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IN-VITRO ANTIMICROBIAL SCREENING OF SOME COMMERCIALIZED REGISTERED HERBAL MIXTURES SOLD IN ANAMBRA AND ENUGU STATES, NIGERIA.

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ABSTRACT

The antimicrobial activities of some herbal mixtures used for treating typhoid fever, gastrointestinal infections, bronchitis, dental carries and venereal diseases were investigated. Fifteen registered herbal mixtures (coded P_1-P_{15}) purchased from Enugu and Anambra States in Nigeria, were screened for antimicrobial activity against clinical isolates of *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Salmonella typhi, Streptococcus mutans and Candida albicans* using the Kirby-Bauer disc diffusion method. Ciprofloxacin (5µg/ml) and fluconazole (25µg/ml) were used as standards for comparison. Results showed P_1, P_2, P_6 and P_7 were active against one or more of the test organisms. The herbal mixtures P_3, P_4, and P_5 showed only very little activity with inhibition zone diameter ranges of 6.5±0.92-10.5±1.29mm whereas P_8-P_{15} exhibited no activity. P_1 inhibited all the test microorganisms with its highest activity (24.25±1.71mm) against *Staphylococcus aureus*. Also, P_7 had the least inhibiting only *Staphylococcus aureus* with inhibition zone diameter of 16.0±0.67mm. The highest zone of inhibition (25.75±1.71mm) in this sturdy was produced by P_2 against *Staphylococcus aureus* with MIC and MBC of 0.156mg/ml and 1.25mg/ml respectively. *Streptococcus mutans* and *Candida albicans* were resistant to all the mixtures except P_1. *Staphylococcus aureus* was the most sensitive. Statistical analysis showed significant difference ($P<0.05$) in the sensitivity of

test organisms to the herbal mixtures. However, most of the herbal mixtures showed no activity as claimed. This sturdy has shown herbal mixtures possess some antimicrobial activity against some human pathogens but there is need to standardize, monitor and regulate herbal mixtures available in the Nigerian markets for better results.

KEY WORDS: Antimicrobial activity, herbal mixtures, registered, human pathogens.

INTRODUCTION

The search for components with antimicrobial activities has gained increasing importance in recent times, due to growing worldwide concern about the alarming increase in the rate of infection by antibiotic-resistant microorganisms.[1]

Herbal remedies or medicines consist of portions of plants or unpurified plant extracts containing several constituents with different pharmacological targets involved in the therapeutic action.[2] This characteristic may be an added advantage compared to single isolated compounds, especially when the underlying disease has a multi-factorial etiology. Herbal therapy is becoming increasingly popular among patients and physicians, making plants to remain the most common source of antimicrobial agents. Their usage as traditional health remedies are the most popular for 80% of the world population in Asia, Latin America and Africa and is reported to have minimal side effects.[3,4] Many herbal preparations are marketed to the public for various ailments from outlets ranging from health, food stores, trade fares and Internet sites. Currently in the United States especially, herbal products are not regulated as they are considered dietary supplements therefore there is no standardization of active ingredients, purity and concentration, and there is also no regulations governing which herb can be marketed for various ailments.[5] The problem has been the development of increasingly strict criteria of proof that a medicine really does what it is claimed to do.[6]

A critical evaluation of their efficacy is therefore important to make sure that plant samples used for herbal mixtures in the market are stored under desirable conditions. Thus, this sturdy strives to evaluate the antimicrobial activities of some herbal mixtures (sold in Enugu and Anambra States of Nigeria) against some human pathogens.

MATERIALS AND METHOD

Sample Collection: Fifteen (15) registered herbal mixtures/products were purchased from pharmaceutical shops and herbal mixture vendors in Enugu and Anambra States, Nigeria.

These herbal mixtures are used for the treatment of typhoid fever, diarrhea, bronchitis, dental carries and venereal diseases.

Table 1: Details of the herbal products

Herbal Product Code	Herbal Product Name	Batch Number	NAFDAC Number	Manufactured Date	Expiry Date
P_1	Golden root dental mixture	005	A7-1104L	June, 2014	June, 2017
P_2	Guaranty total flush for STD	NI	0402	NI	NI
P_3	Katoka herbal mixture	NI	04-4763L	NI	NI
P_4	Tree that sustains life	001	001 04-8072L	May, 2011	May, 2025
P_5	Beta cleanser bitters	008	A7-0738L	Sept., 2014	Aug., 2017
P_6	Omega roots and ginseng	04	04-8618L	Jan., 2015	Feb., 2018
P_7	Goodwills herbal mixture	02230	A7-0246L	2015	2018
P_8	Gabson herbal mixture	44	A7.11571L	Sept., 2013	Oct., 2016
P_9	Quick herbal mixture	001	04-88072L	July, 2009	July, 2020
P_{10}	Opat herbal wonder for teeth germs	NI	11617	June, 2014	Dec., 2017
P_{11}	Goko herbal cleanser herbal mixture	0005	A7-0804L	March, 2014	Feb., 2017
P_{12}	New hope Aloe vera antibiotics	NI	09840	Feb., 2015	Feb., 2018
P_{13}	Bernacine natural liquid	NI	A7.77854L	Jan., 2013	Jan., 2016
P_{14}	Africa herbals Nigeria	NI	A7-0320	2014	2017
P_{15}	Joe best herbal mixture	NI	04.721	Aug., 2014	July, 2017

Key

NI = Not indicated

NAFDAC = National Agency for Food and Drug Administration and Control.

Concentration of Herbal Mixtures: Total of 20ml each of aqueous herbal mixtures were concentrated using hot water bath at 30˚C for 6 hours. The concentrated herbal mixtures were put in different clean sterile labeled sample bottles and stored in the refrigerator until they are needed.

Sterility Testing: The mixtures were checked for sterility by streaking on sterile nutrient agar and Sabouraud Dextrose Agar (SDA) plates and incubated for 24-48 hours. Uninnoculated sterile Nutrient Agar and Sabouraud Dextrose Agar plates were kept for media sterility control.

Source of Test Organisms: Clinical bacterial and yeast isolates which include *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Salmonella typhi, Streptococcus mutans*

and Candida albicans were obtained from Nnamdi Azikiwe University Teaching Hospital (NAUTH), Nnewi, Anambra State, Nigeria. Confirmation tests were carried out on the organisms according to the method of.[7]

Culture Media

Eosin Methylene Blue (EMB) Agar and mannitol Salt Agar (MSA) were used in the confirmatory test for *Escherichia coli* and *Staphylococcus aureus* respectively. *Salmonella-Shigella* Agar (SSA) and slants of Triple sugar iron Agar (TSIA) were used for *Salmonella typhi*. *Streptococcus mutans* and *Klebsiella pneumoniae* were respectively inoculated on blood agar and MacConkey agar whereas Chromogenic Candida agar was employed for the confirmation of *Candida albicans*. The stock cultures were stored at 4˚C in Nutrient Agar slants (for the bacteria) and Sabouraud Dextrose Agar slants (for the yeast).

Preparation of Turbidity Standard

A 0.5 McFarland Standard was prepared by adding 0.5ml of 0.048M $Bacl_2$ (1.17% w/v $Bacl_2 2H_2O$) to 99.5 ml of 0.18 M H_2SO_4 (1% v/v) with constant stirring. A barium sulphate precipitate was checked for optical density using matches curvettes with 1 cm path and distilled water as a blank standard. A UV-Vis spectrophotometer was used to measure the absorbance at 625nm. An absorbance of 0.1 was obtained which was in the accepted range of 0.08-0.13. The approximate cell density corresponding to 0.5 McFarland is 1×10^6 cells/ml.

Standardization of the Test Organisms

The test organisms were inoculated into Nutrient broth and Sabouraud dextrose broth (SDB) and incubated for 24 hours. The resulting turbidity was adjusted to 0.5 McFarland turbidity standard using the same broth medium. The broth culture was diluted 1:200 by mixing 0.1ml of the inoculums and 19.9ml of the broth. This gives working inoculums that should contain 10^5-10^6 cells/ml within the 30 minutes it was used.

Sensitivity Screening: Sensitivity screening was carried out by the method of.[8] Antimicrobial activities of the herbal mixtures were tested using Mueller-Hinton Agar (MHA) and Sabouraud Dextrose Agar supplemented with 0.05mg/ml of chloramphenicol. Sterile discs (6mm in diameter) were made from Whatman No 1 filter paper impregnated with 0.2ml of 100mg/ml of each herbal mixture for 24 hours. The discs were allowed to air dry under aseptic condition. Sterile swab sticks were used to inoculate the standardized test organisms evenly on solidified Mueller-Hinton Agar plates and Sabouraud Dextrose Agar

plates for bacteria and yeast respectively. The inoculated plates were allowed to dry for ten minutes. Then sterile forceps was used to place the impregnated discs on the surface of the solidified agar. This was done in duplicate.[9,10] Discs impregnated with ciprofloxacin (5µg/ml) for the bacterial test organisms and fluconazole (25µg/ml) for the yeast served as positive control where as discs saturated with sterile water served as negative control. Zones of clearance were measured in (mm) using a ruler.

Determination of Minimum Inhibitory Concentration (MIC)

The MIC values were determined by broth dilution assay. Sterile reconstituted herbal mixtures were serially diluted (two-fold) in sterile Nutrient broth and Sabouraud dextrose broth supplemented with 0.05mg/ml of chloramphenicol in test tubes for bacteria and yeast respectively to obtain a concentration range of 10mg/ml to 0.156mg/ml. Then 0.1ml of each standardized test organism was added to each of the test tubes and the preparation was incubated at 37^0C for 24 hours for bacteria and 48 hours for the yeast. Negative controls were equally set up using broth cultures of test organisms without herbal mixtures. Tubes with medium only were set as controls for sterility of the medium. Test tubes were evaluated for the presence or absence of visible turbidity in the broth after the incubation period. The lowest concentration (highest dilution) of the mixture preventing appearance of turbidity (growth) was considered and recorded as the MIC.[11]

Determination of Minimum Bactericidal and Fungicidal Concentrations (MBC and MFC)

From the tubes showing no visible growth or turbidity in MIC, 0.1 ml of the suspension was inoculated onto sterile nutrient agar and Sabouraud Dextrose Agar. The plates were incubated at 37^0C for 24hours and 48 hours for bacteria and yeast respectively. The least concentration that did not show any visible growth of the test microorganism was considered as the MBC for the bacterial organisms and MFC for the yeast. A plate with media only was set as negative control to check the sterility of the media.[12]

Statistical Analysis

The tests were carried out in quadruplet and values for the diameter of the zone of inhibition reported as mean ± standard deviation. Also, the data obtained were subjected to one- way ANOVA using Statistical package for Social Science (SPSS) 15.0 for Windows Evaluation, Version 2006. P-values< 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

The disc diffusion method was used to determine the antimicrobial potency of fifteen (15) registered herbal mixtures (coded as P_1-P_{15}) against clinical isolates of *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Salmonella typhi, Streptococcus mutans and Candida albicans*. Details of the herbal mixtures/products used are shown in table 1. The result of the antimicrobial activity of the mixtures against the test microorganisms is presented in table 2. Seven (7) out of the fifteen (15) registered mixtures had varying degrees of activities against the test organisms. *Staphylococcus aureus* showed the highest sensitivity (with an inhibition zone diameter of 25.75±1.71mm) followed by *Escherichia coli* (24.0±1.58 mm), *Klebsiella pneumoniae* (21.74±1.71 mm), *Streptococcus mutans* (20.5±1.11mm), *Salmonella typhi* (17.75±1.70 mm) and *Candida albicans* (17.75±1.70mm). This agrees with a similar work carried out by[13] and that by[14] in which *Staphylococcus aureus* showed the highest sensitivity followed by *Escherichia coli*.

Table 2: In-vitro antimicrobial activity of the mixtures against the test microorganisms.

Herbal mixtures	Inhibition Zone Diameter (mm)*					
	E. coli	***S. aureus***	***K. pneumoniae***	***S. typhi***	***S. mutans***	***C. albicans***
P_1	23.75±1.81	24.25±1.71	19.0±2.23	17.75±1.70	20.5±1.11	17.75±1.70
P_2	24.0±1.58	25.75±1.71	21.74±1.71	19.5±0.96	--	--
P_3	7.5±1.29	9.25±1.66	--	7.5±0.58	--	--
P_4	6.5±0.92	8.5±1.29	--	8.0±0.82	--	--
P_5	--	10.5±1.29	--	--	--	--
P_6	18.5±1.29	21.5±1.29	--	--	--	--
P_7	--	16.0±0.67	--	--	--	--
P_8	--	--	--	--	--	--
P_9	--	--	--	--	--	--
P_{10}	--	--	--	--	--	--
P_{11}	--	--	--	--	--	--
P_{12}	--	--	--	--	--	
P_{13}	--	--	--	--	--	--
P_{14}	--	--	--	--	--	--
P_{15}	--	--	--	--	--	--
CPX	26.5±1.29	27.5±1.29	20.25±1.71	24.0±1.82	9.75±1.71	--
FLU	--	--	--	--	--	21.0±2.58

Key

-- = No zone of inhibition

* = Mean ± standard deviation

CPX = Ciprofloxacin (5µg/ml)

FLU = Fluconazole (25µg/ml)

Of the 15 (fifteen) herbal mixtures, only P_1 showed activity against all the test microorganisms including the fungus, *Candida albicans*. Therefore, the claim of the use of P_1 to treat infections specifically caused by these microorganisms can be justified and thus P_1 can be recommended for use to treat such infections. The inhibition zone diameters produced by P_1 compared favourably with those of the antibacterial positive control drug (ciprofloxacin) and the antifungal positive control drug (fluconazole). However, the reaction of the test fungal microorganism (*Candida albicans*), to P_1 is regarded as intermediate since the inhibition zone diameter is in the range 15.0-18.0mm with reference to fluconazole.[15] This implies that P_1 can be used to treat infections caused by *Candida albicans* but this will be dose dependent. The herbal mixture, P_1 showed a much higher antibacterial activity (20.5±1.11mm) against *Streptococcus mutans* than ciprofloxacin (9.75±1.71mm). Such cases were a plant extract exhibits a higher antibacterial effect than a standard antibiotic have been documented.[16] Inhibition zone diameter ≤ 15mm produced by ciprofloxacin (5µg/ml) is considered resistant.[17] The bacterial test organisms, except *Streptococcus mutans* were all susceptible to ciprofloxacin. This finding agrees with that of[13] in which all the bacterial test organisms including *Escherichia coli, Staphylococcus aureus* and *Salmonella typhi* were susceptible to ciprofloxacin. The herbal mixture, P_2 was active against *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae* and *Salmonella typhi* while P_6 exhibited antimicrobial activity only against *Escherichia coli* and *Staphylococcus aureus*. Thus, P_2 and P_6 can be used to treat diseases in which these microorganisms are implicated since the primary objective of *in vitro* susceptibility testing is to predict the impact of administration of the tested agent on the outcome of infection caused by the tested organism or similar organisms.[18]

Though P_3, P_4 and P_5 showed some degree of activity against the bacterial test organisms, yet the organisms are considered as being resistant to these herbal mixtures in comparison with ciprofloxacin since the inhibition zone diameters ≤ 15mm.[17] Most of the herbal mixtures (P_8-P_{15}) showed no activity against any of the test microorganisms (table 2). This, however, does not imply these herbal mixtures do not possess some phytochemical substances that can exert some antimicrobial activity. The antimicrobial activity of the herbal mixtures can be influenced by the method of their preparation as well as the choice of solvents used.[19,20] Also, it has been documented that the time (season) of harvest of a particular plant together with the age of the plant at the time of harvest can determine the amount of active constituents and thus, the potency of the plant.[21,22]

The minimum inhibitory concentration (MIC) and the minimum bactericidal/fungicidal concentration of the herbal mixtures are respectively presented in tables 3 and 4. The herbal mixture (P_1) had a bactericidal/fungicidal effect against all test microorganisms with minimum bactericidal/fungicidal concentration (MBC/MFC) values ranging from 1.25mg/ml-2.50mg/ml. It was only bateriostatic against *Streptococcus mutans* with MIC value of 1.250mg/ml. The herbal mixture, P_2 showed a bactericidal effect against *Escherichia coli, Staphylococcus aureus, Klebsiella pneumonia* and *Salmonella typhi* with MBC value of 1.250-10.00mg/ml. While P_6 was bactericidal against *Staphylococcus aureus* (MBC value of 5.00mg/ml), it was only bacteriostatic against *Escherichia coli* with MIC value of 5.00mg/ml. Also, P_7 was only bateriostatic against *Staphylococcus aureus* with MIC value of 10.00mg/ml.

Table 3: The minimum inhibitory concentration (MIC) of the herbal mixtures against the test microorganisms.

Herbal mixtures	Minimum inhibitory concentration (mg/ml)					
	E. coli	***S. aureus***	***K. pneumoniae***	***S. typhi***	***S. mutans***	***C. albicans***
P_1	1.250	0.625	1.250	1.250	1.250	0.625
P_2	0.312	0.156	0.625	0.625	-	-
P_6	5.000	1.250	-	-	-	-
P_7	-	10.000	-	-	-	-

Key

- = No antimicrobial activity

Table 4: The minimum bactericidal/fungicidal concentration of the herbal mixtures against the test microorganisms.

Herbal mixtures	Minimum bactericidal/fungicidal concentration (mg/ml)					
	E. coli	***S. aureus***	***K. pneumoniae***	***S. typhi***	***S. mutans***	***C. albicans***
P_1	2.50	1.250	2.50	2.50	> 10	2.50
P_2	2.50	1.250	5.00	10.00	-	-
P_6	>10	5.000	-	-	-	-
P_7	-	>10	-	-	-	-

Key

- = No antimicrobial activity

CONCLUSION

From the results of this sturdy, it was observed that the herbal mixtures had some activity, especially antibacterial activity against the test microorganisms since the only fungus (*Candida albicans*) was resistant to all the herbal mixtures but one. Based on the results of

this sturdy, some herbal mixtures can be used for the treatment of infections caused by microorganisms used in this sturdy since some herbal mixtures can have more potent antibacterial activity against some bacteria than even some standard antibiotics as revealed in this sturdy.

Most of the herbal mixtures used in this sturdy, showed no activity against the test microorganisms. This does not necessarily imply they possess no substances that can exert some antimicrobial activity. The method of preparation as well as the age and time of harvest of the plants used for the herbal mixtures can influence their potency. It is thus, recommended that a regulatory body be instituted which will not only regulate and monitor the production of these herbal mixtures, but will also aid in the standardization of the herbal mixtures.

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