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ANTIFUNGAL AND PHYTOCHEMICAL CONSTITUENTS STUDY OF CLOVE OIL FROM *SYZYGIUM AROMATICUM*

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ABSTRACT

Introduction: Phytochemical screening and antimicrobial sensitivity of clove flower (*Syzygium aromaticum*) was studied. **Materials and methods:** Clove oil from *Syzygium aromaticum* showed strong activity against all the tested fungal isolates at various concentrations of 100mg/ml, 50mg/ml, 25mg/ml, 12.5mg/ml and 6.25mg/ml. The tested fungal isolates were *Aspergillus niger* and *Candida albicans*. **Results:** All the isolates were sensitive to the aqueous extract of Cloves flower (*S. aromaticum*). The highest sensitivity of 27 mm was observed in *Aspergillus niger* at 100mg/ml while at concentration of 12.5 mg/ml showed the same sensitivity against both *Aspergillus niger* and *Candida albicans*. The Minimum Fungicidal Concentration (MFC) of 6.25 mg/ml was observed for all fungi isolates used. Various phytochemicals tested were present in *S. aromaticum* these include flavonoids, tannins, saponins, anthraquinones and triterpenes. The results of this study showed that the extract of *S. aromaticum* has potent bioactivity against all fungi isolates studed. The presence of phytochemical in the extracts may have been responsible for the antifungal activity exhibited by the plant extract.

KEYWORDS: *Syzygium aromaticum*, antimicrobial activity, antifungal activity, phytochemical screening, volatile oils.

INTRODUCTION

Medicinal plants are used for treating various diseases since prehistory. Around, 250 to 500 thousand plant species are estimated to be present on the planet, out of which 1 to 10% of them are used as food by human beings and other animals.[1] According to World Health Organization (WHO), 80% of the world's population depends mainly on traditional medicines and use plant extracts in traditional treatments.[2] This is commonly found in rural areas where synthetic drugs are not available or too expensive to purchase. Some of the crude drugs used in past are still in use in phyto-therapeutics. Cinchona plant is often used in its natural and unrefined form to treat malaria,[3] although some other herbal drugs are refined by isolating their pure active compounds and their active principles, helped in the advancement of scientific medicine. 20th century, the antibiotic era substantially reduced the threat of infectious diseases, but over the years there is a decrease in microbial susceptibility to existing microbial agents, responsible for drug resistance which is exerting global problem today. In fact, the theme of World Health Day 2011 was: no action today, no cure tomorrow. There is an urgent need of new antimicrobial agents to overcome this global problem.[4] Higher plants are a rich source of antibiotics,[5] the antibiotic action of allinine from Allium sativum (garlic), or the antimicrobial action of berberine from goldenseal (Hydrastis canadensis). During the period (1981- 2006), 109 drugs were approved in which 69% were from natural products and 21% of the antifungal drugs were refined natural derivatives.[6] The promising potential of antimicrobial plant derived substances have attracted the attention of pharmaceutical and scientific communities during the last few years.[7] The primary benefit of plant derived medicines is that they are relatively safer than their synthetic counterparts and offer profound therapeutic benefits and more affordable treatment.

Cloves (*S. aromaticum*) are dried aromatic unopened floral buds of an evergreen tree 10-20 m in height, belonging to the family *Myrtaceae*, indigenous to India, Indonesia, Zanzibar, Mauritius and Ceylon. They are esteemed as a flavoring agent and also used as a spice for scenting, chewing tobacco and an ingredient of betel

chew. Cloves have many therapeutic uses; they control nausea and vomiting, cough, diarrhea, dyspepsia, flatulence, stomach distension and gastro intestinal spasm, relieve pain, cause uterine contractions and stimulate the nerves.[8,9,10,11,12] In addition, the cloves are highly antiseptic,[13] antimutagenic,[14] anti-inflammatory,[15] antioxidant, antiulcerogenic,[16,17] antithrombotic,[18] antifungal,[19,20] antiviral[21] and antiparasitic.[22] Spices have been traditionally used since ancient times, for the preservation of food products as they have been reported to have antiseptic and disinfectant properties.[23] *S. aromaticum* has been shown to be a potent chemo preventive agent, used by the traditional Ayurvedic healers of India since ancient times to treat respiratory and digestive ailments.[24,25] Eugenol is the main volatile compound extracted from clove bud (*S. aromaticum*) and used in traditional medicine, as a bactericide, fungicides and anesthetic.[26]

OBJECTIVES

To determine antimicrobial (antifungal) activity of test plant extracts on selected microorganisms and to compare zones of inhibition of the extract with that of the commercial antibiotics on selected microorganisms.

MATERIALS AND METHODS

Collection, identification and Preparation of Plant Material

Syzygium aromaticum flower were purchased from Market, the plant was identified by a taxonomist at medicinal and aromatic plants institute, National Center for Research Khartoum, Sudan.

Volatile oil Distillation

Distillation of volatile oils was carried out using the method described by:[27] 500 g of the plant sample was placed in 3000 ml rounded bottom capacity flask. 2000 ml of distilled water was added and the Clevenger receiver heavier than water) (Duran West Germany) and condenser attached to the top of the flask. System was heated at 100 C for about four hours till the volume of oil above water layer at the receiver constant. Oil was pipetted, dried over sodium sulphate anhydrous and stored in a dark container in a refrigerator till used. Yield percentages were calculated as followed:

Volume of oil / weight of plant sample * 100.

Table 1: Yield % of extracts.

Sample	*Weight of sample*	*Volume of oil*	*Yield %*
Clove	*500 g*	*41 ml*	*8.2 %*

1. Phytochemical screening[28,29,30,31]

Phytochemical screening for the active constituents was carried out using the methods described by (Martinez & Valencia (1999), Sofowora (1993), Harborne (1984) and Wall et al (1952)) with many few modifications.

1.1. Identification of tannins

0.5 g of the extract was washed three times with petroleum ether, dissolved in 10 ml hot saline solution and divided in two tests tubes. To one tube 2-3 drops of ferric chloride added and to the other one 2 – 3 drops of gelatin salts reagent added. The occurrence of a blackish blue colour in the first test tube and turbidity in the second one denotes the presence of tannins.

1.2. Test of sterols and triterpenes

0.5 g of the extract was washed three times with petroleum ether and dissolved in 10 of chloroform. To 5 ml of the solution, 0.5 ml acetic anhydride was added and then 3 drop of conc. Sulphuric acid at the bottom of the test tube. At the contact zone of the two liquids a The gradual appearance of green, blue pink to purple color was taken as an evidence of the presence of sterols (green to blue) and or triterpenses (pink to purple) in the sample.

1.3. Test for Alkaloids

0.5 g of the extract was heated with 5 ml of 2N Hcl in water bath and stirred for about 10 minutes, cooled filtered and divided into two test tubes. To one test tube few drops of Mayer's reagent was added while to the other tube few drops of Valser's reagent was added. A slight turbidity or heavy precipitate in either of the tow test tubes was tanked as presumptive evidence for the presence of alkaloids.

1.4. Tests for Flavonoids

0.5 g of the extract was washed three times with petroleum ether and dissolved in 30 ml of 80% ethanol.

The filtrate was used for following tests:

A/ to 3 ml of the filtrate in a test tube 1ml of 1% aluminum chloride solution was in methanol was added. Formation of a yellow color indicated the presence of Flavonoids. Flavones or and chalcone.

B/ to 2 ml of the filtrate 0.5ml of magnesium turnings were added. Producing of defiant color color to pink or red was taken as presumptive evidence that flavonenes were present in the plant sample.

1.5. Test for Saponins

0.5 g of the extract was placed in a clean test tube. 10 ml of distilled water was added, the tube stoppered and vigorously shaken for about 30 seconds. The tube was then allowed to stand and observed for the formation of foam, which persisted for least an hour, was taken as evidence for presence of saponins.

1.6. Test for Cumarins

0.5 g of the extract was dissolved in 10 ml distilled water in test tube and filter paper attached to the test tube to be saturated with the vapor after a spot of 0.5N KoH put on it. Then the filter paper was inspected under UV light, the presence of coumrins was indicated if the spot have found to be adsorbed the UV light.

1.7. Test for Anthraquinone glycoside

0.5 g of the extract was boiled with 10 ml of 0.5N KoH containing 1ml of 3% hydrogen peroxide solution. The mixture was extracted by shaking with 10 ml of benzene. 5ml of the benzene solution was shacked with 3ml of 10% ammonium hydroxide solution and the two layers were allowed to separate. The presence of anthraquinones was indicated if the alkaline layer was found to have assumed pink or red color.

2. Preparation of standard fungal organisms

The fungal standard cultures were obtained from the department of Microbiology and Parasitology, Medicinal and Aromatic Plant Research Institute, Khartoum and were maintained on Sabouraud dextrose agar, incubated at 25○C for 4 days. The fungal growth were harvested and washed with sterile normal saline and finally suspended in 100 ml of sterile normal saline and the suspension was stored in refrigerator till used. In vitro testing of extract for antifungal activity.

The cup-plate agar diffusion method (Kavanagh, 1972),[32] was adopted, with some minor modifications; to assess the antifungal activity of the prepared extracts (NCCLS 2000). In accordance with this method one ml of the isolated standardized and fungal stock suspension (108-109 C.F.U per ml) were thoroughly mixed with 100 ml of sterile molten Mueller- Hinton agar which was maintained at 45○C. Twenty ml aliquots of the inoculated Mueller-Hinton agar were distributed onto sterile Petri-dishes. The agar was left to set, and in each of these plates, four cups (10 mm in diameter) were cut using a sterile cork borer (NO.4) and the agar discs were removed. Alternate cups were filled with 100µL of samples of each of the extract, using standard fine adjustable automatic pipette and allowed to diffuse at room temperature for two hours. The plates were then incubated in the upright position, at 37○C for 18 hours. Two replicates were carried out for each extract against each of the tested organisms. Simultaneously, positive control involving the addition of methanol instead of the extract was included. Upon the completion of incubation the diameter of the resultant inhibition zones were measured, averaged and then the mean values were tabulated.

3. Determination of Minimum Inhibitory Concentrations (MIC) by agar well dilution method

Both extracts were prepared in the series of decreasing concentrations in the following order 50, 25, 12.5, 12.5, 6.25 and 1.56 mg / ml. MIC is the least concentration of antifungal agent that completely inhibits the growth. Results were reported as MICs.

4. Antifungal activity of reference drugs

The antifungal drugs were also tested at different concentrations obtained by taking 0.1 g of each powdered drug and dissolved in 100 ml sterile distilled water to give a concentration of 1000 µg/ml followed by serial dilutions to give concentrations of 12.5, 25 and 50 µg/ml Nystatin against reference fungi Candida albicans and Aspergillus niger. 5, 10 and 20 µg/ml Clotrimazole against the same organisms.

RESULTS

The result of the antifungal activity of Cloves (*S. aromaticum*) extract at various concentrations such as 100mg/ml, 50mg/ml, 25mg/ml, 12.5mg/ml and 6.25mg/ml showed their activities against microbial isolates which are *Aspergillus niger* and *Candida albicans* (Table 3).

It also indicates minimum fungicidal concentration (MFC) of 6.25 mg/ml for all fungal isolates with *Syzygium aromaticum* extract. The result of the antifungal activity of *S. aromaticum* extract compared to antifungal drugs clotrimazole and nystatin are shown in (Table 3). The result of phytochemical screening of the extract of *S. aromaticum* is presented in (Table 2). The analysis revealed the presence of saponins, cumarins, anthraquinones, tannins, flavonoids and triterpenes in the extract. And absence of alkaloids and sterols.

Table 2: Phytochemical screening of the *S. aromaticum* extract.

Test	Results	Observation
Saponins	++	Foam
Cumarins	+	UV absorption
Alkaloids	-	No observation
Anthraquinones	++	Pink colour
Tannins	+++	blue colour
Flavonoids	+++	Yellow color
Sterols	-	No observation
Triterpenes	++	Purple colour

Table 3: Antifungal activity of the extract against Standard Organisms.

Sample	Concentration	Standard tested organisms* /M.D.I.Z (mm)**	
		C. a	A. n
Clove oil	10 %	26	27
	5 %	23	25
	2.5 %	21	21
	1.25 %	20	20
	0.65 %	17	18

*Standard organisms tested:, *A.n= Aspergillus niger, C.a= Candida albicans*

Table 4: Antifungal activity of reference antibiotics against standard microorganism.

S. No	Drugs	Concentrations (µg/ml)	Standard microorganisms used MDIZ* (mm)	
			Tested fungi used(M.D.I.Zmm)	
			A.n	**C.a**
3	Clotrimazole	40	30	42
		20	22	40
		10	19	33
		5	16	30
4	Nystatin	50	28	17
		25	26	14
		12.5	23	-

DISCUSSION

Extract of Cloves flower (*Syzygium aromaticum*) showed activity against all the tested microbial isolates at concentrations of 100mg/ml, 50mg/ml, 25mg/ml, 12.5mg/ml and 6.25mg/ml (Table 3). The antifungal activity of Cloves flower (*S. aromaticum*) extract was read from the diameter zone of inhibition. It could be observed that Clove flower (*S. aromaticum*) extract had broad spectrum of activity on the test organisms. The tested microbial isolates they were sensitive to extract of Clove flower (*S. aromaticum*). The highest sensitivity was observed in *Aspergillus niger* as 27 mm at 100mg/ml and 18 mm at 6.25mg/ml, while *candida albicans* showed the lowest sensitivity of 26 mm at 100 mg/ml, and 17 mm at 6.25mg/ml.

The antimicrobial activity of plant extracts has been linked by many researchers to be due to the presence of phytochemicals in them.[33,34] Reported that crude extracts of plant materials may contain inactive substances which may also antagonize the antimicrobial actions of one another. The MIC of the extract is 6.25mg/ml for all organisms used. Comparison of antifungal drug such as Nystatin used in this study with the extract of Cloves flower (*S. aromaticum*) showed that Nystatin was more effective in the treatment of *candida* infection with inhibition zone 28 mm at concentration 50 µg/ml.

Nystatin showed no activity against *Aspergillus niger* at concentration of 12.5 µg/ml (Table 4), while the extract predictable activity.

Against *Aspergillusniger*. Not much has been reported on the antifungal activity of *S. aromaticum* extracts, except.[35] who reported on the *anticandida* activity extract of *S. aromaticum*. The results of the phytochemical analysis are presented in (Table 2). saponins, cumarins, anthraquinones, tannins, flavonoids and triterpenes were detected in extract of Clove flower (S. aromaticum). Literature related to antimicrobial and phytochemical constituents of *S. aromaticum* extracts is scanty. This might be attributed to the fact that *S. aromaticum* is a tree indigenous to West Africa and therefore research on the plant is scanty and claims by traditional herbalists on the usefulness of the plant as medicinal mostly centered on the use of the stem bark, root and leaf.

CONCLUSION

The results of this study showed that the extract of *S. aromaticum* has antifungal activity. Perhaps its potential activity on *Aspergillus niger* and *Candida albicans* coupled with its low MIC on both fungal isolates tested might give it an impetus as a potential fungal agent particularly against *Aspergillus niger* infections. The presence of phytochemicals in the extract may have been responsible for the activity possessed by the plant extracts and can be used as phytosubstrate in development and drug discovery.

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