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Research Article

ANTIFUNGAL AND PHYTOCHEMICAL PROPERTIES OF *SYZYGIUM AROMATICUM* ESSENTIAL OIL AGAINST SOYBEAN SEED-BORNE FUNGI

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A B S T R A C T

The effect of *Syzygium aromaticum* L. essential oil extract on soybean seed-borne fungi was investigated. The essential oil was characterized thorough phytochemical analysis, thin layer chromatogram (TLC), column chromatography and gas chromatography-mass spectrometry (GC-MS). Eugenol was found to be the major compound present in the extract. Soybean seed-borne fungi such as *Aspergillusflavus, Cladosporium cladosporioides, Fusarium oxysporum* and *Macrophomina phaseolina* were isolated and identified with served as a test fungi. The maximum inhibition was observed in *F. oxysporum* and *M. phaseolina* (100%) followed by *A. flavus* (87%) and *C.cladosporioides* (84%). The result suggests that the eugenol extracted from *Syzygium aromaticum* is capable of rendering antifungal efficacy and hence, has a great potential in reducing soybean seed-borne fungal diseases with mycotoxin contamination.

INTRODUCTION

Soybean [*Glycine max* (L.) Merr.] is one of the most important worldwide legume crops as a predominant source of both animal feed protein and cooking oil. It is now the first legume species with a complete published draft genome sequence (Schmutz *et al*., 2009). Botanically soybean belongs to the order *Fabales* and family *Fabaceae.* Soybean protein contains most of the essential amino acids necessary for the good health. This makes soybean protein a complete protein, similar to the protein found in animal foods (Kaushik *et al*., 1995; Preece *et al*., 2017). Soybean is cultivated in India mainly as a Kharifcrop (De *et al*., 2001). The plants flourished in hot and humid summer weather conditions of India which is also favored by fungi (Bharathi *et al*., 2007).

In developing countries such as India, most of the crop yield is reduced due to pathogenic mycoflora and quality of thecrop is affected by mycotoxin tarnishing (Richard, 2007; Wrather *et al*., 2001).

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The grains stored for consumption purposes cannot be treated with fungicides, pesticides and bactericides due to resistance developed by the pathogens, non-biodegradable and extremely toxic in natureis driving attention toward natural alternatives (Calhelha *et al*., 2006, Knight *et al*., 1997). Plant extracts have increasingly gained the interest of researchers as potential antimicrobial agent's alternatives to chemical preservatives (Gyawali and Ibrahim, 2014; Hammer *et al*., 1999; Mahendra *et al*., 2016). Nevertheless, huge efforts are being made to all over the world to isolate bioactive principles from medicinal plants for their conceivable utility in the improvement of plant-based eco-friendly fungicides (Carocho and Ferreira, 2013; Jayalakshmi *et al*., 2015). Noteworthy level of antifungal activity has been demonstrated by crude extracts of innumerable medicinal plants.

Syzygium aromaticum L. is an evergreen tree that grows to a height ranging from 8–12 m, which produces flower buds that has numerous medicinal properties (Chaieb *et al*., 2007). Essential oil obtained from buds has been traditionally used as a source of antimicrobial agents against oral bacteria that are commonly associated with dental caries and also finds use in fragrance and flavoring industries (Cai and Wu, 1996). The main constituents of *S. aromaticum* buds are eugenol,

carvacrol, thymol, and cinnamaldehyde (Bakkali *et al*., 2008). *S. aromaticum* in precise has engrossed the consideration owing to the potent antioxidant antimycotoxigenic and antimicrobial activities standing out among the other spices (Deans *et al*., 1995; Ghaffar *et al*., 2017; Kheawfu *et al*., 2017). Essential oils are aromatic volatile oily hydrophobic liquid distillates that are mined mostly from medicinal plant parts, such as flowers, buds, seeds, leaves, twigs, bark, wood, fruits, roots and whole plant. These essential oils are highly complex mixtures of 20 to 60 volatile compounds, albeit some may contain more than 100 different components.The aim of the current research effort was to evaluate the efficacy of *S. aromaticum* essential oil against soybean seed-borne fungi.

MATERIALS AND METHODS

Collection of plant material and oil extraction

Commercially available buds of *S. aromaticum* were obtained from local market Bangalore, India (12°59′0”N 77°35′0”E) in the month of January (2017). The buds were shade dried and pulverized using an aseptic electric blender to obtain a powdered and stored in sterile polyethylene sample bag prior to use. The powdered sample were subjected to steam distillation unit fig. 01. for 6 hours (Dorman and Deans, 2000). The essential oils were dried over anhydrous sodium sulfate and stored in opaque sterile bottle at 0-4°C.

Phytochemical analysis

Qualitative phytochemical test to detect the presence of different phytochemicals such as alkaloids, flavonoids, tannins, and terpenoids was carried out using standard protocols (Harborne, 1998).

Determination of alkaloids

To the 0.5 ml of thetest solution, a drop of Dragendorff’s reagent (solution of potassium bismuth iodide) was added. Brown precipitate indicated the presence of alkaloids. In the test solution, a drop of Mayer’s reagent (potassium tetraiodomercurate solution) was added. A white precipitate showed the presence of alkaloids (Sreevidya and Mehrotra, 2003).

Determination of flavonoids

Thetest solution of 0.5 ml was mixed with concentrated hydrochloric acid (HCl) and the color was observed. Red color indicated the presence of flavonoids. Magnesium ribbon was added to the test solution followed by the addition of a drop of concentrated hydrochloric acid. A resulting color ranging from orange to red further confirmed the presence of flavonoids (Yadavand Agarwala, 2011).

Determination of tannins

The tannin determination was performed by thefollowing method. Ten percent (w/v) ferric chloride solution was added to the test solution. A bluish-blackcolor, which disappeared on theaddition of dilute sulphuric acid followed by a yellowish brown precipitate, indicated the presence of tannins.

Determination of terpenoids

A test solution of 2 ml extract was mixed with 2 ml of chloroform and concentrated sulphuric acid was carefully added to form a layer. A reddish brown indicates the presence of terpenoids (Raghunandan *et al*., 2010).

Thin layer chromatogram profiling of Syzygium aromaticum

Reference solution

The standard eugenol was purchased from Sigma- Aldrich, USA and prepared at a concentration of 50 µl/ml in hexane.

Solvent system

The solvent systems of different polarities were prepared and TLC studies were carried out to select the solvent system capable of showing better resolution. The best solvent system selected includes Toluene: Ethyl acetate at (9.5: 0.5) ratio.

Procedure

The 5µl of thetest solution and 3µl of standard solution was applied separately on precoated silica gel 60 F_{254} TLC plate of a uniform thickness of 0.2mm (E. Merck, India). The plate was activated by heating for 5 to 10 min. The chromatogram was developed in a suitable solvent system in a chamber saturated with solvent vapors and allowed to run a distance of 8 cm.

Analyte detection

The developed TLC plates were air dried and visualized under visible light. They were observed under ultraviolet (UV) light at both 254 nm (shortwave) and 366 nm (long wave). The chromatogram was later derivatized in anisaldehyde-sulphuric acid reagent (Tanaka *et al.*, 1996) and heated at 105°C for 5 to 10 min. The movement of the analyte was expressed by its retention factor (R_f) and calculated as mentioned below. The R_f value and color of separated bands were recorded.

$$R_f = \frac{\text{Distance traveled by the solute}}{\text{Distance traveled by the solvent front}}$$

Column chromatography

Column chromatography was used to elute a large quantity of pure samples from the crude extract. Silica gel (60-120) was used as stationary phase in column chromatography. Cotton was inserted first in the column towards the outlet and fixed to the clamp. Sea sand of 1 cm bed was decanted into the column and allowed to settle at the bottom. Then silica gel powder was added to the column to fill up to 10 cm height from the neck of the column. Hexane was run in the column up to the bed to make the column entirely wet. Excess of solvent was added on the top of the silica gel bed. The column was gently tapped with soft materials to avoid the formation of air bubbles in the bed. The test solution was mixed with little silica gel and loaded to the column and eluted at a flow rate of 41drops/min with the mobile phase combination, which gave the best separation of compounds from TLC analysis.

GC-MS Analysis

The Clarus 680 GC was utilized as a part of the study with minor modifications. Fused silica column, packed with Elite-5MS (5% biphenyl 95% dimethylpolysiloxane, 30 m × 0.25 mm ID × 250µm df) and the components were separated using Helium as carrier gas at a constant flow of 1 ml/min. The injector temperature was set at 260°C during the chromatographic run. The 1µL of extract sample injected into the instrument the oven temperature was as follows: 60 °C (2 min); followed by 300 °C at the rate of 10 °C min^{-1}; and 300 °C, where it was held for 6 min. The mass detector conditions were: transfer line temperature 240 °C; ion source temperature 240 °C; and ionization mode electron impact at 70 eV, a scan time 0.2 sec and scan interval of 0.1 sec. The fragments from

40 to 600 Da. The spectrums of the components were compared with the database of spectrum of known components stored in the GC-MS NIST (2008) library.

Antifungal activity

Test fungi

Test fungi such as *A. flavus, C. cladosporioides, F. oxysporum* and *M. phaseolina* were isolated from soybean seed sample (JS 335) by standard blotter paper method (Lakshmeesha *et al*., 2014).

Blotter paper method

Three layers of blotting paper (9 cm diameter) were cut according to the size of petri plate, placed at the bottom and moistened with sterile distilled water. Excess water was drained-off and petri plates were autoclaved for 121°C for around 20 min. at 15 psi. Seed samples were surface disinfected with 1% sodium hypochlorite solution for about 2 min. at room temperature and placed 10 seeds per petri plates. The petri plates were incubated for seven days at 25±2°C in alternating cycles of 12 hours darkness and 12 hours light. After seven days of incubation, fungi were detected by their growth and spore morphology by following the keys outlined by various researchers (Barnett and Hunter, 1972, Watanabe, 2010).

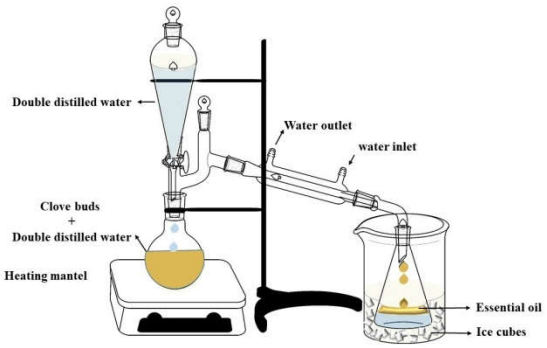


Figure 1 simple steam distillation unit

Poison-food method

Antifungal activity of *S. aromaticum* extract was determined by posion food method (Sridhar*et al*., 2003) as described earleir. Sabouraud agar supplemented with different concentrations (8 mg/ml, 4 mg/ml, 2 mg/ml and 1 mg/ml) was poured in petri plates under aseptic condition. After solidification, aseven-day-old mycelial disc of test fungi was transferred aseptically to the center of petri plates. The SDA media devoid of the extract served as control. Captan was used as positive control (50 µg/ml). All the treatments were replicated three times. The petri plates were incubated at 28±1°C for seven days. Fungal toxicity of the extract was measured after seven days of incubation period in terms of percent mycelial growth inhibition.

$$\text{Percentgrowth inhibition} = \frac{dc-dt}{dt} \times 100$$

Where dc = Average increase in mycelia growth in the control.
dt = Average increase in mycelia growth in the treatment.

Statistical analysis

Antifungal experiments data were analyzed by using univariate analysis. Observations were expressed as amean ± standard error, (n=3). Means were separated by Tukey's HSD multiple range test at 0.05 significant (P <0.05) using SPSS software (version 19).

RESULTS AND DISCUSSION

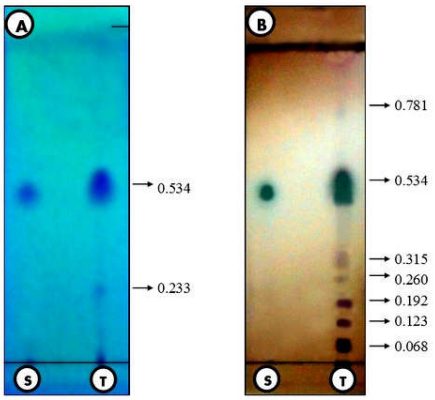
Phytochemical screening of *S. aromaticum* extracts showed that solvent extracts contain most of the phytochemicals such as tannins and terpenoids (Table 1).

Table 1 Phytochemical constituents in hexane extract of *Syzygium aromaticum.*

Sl. No:	Phytochemical test	Hexane extract
1	Alkaloids	
	a)Mayer's test	-
	b)Dragendorff's test	-
2	Flavonoids	
	a)Magnesium-HCl reduction	-
3	Tannins	+
4	Terpenoids	+

(+): Presence of phytochemical; (-): Absence of phytochemical.

Average yields of essential oil contents were calculated relative to thedry *S. aromaticum* buds. TheTLC profiling of these extracts yielded an impressive result that directs towards the presence of different types of phytochemicals with different R_f values in various solvent systems. The best solvent system was standardized and variation in R_f values of phytochemicals provided a very important hint in understanding their polarity and also helped in the separation of pure compounds by column chromatography. *S. aromaticum* extracts resolved bands were recorded in Table 2 and Figure 2 indicates the chromatogram of TLC plates.



Track S- Eugenol standard: Track T- Test solution

Figure 2 Thin layer chromatogram profile on hexane extract of *S. aromaticum* flower buds, (A) Under short wave UV 254 (nm) and (B) after derivatization in Anisaldehyde-Sulphuric acid reagent.

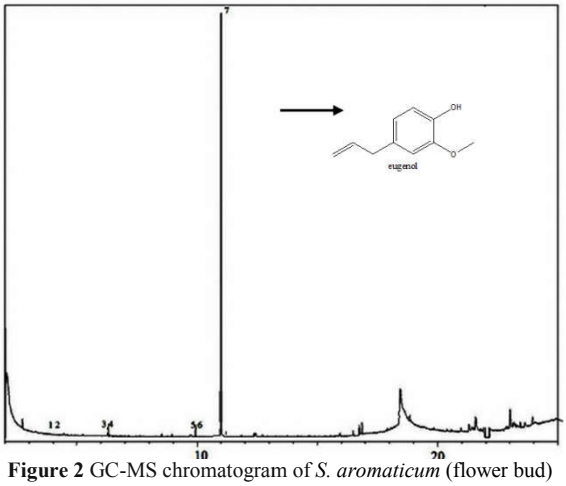


Figure 2 GC-MS chromatogram of *S. aromaticum* (flower bud) extractshowing eugenol as the major chemical constituent.

Table 2 TLC of hexane extract from *Syzygium aromaticum* flower buds under UV (254 nm and 366 nm) and after derivatization of chromatogram.

R_f value			
Short wave UV (254 nm)	**Long wave UV (366 nm)**	**After derivatization with anisaldehyde-H_2SO_4**	**Colour of the band after derivatization**
0.233	No bands detected	0.068	Dark violet
0.534 (matching with Eugenol standard)		0.123	Violet
		0.192	Violet
		0.26	Pink
		0.315	Pink
		0.534 (matching with Eugenol standard)	Purplish green
		0.781	Light blue

A band (R_f 0.534) corresponding to eugenol was visible in both the standard and test solution tracks. Upon derivatization with ananisaldehyde-H_2SO_4 acid reagent, seven bands with R_f 0.068, 0.123, 0.192, 0.260, 0.315, 0.534 (matching with standard eugenol) and 0.781 were visualized in *S. aromaticum* extract. The test sample subjected to GC-MS revealed the presence of eugenol (Figure 3a and 3b) as major peak from the GC-MS database (Table 3). Compounds were identified by comparison of retention indices for all the compounds with those reported in the literatur(Bullerman *et al*., 1977;Jennings, 2012). The mass of the test samples was compared with an authentic specimen and by matching their mass spectral fragmentation patterns with those stored in the spectrometer database, using NIST (2008) library.

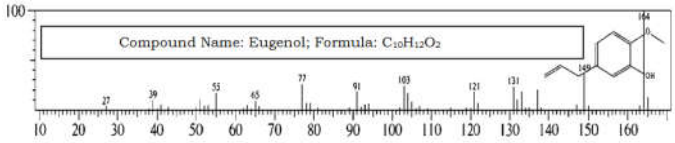


Figure 3 GC-MS analysis of *S. aromaticum* flower bud extract showing the major base peak at 164.

Table 3 Phytochemical compositions of *S. aromaticum* flower bud extract by GC-MS analysis.

Peak No.	R. Time	Relative %	Compound
1	2.731	0.2	Hexanal (CAS) n-Hexanal
2	4.447	0.04	Pentanoic acid (CAS) Valeric acid
3	5.237	0.02	1-Hexanol, 2-ethyl- (CAS) 2-Ethylhexanol
4	6.302	0.32	Nonanal (CAS) n-Nonanal
5	8.529	0.09	Nonanoic acid (CAS) Nonoic acid
6	8.952	0.06	1-methoxy-4-(1-E-propenyl) benzene
7	10.924	97.03	Eugenol
8	12.375	0.1	Decanoic acid (CAS) Capric acid
9	12.433	0.09	3-Heptanol, 2,6-dimethyl-
10	16.744	0.34	Hexadecanoic acid (CAS) Palmitic acid
11	16.858	0.31	Dibutyl phthalate

The results of poison-food technique at four different concentrations (8, 4, 2 and 1 mg/ml) of eugenol was tabulated in Table 4. Results represented in Table 4 indicated that eugenol showed broad-spectrum activity against the tested fungi (Figure 4-5).

Table 4 Antifungal activity of eugenol at different concentration on four soybean seed-borne fungi

Organism	Concentration of eugenol (mg/ml)					
	8	4	2	1	NC	PC
A. flavus	86.91±0.74^a	83.73±0.51^b	75.87±0.78^c	69.41±1.06^d	0	100
C. cladosporioides	84.2±0.93^a	79.41±0.49^b	72.73±0.28^c	67.066±0.11^d	0	100
F. oxysporum	100±0.0^a	100±0.0^a	92.48±0.60^b	88.63±0.46^c	0	100
M. phaseolina	100±0.0^a	100±0.0^a	91.74±0.93^b	85.953±0.85^c	0	100

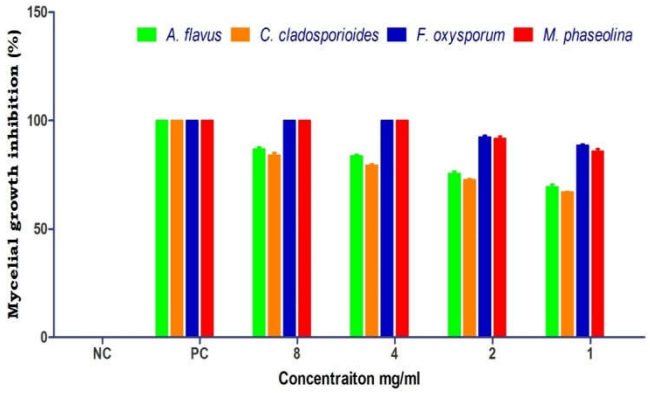


Figure 4 *In vitro* antifungal activity of eugenol on fourfungal isolates.

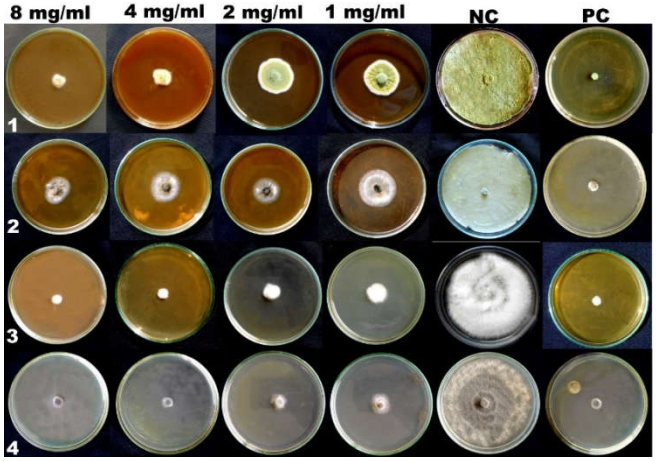


Figure 5 Inhibitory effect of eugenol on four soybean seed-borne fungi.

NC, Negative control: PC, Positive control: 1, *A. flavus:* 2, *C. cladosporioides:* 3, *F. oxysporum:* 4, *M. phaseolina*

The above mentioned readings were exclusive of the disc diameter. Observations were expressed as mean ± standard error, (n=3). The values followed by different alphabets differ significantly when subjected to Tukey HSD test, P value ≤0.05.

Two fungi *viz., F. oxysporum* and *M. phaseolina* was completely (100%) inhibited by eugenol 4mg/ml whereas *A. flavus* and *C. cladosporioides* showed 87% and 84% inhibition. This finding confirmed the documented antifungal activity ofmany plant extracts against *Fusarium* spp. These results are consistent with the previous reports on related fungi (Bakkali *et al*., 2008). Additionally, outcomes of the present research are in agreement with the study conducted by Rana *et al*., (2011) for the antifungal activity of *S. aromaticum* essential oil against *F. moniliforme, F. oxysporum,* and *Aspergillus* sps. and testified that these fungal species were inhibited by the oil when tested and highest sensitivity was reported for*F. oxysporum*.

The main chemical components of clove oils are eugenol, acetyl eugenol, iso-eugenol,and β-caryophyllene (Hemalatha *et al*., 2016;Sebaaly *et al*., 2016). These phenolic compounds are

responsible for the antibacterial and antifungal properties of essential oil (Omidbeygi *et al*., 2007). The oils with high levels eugenol are usually having strong inhibitory properties against *F. oxysporum, F. verticillioides* and *F. avenaceum*, *Macrophomina phaseolina* and, *Aspergillus flavus* and *Aspergillus versicolor* (Bilgrami *et al*., 1992; Dubey,1991; Lang and Buchbauer, 2012). The GC-MS data showed eugenol as the major constituent of clove oil and mass spectral data for eugenol showed the molecular ion at 164 which corresponds to the relative molecular mass of eugenol(Ayoola *et al*., 2008). The essential oil components examined and confirmed, including eugenol as toxic effects on the *in vitro* mycelium growth against several phytopathogenic and mycotoxigenic fungi such as *Penicillium, Fusarium, Aspergillus species and Alternaria alternate* (Abbaszadeh *et al*., 2014;Campaniello *et al*., 2010). The results substantiated that eugenol possessed good potential as natural antifungal additives to inhibit the growth of *A. flavus, M. phaseolina, F. oxysporum* and *C. cladosporioides*.

Essential oils derived from medicinal plants have a long history of use as natural antimicrobial agents and recently have been used in large quantities of pharmaceutical, food, industrial and cosmetic products since these oils effectively inhibit the growth of a wide range of pathogens. Exhaustive literature review demonstrated that *S. aromaticum* as a medicinal and aromatic plant with anenormous spectrum of pharmacological activities having considerable importance in agricultural and consumer products. *S. aromaticum* extract was successfully effective in suppressing *Macrophomina phaseolina* and *Fusarium oxysporum* growth *in vitro* effectively. This extract could be promising as a source of natural eco-friendly fungicidal compounds for *in vivo* applications. This research emphasizes the potential of *S. aromaticum* be used as new fungicidal agents and also provides sufficient baseline information for future works and commercial exploitation.

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