

Phytochemical Content, GC-MS Analysis, In Vitro Antioxidant and Antibacterial Efficacy of Methanolic Extract from Clove Flower Buds (*Eugenia Caryophyllus*)

Archana Dixit* Rachna Prakash Srivastav and Mahendra Kumar

Department of Chemistry, Dayanand Girl's P.G. College, Kanpur

Abstract: Plant-derived pharmacologically active compounds have become significant in the food and pharmaceutical sectors. The objective of this work is to identify and examine the antioxidant and antibacterial effects of the phytochemicals found in the crude extract of *Eugenia caryophyllus* flower buds. The antioxidant activity of the methanol, acetone, and chloroform extracts was assessed using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) test. The methanol extract demonstrated superior radical scavenging efficacy compared to the other chosen solvents. A preliminary screening of phytochemicals was conducted on the methanol extract, revealing a significant total phenol concentration. The antibacterial activity was assessed using a well diffusion experiment, revealing that the methanol extract was efficient against *Klebsiella pneumoniae*. GC-MS analyses reveal the existence of aromatic chemicals, with eugenol and eugenyl acetate identified as the primary constituents. The findings of this study suggest that *Eugenia caryophyllus* flower bud extract may be regarded as a health nutrient in the food and pharmaceutical sectors.

Keywords: *Eugenia caryophyllus*. Phytochemicals. Antibacterial agent. Antioxidant efficacy. Eugenol

Introduction

Spices significantly enhance the flavor and aroma of food in the human diet. It has been utilized in traditional medicine for several years across various countries. In addition to serving as flavoring agents in food, it functions as an antioxidative and antimicrobial agent. *Eugenia caryophyllus*, commonly referred to as the clove plant, is a member of the Myrtaceae family. Clove bud essential oil exhibits biological activities, including antibacterial, antifungal, insecticidal, and antioxidant properties, and has been traditionally utilized as a flavoring and antimicrobial agent in food products [1-2]. The antibacterial activity of clove oil is primarily attributed to its high eugenol content. Clove oil exhibits various therapeutic effects, including anti-inflammatory, antiemetic, analgesic, antispasmodic, carminative, and antiseptic properties [3]. Free radicals, or reactive oxygen species (ROS), generated by living systems, can initiate disease by damaging biomolecules [4-5]. Antioxidants protect against cell damage caused by free radicals by inhibiting or slowing the oxidative reactions occurring within cells. Synthetic antioxidants, including butylated hydroxyl-toluene (BHT), are effectively employed in industrial applications; however, they may pose health risks to humans [6-7]. Plant-based natural

antioxidants have garnered significant interest recently and are utilized as alternative antioxidant substances. Plants contain various anti-oxidative compounds, including tannins, phenolics, carotenes, and vitamins. Plant phenolics exhibit significant antioxidant potential due to their high redox activity [9-11]. Mihara and Shibamoto [12] reported that eugenol in clove oil exhibits high antioxidant activity and phototoxicity due to its active involvement in photochemical reactions. It was reported that the generation of hydroxyl radicals can be inhibited by eugenol, and that clove oil demonstrates a comparable antioxidant effect at low concentrations when compared to BHT [13]. Earlier research has documented the antibacterial activity of *E. caryophyllus* extracts against various pathogenic bacteria, including *E. coli*, *Salmonella*, *Listeria*, and *Staphylococcus* [14-17]. Earlier research indicates that clove essential oil has been extensively investigated for its antioxidant and antimicrobial properties. This study investigates the antioxidant and antimicrobial potential of clove powder. This study aimed to investigate the efficiency of various solvents for phytochemical extraction from *E. caryophyllus* and to evaluate the antioxidant and antibacterial efficacy of the extract. Additionally,

secondary metabolites and functional groups of the phytochemicals were analyzed using GC-MS and FTIR, respectively.

Materials and methods

Clove bud powder preparation

The flower buds of fresh *E. caryophyllus* used in this study were acquired at the local market in Chengalpet. Clove buds were carefully air-dried for one week and ground to fine powder. Clove bud powder was stored in air tight container at room temperature.

Preparation of *E. caryophyllus* extract

The crude extract of clove bud was obtained by direct extraction with chloroform, acetone and methanol [5]. In brief, 10 g of finely ground bud powder was extracted with 100 mL of chloroform, acetone and methanol in separate conical flasks in shaking condition.

Estimation of total phenolic content

Total phenolics present in the methanolic extract of *E. caryophyllus* were determined using the Folin-Ciocalteu reagent [18]. In brief, 0.5 mL of extract along with 0.1 mL of 0.5 N Folin-Ciocalteu's reagent was incubated at room temperature for 15 min. Then 2.5 mL saturated sodium carbonate solution was added and further incubated for 30 min at room temperature. The absorbance was measured at 760 nm in UV-Vis spectrophotometer. Gallic acid was used as a positive control. Total phenol values were expressed in terms of gallic acid equivalent (mg/g of extracted compounds). The assay was carried out in triplicates and expressed as mean $\pm$ SD.

Estimation of total flavonoid content

Total flavonoids in the methanol extract of *E. caryophyllus* flower buds was determined by aluminium chloride colorimetric method (Chang et al. 2002). The reaction mixture 3 mL consisting of 1 mL of sample (1 mg/mL), 0.5 mL of (1.2 %) aluminium chloride and 0.5 mL (120 mM) potassium acetate was incubated at room temperature for 30 min. The absorbance of the sample was measured at 415 nm and Quercetin was used as positive control. Flavonoid content was expressed in terms of quercetin equivalent

(mg/g of extracted compound). The assay was carried out in triplicates and expressed as mean $\pm$ SD.

Evaluation of antioxidant potential of *E. caryophyllus* extract by DPPH assay

Radical scavenging capacity of *E. caryophyllus* crude extract prepared by three solvents was determined and compared with α -tocopherol by using DPPH. [19] The ability to scavenge DPPH radical was calculated by the following formula.

$$\text{DPPH radical scavenging activity(\%)} = \frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}}} \times 100$$

It is repeated thrice with the same material but using fresh solvent and each time the extract was decanted into pre-weighed glass vials. The solvent in the extract was removed by condensation. The extracted residues were weighed and dissolved in DPPH solvents to yield 10 mg/mL solutions for further analysis.

Phytochemical analysis

The phytochemicals present in the crude extract were screened by the qualitative assays for plant secondary metabolites. Qualitative phytochemical tests for alkaloids, phenols, glycosides, saponins, flavonoids, tannins, reducing sugars were performed [20,12]. The colour intensity was used as response for these tests. Acontrol is the absorbance of DPPH radical and ethanol. Asample is the absorbance of DPPH radical and extract. Measurements were done in triplicate and the values were corrected for radical decay using blank solutions.

Thin layer chromatography

Thin layer chromatography (TLC) is used to separate the compounds present in the crude extract of *E. caryophyllus* [1]. Methanol and chloroform (1:9) was used as mobile phase. The sample with the concentration of about (1 mg/mL) was spotted on the TLC plates and dried. The spots were identified in long UV, short UV and also in the iodine chamber. Rf value was calculated to find the active metabolites. Rf value is distance travelled by the solute to the distance traveled by the solvent. Developed standardized chromatography plates of *E. caryophyllus* crude extract were sprayed with DPPH (dissolved in

DMSO) and the zone of inhibition was observed. The specific compounds (band) which possess antioxidative properties shows clear zone.

Gas chromatography-mass spectrometry (GC-MS) analysis

The methanol extract of *E. caryophyllus* was analyzed using GC-MS (SHIMADZU QP2010). The GC specifications were as follows: column oven temperature: 70 °C, injector temperature: 200 °C, injection mode: Split, Split Ratio: 40, Flow control mode: Linear velocity, Column Flow: 1.51 mL/min, Carrier Gas: Helium 99.99 % purity. Column oven temperature program: rate temperature (°C) hold time (min) -70 2, (35.0 min). column: VF-5 ms: length:

30.0 m, diameter: 0.25 mm. The MS specifications were as follows: Ion source temp: 200 °C, interface temp: 240 °C, scan range: 40–1000 m/z, event time: 0.5 s, solvent cut time: 5 min, start time: 5 min, end time: 35 min, ionization: EI (–70 eV) [3].

Antimicrobial activity of *E. caryophyllus* extract

The antimicrobial activity of *E. caryophyllus* extract against different bacterial pathogens was evaluated using well diffusion assay [2]. Bacterial strains were maintained in nutrient agar plates and subcultured at regular intervals. For evaluating antibacterial efficacy of *E. caryophyllus* extract, two Gram-positive bacteria (*Bacillus subtilis*, *Staphylococcus aureus*) and two Gram-negative bacteria (*Klebsiella pneumoniae*, *Vibrio cholerae*) were tested in well diffusion assay. The wells (8 mm diameter) were made in the nutrient agar plate using a cork borer. Stock solution of each plant extract was prepared at a concentration of 1 mg/mL in different plant extract. About 100 µL of different concentrations of plant solvent extracts were added with sterile syringe into the wells and allowed to diffuse at room temperature for 2 h. Control experiments comprising inoculums without plant extract were set up. The plates were incubated at 37 °C for 18–24 h and the diameter of the inhibition zone (mm) was measured.

Results and Discussion

Extraction and analysis of phytochemicals

This study utilized three solvents—acetone, chloroform, and methanol—for the extraction of phytochemicals, and the extractive values were assessed. Methanol extractions produced 3.7 g, while acetone and chloroform yielded 3.0 g and 2.4 g, respectively. Methanol demonstrated superior extractive efficiency compared to other solvents, a finding consistent with existing literature [21,11]. The extract of *E. caryophyllus* underwent phytochemical analysis utilizing both qualitative and quantitative methods. Qualitative analysis involved conducting various tests for phytochemicals, which confirmed the presence of alkaloids, phenols, flavonoids, tannins, and saponins in the crude extract. The identified phytoconstituents in the extract may account for its antioxidant and antimicrobial properties [27]. In quantitative analysis, the phytochemicals assessed in the methanol extract of *E. caryophyllus* were ranked as total phenolics > flavonoids, with respective values of 596.6 mg/g and 62.6 mg/g of extract. The high concentration of phenolic compounds indicates that *E. caryophyllus* extract possesses significant antioxidant capacity due to its ability to scavenge free radicals and reactive oxygen species [22].

Assay for antioxidant activity

The radical scavenging activity of acetone, chloroform, and methanol extracts of *E. caryophyllus* was evaluated using the DPPH assay, with α -tocopherol serving as the standard. All solvent extracts demonstrated antioxidant activity within a concentration range of 20–200 µg/mL (Table 1). Among the three extracts analyzed, methanol demonstrated the highest antioxidant capacity at 93.2%, while acetone and chloroform exhibited antioxidant activities of 85.4% and 81.4%, respectively. This result indicated that methanol exhibits greater extractive efficiency compared to the other two solvents [23] (Tepe 2008).

Table 1: Antioxidant Activity of Acetone, Chloroform, and Methanolic Extracts of *E. caryophyllus* Flower Buds

Concentration ($\mu\text{g/ml}$)	Radical activity	scavenging(%)		
	α -tocopherol	Acetone extract	Chloroform extract	Methanol extract
20	17.16 ± 0.76	$75.20 \pm 0.20^*$	$65.56 \pm 0.51^*$	$81.8 \pm 0.72^*$
40	34.73 ± 0.25	$76.10 \pm 0.15^*$	$71.06 \pm 1.0^*$	$82.8 \pm 0.80^*$
60	46.50 ± 0.70	$77.00 \pm 0.20^*$	$71.5 \pm 0.51^*$	$83.8 \pm 0.72^*$
80	49.30 ± 1.12	$78.20 \pm 0.20^*$	$72.6 \pm 0.62^*$	$85.3 \pm 0.30^*$
100	54.33 ± 0.61	$79.50 \pm 0.26^*$	$73.8 \pm 0.76^*$	$86.4 \pm 0.51^*$
120	58.63 ± 0.40	$80.06 \pm 0.30^*$	$76.6 \pm 0.52^*$	$87.5 \pm 0.51^*$
140	65.80 ± 0.34	$81.50 \pm 1.00^*$	$77.0 \pm 0.60^*$	$88.9 \pm 0.36^*$
160	72.80 ± 0.72	$82.20 \pm 0.25^*$	$78.7 \pm 0.25^*$	$90.0 \pm 0.50^*$
180	73.70 ± 0.30	$84.10 \pm 0.23^*$	$79.9 \pm 1.05^*$	$91.6 \pm 0.57^*$
200	84.10 ± 0.17	$85.30 \pm 0.15^*$	$81.4 \pm 0.45^*$	$92.2 \pm 0.64^*$

The agar well-diffusion method was employed to assess antimicrobial activity. The antimicrobial activities of *E. caryophyllus* flower bud extracts were assessed against four bacterial strains, comprising two Gram-positive and two Gram-negative organisms. The methanolic solutions of the extracts demonstrated significant antimicrobial activity against all tested Gram-positive and Gram-negative bacteria, as illustrated in Fig. 1 through the well diffusion assay. Clove extract demonstrated a wide range of

antimicrobial activity, with a minimum zone diameter of 17 mm against *Vibrio cholerae* and *Klebsiella pneumonia*, and a maximum zone diameter of 26 mm. The methanol extract demonstrated moderate activity against *Vibrio cholerae*, *Bacillus subtilis*, and *Staphylococcus aureus* (see Table 2 and Fig. 1). The inhibition zone diameter (mm) was measured, and the activity index was calculated. Recent reports indicate that ethanolic extract of clove exhibits antimicrobial properties.

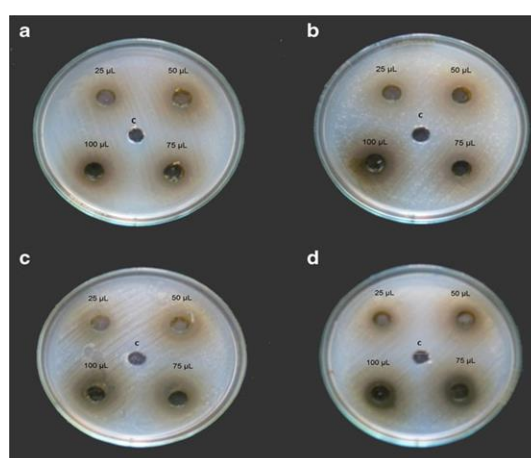


Fig. 1 Antibacterial activity of *E. caryophyllus* extract on a *Bacillus subtilis*, b *Staphylococcus aureus* c *Klebsiella pneumonia* and d *Vibrio cholerae*

Table 2 Effect of *E. caryophyllus* methanol extract against different pathogens

Bacteria	Zone of inhibition (mm)		Positive control(taxim)		
	Negative control (methanol)				
	25 µL	50 µL	75 µL	100 µL	
<i>Bacillus subtilus</i>	18.3 ± 0.57	21.3 ± 0.57	23 ± 1.00	24.3 ± 0.57 ^c	17 ± 1.00
<i>Staphylococcus aerus</i>	18 ± 1.00	22 ± 1.00	22 ± 0.57 ^b	25 ± 1.00 ^c	16 ± 1.00
<i>Klebsiella pneumonia</i>	19.33 ± 0.57	22.6 ± 0.57	24.33 ± 1.52	26 ± 1.00 ^d	15.6 ± 1.15
<i>Vibrio chlorea</i>	17.33 ± 0.57	18.0 ± 1.00	21.0 ± 1.00	24 ± 1.00	17 ± 1.00

Nazrul et al.[21] and Siddiqua et al. [24] reported that the combination of clove oil with cinnamaldehyde exhibited enhanced antibacterial activity against foodborne pathogens. Thin layer chromatography (TLC)

The methanol extract at a concentration of 1 mg/mL revealed four major compounds with Rf

values of 0.48, 0.62, 0.71, and 0.8, as visualized under iodine chamber and UV light, as shown in Fig. 2a and b. A higher Rf value of a compound correlates with a greater distance traveled on the TLC plate.

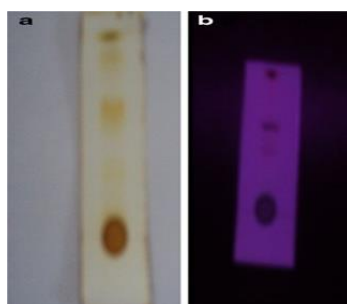


Fig. 2 TLC plate showing the phytochemicals separation in an iodine chamber and b under UV light

Gas chromatography-mass spectrometry (GC-MS)

The GC and MS running time for the methanol extract of *E. caryophyllus* were 36 min and spectrum is shown in Fig. 4.

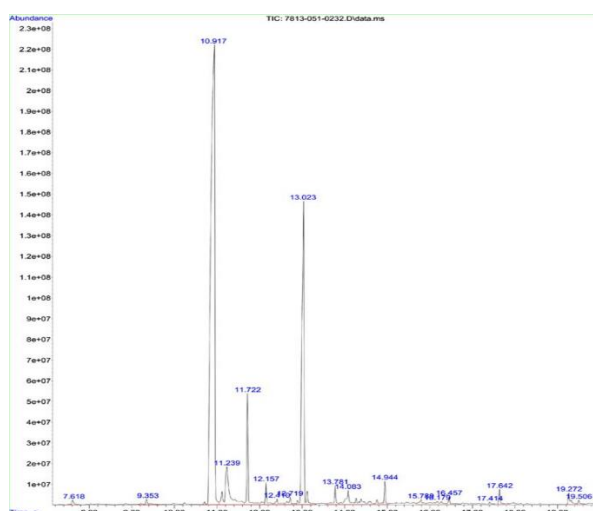


Fig. 4 GC-MS chromatograph for methanolic extract of *E. caryophyllus* powder

The interpretation of the mass spectrum (GC-MS) was conducted utilizing the database from the National Institute of Standards and Technology (NIST). The spectrum of the unidentified component was analyzed in relation to the spectra of established components archived in the NIST library. The analysis of GC-MS results encompasses the active principles along with their retention time, molecular formula, molecular weight, and composition found in the methanol extracts of *E. caryophyllus*. The identified compounds in the extract, along with their percentage composition, are presented in Table 3. The results indicated that the extracts comprised a complex mixture of various compounds, many of which were found in trace amounts. Eugenol (54.88 Phenol, 2-methoxy-

4-(2-propenyl) (19.46%), 1,2,3-Benzenetriol (5.48%), and caryophyllene (4.48%) were identified as the prominent components in the clove extract, with their structures illustrated in Table 4. In addition to the presence of other phytochemicals like eugenyl acetate, caryophyllene, and 1,2,3 benzenetriol, eugenol is essential for its antioxidant and antibacterial properties. Eugenol is the primary component in clove extract and aligns well with the findings reported by Jasna et al.[25]. The elevated levels of eugenol in clove extract suggest its potential for antibacterial and antioxidant properties. Research on the antibacterial properties of eugenol indicates that it interferes with the cell

Table 3 GC-MS analysis report for the methanol extract of *E. caryophyllus*

Retention time		Name	Molecular formula	Molecular
weight	Area (%)			
	7.618	4H-Pyran-4-one,2,3-dichloro-3,5-dihydroxyl-6-methyl		
	C9H15Cl6O4P	430.9	0.34	
9.352	Benzaldehyde,4-ethyl	C9H10O	134.19	0.47
10.90	Eugenol	C10H12O2	164.1	54.88
11.238	1,2,3-Benzenetriol	C6H6O3	126.12	5.47
11.721	Caryophyllene	C15H24	204.36	4.48
12.156	1,4,7-Cycloundecatriene,1,5,9,9-tetramethyl-z,z,z		C15H24	204.36 0.87
12.411	Gamma-murolene	C15H24	204.34	0.48
12.718	Alpha-farnesene	C15H24	204.34	0.61
13.022	Phenol,2-methoxy-4-(2-propenyl)	C12H14O3	206.24	19.45 13.782
	Caryophyllene oxide	C15H24O	220.36	0.95
14.082	Asarone	C12H16O3	208.25	2.53
14.943	2',3',4'-Trimethoxyacetophenone	C11H14O4	210.23	1.15
15.781	Benzeneacetamide,N-(aminocarbonyl)-4-hydroxy-3-methoxy		C19H29NO3	319.43 0.45
16.178	Phenol,2-methoxy-4-(methoxymethyl)	C9H12O3	168.18	0.75
16.456	Farnesol,acetate	C17H28O2	264.42	0.39
17.415	2,4,4-Trimethyl-3-(3-methylbutyl)cyclohex-2-enone		C14H24O	208.32 0.13
17.641	n-Hexadecanoic acid	C16H32O2	256.41	0.56
19.271	9,12-octadecadienoic acid(z,z)	C18H32O2	280.44	0.76
19.505	Octadecanoic acid	C18H32O2	284.47	0.22

membrane and augments its non-specific permeability (Gill and Holly 2006). The lipophilicity of eugenol facilitates the disruption of cellular structure by integrating with the lipopolysaccharide layer of the bacterial cell membrane, resulting in the leakage of intracellular components and subsequent cell death. [7,26] investigated the impact of eugenol on *Enterobacter aerogenes* and concluded that eugenol exhibits enzyme inhibitory activity through the binding of its hydroxyl group to proteins. In addition to its antibacterial properties, eugenol also exhibits possible antioxidant capabilities by donating a hydrogen atom and stabilizing phenoxyl radicals to impede oxidation. The conjugation of the carbon chain with the aromatic ring of eugenol plays a role in the stability of the phenoxyl radical by resonance [10]. Eugenol can reduce multiple DPPH radicals by creating dimers. Pyrogallol, or 1,2,3-benzenetriol, also has antibacterial properties. It is a hydroxylated phenol with three hydroxyl groups connected to the phenolic ring, which contributes to its heightened toxicity against bacteria.

Conclusions

In conclusion, methanol was identified as the optimal solvent for the extraction of phytochemicals from *E. caryophyllus*. The optimal findings for radical scavenging activity of methanol were achieved with a maximum of (92.2 ± 0.64) , closely approximating the radical scavenging activity of the reference α -tocopherol (84.06 ± 0.901). The methanol extract exhibited the highest quantity of bioactive components in early phytochemical analysis and a substantial concentration of total phenolics in the antioxidant activity assessment. The antibacterial testing findings indicate that *E. caryophyllus* extract was much more efficient against *K. pneumonia* than the other tested bacterial strains. Bioautography study indicated that the whole extract has free radical scavenging capabilities. GC-MS analyses identified a significant quantity of bioactive metabolites, including eugenol, caryophyllene, and phenol, 2-methoxy-4-(2-propenyl), in the extract. The findings of this study indicated that *E. caryophyllus* exhibited superior antioxidant and antibacterial properties, which may be utilized in dietary and medicinal applications.

Table 4 Major phytochemical constituents present in the tested sample of *E. caryophyllus*

Name	Structure
Eugenol	
Eugenyl acetate (Phenol,2-methoxy-4-(2-propenyl))	
Caryophyllene 1,2,3 Benzenetriol (Pyrogallol)	

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