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Phytochemical Screening, Antifungal and Antibacterial Activity of Aqueous and Ethanolic Leaf and Stem Extracts of *Gnetum africanum* Welw

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ABSTRACT

Aqueous and ethanolic leaf and stem extracts of *Gnetum africanum* were investigated for the presence and composition of these phytochemicals (alkaloid, flavonoid, tannin, phenol, saponin, sterol, terpenoid and cyanogenic glycoside) and their antifungal and antibacterial activities at various concentrations against some selected clinical microbes (fungal strains: *Candida albicans* and *Aspergillus niger* and bacterial strains: *Staphylococcus aureus*, *Escherichia coli* and *Salmonella typhi*) using standard methods. Analysis of variance (ANOVA) was employed in data analysis. Qualitative and percent quantitative phytochemical results showed that both the aqueous and ethanolic leaf and stem extracts contained these phytochemicals assayed but at varied quantities except cyanogenic glycoside. Antifungal and antibacterial studies indicated that both the aqueous and ethanolic leaf and stem extracts of *Gnetum africanum* inhibited the growth of the microbes but at varied levels and the inhibition was extracts concentration dependent. However, both the aqueous and ethanolic leaf and stem extracts showed no inhibition against the bacterial strains at 50 mg mL^{-1} of the extracts. The extracts showed higher inhibition against the fungal strains than the bacterial strains. Inhibitory effect of the leaf extract was significantly higher than those of the stem extract. The ethanol extract showed significantly higher inhibition than the aqueous extract. Antibiotics had a better activity when compared to the extracts at the same concentration. *Gnetum africanum* extracts were biostatic in their action, when purified will give a product with higher activity. The data obtained from the study indicated that the plant possessed antifungal and antibacterial potentials especially antifungal and could be used as natural fungicides.

Key words: Antifungal, antibacterial, ethanol, aqueous, *Gnetum africanum*, fungal strains, bacterial strains

INTRODUCTION

The use of medicinal plants for treatment of microbial diseases is well known and has been documented since ancient times. Medicinal plants have pharmaceutical and antibacterial properties (Bari *et al*., 2010). Plants synthesize many components, which act as defensive agent, helping to protect them from microbial infection and other diseases. Those compounds are bioactive and can be medicinal, intoxicating or toxic depending on circumstance. Several plants species have been tested for antimicrobial properties but vast majorities have not yet been adequately evaluated

(Azoro, 2002). Various studies have been published, investigating the antifungal and antibacterial activities of plant derived compounds against a range of pathogens (Tassou *et al*., 2000; Friedman *et al*., 2002; Momtaz and Abdollahi, 2010; Ara *et al*., 2009; Manikandan *et al*., 2011). Antimicrobial compounds derived from plants might inhibit bacteria through different mechanisms and provide clinical values for the treatment of infection caused by resistant microbes (Stein *et al*., 2005). Different substances have been identified in medicinal plants which are believed to be the antimicrobial agent and these include; different forms of alkaloids, diterpenes, saponins, flavonoids, sterols, quinines, different forms of other proteins as well as lipids (Sofowora, 1993). Evidences have been accumulated to demonstrate the promising potential of medicinal plants used in various traditional, complementary and alternative systems of treatment of human diseases. Antibiotics are one of the most important weapons in fighting bacterial and fungal infections and have greatly benefitted the health related quality of human life, since their introduction. Synthetic antibiotics accumulate in the body causing liver damage and other tissue problems. Such problems are not seen, when natural antibiotics extracted from plants are used. These extracts are safe and potentially effective (Aparadh *et al*., 2012). The increasing prevalence of multidrug resistant strain of bacteria and fungi and recent appearance of strain with reduced susceptibility to antibacterial and antifungal raises the specter of untreated bacterial and fungal infections. In an effort to expand the search for the new antimicrobial agents from natural sources *Gnetum africanum* Welw. of the family Gnetaceae has been evaluated in this study.

Gnetum africanum Welw. is traditionally a wild vine found mainly in the humid tropical forest region of Nigeria, Central African Republic, Cameroon, Gabon, Democratic Republic of the Congo and Angola (Mialoundama, 1993). *Gnetum africanum* has wide variety of uses which ranges from food, feeds, medicines and commercial uses. It is one of the most popular green leafy vegetable in Nigeria and is gaining equal popularity as a delicious food leaf in other African countries. Primarily, *Gnetum africanum* leaves are valued as tasty vegetables when finely shredded and incorporated into soup or stew, or made into condiments or even taken raw as salad (Iweala *et al*., 2009). Nutritionally, *Gnetum africanum* is very rich in proteins and minerals. The leaves contain high nutritional values and these suggest the potential role of this species in the fight against malnutrition. The leaves are added to soups and native salad to promote fertility in women. Both the leaf and the seed have been shown to be effective in the treatment of enlarged spleen, sore throat and cough and as a cathartic in Nigeria (Ndam *et al*., 2000). The antifungal and antibacterial activity of plant extracts is shown to be a function of their phytochemicals composition and *Gnetum africanum* leaf extract has shown to contain these phytochemicals (tannin, flavonoid, Terpenoid, alkaloid, saponin, phenol) (Iweala *et al*., 2009). The objective of this study was to evaluate the phytochemical, antifungal and antibacterial activity of aqueous and ethanolic leaf and stem extracts of *Gnetum africanum* against pathogenic bacteria and fungi to determine their potentials as antifungal and antibacterial agent.

MATERIALS AND METHODS

Collection of plant samples: The plant samples were collected between June-July, 2014 from Umuchu in Aguata local government area, Anambra state. The *Gnetum* species was authenticated at Department of Botany, Nnamdi Azikiwe University, Awka where the voucher specimen was deposited.

Preparation of plant samples: The leaves and stems of *Gnetum africanum* were cut into bits with knife and oven dried at 65°C for 12 h to remove all moisture. The samples were then ground into fine powder.

Extraction

Aqueous extraction: The aqueous extract of the plant was prepared by adding the ground sample of leaf and stem in 100 mL of distilled water. The concentration of each extract was determined by adding 50, 75, 100 and 150 g in 100 mL of distilled water. The experimental set-up was left for 24 h at room temperature and thereafter filtered using No. 1 Whatman filter paper. The extract was then concentrated by heating on water bath to 50 mL of the original volume of the extract.

Ethanol extraction: The ethanolic extract of the plant was prepared by soaking the ground sample of the leaf and stem in 100 mL of ethanol. The concentration of each extract was determined by adding 50, 75, 100 and 150 g in 100 mL of ethanol. The experimental set-up was left for 24 h at room temperature and thereafter filtered using No. 1 Whatman filter paper. The extract was then concentrated to 50 mL of the original volume of the extract and stored in an air tight container in a refrigerator at 4°C until when needed.

Preliminary phytochemical screening: Qualitative phytochemical screening of the extracts was conducted to determine the presence of phytochemicals such as tannins, saponins, flavonoids, alkaloids, sterols, phenols and cyanogenic glycoside. This was done using standard procedure as described by Harborne (1973).

Test microorganisms: The following microorganisms: Bacterial strains *Staphylococcus aureus* (NR 201), *Escherichia coli* (NR 202), *Salmonella typhi* (NR 204) and Fungal strain *Aspergillus niger* (NR 241) and *Candida albican* (NR 242), were collected based on their clinical and pharmacological importance.

Sources of test microorganisms: The pure cultures of the microorganisms were obtained from the pathology Department of National Root Crop Research Institute, Umudike, Abia State. The isolates were checked for purity and are maintained on nutrient broth at 4°C in the refrigerator until when required.

Antimicrobial activity: The zone of inhibition of the extracts was determined using agar diffusion method as described by ICMSF (1998). Both bacteria and fungi pathogen were grown first in nutritional bath before use. The microorganisms were later sub-cultured in Mueller Hinton Agar. Wells were then bored into the agar medium using a sterile 6 mm cork borer. The wells were then filled up with 0.02 mL of the extract and care was taken not to allow the solution to spill on the surface of the medium. The plates were allowed to stand on the laboratory bench for between 1-2 h for proper absorption of the solution into the medium. The plates were turned inside upside down and the wells labelled with a marker. The plates were incubated aerobically at 37°C for 24 h. Sensitivity of the organisms to the extract was recorded by measuring the zone of inhibition. The extent of inhibition was expressed in terms of the diameter of the inhibition zone as measured with a transparent meter rule. The effects of the extracts on bacteria and fungi pathogens were compared with those of the standard antibiotic ampicillin fungabacter for bacteria and fungi as standard control, respectively.

Statistical analysis: The results were analyzed using ANOVA. The Duncan's multiple range test significance was use to test the difference among treatments. All analyses were carried out at 5% level of significance.

RESULTS

The results of the study were shown in Table 1-6 and Fig. 1. Qualitative phytochemical screening of leaf and stem extracts of *Gnetum africanum* in aqueous and ethanolic solvents is presented in Table 1. It was found from the result that all phytochemical (alkaloid, flavonoid, phenol, saponin, sterol, terpenoid and cyanogenic glycoside) assayed except cyanogenic glycoside was present in both solvent extracts of *Gnetum africanum*.

Table 1: Qualitative phytochemical screening of aqueous and ethanol stem and leaf extracts of *Gnetum africanum*

	Phytochemicals							
Solvents extracts	Alkaloid	Flavonoid	Saponin	Phenol	Sterol tannin	Terpenoid	Cyanogenic	Glycoside
Aqueous extracts								
Leaf	+	+	+	+	+	+	+	-
Stem	+	+	+	+	+	+	+	-
Ethanol extracts								
Leaf	+	+	+	+	+	+	+	-
Stem	+	+	+	+	+	+	+	-

Positive +ve: Presence and Negative -ve: Absence

Table 2: Percent Quantitative phytochemical screening of aqueous and ethanolic stem and leaf extracts of *Gnetum africanum*

	Phytochemicals						
Solvents extracts	Alkaloid	Flavonoid	Saponin	Phenol	Sterol tannin	Terpenoid	Cyanogenic
Aqueous extracts							
Leaf	0.59±0.00^{c}	0.33±0.01^{b}	0.61±0.02^{b}	0.02±0.00^{a}	0.01±0.00^{b}	0.49±0.01^{c}	0.20±0.00^{b}
Stem	0.54±0.00^{b}	0.27±0.02^{a}	0.48±0.00^{a}	0.03±0.00^{b}	0.01±0.00^{a}	0.18±0.01^{b}	0.13±0.00^{a}
Ethanol extracts							
Leaf	0.60±0.00^{d}	0.35 0.01^{b}	0.64±0.02^{b}	0.02±0.00^{a}	0.01±0.00^{c}	0.51±0.01^{c}	0.25±0.01^{c}
Stem	0.49±0.00^{a}	0.31±0.02^{ab}	0.52±0.00^{a}	0.03±0.00^{b}	0.01±0.00^{ab}	0.14±0.00^{a}	0.18±0.02^{b}

Results are in Mean±Standard Deviation, *Columns with the same superscript are not significantly different, **There is significant difference ($p<0.05$)

Table 3: Antibacterial and antifungal activity of aqueous and ethanol extracts of *Gnetum africanum* at 50 g/100 mL of the extracts (zone of inhibition)

	Zone of inhibition of microbes (mm)*					
	Aqueous extracts			Ethanol extracts		
Microorganisms	Stem	Leaf	Control	Stem	Leaf	Control
Staphylococcus aureus	-	-	12.58±0.18	-	-	12.58±0.18
Salmonella typhi	-	-	8.46±0.06	-	-	8.15±0.06
Escherichia coli	-	-	9.17±0.01	-	-	9.17±0.01
Candida albican	0.85±0.00^{a}	1.34±0.00^{b}	14.60±0.14^{c}	1.16±0.00^{a}	1.79±0.01^{b}	14.60±0.14^{c}
Aspergillus niger	1.06±0.00^{a}	1.82±0.00^{b}	15.67±0.020^{c}	1.78±0.03^{a}	2.37±0.04^{b}	15.67±0.02^{c}

Result values are in Mean±Standard Deviation, *Columns followed by the same superscript are not significantly different, **Significant difference exist $p<0.05$, Control: Ampicillin and fungabacter for bacteria and fungi, respectively

Table 4: Antibacterial and antifungal activity of aqueous and ethanol extracts of *Gnetum africanum* at 75 g/100 mL of the extracts (zone of inhibition)

	Zone of inhibition of microbes (mm)*					
	Aqueous extracts			Ethanol extracts		
Microorganisms	Stem	Leaf	Control	Stem	Leaf	Control
Staphylococcus aureus	2.41±0.01^{a}	3.44±0.02^{b}	15.56±0.05^{c}	3.44±0.05^{a}	3.54±0.02^{a}	15.56±0.05^{b}
Salmonella typhi	2.79±0.01^{a}	3.88±0.03^{b}	11.30±0.00^{c}	2.37±0.04^{a}	4.31±0.01^{b}	11.30±0.00^{c}
Escherichia coli	3.41±0.01^{a}	3.61±0.01^{a}	12.62±0.11^{b}	2.62±0.12^{a}	3.64±0.02^{b}	12.62±0.11^{c}
Candida albican	4.76±0.06^{a}	5.52±0.00^{b}	17.87±0.10^{c}	5.41±0.01^{a}	6.72±0.03^{b}	17.87±0.10^{c}
Aspergillus niger	5.22±0.03^{a}	6.66±0.06^{b}	19.32±0.12^{c}	5.71±0.13^{a}	7.63±0.04^{b}	19.32±0.12^{c}

Result values are in Mean±Standard Deviation, *Columns followed by the same superscript are not significantly different, ** Significant difference exist $p<0.05$, Control: Ampicillin and fungabacter for bacteria and fungi, respectively

Table 5: Antibacterial and antifungal activity of aqueous and ethanol leaf and stem extracts of *Gnetum africanum* at 100 g/100 mL of the extracts (zone of inhibition)

	Zone of inhibition of microbes (mm)*					
	Aqueous extracts			Ethanol extracts		
Microorganisms	Stem	Leaf	Control	Stem	Leaf	Control
Staphylococcus aureus	5.32±0.02^{a}	6.23±0.03^{b}	18.30±0.14^{c}	5.77±0.04^{a}	6.63±0.24^{b}	18.30±0.14^{c}
Salmonella typhi	4.63±0.24^{a}	5.66±0.05^{b}	15.47±0.02^{c}	4.81±0.01^{a}	7.29±0.01^{b}	15.47±0.02^{c}
Escherichia coli	5.43±0.03^{a}	5.53±0.04^{b}	17.60±0.00^{c}	4.66±0.05^{a}	6.32±0.05^{b}	17.60±0.00^{c}
Candida albican	8.62±0.00^{b}	8.16±0.00^{a}	21.77±0.04^{c}	7.53±0.10^{a}	9.61±0.01^{b}	21.77±0.04^{c}
Aspergillus niger	8.53±0.24^{a}	9.61±0.01^{b}	23.43±0.06^{c}	7.82±0.02^{a}	10.51±0.16^{b}	23.43±0.04^{c}

Result values are in Mean±Standard Deviation, *Columns followed by the same superscript are not significantly different, ** Significant difference exist p<0.05, Control: Ampicillin and fungabacter for bacteria and fungi, respectively

Table 6: Antibacterial and antifungal activity of aqueous and ethanol leaf and stem extracts of *Gnetum africanum* at 150 g/100 mL of the extracts (zone of inhibition)

	Zone of inhibition of microbes (mm)*					
	Aqueous extracts			Ethanol extracts		
Microorganisms	Stem	Leaf	Control	Stem	Leaf	Control
Staphylococcus aureus	7.75±0.07^{a}	9.61±0.01^{b}	21.53±0.10^{c}	7.78±0.03^{a}	9.77±0.09^{b}	21.53±0.10^{c}
Salmonella typhi	7.75±0.00^{a}	8.76±0.05^{b}	19.39±0.00^{c}	6.81±0.01^{a}	10.56±0.06^{b}	19.39±0.01^{c}
Escherichia coli	7.61±0.01^{a}	8.88±0.03^{b}	20.81±0.16^{c}	6.89±0.07^{a}	9.60±0.00^{b}	20.81±0.16^{c}
Candida albican	10.56±0.08^{a}	11.62±0.00^{b}	23.63±0.24^{c}	9.71±0.10^{a}	12.58±0.31^{b}	23.63±0.24^{c}
Aspergillus niger	10.77±0.04^{a}	11.57±0.09^{b}	24.56±0.09^{c}	9.29±0.01^{a}	12.43±0.04^{b}	24.56±0.09^{c}

Result values are in Mean±Standard Deviation, *Columns followed by the same superscript are not significantly different, ** Significant difference exist p<0.05, Control: Ampicillin and fungabacter for bacteria and fungi, respectively

Fig. 1(a-b): *Gnetum africanum* in it natural habitat

Quantitative phytochemical screening of leaf and stem extracts of *Gnetum africanum* in aqueous and ethanolic solvents is presented in Table 2. The table shows that the mean alkaloid, flavonoid, tannin, saponin and terpenoid composition is highest in ethanol extract of the leaf. Ethanol extract of the stem contain the lowest composition of alkaloid and tannin, while the aqueous extract of the stem contain the lowest composition of flavonoid, saponin and terpenoid. The aqueous and ethanolic extract of the leaf and stem contain significantly equal composition of sterol and phenol. Generally, the leaf extract has higher composition of all the phytochemical assayed except in sterol and phenol, where it was statistically at par with the stem. Ethanol solvent gave higher yield of most phytochemicals assayed.

Antibacterial and antifungal results: Antibacterial and antifungal results are presented in Table 3-6. Antibacterial and antifungal activity of aqueous and ethanol extracts (Leaf and Stem) of *Gnetum africanum* were studied at different concentrations (50, 75, 100, 150 g/100 mL) against three pathogenic bacterial strains (*Staphylococcus aureus, Escherichia coli*, *Salmonella typhi*) and two fungal strains (*Aspergillus niger* and *Candida albican*). Antibacterial and antifungal potentials of extracts were assessed in terms of zone of inhibition of microorganisms' growth.

Table 3 indicates that both the aqueous and ethanolic leaf and stem extract of *Gnetum africanum* at 50 g/100 mL showed inhibitory effect against the fungal strains (*C. albicans* and *A. niger*) but had no inhibition on the bacterial strains (*S. aureus, S. typhi* and *E. coli*). However, the aqueous and ethanolic leaf extract showed significantly higher inhibition than the stem. Similarly, in comparison with the control, the inhibition is significantly higher in the control than in plant extract (both aqueous and ethanol).

As shown in Table 4, both the aqueous and ethanolic leaf and stem extracts of *Gnetum africanum* at 75 g/100 mL showed anti bacterial and antifungal activity. The leaf showed significantly higher inhibitory effect against the microbes in both the aqueous and ethanol extract than the stem. However, in comparison with the control, the inhibition of the microbes is significantly higher in the control than in plant extract (both aqueous and ethanol). Generally, the susceptibility of *S. aureus*, *C. albicans* and *A. niger* were higher in the ethanol extract while the susceptibility of *E. coli* was higher in the aqueous extract.

The result in Table 5 indicates that, both the aqueous and ethanolic leaf and stem extracts of *Gnetum africanum* at 100 g/100 mL showed anti bacterial and antifungal activity. The leaf showed significantly higher inhibition against all microbes in both the aqueous and ethanol extract than the stem except in *C. albican*, where the aqueous extract of the stem showed higher inhibition than the leaf. However, in comparison with the control, the inhibition of the microbes is significantly higher in the control than in plant extract (both aqueous and ethanol). Generally, the susceptibility of microbes was higher in the ethanol extract than in the aqueous extract.

Table 6 indicates that the aqueous and ethanolic leaf and stem extracts of *Gnetum africanum* at 150 g/100 mL showed both anti bacterial and antifungal activity. The leaf showed significantly higher inhibition against all microbes in both the aqueous and ethanol extract than the stem. However, in comparison with the control, the inhibition of the microbes is significantly higher in the control than in plant extract (both aqueous and ethanol). The susceptibility of microbes was higher in the ethanol extract than in the aqueous extract except in *Aspergillus niger*, where the reverse was the case.

DISCUSSION

Phytochemical screening revealed that both aqueous and ethanolic leaf and stem extracts of *Gnetum africanum* contained all the phytochemical assayed (alkaloid, tannin, saponin, sterol, flavonoid, Terpenoid and cyanogenic glycoside) except cyanogenic glycoside (Table 1-2). The phytochemicals were present varied quantities. Tannins are astringent in taste and help in healing of wounds and inflamed mucous membrane (Njoku and Akumefula, 2007). Tannins is potential metal ion chelator, proton precipitating agents and biological antioxidant (Okonkwo, 2009). Flavonoids are most commonly known for their antioxidant activity and act as transformers which modify the body's reactions to carcinogens, viruses and allergens. They possess anti-cancerous, anti-inflammatory, anti-microbial and anti-allergic activity (Balch and Balch, 2000) and may, therefore be useful in therapeutic roles (Jisaka *et al*., 1992). Terpenoids are antifungal and

antibacterial which is attributed to their membrane disruption action and inhibitory action on bacterial cell or fungus (Cichewicz and Thorpe, 1996). Many alkaloids for example are known to have effect on the central nervous system and act as antipyretic such as morphine, a painkiller. Similarly, Saponins which are a special class of glycosides have been found to possess antifungal activity (Ogu *et al*., 2012). Saponins have been reported to have a wide range of pharmacological and medicinal activities. Interestingly, they have been indicated to usually have low oral toxicity in humans (Sparg *et al.,* 2004). Plants containing saponins are used to heal wounds (Okwu and Josiah, 2006) because saponins have the ability to precipitate and coagulate Red Blood Cells (RBCs) (Sood *et al*., 2012). Sterols have been used in medicine to treat variety of conditions ranging from endocrine hormonal alteration to coronary insufficiency (Clifford *et al*., 1973). Phenols are known to inhibit the mutagenity of cell DNA and neutralize free radicals (Heinonen *et al.,* 1998). They also function as antimicrobial compounds produced by some plants to protect them from pathogens.

The leaf extract showed significantly higher composition of most chemicals assayed (alkaloid, flavonoid, tannin, sterol, saponin and terpenoid) than the stem and therefore could serve as better source of these compounds for medicinal purposes. The result showed that the ethanol extract had the highest yield of most of the compounds (flavonoid, tannin, sterol, phenol, saponin and terpenoid) while aqueous extract had only the highest yield of alkaloid (Table 2). This could be attributed to the fact that ethanol although classified as a polar solvent, is not very polar as water (Cheremisinoff, 2003). This shows that ethanol solvent will be miscible in water and will extract mostly the ionic compounds from *Gnetum africanum* than water.

Antibacterial and antifungal activity indicated that, both the aqueous and ethanolic stem and leaf extracts of *Gnetum africanum* all showed antibacterial and antifungal activity except at 50 g/100 mL, where the extracts showed no antibacterial activity and the inhibition was extract concentration dependent. This could be attributed to the presence of chemical compounds (alkaloid, flavonoid, tannin, sterol, saponin and terpenoid) in the extracts (Table 3-6). These phytochemicals are known to have medicinal properties. However, the leaf extract showed higher antibacterial and antifungal activity against the microbes than the stem extract. This according to Hassan *et al.* (2009) could be attributed to presence of higher bioactive compounds in leaf extracts. Furthermore, the sensitivity and susceptibility of the microbes to the plant extracts varied. In particular, the fungal strains were highly sensitive and susceptible to the plant extracts than the bacterial strains. The difference according to Ogu *et al. (*2012) is due to the fact that gram positive bacteria such as *Escherichia coli* develop resistant to inhibition caused by plant extract.

The ethanol and aqueous extracts inhibition against the test microbes varied significantly. However, the activity of the ethanol was more effective when compared to that of the aqueous. This could be attributed to the fact that ethanol have a better dissolving capacity than water (Cheremisinoff, 2003).

CONCLUSION

Microbial resistance is a world concerned problem. Efforts are being made to discover new antimicrobial agents from various sources. Constant research investigations may result in the discovery of novel effective agents. This study revealed that the plant extracts possessed bioactive compounds that have antibacterial and antifungal activities against some human pathogens, which justified their use in ethnomedicine for treatment of infectious diseases. The ethanol extract showed significantly higher inhibition than the aqueous extract in all concentrations except at

75 g/100 mL where the aqueous extract showed higher inhibition against *E. coli* and at 150 g/100 mL where it also had higher inhibition against *A. niger*. Furthermore, both solvent extracts showed no inhibition against bacterial strains at 50 g/100 mL of the plant extracts. The *Gnetum africanum* extracts both showed antibacterial and antifungal activities however, the leaf extract showed better inhibition than the stem extract indicating that it is a better antimicrobial agent than the stem. The data obtained from the study indicated that the plant possessed antibacterial and antifungal potentials especially antifungal.

Furthermore, before use in human being isolation of pure compound, toxicological study and pharmacological activity should be carried out thereafter.

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