Phytochemical investigation and *In-vitro* antioxidant, antibacterial activity of hydro-ethanol extract of *Lavandula angustifolia flower*

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Abstract

Investigation of phytochemical properties enables scientific database for further exploration of therapeutic uses. The aim of the present study is to carryout quantitaive, qualitative, antioxidant and antibacterial study of hydro-ethanol extract of *Lavandula angustifolia* flower. The hydro-ethanol extract of *Lavandula angustifolia* flower was done by maceration and its preliminary qualitative, quantitative studies were carried out. Further, it was subjected to *in-vitro* anti-oxidant and anti-bacterial studies. The phytochemical investigation of hydro-ethanol extract of *Lavandula angustifolia* flower showed the presence of alkaloids, glycosides, pholyphenols, flavonoids, tannins, saponins, terpenoids and essential oil. And the qunatitaive estimation showed the total phenol content, total flavonoids and total tannin content. The hydro-ethanol extract of *Lavandula angustifolia* flower showed significant anti-oxidant activity and anti-bacterial activity.

Keywords: *total phenolic content, total flavonoid content, total tannin content,anti-oxdant activity.*

1 Introduction

Most of these treatments use plant extracts and their active substances, and according to the WHO, 80% of the world's population relies on herbal medicinal items for their fundamental health care needs.(1) Therefore, the thorough phytochemical investigation of medicinal plants is needed to prove their potential, efficacy in various diseases with less or no side effects. The ethnobotany and ethno-pharmacognosy serve as a guide for chemical, pharmacological, and physiological investigations that enable the establishment of a scientific basis for their therapeutic properties. The findings from these plants will support for the pharmacological potential of therapeutic herbs.(2)

Lavandula species is a flowering plant that is widely used in the fields of cosmetics, perfumes, foods, and aromatherapy. It has been used to treat colic and chest ailments, worrisome headaches, and biliousness, and in cleaning wounds. It has antifungal, antibacterial, neurologic, antimicrobial, anti-parasitic, anti-diabetic, and analgesic effects among others. Lavandula species has prospects for various biological applications, especially with its dermatological application. Advances in drug development would enable characterization of various bioactive constituents; thus, its development and application can have a more positive impact on humanity (2). Therefore, we found interesting to determine the amount of secondary metabolites present in *Lavandula angustiolia* flower buds and to test it against *S. aureus* bacteria in order to estimate their antimicrobial effect.

2 Materials and Methods

2.1 Drugs and Chemicals: *Staphylococcus aureus* bacterial culture was collected from Biotechnology department of Jain Institute of Technology, Bengaluru and all the other chemicals and reagents were used of analytical grade.

Plant material and extraction: The dried *Lavandula angustifolia* flower bud (LA) was collected from Ravina Candle Industry, Delhi and identified by Mr. Nagaraj, DGM Horticulture and Rural Development Vasavadatta Cement, Sedam, India. Hydro-ethanol extract of LA (LAE) was prepared with ethanol : water (70:30) by maceration at room temperature for 3 days with occasional shaking. It was filtered and the filtrate was pooled, evaporated and stored for further studies. (3)

2.2 Preliminary phytochemical studies

Hydro-ethanol extract of LAE was subjected to various phytochemical tests for the identification of phytoconstituents present in it by standard procedures (3).

a) **Alkaloids:**
   Dragendorff's test: To 1 ml of the extract, 1 ml of Dragendorff's reagent was added and mixed. The resulting solution turns into orange-red precipitate indicates the presence of alkaloids.
   Mayer's test: To 1 ml of the extract 1 ml of Mayer's reagent was added. Formation of whitish yellow or cream-colored precipitate indicates the presence of alkaloids.
b) **Glycosides (Legal test):** The extract was dissolved in pyridine and sodium nitroprusside solution was added to make it alkaline. The formation of pink red or red colour indicates the presence of glycosides.
c) **Polyphenols:**to 2 mL portion of the flower extract, two drops of alcoholic solution of 2 % ferric chloride were added. The appearance of a more or less dark blackish-blue or green colour indicates the presence of polyphenolic compounds.
d) **Flavonoids (Shinoda test):** to a few drops of extract and a few fragments of magnesium and then add 0.5 mL of hydrochloric acid was added. The reddish color indicates the presence of flavonoids.
e) **Anthocyanins:** to 2 mL of flower extract 2 mL of 2 N HCl was added. The appearance of a pink-red colour that turns purplish blue after addition of ammonia indicates the presence anthocyanins.
f) **Tannins:**
   To small quantity of extract, lead acetate solution was added. The formation of white precipitates indicates the presence of tannins.
   To 1 mL of extract, few drops 5% of ferric chloride was added. A bluish black or greenish coloration was observed. It was an indication of pyrogallol tannins or catechol, respectively.
g) **Saponins:** The saponins was identified by adding 10 mL of the extract to a test tube. After stirring vertically for about 15 sec and left to stand for about 15 min, the formed foam height was measured. A greater than 10 mm foam height indicates the presence of saponins.
h) **Test for terpenoids (Salkowski test)**: 2mL of chloroform was mixed with 0.5mL of extract. Then 3mL of conc. H_2SO_4 was added carefully to form layer. The red colour appearance indicates the presence of terpenoids.
i) **Carbohydrates:**
   Molisch's test: To 2 ml of the extract, 1ml of α-naphthol solution, and concentrated sulphuric acid was added through the side of the test tube. Purple or reddish violet color at the junction of the two liquids reveals the presence of carbohydrates.
   Benedict's test: To 5 ml of Benedict's reagent, 1ml of extract solution was added. Then boil for 2 minutes and cool. The formation of red precipitate shows the presence of sugars.
j) **Cardiac Glycoside (Keller-Kiliani test):** In a test tube 2 mL of glacial acetic acid

containing 1 – 2 drops of 2% solution of $FeCl_3$ was mixed with extract and poured into another test tube containing 2 mL of conc. H_2SO_4. Brown rings are formed which indicates the presence of cardiac glycosides.

k) **Coumarins:** In a test tube, 1 g of methanolic extract was placed and covered with filter paper moistened with dilute NaOH, then heated on water bath for a few minutes. The filter paper was examined under UV light. Fluorescence was detected by the UV test (365 nm), yellow fluorescence regarded as positive for the presence of coumarins.

l) **Steroids (Liebermann-Burchard test):** In a test tube, 1 mL of acetic acid anhydride was added to 1 mL of extract, the solution was cooled well in ice followed by the addition of conc. H_2SO_4 carefully. The appearance of color development from violet to blue or bluish green was an indication for the presence of steroids.

m) **Protein (Millon's test):** About 1 mL of extract was mixed with 2 mL of Millon's reagent (mercuric nitrate in nitric acid containing traces of nitrous acid), white precipitate formed, which transformed to red upon gentle heating. It indicates the presence of amino acids and protein.

2.3 Quantitative Phytochemical Analysis

a) **Total Phenolic Content (TPC):** The total phenolic content was estimated by Folin Ciocalteu method, which was developed in 1927 for the measurement of tyrosine. Folin Ciocalteu reagent is a mixture of sodium molybdate and sodium tungstate and undergoes reaction with poly phenols. This reaction develops blue color, which shows the absorbance at 765nm [4,5].

For 1ml of standard gallic acid (10-100 µg/ml), sample and blank, 5 ml 10% Folin-Ciocalteu reagent and 4 ml of 20% sodium carbonate solution were added. The mixture was allowed to stand for 30 min and absorbance was measured at 765 nm with the help of a UV spectrophotometer (1700 Shimadzu). All samples were analyzed in triplicate. The total phenolic contents of the sample were calculated in terms of Gallic acid equivalent [GAE].

$$C = \frac{xV}{m}$$

Where,
C= Total phenolic compound in mg/gm of the sample
x = Concentration of gallic acid (established via calibration curve)
V = Volume of sample (ml)
m = Weight of sample (gm)

b) **Total Flavonoid Content (TFC):** The total flavonoid content was estimated by Aluminum chloride method. Aluminum chloride forms acid stable compound with keto and hydroxyl group of flavones and flavanols. It also forms acid labile complexes with flavonoids [5,6].

For 1ml of standard Quercetin (50-400 µg), sample and blank, 4 ml of water and 0.3ml of 5% sodium nitrite solution were added. After 5 minutes, 0.3 ml of 10% aluminium chloride and 2 ml of 1 M sodium hydroxide solution was added. The final volume was made up to 10 ml by using distilled water. The absorbance of the resulting mixture was measured at 510 nm with the help of a UV spectrophotometer (1700 Shimadzu). The experiment was conducted triplicate for the samples. The total flavonoid content of the sample was calculated in terms of quercetin equivalent (QE).

$$C = xVM$$

Where,
C = Total flavonoid content compounds in mg QE/g,
x = Concentration of quercetin (established via calibration curve),
V = Volume of extract in ml and M = weight of sample in gm.

c) **Total Tannins Content (TTC):** Quantitative tannin content in the extract was tested according to the method suggested by Mital and Jha. 1 ml of extract was mixed with 0.5 ml of 10% Folin reagent, and then it was saturated with 1 ml of Na_2CO_3 and 8 ml of distilled water was added to the final mixture. The solution, which was incubated for 30 min, was centrifuged and the supernatant was analyzed at 725 nm. Tannic acid was used as a standard and tannin content was expressed as mg tannic acid equivalent (TAE)/g [7].

2.4 *In-vitro* Antioxidant Studies

The antioxidant activity of extract was assessed by measuring the scavenging abilities of 2,2-diphenyl 1-picrylhydrazyl stable radical. The DPPH method works on the principle of DPPH reduction facilitated by the presence of an antioxidant capable of donating hydrogen. Extract reduced the color of DPPH by donating hydrogen. DPPH, has proton free radical with a characteristic absorption, which decreases significantly on exposure to proton radical scavengers [8].

1ml of standard ascorbic acid (20-120 µg), sample, positive control and blank were taken in test tubes. 1ml of 0.2 mM DPPH solution was added in all the test tubes. Shake it variously. Keep it in a dark room for 30 min. Measure the absorbance at 517 nm by using UV- visible spectrophotometer (1700 Shimadzu). The experiment was performed triplicate for sample. Percentage of inhibition was calculated by:

$$\text{Percentage inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} x100$$

2.5 Anti-bacterial activity (Agar well diffusion method)

Mannitol salt agar (MSA) was prepared and poured into sterilized Petri dishes. *Staphylococcus aureus* was cultured on MSA plates, and wells (6mm diameter) were created. Standard (Streptomycin), 20%, and 40% w/w LAE CI were added to respective wells. Petri dishes were sealed and incubated at 37 ºC for 24 hours. Later, the zone of inhibition diameters was measured to assess antibacterial efficacy. A control MSA plate without inoculation was maintained for comparative analysis and to monitor potential contamination or agar antimicrobial properties (9,10).

2.6 Statistical analysis: GraphPad Prism (version 10.03) software was used for analyzing the raw data collected in the experiment. Statistical comparison were made by one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons.

3 Results

3.1 Plant Extraction

The hydro-ethanol extract of *Lavandula angustifolia* flower buds yield was found to be 20 % w/w. The preliminary phytochemical investigation of LAE showed the presence of tannins, saponins, alkaloids, flavonoids, steroids, glycosides, terpenoids, phenols, proteins, Anthraquinones and carbohydrates.

3.2 Preliminary phytochemical studies

The preliminary qualitative phytochemical studies of LAE confirmed the presence of alkaloids, glycosides, polyphenols, flavonoids, tannins and terpenoids.

Table 1. Preliminary phytochemical studies of hydro-ethanol extract of *L. angustifolia fower bud*

Phytochemicals	Observation	Results
Alkaloids	orange-red precipitate	+
	cream-colored precipitate	+
Glycosides	blackish-blue colour	+
Polyphenols	reddish color	+
Flavonoids	purplish blue	+
Tannins	white precipitates	+
Saponins	greater than 10 mm foam height	+
Terpenoids	Red colour	+
Carbohydrate	Purple violet color at the junction of the two liquids	+
	red precipitate	+
Cardiac glycoside	Brown rings are formed at the junction of two liquids.	+
Coumarins	yellow fluorescence	+
Steroids	blue colour	+
Proteins	Red colour	+
Anthocyanins	purplish blue	+
Essential oils	Foam	+

3.3 Quantitative phytochemical analysis

a) Total Phenolic content

The total phenolic content was determined in terms of gallic acid equivalent (GAE) in mg/gm of the sample. The standard curve of gallic acid equation was y = 0.0086x - 0.0613, where R^2 = 0.9909. The total phenolic content of extract was found to be 10.14 mg of GAE/gm of LAE.

b) Total Flavonoid Content:

The total flavonoid content was determined in terms of quercetin (QE) in mg/gm of the sample. The standard curve of quercetin equation was y = 0.0005x + 0.0102, where R^2 = 0.9962. The total flavonoid content of extract was determined as 73.36 mg of QE/gm of LAE.

c) Total Tannins Content:

The total Tannin content was determined in terms of Tannic acid equivalent (TE) in mg/gm of the sample. The tannic acid standard curve equation was y = 0.0056x + 0.0036, where R^2 = 0.9785. The total Tannins content of extract was calculated to be 6.68 mg of TE/gm of LAE.

3.4 Anti-oxidant activity

The standard ascorbic acid showed the strongest free radical scavenging activity with 25.46 µg and hydro-ethanol extract of *L. angustifolia* flower showed the free radical scavenging activity with IC_{50} value 270.06mg. The standard ascorbic acid and ethanol extract of CI showed concentration-dependent activity. The results of 3.2 and 3.3 were shown below-

Table 2. Antioxidant activity of hydro-ethanol extract of *L. angustifolia fower bud*

Compounds	*Concentration*	*% Inhibition*	*IC_{50}*
Ascorbic acid (µg/ml)	10	35.27±2.347	25.46 µg
	20	47.07±2.024	
	30	60.22±1.294	
	40	74.66±1.063	
	50	84.05±0.988	

	60	95.44±0.525	
Hydro-ethanol extract of L. angustifolia flowers (mg/ml)	100	0	175.42 mg
	200	23.05±2.57	
	300	52.51±0.39	
	400	58.96±0.62	
	500	58.11±1.07	
	600	78.17±0.62	
	700	88.76±0.29	
	800	98.69±0.01	
Values are expressed as Mean ±SEM with three replications (n=3) for each experiment. *Ascorbic acid as standard chemical, DPPH = 1,1- diphenyl-2-picrylhydrazyl			

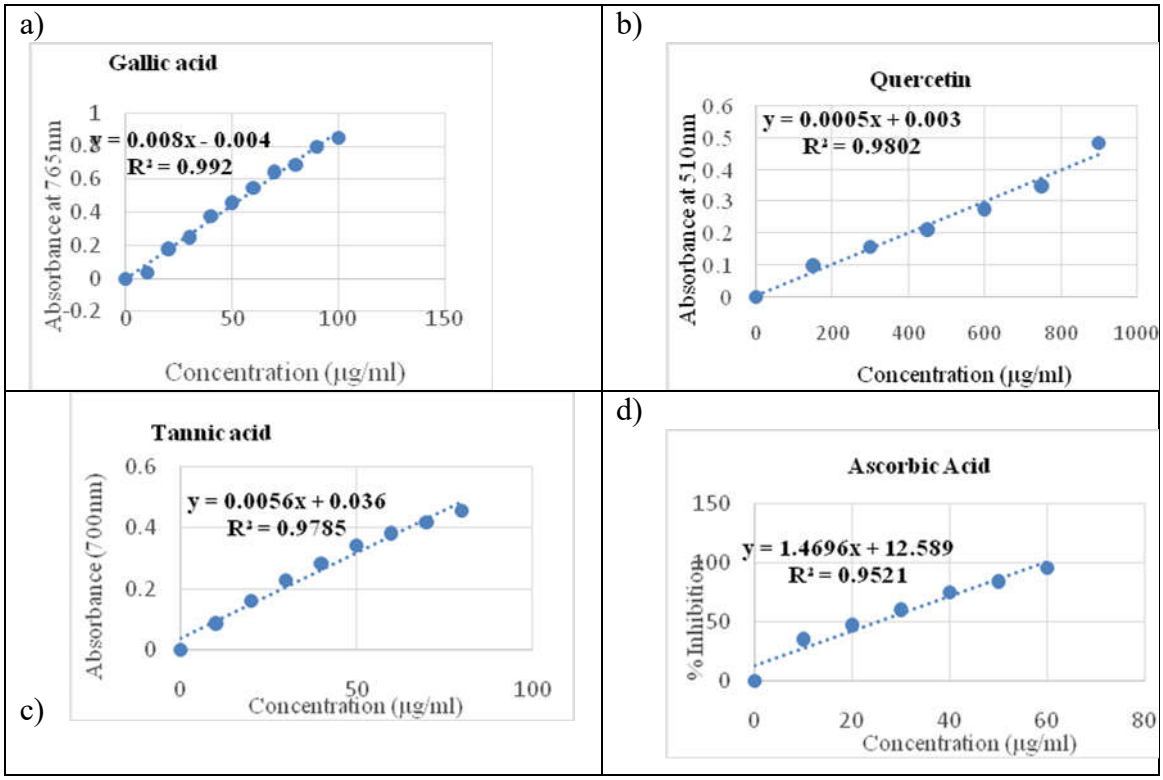

Figure 1. The graph represents the standard graph of a) Gallic acid b) Quercetin c) Tannic acid and d) Ascorbic acid

3.5 Anti-bacterial activity (Agar well diffusion method):

Hydro-ethanol extract of *L. angustifolia* flower bud inhibited the *Staphylococcus aureus* bacteria. The diameter of zone of inhibition of standard was 20.13 mm and test samples 20% extract were 22.28 mm and 21.31 mm for 40% extract.

4 Discussion

Medicinal plants are having major role in global medicine, but standardized quality control is inadequate. Herbal remedies, effective for conditions like wounds and inflammation, require evaluation due to lack of scientific evidence. Traditional cultures highlight the significance of herbal medicine in wound care. The symbiotic relationship between the body and bacteria can be disrupted by wounds, leading to pathogenic microbial growth. This risk increases with contamination from various sources, creating a unique microbial ecosystem. Therefore, here we have attempted to explore the phytochemical investigation of *L. angustifolia* flowers bud and also its in-vitro anti-oxidant and anti-bacterial properties.

The LA flowers extraction was carried out using water-ethanol as it was depicted that the ethanol extract consisted maximum number of phytoconstituents as compared to other solvent. Therefore, the ethanol was used as solvent for extraction.

The ethanol extract of flowers was screened for the presence of preliminary phytochemicals which showed the presence of flavonoids, glycosides, alkaloids, saponins, steroids, terpenoids, polyphenols, carbohydrates and tannins. Quantitative analysis of LAE was carried out for estimation of total phenolic, flavonoids and tannins contents. The ethanol flower extract of *CI* illustrated the highest total flavonoids (10.14 mg GAE/gm) when compared to phenols (73.36 mg GAE/gm) and tannins (6.68 mg TE/gm). The reported articles suggest that flavonoids, polyphenols and tannins have potent anti-oxidant and anti-bacterial property. The plant extract successfully reduced the purple-coloured stable free radical DPPH to its yellow-coloured form, diphenyl picrylhydrazine. This observation provides strong evidence that *L. angustifolia* flowers extract contains active components capable of supplying hydrogen to neutralize the unpaired electron. The DPPH radical scavenging method is particularly reliable because its outcomes remain consistent regardless of substrate polarity. The *L. angustifolia* flowers extract scavenging ability demonstrates its potential to reduce the concentration of DPPH, indicating its effectiveness as an antioxidant.

In-vitro antimicrobial activity of LAE flowers extract against *S. aureus* (Gram-positive bacterium) using the agar well diffusion method revealed that the extract exhibited significant zones of inhibition, measuring 22.28 mm for the 20% w/w formulation and 21.31 mm for the 40% w/w formulation, both outperforming the standard Streptomycin, which had an inhibition zone of 20.13 mm, further confirming antimicrobial efficacy.

5 Conclusion

The present study revealed that *L. angustifolia* flower buds exhibited significant anti-oxidant and anti-bacterial activity, which can be attributed to the presence of phenoilic compounds. The finding provided scientific evidence for the use in traditionally used medicinal plants.
The findings support scientific evidence for the usage of LA as groundwork in traditional knowledge and point to a bright future for antibacterial drug research. Further pharmacological studies are required to be conducted using other microbial strains. Toxicological tests, in vivo bioactivity studies, and molecular characterization should be conducted to exhibit its significant activity.

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7 Conflict of Interests- The authors declare that they have no conflict of interests.

8 References

1. *G. A. Rather, A. Nanda, E. Raj, N. Mathivanan, K. Thiruvengadam, M. A. Sofi, et al., Determination of Phytochemicals, in vitro Antioxidant and Antibacterial activity of Lavandula angustifolia Mill. Res J Pharm Technol., vol 16, no 3, (2023), pp. 1161–1166.*
2. *G. E. Batiha, J. O. Teibo, L. Wasef, H. M. Shaheen, A. P. Akomolafe, T.K A. Teibo, et.al., A review of the bioactive components and pharmacological properties of Lavandula species. Naunyn-Schmiedeberg's archives of pharmacology, vol 396, no. 5, (2023), pp. 877–900.*
3. *N. Dobros, K. Zawada, K. Paradowska, Phytochemical Profile and Antioxidant Activity of Lavandula angustifolia and Lavandula intermedia Cultivars Extracted with Different Methods. Antioxidants, vol 11, no. 4, (2022), pp 1-8.*

4. *Siddiqui N, Rauf A, Latif A, Mahmood Z. Spectrophotometric determination of the total phenolic content, spectral and fluorescence study of the herbal Unani drug Gul-e-Zoofa (Nepeta bracteata Benth). J Taibah Univ Med Sci. 2017 Aug 1;12(4):360–3.*
5. *Pandey LK, Sharma KR. Analysis of Phenolic and Flavonoid Content, α -Amylase Inhibitory and Free Radical Scavenging Activities of Some Medicinal Plants. Scientific World Journal. 2022;2022.*
6. *Shraim AM, Ahmed TA, Rahman MM, Hijji YM. Determination of total flavonoid content by aluminum chloride assay: A critical evaluation. LWT. 2021 Oct 1;150.*
7. *Pulipati S, Anil V, Chandu K, Begum R, Babu S. International Journal of Biological & Pharmaceutical Research Quantitative Determination Of Tannin Content And Evaluation Of Antibacterial Activity Of Crossandra Infundibuliformis (L) Nees Against Uti Pathogens. Vol. 5, International Journal of Biological & Pharmaceutical Research. 2014.*
8. *Baliyan S, Mukherjee R, Priyadarshini A, Vibhuti A, Gupta A, Pandey RP, et al. Determination of Antioxidants by DPPH Radical Scavenging Activity and Quantitative Phytochemical Analysis of Ficus religiosa. Molecules. 2022 Feb 1;27(4).*
9. *Tufa TB, Guta A, Tufa TB, Nigussie D, Beyi AF, Gutema FD, et al. Efficacy of Penicillin–Streptomycin Brands against Staphylococcus aureus: Concordance between Veterinary Clinicians' Perception and the Realities. Antibiotics. 2023 Mar 1;12(3).*
10. *Pulipati S, Vagdevi G, Prudhvi S, Babu S. International Journal of Biological & Pharmaceutical Research Evaluation Of Phytochemical And Antimicrobial Properties Of Crossandra Infundibuliformis (L) Nees Flower Extract. Vol. 4, International Journal of Biological & Pharmaceutical Research. 2013.*