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

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ABSTRACT

This study was carried out to screen the antimicrobial activities of some herbal mixtures used for treating typhoid fever, gastrointestinal infections, bronchitis, dental carries and venereal diseases. The Kirby-Bauer disc diffusion method was used to screen fifteen unregistered herbal mixtures (coded H_1-H_{15}) purchased from Enugu and Anambra States in Nigeria, against clinical isolates of *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Salmonella typhi, Streptococcus mutans and Candida albicans*. Ciprofloxacin (5µg/ml) and fluconazole (25µg/ml) were used as standards for comparison. Results showed H_1, H_2, H_3, H_4 and H_5 were active against one or more of the test organisms. Herbal mixtures H_6-H_{15} exhibited no activity. The highest zone of inhibition (21.25±2.20mm) in this sturdy was produced by H_5 against *Staphylococcus aureus* with MIC and MBC of 1.25mg/ml and 5.00mg/ml respectively while H_1 showed the least activity (9.50±1.29mm) against *Salmonella typhi*. The herbal mixtures H_3 and H_4 respectively produced inhibition zone diameters of 20.50±2.38mm and 20.0±2.16mm against *Candida albicans* which favourably compared with that (21.0±2.58mm) of the standard drug fluconazole. *Streptococcus mutans* and *Klebsiella pneumoniae* exhibited resistance to all the herbal mixtures except H_4 to which *Klebsiella pneumoniae* was sensitive. *Staphylococcus aureus* was the most sensitive with an overall sensitivity of 35.0%. Statistically, there was a significant difference ($P<0.05$) in the sensitivity of test organisms. However, 67% of the herbal mixtures showed no activity as claimed. This study has shown herbal mixtures possess some antimicrobial activity against some human pathogens and thus, recommends the regulation and monitoring of their production process to aid standardization.

Keywords: Antimicrobial activity, herbal mixtures, unregistered, human pathogens

1. INTRODUCTION

Herbal medicine or herbalism is the use of herbs or herbal products for their therapeutic or medicinal value. They may come from any part of the plant but are most commonly made from leaves, roots, bark, seeds, and flowers and are eaten, swallowed, drunk, inhaled, or

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applied topically to the skin. Herbal products often contain a variety of naturally-occurring biochemicals from plants, many of which contribute to the plant's medicinal benefits [1].

In the past ten years, 62% of the new anticancer-agents have been natural products or based on natural products models. Since then, it marked the birth of the study on purification and the effects of drugs from natural products [2]. Drugs derived from natural sources play a significant role in the prevention and treatment of human diseases. In many developing countries, traditional medicine is one of the primary healthcare systems [3,4]. Herbs are widely exploited in the traditional medicine and their curative potentials are well documented [5].

There is increasing awareness and general acceptability of the use of herbal drugs in today's medical practice. Although, most of these applications are unorthodox, it is however a known fact that over 80% of the world population depends on herbal medicines and products for healthy living [1,6].

Although therapies involving these agents have shown promising potential with the efficacy of a good number of herbal products clearly established, many of them remain untested and their use are either poorly monitored or not even monitored at all. The consequence of this is an inadequate knowledge of their mode of action, potential adverse reactions, contraindications and interactions with existing orthodox pharmaceuticals and functional foods to promote both safe and rational use of these agents [7]. All medicines, whether they are synthetic or of plant origin, should fulfill the basic requirements of being safe and effective [8].

Like conventional medicines, licensed herbal medicines hold a product license based on safety, quality, and efficacy [9]. On the other hand, due to insufficient evidence of reproducible efficacy to meet regulatory standards, license cannot be obtained for some herbal medicines to sell these products [10]. In developed countries like UK, herbal medicinal products are required to meet specific standards of safety and quality, agree upon indications for use based on their traditional use and also provide information in a leaflet to promote safe use of the product by the purchaser [9]. However, this is not the case in many other parts of the world, especially in the developing countries where many unregistered and poorly regulated herbal products are sold freely in the market with little or no restraint. Because of this, it becomes necessary to substantiate the efficacy of some unregistered herbal mixtures. Thus, this study was undertaken to evaluate the antimicrobial activities of some unregisetered herbal mixtures (sold in Enugu and Anambra States of Nigeria) against some human pathogens isolated from clinical sources.

2. MATERIALS AND METHODS

2.1 Sample Collection

Fifteen (15) unregistered 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.

2.2 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.

2.3 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. Uninoculated sterile Nutrient Agar and Sabouraud Dextrose Agar plates were kept for media sterility control.

2.4 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 [11].

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Table 1: Details of the unregistered herbal products

Herbal Mixture Code	Herbal Mixture Name	Batch Number	Manufactured Date	Expiry Date
H_1	Mama and Papa Ejima	NI	NI	NI
H_2	Isimmili herbal mixtures	NI	NI	NI
H_3	Madam F. Kayes bitters	NI	May, 2013	June. 2017
H_4	Asheitu Adams (S.T.D)	NI	December, 2014	December, 2016
H_5	Simaco Herbal mixture	003/12	October, 2014	October, 2017
H_6	Asheitu Adams bitters	NI	January, 2015	January, 2017
H_7	Miracle herbal mixture	NI	January, 2015	January, 2018
H_8	Ide herbal mixture	NI	NI	NI
H_9	Mama Umuchu Herbal mixture	NI	NI	NI
H_{10}	Ngene herbal mixtures	NI	NI	NI
H_{11}	Nwanyi Idemili Herbal mixtures	NI	NI	NI
H_{12}	Majestic Dental Solution	001	2013	2017
H_{13}	Dr Nwakor's Herbal mixture	45D	May, 2013	April, 2016
H_{14}	Angel herbal mixture	0906	NI	NI
H_{15}	Nwanyi Ocha Herbal remedy	NI	NI	NI

Key
NI = Not indicated

2.5 Culture Medium

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).

2.6 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.

2.7 Standardization of 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.

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2.8 Sensitivity Screening

Sensitivity screening was carried out by the method of [12]. 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.

2.9 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 [13].

2.10 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 [14].

2.11 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.

3. RESULTS AND DISCUSSION

3.1 Susceptibility of the Test Microorganisms to the Herbal Mixtures

The disc diffusion method was used to determine the antimicrobial potency of fifteen (15) unregistered herbal mixtures (coded as H_1-H_{15}) against clinical isolates of *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Salmonella typhi, Streptococcus mutans and Candida albicans*. Table 1 shows the details of the herbal mixtures used in this study. The result of the antimicrobial activity of the mixtures against the test microorganisms is presented in table 2. Five (5) out of the fifteen (15) unregistered mixtures had varying degrees of activities against the test organisms. As can be seen in table 2, *Staphylococcus aureus* showed the highest susceptibility (with an inhibition zone diameter of 21.25±.20mm) among the test microorganisms. Similar findings have been document by some other researchers [15,16,17]. In this present study, *Candida albicans* exhibited the second highest susceptibility with an inhibition zone diameter of 20.5±2.38mm. This, however, does not agree with a similar work carried out by [15] and that carried out by [16] in which *Escherichia coli* showed the second highest sensitivity. These differences might be as a result of the active ingredients found in the different herbal mixtures [18]

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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*
H_1	16.25±1.26	13.0±0.82	--	9.5±1.29	--	--
H_2	--	15.0±1.83	--	--	--	--
H_3	17.75±1.00	19.0±1.83	--	--	--	20.5±2.38
H_4	15.75±0.92	18.5±1.29	18.0±0.82	14.0±1.83	--	20.0±2.16
H_5	16.0±1.63	21.25±2.2	--	--	--	14.5±1.29
H_6	--	--	--	--	--	--
H_7	--	--	--	--	--	--
H_8	--	--	--	--	--	--
H_9	--	--	--	--	--	--
H_{10}	--	--	--	--	--	--
H_{11}	--	--	--	--	--	--
H_{12}	--	--	--	--	--	--
H_{13}	--	--	--	--	--	--
H_{14}	--	--	--	--	--	--
H_{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)

3.2 Activity of the Herbal Mixtures and Control Drugs Against the Test Microorganisms

The herbal mixture, H_4 showed activity against all the test microorganisms except *Streptococcus mutans*. This herbal mixture can thus, be recommended for use to treat infections specifically caused by these microorganisms as claimed. The herbal mixtures H_3 and H_4 produced inhibition zone diameters of 20.50±2.38mm and 20.0±2.16mm respectively, favourably comparable with that (21.0±2.58mm) of the standard antifungal positive control drug (fluconazole) against *Candida albicans*. Thus, the fungus, *Candida albicans* is said to be susceptible to H_3 and H_4 with reference to fluconazole [19]. Though H_1 showed some antibacterial activity against *Staphylococcus aureus* and *Salmonella typhi*, (with inhibition zone diameters of 13.0±0.82mm and 9.5±1.29mm respectively), these microorganisms are considered resistant to H_1. This is because inhibition zone diameter ≤ 15mm with reference to ciprofloxacin (5µg/ml) is considered resistant [20]. *Streptococcus mutans* and *Klebsiella pneumoniae* were resistant to all the herbal mixtures except H_4 to which *Klebsiella pneumoniae* was sensitive. This finding agrees to a reasonable extent to those of [15] in which *Streptococcus mutans* was resistant to all the herbal mixtures used except one.

All the bacterial test organisms, except *Streptococcus mutans* were susceptible to ciprofloxacin. This also agrees with the findings of a similar study carried out by [15]. Though ciprofloxacin exhibited some antibacterial property (inhibition zone diameter of 9.75±1.71mm) against *Streptococcus mutans*, the organism is considered to be resistant. This is because inhibition zone diameter ≤ 15mm produced by ciprofloxacin (5µg/ml) is considered resistant [20]. However, the reaction of the test fungal microorganism (*Candida albicans*), to H_5 is regarded as intermediate since the inhibition zone diameter is in the range 15.0-18.0mm with reference to fluconazole [19]. The implication here is that H_5 can be used to treat infections caused by *Candida albicans* but this will be dose dependent. The herbal mixtures, H_3 and H_5 were active against the bacterial organisms *Escherichia coli and Staphylococcus aureus*, while H_1 exhibited antimicrobial activity only against *Escherichia coli*. 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 [21], these herbal mixtures (H_1, H_3 and H_5) can thus, be recommended for used to treat diseases in which these microorganisms are implicated.

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Most of the herbal mixtures (H_6-H_{15}) showed no antimicrobial activity against any of the test microorganisms (table 2).This does not imply these herbal mixtures do not contain any phytochemical substances that can exert some antimicrobial activity. 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 phytochemical substances and thus, the potency of the plant [22,18]. Also the antimicrobial activity of the herbal mixtures can be influenced by the method of their preparation as well as the choice of solvents used [23,24].

3.3 The Minimum Inhibitory Concentration and Minimum Bactericidal/Fungicidal Concentration of the Herbal Mixtures

Tables 3 and 4 respectively show the minimum inhibitory concentration (MIC) and the minimum bactericidal/fungicidal concentration of the herbal mixtures. The herbal mixture (H_1) had a bactericidal effect (MBC value of 10mg/ml) against *Escherichia coli* and was only bacteriostatic against *Staphylococcus aureus* with an MIC value of 5.00mg/ml. H_3 showed a bactericidal/fungicidal effect against *Escherichia coli, Staphylococcus aureus* and *Candida albicans* with minimum bactericidal/fungicidal concentration (MBC/MFC) values ranging from 5.00mg/ml-10.00mg/ml. The herbal mixture, H_4 exhibited bactericidal/fungicidal effect (MBC/MFC value of 10.00mg/ml) against *Klebsiella pneumonia, Salmonella* and *Candida albicans* but it was only bateriostatic against *Escherichia coli* and *Staphylococcus aureus* with MIC values of 10.00mg/ml and 5.00mg/ml respectively. None of the herbal mixtures had any cidal effect against *Streptococcus mutans*. This is in agreement with a similar work carried out by [15].

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*
H_1	2.500	5.000	-	-	-	-
H_2	-	5.000	-	-	-	-
H_3	1.25	1.25	-	-	-	0.63
H_4	10.00	5.000	10.00	10.00	-	1.50
H_5	10.00	1.25	-	-	-	10.00

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*
H_1	10	>10	-	-	-	-
H_2	>10	-	-	-	-	-
H_3	5.000	5.000	-	-	-	2.50
H_4	>10	>10	10.00	10.00	-	10.00
H_5	>10	5.00	-	-	-	>10

Key
- = No antimicrobial activity

4. CONCLUSION

The results of this present study revealed that the herbal mixtures had some antimicrobial activity against the test microorganisms. Two of the herbal mixtures (H_3 and H_4) compared favourably with fluconazole (25mg/ml) in their antifungal activity against the only fungus (*Candida albicans*) used in the study. Based on the results of this study, some herbal mixtures can be used for the treatment of infections caused by microorganisms used in this sturdy.

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Though about 67% of the herbal mixtures used in this study, showed no activity against the test microorganisms, it does not necessarily imply they do not possess any antimicrobial potential. The method of preparation (with regards to the solvent used) as well as the age and time of harvest of the plants used for the herbal mixtures can influence their potency. This study thus, recommends the regulation and monitoring of the production process of these herbal mixtures. This will not only aid in the standardization of the herbal mixtures, but will also reduce the problems militating against recognition of traditional medicinal practices by orthodox medical practitioners.

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