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RESEARCH ARTICLE

IN VITRO EVALUATION OF THE COMBINED EFFECTS OF METHANOL EXTRACTS FROM CASSYTHA FILIFORMIS AND CLEISTOPHOLIS PATENS AGAINST PSEUDOMONAS AERUGINOSA AND ESCHERICHIA COLI

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Abstract

Traditional medical practitioners often combine two or more different plants' parts for the treatment of ailments. This study was conducted to evaluate the combined effects of extracts from aerial parts of *Cassytha filiformis* Linn and the root back of *Cleistopholis patens* (Benth) against some clinical isolates of *Pseudomonas aeruginosa* and *Escherichia coli*. Both plants' parts were collected, washed, shade-dried, pulverized and extracted with methanol in a Soxhlet apparatus.The extracts were, then, concentrated by evaporation to dryness using rotary evaporator at temperature of 40-45^{0}C. Checkerboard method was used to study the interactions of the methanol extracts of the two plants' parts. The interaction studies showed that the combined effects of the extracts were predominantly indifference and antagonisms against the test isolates. Our findings, therefore, discourage the combination of the methanol extracts of these plants' parts for the treatment of the infections caused by the organisms.

Introduction

Cassytha filiformis (Family- Lauraceae) is a perennial and twinning plant found on all tropical shores and common throughout Southwest Asia and West Africa including Nigeria. In these areas and many other parts of the world, *Cassytha filiformis* is used in the treatment of many diseases . In India, it is used in cleaning ulcer and in an eye-wash and in java, the pounded stems are used for intestinal ailment and during epilepsy attack (Ibe and Nwufo, 2005). Its common names are devil's gut, love vine, laurel dodder, pololo, malolo, woe vine kaunaoa pehu, gbanigerige depending on the locality. The phytochemical screening of the aerial parts of *Cassytha filiformis* revealed the presence of flavonoids, alkaloids, glycosides, terpenoids, steroids, resins and tannins, saponins and lipids (Adonu et al, 2013a). The presence of these phytochemicals explains the antimicrobial/medicinal potentials inherent in the *Cassytha filiformis* extracts(Chang et al, 1997, Wu et al, 1998).

Cleistopholis patens (Family-Annonaceae) is a tree of about 30 m tall, with a trunk up to 10m tall and 20 –90 cm wide, bark is grayish-white, smooth fibrous or furrowed. It is fast growing, always seen colonizing abandoned areas (Burkill, 1985). In Nigeria and other parts of African countries, the bark and the leaves of these plants are used in the treatment of typhoid fever and urogenital infections. The root bark of *Cleistopholis patens* collected in Ghana yielded two sesquiterpenes and five alkaloids (Burkill,1997). In addition, modern medical research has resulted in the isolation of

secondary metabolites that are antimicrobials. For instance, the steroidal, glycosidal and alkaloidal fractions of *Cleistopholis patens* are effective against *Klebsiella pneumoniae (*Ebi and Kamalu, 2001). The anti bacterials- oligorhamnosides- have been isolated from Cleistopholis patens (Hu et al ,2006) .The phytocemicals- sesquiterpenes and alkaloids (1,2) isolated from the stem and root bark of *Cleistophis patens* are potent anticandidal agents (Hufford, et al 1987). Moreso, alkaloids, (eupolauridine) and 3-methoxy champangine, from the ethanolic extract of *Cleistopholis patens (Benth) Engl and Diels* are potent anti-fungal agents (Liu, et al 1990).

Escherichia coli and Pseudomonas aeruginosa are among the major cause of non-sexually transmitted urogenital infections, nosocomial infections of bed sores, iatrogenic wound infections and other infections in immunocompromised men, women and children. Urinary tract infection caused by one or more of these Gram negative organism is the most common bacterial infection managed in general medical practice and accounts for 1-3% of consultations (Goddard, et al 2006). Those bacteria that cause urogenital infections often emerge from the intestinal microbiota and ascend along perineum to the vagina and then to the urethra and bladder (Reid and Bruce 2006). The frequency with which these Gram negative bacilli are acquiring resistance to one or more antimicrobial agents traditionally used for treatment of these infections is a matter of great concern. Obviously, extended spectrum beta-lactamase (ESBL) production by Gram negative bacilli was considered as the most important threat to clinical therapeutics (Livermore,1998, Mathur P et al, 2002). Consequently, combination of two antimicrobials are encouraged especially those with established synergistic effects when combined together. Traditionally, these two plants are used either singly or in combination for the management and treatment of many infections and ailments in Nigeria and some other African countries. This study was conducted to evaluate the combined effects of the extracts from aerial parts of *Cassytha filiformis* Linn and root backs of *Cleistopholis patens* (Benth) against some clinical isolates of *Pseudomonas aeruginosa* and *Escherichia coli*.

MATERIALS AND METHODS

PLANT MATERIALS

Authenticated fresh root back of *Cleistopholis patens (Benth)* and aerial parts of *Cassytha filiformis Linn* were used for the study. The two plant parts were collected from Afikpo in Ebonyi State and Abraka from Delta State between September and December, 2012.

Test micro organisms

A total of fourty eight (48) pathogens were used. They include 28 isolates of *Escherichia coli* and 20 isolates of *Pseudomonas aeruginosa.* They were collected from Pharmaceutical and Medical microbiology units of Pharmaceutics and Microbiology Departments of University of Nigeria Nsukka, Enugu State University Teaching Hospital and National orthopaedic Hospital, Enugu ,Enugu State, Nigeria. The bacteria were preserved in refrigerator temperature (2-8^{o}C) on nutrient agar (NA) slant.

Culture Media

The culture media used were, Nutrient agar (N.A), Blood agar (B.A) MacConkey agar (M.A), Nutrient broth, all from Fluka Biochemika (Germany], and Kliglier Iron agar (K.A) [BIOTECH, United Kingdom.]

Solvent for extraction

The extraction solvent used was methanol (Sigma Aldrich, Germany).

Reference antibiotic

The standard antibiotc used was Ciprofloxacin (Cipxin$^{(R)}$-500, Zim Lab Ltd, India)

Extraction of plant materials

The chopped root back of the *Cleistopholis patens (Benth)* and aerial parts of the *Cassytha filiformis* Linn were collected fresh, washed thoroughly with tap water,then, rinsed several times with sterile water and shade-dried for 3 weeks. The dried plant materials were pulverized into powder. The methanol extracts of the two plants were obtained by Soxhlet extraction. A 250gm of *Cassytha filiformis* and 300gm of *Cleistepholis patens* powder were extracted with 1.5L and 1.6L of methanol respectively.

Maintenance and standardization of stock cultures.

The stock culture of each clinical isolate was stored in nutrient agar slants at 2-8^{0}C. Prior to use, the cultures were activated by successive daily sub-culturing first into MacConkey agar plates and then into nutrient agar slant for a period of 3 days. The standardization of innoculum was carried out according to the method described by Arora (1999). The tops of 5-10 similar appearing, well isolated colonies on an agar plate were touched with a sterilized straight wire and then, inoculated in a nutrient broth medium. These broth bottles were incubated at 37 ^{0}C for 4 – 6 h to obtain the growth at logarithmic phase. The density of the organisms (bacteria) was adjusted to approximately 10^{8} colony –

forming units (CFU)/mL by comparing its turbidity with that of 0.5 McFarland opacity standards.

Preparation of turbidity standard equivalent to O.5 McFarland

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

Sensitivity Test

Preliminary antibacterial screening of the extracts of both plants and standard antibiotic-Ciprofloxacin-against the bacteria was done by the method of the agar diffusion (Mirjana, et al (1979) with little modification. A 100 mg/ml of each of the extracts in DMSO was double diluted serially with sterile distilled water. Molten nutrient agar (20 ml each) was seeded with 0.1 ml of standardized broth cultures of bacteria .With a sterile cork borer, a total of 5 wells, 8 mm in diameter were made in the agar. 0.08 ml each of the two-fold dilutions was added into each labelled hole using a sterile pipette. As a negative procedural control, 0.08 ml DMSO was put in the centre well. Similarly, doubling dilutions of 0.100 mg/ml of Ciprofloaxin were added into respective agar-wells as positive controls. The plates were left for 1hour at room temperature for diffusion after which they were incubated at 37^0C for 24 h for bacteria. Diameters of the zones of inhibition (IZD) were measured at the end of the incubation period. The average of triplicate determinations was taken.

Evaluation of the MIC of the extracts and their interactions

The MIC values of the extracts were determined using agar dilution method (NCCLS, 1999). The effects of the extracts in combination against the test bacteria 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 4 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 of the test bacteria. 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.

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

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

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

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 percentage yield, sensitivity tests and the interactions of the methanol extracts of the two plants are shown in the **Tables 1-4.** The extraction yields of the extracts are 12.5% and 10.3% for *Cleistopholis patens and Cassytha filiformis linn* respectively(**Table 1**). In **Table 2**, the results of the preliminary susceptibility tests and the MIC values of the extracts against the two organisms are shown. The test bacteria were sensitive to both the extracts and the standard antibiotic Ciprofloxacin and this was reported as +. The respective MIC values (mg/ml) of the extracts against *Pseudomonas aeruginosa* strains and *Escherichia coli* strains were 25.67 ± 7.33(for *Cleistopholis patens*), 15.50 ± 3.00(for *Cassytha filiformis linn*) and 27.99 ± 3.09(*Cleistopholis patens*), 18.50 ± 5.37 (*Cassytha filiformis linn*). In **Table 3**, no synergistic effect was recorded with all the ratios of combination of the two extracts against *Pseudomonas aeruginosa* strains. So also was the zero values of the additive effects at combination ratios of 2:8, 3:7, 4:6 7:3, 8:2 and 9:1. The combination of the two extracts at ratios 5:5 and 6:4 produced additive effects with small number of strains of the test organism. Indifference and antagonistic effects were recorded with all the combination ratios against the *Pseudomonas aeruginosa* strains. In **Table 4**, no synergistic effect was recorded with all the ratios of combination of the two extracts against *Escherichia coli* strains. So also was the zero values of the additive effects at combination ratios of 1:9, 2:8, 3:7, 4:6, 8:2 and 9:1. The combination of the two extracts at ratios 5:5, 6:4 and 7:3 produced additive effects with small number of strains of the test organism. At ratios 2:8 and 5:5 no indifference result was got whereas the rest of the ratios resulted in indifference. Antagonistic effects were recorded with all the combination ratios against the *Escherichia coli* strains.

Table 1 Extraction yield

Plant	Part	Percentage yield of the extract (%)
		Methanol extracts
Cassytha filiformis linn	Aerial parts	10.3
Cleistopholis patens (Benth)	Root back	12.5

Table 2. Preliminary sensitivity results and the MIC values of the extracts against the test bacteria

Test bacteria	Sensitivity Result			MIC(mg/ml)	
	Moh.P	Moh.F	Cipixin	Moh.P	Moh.F
Pseudomonas aeruginosa	+	+	+	25.67 ± 7.33	15.50 ± 3.00
Escherichia coli	+	+	+	27.99 ± 3.09	18.50 ± 5.37

Key:

Moh.P = Methanol root back extracts of *Cleistopholis patens (Benth)*
Moh. F= Methanol extracts of *Cassytha filiformis Linn*
+ = Sensitive

Table 2: Combined effects of Moh.P and Moh.F as determined by checkerboard against *Pseudomonas aeruginosa*

	Number of Strains (%)			
Ratio of combination MohP: MohF	Synergism (≤ 0.5)[a]	Additive (>0.5-1)[a]	Indifference (>1-2)[a]	Antagonism (>2)[a]
0 : 10	-	-	-	-
1 : 9	0	1(11.1%)	7(77.8%)	1(11.1%)
2 : 8	0	0 (0%)	5(55.6%)	4(44.4%)
3: 7	0	0	6(66.7%)	3(333.3%)
4: 6	0	0	2(22.2%)	7(77.8%)
5: 5	0	2(22.2%)	1(11.1%)	6(66.7%)
6: 4	0	2(22.2%)	1(11.1%)	6(66.7%)
7: 3	0	0	2(22.2%)	7(77.8%)
8: 2	0	0	2(22.2%)	7(77.8%)
9: 1	0	0	3(33.3%)	6(66.7%)
10 0	-	-	-	-
Mean percentage		6.2%	35.8%	58.0%

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

Moh.P = Methanol root back extracts of *Cleistopholis patens (Benth)*

Moh. F= Methanol extracts of *Cassytha filiformis Linn*

Table 3: Combined effects of Moh.P and Moh.F as determined by checkerboard agar dilution against *Escherichia coli strains*

Ratio of combination MohP: MohF	Number of Strains (%) Synergism (≤ 0.5)[a]	Additive (>0.5-1)[a]	Indifference (>1-2)[a]	Antagonism (>2)[a]
0 : 10	-	-	-	-
1 : 9	0	0	1(7.7%)	12(92.3%)
2 : 8	0	0	0	13(100%)
3: 7	0	0	1(7.7%)	12 (92.3%)
4: 6	0	0	1(7.7%)	12 (92.3%)
5: 5	0	1(7.7%)	0	12 (92.3%)
6: 4	0	1(7.7%)	1(7.7%)	11 (84.6%)
7: 3	0	1(7.7%)	1(7.7%)	11 (84.6%)
8: 2	0	0	1(7.7%)	12 (92.3%)
9: 1	0	0	4(30.8%)	9 (69.2%)
10 0	-	-	-	-
Mean percentage		2.6%	8.6%	88.8%

Key: [a]Fractional inhibitory concentration (FIC) Index.
Moh.P = Methanol root back extracts of *Cleistopholis patens (Benth)*
Moh. F= Methanol extracts of *Cassytha filiformis Linn*

Discussion

The percentage yield of the root back extract of *Cleistopholis patens* (Benth) was greater than the extract from the aerial parts of the *Cassytha filiformis* linn. The methanol aerial parts extract of *Cassytha filiformis* linn collected at different locations and time of the year investigated upon by the same author and other scientists showed that the present study produced higher yield of the plant extract(.Adonu et al, 2013b). The root back extract of *Cleistopholis patens (Benth)* was active against the test bacteria(**Table 2**). This result agrees with the findings of other scientist on the methanol extracts of

different parts of the same plant under study(Hu, et al 2006). So also was the extracts from *Cassytha filiformis Linn* (Moh.F). The interactions between the methanol root back extract of *Cleistopholis patens (Benth)* (Moh.P) and that of the *Cassytha filiformis Linn* (Moh.F) aerial parts against *Pseudomonas aeruginosa* and *Escherichia coli* strains are shown in **Tables 3** and **4** respectively. No synergistic effect was observed with the two extracts combined against all the strains of both *Pseudomonas aeruginosa* and *Escherichia coli*. With the isolates of *Pseudomonas aeruginosa*, ratios (Moh.P: Moh.F) of 1:9, 5:5 and 6:4 showed additive effect (mean= 6.2%). The effects of the interaction between the two extracts against these strains were predominantly indifferences (mean 35.8%) and antagonistic (mean = 58%). With the isolates of *Escherichia coli*, ratios (Moh.P: Moh.F) of 5:5, 6:4, and 7:3 showed additive effect (mean= 2.6%). In addition, small number of strains (mean 8.6%) of these species showed indifference effect with both extracts leaving the greatest percentage of the strains (mean = 88.8%) antagonistic with the extracts. These combination studies discourage the possibility of combining the extracts in the treatment of the infections caused by *Escherichia coli* and *Pseudomonas aeruginosa*. However, when used singly, these extracts have a good antimicrobial activity against *Ps. aeruginosa* and *E. coli*(Adonu, et al,2013c). Possible explanations of this decreased activity in the combination are as follows firstly, some ingredients in one extracts could be biocidal while in the second extract, biostatic. Secondly, the ingredients present in the two extracts could have reacted and produced an agent with reduced activity against test organisms. Again, the products of such interactions could be such products with diffusion problem. It is obvious from the results of this study that more pronounced antimicrobial activities could have been observed if pure compounds from these extracts were used.

CONCLUSION

In combination, these extracts do not seem promising against the test pathogens. Our findings in this work disagree with the use of these plants in combination in the ethnomedicinal treatment of infections caused by the test organisms.

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