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ANTIMICROBIAL AND TOXICOLOGICAL STUDIES OF EPA-IJEBU. A "WONDER – CURE" CONCOCTION USED IN SOUTH-WEST,NIGERIA.

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Abstract

'Epa-ijebu' is regarded as a "wonder cure" concoction used in curing many diseases and as an antidote to scorpion and snake bites among the Yoruba's in South West, Nigeria. Initial report had indicated antibacterial activity of the concoction against some common bacterial pathogens. This present study screened for fungicidal activity against *Candida albicans*, *Microsporum* spp, *Trichophyton mentagrophytes* and *Aspergillus fumigatus* as well as bactericidal activity against *Helicobacter pylori*, *Salmonella Typhi*, *Salmonella Enteritidis*, *Shigella flexneri*, and Enteroheamorrhagic *Escherichia coli* . Toxicity of the concoction was also tested . The disc diffusion method and the Agar well diffusion technique were employed for screening the Epa-Ijebu against clinical isolates of the four fungi .Both the Minimal Inhibitory Concentration (MIC) and Minimal Fungicidal Concentration (MFC) were also determined. A comparison between the MFC of the Epa-Ijebu and three marketed antifungal drugs (Griseofulvin, Nystatin and Itraconazole) was made. Minimal Inhibitory Concentration (MIC) and Minimal Bacterial Concentration (MBC) were determined using serially diluted Epa-Ijebu concoction. Acute toxicity of the Epa- Ijebu was also tested by feeding it to one week old laboratory mice. The Epa-Ijebu concoction at 25.0 – 50.0 mg/ml inhibited the growth of the fungi and was recorded to be fungicidal at 50.0 – 100.0 mg/ml for all the fungi screened. These figures compare favorably with the known antifungals. The concoction inhibited the growth of all the bacteria at the concentration used and was found to have an MIC of between 15.6 - 125 mg/ml and MBC of between 31.25 and 250mg/ml. However, toxicological assays showed that the concoction was toxic to the animals at high concentrations of 0.2 -0.8 g/ml leading to the deaths of the animals within 24hrs of being fed. Histological examination of the stomach, liver and kidney showed that profound erosion of the tissues with marked area of karyolysis and karyorrhesis. The study confirms the antifungal and antibacterial properties of the "wonder cure" concoction but its use as an antidote to many ailments need to be moderated because of its toxicity.

Introduction

In Africa, the reliance on folk medicine has been encouraged due to the presence of abundant and diverse vegetation, and also due to the present shortage of conventiontional drugs, or the exorbitant price of such drugs. Also the World Health Organization is taking an official interest in herbal medicine in order to fulfill its aim of making health care available to all by the year 2010. Generally, common ailments in rural areas are treated by some village old people who possess the knowledge of the local plants. In Nigeria notable contributions towards the folk medicinal have been made (Olaniyi, 1974; Gbile, 1986; Adjanohoun et al., 1991; Sofowora, 1982) among many others.

Among the Yoruba in the western part of Nigeria the use of a "wonder-cure concoction" named Epa-Ijebu is widespread. This is regarded as a potent medicament in healing many diseases and as an antidote to scorpion and

snake bites. It is very popular among the Yorubas, in the South western part of Nigeria. According to information received from local herbalists, the recipes include juice from *Citrus aurantifolia*, *Citrus aurantium*, and fruit of *Aframonium melegueta* as well animal parts including a type of rat *Rattus norvegium*, snake heads (various types) and scorpion. All the animal parts are dried and ground into powder. The concoction is prepared by mixing all the ingredients in a large pot and cooked until the materials are reduced by half and allowed to cool. Thereafter, it is dispensed into small bottles and labeled for sale. Small quantities of the paste is added and mixed with palp (a slurry of milled corn prepared in boiled water) and drank . It is said to be very efficacious in treating various ailments. Initial reports (Adeleye et al.,2008) showed that the Epa-ijebu had inhibitory activities against some bacterial pathogens even more than any of twelve other plant extracts screened.

The phytochemical analysis conducted on Epa-Ijebu by same worker(s) (Adeleye et al.,2008) revealed the presence of bioactive compounds including tannin, flavonoid, alkaloids, phlobatannin, anthrocyanin, reducing sugar, Saponin and anthraquinone in the concoction. This present study was designed to achieve the following objectives: (i) Screen the Epa-Ijebu concoction for antifungal activity, (ii)Test same for broad spectrum antibacterial activity by screening against more pathogenic bacteria, and (iii) Assess the toxicity of the concoction *in vivo.*

Materials and Methods

Procurement of "wonder cure" concoction (Epa-ijebu)

Bottles containing the "wonder-cure" concoction (Epa-Ijebu) were purchased from the local herbalist (Elewe-omo) at Mushin market, Lagos. In addition some quantities were purchased from hinterland in Ondo State which were believed to be more potent and "original".

Screening for antifungal activity

Test organisms

The test organisms used for screening the antifungal activity of the 'wonder-cure concoction' (Epa-Ijebu) were *Trichophyton mentagrophyte*, *Candida albicans*, *Aspergillus fumigatus* and *Microsporum* Spp. They were clinical isolates obtained from the Microbiology Laboratory of the Lagos University Teaching Hospital, Idi-Araba, Lagos. *Candida albicans* was cultured overnight on SDA while the others were cultured on SDA maintained at 25^{O}C for 72 hrs. Their identities cofirmed by Dr. Adekunle, a Mycologist in the Department of Botany and Microbiology, University of Lagos). *A.fumigatus* was isolated from superficial mycotic skin infection.

Punch hole technique or Agar well technique

Sterile SDA plated were seeded with 10^3 spores /ml of the test organisms, thereafter the concoction (which was in paste form) was reconstituted using sterile distilled water as solvent to get 100 mg/ml concentration. A 1ml micropipette was adjusted to deliver 0.1 ml of the concoction in different wells (5 mm diameter) borne on the surface of the SDA plates. The plates were incubated at 25^{O}C for 72 hrs. The presence of zones of inhibition around each of the well after the incubation period was regarded as an indication of antifungal activity of the "Epa-Ijebu" while the absence of any measurable zones of inhibition was interpreted as absence of fungicidal activity.
Control consisted of the test organisms grown on SDA without inclusion of the concoction but with sterile distilled water as blanks Known antifungals, Itraconzole, Nystatin and Griseofulvin were used as positive control for comparison.

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Disc diffusion technique

The disc diffusion technique of Taylor et al. (1996) was used. Discs were prepared by impregnating sterile discs with 100 mg/ml concentration of the concoction and dried overnight (at 35 C). The discs were applied to sterile SDA plates previously seeded with 10^3 spores/ml of the test organisms. All plates were incubated at 25^{o}C for 72hrs and later observed for zones of inhibition of fungal growth and the diameter of zones measured in mm using a meter rule. Control experiments were set up using the test organisms grown on SDA without inclusion of the concoction but with sterile distilled water as blanks. Known antifungals (Itraconazole,Nystatin and Griseofulvin) were used as positive controls for comparison.

Minimal Inhibitory Concentration (MIC)

The MIC of the concoction was determined by incorporating varying concentrations of the concoction solutions (12.5 – 100mg/l) into sets of test tubes containing sterile normal saline. Using a micropipette 0.01ml of the 10^3 spores of the fungi was delivered into each of the test tubes containing the concoction while the normal unseeded normal saline was set up as the control. All tubes were incubated at 25^OC for three days. The MIC was regarded as the lowest concentration of the extract that did not permit any visible growth when compared with that of the control tubes.

Minimal Fungicidal Concentration (MFC)

0.10ml samples from the test tubes used in the MIC test which did not show any visible growth after the period of incubation were streaked onto a freshly prepared SDA medium. The MFC was taken as the lowest concentration of the concoction that did not permit any fungal colony growth after 72 hrs of incubation on solid medium

Antibacterial assay

For the antibacterial activity the test organisms were *Helicobacter pylori, Salmonella Typhi, Salmonella Enteritidis, Shigella flexneri,* and Enterohaemorrhagic c*oli (*EHEC). All organisms were obtained from Nigerian Institute of Medical Research (NIMR) Yaba, Lagos and sub-cultured unto fresh plates 24 hrs before use. 0.5ml Macfarlan standard of each test bacteria was aseptically swabbed on the surface of Mueller Hington agar plates. 20 microliter of the Epa-Ijebu (in paste form) was applied to the surface of the agar plate at four spots equi-distance from each other. For control, disc containing ampicillin was applied to the surface of the seeded agar plates. All plates were at 37^0C for 24hrs. except plates containing *H. pylori* which were incubated at 37^0C for 48hrs.There after, zone of inhibition was measured with a metre rule.

Minimal Inhibitory Concentration

1.0ml of Epa –Ijebu was double – diluted in 2ml of sterile Mueller–Hington broth to give the following concenhtrations: 1/2, 1/4, 1/8, 1/16, 1/32, 1/64 respectively. 20 microliter of the standardized test organisms (Macfarland standards) (except *Helicobacter pylori),* was inoculated into the serially diluted concoction. For *H. pylori* brain heart infusion broth supplemented with isovitalex was used as dilution broth for the concontion.The culture tubes were incubated at 37^oC for 24hrs (48hrs for *H. pylori*). The lowest concentration of the concoction that showed no growth after incubation was taken as Minimal Inhibitory Concentration

Minimal Bacteriocidal Concentration

The serially diluted culture tubes containing Epa – Ijebu and test organism that showed no growth after incubation (above) were sub-cultured unto sterile unto sterile MacConkey agar plates and incubated at 37^oC for 24hrs (except for *H. pylori* which was incubated for 48hrs). The lowest concentration of the concoction which did not allow the growth of the test bacteria was taken as the Minimal Bactericidal Concentration (MIC).

Toxicological and histological studies

One week old laboratory mouse in six groups of 10 each were fed orally with 0.025mg/ml – 0.8g/ml of the freeze-dried concoction. They were allowed to drink water *ad-libitum* and monitored for death. Just about the time death was recorded in some, the living animals in the group were sacrificed by suffocating with 10% chloroform and cut open bilaterally to extract the liver, kidney and stomach specimens. The tissues, cut into bits, were immediately placed in 10% formal saline for 2hrs to prevent post-mortem changes. Thereafter, the tissues were fixed for 24 – 48 hrs. They were then cut into blocks with each measuring 5 x 3 x 2cm and then processed in the tissue processor. They were dehydrated through ascending grades of alcohol. Cleared in two changes of xylene and impregnated with wax. The processed tissue blocks were then trimmed and four (4) microns thick sections were cut, dried and stained in Cole's haematoxylin and rinsed in water. It was then differentiated in 1% HCl in 70% alcohol, rinsed in water and Blued in tap water. They were counterstained with 1% Eosin, rinsed in water, dehydrated, cleared and mounted in DPX (Diethylene Phtalate Xylene). The slides were viewed under the microscope and pictures of the tissues taken at X400 magnification respectively.

Results

Table Ia shows the antifungal activity of the concoction at different concentrations using the disc diffusion and or agar well techniques respectively. At lower concentrations (10^{-3}–10^{-5} mg/ml) the concoction did not show any fungicidal activity in the disc diffusion and 10^{-4} – 10^{-5} mg/ml in the punch hole technique. At higher concentration of 10^{-1} mg/ml, the plates with the punch hole technique showed no fungal growth. The results also showed that the agar well technique was more effective than the disc diffusion technique and the zones of inhibition ranged between 2mm-18mm for both techniques.

Table 1b shows the result of Minimal Inhibitory Concentration (MIC) and Minimal Fungicidal Concentration (MFCs) measured in mg/ml of the concoction against the four fungi species screened. The range of MIC was between 25.0 mg/ml – 50.0 mg/ml and 50 mg/ml-100 mg/ml for MFC. The concoction was unable to inhibit the growth of the organism at low concentrations. It was observed that the concoction was fungicidal against all the fungi but only at high concentrations (50.0mg/ml – 100.0mg/ml).From the results the concoction had comparable fungicidal properties as the known antifungals used as control.

The results of the antibacterial assay of Epa –Ijebu is shown in Table 2a, All the four bacteria were inhibited by both crude and 10^{-1} diluted Epa- Ijebu. The zone of inhibition displayed was comparable to the standard antibiotic. Table 2b showed the result of the Minimal Inhibitory Concentration (MIC) and Minimal Bacteriocidal Concentration (MBC) of the Epa- Ijebu on the five bacterial isolates tested. Except for *H. pylori* MIC ranged between 15.6 and 31.25 mg/ml while the MBC was between 31.5 and 125mg/ml. The MIC and MBC for *.H. pylori was* 125mg/ml and 250mg/ml respectively

Table 3 shows the level of degeneration and erosion on the animal tissues. The histological section showed a wide sectional layer of stomach tissue, kidney tissue, liver tissue, with thick layer of muscularus externa, well distributed renal corpuscles with clear area of epithelial lining and well arranged hepatocyte radiating from the central vein, the portal triad well displayed in the stomach, kidney and liver tissues of the laboratory animals fed with 0.025 mg/ml of the concoction indicating an absence of degeneration (Plates 1- 3). Plates 4 – 6 showed a marked area of degeneration, with an area of karyolysis and karyorrhexis on tissues of mice fed with concentrations ranging from 0.2-0.8mg/ml. There was evidence of erosion of the mucosa lining especially in the stomach tissues.

Table 1a: Mean result of antifungal activity of Epa-Ijebu concoction using the paper disc^{+} and punch hole^{++} methods.(measured in mm as diameter of zones of inhibition)

Test Organisms	**Concentrations of Epa- Ijebu (mg/ml)**				
	10^{-1}	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**
C. albicans	10^{+} NG^{++}	6^{+} 18^{++}	0^{+} 8^{++}	0^{+} 0^{++}	0^{+} 0^{++}
T. mentagrophytes	10^{+} NG^{++}	4^{+} 12^{++}	0^{+} 8^{++}	0^{+} 0^{++}	0^{+} 0^{++}
A. fumigatus	8^{+} NG^{++}	0^{+} 12^{++}	0^{+} 2^{++}	0^{+} 0^{++}	0^{+} 0^{++}
Microsporum spp	8^{+} NG^{++}	6^{+} 14^{++}	0^{+} 6^{++}	0+ 0^{++}	0^{+} 0^{++}

Table 1b: Comparism between the fungicidal activity of the Epa-Ijebu and that of Griseofulvin, Nystatin and Itraconazole (Zone of inhibition measured in mm)

Organisms	**Epa-Ijebu(100mg/ml)**		**Griseofulvin (10mg/ml)**		**Nystatin (10mg/ml)**		**Itraconazole (10mg/ml)**	
	MIC	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**
Candida albicans	25.0	50.0	25.0	25.0	25.0	25.0	25.0	25.0
T. mentagrophtes	25.0	50.0	25.0	25.0	25.0	25.0	25.0	25.0
Aspergillus fumigatus	50.0	100.0	25.0	25.0	25.0	50.0	25.0	50.0
Microsporum spp	50.0	50.0	25.0	50.0	25.0	50.0	25.0	50.0

Table 2a: Mean result of antibacterial activity of Epa-Ijebu concoction (concentrated and diluted) measured in mm as zone of inhibition

Test organism	**Concentrated Epa-Ijebu**	**10^{-1} dilution**	**Control (Ampicilin)**
H. pylori	22^{+}	22^{+}	20
S.Ttyphi	18^{+}	14^{+}	16
S. Enteritidus	11^{+}	16^{+}	15
S flexneri	17^{+}	3^{+}	15
Enterohaemorrhagic *E. Coli* (EHEC)	13^{+}	0^{+}	17

Table 2b: Minimal Inhibitory Concentration (MIC) and Minimal Bacteriocidal Concentration (MFC) in mg/ml of Epa-Ijebu concoction on bacterial isolates

Organisms	**MIC (mg/ml)**	**MBC (mg/ml)**
H. pylori	125	250
S.Typhi	15.625	31.25
S. Enteritidis	31.25	62.50
S. flexneri	15.625	31.25
Enterohaemorrhagic *E. Coli* (EHEC)	31.25	125.0

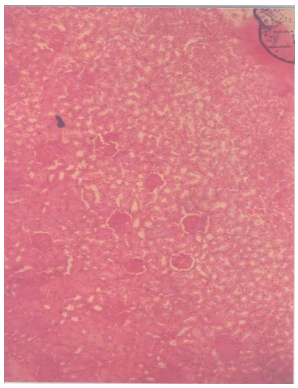

Plate I: Histologic section of the normal stomach tissue of the laboratory mouse fed with 0.025 g/ml of the freeze dried concoction

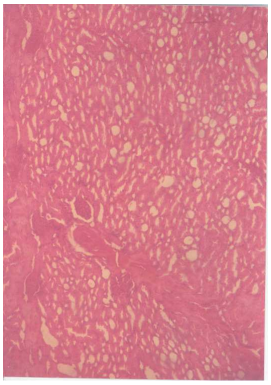

Plate II: Histologic section of the normal kidney tissue ofthe laboratory mouse fed with 0.025 g/ml of the freeze dried concotion.

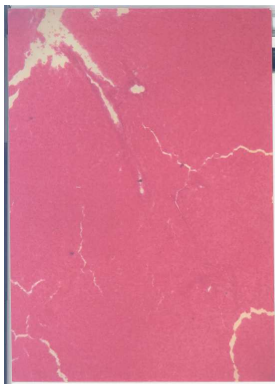

Plate III: histogy section of the normal liver tissue of thelaboratory mouse fed with 0.025 g/ml of the freeze dried concoction

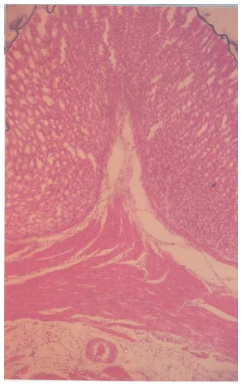

Plate IV : Histologic section of the eroded kidney tissue of a laboratory mouse fed with 0.8 g/ml of the freeze dried concoction

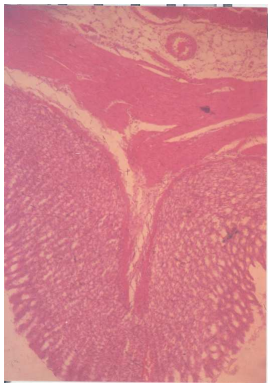

Plate V: histology section of an eroded liver tissue of the laboratory mouse fed with 0.8 g/ml of the freeze dried concoction.

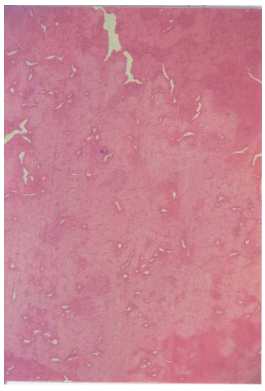

Plate VI: histology section of an eroded stomach tissue of a laboratory mouse fed with 0.8 g/ml of the freeze dried concoction

Table 3: Histological examination of dead and sacrificed animal tissues

ORGANS		LIVER			KIDNEY			STOMACH				
Group	Average body weight	Level of degradation	Erosion	Areas affected	Level of degeneration	Erosion	Areas affected	Level of degeneration	Erosion	Area affected	Significant/ Non Significant	comment
Control	17.03	-	-	Total	-	-	Total	-	-	Total	NS	Normal
Group 1(0.025g/ml)	19.73	-	-	Total	-	-	Total	-	-	Total	NS	Normal
Group II (0.05g/ml)	18.56	-	-	Total	-	-	Total	-	-	Total	NS	Normal
Group III (0.10g/ml)	20.69	-	-	Total	-	-	Total	-	-	Total	NS	Normal
Group IV (0.20g/ml)	20.36	+	-	Total	-	-	Total	-	-	Total	S	Abnormal
Group V (0.40g/ml)	17.74	++	+	Total	++	++	Total	++	++	Total	S	Abnormal
Group VI (0.80g/ml)	19.34	++	++	Total	++	++	Total	++	+ ++	Total	S	Abnormal

Discussion

This present study established the antifungal and antibacterial activities of Epa-ijebu, a native "cure- all" concoction. Its fungicidal activities compared favorably with that of established drugs. There had been no previous study on the fungicidal activities of this native concoction although there had been numerous reports of herbal treatment for fungal infections. (Adtunbi et al.,1986; Oloke and Kolawole, 1998; Ajayeoba and Ekundayo, 1999; Adeleye et al., 2003; Elujoba et al., 2005). Similarly the concoction was found to be inhibitory to all the bacteria studied. This is in line with the previous findings by Adeleye et al (2008) where the concoction was screened for activity against *S. aureus*, *E. coli*, *P. vulgaries* and *S. sonnei*. This current study obviously confirmed the broad spectrum antimicrobial activity of the concoction. The presence of bioactive compounds including tannin, alkaloids anthraquinone and saponin as reported by Adeleye et al. (2008) may be responsible for this antimicrobial actvity. The effect may also be due in part to the presence of *citrus aurantifolia* and *C. aurantium* (juice) and fruit of *Aframonium melegueta* in the recipe used for compounding the concoction. These three plants have been established to have antibacterial effects (Elujoba et al., 2005)

Toxological studies carried out showed that the concoction may be toxic at concentrations above 0.2mg/ml causing the death of all the mice. Histological studies examination showed extensive tissue degeneration in the stomach, kidney and liver of the mice (Plates 4-6). This is consistent with ingestion of a poisonous material. This may be attributed to the presence of snake head, scorpion and poisonous rats (dried and ground) in the recipe. These substances are known to have some level of toxin which may induce the tissue erosion and egeneration noticed. Thus, much as the concoction has been proved to have antimicrobial properties by this current study, the safety level is questionable when taken at high concentrations.

Our findings offer a scientific basis for the use of the Epa – Ijebu as a remedy in treating infections caused by fungi and bacteria since it was found to have antifungal and antibacterial properties. However, taking it at a concentration higher than 0.2mg/ml may not be safe since it was found to be toxic to laboratory mice at these concentrations.

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