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Research Article

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ETHANOLIC EXTRACTION AND PHYTOCHEMICAL SCREENING OF TWO NIGERIAN HERBS ON PATHOGENS ISOLATED FROM WOUND INFECTIONS

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ABSTRACT

The study was aimed at assessing extracts of two Nigerian herbs: *Chromolaena odorata (leaf), Citrus sinensis* (ripe and unripe peels), as veritable alternative therapies for treatment of wound infections. The study was carried out in four phases comprising isolation of different bacterial pathogens from clinical wound samples, crude extraction of plant materials, Kirby Bauer method of sensitivity testing, using the extracts and finally, phytochemical analysis of the extracts. Microbiological analysis of wound specimens, using the streak-plate technique, revealed the presence of bacteria involving in descending order of prevalence *Klebsiella* spp (25.4%), *Staphylococcus aureus* (24.1%), *Pseudomonas* spp (18.2%), *Escherischia coli* (18.0%), and finally *Streptococcus pyogenes* (14.1%). The antimicrobial assay of the extracts showed that *C. odorata* produced zones of inhibition which ranged from 9.0 – 12.0mm with *Pseudomonas* spp (12mm), observed as the most sensitive and least, *S. aureus* (9mm), but no effect on *Klebsiella*; *C. sinensis* unripe peel produced zones ranging from 10.0 – 20.0mm with the most sensitive organism being *S. pyogenes* (20mm), with least sensitive *S. aureus* and *Klebsiella* (10mm each). Extracts from ripe *C. sinensis* did not show any zones of inhibition on any of the isolates. Phytochemical analysis of the plant materials revealed the presence of: alkaloids, flavonoids, cyanogenic glycosides, cardiac glycosides, tannins and saponins as the phytochemical components present in the leaves of both herbs. Concern over abuse of antibiotics and the resultant resistance of pathogens to these comparatively costly conventional drugs, has prompted several World-wide studies including the present one aimed at evaluating plant materials, as possible alternative, effective easily affordable sources in wound diseases treatment.

Keywords: Nigerian herbs, wound infection, pathogens, phytochemicals, zone of inhibition.

INTRODUCTION

Secondary metabolites present in plants have been linked with the healing properties of plants. In addition to their active ingredients, plants contain minerals, vitamins, volatile oils, glycosides, alkaloids, bioflavonoids, and other substances that are important in supporting a particular herb's medicinal properties.[1]

The World Health Organization (WHO) estimates that 4 billion people (80%) of the world's population presently use herbal medicine for one form of primary healthcare or another. Its history is inextricably intertwined with that of modem medicine, but Pharmacologists, rather than use a whole plant identify, isolate, extract and synthesize individual components, thus capturing the active properties as against the herbalist who considers that the power of a plant lies in the interaction of all its ingredients.[1]

It has been reported that plants used as medicine offer synergistic interactions between both known and unknown properties[2], since these medicinal plants have different actions for varied purposes. Herbs that play a role in wound healing for instance, encourage blood clotting, fight infections and accelerate the wound healing process.[3]

The wound healing process in itself consists of integrated cellular and biochemical events leading to the re-establishment of structural and functional integrity, with regain of strength of injured tissue. Treating a wound therefore is aimed at either shortening the time required for healing or to minimize undesired consequences and so, attention is directed towards discovering an agent which will accelerate wound healing, either when it is progressing normally or when it is being treated.[4]

Due to resistance of wound pathogens to existing antibiotics, attention is now shifted to alternative sources of cure. This prompted the investigation of two herbs - *Chromolaena odorata* and *Citrus sinensis* for their possible antimicrobial effect on wound pathogens.

Chromolaena odorata is an invasive weed of field crops in Africa and Asia, where it has been introduced. Sometimes grown as an ornamental or medicinal plant, the herb is

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used in traditional medicine where the young leaves are crushed and the decoction used in treating skin wounds.[5] On the other hand, the *Citrus sinensis* peel was initially valued by Europeans, mainly for medicinal purposes before the *Citrus* was adopted as a luscious fruit.[6] The orange peel contains volatile essential oils which are said to be effective in inhibiting microbial growth and in disinfecting wounds; among its other medicinal capabilities.[6]

MATERIALS AND METHODS

Collection of plant materials

Chromolaena odorata leaves were collected in the premises of Federal University of Technology, Owerri (FUTO), Nigeria and taken to the laboratory. Care was taken to collect only fresh, healthy and not dried or mottled leaves. They were then washed and allowed to air dry, before they were dried under mild sun for 7 days.

The peels of both ripe and unripe fresh oranges *(Citrus sinensis)* were also collected from oranges bought at Ekeonunwa Market, Owerri, Nigeria. They were washed and peeled. The peels were dried separately (ripe and unripe) under mild sun for 7 days.

The dried plant materials were each macerated and ground into a fine powder, using clean, dry SUNIK electric blender, disinfected with 95% ethanol.

Extraction of plant materials

The ground *Chromolaena odorata* (leaves) and *Citrus Sinensis* (peels) were transferred into round bottom flasks and submerged in 95% ethanol. The flasks were stoppered and left to stand for 24 hours. The extracts were then filtered using Whatman No 1 filter paper. The filtrates were concentrated to a powdered form through complete evaporation of the extraction solvent at 80^{o}C, using gentle heat. The resultant residue was dried to a constant weight of 65°C in an oven, weighed and blended into tiny bits from where portions of graded doses of 5, 10 and 15mg/ml were prepared by soaking (reconstituting) the respective weights in the equivalent of 1 ml of physiological saline for 12 hours before filtering with millipore filter into khan tubes. The filtrates were then stored in the freezer at 5°C until needed for the various studies. This method was adapted from Pavia *et al*.[7]

Collection and isolation of wound samples

Wound swab samples were collected on consent, from Surgical and Orthopedic Wards of Federal Medical Centre, Owerri, and Male Medical Ward, Abia State University Teaching Hospital, Aba, Nigeria, using Antec Medical Products sterile swab sticks. The samples were immediately taken to the laboratory for culture and isolation of bacterial species using standard microbiological procedures involving inoculating the swab unto blood agar, cysteine lactose electrolyte deficient (CLED) agar and Mackonkey media using the streak plate method. The inoculated plates were incubated for 24 hours at 37°C after which they were read and isolates identified and recorded.

Antimicrobial susceptibility test

The procedure used in testing for the antimicrobial activity of the plant extracts was the Kirby Bauer Disc diffusion method. A wire loop was flamed and allowed to cool before it was used to carefully pick up discrete colonies which were inoculated onto surface of dried, fresh nutrient agar plates. Using streak plate method, the colonies were streaked evenly over the surfaces of the agar plates. The plates were then labeled and allowed to dry slightly.

Using sterile forceps, sterile 5mm discs (cut from Whatman No. 1 filter paper with a paper punch device and sterilized before use) were picked and submerged in each of the graded concentrations of extracts, namely; ripe and unripe peels of *Citrus sinensis* and leaves of *Chromolaena odorata.* Using the forceps again, the discs were picked, laid and pressed gently on the nutrient agar medium already seeded with each of the isolated wound pathogens. Commercially prepared Gentamicin disc was used as a positive control and discs soaked in distilled water as a negative control in each agar plate. The plates were allowed to stand for 30minutes and then incubated at 37°C for 24 hours. Antimicrobial activity of each extract against the test organisms were indicated by a growth-free zone around the respective discs and the diameters of the zones of inhibition to the nearest millimeter with a ruler were obtained by measuring the distance from one end of the inhibition zone, across the disc to the other end, as reported by Nwanebu *et al*.[8]

Phytochemical screening of the plant extracts

A quantity of the samples of extracts of leaves of *Chromolaena odorata* and peels of *Citrus sinensis* was first reconstituted in the solvent used for its extraction (95% ethanol).

Test for alkaloids: This will be carried out according to the method of Magadula and Tewtrakul.[9] In this test, 1ml of each plant extract was shaken with 5ml of 27% HCl and heated gently in a steam bath for 1 minute. Then 0.5ml of Wagner's Reagent was added to each mixture and observation made for a brick red colouration which indicated a positive result or non which indicated negative.

Test for saponins (frothing test): This was carried out according to the method of Horbone.[10] 3.0 ml of each plant extract was added into test tubes and shaken vigorously. They were then allowed to stand on the bench for 1 minute and observation made for the formation of stable froths which indicated positive results.

Test for cardiac glycosides: In this test, 2ml of each plant extract was pipetted into test tubes and mixed with carbon tetrarchloride and 1ml of concentrated H_2SO_4 which was run down the side of the tube. Observation was made for a brown colouration which indicated a positive result.[10,11]

Test for flavonoids: To 1ml of each of the plant extract in a test tube was added 1ml of 5% lead acetate and the mixture was allowed to stand on the bench. The formation of precipitates in any of the samples showed that the extract contained flavonoids.[12]

Test for tannins: Each extract (1 g) was dissolved in 20 ml of distilled water and filtered. Three drops of 10% of $FeCl_3$ were added to 2ml of the filtrate. The appearance of blackish-blue or blackish-green colouration was indicative of tannins. Some 2 ml of the filtrate was added, 1 ml of bromine water and a precipitate was taken as positive for tannins.[9]

Test for cyanogenic glycosides: 2ml of each plant extract was introduced into separate conical flasks; thereafter a dry strip of alkaline picurate paper was suspended from the mouth of the conical flasks care being taken to ensure that the paper did not touch the samples. Then the mouth of the conical flasks was plugged with cotton wool and the mixtures heated for 1 hour in a water bath. Any cyanide

present in any of the samples will react with the picurate paper which is yellow and change it to brick red to indicate a positive result.[10,11,13]

RESULTS AND DISCUSSION

From the 13 wound samples collected from Federal Medical Centre Owerri (Surgical and Orthopaedic wards) and Abia State University Teaching Hospital, Aba (Male Medical Ward), a total of 5 bacterial isolates were obtained. Table **1** shows the mean total bacterial count from the wound cultures and the organisms identified within one month. It also shows percentage prevalence of individual isolates. The highest isolations were made in week 1 with 497 (29.8%), while the least was made in week 4 with 308 (18.5%). *Klebsiella* spp occurred highest, 423 (25.4%), while *streptococcus pyogenes,* 233 (14.1%) prevailed the least.

Table 1. Mean total bacterial count of identified isolates (Figures in parentheses represents percentages)

Organisms	Week 1	Week 2	Week 3	Week 4	Total
Klebsiella spp	160 (9.6)	55(3.3)	105 (6.3)	103 (6.24)	423 (25.4)
Staphylococcus aureus	-	198 (11.9)	85 (5.1)	120 (7.2)	403 (24.1)
Pseudomonas spp	149 (8.9)	46 (2.8)	25 (1.5)	85 (5.1)	305 (18.2)
Eschericia coli	188 (11.2)		113 (6.8)	-	301 18.0)
Streptococcus pyogenes	-	165 (9.9)	70 (4.2)	-	235 (14.1)
Total	**497 (29.8)**	**464 (27.8)**	**398 (23.9)**	**308 (18.5)**	**1667 (100)**

Table **2** shows the effect of the ethanolic extraxt of *Chromolaena odorata* leaf and *Citrus sinensis* peel (ripe and unripe) on the isolates including the positive control used (the antibiotic gentamicin and distilled water) on the wound isolates. The table shows the diameter of zones of inhibition on the organisms (mm).

Table 2. Zones of inhibition (mm) of ethanolic extract of *Chromolaena odorata, Citrus sinensis* and the controls on the wound isolates

Test Organism	*C. odorata*	*C. sinensis* (unripe)	*C. sinensis* (ripe)	Gentamicin	Distlled water
Klebsiella spp	0.00	10.00	0.00	15.00	0.00
Escherichia coli	10.00	0.00	0.00	9.00	0.00
Streptococcus pyogenes	10.00	20.00	0.00	24.00	0.00
Pseudomonas spp	12.00	13.00	0.00	12.00	0.00
Staphylococcus aureus	9.00	10.00	0.00	21.00	0.00

A total of 14 swab specimens were screened for possible wound pathogens, out of which two (2) showed no growth while twelve (12) gave mixed growths with a total of 5 bacterial species isolated. Incubation was carried out aerobically, giving no possible room for anaerobes to grow. The lack of growth observed in the two samples could have been as a result of the patients being on antibiotics treatment prior to sample collection[14] or the organisms could be strict anaerobes.

Table **1** shows the mean total bacterial count of the identified bacterial species and their isolation rates. In each week, at least three bacterial species were isolated. The first week recorded the highest number of isolates with 497(29.8%), followed by week two which recorded 464(27.8%). Week three with 398(23.9%) was next while week four with 308(18.5%) recorded the least. From the Table also it could be deduced that *Klebsiella* spp with 423(25.4%) was the most prevalent organism in the wound samples, followed by *Staphylococcus aureus* with 403(24.1%); *Pseudomonas* spp was next, with 305(18.2%), then *Escherichia coli*, with 301(18.0%) and finally *Streptococcus pyogenes* with the least percentage of 235(14.1%). In a similar study elsewhere, it was reported that *Staphylococcus aureus* recorded the highest isolation rate of 41% while *E. coli* recorded the least with 20%.[15] It is possible that the type of environment and the state of the wound at any particular time influence the type and prevalent of organisms isolated from a given wound sample.

Tables **3** and **4** show the antibacterial activity of the ethanolic extracts of *Chromolaena odorata* leaf and ripe and unripe peels of *Citrus sinensis* including that of the positive control – gentamycin, on the bacteria isolates. It could be observed that the extract of *Chromolaena odorata* showed weak inhibition potential against *E. coli* (10.00mm), S. *pyogenes* (10.00mm) and *S. aureus* (9.00mm). However against *Pseudomonas* spp, it produced its highest inhibition of 12.00mm, but had no effect on the growth of *Klebsiella* spp. Similarly, unripe orange peel extract showed strong inhibition on the isolates. Its highest inhibition was on *S pyogenes* (20.00mm), moderate inhibition on *Pseudomonas* spp (l3.00mm), equal zones of 10.00 mm on *Klebsiella* spp and *Staph aureus* but no effect on *E. coli.* Ripe orange peel had no effect whatsoever on any of the wound pathogens while gentamycin inhibited all the pathogens in levels ranging from weak, moderate to strong with the most susceptible being *S. pyogens* and the least *E. coli*.

Table 3. Levels of sensitivity of the isolated organisms to the extracts and the controls

Test Organisms	*C. odorata*	*C. sinesis* (unripe)	*C. sinesis* (ripe)	Gentamicin	D/w
Klebsiella spp	-	+	-	++	-
Escherichia coli	+	-	-	+	-
Streptococcus pyogenes	+	+++	-	+++	-
Pseudomonas spp	++	++	-	++	-
Staphylococcus aureus	+	+	-	+++	-

- = Negative; + =Weak; ++ = Moderate; +++ = Strong

Table 4. Phytochemical contents of *C. odorata* and *citrus sinensis* (unripe)

Plant material	Alkaloids	Saponins	Cardiac glycosides	Flavonoids	Tannins	Cyanogenic glycosides
C. odorata	+		+	+	+	+
C. sinensis	+	+	+	+	+	+

+ = present; - = absent

This result has confirmed earlier findings that gram negative organisms are generally more resistant to antimicrobial agents; probably due to their complex cell wall structures as well as possession of antibiotic resistance plasmids by some such as *P. aeruginosa*[16] and production of enzymes called Extended Spectrum Beta-

Lactamases (ESBL) by others such as *E. coli and Klebsiella*.[17] The reduced susceptibility of *Klebsiella, E. coli* and *Pseudomonas* to the plant extracts and even to gentamycin could therefore have been as result of the reasons adduced.[18] Gram positive organisms which lacked these basic qualities recorded the highest zones of inhibition as this study has shown with *C. sinensis* (unripe) producing zones as high as 20.00mm on *S. pyogenes.*

In addition to the antibacterial activity observed for *C. odorata* in this study, a similar one conducted by Arce and Barroga[19], revealed that the stem of C. *odorata* has moderate antimicrobial effect against *Candida albicans* and weak activity against *S. aureus* and *P. aeruginosa.* The study also revealed that C. *odorat.a* leaf has coagulative effect on wounds which may be due to the presence of tannins which precipitate proteins as one its constituents.

Ripe extracts of *C. sinensis* did not show any antibacterial activity throughout the study. This may be due to the higher concentration of aliphatic aldehydes and oxygen-contaminating mono-terpenes and sesquiterpenes which has little antimicrobial potentials than the peels of fully developed unripe oranges.[20]

Table **4** shows the results of the phytochemical screening of the extracts. As observed, both extracts contain alkaloids, cardiac glycosides, flavonoids, tannins and cyanogenic glycosides. Saponins were absent in C. *odorata* but present in *C. sinensis*. Tannins have been reported to reversibly form complexes with proline-rich proteins, resulting in the inhibition of cell protein synthesis as well as production of typical tanning effect which is important in treating inflamed or ulcerated tissues, burns, wounds, pneumonia, and dysentery. Indeed plant parts that contain tannins are astringent in nature and have important roles as stable and potent antioxidants.[21] Terpenoids, saponins and flavonoids in plant materials exert antibacterial properties and together with alkaloids and tannins in synergistic manner are responsible for growth inhibition of the pathogens.[22]

The wound healing ability of both extracts could therefore be attributed to saponins, flavonoids, alkaloids and tannins.[22,23] Studies have shown that flavonoids are mainly responsible for the activities of C. *odorata* in wound healing and antioxidant activity.[24] Elsewhere they have also been reported to have antimicrobial activity[5], while flavonoids in orange peel have anti-inflammatory, bactericidal and antifungal actions. Orange essential oil also has anti-oxidant activity which helps to promote wound healing by enhancing proliferation of fibroblasts endothe1ial cells, and keratinocytes stimulation of keratinocyte migration.[20]

CONCLUSION

Finally, since *Chromolaena odorata* leaves and unripe peels of *Citrus sinensis* were found in this study to have antimicrobial activity ranging from weak, moderate to strong, against isolated wound infecting organisms, and was also compared to a conventional antibiotic (gentamicin), their ready availability locally and the closeness of their efficacy to the conventional gentamicin, will surely enhance their application among other uses, as potent alternatives to antibiotics for effective treatment of bacterial wound infections.

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