus spp. diphtheroids* and *Staphylococcus epidermidis* (27). *S. epidermidis* is an opportunistic pathogen that can cause vaginitis under favorable physiological conditions. The proximity of the female genitalia to the bowel opening could lead to the contamination of the vaginal area with *Escherichia coli* thus might lead to vaginal infections.

In the vagina, sharp co-operative relationship of microbes with the host provides first line of defense against the migration of opportunistic pathogens. Dysbiosis of vaginal micro biome leads to damage or fall in number of beneficial *Lactobacillus* species, thus increasing host susceptibility to opportunistic pathogens either normally present in human vagina in lower quantity such as *S. epidermidis* and *S. aureus* micro biome or are sexually transmitted. As this nasty cycle remains unchecked for long period, it may magnify the effect of dysbiosis leading to persistent imbalance with chronic inflammation i.e. vulvovaginitis (7).

Complementary and alternative medicine (CAM) is the complement for conventional medical therapy which is becoming increasingly prevalent among women with genital infections. CAM is less invasive, safe, effective, economical, and convenient (28). Blue vitriol and brimstone are good alternatives to conventional antibiotics in treatment of vulvovaginitis.

Staphylococcus epidermidis was susceptible to both blue vitriol and brimstone. Blue vitriol gave the best inhibition zone diameter (IZD) of 30.00±0.00mm while brimstone gave IZD of 20.50±2.12mm as shown in table 1. At 50mg/ml, *Escherichia coli* was resistant to brimstone; but at 200 mg/mL, blue vitriol gave the best IZD of 30.00±0.00mm while brimstone (19.00±1.41mm) as shown in table 2. Blue vitriol (200 mg/mL) gave IZD of 29.00±1.41mm; while brimstone (200 mg/mL) gave IZD of 16.50±2.12mm against *Lactobacillus spp* as shown in table 3. Table 4 shows the inhibition zone diameter of blue vitriol and brimstone against *Staphylococcus aureus*. Blue vitriol gave the best IZD of 31.50±2.12mm at 200 mg/mL while only 200mg/ ml of brimstone was able to inhibit the growth of *Staphylococcus aureus* with IZD of 11.00±0.00mm. The positive control ciprofloxacin gave IZD ranging from 17.50±2.12mm to 21.50±2.12mm as shown in tables 1 – 4. The inhibition zone diameter increased with concentration.

Nwachukwu *et al*. (29) stated that brimstone is one of the substances whose biocide properties (including antibacterial, antifungal and protocidal) have been indeed recognized for a long time. In medicine, blue vitriol has been used as a locally applied fungicide, bactericide, and astringent (12, 14, 16). The findings above support the antimicrobial activity results obtained in this study.

The MIC of blue vitriol against all the isolates was 12.5 mg/mL; while the MIC of brimstone against the isolates ranged between 100 mg/mL – 200 mg/mL, as shown in tables 5 – 8. Similarly, the MBC of blue vitriol against the isolates ranged between 25 mg/mL – 50 mg/mL and Brimstone 50 mg/mL – 200 mg/mL as shown in tables 9 – 12. The MIC and MBC of the positive control, Ciprofloxacin was 25 mg/mL – 100 mg/mL as shown in tables 5 -12. The findings above agree with the report of Udemezue *et al.*, (30) who noted the antimicrobial effects of blue vitriol and brimstone against *Streptococcus mutans, Staphylococcus aureus, Lactobacillus spp and Candida tropicalis* isolated from dental caries.

CONCLUSION

Development of new antimicrobials is very essential in healthcare system in order to address public health concerns such as poor efficacy, resistance, toxicity and adverse effects of conventional antimicrobial agents. Thus, there is urgent need for cheaper, less toxic and more effective antimicrobial agents. The *in vitro* antimicrobial effectiveness of blue vitriol and brimstone has been established in this research.

RECOMMENDATION

Further studies on purification, structural elucidation and commercialization of blue vitriol and brimstone are strongly recommended. The application of nanotechnology for optimal drug delivery to target sites is also recommended.

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