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NANOENCAPSULATED EXTRACT FROM *LEPTADENIA HASTATA* (PERS.) DECNE LEAF EXHIBITED ANTIMICROBIAL POTENTIALS AGAINST *ASPERGILLUS NIGER* ATCC 11414

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ABSTRACT

Leptadenia hastata is a leafy liana vegetable plant found in northern Nigeria. Its whole parts have been used in ethnomedicine to manage hypertension, diabetes, nasal congestion, skin infections and other diseases. Methanol and aqueous extracts of *Leptadenia hastata* were screened for the presence some metabolites while, chitosan nanoparticles loaded with *Leptadenia hastata* leaf extracts were tested against a fungus *Aspergillus niger* ATCC 11414 *in vitro* to determine the diameter zones of inhibition at concentrations of 0.5, 1, 2, 4 and 8µg/mL using agar well diffusion. The results showed that the leaf extracts contained carbohydrates, flavonoids, alkaloids, tannins and cardiac glycosides while saponins were not detected. Nanoparticulate drug delivery of extracts and standard antifungal drug fluconazole showed that chitosan nanoparticle encapsulated drugs inhibited the growth of the fungus in concentration dependent fashion after 72h incubation. These results were significantly different from extracts and drug that were not encapsulated in chitosan nanoparticles. The study therefore showed that chitosan encapsulated antifungal agents displayed potent antimicrobial activity against *Aspergillus niger* ATCC 11414. Hence, nanoencapsulated *Leptadenia hastata* leaf extract possessed great antifungal activity against resistance *Aspergillus niger* ATCC 11414. Thus, our research revealed an alternate therapeutic measure against the fungus.

KEYWORDS

Chitosan nanoparticles, *Leptadenia hastata*, Metabolites, *Aspergillus niger* ATCC 11414 and Nanoencapsulation.

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INTRODUCTION

Chitosan nanoparticle is a bio-degradable, bio-compatible microsphere affirmed as being safe for human nutritious uses and used for treating wounds. It has been used as a carrier as polymeric nanoparticulate drug delivery via various routes of administration. Chitosan has chemical functional groups that can be modified to achieve specific goals, thus making it a polymer with an amazing range of potential applications. It is the most

important derivatives of chitin which is derived from crustacean shells such as those from prawns or crabs, as well as from the cell walls of fungi. It is produced by removing the acetate moiety from chitin. (Munawar *et al*, 2017)[1].

Aspergillus niger is a fungus which belongs to the family *Trichocomaceae* which reproduces by conidiophores. It is a type of mould which can cause pneumonia sometimes. It has been reported to also cause black mouldon certain foods like breads, flour, tomatoes, onions, grapes and some vegetables. Some strains of *Aspergillus niger* are known to secrete mycotoxins which can result in nephrotoxicity and renal tumours in animals as well as potentially hazardous to human health through their consumption. This is why *Aspergillus Niger* was referred to food spoilage microbe (Hinton-Sheley, 2018)[2].

Leptadenia hastata (Pers) Decne, (Asclepidiaceae) is a leafy vegetable plant. It is a liana with a pale white, soft and grooved stem. Leaves are simple acuminate and ovate shaped and exude white latex when crushed. Flowers are green or yellowish green and fruits are dehiscent, two-halved releasing cotton winged seeds. (Burkill, 1985[3], Dalziel *et al*, 1984[4]). It is widely distriuted in tropical Africa including northern Nigeria (Jansen, 2004)[5]. The plant is locally known in Nigeria as "Yaadiyaa", "Dan Bakwa" (in Hausa), "kalimbo", "Njera" (in Kanuri), "*Isanaje*" (in Igbo) and "*Ogbofunfun*" (in Yoruba). In Nigeria, the plant is used as a spice and in preparation of sauces (Ibrahim *et al*, 2012)[6] and considered as a famine food in some part of Africa (Freiberger *et al*, 1998[7], Jansen, 2004[5]).

The plant has beenused in the Nigerian ethnomedicine to manage hypertension, catarrh and skin diseases. (Dan-Batta and Aliyu, 2011)[8], gonorrhoea and stomach ache in children (Tamboura *et al*, 2005)[9]. Pharmacological studies have shown that the plant has antimicrobial (Aliero and Wara, 2009)[10], antiandrogenic (Bayala *et al*, 2011)[11], anti-inflammatory and wound healing (Nikiema *et al*, 2001)[12] and anti-diabetic activities. It was reported to contain compounds such as lutein, lupeol, β-carotein, kidjolanin, cyananforid and gagaminin (Aquino *et al*, 1996[13], Bello *et al*, 2011[14]).

MATERIAL AND METHODS

Collection and identification of plant material

The leaves of *Leptadenia hastate* were freshly collected from the Mohamed Lawan's College of Agriculture farm Maiduguri on the early hours of 22nd January, 2020 and authenticated with the herbarium voucher number UMM/FPH/APN/003 deposited at the herbarium of the Department of Pharmacognosy, Faculty of pharmacy, University of Maiduguri.

Preparation of plant material

The leaves of *Leptadenia hastata* collected were washed using tap water and shade dried at room temperature for two weeks. The dried leaves were ground into fine powder using a wooden pestle and mortar.

Extraction of plant material

The powdered leaf was weighed and subjected to cold maceration technique with methanol for 24h. After extraction with methanol, the marc was then subjected to another extraction using water to obtain the aqueous fraction.

Preliminary phytochemical screening of extract from various solvents

Methanol and water extracts of *Leptadenia hastata* were used for the assays. The preliminary phytochemical analysis of the leaves extract was carried out according to standard method of Trease and Evans (2006)[15].

Test for carbohydrates

a) *Molisch's* test

Extract was treated with few drops of alcoholic alpha naphthol and 0.2mL of conc. Sulphuric acid was added slowly along the sides of the test tube. A purple to violet colour ring was observed at the junction.

b) *Fehling's* test

The Fehling A and Fehling B solutions were mixed and few drops of extract was added and boiled. A brick-red coloured precipitate of cuprous oxide formed was observed thus confirms the presence of carbohydrates.

c) *Barfoed's* test

1mL of aqueous filtrate of the extract was mixed with 1mL of Barfoed`s reagent in a test tube. The test tube was then heated on a water bath for a few minutes. A red precipitate of cuprous oxide indicated the presence of a monosaccharide sugar.

Test for cardiac glycosides

a) *Salkowski test for steroidal nucleus*

Small amount of the two extract was dissolved separately in 2mL chloroform each followed by the addition of conc. H_2So_4 to form a layer. A reddish-brown colour at the interphase indicates the presence of a steroidal nucleus (Sofowora, 1993)[16].

b) *Keller-Killiani* test

In 2mL of each of the plant extracts (methanol and aqueous) a glacial acetic acid one drop of 5% $FeCl_3$ and conc. H_2SO_4 were added. Reddish brown colour appears at the junction of the two liquid layers and upper layer appear bluish green, confirming the presence of glycosides.

c) *Libermann-Buchard`s* test

Using a portion of the extract transfer into test tube, acetic anhydride was added. In a slanting position add conc. H_2SO_4 so that it will enter by the wall of the test tube. A reddish layer at the interphase and a greenish or light green upper layer is responsible for the steroid nucleus.

Test for tannins

a) *Ferric-chloride* test

About 5mL of distilled water was added to the extract and boiled on the water bath for about two min and then filtered. To 2mL of the filtrate, few drops 10% alcoholic ferric chloride solution were added. Effervescence occurred and the dark brown solution changed to green, blue to violet coloration indicates the presence of phenolic group (OH group).

b) *Lead acetate* test

A mixture of equal volume 10% lead acetate was observed indicating the presence of tannins (Sofowora, 1993[16], Trease and Evans, 2002[15]).

Test for flavonoids

a) *Shinoda`s* test

Magnesium chip weighing 0.1g and a few drops of conc. HCl are added to the extract dissolved in ethanol, warmed and then filtered. The reaction mixture gives a rose red coloration or red to purple colour, indicating the presence of flavonoids.

b) *Sodium hydroxide* test

2mL of the filtrate extract was dissolved in 10% NaOH of the solution to give a yellow colour. A change in colour from yellow to colourless on addition of dilute hydrochloric acid indicates the presence of flavonoids.

Test for alkaloids

a) *Dragendorff's reagent*

2mL of the extract was stirred with 5mL of 1% aqueous HCl on water and filtered. The filtrate was divided into two portions. To filtrate A, few drops Dragendorff's solution was added. The presence of orange red precipitate indicates the presence of alkaloids.

b) *Wagner's reagent*

To portion B, add few drops Wagner`s reagent. The formation of reddish-brown precipitate indicates the presence of alkaloids.

Test for saponins

A small quantity of methanol and aqueous leaves extract of *Leptadenia hastata* was shaken with water in a test tube. A honey comb froth which persists on standing was taken as preliminary evidence for the presence of saponins.

Preparation of chitosan nanoparticles (CSNPs) loaded *Leptadenia hastata* leaf extract

Chitosan NPs encapsulated *Leptadenia hastata* leaf extract was prepared by spray drying method. Briefly, 1g of methanol and aqueous extract was dissolved in 10mL deionized water. Similarly, 2g of chitosan power (90+ % (100 G deacetylated) made from shrimp shells (Chem Savers Inc, USA), was accurately weighed on an analytical balance and transferred into a clean 500mL beaker and dissolved with 20mL deionized water. The dissolved extract was added to the beaker containing the dissolved chitosan powder under a constant stirring at 3000rpm for 2h. Glutarylaldehyde (GA) solution 500µLwas added to the mixture in drop wise using a pipette. The above procedure was followed in preparation for both the methanol and aqueous extracts. The resulting mixtures were subjected to

dry in an oven over night at a controlled temperature at 45ºC. CSNPs without any plant extract was used as control, while the characterization of the CSNPs was previously described (Ukwubile *et al*, 2021)[17].

Antimicrobial evaluation of extracts

***Aseptic* techniques**

All work surfaces were mopped with moist rag and disinfected with cotton wool socked in dettol (disinfectant) to prevent contamination during the process a hot air oven was used to sterilize the conical flasks, forceps, wire loop and beakers at 160ºC for 45 min. Every material used in this stage were adequately sterilized using an autoclave. Materials such as glass wares were properly washed with detergent and water to remove dirt and contaminants and were allowed to dry prior to usage. These materials were then sterilized in portable laboratory autoclave at 120ºC for 15 min (Umaru *et al*, 2018)[18].

Preparation of nutrientagar culturing plates

This was carried out by dissolving 28g of nutrient agar in 1L of distilled water and then covered with aluminium foil. The media was boiled to dissolution and sterilized at 121ºC for 15 min. The media was allowed to cool to 45ºC and 20mL of the sterilized medium was poured into the sterile petri dish and allowed to cool and solidify. The plates were dried at 37ºC for 30min. The microbes were spread evenly over the surface of the medium using separate sterile swab stick for each of the microorganisms (Umaru *et al*, 2018)[18].

Antimicrobial screening of *Leptadenia hastate* leaf extracts by disc diffusion assay

Each of the extracts was dissolve in 10mL sterile distilled water to make an initial concentration of 1000mg/mL. These were then serially diluted using 1:10 to produce concentrations of 8.0µg/mL, 4.0µg/mL, 2.0µg/mL, 1.0µg/mL and 0.5µg/mL. Blank disc of 6mm diameter was immersed in various concentration of the extracts and placed in five positions on the plate with another one immersed in the standard drugs. The fungus plates was incubated at 37ºC for 72 h, before the growth was observed. The diameter zones of inhibition were measured using a transparent ruler and results recorded in millimetres. Sterile distilled water was used as negative control. The experiment was done in triplicate (Umaru *et al*, 2018[18], Ram *et al*, 2010[19]).

Statistical analysis

Raw data obtained were subjected to analysis of variance (one-way ANOVA) to determine the level of significance between treated and control experiment, while the variations in means were determined using Duncan multiple range test (DMRT). The value of $p \leq 0.5$ was taken as statistical significance analysed by Jamovi 2 statistical software.

RESULTS AND DISCUSSION

The result in Table No.1 showed the aqueous extract (AE) extracted more of the metabolites than the methanol extract (ME). Metabolites such as carbohydrates, flavonoids, alkaloids, tannins and cardiac glycosides were detected in large amount while saponins were not detected in the AE. These metabolites unarguably were responsible for most of the biological activities of the leaf of *Leptadenia hastata*.

For instance, plant metabolites have received an increasing attention because of interesting new discoveries from their biological activities (Cho *et al*, 2003)[20]. Similarly, flavonoids have been reported to possess anticancer, antioxidant and antimicrobial potentials by dissolving the cell membranes of most pathogens while saponins were used as sapogenins as arrow poisons. Some of these roles played by these metabolites were further confirmed from our study especially antifungal activity against *Aspergillus niger*. It is possible that these metabolites were achieve their therapeutic action of the fungus by binding to the protein receptors on its surface resulting in fungicidal effects as seen from the study (Isaac *et al*, 2018[21], Kauffman and Carver, 2008[22]).

Aspergillus niger have been known to cause a fungal disease called aspergillosis. Other types of health complications caused by this fungus as body allergies, lung inflammation and organ infections.

Because of resistance posed by the fungus, the treatment is usually difficult using the current chemotherapeutics. For example, the treatment of invasive aspergillosis and chronic necrotizing pulmonary aspergillosis requires intravenous antifungal therapy.

Voriconazole is usually first-line therapy, sometimes in combination with other agents, like caspofungin. Similarly, triazole antifungal agent, is avuconazole, is also indicated for invasive aspergillosis. Amphotericin may sometimes be prescribed in treatment failures. Moreso, allergic bronchopulmonary aspergillosis (ABPA) is a hypersensitivity reaction treated with corticosteroids. The addition of oral antifungal therapy with itraconazole may be beneficial in the management of ABPA. Despite of these treatment measures, none have been to effectively remove the infections by this mold. From the current study, chitosan nanoparticulate encapsulated *Leptadenia hastata* leaf extract greatly inhibited the growth of *Aspergillus niger* when compared with the standard drug fluconazole ($p \leq 0.05$; one-way ANOVA). From our study also, nanoecapsulation of fluconazole produced the highest diameter zone of inhibition against the fungi at various concentrations (Table No.2, Figure No.1). Chitosan loaded plant extracts also showed higher diameter zones of inhibitions against the fungus than extracts delivered ordinary in concentration and time dependent fashion (Figure No.1 and Figure No.2).

Fluco (standard drug fluconazole), MECSNPs (chitosan-loaded *Leptadenia hastata* methanol extract), AECSNPs (chitosan-loaded *Leptadenia hastata* aqueous extract), ME (methanol extract of *Leptadenia hastata* leaf), AE (aqueous extract of *Leptadenia hastata* leaf), Fu/CS (chitosan loaded fluconazole), CSNPs (chitosan nanoparticles).

Table No.1: Phytochemical constituents of methanol and aqueous leaf extracts of *Leptadenia hastate*

S.No	Phytochemical constituents	Test	Observation		Inference	
			ME	AE	ME	AE
1	Carbohydrate	Molisch's	Purple Color	Purple Colour	++	++
		Fehling's	Brick Red Color	Brick Red Color	++	++
2	Flavonoids	Shinoda's	Red Colour	Red Colour	+	++
		NaOH	Yellow Colour	Yellow Colour	+	++
3	Alkaloids	Mayer's	Orange Colour	Orange Colour	+	++
		Wagner's	Red Colour	Red Colour	+	++
4	Saponins	Frothing	No Frothing	No Frothing	—	—
5	Tannins	$FeCl_3$	Blue Colour	Blue Colour	++	++
		Lead Acetate	Blue Colour	Blue Colour	++	++
6	Cardiac Glycoside	Salkowoski	Red Colour	Red Colour	++	++
		Keller-Kelliani	Red Colour	Red Colour	++	++

ME (methanol extract), AE (aqueous extract), + (detected in small amount), + + (detected in large amount), - (not detected).

Table No.2: Antimicrobial activity of extracts of *Leptadenia hastate* leaf and drug-loaded chitosan NPs on *Aspergillus niger* ATCC 11414

S.No		Concentration Diameter Zone of Inhibition (mm) (µg/mL)						
		Flu	MECSNPs	AECSNPs	ME	AE	FuCS	CSNPs
1	0.5	8	10	10	0	8	20	0
2	1.0	14	13	15	8	12	30	8
3	2.0	20	20	22	14	15	28	8
4	4.0	23	23	24	15	15	30	8
5	8.0	25	35	30	15	15	35	10

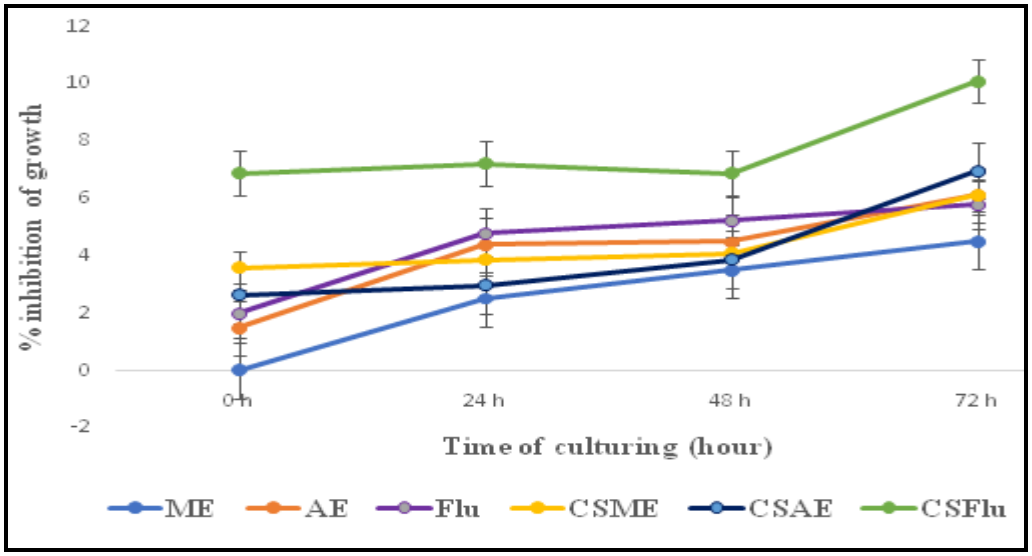

Figure No.1: % fungal growth inhibition at different concentrations from 0-72 hours. ME (methanol extract), AE (aqueous extract), Flu (fluconazole), CSME (chitosan loaded with ME), CSAE (chitosan loaded with AE), CSFlu (chitosan loaded with flu)

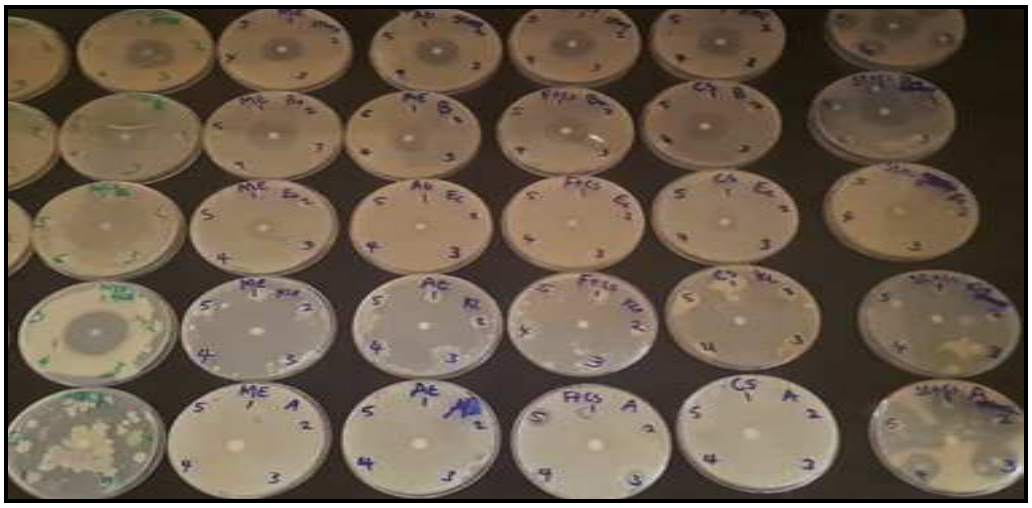

Figure No.2: Culturing petri dishes for *Aspergillus niger ATCC 11414* after 72 h

CONCLUSION

Our study showed that nanoencapsulated drugs reduced significantly the growth of *Aspergillus niger ATCC* 11414 at different concentrations in concentration-dependent fashion and time. The study further showed that chitosan nanoparticulate delivery of antifungal drugs is very promising and safe. This aspect of research should be further be extended to other pathogenic fungi for comparing results.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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