RESEARCH ARTICLE

Molecular characterization and evaluation of antimicrobial activity of essential oils derived from *Syzygium aromaticum*

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Abstract

Objective: This work is designed to study and evaluate the antibacterial potency of phytochemicals from *Syzygium aromaticum* (clove) contrary to foodborne pathogens. In the present research, the extract of *S. aromaticum* was characterized using thin-layer chromatography, gas chromatography–mass spectrometric (GC–MS), and Fourier-transform infrared (FT-IR). **Methods:** The extraction was done by hydrodistillation process and the existence of essential oils was detected by analytical and chromatographic techniques. The conformation of the presence of eugenol in the extracted sample was achieved with high-performance thin-layer chromatography in comparison with the standard. The extract was subjected to GC–MS and FT-IR for quantification and structure prediction, respectively. Antibacterial assay was performed with different concentration of clove extracts (25%, 50%, and 75%) against different foodborne pathogens such as *Escherichia coli, Listeria monocytogenes, Bacillus cereus, Staphylococcus aureus, Klebsiella oxytoca,* and *Staphylococcus epidermidis*. ANOVA analysis with single factor was carried out to determine the significance of procured results. **Results:** The existence of higher concentration of eugenol along with caryophyllene and alpha-caryophyllene in the clove extract was manifested with GC–MS analysis. FT-IR analysis determined the major functional moieties and also confirmed the structure of bioactive compounds. After 24 h, the clove extract has shown to have antibacterial activity against all bacteria used in this study except for *S. epidermidis*. *P* value was found to be <0.05 which revealed the statistical significance of results. **Conclusion:** From this investigation, it is appraised that clove extract using the organic solvent dichloromethane has effective antibacterial property over foodborne pathogens. This study illustrates that organic compounds present in *S. aromaticum* could be an potent barrier against the microorganism tested and also could be used for the advancement in developing different drugs in Pharma industries.

Key words: Eugenol, Fourier-transform infrared, gas chromatography–mass spectrometric, high-performance thin-layer chromatography, hydrodistillation, *Syzygium aromaticum*, thin-layer chromatography

INTRODUCTION

The plant products are holding their priority in today's pharmaceutical industry. The natural medicines take the head because of the reaction caused by the synthetic medicines in our system. Nearly, one-third of the synthetic medicines have been replaced by natural drugs and still researchers are behind new plant products.[1] Almost every plant part produces bioactive compounds called phytochemicals. These phytochemicals incorporate with other nutrients formed the guard organization across the discrete number of diseases and environmental factors.[2] Amino acids, sugars, proteins, and chlorophyll are regarded as primary metabolites. All other phytochemicals such as alkaloids, flavonoids, tannins, terpenoids, saponins, and essential oils are classified under secondary metabolites. All the above quoted compounds are synthesized in greater number of plants which are recognized for their therapeutic activity.[3]

Syzygium aromaticum (cloves) is the flower bud of a tree associated to the genealogy of *Myrtaceae*. The tree raise

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Received: 23-12-2017
Revised: 12-01-2018
Accepted: 12-02-2018

up to 8–12 m tall, with broad leaves and crimson flowers associated into bunch at terminal end. Cloves are frequently used as spices in different cuisines such as Asian, African, and Middle East. Cloves are observed as time-honored biologic medication for local analgesics, anthelmintics (antiparasitic),[4] and in dental treatment. Topical administration of clove extract over the bowel region is used to cure abdominal cramps. The preeminent component in clove extract is eugenol (70–90%). The IUPAC name of eugenol is 4-allyl-2-methoxyphenol with molecular formula $C_{10}H_{12}O_2$. It has boiling point of 254°C. Eugenol combine with zinc oxide is used in filling dental cavities.[5,6] Additional to eugenol, cloves contain acetyl eugenol, beta-caryophyllene, and vanillin. Cloves also contain some non-volatile substances such as tannins (10–13%), triterpenes (2–4%), sterols, and flavonoids such as isobiflorin and biflorin.[7] Besides, it also shows better antifungal property against *Candida aspergillus* and *Dermatophyte* species.[8] The present study focuses on developing new remedy for foodborne diseases and also providing an alternative for synthetic food preservatives.

MATERIALS AND METHODS

Collection and Preparation of Sample

Clove flower buds (*S. aromaticum*) were purchased from local market in Sathyamangalam. About 50 g of cloves was measured and crushed using mortar and pestle. The powdered cloves were stored in airtight container for future use.

Extraction of Essential Oil from Clove

The components present in clove are said to be a volatile compound and are extracted using steam distillation.[8] Initially, 10 g of powdered cloves was measured and 80 ml of dist. H_2O was added to the round bottom flask of the steam distillation apparatus, and it is carried out for 2 h. About 20 ml of dist. H_2O was added every 30 min to avoid burning of the sample. The distillate was collected and then subjected to liquid-liquid extraction. Liquid-liquid extraction was performed by adding three portions of 15 ml of dichloromethane (DCM) to 50 ml of distillate in the separating funnel as per standard protocol. The collected extract was dehydrated using anhydrous sodium sulfate.[7] Then, the extract is dealt with rotary evaporator at 40°C to evaporate the solvent and the pure extract was concentrated.

Analysis of Clove Extract through Thin-layer Chromatography (TLC)

TLC was performed to analyze the clove extract in correlation with the standard protocol. TLC *silica* gel plate used is of 1mm with aluminum sheet cover. Sample extract was made by dissolving 30 µl of sample in 70 µl of dichloromethane. With the help of capillary pipettes, the sample and the standard were applied in TLC plate. The starting line of the sample and the standard was 2 cm from radical and 1 cm from the sides of the TLC sheets. Then, TLC sheets were developed with hexane and acetone in the ratio of 9:1.[9] After 30 min, the TLC sheets were examined under ultraviolet (UV) of short wavelength.

$$R_f \text{Value} = \frac{\text{Distance from the starting point to the center spot of the sample}}{\text{Distance from the starting point to the solvent moved at the end}}$$

High-performance Thin-layer Chromatography (HPLC) Technique for Detection of Bioactive Compound

Analysis of clove oil for the detection of bioactive components carried out through HPLC (Agilent) using C18 column with isocratic type pump, and Empower software is used for data analysis mobile phase: Methanol:water: acetonitrile (10:40:50), flow rate of 0.7 ml/min, UV detection range:280 nm. About 20 µl of sample injected through injector loop and analyzed for 30 min.[10]

Gas Chromatography–mass Spectrometric (GC–MS) Analysis for Determination of Essential Oil from *S. aromaticum*

The sample analysis was performed to detect the existence of essential oils in clove extract using GC–MS (Perkin Elmer model: Clarus 680) and also it is equipped with mass spectrometer (Clarus 600 (EI) analyzed using (TurboMassver 5.4.2) software. This equipment employed an integrated *silica* column, packed with Elite-5MS (5% biphenyl 95% dimethylpolysiloxane, 30 m × 0.25 mm ID × 250 µm df) and helium is regarded as a carrier gas to separate the components with steady flow rate of 1 ml/min.

During chromatographic run, the temperature for GC–MS column was entrenched at 260°C. Sample of about 1 µL is injected to the instrument where the oven temperature is fixed by 300°C at which it is increased at the rate of 10°C/min from the initial temperature of about 60°C. At 300°C, column is retained the temperature for 6 min.

Mass detector settings were noted as transfer line temperature of about 240°C, ion source temperature as 240°C, and ionization mode electron impact set as 70 eV. The chromatogram obtained from GC–MS study was related with the known database of components which is stored in the GC–MS NIST (2008) library.

Structural Prediction of Essential Oils Components using Fourier-transform Infrared (FT-IR)

Extracted sample was blended with KBr in the ratio of 1:9 and made into a pellet in the KBr hydraulic press of pressure about 10 tons. The FT-IR was used to analyze the functional moieties in the pellet which was put up in the sample holder in the range of 4000–400/cm.

Antibacterial Activity of Dichloromethane Clove Extract

Antibacterial assay of *S. aromaticum* extract (eugenol) contrary to foodborne organisms was done by well diffusion method.[11] The foodborne pathogens used to resolve the antibacterial efficacy of eugenol were *Bacillus cereus, Staphylococcus aureus, Listeria monocytogenes, Klebsiella oxytoca, Escherichia coli, and Staphylococcus epidermidis*. The bacterial strains were obtained as glycerol stock and cultured in brain heart infusion broth and subcultured periodically. The cultured bacteria were plated in nutrient agar and wells were made using gel puncture onto the agar for analyzing the effectiveness of the extract on the bacterial strains. The clove extract was added into the wells in different concentration (25%, 50%, and 75%). Control was made without adding eugenol in the well. After certain fecundation period of 24 h at 37°C, the zone of inhibition was observed.

Statistical Analysis of DCM Extract

Data obtained from the result was grouped and evaluated using statistical formula. For retrieved values, mean and standard deviation were calculated. ANOVA test for the single factor is done using excel document. Biologically, $P < 0.05$ was noted to be significant and correspond to that mean and standard error value were noted.

RESULTS AND DISCUSSION

The clove extract (15 ml) was obtained as a result of steam distillation with 50 g of clove powder followed by liquid-liquid extraction using DCM. TLC is used to examine the existence of desired compound in the extract. R_f value is calculated for sample (right) and standard (left) and it was seen 0.43 and 0.45, respectively [Figure 1]. From the R_f value, it is able to affirm the existence of the target compound in the sample, and it is correlated with the currently available report[11] which gives us a clear view for proceeding future studies. The dichloromethane extract was analyzed using HPLC and its retention time found to be 14.2 min which confirms the presence of essential oil eugenol comparing with the standard protocol.[10]

S. aromaticum extract was interpreted using GC–MS. In this instrumental analyze, sample was separated using column Elite-5MS. The peak obtained was analyzed with the help of NIST (2008) library. This analysis revealed the presence of three compounds such as eugenol, caryophyllene, and alpha-caryophyllene in the extract [Figure 2]. Among these, eugenol (83.785%) was recognized to be present in large amount compare to others such as caryophyllene (5.191%) and alpha-caryophyllene (11.024%) with respect to their individual retention time as 11.657, 11.847, and 13.268 min, respectively. The structure for the above molecules is reported in Table 1. The extraction process also carried out by supercritical fluid extraction method as reported,[12] but this possess some disadvantages when compared with solvent extraction which is considered more economical. Essential oil components procured from clove have the habit of inhibiting the action of enzymes, especially in Type 2 diabetes disorder.[13] Therefore, eugenol is regarded as the elementary source in clove, which exists in high amount in the extract. Many literatures provide us detailed study on antimicrobial and antioxidant activity of eugenol but against limited pathogens.

Figure 1: Thin-layer chromatography analysis of the presence of EO in clove extract

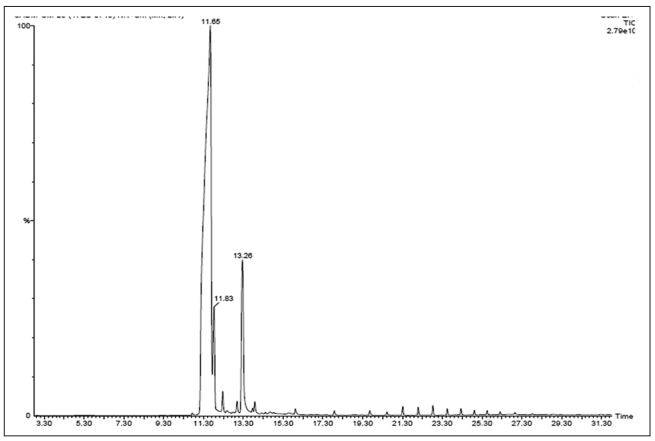
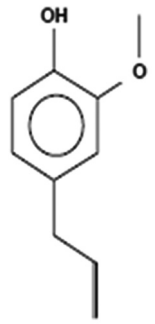
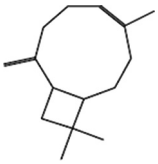
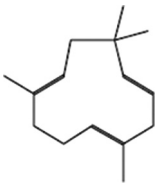


Figure 2: Gas chromatography–mass spectrometric chromatogram for analysis of extract from clove

Table 1: Structural prediction of bioactive components from GC–MS analysis of *S. aromaticum* extract

Retention time	Compound name	Molecular formula	Molecular weight	Area %	Structure
11.657	Eugenol	$C_{10}H_{12}O_2$	164	83.785	
11.847	Caryophyllene	$C_{15}H_{24}$	204	5.191	
13.268	Alpha Caryophyllene	$C_{15}H_{24}$	204	11.024	

GC–MS: Gas chromatography–mass spectrometric, *S. aromaticum: Syzygium aromaticum*

The FT-IR results were retrieved and analyzed with regard to the wave numbers provided in the graph. Accordance with the wave number, their functional group was noted. This will provide us the structural properties of the above-mentioned phytochemical components [Figure 3]. The wave number (cm^{-1}) retrieved from the clove sample was noted such as 1037, 1624, 2368, 2874, and 2930 which confirms the existence of various groups such as alcohols, ether, ester (C-O), carboxylic acids (O-H), anhydrides, alkanes (C-H), amide (C=O), and alkenes (C=C). The above values and their functional moieties were determined with the aid of introduction to spectroscopy written by Donald *et al*.[14] From obtained FT-IR spectrum, numerous studies were conducted with the help of Booyens and Thantsha[15] and Devi *et al*.[16] to evaluate the nature of eugenol sample spectrum with standard.

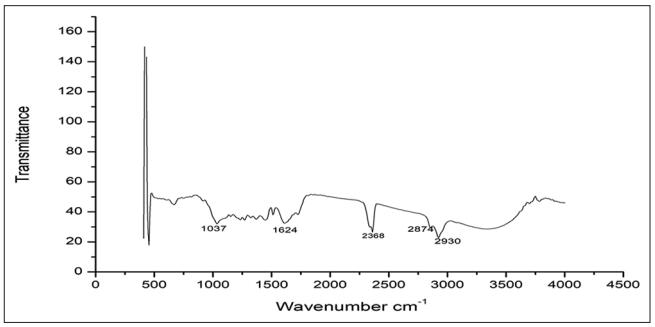


Figure 3: Fourier-transform infrared spectra for analysis of clove sample

The extract of *S. aromaticum* was used to explore the response of eugenol on various foodborne organisms such as *B. cereus, S. aureus, L. monocytogenes, K. oxytoca, E. coli,* and *S. epidermidis*. Distinct eugenol concentrations such as 25%, 50%, and 75% were added to the prepared well in the agar plates. Antimicrobial activity of eugenol contrary to *Helicobacter pylori* was earlier reported.[17] Antifungal activity was also evaluated and interpreted for eugenol.[18] Effect of eugenol and other essential oils contrary to distinct pathogens were assessed; recent review work[19] gives exhaustive perspective on numerous process of evaluating the antimicrobial activity of eugenol and eugenol containing essential oils by disc diffusion, broth dilution, and agar diffusion methods. Zone of inhibition for each of the given concentration was measured, and it was found that among six selected organisms five showed susceptibility toward eugenol, only one showed resistance [Figure 4]. The action of inhibition varying with given concentration was found [Figure 5].

Statistical analysis of the DCM extract furnishes the satisfactory results which revealed the procured *P* value from the ANOVA table found to be <0.05. Student's *t*-test of the antibacterial activity in distinct concentration of the clove extract provides the significant results at 5% level of difference.

CONCLUSION

From this study, it is concluded that the organic solvent DCM gives the better extraction of essential oils from

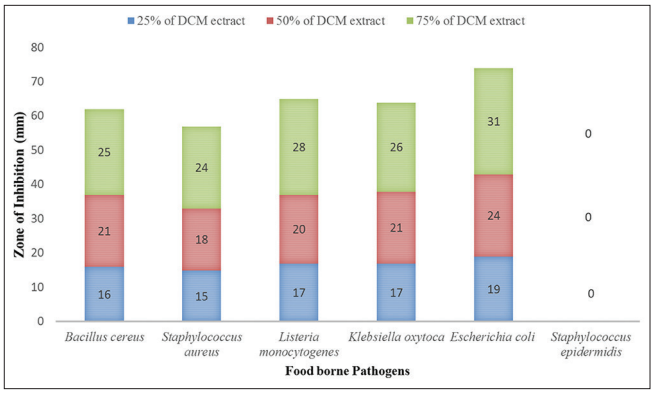


Figure 4: Graphical representation of antimicrobial activity of clove extract

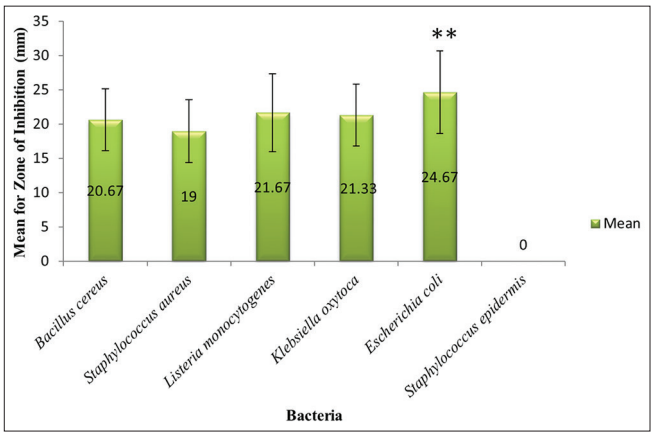


Figure 5: Overall representation of activity of clove extract with their error bar

S. aromaticum. The extract provides better result compared to the other solvents. GC–MS and FT-IR results procured from this extract provide the confirmation of higher concentration of eugenol. Hence, this research exhibits that DCM extraction regarded as the best method for the separation of essential oils components. Functional moieties obtained from FT-IR spectrum provide clear view about the existence of the desired compound in the extract. The extracted solution was subjected to evaluate the antimicrobial activity contrary to foodborne pathogens in distinct concentrations. In this evaluation, *S. epidermidis* showed resistance toward the extract than other strains. Therefore, this investigation affords the detailed view on the effect of eugenol against a variety of pathogens and in the development of various strains toward susceptibility in the environment against these naturally derived components.

ACKNOWLEDGMENT

This research is a part of B. Tech Project work of the second, third, and fourth authors. Authors gratefully acknowledge AICTE, New Delhi, No 20/AICTE/RIFD/RPS (Policy-1) 28/2012-13 sponsored Molecular Diagnostics and Bacterial Pathogenomics Research Laboratory, Department of Biotechnology and Bannari Amman Institute of Technology, for providing an ambient environment for the successful completion of the project. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed. No writing assistance was utilized in the production of this manuscript.

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Source of Support: Nil. **Conflict of Interest:** None declared.