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Research Article

Bioactivity of leaf extracts from *Hyptis suaveolens* against Storage Mycoflora

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Abstract: The Cereals, pulses, oilseeds etc. are very important products for storage. A safe storage place must be provided for the grain produced until it is needed for consumption and multiplication purposes. Since grain production is seasonal, and consumption is continuous, safe storage must maintain grain quality and quantity. This means that grains have to be protected from weather, molds and other microorganisms, moisture, destructively huge temperatures, insects, rodents, birds, objectionable odors and contamination, and from unauthorized distribution. The protection of crops, stored food grains and pest control in the public health sector continues to place heavy reliance upon the use of chemicals. The history of pesticide development has been instructive to us in terms of benefits derived as well as the hazards, which accompany indiscriminate use of these poisons. The volatile substances obtained from higher plants have proved their usefulness in controlling biodetioration as they form a vast cornucopia of defence chemicals, toxicant and antimicrobial agents. In this communication bioactivity of leaf extracts from *Hyptis suaveolens* was tested against *Aspergillus flavu*s and *Aspergillus parasiticus.*
Keywords: Antimicrobial, Phytochemical, Bioactive compounds, *Hyptis suaveolens*.

INRODUCTION

Food grains form an important part of the vegetarian Indian diet. Grain production has been steadily increasing due to advancement in production technology, but improper storage results in high losses in grains. According to World Bank Report, post-harvest losses in India amount to 12 to 16 million metric tons of food grains each year, an amount that the World Bank stipulates could feed one-third of India's poor. The monetary value of these losses amounts to more than Rs 50,000 crores per year [1]. Natural contamination of food grains is greatly influenced by environmental factors such as type of storage structure, temperature, pH, moisture, etc [2]. Types of structure used, length and purpose of storage, grain treatment (e.g. parboiling) and pre-storage practices are all important variables affecting storage losses. The importance of these regional and crop variations immediately determines certain necessary characteristics of crop storage research [3]. During storage, quantitative as well as qualitative losses occur due to insects, rodents, and micro-organisms. The ecosystem within stored grain structures is limited in microbial species because of human efforts to maintain grain quality [4] [5]. Of the more than 70,000 fungi species that have been described [6], relatively few are found on grain. At harvest, grain contains populations of field microbes. Once the grain is placed into a storage facility, a succession of new microbial species begins to grow. Without intervention, microbial respiration will increase temperature and moisture, providing optimum growth conditions for even more diverse fungal species.

The micro flora of cereals (including oilseeds) is made up of a wide variety of fungi, bacteria and actinomycetes. These microorganisms are the same as those found in soil, air, and on or in living or dead plants and animals [7]. [8**]** Reported that more than 150 species of fungi have been isolated from within seeds or kernels. Fungi are generally ubiquitous and omnivorous [9].

However, the exact mechanism and time of fungal invasion of seeds is unclear. [10] grouped fungi that invade cereals into two categories: (1) field fungi and (2) storage fungi. This division is not taxonomically valid but is based primarily upon moisture requirements. Storage fungi are predominantly species of *Aspergillus and Penicillium.* "Species" of *Aspergillus* are not always well defined and are sometimes referred to as "groups." The major storage fungi consist of five or six groups of *Aspergillus,* plus several species of *Penicillium* which are common until deterioration is well advanced [11]**.** Certain other species of *Penicillium* are considered field fungi [12]**.**

A major problem of agricultural production is loss of grain during and after harvest. Microorganisms, insects, and rodents contribute greatly to these postharvest losses. A commonly quoted estimate provided by the FAO for worldwide losses for all cereals, leguminous seeds, and oilseeds is 10% [13]. [11] state that fungi are the major cause of spoilage in stored grains and seeds. Consequently, it is well-established that fungi destroy food and feed. However, the basic problem remains of implementing effective measures to reduce fungal losses.

The apprehension that large-scale use of plant-based fungicides/insecticides may lead to resistance among pathogens has not substantiated. Unlike conventional pesticides based on a single active ingredient, the bioactive components in the natural plant product will be made up by a complex array of novel chemicals that affect not only one physiological function but rather act in concert on several processes [14]. Such products from higher plants are relatively bio-efficacious, economical and environmentally safe and can be an ideal candidates for use as agrochemicals [15] [16]. Among these, essential extract [17] from a number of plants have been reported to show bioactivity against a wide array of plant pathogenic and storage mycoflora [18] [19].

However, as yet not many work has been carried out to explore the fungicidal activity of the leaf extract *from Hyptis suaveolens* (L) Poit. an annual member of family Lamiaceae, growing abundantly as a weed in India. Thus in this present communication, the effect of leaf extract obtained from *Hyptis suaveolens* on fungi causing storage rot was studied.

MATERIALS AND METHODS

Survey and Collection of Sample:

To obtain a representative sample, all the pulses samples Peanut (*Arachis hypogea*), Soyabean (*Glycine max*), and Bean (*Phaseoles vulgaris*), were collected by random sampling method from Madurai east region Fig 1. The collection areas comprised of regulated market yards.

Isolation, Purification and Identification:

Two standard methods of isolation (namely agar plate and blotter methods) were used for tested these seeds (Neergard, P., 1977).Every seed sample was divided into two groups. The first group was disinfected by soaking in 1% sodium hypochlorite solution for 2 min, then washed several times with sterilized water and dried between two sterilized filter papers. The second group was untreated (non-disinfected). Some of this seeds (disinfected or not) were transferred onto potato dextrose agar (PDA) plates (fig 2) but other seeds were plated on three sterilized filter papers with enough moisture in sterilized Petri dishes [blotter method] . Five seeds / dish and 4 dishes for each as replicate. All dishes were incubated at 25±2°C under darkness for 7 days. Seeds were examined every day. Any fungal growth was transferred and purified using hyphal tip and/or single spore techniques onto PDA medium in the presence of antibiotic (Streptomycin). Developing fungi were transferred to PDA slants. Fungal cultures (15 days old) were identified in the, Centre for Botanical Research Department, Madurai. Cultures were kept at 5°C for further studies.

Collection of Plant material

The fresh plant organ (Leaves) of *Hyptis suaveolens* were collected from Pasumalai, Madurai (Fig 3). The voucher specimen is preserved in the Department of Botany, The Madura College, Madurai, Tamilnadu.(India). The collected plants were washed repeatedly with tap and finally with distilled water. Then sliced and they were dried and powdered with help of grindings and filtered through sieves and stored for extraction.

Preparation of Plant extract

Plant materials belonging to botanical families included in this study were collected, washed with tap water, disinfected by Immersion in 2% sodium hypochlorite solution for 30 min rinsed with sterile distilled water to eliminate residual hypochlorite and dried in shade. The shade-dried leaf material of plant species was grounded into a powdered material using a blender to pass 100 mm sieve and the mince was sealed in polyethylene bags until extraction. For preparation of methanolic extracts, 50 g of dry powder plant leaf material from plant species was soaked in methanol (10 ml of methanol/g of plant material) with stirring for 48 h then filtered through double layers of muslin, Centrifuged at 9000 rpm for 10 min and finally filtered again through What man filter paper No. (41) to remove debris and obtain a clear filtrate. The filtrates were evaporated and dried under reduced pressure and temperature below 40°C (Fig 4).

GCMS Analysis

Analytical gas chromatography was carried out on a Perkin Elmer 115 gas chromatograph fitted with a 30 m × 0.25 mm ID, 0.25 μm film thickness. Column temperature was initially kept at 70°C for 2 min, then gradually increased to 120°C at 2°C min-1 rate, held for 2 min and finally raised to 250°C at 5°C min-1. Diluted samples (1/100 v/v, in hexane) of 1 μl were injected at 250°C. Flame ionization detection (FID) was performed at 280°C. GC-MS analysis was performed on an Agilent 6850 Series II apparatus, fitted with a fused silica HP-5MS non-polar capillary column (30 m × 0.25 mm ID, film thickness 0.25 μm). Carrier gas was helium at a flow rate of 1 ml/min. Injector and MS transfer line temperatures were set at 230 and 270°C, respectively. Ion source temperature was 200°C. The injection volume was 0.1 μl with a split ratio of 1:50. Mass range was from m/z 40 to 550 amu. Identification of components of the essential extracts was based on GC retention indices and computer

matching with the Wiley Library as well as by comparison of the fragmentation patterns of the mass spectra with those reported in the literature.

Test Fungi:

Two species of *Aspergillus* viz *Aspergillus flavus* and *Aspergillus parasiticus* which were frequently associated with Peanut seeds with higher percentage were selected for antifungal activity assay of methonolic extract of test plant.

Antifungal activity test

Antifungal activity was screened by agar well diffusion method [7]. The methanol extracts of *Hyptis suaveolens* plant leaf were tested against *Aspergillus flavus* and *Aspergillus parasiticus* . The PDA medium was poured in to the sterile petriplates and allowed to solidify. The test fungal culture was evenly spread over the media by sterile cotton swabs. Then wells (6 mm) were made in the medium using sterile cork borer. 200µl of each extracts were transferred into the separate wells. The plates were incubated at 27°C for 48 – 72 hrs. After the incubation the plates were observed for formation of clear incubation zone around the well indicated the presence of antifungal activity. The zone of inhibition was recorded.

Fig-1 : Collection of Sample

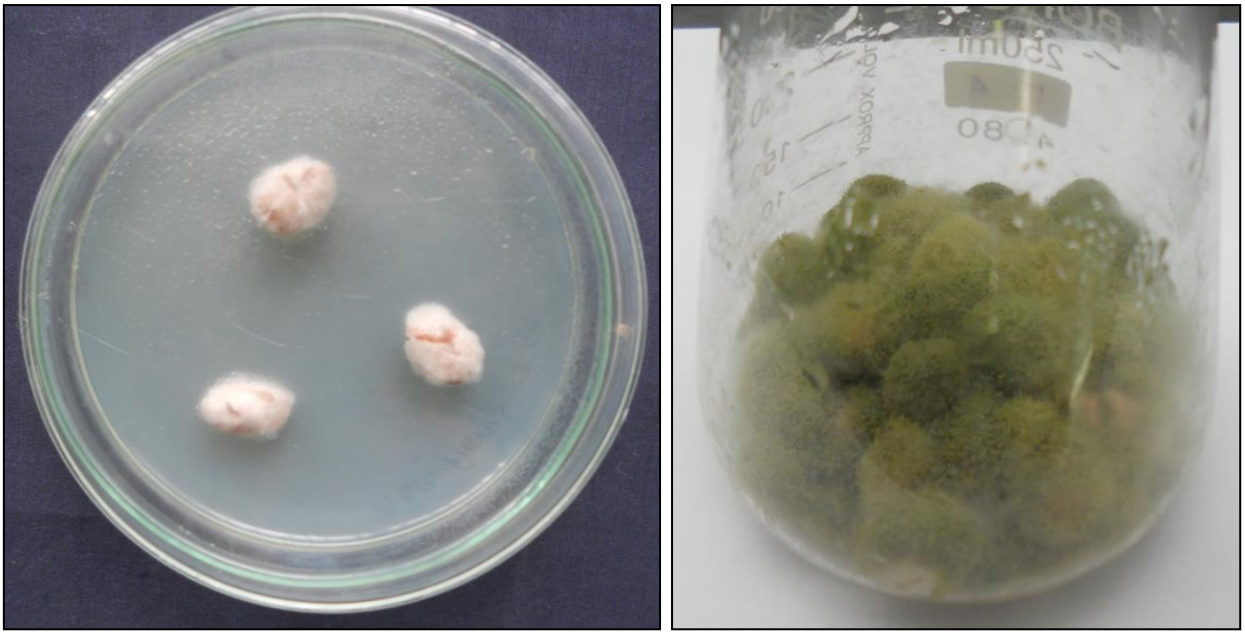


Fig- 2: Isolation and Identification of sample

Fig- 3: Collection of Plant material *(Hyptis suaveolens)*

Fig -4: Preparation of Plant extract

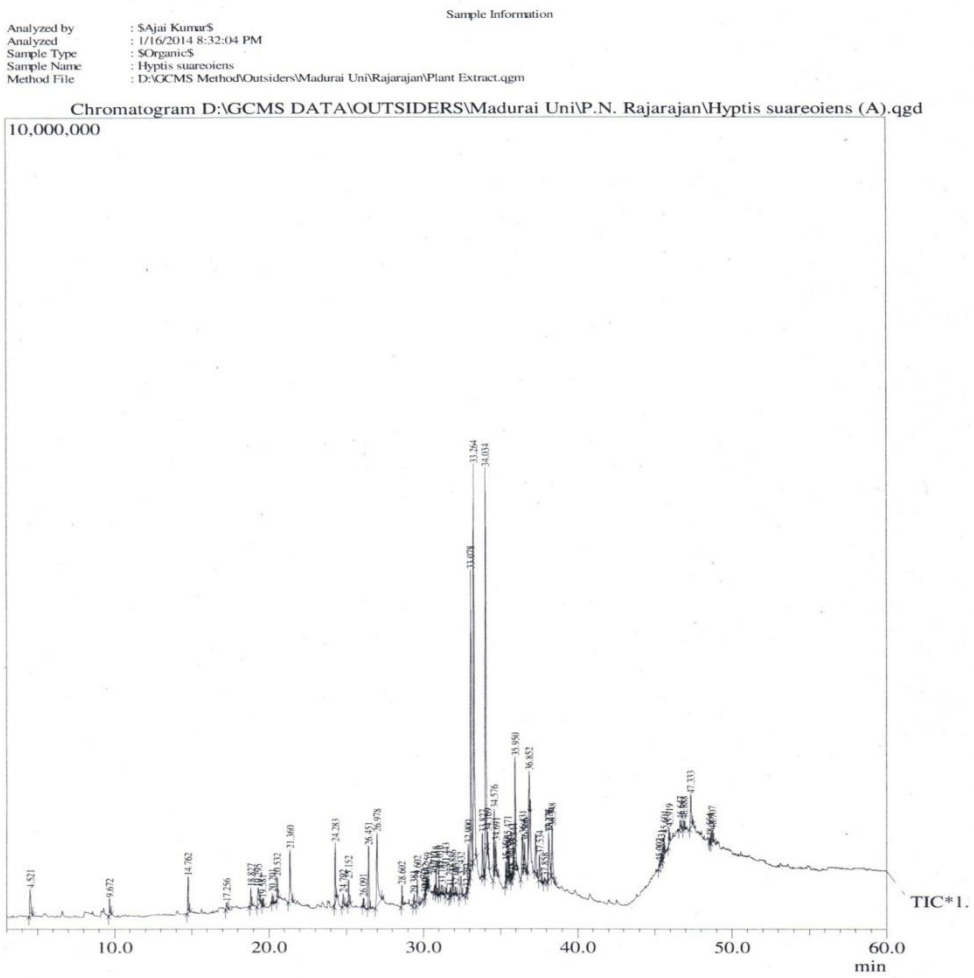


Fig-5: Total ion chromatogram (TIC) of leaf of *Hyptis suaveolens*

Table 1. Chemical composition of *Hyptis suaveolens*

Peak Report TIC

Peak#	R.Time	Area	Area%	Name
1	4.521	2103752	1.67	Eucalyptol
2	9.672	767499	0.61	2-Oxabicyclo[2.2.2]octan-6-ol, 1,3,3-trimethyl-
3	14.762	1585669	1.26	Caryophyllene
4	17.256	368139	0.29	SESQUISABINENE HYDRATE
5	18.827	756188	0.60	Caryophyllene oxide
6	19.295	1220867	0.97	Diethyl Phthalate
7	19.581	276587	0.22	Selina-6-en-4-ol
8	20.201	379038	0.30	NAPHTHALENE, 1,2,3,5,6,8A-HEXAHYDRO-4,7-DIMET
9	20.532	905723	0.72	Selina-6-en-4-ol
10	21.360	3050064	2.42	Bergamotol, Z-.alpha.-trans-
11	24.283	2515034	2.00	2,6,10-TRIMETHYL,14-ETHYLENE-14-PENTADECNE
12	24.792	594932	0.47	2,6,10-TRIMETHYL,14-ETHYLENE-14-PENTADECNE
13	25.152	1065551	0.85	2,6,10-TRIMETHYL,14-ETHYLENE-14-PENTADECNE
14	26.091	295361	0.23	HEXADECANOIC ACID, METHYL ESTER
15	26.451	3060708	2.43	
16	26.978	5077680	4.03	n-Hexadecanoic acid
17	28.602	746518	0.59	7-Isopropyl-1,1,4a-trimethyl-1,2,3,4,4a,9,10,10a-octahydroph
18	29.381	356660	0.28	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-
19	29.602	1389764	1.10	2-HEXADECEN-1-OL, 3,7,11,15-TETRAMETHYL-, [R-[R
20	30.067	145046	0.12	1R,4s,7s,8R,11R-2,2,4,8-Tetramethyltricyclo[5.3.1.0(4,11)]ur
21	30.142	196110	0.16	d-Norandrostane (5.alpha.,14.alpha.)
22	30.259	650474	0.52	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-
23	30.717	209023	0.17	Phytol, trimethylsilyl ether
24	30.818	843891	0.67	KAUR-16-ENE
25	31.010	900891	0.71	1H-Benzocycloheptene-7-ol, 2,3,4,4a,5,6,7,8-octahydro-1,1,4a,
26	31.187	506465	0.40	Naphthalene, decahydro-1,1,4a-trimethyl-6-methylene-5-(3-me
27	31.443	491292	0.39	03027205002 FLAVONE 4'-OH,5-OH,7-DI-O-GLUCOSIDE
28	31.722	284927	0.23	CYCLOHEXAN, 3,3,5,5-TETRAMETHYL-1-(3,3,5,5-TETR
29	31.886	1120772	0.89	2.BETA.-HYDROXY-9-OXOVERRUCOSANE
30	32.083	501556	0.40	Silane, trimethyl[[(3.beta.,4.alpha.,5.alpha.)-4-methylcholesta-
31	32.432	1306483	1.04	Lanosterol
32	32.792	245759	0.20	SCLARAL
33	32.900	1629225	1.29	Lanosterol
34	33.078	14044292	11.15	Bicyclo[3.1.1]hept-2-ene, 2,2'-(1,2-ethanediyl)bis[6,6-dimethy
35	33.264	19729228	15.66	Cholest-14-en-3-ol, (3.beta.,5.alpha.)-
36	33.827	1909239	1.52	10,12,14-Nonacosatriynoic acid
37	34.034	17198527	13.65	1-Phenanthrenemethanol, 1,2,3,4,4a,9,10,10a-octahydro-1,4a-
38	34.169	1052374	0.84	3-BROMOCHOLEST-5-ENE #
39	34.576	2767063	2.20	2-[4-methyl-6-(2,6,6-trimethylcyclohex-1-enyl)hexa-1,3,5-trien
40	34.691	1294251	1.03	TRICYCLO[5.4.3.0(1,7)]TETRADECANE-3,6-DIOL, 4-FO
41	35.350	901338	0.72	3-BUTEN-2-ONE, 4-(2,5,5-TRIMETHYL-3-OXATRICYCL
42	35.471	1580172	1.25	Stigmasterol
43	35.580	587751	0.47	SPIRO[TRICYCLO[4.4.0.0(5,9)]DECANE-10,2'-OXIRANE
44	35.743	351278	0.28	
45	35.844	919283	0.73	22,23-Dibromostigmasterol acetate
46	35.950	5257386	4.17	4,8,13-Cyclotetradecatriene-1,3-diol, 1,5,9-trimethyl-12-(1-me
47	36.431	2355291	1.87	9,19-Cyclolanost-23-ene-3,25-diol, 3-acetate, (3.beta.,23E)-
48	36.566	1437747	1.14	Thunbergol
49	36.852	3504435	2.78	2-(1,4,4-TRIMETHYL-2-CYCLOHEXEN-1-YL)ETHANOL
50	37.275	2214748	1.76	Pregnan-20-one, 3-hydroxy-, (3.beta.)-
51	37.534	1481317	1.18	2-[4-methyl-6-(2,6,6-trimethylcyclohex-1-enyl)hexa-1,3,5-trien
52	37.858	188248	0.15	
53	38.136	3795682	3.01	2-[4-methyl-6-(2,6,6-trimethylcyclohex-1-enyl)hexa-1,3,5-trien
54	38.348	2877621	2.28	4,4-DIMETHYL-3-OXOANDROST-5-EN-17-YL ACETATE
55	45.292	156072	0.12	.gamma.-Tocopherol
56	45.431	255341	0.20	Cholesta-4,6-dien-3-ol, (3.beta.)-
57	45.601	794535	0.63	STIGMAST-5-EN-3-OL, OLEAT
58	45.919	542776	0.43	.alpha.-Tocopherol-.beta.-D-mannoside
59	46.647	459400	0.36	ERGOST-5-EN-3-OL, (3.BETA.,24R)-
60	46.888	445050	0.35	STIGMASTA-5,23-DIEN-3-OL, (3.BETA.)-
61	47.333	1532950	1.22	STIGMAST-5-EN-3-OL, (3.BETA.)-
62	48.564	174423	0.14	STIGMAST-4-EN-3-ONE
63	48.707	646817	0.51	3,5,6,9,11,12,13,14,17,18,21,22,23,24,25,26-HEXADECAH
		126002283	100.00	

RESULTS

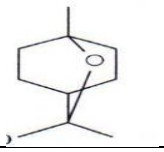
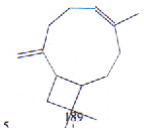
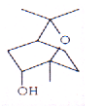
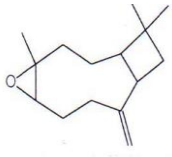
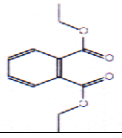
The Phytochemical investigation shows that the plant "*Hyptis suaveolens*" contains major components mainly in leaves. *Hyptis suaveolens* contains many diverse Phytochemical like α-Phellandrene , which is a monocyclic terpene with a pleasing aroma, α - pinene a terpene having very reactive four membered rings,4,11,11-Trimethyl-8-Methylene- Bicyclo{7.2.0}-Undec-4-ene , α-Caryophyllene , 3-cyclohexen-1-carboxaldehyde, 5α-androst-2,11-dione, 5α-androst-9(11)-en-12- one , 4-methyl-1-(1-methylethyl)-3- cyclohexen-1-ol , 2-

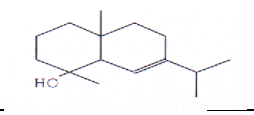
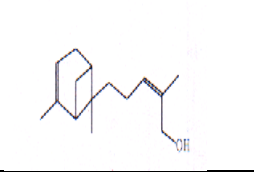
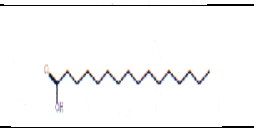
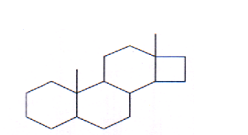
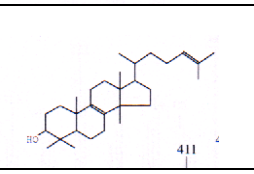
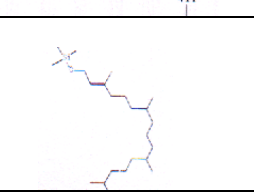
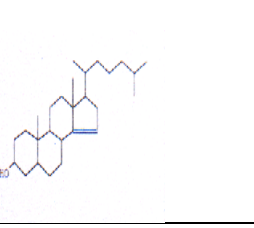
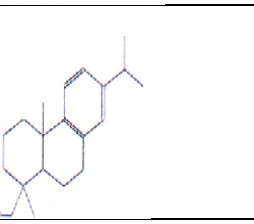
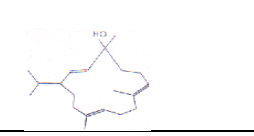
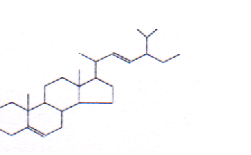
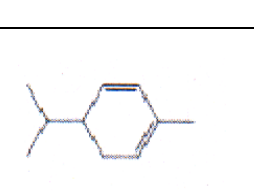
Oxabicyclo octan-6-ol Thujane , 1 8 cineole , 3,7-dimethyl-1,6-octadien-3-ol, 2,5- dimethyl-3-methylene-1,5-heptadiene , Diethyl Phthlate,1,3,3-trimethylbicycloheptan-2-ol ,α-cymene , Caryophyllene oxide, Bergamotol . The presence of many terpenoids identified with the help of GC-MS analysis. Limonene, thujane ; α- pinene ; α-phellandrine ; 4-methyl-l-(lmethylethyl)- 3-cyclohexen-l-ol ; 3- cyclohexen-1-carboxyaldehyde ; 4, 11, 1 l-trimethyl-8-methylene bicycle [7.2.0] undec-4-ene ; n-Hexadecanoic acid , Phytol, trimethylsilyl ether, 1 – Phenanthrenemethanol, octahydro-1, 4- dimethylazulene ; Selina-6-en-4-ol, Thunbergol , Stigmasterol, 5α, 8β, h-9β, h-10a- labd-14-ene; 5α-androst-9(l l)-en-12-one and 5α- androstan-2,11-dione 1, 8-cineole and β-caryophyllene as main constituents with minor concentration of β-Pinene , Sabinene, Fenchol , 4- terpinenol, eugenol, α-copaene , β- elemene, α-Humulene (16b), α-Bergamotene, aromadenedrene , γ-cardinene, δ-cadinene, α phallandrene , myrcene , linalool , α-terpinolene, γ – terpinene . They reported the absence of α-Terpinene, p-Cymene, Limonene .

The main constituents were Cholest- 14-en-3-ol ,1–Phenanthrenemethanol 1, 8 – cineole (32%) & B-caryophyllene (29%), Organic compounds like alkaloids – 10.32%, Tannins – 0.52%, Crude protein – 14.22%, Flavonoids – 12.54%, crude fibre – 9.04%, Phenols – 0.05%, ash – 5.68%, Saponins – 0.3%, carbohydrate – 66.6%, Lipid – 4.46%, Food energy – 363.43 mg/cal, Moisture content – 4.7%, Dry matter – 95.3%. Minerals line K – 1.8%, P – 0.79%, N – 2.28%, Ca – 1.06%, Mg – 0.67%, Na – 0.46% was identified. From the results of GC-MS **(Fig.5 and Table 1, 1.1)** it was observed that AllylOctadecanoate, Bicyclo hept-2-ene,2,2 –(1,2- ethanediyl)bis,Octadec-9-Enoic Acid is the dominant compounds in the leaf methanol extract of *Hyptis suaveolens* which are tri-terpenoids. In the aqueous extract neophytadiene was the major compound isolated. The medicinal properties of the plant i.e. antibacterial, antifungal, insecticidal, antifertility, anticancerous properties may be due to the above compounds.

Antifungal activity of *Hyptis suaveolens* was screened against aflatoxigenic fungi *Aspergillus flavus* and *Aspergillus parasiticus*. The extracts showed varying of inhibitory effects. The inhibitory effects of extracts were directly proportional to the increasing concentration of field grown leaf. The Methonolic extract produced good antimicrobial activity at 200µg/ml. The Methanolic extracts showed a 25mm zone of inhibition against *Aspergillus flavus* and 24mm zone of inhibition against *Aspergillus parasiticus* (Table 2 and fig 6).

Table 1.1. Chemical nature of bioactive components from *Hyptis suaveolens* (Identified by GC-MS Study

S.NO	Name of Compound	M.F	M.W	Structure
1	Eucalyptol	$C_{10}H_{18}O$	154	
2	Caryophyllene	$C_{15}H_{24}$	204	
3	2-Oxabicyclo octan-6-ol	$C_{10}H_{18}0_2$	170	
4	Caryophyllene oxide	$C_{15}H_{24}O$	220	
5	Diethyl Phthlate	$C_{12}H_{14}O_4$	222	

6	Selina-6-en-4-ol	$C_{15}H_{26}O$	222	
7	Bergamotol	$C_{15}H_{24}O$	220	
8	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	
9	d –Norandrostane	$C_{18}H_{30}$	246	
10	Lanosterol	$C_{30}H_{50}O$	426	
11	Phytol, trimethylsilyl ether	$C_{23}H_{48}OSi$	368	
12	Cholest- 14-en-3-ol	$C_{27}H_{46}O$	386	
13	1 – Phenanthrenemethanol	$C_{20}H_{30}O$	286	
14	Thunbergol	$C_{20}H_{34}O$	290	
15	Stigmasterol	$C_{29}H_{48}O$	412	
16	α- Phellandrena	$C_{10}H_{16}$	136	

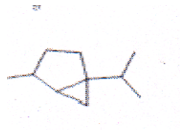
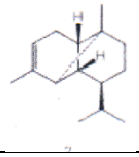
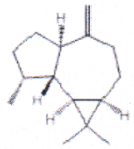
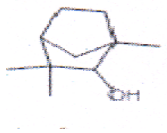
17	Thujane	$C_{10}H_{18}$	138	
18	α- Copaene	$C_{15}H_{24}$	204	
19	Aromodendrene	$C_{15}H_{24}O$	220	
20	Fenchol	$C_{10}H_{18}O$	154	

Table2: Inhibition of growth of *Fungus* by plant leaf extracts at the different concentrations

S.No	Test Microorganism	Zone of inhibition (mm) in different concentration				
		Control	Fluconazole (10µg/ml)	100µg/ml	150µg/ml	200µg/ml
1	*Aspergillus flavus*	C	23	22	24	25
2	*Aspergillus parasiticus*	C	21	20	22	24

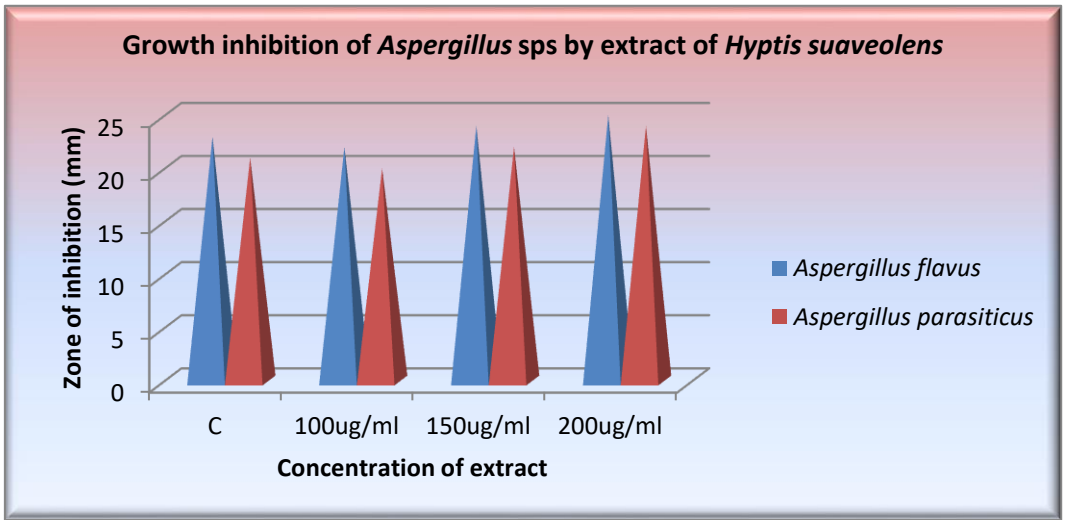


Fig-6.: Inhibition of growth of *Aspergillus flavus and Aspergillus parasitcus* by *Hyptis suaveolens* leaf extracts at the three concentrations.

DISCUSSION

As this study was designed to find out the possibility of utilizing volatile constituents of higher plants as preservatives of food commodities against fungal deterioration, *Aspergillus flavus* and *Aspergillus parasiticus* were selected as the test organisms since they were found to be the most common bio-deteriogens during the survey .

The methonolic leaf extract of *Hyptis suaveolens* was also analysed by GC-MS . Different compounds were identified in crude extract and 60 compounds were isolated from extract. Of these compounds Cholest-14-en-3-ol and Phenanthrenemethanol were in major amounts. The fungicidal activity is attributed to Thunbergol, 1,8-cineole , β-caryophyllene found in major concentration . The plant extract can be used against fungal infections, digestive problems and also as an appetizer.

The *Hyptis suaveolens* leaves revealed a fast and steady anti-*Aspergillus flavus* property with a strong inhibition of the mycelial growth, fungi spore germination and aflatoxins production. Based on this study, it can be concluded that the essential leaf extract from *Hyptis suaveolens* leaves, if applied in sufficient amounts, possesses fungitoxic activities, inhibiting the growth of *Aspergillus flavus* and *Aspergillus parasiticus* and thus could be considered as an alternative inhibitor of the survival of this fungi in foodstuffs, in addition to offer some protective effect to the production of aflatoxins.

The antifungal activity of *Hyptis suaveolens* against aflatoxic fungal strains obtained by the well diffusion method. Various concentrations of methanolic extract used for this present study. The 200µg/ml concentration of plant extract exhibited a good inhibition against all the fungal strains when compared to control.

Based upon the present study it could be concluded that leaf extract from *Hyptis suaveolens* and its major constituents possess fungitoxic activity worth exploiting for the management of spoilage of stored commodities. In pilot experiments it can be concluded that this leaf extract can serve as a natural fungicide or possibly as a template for the synthesis of novel fungicides. The findings suggest that the *Hyptis suaveolens* leaf extract can be exploited as a potent and ecofriendly fungitoxic fumigant against storage mycoflora because of its high yield, strong and durable fungitoxicity and thermostability.

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