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RESEARCH ARTICLE

HEAVY METAL ANALYSIS AND PHYTOCHEMICAL SCREENING OF TWO INDIGENOUS SPECIES (*Zingiber officinale* AND *Centrosema pubescens*) FROM NIGERIA

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ABSTRACT

The plant species (Rhizome of *Zingiber officinale* and leaves of *Centrosema pubescens*) were tested for antimicrobial activity on four bacterial, *Staphylococcus aureus*, Salmonella typhi, *Pseudomonas aeruginosa* and *Escherichia coli* and one fungi (*Candida albican*) of clinical importance. Paper disc assay was employed in the test. Results showed that the ethanolic extracts of both plants were active against the bacteria and the ethanolic extract of *Centrosema pubescens* exhibited a significant activity on Candida albicans. The phytochemical analysis carried out on *Zingiber officinale* and *Centrosema pubescens* revealed the presence of alkaloids, tannins, steroids, flavonoids, terponoid and cardial glycoside. The presence of these phytochemical supports the use of this plants as antimicrobial agent. The heavy metal analysis carried out revealed the presence of Manganese, Lead, Cadmium, Zinc and Chromium which are all within the maximum recommended level by WHO.

INTRODUCTION

Medicinal plants may be defined as any plant that can be put to culinary or medicinal uses and include those we associate with, such as garlic. The drugs of the past were substances with a particular therapeutic action extracted from plants (Ekwenye and Elegalam, 2005). The use of herbs and medicinal plants as the first medicines is a universal phenomenon. Every culture on earth, through written or oral tradition, has relied on the vast variety of natural chemistry found in healing plants for their therapeutic properties (Nweze et al., 2004). Researchers are increasingly turning their attention to natural products looking for new leads to develop better drugs against cancer, as well as viral and microbial infections Hoffmann *et al.,* 1993., Srinivasan *et al.,* 2001). Sofia *et al.,* 2007 reported that the ginger extract showed insignificant antibacterial activity against *Escherichia coli, Staphylococcus aureus* and *Bacillus cereus.* In 2005, Lopez et al2005 reported that essential oil of ginger showed weakest inhibition against selected bacteria and fungi whilst Rath *et al.,* 2002 reported that essential oil of ginger did not show any inhibition on the tested pathogens in their study. *Centrosema pubescens* is a shrub that belong to the family *Fabaceae* it is perennial, trailing – climbing herb with strong tendency to root at nodes of trailing stems. According to world health organization (WHO), more than 80% of the world's population relies on traditional medicines for their primary health care needs. The medicinal value of plants lies in some chemical substances that produce a definite physiologic action on the human body. The most important of these bioactive compounds of plants are alkaloids, flavonoids, tannins and phenolic compounds. The phytochemical research based on ethno-pharmacological information is generally considered an effective approach in the discovery of new anti-infective agents from higher plants (Duraipandiyan, Ayyanar and Ignacimuthu, 2006). The objective of this present work was to study the antibacterial effects of the medicinal plant extracts of *Zingiber officinale* and *Centrosema pubescens* on selected microorganisms, *Staphylococcus aureus* : gram positive, *Escherichia coli:* Gram negative, *Salmonella typhi*: Gram negative, *Pseudomonas aeruginosa* Gram negative, and a fungi, *Candida albicans*. And to investigate the concentration of selected heavy metals in *Zingiber officinale* and *Centrosema pubescen.*

MATERIALS AND METHODS

COLLECTION AND IDENTIFICATION OF PLANTS

The two plants *Zingiber officinale* and *Centrosema pubescens* were purchased from Mushin and Oyingbo market, a native medicinal plant market in Lagos, Nigeria. *Zingiber officinale* was purchased dried already while *Centrosema pubescens* was purchased fresh and was allowed to shade dry at room temperature at about 20-28^{0}c for fourteen days (14), after which the both plants were separately grinded into powdery form and was weighed. The plants were identified in the department of Biological sciences, Yaba College of Technology, Lagos. These medicinal plants were chosen based on their traditional medicinal use and reported biological activities.

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EXTRACTION TECHNIQUE

Ethanol extraction of *Zingiber officinale* (Ginger)

400g of the powdered ginger was soaked in 200mls of 95% ethanol for 72 hours and the extract was obtained and dried with oven at low temperature of 35-40^{0}C and was stored in the refrigerator in a serile bottle till used.

Aqueous Extraction of *Zingiber officinale*

400g of the ginger was soaked in 300mls of distilled water for 48 hours and was shaken vigorously to allow for proper extraction and was filtered using a sterile muslin cloth after which the extract was obtained, oven dried at a low temperature and stored in a refrigerator at 4^{0}C until required.

Ethanol Extraction of *Centroseme pubescens*

400g of *Centrosema pubescens* (grinded) was soaked in a 200mls of ethanol 95% for 72 hours and the extract was obtained, dried using hot plate and oven at a very low temperature was weighed after which and was stored in a refrigerator until used.

The Aqueous Extraction of *Centrosema pubescens*

It was carried out with the same method used in Ethanol extraction above. TEST ORGANISMS The test organisms, *Escherichia coli (21922), Salmonella typhi (28225), Staphylococcus aureus* and *Pseudomonas aeruginosa (27853)* were collected from the National Institute of medical Research, Yaba, Lagos (NIMR). The bacteria were collected in a gelled MacConkey agar and cultured in nutrient agar while the fungi (*Candida albicans ,ATCC 90028)* was cultured using subauraud dextrose agar.

STERILIZATION METHOD

The materials used such as agar both, were asceptically sterilized in an autoclave at 121^{o}C for 15 minutes. And also beakers, McCartney bottles, peteridishes, pipette, folter papers, test tubes and many other glass wares (apparatus as well as metallic apparatus such as spatirk, and forcepts were sterilized using hot air oven at a temperature of 160^{o}C for 1 hour. The wire loops were sterilized by flaming through the blue flame using either spirit lamp or Bunsen burner until red hot and is allowed to get cool before using for innocation. Alcohol (Ethanol) was used to clearn the working bench mostly the working area for sanity in order to prevent contamination of the work and all the procedures were aseptically down in order to avoid contamination.

PREPARATION OF AGAR (CULTURE MEDIA)

All culture media including nutrient agar and subourauds dextrose agar for inoculating fungi while the nutrient agar is for bacteria. For the preparation of nutrient agar, 28g of the agar was being dissolved in 100ml of water, it was stired with stirring rod and plugged with cotton wool with aluminum foil covering the mouth and sterilized in the autoclave. The sterilization was done with the autoclave at 121^{o}C, 1 at 15 minutes. After cooling the sterilized agar it was poured with the numbers of petridishes required and was done asceptically to avoid contamination. The plates were stired gently to mix the component properly. The agar were allowed to solidify after which the plates were turned upside down. Preparation of sabouraud dextrose after 68g of the suboraud dextrose agar was dissolved in 100ml when stired and dissolved under flame for adequate temperature, it was plugged with cotton wool and aluminum foil and sterilized in autoclave at 121^{o}C at atm for 15 minutes. After cooling, 1ml of antibiotics was added into 25ml prepared agar to inhibit growth of other microorganisms. The agar is then poured into required petridish and stirred gently to mix the component properly.

PREPARATION OF DISCS

Whatman filter paper was perforated into small circular pieces of about 6mm in large quantities.
They were sterilized in autoclave putting them in a beaker at 121oC covered with aluminum foil paper at 121^{o}C, 1 atm for 15 minutes. They were then soaked into the different concentrations of extracts of ginger and *Centrosema pubescens* for 24 hours before placing them on the different inoculated agar using forcepts.

RECONSTITUTION OF EXTRACTS

The different extracts obtained in the steric universal bottles were later reconstituted with dimethyl formamide (DMF). The dried extracts were reconstituted by dissolving in 50mg, 100mg ad 150mg each in 1ml dimethyl formamide solutions in 5ml vial bottle. Also, central measure was obtained using 100mg of ciprofloxacin powder in 1ml of the dimethyl formamide solution. This was used as positive control while 1ml of dimetyl formamide solvent was used as negative standard control.

SENSITIVE TESTING

DISC DIFFUSION METHOD
The cultured media prepared (the nutrient agar and the sabouraud dextrose agar/were prepared and poured accordially into the different petridishes and was allowed to solidity after which, the tested organism were placed on the solidified agar and was sprayed all over the surface of the agar using siring and the prepared disc of each concentration of extracts were then gently placed aseptically using forcepts on the surface of the agar, and was then incubated at 37^{o}C. the susceptibility of the organism, were observed after 24 fours and progressively for 3 days of incubation and the diameter of the clear zone of inhibition for each concentration of extracts were recorded. Also the positive control were notice for clear zone of inhibition.

PHYTOCHEMICAL SCREENING

Chemical tests were carried out on the aqueous and ethanol extracts of the produced specimen using standard procedure to identify the constitutions as described by Sofowora 1993, Trease and Evang 1989 and Harborn (1973).

QUALITATIVE ANALYSIS OF THE CONSTITUENTS

Test For Flavonoid 5ml of 10% dilute ammonia solution was added to a portion of the aqueous fiterate of the plant extracts, followed by addition of concentrated H_2S0_4. A yellow colourable observed in the extract indicating the presence of flavonoid. Test for saponins 2g of the powderd sample was boiled in 20ml of distilled water in a water bath and foltered. 10ml of the filtrate was mixed with 5ml destilled water and shaken vigorous for stable persistent froth. The frothing was mixed with 3 drops of olive oil, shaken vigorously for uniformly and was then observed for the formation of emulsion. Test for tannin 0.5g of the dried powdered sample was boiled in 20ml of water in a test tube and then filtered. A

few drops of 0.1% ferric chloride was added and observed for brownish green to a blue-black colouration. Presence of brownish green or blue-black colouration conformed the presence of tannin. Test for cardial glycosides 5ml of the extracts were treated with 2ml of glacidal acetic acid containing 1 drop of ferric chloride solution (0.1%). This was under large with 1ml of concentrated H_2 50_4. A browning of the interface suggest a deoxylsugar characteristics of cardenolodes. A violet ring may appear below the broming, while in the acetic layer, a greenish ring may form gradually though out thin lager. Test for phlobatannin An aqueous extract of the plant sample was boiled with 1% aqeous hydrochloric acid and deposition of red precipate was taken as evidence for the presence of phlobatannins. Test for Terpenoids 5ml of each extract was mixed in 2ml chloroform, and concentrated H_2 50_4 9 (3ml) was carefully added for form a layer. A reddish brown colouration of the interface was formed to suggest positive results for the presence of terpenoids. Test for steroids 2ml of acetic anhydride was added to 0.5g ethanolic extract of each of the sample with 2ml H_250_4. The colour changed from violet to blue or green indicating the presence of steroids.

QUANTITATIVE ANALYSIS OF THE CONSTITUENT

Determination of total phenol by spectrophotometer method The fat free sample was boiled with 50ml of ether for the extraction of the phenolic component for 15 minutes. 5ml of the extract was pipetted into a 50ml flask and then 10ml of distilled water was added. 2ml of ammonium hydroxide solution and 5ml of concentrated amylachohol were made up to mark and left to react for 30min for colour development. This was measured at 505nm. Alkaloid determination using harborne (1973) method 5g of the sample was weighed into a 250ml beaker and 200ml of 10% acetic acid in ethanol was added and covered and allowed to stand for 4 hours. This was filtered and the extract was concentrated on a water bath to one quarter of the original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitate was collected and washed with dilute ammonium hydroxide and then filtered. The residue is the alkaloid, which was dried and weighed. Tannin determination by van-burden and robinson (1981) 500mg of the sample was weighed into a 50ml and shaken for 1 hour in a mechanical shaker. This was filtered into a 50ml volumetric flask and made up to the mark. Then 5ml of the filtered was pipitted out into a test tube and mixed with 2ml of 0.1M $FeC1_3$ in 0.1M HC1 and 0.008m potassium Ferrocyanide. The absorbance was measured at 120nm within 10min. Saponin determination The method used was that of Obadoni and Ochuko (2001).

The samples were ground and 20g of each were put into a conical flask and 100cm3 of 20% aqueous ethanol were added. The samples were heated over a hot water bath for 4 hours with continuous stirring at about 55^0C. The mixture was filtered and the residue re-extracted with another 200ml 20% ethanol. The combined extract was reduced to 40ml over water bath at about 90^0C. The concentrate was transferred into a 250ml separatory funnel and 20ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated. 60ml of n-butanol was added. The combined n-butanol extracts were washed twice with 10ml of 55% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation the samples were dried in the oven to constant weight, the saponin content was calculated as percentage. Flavonoid determination by the method of Boham and Kocipaiabyazan (1974) 10g of the plant sample was extracted repeatedly with 100ml of 80% aqueous methanol at room temperature. The whole solution was filtered through Whatman filter paper No. 42 (125mm). The filtrate was later transferred into a crucible and evaporates into dryness over water both and weighed to a constant weight.

HEAVY METAL ANALYSIS

The metals analysed in the crude powdered samples (*Zingiber officinale* and *Centrosema pubescens*) include manganese, Lead, Cadmium, Zinc, Chromium.

Digestion of the samples

About 5g of the air-dried powdered samples was weighed and placed in a 250ml conical flask and gently heated on a hot plate. Heating was then continued until enough water was driven off for partial carbonization to occur. The beaker was then placed in an electric furnace and heated at 555^0C for one hour to ash. The sample was then removed and allowed to cool. A ratio 1:1 mixture of HNO_3/H_2O was prepared, and about 5ml of the mixture was added to the ashed sample, and warned slightly for about 5minutes. The mixture was then filtered for Atomic Absorption Spectroscopy (AAS) using whatman filter papers. The stock standard solution prepared was then used to calibrate the instrument after which the digested sample was introduced into the AAS for analysis.

RESULTS AND DISCUSSION

The results for the antibacterial screening have shown that the ethanolic extracts and not water extracts have antibacterial activity. Investigation in the past has also clearly shown that ethanolic extracts were more effective than water extracts due to some element constituent present which are extracted into ethanol and are absent in water extract (Ibekwe *et al.,* 2001 and Dulta, 1993). They have attributed this observation to the high volatility of Ethanol which tends to extract more active compound from the sample than water, hence, this study follow similar trends. The results of the inhibition of bacterial

Table 1. Zones of inhibition of Ethanolic extracts of *Zingiber officinale* on Tested Organisms

Mean Zones of inhibition (mm)

Tested Organisms	Concentration (mg)/ml			Ciprofloxacin +ve control 100mg/ml	DMF –ve Control 1ml
	50	100	150		
Salmonella typhi	15.0	16.0	20.0	21.0	0.0
Staphylococcus aureus	7.0	10.0	10.0	19.0	0.0
Pseudomonas aeruginosa	5.0	7.0	6.0	24.0	0.0
Escherichia coli	10.0	10.0	12.0	10.0	0.0
		Ketaconazole	+ve control		
Candida albicans	3.0	2.0	2.0	3.0	0.0

growth have shown that the extracts are active at high concentration and inactive at very low concentrations, this is similar to the result of Malu *et al.*(2009). Thus this study may suggest that the inhibition of bacterial growth activity of the extracts is dose dependent. The antibacterial activity and inhibition activity of ginger extracts could be attributed to the chemical properties of ginger. According to O'Hara *et al.,* (1998), the main constituents of ginger are

Table 2. Mean Zones of inhibition of *Zingiber officinale* Aqueous extracts on Tested Organisms

				Mean Zones of inhibition (mm)	
Tested Organisms	Concentration (mg)/ml)			Ciprofloxacin +ve control 100mg/ml	DMF –ve Control 1ml
	50	100	150		
Salmonella typhi	16.0	12.0	7.0	25.0	0.0
Staphylococcus aureus	8.0	5.0	6.0	21.0	0.0
Pseudomonas aeruginosa	1.0	5.0	6.0	11.0	0.0
Escherichia coli	7.0	5.0	1.0	10.0	0.0
	Ketaconazole +ve control				
Candida albicans	1.0	4.0	2.0	4.0	0.0

Table 3. Mean Zones of inhibition of ethanolic extracts of *Centrosema pubescens* on Tested Organisms

				Mean Zones of inhibition (mm)	
Tested Organisms	**Concentration(mg)/ml)**			**Ciprofloxacin +ve control 100mg/ml**	**DMF –ve Control 1ml**
	50	**100**	**150**		
Salmonella typhi	9.0	7.0	9.0	7.0	0.0
Staphylococcus aureus	16.0	10.0	7.0	9.0	0.0
Pseudomonas aeruginosa	10.0	13.0	13.0	20.0	0.0
Escherichia coli	10.0	7.0	9.0	6.0	0.0
	Ketaconazole +ve control				
Candida albicans	5.0	7.0	4.0	10.0	0.0

Table 4. Zones of inhibition of Aqueous extracts of *Centrosema pubescens* on Tested Organisms

				Zones of inhibition (mm)	
Tested Organisms	Concentration (mg)/ml)			Ciprofloxacin +ve control 100mg/ml	DMF –ve Control 1ml
	50	100	150		
Salmonella typhi	6.0	7.0	12.0	15.0	0.0
Staphylococcus aureus	10.0	10.0	10.0	10.0	0.0
Pseudomonas aeruginosa	9.0	9.0	10.0	15.0	0.0
Escherichia coli	6.0	2.0	10.0	15.0	0.0
	Ketaconazole +ve control				
Candida albicans	5.0	7.0	2.0	5.0	0.0

Table 5. The phytochemical compound present in the crude extract of *Zingiber officinale* and Centrosema

Active components	*Zingiber officinale*	*Centrosema pubescens*
Alkaloid	+	+
Saponin	-	-
Flavonoid	-	+
Steroid	+	+
Terpernoids	+	+
Cardiac glycoside	-	+
Tannin	+	+
Phlobatanis	-	-

Table 6. Percentage composition of active ingredients of the crude extracts of *Zingiber officinale* and

Components	*Zingiber officinale* % composition	*Centrosema pubescens* % composition
Saponin	1.60	1.27
Flavonoid	0.08	0.16
Alkaloid	2.07	1.59
Total phenol %	0.19	1.78

Table 7. Concentrations of Heavy metals in *Zingiber officinale* and *Centrosema pubescens*

Metal	***Zingiber officinale***	***Centrosema pubescen***	**Maximum recommended level**
Manganese (Mn)	0.017	0.026	500
Lead (Pb)	ND	0.314	0.3
Cadmium (Cd)	ND	ND	0.2
Zinc (Zn)	0.112	0.032	99.40
Chronium (Cr)	0.016	0.020	2.30

sesquiterpenoids with zingiberene as the main component. Other components include β- sesquiphellandrene, bisabolene and farnesene, which are sesquiterpenoids, and trace monoterpenoid fraction, (β sesquiphellandrene, cineol and citral. Our phytochemical screening revealed the presence of terpenoid, alkaloid, tannin and steroid. The terpenoids are of important in pharmacy due to their relationship with such compounds as vitamin A and could be of immense medical applications. Terpenoids are reactive compounds (Ekam and Ebong, 2007). The extract of rhizomes of *Z. officinale* has been shown to have pronounced inhibitory activities against *Candida albicans*. (Atai *et al.,* 2009) .This result is in good agreement with our investigation which shows that the ginger extract was effective in inhibiting the growth of Candida albicans with zones of inhibition ranging from 2.0–3.0mm, the zones of inhibition of ketaconazole (positive control) was 3.0mm. *Salmonella typhi* was greatly inhibited by *Zingiber officinale* and *Centrosema pubescens* with zones of inhibition ranging from 15.0 – 20.0mm and 7.0 -16.0mm respectively. The most pronounced activity shown by ethanolic extract of *Zingiber officinale* was against Salmonella typhi at concentration 150 mg/ml and inhibition zone of 20.0mm which was close to the inhibition zone of the drug Ciprofloxacin 21.0 mm. The results revealed that the both plant extract have antimicrobial activity against the microorganism tested. The result of this work showed that plant extracts of *Zingiber officinale* and *Centrosema pubescens* inhibited the growth of the bacteria tested (Table 1

to 4), this suggests that the plant extract is broad spectrum in activity. Antimicrobial activity of the plant extracts was observed on Salmonella typhi, Pseudomonas aeruginosa and Staphylococcus auerus at high concentration value, this is similar to the earlier result obtained by (Oladunmoye, 2007). Ethanolic extract of *Centrosema pubescens* had a remarked sensitivity towards *Staphylococcus aureus* with inhibition zones of 16.0mm at 50 mg/ml , this was higher than the inhibition zone of Ciprofloxacin 9.0mm. the inhibition zone was highest for the fungi Candida albicans at 100mg/ml with 7.0mm while the positive control had inhibition zone of 10.0mm,Table 3. The antimicrobial effects of *Zingiber officinale* and *Centrosema pubescens* is due to their Phytochemical constituents present in them. Ginger extracts are rich in phytonutrient such as alkaloids, tannins, steroids, terpernoids and phenolic compounds while *Centrosema pubescens* is rich in alkaloids, tannins, flavonoids, steroids, terpernoids cardiac glycosides and phenolic compound. The biological function of flavonoids which *Centrosema pubescens* contains includes protection against allergies, inflammation, free radicals, platelet aggregation, microbes, ulcer, hetatoxins and tumors (Okwu, 2004). This may be the reason behind the use of extracts of this plant in the treatment of intestinal troubles in herbal medicine (Okwu, 2004). The presence of phenolic compounds in the extract indicates that the plants might be an antimicrobial agent. The presence of tannin in this plants strongly supports their use in treating wounds, burns and hemorrhoids in herbal medicine. The presence of phytochemical components supports it uses as antimicrobial agent. Hence it can be concluded that the ethanolic extract of both plants possess a significant antimicrobial activities. This also stands as a scientific support for the usage of this plant for treating fever in traditional medicine. The phytochemical analysis revealed the presence of Tannin, Flavonoid, steroid, terpernoids, cardiac glycoside, alkaloid and phenol as shown in Table 5 . The percentage constituent of *Zingiber officinale* and *Centrosema pubescens* are shown in Table 6 . The result of heavy metal analysis shows that both plants contains varying levels concentrations of heavy metals (Table 7). The concentration of lead in *Centrosema pubescens* was 0.314ppm which was within the maximum recommended level of 3.0ppm by WHO. Lead was not detected in *Zingiber officinale*. Zinc had concentrations of 0.112ppm and 0.032ppm for *Zingiber officinale* and *Centrosema pubescens* respectively. Concentrations of manganese for *Zingiber officinale* and *Centrosema pubescens* was 0.017ppm and 0.026ppm,maximum recommended level for Mn is 500ppm.

CONCLUSION

The safe level of heavy metals in the plants suggests it is safe for comsumption. Both the ethanolic and aqueous extracts of *Zingiber officinale* and *Centrosema pubescens* contains antibacterial properties which inhibited the growth of the selected pathogens due to the active component they contain. It could be concluded from this study that the extracts from the two plants (*Zingiber officinale* and *Centrosema pubescens*) showed antimicrobial activity against the tested isolates and probably justify its use as antimicrobial agents. Our results suggest the ginger components as promising candidates for development of antifungal agents for topical applications.

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