Original Article

Phytochemical Analysis, Antimicrobial and Anti-Inflammatory Efficacy of *Leucas aspera* Leaf Extracts

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ABSTRACT

Medicinally, *Leucas aspera* has been confirmed to comprise broader pharmacological effectiveness *viz.*, antioxidant, insecticide, antipyretic, chronic rheumatism, and cytotoxic activity *etc.* This plant is traditionally used in the treatment of common infections viz., sore eyes and nose, fever, cough, skin eruptions, cold, wounds and sore throat. In this study, we intended to screen the phytochemical constituents, evaluate the antimicrobial and anti-inflammatory capacities of different solvent extracts of *Leucas aspera* leaves. *Leucas aspera* leaves were collected, shade dried, fine powdered and subjected to phytochemical extraction using methanol, ethanol, water and hydroalcohol. From the extracts, phenolic content was estimated by Folin-Ciocalteau reagent method followed by antimicrobial activity by Kirby-Bauer and Micro dilution assay with four different pathogenic bacteria. Later, anti-inflammatory activity was performed by various enzymatic assays. Phytochemical screening of *Leucas aspera* extract confirmed the presence of alkaloids, flavonoids, phenols, and tannins. The hydroalcoholic (MIC:12.5 µg/ml; MBC: 25µg/ml) and ethanolic (MIC:6.25 µg/ml; MBC:12.5 µg/ml) extracts presented effective and potent antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus mutants*, and *Propionibacterium acne*. Among the *in vitro* anti-inflammatory assays, hydroalcoholic extracts offered effective albumin denaturation (183.8±31.6µg/ml), heat induced hemolysis (213.4±22.3µg/ml) and considerable hypotonicity induced hemolysis (277.8±29.9µg/ml). The results were expressed as mean ± standard deviation, and statistical interpretation was based on two-tailed tests at a $p \leq 0.05$ significance level. In this current study, it was observed that *Leucas aspera* holds a variety of valuable secondary metabolites, which includes strong antimicrobial and anti-inflammatory activities, however further studies are necessary to assess its therapeutic use. Based on the existing experiments, corresponding results may set the foundation for future research.

Keywords: Anti-Inflammatory, Anti-microbial, *Leucas aspera*, Medicinal plant, Phytochemical.

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1. Introduction

Leucas aspera ('Thumbai') is a common weed in India and the Philippines, belonging to the *Lamiaceae* family and, which includes about 80 species (1). It is an aromatic, small, erect herb and has a flowering season between August and September. The pharmaceutical drugs such as linoleic acid, glucosides, linolenic acid, oleanolic acid, nicotinic acid, saponins, sterols, stearic acid, tannins, ursolic acid *etc.* have already been isolated and reported from the leaves, roots, flowersand seeds of this plant (2). This plant is traditionally used in the treatment of common infections *such as* sore eyes and nose, fever, cough, skin rashes, cold, wounds and sore throat (3, 4). The development and spread of multidrug resistant strains of pathogenic bacteria such as *Acinetobacter baumannii, Enterococcus faecium, Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus, and Enterobacter sps*. Have become a threat to public health, and there are only few or even sometimes no potent antimicrobial agents available against them (5). On other hand, inflammation - a pathogenic dysfunction, the root cause of a wide range of diseases such as immune-mediated conditions, diabetes, rheumatic diseases and cancer, is associated with pain, and involves increased protein denaturation, increased vascular permeability and membrane alteration (6). It is a self-defense activity of our body against harmful stimuli such as allergens or tissue injury; resulting in the migration of leukocytes from the venous system to the site of damage, and the release of cytokines (7). There are drugs like steroidal, non-steroidal, anti-inflammatory and immunosuppressive drugs to control and suppress the inflammation but they are associated with various side effects *viz.*, diarrhea, decreased appetite, headache, kidney and liver dysfunction, ulcers and prolonged bleeding after injury or surgery, gastric erosions (8). Therefore, it is essential to incorporate natural anti-inflammatory factors into medication therapy in order to obtain drugs with enhanced pharmacological response and show minimal side effects. Plant extracts have been used by ancient civilizations for the treatment of various ailments, and roughly 30% of the world's pharmaceutical sales are based on natural products. According to the World Health Organization (WHO), medicinal plants are the best sources to obtain a variety of drugs, and surveys conducted by the WHO report that more than 80% of the world's population depends on traditional medicine to treat various diseases (9). Although countless studies are conducted since many years, yet there is no notable report to evaluate the antimicrobial and anti-inflammatory efficacy of *Leucas aspera* plant. Our primary objective is to evaluate phytochemical constituents and to report the anti-inflammatory efficacy followed by antimicrobial properties of *Leucas aspera* leaf extract.

2. Materials and Methods

2.1. Collection and Authentication of Plant Sample

The *Leucas aspera* plant sample (leaves) was collected and authenticated by Dr. Harsha Hegde, Scientist D, Indian Council of Medical Research - National Institute of traditional medicine (ICMR-NITM), Belagavi, Karnataka. The leaves were thoroughly washed dried in the shade. They were then crushed into fine powder and stored at 4°C till further use.

2.2. Phytochemical Extraction and Analysis

The course powder was subjected to Soxhlet extraction using 4 different solvents *namely*, methanol, ethanol, hydroalcohol (ethanol: water 1:1) and water/aqueous. The extracts were collected and the with excess solvents were separated by rotary evaporation. After rotary evaporation, the extracts were kept on a water bath for drying. Later, the dried extracts were stored at 4°C in the air-tight containers. These extracts were qualitatively tested for different phytochemical constituents such as alkaloids, phenols, flavonoids, tannins, lignins, and sterols with slight modifications of the standard methodology (10).

2.3. Estimation of Total Phenolics

The total phenolic content of *Leucas aspera* leaf extracts was estimated by the Folin-Ciocalteau method with slight modifications (11). The dilution of 1mg/ml concentration was prepared for all the solvent extracts including gallic acidas a standard. In a clean and dry test tube, 200µl of each extract was added followed by 200µl of Folin-Ciocalteau reagent and allowed to stand for 10 min. Then 1.25 ml of 10% NaOH, 1 ml of distilled water was added and incubated for 1 hour and 30 minutes at 37°C. The absorbance of the color is read spectrophotometrically at 760nm. The standard graph is plotted and the total phenolic content is estimated and expressed as mg/g of gallic acid equivalent.

2.4. Antimicrobial Assays

2.4.1. Agar WellDiffusion/Kirby-Bauer Method

Antimicrobial susceptibility testing was performed using the agar welldiffusion method, which is accepted and approved by the Clinical and Laboratory Standards Institute (CLSI) for bacterial testing (12). Autoclaved Müller-Hinton agar (MHA) was poured into a petridish and allowed to solidify. After the media solidified , the wells were carefully punched and 100µl of inoculums (*Escherichia coli*, *Staphylococcus aureus*, *Streptococcus mutants*, *Propionibacterium acne*) corresponding to the turbidity of 0.5 McFarland standards, were evenly distributed. Approximately 100µl of different concentrations (60, 70 and 80mg/ml) of the extract were added to the wells and incubated at 37°C for 24 hours. After incubation, the zone of inhibition was recorded. The above procedure was repeated for the rest of the extracts.

2.4.2. Microdilution Assay: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

MIC is defined as the lowest concentration of an antimicrobial agent at which it prevents the visible growth of bacteria.[19]. The pure cultures of *Escherichia coli, Staphylococcus aureus, Streptococcus mutants and*

Propionibacterium acne were grown overnight and diluted in Mueller-Hinton broth to achieve turbidity corresponding to 0.5 McFarland standard. All extracts were inoculated into test tubes at 1:1 dilution with equal volumes of the specified microorganism and incubated at 37°C for 24 hours. After incubation, the MIC tubes were plated on the agar plates including a positive and negative control and incubated for 24 hours to check for microbial growth. To determine the MBC, selected MIC were plated to determine viable CFU/ml (13).

2.5. *In vitro* Anti-Inflammatory Assays

2.5.1. Inhibition of Albumin Denaturation

Inhibition of albumin denaturation was tested with different concentrations of *Leucas aspera* leaf extracts. 1ml of 1% aqueous solutions of bovine albumin fraction was mixed with 1ml of different concentrations of leaf extracts and incubated at 37 °C for 20 min. Later it was heated to 51ºC for 20 min and the turbidity was measured at 660 nm (14, 15). Diclofenac sodium was used as the standard drug.The percentage inhibition of albumin denaturation was calculated as follows:

Percentage inhibition = (Abs Control –Abs Sample) X 100/ Abs control.

2.5.2. Anti-proteinase Activity

The anti-proteinase activity of *Leucas aspera* was tested with different concentrations of leaf extracts. In each tube containing 0.6 mg of trypsin and 1 ml of leaf extract, 1 ml of 20 mM Tris HCl was added. The mixture was incubated at 37°C for 5 min and then 1 ml of 0.8% (w/v) casein was added and the mixture was incubated again for 20 min. Then 2 ml of 70% perchloric acid was added to all the test tubes and allowed to stand. The tubes were centrifuged and the absorbance of the centrifugate was read at 210 nm against buffer as blank (14, 15). Diclofenac sodium was used as the standard drug. The anti-proteinase activity was calculated using the following formula:

Percentage of Anti-proteinase activity = (Abs control –Abs sample) X 100/ Abs control.

2.5.3. Membrane Stabilization Assay

2.5.3.1. Blood Sample Collection

Blood samples were obtained from healthy volunteers who had not taken any non-steroidal anti-inflammatory drugs for 2 weeks and prior to the experiment consent forms were collected r from volunteers. Ethical clearance was obtained from Institutional Ethics Committee (Letter No. KLESKF/IEC/017, Date: 20.01.2018).

2.5.3.2. Preparation of Red Blood Cells (RBCs) Suspension

The tubes containing blood sample are centrifuged at 3000rpm for 10 min, the supernatant was discarded and the pellet was washed 3 times with equal volume of normal saline and later 10% v/v suspension is made with normal saline (14, 15).

2.5.3.3. Heat-induced hemolysis

Heat- induced hemolysis of *Leucas aspera* leaf extracts was tested by adding 1 ml of 10% RBC's suspension to all the test tubes containing 1ml of leaf extract of different concentrations. All the centrifuge tubes containing the reaction mixture were incubated in a water bath at 56 ºC for 30 min and the absorbance of the centrifuge was read at 560 nm. Aspirin was used as a standard drug (14, 15). The percentage of heat-induced hemolysis was calculated using the following formula:

Percentage inhibition = (Abs control –Abs sample) X 100/ Abs control.

2.5.3.4. Hypotonicity Induced Hemolysis

Hypotonicityinduced hemolysis of *Leucas aspera* leaf extracts was tested by adding 1 ml of phosphate buffer to 1 ml of leaf extract of different concentrations, to which 2 ml of hyposaline and 0.5ml of RBC suspension were added. All the test tubes were incubated at 37 ºC for 30 minutes and the tubes were centrifuged at high speed for 2- 3 minutes. The centrifuged supernatant was discarded assayed using a spectrophotometer at 560 nm. Diclofenac sodium was used as the standard drug (14, 15). The percentage of Hypotonicity induced hemolysis was calculated using the following formula:

Percentage inhibition = (Abs control –Abs sample) X 100/ Abs control.

2.6. Statistical Analysis

The data obtained were statistically interpreted and expressed as the mean and standard deviation of the mean using the statistical program IBM SPSS Statistics software Inc., version 20.0 (Armonk, NY: IBM Corp.). In vitro assay data were analyzed using GraphPad prism to determine IC_{50}. Statistical interpretation was based on two- tailed tests at a significance level of $p \leq 0.05$.

3. Results

3.1. Phytochemical Analysis

The total yield of crude extracts from *Leucas aspera* leaves with different solvents were as follows: methanol (29.36g), ethanol (20.35g), hydro-alcohol (18.79g) and aqueous (14.5g). Preliminary phytochemical screening of *Leucas aspera* leaf extracts revealed the presence of alkaloids in all the solvent extracts, while the absence of carbohydrates and proteins. The content of flavonoids, phenolics and Tannins were high in hydro alcoholic and ethanolic extracts compared to methanolic and aqueous extracts (Table 1).

3.2. Total Phenolic

The total phenolic content of different extracts of *Leucas aspera* was determined by the Folin-Ciocalteau reagent method and the phenolic content is expressed as GAE/g of plant extract. The phenolic content of methanolic, ethanolic, hydroalcoholic, and aqueous extracts were 35.26 ± 1.19mg/g, 44.36 ± 1.06 mg/g, 38.4 ± 0.96 mg/g and 37.6 ± 1.51 mg/g GAE, respectively.

3.3. Antimicrobial Assays

3.3.1. Agar WellDiffusion/Kirby-Bauer Method

Among them, only hydroalcoholic extract followed by ethanolic extract showed a significant antimicrobial activity against all four pathogenic bacteria. The methanolic extract had an antimicrobial activity against *S. aureus* and *S. mutants*, but the aqueous extract didn't show any antimicrobial activity (Figure 1) (Table 2).

Table 1. Preliminary phytochemical screening of *Leucas aspera* leaves extract.

Constituent	Test	Methanol	Ethanol	Hydroalcohol	Aqueous
Alkaloids	Mayer's test	+	+	+	+
	Wagner's test	+	+	+	+
Carbohydrates	Molisch test	-	-	-	-
	Fehling's test	-	-	-	-
Proteins	Biuret test	-	-	-	-
	Xanthoproteic test	-	-	-	-
Flavonoids	Lead acetate	+	+	++	+
	Zn-HCl reduction	+	++	+	+
	NaOH test	+	++	++	+
Phenols and Tannins	Gelatin test	+	+	+	+
	Lead acetate	+	+++	++	+
	Alkaline reagent	+	+++	++	+
Saponins	Haemolysis test	+	++	+++	++
	Froth test	+	+	+	+

-Absent; + present; ++ moderately present; +++ high presence.

Table 2. Agar well diffusion of *Leucas aspera* leaves extracts.

Microorganisms	Concentration (mg/ml)	Methanol (mm)	Ethanol (mm)	Hydroalcohol (mm)	Aqueous (mm)
Escherichia coli	60	-	10	15	-
	70	-	11	16	-
	80	-	12	16	-
Staphylococcus aureus	60	12	13	12	-
	70	13	14	15	-
	80	14	15	16	-
Streptococcus mutants	60	13	12	12	-
	70	14	13	14	-
	80	-	14	15	-
Propionibacterium acne	60	-	-	15	-
	70	12	-	12	-
	80	-	-	15	-

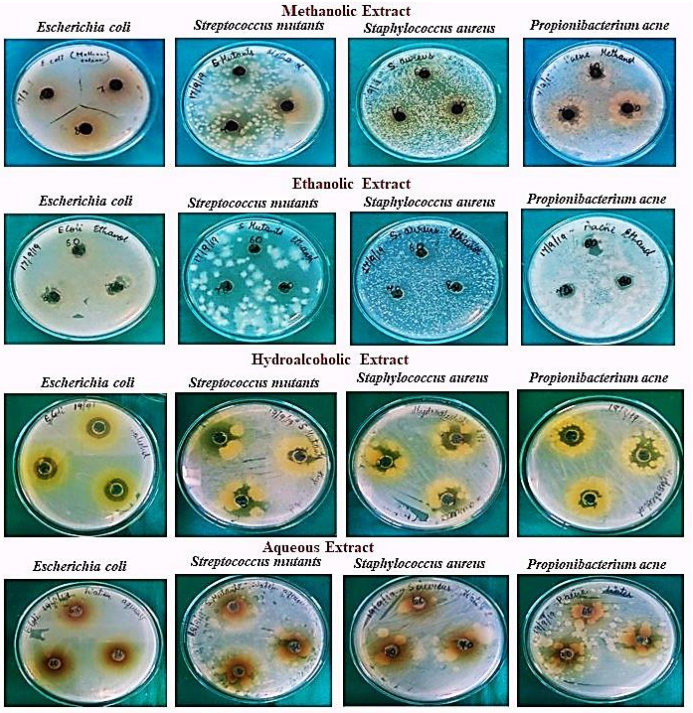

Figure 1. Agar well diffusion of Methanolic, Ethanolic, Hydroalcoholic and Aqueous extract against *E. coli*, *S. aureus*, *S. mutants*, *P acne*.

3.3.2. Microdilution Assay: MIC and MBC

For the MIC and MBC, three extracts *viz.*, methanol, ethanol and hydroalcohol of leaf showed prominent results against selected pathogens (Figure 2). Ethanolic (MIC:6.25 µg/ml; MBC:12.5 µg/ml) followed by hydroalcoholic (MIC:12.5 µg/ml; MBC: 25µg/ml) leaf extracts had significant antimicrobial activity.

3.4. *In vitro* Anti-Inflammatory Assay

3.4.1. Inhibition of Albumin Denaturation

Inhibition of albumin denaturation by all the extracts with IC_{50} values was as follows: methanolic (271.1±48.2µg/ml), ethanolic (356.5±42.4µg/ml), hydroalcoholic (183.8±31.6µg/ml) and aqueous (282.5±25.0µg/ml) with standard (Diclofenac Sodium) having an activity of 273.3 ± 27.3µg/ml. Amongall, the hydroalcoholic extract was found to be effective (Figure 3).

3.4.2. Anti-Proteinase Activity

The anti-proteinase activity of all the extracts with IC_{50} values were as follows: methanolic (415.2±36.7µg/ml), ethanolic (273.0±28.5µg/ml), hydroalcoholic (320.7±23.9µg/ml) and aqueous (363.7±32.7 µg/ml) with standard (Diclofenac Sodium) having an activity of 197.2±34.6 µg/ml. Among all, ethanolic extract was found to be effective (Figure 4).

3.4.3. Heat Induced Hemolysis

Among all,the hydroalcoholic extract showed a better heat induced hemolysis activity with IC_{50} value 213.4±22.3µg/ml. Rest others were having IC_{50} values were as follows: Aqueous (339.8±32.8µg/ml), ethanolic (398.6±37.5µg/ml) and methanolic (466.7±22.3µg/ml) when compared with standard *i.e.,* Diclofenac Sodium with IC_{50} value of about 205.5±29.1µg/ml.

3.4.4. Hypotonicity Induced Hemolysis

Among all, the hydroalcoholic extract showed a better heat induced hemolysis activity with IC_{50} value 277.8±29.9µg/ml. Rest others were having IC_{50} values were as follows: Aqueous (281.8±37.3µg/ml), ethanolic (333.5±41.5µg/ml) and methanolic (403.6±31.6µg/ml) when compared with standard *i.e.,* Diclofenac Sodium with IC_{50} value of about 268.3±21.1µg/ml.

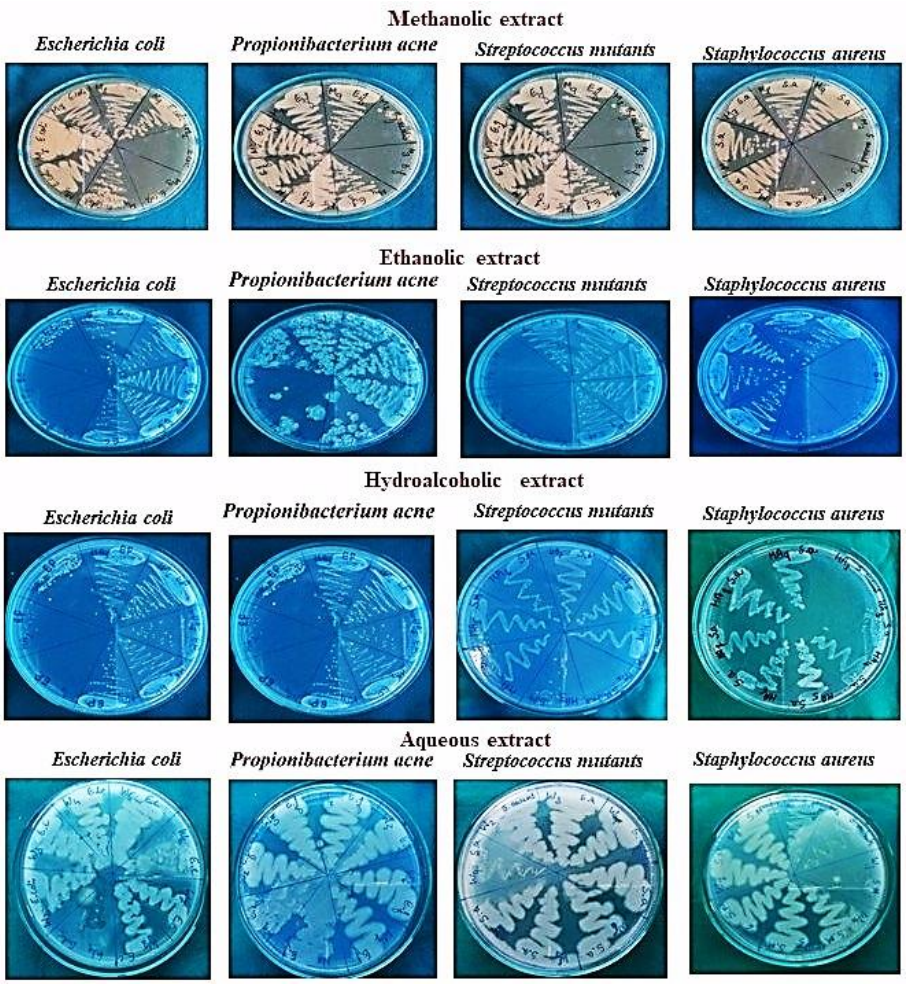

Figure 2. Minimum Bactericidal Concentration of Methanolic, Ethanolic, Hydroalcoholic and Aqueous extract against *E. coli*, *S. aureus*, *S. mutants*, *P acne*

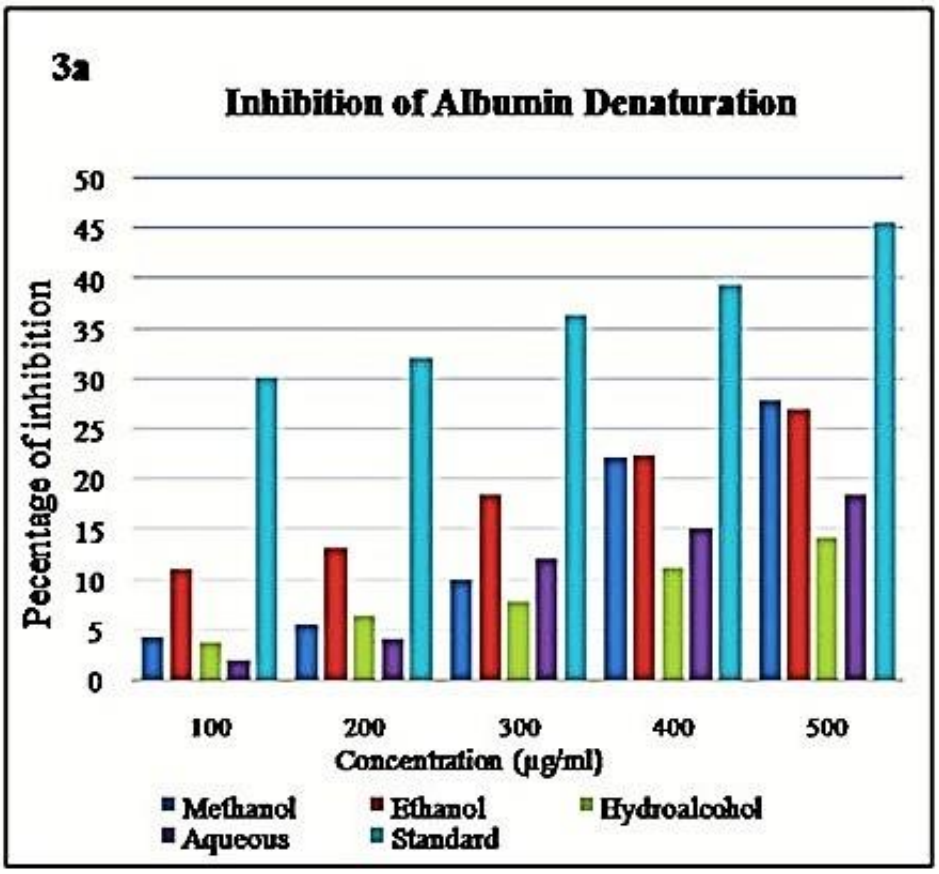

Figure 3. Percentage inhibition of albumin denaturation.

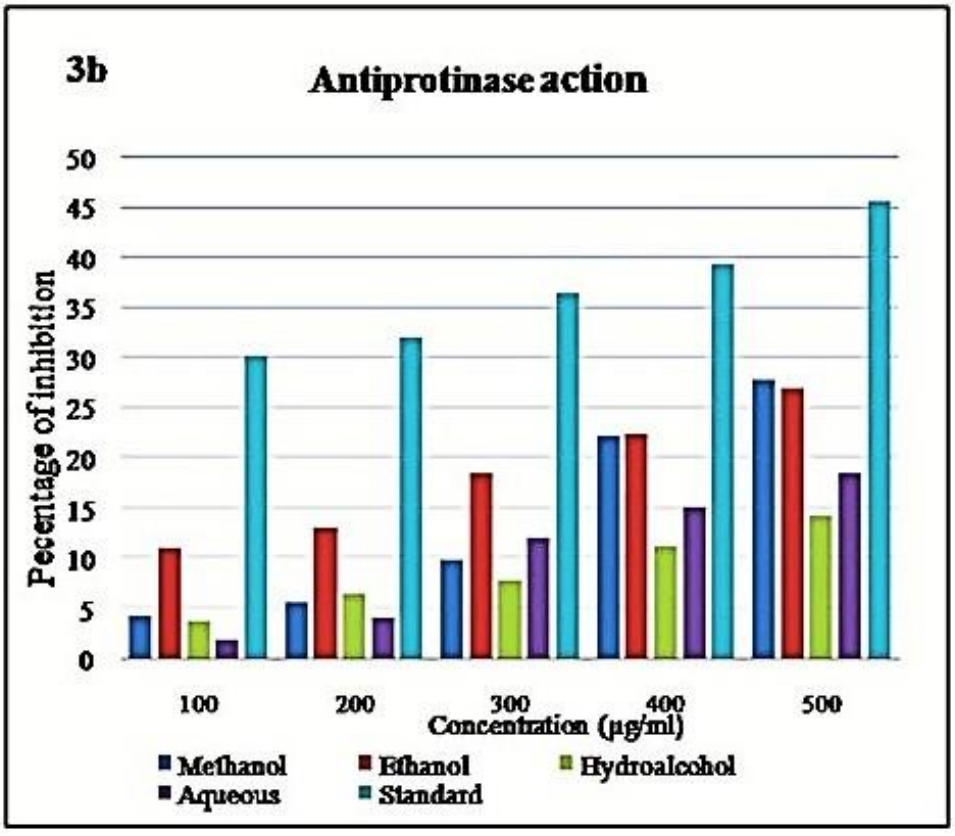

Figure 4. Percentage inhibition of Anti-proteinase action.

4. Discussion

The drugs from the plants are safe, efficient, less expensive, and rarely have some side effects, for which people prefer herbalbased drugs, resulting in exploration of medicinally important phytochemicals from plants (16, 17). In earlier report, phytochemical screening of *Leucas aspera* leaf extract was carried out using chloroform, aqueous-ethanol and water which showed presence of sterols, alkaloids, flavonoids, galactose, oleanolic acid, ursolic acid, aerial parts contain α and ß-sitosterol (18). The previous studies have shown that ethanolic extract of *Leucas aspera* whole plant has higher amount of phytoconstituents and a significant anti-oxidant activity (19). Supportively, in our study ethanolic and hydroalcoholic extracts were possessing higher phytoconstituents. It is also reported that, *Leucas aspera* whole plant extract has shown a significant resistance against the growth of *Escherichia coli, Staphylococcus aureus, Bacillus subtilis, Streptococcus pyogenes, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (20), which holds good with our study by Kirby-Bauer and Microdilution assay where hydroalcoholic extract showed substantial antimicrobial activity against *Escherichia coli, Staphylococcus aureus, Streptococcus mutants,* and *Propionibacterium acne*. Similarly, methanolic extracts of *Leucas aspera* flowers (21), and root (22) have been reported to have antifungal and antioxidant activity respectively. The presence of catechins, flavonoids, and phytosterols compounds highlights the antioxidant and anti-inflammatory potential of *Leucas aspera* aerial parts extract (23). In the present study, hydroalcoholic leaf extract of *Leucas aspera* exhibited a significant anti-inflammatory activity, which convinces the previous report where alcoholic extracts proved to have an anti-inflammatory activity in rat models (24, 25). In present study, it is observed that *Leucas aspera* holds a variety of valuable secondary metabolites, which includes a strong antimicrobial and anti-inflammatory activities. Based on the existing experimentations, corresponding results may set the foundation for future research.

Acknowledgment

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Authors' Contribution

Samprita Sungar supported study concept, design, and acquisition of data, Sridevi I. Puranik and Mujeeb M. A. completed analysis and interpretation of data, Makhadumsab M. Toragall performed statistical analysis and drafted the manuscript, Administrative and technical support was provided by Aimen Akbar A and Ravindranath Aladkatti and Shridhar C. Ghagane supervised the study.

Ethics

This article contain studies with human participants. Ethical approval was obtained from Institutional Ethics Committee (Letter No. KLESKF/IEC/017, Date: 20.01.2018).

Conflict of Interest

Samprita Sungar, Makhadumsab M. Toragall, Mujeeb M. A., Sridevi I. Puranik, Aimen Akbar A., Ravindranath Aladkatti and Shridhar C. Ghagane declare that they have No conflicts of interest.

Data Availability

The data that support the findings of this study are available on request from the corresponding author.

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