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EVALUATION OF THE COMBINED EFFECTS OF *CASSYTHA FILIFORMIS* AND *CLEISTOPHOLIS PATENS* AGAINST SOME CLINICAL ISOLATES OF *STAPHYLOCOCCUS AUREUS* BY *IN VITRO* METHOD

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ABSTRACT

Context: Herbalists use *Cassytha filiformis* Linn and *Cleistopholis patens* (Benth) for the treatment of urogenital infections.

Objectives: The aim of this study was to evaluate the interactions of extracts from *Cassytha filiformis* and *Cleistopholis patens* leaves against *Staphylococcus aureus*

Method: Both plants' parts were colleccted ,shade-dried, pulverized and extracted with sterile distilled water and methanol. Extraction with water was done by 48 h maceraion while methanol was done in a Soxhlet apparatus. The extracts were concentrated and dried separately under vacuum. Gentamicin (Gentalek [R]) was used as a reference standard antibiotic. Preliminary antimicrobial screening of these antibacterials was carried out using agar-well diffusion techniques. Checkerboard method was used to study the combined effects of these extracts.

Results: The preliminary sensitivity test showed that the water extract of *Cleistopholis patens* was inactive against the strains of *Stapylococcus aureus* tested whereas the *Cassytha filiformis* extracts were active against the organisms. The respective mean MIC values of the methanol extracts from *Cassytha filiformis* and *Cleistopholis patens* against *Staph. aureus* were 15.21 ± 0.68 and 24.62 ± 1.56 mg/ml while the water extract from *Cassytha filiformis*

was 11.38 ± 0.99 mg/ml. The interaction studies showed that the combined effects of the extracts were predominantly indifference and antagonisms against *Stapylococcus aureus.*

Conclusion: The extracts from these two plants should not be combined, however, can be used singly for the treatment of infections caused by *Staph. aureus*

Keywords: Combined effects, *Cassytha filiformis Linn, Cleistopholis patens, Staphylococcus aureus,* MIC, Checkerboard method.

INTRODUCTION

Cleistopholis patens (family: Annonnacaea) is a sun-loving tree about 20-35 m tall found in many parts of African countries where it has several applications. Such countries are Angola, Cameroon, Congo, Ghana, Ivory Coast, Liberia, Nigeria, Sierra Leone, Togo Uganda, Zaire. Its common names are "salt and oil tree" and its local names are: in Nigeria; Edo (Otu), Nsukka(ogwu odenigbo), Igbo (Ojo), Yoruba (Apako) or Oke. In Ghana; Akan-Akyem (Fifiriwa) Asante(Afirifiriwa),Nzema (Aheri). In Ivory Coast: Abure (Owua),Akan-Asante (Autie). In Liberia Krus-Basa (Nee-wahn-johr),Mende (Moigbwama) (Burkill (1985). Phytochemical sceening of *Cleistopholis patens* revealed the presence of glycosides, alkaloids, steroids, saponins, terpenoids, favonoids and carbohydrates(Adonu et al.,2013).

Cassytha filiformis is a leafless, perennial, climbing, twinning, vine-like plant in the family lauracaea. In traditional medicine, the plant is pounded and the water extracts used in the treatment of difficulty in urination. In modern medical research, *Cassytha filiformis* extracts have been seen to have diuretic activity (Kirtikar and Basu (1991), Sharma et al (2009). Pytochemical screening reveals that the aerial parts of *Cassytha filiformis* consists of alkaloids ,flavonoids ,triterpenoid, and steroids (Sharma et al, 2009). Other reports show that some of the isolated compounds from this plant are lignan, cassyformin, filiformin, apomorphine alkaloids, actinodophine and octeine (Chang Fang-Rong et al, 1998). The presence of these phytochemicals explains the antimicrobial/medicinal potentials inherent in the *Cassytha filiformis* extracts(Chang et al 1997, Wu et al 1998).

Staphylococcus aureus strains are Gram positive bacteria of the family *Micrococcus*(Arora 1999) .About 35-45 % of normal adults carry *S.aureus* in the anterior nares. *Staphylococci* shed by patients and carriers contaminate fomites such as towel, handkerchief, bed linen, door cottons and blankets and may persist on them for weeks. In a study conducted by Chigozie J, et al (2010), Uneke C.J., et al (2008), and Gurjeet S., et al (2013), the greatest

number of microorganisms isolated from stethoscopes used by health workers in hospitals is *S. aureus.* *S. aureus* is an opportunistic pathogen as it causes infection most commonly at sites of lowered host resistance eg damaged skin or mucosa. *S. aureus* causes 1-5% of cases of urinary tract infection (Arora, 1999). *Staph. aureus* also cause apportunistic infection of genital tract. A depletion of vaginal Lactobacillus has been directly associated with an increase in the incidence of genito-urinary infection caused by this organism (Hawes et al 1996). Other infections caused by *Staph.aureus* include wound and burn infections (Wu and Liu 1994),boils, impetigo, furuncles, inflammatory conditions like osteomyelitis, tonsilitis, pharyngitis, endocarditis, bronchopneumonia and Staphylococcal scalded skin syndrome(SSSS). SSSS occurs mainly in neonates and the mortality rate in adults is as high as 50%(Arora ,1999). This study was conducted to evaluate the combined effects of extracts from *Cassytha filiformis Linn* and *Cleistopholis patens* against *Staphylococcus aureus* isolated from clinical specimens.

MATERIALS AND METHODS

Test bacteria

A total of 85 isolates of *Staph. aureus* were used for the study. They were isolated from midstream urine, high vaginal swab (HVS), urethral swab (US), earswab(ES), pus and wound of patients attending out-patient departments (OPD) of both Nsukka General Hospital and Bishop Shanahan Hospital, both at Nsukka, Enugu State, Nigeria. These patients were manifesting signs and symptoms of bacterial infections(Arora ,1999).

Culture Media

The culture media used were Nutrient agar ,Nutient broth, Blood agar (B.A), MacConkey agar (M.A) , all from Fluka Biochemika, (Germany).

Solvents for extraction

The extraction solvents used were methanol (Sigma Aldrich, Germany), and distilled water (Water Resources Management Laboratory Limited, University of Nigeria, Nsukka, Enugu State).

Plant Materials

The aerial parts of *Cassytha filiformis Linn* and the leaves of *Cleistopholis patens* (*Benth)* were collected from Nsukka in Enugu State between May and August 2011. The identity of the two plants were authenticated by Mr. A .O .Ozioko of the Bioresource Development and

Conservation programme (BDCP) Nsukka, Enugu State. The two plants were dried under the shade for 12 days, pulverized and stored at room temperature for some days before extraction.

Extraction of plant materials

The plants parts were shade - dried and pulverized to coarse powder using an electrically operated mill and the powder weighed . A 600g quantity of *Cleistopholis patens* and a 300g quantity of *Cassytha filiforms Linn* were macerated with 4.0 L and 3.0 L of water respectively and allowed to stand at room temperature for 48 h. The extracts were filtered using porcelain sieve and the extracts concentrated and dried separately under vacuum . The methanol extracts of the two plants were obtained by Soxhlet extraction.

A 400g each of *C. filiformis* and *Cleistepholis patens* powder were extracted with 1.5 L and 1.6 L of methanol respectively.

Preparation and sterilization of media

All the media were prepared according to manufacturers instructions.

Isolation and characterization of test bacteria

The specimens of urine, high vaginal swab, urethral swab, pus, ear and wound swabs (from patient manifesting symptoms of bacterial infections) were immediately inoculated on blood agar plates and MacConkey agar plates. These plates were incubated at 37 ^{0}C for 24 h according to the method described by Cheesbrough (2000). Colonies suspected to belong to micrococci were isolated, Gram-stained and examined microscopically. All such isolates were also subjected to the following standard biochemical tests for micrococci ;Catalase test, Coagulase test, Nitrate test, Indole, (Cowan and Steel, 1993: Cheesbrough, 2000) (Arora and Chugh, 1977).

Maintenance and standardization of stock cultures.

The stock culture of each clinical isolate was stored in nutrient agar slants at 4 ^{0}C. Prior to use, the cultures were activated by successive daily sub-culturing first into blood agar and MacConkey agar plates and then into nutrient agar slant.

The standardization of innoculum was carried out according to the method described by Arora (1999).

Preparation of turbidity standard equivalent to O.5 McFarland

The standard was prepared by using the techniques described by Cheesbrough (2000):

Preliminary sensitivity test

Preliminary antimicrobial screening of the extracts of both plants against the bacteria was done by the method of the cup-plate agar diffusion (Boakye – Yiado 1979).

Evaluation of minimum inhibitory concentration (MIC) and fractional inhibitory concentration (FIC) of the extracts

The MIC of the antimicrobial agents were determined using agar dilution method (NCCLS, 1999). The effects of the extracts in combination against *Staph.aureus* were evaluated using the agar dilution checker board method as described by Mandal et al; (2004). The extracts used for the study were combined by incorporation into molten nutrients agar at concentration 3 x MIC. For each isolate of the test organism, different concentrations of the two combined extracts were prepared and evaluated by combining them at different ratios starting from 0:10 (that is, zero part of the first extract to 10 parts of the second extract), then moving through 1:9, 2:8, 3:7,… and 10:0. The same procedure was repeated for all the isolates. For each isolate, the Fractional inhibitory concentrations (FIC) of all the ratios of the combined extracts were determined and then combined. Their sum gives the combined effect. The FIC value for each extract was calculated using the formula.

$$\text{FIC (extract A)} = \frac{\text{MIC of the extract A in Combination}}{\text{MIC of A alone}} \quad \ldots\ldots \quad \ldots\ldots \quad \ldots\ldots \quad \text{equat 1}$$

The addition of FIC_A and FIC_B gives the FIC index from where an inference can be drawn.

$$\text{FIC (index)} = \Sigma\text{FIC} = \text{FIC (A)} + \text{FIC (B..} \quad \ldots \quad \ldots \quad \ldots \quad \ldots \quad \ldots \quad \text{equat 2}$$

The effects of the combinations were classified as synergistic, additive, indifference and antagonistic if the FIC index is ≤ 0.5, >0.5 to 1, > 1 to 2 and >2 respectively (Hayami et al, 1999).

RESULTS

The results of the extracts percentage yield, isolation of the test bacteria, IZDs, MICs and the interations of the extracts against *Staph. aureus* are shown in **Tables 1-5** . The percentage yields of the extracts of the C.patens were greater than those of the C.filiformis**(Table 1) .** The greatest number of the isolates of the Staph aureus was gotten from wound swab, followed by isolates from the urethra and least number was gotten from earswab **(Table 2) . In Table 3**, the results of the activities of the extracts and the gentamicin against *Staph.aureus* showed large inhibition zone diameters. This reveals high efficacy of the

extracts and gentamicin against *staphylococcus aureus.* **Table 4** shows that there exists significant difference in antibacterial activity among the water (wF) and methanol (met.F) extracts of *Cassytha filiformis and* methanol extracts of *C. Patens*(met P) as shown by their MIC values against the bacteria studied. There was no synergistic effects recorded from the interation studies(**Table 5**). The ratios of combinations of the two extracts that produced additive effects(FIC index >0.5-1) are 1:9, 2:8, 3:7, and 5:5 with the highest number of strains seen in ratio 3:7. Large number of the strains showed indifference(FIC index>1-2) and antagonistic results(FIC index>2) in all the ratios tested.

Table 1; Extraction yield

Plant	Part	Percentage yield of the extract (%)	
		Aqueous	Methanol
Cassytha filiformis linn	Aerial parts	18.0	7.9
Cleistopholis patens (Benth)	Leaves	20.2	13.8

Table 2 ;Isolates of Staph. aureus

Bacteria	Sources and number of Strains Isolated						
	MSU	ES	HVS	WS	US	pus	Total
Staphylococcus aureus	12	9	11	22	17	14	85

KEY: MSU= midstream urine , HVS = High Vaginal Swab US = Urethral Swab

ES = Ear Swab WS= wound swab

PUS= aspirated with Sterile syringe

Table 3. The inhibition zone diameter (IZD) of different extracts of *Cleistopholis patens, Cassytha filiformis linn and* the reference drug- gentamicin against eighty five strains of *Staphylococcus aureus*

Concentrations (mg/ml)	*IZD(mm) Mean ± SEM*			
	Met P	wF	met F	Gentamicin[m]
120	22.08 ± 0.36^{a}	23.25 ± 0.45^{a}	23.83 ± 0.43^{a}	18.42 ± 0.64^{b}
60.	17.76 ± 0.34^{a}	20.54 ± 0.53^{b}	17.50 ± 0.29^{a}	12.04 ± 0.23^{c}
30	14.13 ± 0.50^{a}	16.50 ± 0.35^{b}	13.46 ± 1.03^{a}	8.05 ± 0.93^{c}
15.	7.08 ± 1.05^{a}	10.25 ± 0.70^{b}	8.67 ± 1.06^{a}	4.00 ± 0.00^{c}
7.5	0.58 ± 0.42^{a}	4.99 ± 0.85^{b}	1.98 ± 0.00^{a}	0.00 ± 0.00^{a}
3.75	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Keys: a, b and c = levels of significance

Different superscripts in a row indicate significant differences between the groups ($p<0.05$).

met P = methanol extract of Cleistopholis patens

met F = methanol extract of Cassytha filiformis

wF = water extract of Cassytha filiformis

m = IZD values were obtained using conc of gentamicin in µg/ml

Table 4: MIC of the Extracts

MIC (mg/ml)

Bacteria	***Mean ± SEM***		
	Met.P	met.F	w.F
Staph. aureus	24.62 ± 1.56[a]	15.21± 0.68[b]	11.38 ± 0.99[c]

Key: a, b, and c = levels of significance

Different superscripts in a row indicate significant differences between the groups $p<0.05$).

Table 5. Combined effects of met.P and wF as determined by checkerboard agar dilution against *Staph aureus*

Ratio m.P:wf	**Number of strains (%)**			
	Synergism (≤ 0.5)[a]	**Additive (>0.5-1)[a]**	**Indifference (>1-2)[a]**	**Antagonism (>2)[a.]**
0 : 10	-	-	-	-
1 : 9	0	7(8.3%)	53(62%)	25(29.2%)
2 : 8	0	11 (12.5%)	50 (58.3%)	25 (29.2%)
3 : 7	0	21 (25.0%)	64 (75.0%)	0 (0%)
4 : 6	0	0 (0%)	32(37.5)	53 (62.5%)
5 : 5	0	4 (4.2%)	43(50.0%)	38 (45.8%)
6: 4	0	0 (0%)	14(16.7%)	71 (83.3%)
7: 3	0	0 (0%)	21 (25.0%)	64(75.0%)
8: 2	0	0 (0%)	18(20.8%)	67 (79.2%)
9: 1	0	0(0%)	35(41.7%)	50(58.3%)
10: 0	-	-	-	-
Mean percent	0%	5.6%	43.1%	51.3%

Key: [a]fractional inhibitory concentration (FIC) Index.

Met.P = Methanol extracts *of Cleistopholis patens (Benth*)

wF = aqueous extracts of *Cassytha filiformis Linn*

DISCUSSION

The extracts from *Cleistopholis patens (Benth*) and *Cassytha filiformis Linn* have been shown, in several reports to have antimicobial activities(Hu *et al.*, 2006; Liu et al., 1990; Chang et al 1997, Wu et al 1998). The sensitivity of the isolates of Staph. aureus was ascertained by determining the IZD and MIC values of the extracts of both plants(**Tables 3 and 4). In Table 3,** at concentrations 60mg/ml, 30 mg/ml and 15 mg/ml there exist significant statistical difference between metF and wF. This implies that the aqueous extract of *Cassytha filiformis Linn* (wF) with IZD values greater than those of methanol extract of *Cassytha filiformis Linn* (metF) has higher activities against *staph aureus* than the later. It may, therefore, be advisable to use water for the extraction of this plant parts whenever, there is a need to use it in the treatment of bacteria infection caused by *Staph. aureus*. There is a significant difference in antibacterial activity among met.P met.F and wF, against *Staph aureus*. This shows that the met.P (MIC 24.62 ± 1.56 mg/ml) is less potent than met.F(15.21± 0.68 mg/ml) and both are less potent than wF (MIC 11.38 ± 0.99mg/ml) against clinical isolates of *Staph. aureus.* Modern day scientists have investigated the high efficacies of the compounds from *Cleistopholis patens (Benth)* against *Staphylococcus aureus* strains(Hu *et al* 2006, Liu et al 1990). This study has in addition to that, shown the superiority of antibacterial potentials of extracts of aerial parts of *Cassytha filiformis Linn* over *Cleistopholis patens* leaf extracts against *Staph aureus* .The results of this experiment, therefore, support the claims of some traditional medicine practioners for the use of these plants as remedies against genito-urinary infections and wound infections caused by Staph aureus. In **Table 5**, the results of interactions between methanol extract of *Cleistopholis patens (Benth)* leaves (met..P) and the water extract of the *Cassytha filiformis Linn* (wF) showed no synergistic effect against the *staphylococcus aureus* strains. The combined effect was additive against small number of the strains (mean = 5.6%). The combined effects with these *staph aureus* strains were predominantly indifference (mean = 43.1%) and antagonism (mean = 51.3%). The results of this study suggests that the combination of the two extracts in the treatment of staphylococcal infection has no added value, rather, mostly results in one extract antagonizing the effect of the other. This is because no synergistic effect was produced with *Staph aureus*. In addition, antagonistic effects were observed with the organisms; mean of 51%

CONCLUSION

The phytochemicals present in these two plants parts could be responsible for the observed antibacterial effects of the extracts from both parts of the plants against *Staph. aureus*. These

plants, when used singly, had good antibacterial activity against *Staph. aureus*, but in combination represented typical indifference and antagonism.

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DECLARATION OF INTEREST

There is none

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