Journal of Bioresource Management

Volume 9 | Issue 1 Article 3

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Recommended Citation

Kanwal, A., Irshad, S., Akmal, N., & Rizwi, N. B. (2022). Identification and Characterization of Active Ingredient Eugenol from Syzygium Aromaticum (Clove Oil) through HPLC and its Phytochemical Analysis, *Journal of Bioresource Management, 9* (1).
ISSN: 2309-3854 online
(Received: Jul 12, 2021; Accepted: ; Published: Feb 8, 2022)

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Cover Page Footnote

We are extremely grateful to Mr. Waheed U Zamaan from institute of Chemistry, University of the Punjab, for their assistance in oil extraction and HPLC analysis of clove oil.

IDENTIFICATION AND CHARACTERIZATION OF ACTIVE INGREDIENT EUGENOL FROM *SYZYGIUM AROMATICUM* (CLOVE OIL) THROUGH HPLC AND ITS PHYTOCHEMICAL ANALYSIS

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ABSTRACT

Clove, *Syzygium aromaticum*, is one of the most valuable, ancient and premium essential oil which has been used as source of spice in agro-food industry and therapeutics for centuries. The aim of the present study was to analyze biological activities of clove oil as well as characterization of its active component Eugenol to make it worthwhile for different food and pharmaceutical formulations. Clove oil extraction was performed by Soxhlet method. Its phytochemical analysis showed the presence of carbohydrates, flavonoids, coumarins, steroids, saponnins, and tannins while proteins and leucoanthocyanins were absent in it. DPPH (2, 2-diphenyl-1-picrylhydrazyl) scavenging assay was performed which showed an increase in percentage inhibition with an increase in concentration of clove oil which confirmed its antioxidant property. Antimicrobial activity of clove oil was tested against three gram positive strains *Bacillus subtilis, Staphylococcus aureus* and *Bacillus thuringiensis* and one gram negative strain *Escherichia coli* by well diffusion and disc diffusion methods. *E.coli* presented largest zone of inhibition. T-test was applied for statistical analysis of antimicrobial activity. P-value obtained was 0.0215 which is <0.05. Aqueous clove nanoparticles were made and were found to have antimicrobial activity against *E.coli* and *B.subtilis*. Correlation coefficient (R2) through HPLC was found to be 0.973531. Quantitative estimation showed the presence of 740 ppm eugenol. These properties of clove proved it as a valuable spice in pharmaceutical and agro-food sector.

Keywords: Clove oil, eugenol, HPLC, antimicrobial activity, antioxidant activity.

INTRODUCTION

Essential oils are very engrossing plant products, synthesized as a result of secondary metabolism and possess a wide range of biological properties. These are usually characterized by their complex nature, volatility, strong odour, rare colour and solubility in organic solvents. Aromatic plants native to temperate to warm climate, are main source of essential oils. Different parts of plants such as stem, fruit, root, bud, leaves, seed and bark synthesize essential oils whereas secretary cells, epidermis cells and canals store them (Bhowmik et al., 2012). Oil composition, quantity and quality depends on soil structure, plant organ, plant age, climate and vegetative cycle stage (Angioni et al., 2006).

Broad range of applications has been documented for essential oils. Constituents of essential oils are responsible for their unique characteristics e.g. bactericidal, fungicidal, antiviral, anti-inflammatory and antioxidant activities (Purkait et al., 2020). Essential oils are used as preservatives in agro-food industry thereby help in overcoming the growing problem of food spoilage by food borne pathogens. They have not only resolved the social and economic challenges

associated with food spoilage but also proved to be a good replacement of artificial and chemical preservatives. More interest is being developed by scientists toward these natural alternatives because there are many safety concerns related to use of chemical preservatives (Purkait et al., 2020).

Clove, *Syzygium aromaticum*, is one of the most valuable, ancient and premium essential oil, belonging to family Myrtaceae. "Clove" because of its resemblance with nails, derives its name from Latin and French terms *clavus* and *clou* respectively, both of which means "nail". Cloves are dried and closed flower buds of an evergreen medium-sized tree about 10-20m tall with life span upto 100 years (Cortés-Rojas et al., 2014).

Clove oil is an important constituent of cuisines as a flavouring agent in bakery items, confectionary goods, pickles, meats, candies and chewing gums. It also finds its applications in manufacturing perfumes, soaps, toothpaste, and clearing agents (Ju et al., 2018). In dried form, cloves have been used as an ingredient of curry powder, a famous spice mixture of Asian cuisine. Cooking liquids for poultry and meat is also flavoured by whole or ground cloves. Powdered clove is also a source of strong characteristic aroma in Indonesian tobacco "Kretek". While oil form of clove is characterized as a clear yellow liquid that becomes brown with the passage of time (Chomchalow 2001).

Clove is known to be used as a natural antiseptic, antimicrobial, antifungal and antiviral agent. For these properties of clove oil it is used to treat various pathological conditions such as vomiting, nausea, chronic rheumatism, intestinal gas, inflammation of gums and throat. It can also be used as a counter irritant for pain. Pain in muscle cramps and some nerve conditions can also be relieved by using clove oil (Bhowmik et al., 2012).

An active and major component of clove oil is Eugenol, a phenylpropanoid. It is characterized as a pale-yellow liquid having solubility in water but more soluble in organic solvents such as methanol and ethanol. It has specific gravity of 1.531-1.054 (Kamatou et al., 2012). Oil extracted from different parts of clove plant possesses different quantities of eugenol. 60-90% eugenol is present in bud oil, 82-88% in leaf oil whereas 90-95% eugenol is present in stem oil (Kaur and Kaushal 2019). Eugenol possess a wide range of properties such as antimicrobial, antifungal, anti-carcinogenic, anti-oxidant, antibacterial, antiviral as well as anti-mutagenic and anti-inflammatory (Kaur and Kaushal 2019).

In plant extracts, the main candidates possessing antioxidant potential are phenolic compounds. This is because of their chemical structure and oxidation-reduction (redox) potential that helps them to scavenge free-radicals. As *Syzygium aromaticum* and other *Syzygium sp;* are rich in phenolics therefore these are good DNA protectors against oxidative stress (Cui et al., 2018). The chemical profile of clove extract obtained from flower buds of *Syzygium aromaticum*, is largely determined by agronomy and climate of region where it is grown, its processing as well as its storage conditions (Parthasarathy et al., 2008).

The current study has been performed to investigate anti-microbial and anti-oxidant properties of clove extract in order to explore its potential uses in cuisines and therapeutics on scientific basis. For the first time, characterization of Eugenol obtained from bud of clove plant, has been performed through High Performance Liquid Chromatography (HPLC). In future, formation of clove nanoparticles would provide a green and non-energetic route for synthesis of silver nanoparticles. This may be followed up to explore their use as a non-toxic means of cure in therapeutics beyond its typical uses in agro-food sector (Franklyne et al., 2019).

MATERIALS AND METHODS

Cloves were obtained during the months of September-October from different trees present in Lahore region of Pakistan. Clove oil extraction was performed using Soxhlet method. 30 g clove powder was taken and 250 ml of absolute ethanol was used. Extraction procedure was carried out for about 6 hours then concentrated by evaporating the ethanol through Rotary Evaporator at 78°C.

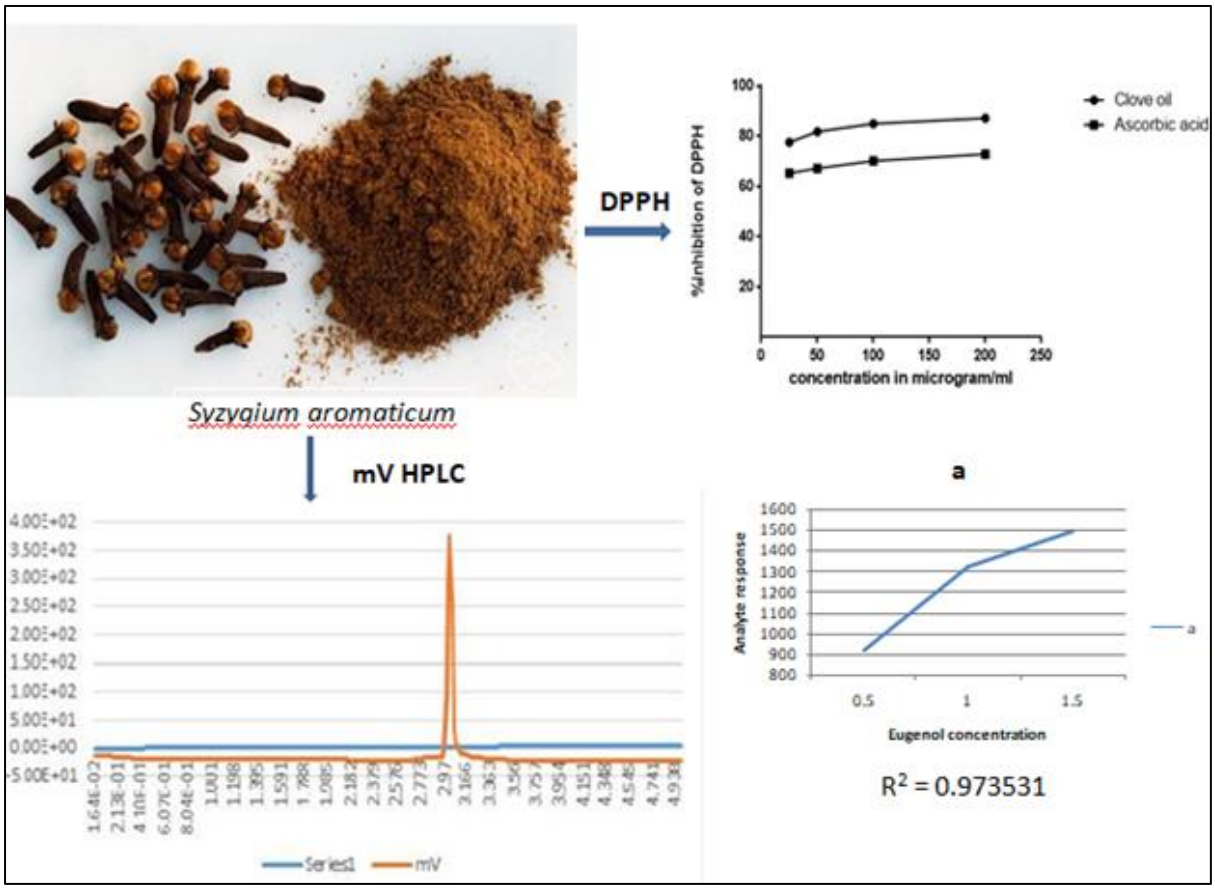

Figure 1: Graphical representation of methodology.

Benedict's test, Xanthoproteic test, lead acetate test, isoamyl alcohol test, alkali test, Salkowski Test, foam test and lead acetate test were used to check the presence of carbohydrates, proteins, flavonoids, leucoanthocyanins, coumarins, steroids, saponnins, steroids, and tannins respectively.

Bromine water, $KMnO_4$ solution and $FeCl_3$ solution was used to check the presence of eugenol in clove oil. 0.002% working solution of DPPH (2,2-diphenyl-1-picrylhydrazyl) was prepared in methanol with different concentrations of clove oil using ascorbic acid as standard. Absorbance was taken at 517nm and used following formula to calculate % inhibition.

% inhibition of DPPH activity = [(OD of control – OD of sample) / OD of control] *100

Disc diffusion and well diffusion methods were used to check antimicrobial activity. Glycerol stocks were activated on Lauria Bertani agar and used to make working plates for further use. Bacterial colony was swabbed on Lauria Bertani agar using sterile cotton swab. Clove oil was applied on autoclaved filter paper discs or wells punctured in Lauria Bertani agar in disc diffusion and well diffusion methods respectively. Plates were then sealed with parafilm and incubated at 37°C overnight. After incubation, zone of

inhibition was measured.

For anti-microbial activity, 10 grams of clove powder was dissolved in 50ml of autoclaved distilled water. Mixture was then boiled for 2 minutes in water bath and filtered by using whatman filter paper. 50ml of 1m M of silver nitrate solution was prepared and heated until it starts boiling. Then 10ml of prepared aqueous clove solution was mixed with 50ml of silver nitrate solution and incubated for 1 hour at room temperature. After synthesis, their antimicrobial activity was tested against bacterial strains by well diffusion method.

Methanol extract of clove (100 mg/ml) along with three dilutions (0.5µl/ml, 1µl/ml and 1.5µl/ml) of standard eugenol were prepared in HPLC grade methanol. In order to inject the sample solution in HPLC system, an appropriate dilution of 2µl/ml was prepared in HPLC grade methanol. Linearity was determined by means of calibration graph. The calibration graph was plotted over different concentration ranges. The absorbance of each analyte was determined at 215nm. The regression equation was calculated by constructing a calibration curve.

RESULTS

Phytochemical analysis of an aqueous extract of clove showed the presence of carbohydrates, flavonoids, coumarins, steroids, saponinns, steroids, and tannins in clove while proteins and leucoanthocyanins were absent in it as shown in table 1.

Table 1: Phytochemical analysis of Syzygium aromaticum (for identification).

Phytochemicals	*Syzygium aromaticum*
Carbohydrates	Present
Protein	Absent
Flavonoids	Present
Leucoanthocynins	Absent
Coumarins	Present
Steroids	Present
Saponinns	Present
Tannins	Present

Presence of eugenol was confirmed by Bromine water, $KMnO_4$ solution and $FeCl_3$ solution tests as shown in table 2.

Table 1: Qualitative analysis of eugenol in clove oil.

Tests	Results
Bromine Test	Positive
Potassium permanganate ($KMnO_4$) test	Positive
Iron chloride ($FeCl_3$) test	Positive

Percentage inhibition was checked against different concentrations of clove oil and ascorbic acid. Significant DPPH scavenging activity has been shown by *Syzygium aromaticum* (88 %) with 200µl/ml when compared with standard ascorbic acid as shown in figure 1.

The antimicrobial activity of clove oil was determined by agar well diffusion and disc diffusion assay against three gram positive bacterial strains *Bacillus subtilis, Staphylococcus aureus* and *Bacillus thuringiensis* and one gram negative bacterial strain i.e. *Escherichia coli*. Largest zone of inhibition i.e 2cm has been found with *E.coli* as shown in figure 2.

After incubation period, the formation of silver nanoparticles was indicated by change in colour of aqueous extract of clove with silver nitrate solution as shown in figure 3.

Nanoparticles formed by aqueous extract of *Syzygium aromaticum* showed zone of inhibition against *Escherichia coli* (0.7cm) and *Bacillus subtilis* (1cm) by Well diffusion method as indicated in figure 4.

Total Chrome 6 software was used to obtain chromatographic peaks of sample used. Data obtained from HPLC graph was computed using MS Excel and their peak areas were calculated. Results of HPLC of sample i.e. methanolic extract of clove as well as different dilutions of standard i.e. eugenol are shown in figure 5.

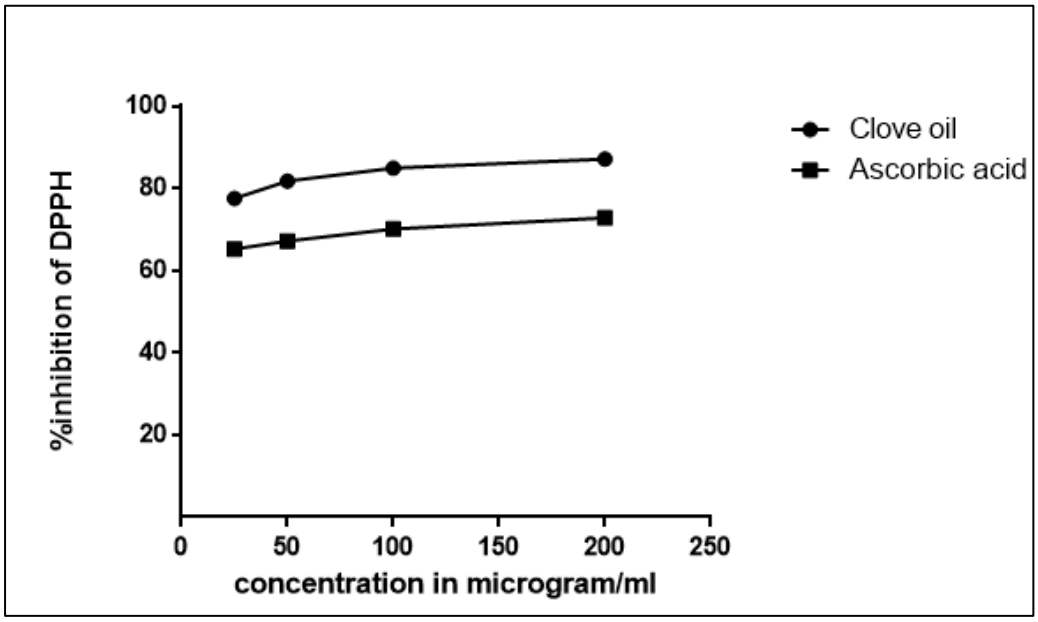

Figure 1: Significant DPPH scavenging activity has been shown by *Syzygium aromaticum* (88 %) with 200µl/m decreases with decreasing concentration when compared with standard ascorbic acid.

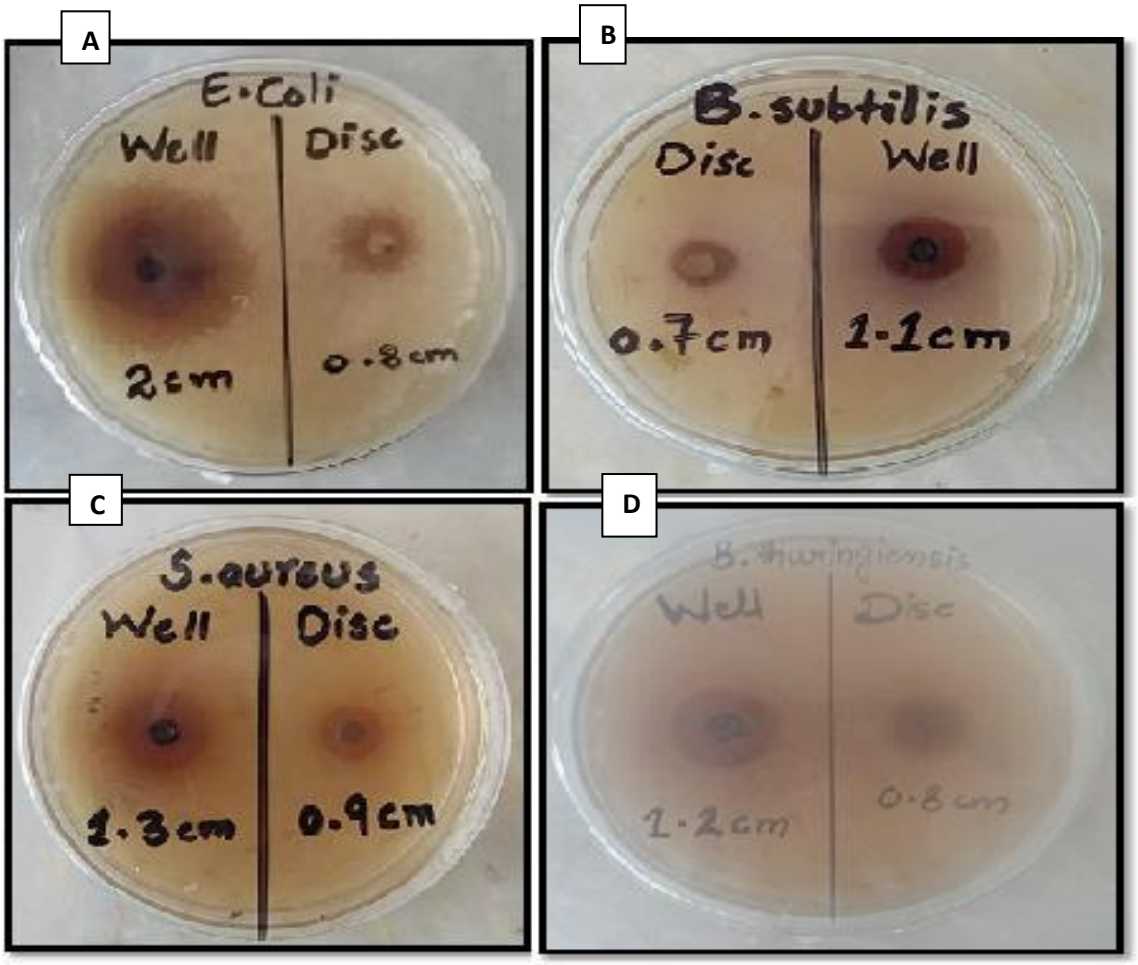

Figure 2: Zones of inhibition by clove oil against (A) *Escherichia coli* (B) *Bacillus subtilis,* (C) *Staphylococcus aureus* and (D) *Bacillus thuringiensis.*

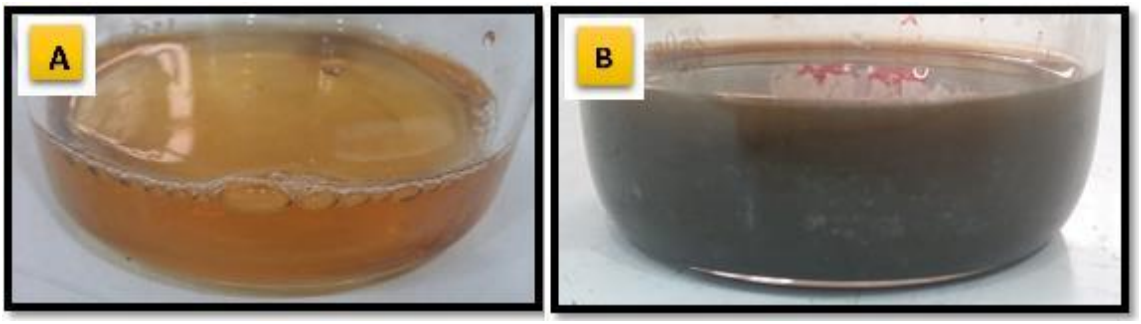

Figure 3: Before incubation period, light yellow colour (A) shows absence of silver nanoparticles. After incubation period (B) dark brown colour indicates presence of silver nanoparticles.

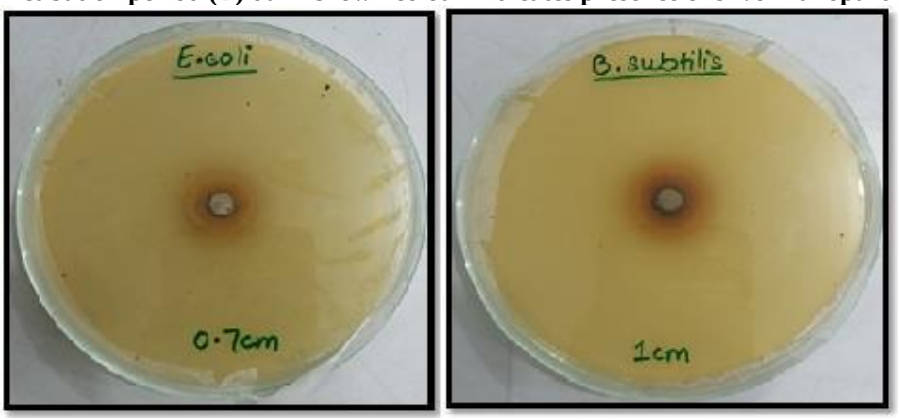

Figure 4: Nanoparticles formed by aqueous extract of Clove showing zone of inhibition against Well diffusion method; *Escherichia coli* (0.7cm) and Bacillus subtilis (1cm).

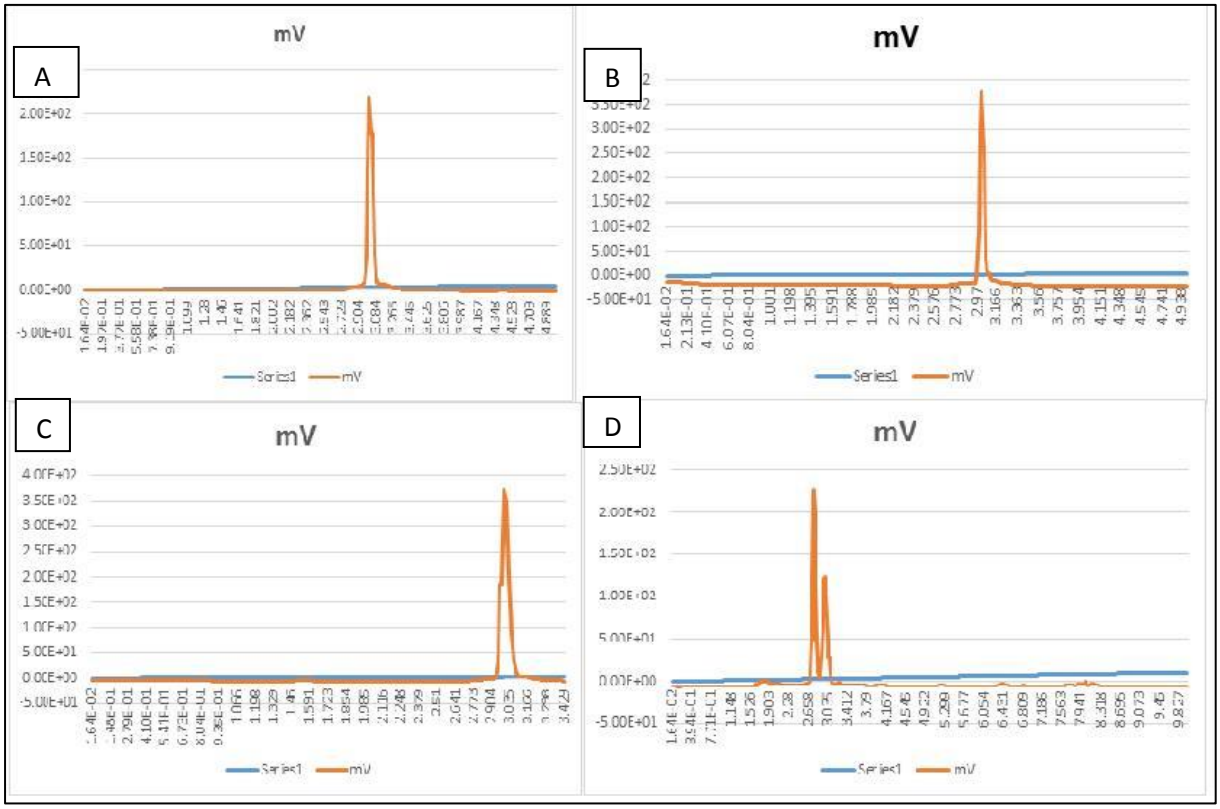

Figure 5: HPLC graph of (A) Standard dilution 1 i.e. (0.5µl/ml), (B) Standard dilution 2 i.e. (1µl/ml), (C) Standard dilution 3: (1.5µl/ml), (D) sample.

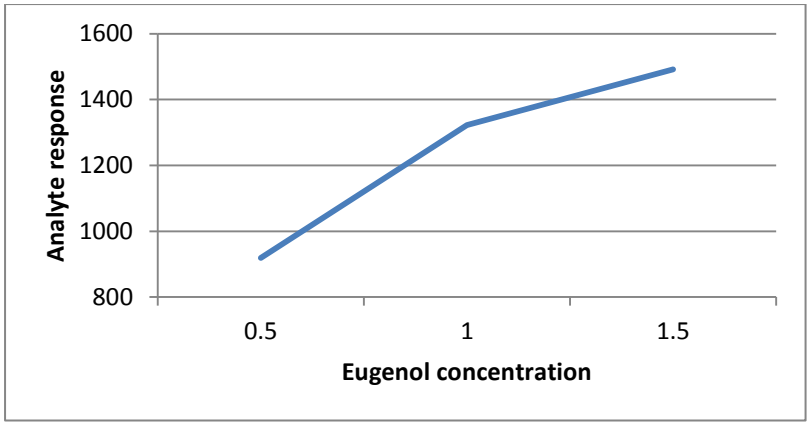

Figure 6: Linearity plot of eugenol.

A good linearity was successfully achieved in concentration range of 0.5µl/ml to 1.5µl/ml. Using MS Excel, correlation coefficient was found to be 0.973531. Relative peak area of sample was used to quantify eugenol in clove formulation used. Quantitative estimation showed that 740 ppm eugenol was present in clove sample used (Figure 6).

DISCUSSION

Different spices are added in food items for flavor enhancement and to ensure long term storage without being spoiled. In current study, a well-known spice clove (*Syzygium aromaticum*) is studied for its several characteristics. Chemical composition, colour and flavor of clove essential oil depends on climate, cultivation techniques, nature of soil, genetic factors and extraction methods (Kaur and Kaushal 2019). Anti-oxidant properties of clove oil were checked by its phytochemical analysis which showed the presence of carbohydrates, flavonoids, coumarins, saponinns, steroids, and tannins whereas presence of carbohydrates, steroids and tannins (Tambe and Gotmare) and tanins, terpenoids, steroids and sterols have already been reported (Kumar et al., 2018). Glycosides, ketones, carbohydrates, aldehydes, and forty- six phenolic compounds are also present in clove oil (Purkait et al., 2020) whereas coumarins have been found in significant quantity in cigrattes synthesized with clove oil (Kumar et al., 2018). Saponins, phytosterols, tannins, glycosides, anthocyanins and emodins are present in smaller quantities (Kadam et al., 2015). Tests for proteins and leucoanthocyanins showed negative results in current study as previously reported (Kumar et al., 2018).

Qualitative tests confirm presence of active ingredient of clove oil Eugenol in *Syzygium aromaticum*as indicated in previous years (Cui et al., 2018). In the present study, antioxidant activity for different concentrations of clove oil was measured taking ascorbic acid as standard. It was clearly observed that increase in concentration of clove oil cause a decrease in absorbance with constant increase in percentage inhibition. Percentage inhibition of different concentrations of clove oil was found to be higher as compared to corresponding concentrations of ascorbic acid as reported by Mohammed and coworkers (Franklyne et al., 2019).

Human DNA protection effect of clove oil was observed against oxidative damage. In present study, intensity of DNA band that was first damaged and then treated with clove was higher than that of untreated damaged DNA. This higher intensity of DNA band implicated that clove oil has very strong DNA protection ability (Ju et al., 2018).

Antimicrobial activity of clove oil showed largest zone of inhibition with *E.coli* whereas previously, largest zone of inhibition was found against *Bacillus*

subtilis (Kumar et al., 2018). Synthesis of amylase and protease in certain bacterial cells is inhibited by eugenol in clove oil ultimately leading to cell death, cell-wall disruption and hindering of enzyme action (Purkait et al.,, 2020).

Aqueous extract of clove was used to make its nanoparticles. Mixing of clove aqueous extract with silver nitrate solution, cause the formation of nanoparticles by reducing Ag^{+} ions to Ag^{0}. Mixing of aqueous extract of clove with silver nitrate solution is followed by incubation that cause change in color of solution from yellow to dark brown (Tambe and Gotmare).

Presence of carbohydrates, sapponins and tannins revealed its affinity for use in different industrial formulations. Eugenol is responsible for anti-inflammatory, anti-microbial, and anti-viral properties of *Syzygium aromaticum* depicting its usefulness in different pharmaceutical industries. Antioxidant property shows DNA protection ability of clove oil thereby it can be used for the treatment of a number of disorders especially against *E.coli.* In future, nanoparticles synthesis from clove oil might be a useful approach for the preparation of different drugs for treatment of disorders. However, further studies will be needed to optimize concentration of these nanoparticles to cure human disorders and to determine its mechanism of inhibitory action for human welfare beyond its typical uses in agro-food sector as flavoring agent and natural preservative.

Declaration of Interest Statement

Authors have declared no conflict of interest.

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