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Research Article

Comparative Evaluation of Aqueous and Ethanolic Extracts of *Piper cubeba*: Phytochemical, Antioxidant, Anti-inflammatory and Antimicrobial Activities and Herbal Oil Formulation

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Abstract

Background: *Piper cubeba* is a medicinal plant rich in bioactive phytochemicals with reported antioxidant, antimicrobial and anti-inflammatory properties. However, comparative evaluation of aqueous and ethanolic seed extracts and their potential for topical formulation remains limited. **Objective:** To compare the phytochemical composition, antioxidant, anti-inflammatory and antimicrobial activities of aqueous and ethanolic *P. cubeba* seed extracts and to develop a preliminary herbal-oil formulation for topical application. **Materials and Methods:** Seeds were extracted separately with water and ethanol. Qualitative phytochemical screening, TLC, total phenolic and flavonoid content, total antioxidant capacity, ferric reducing potential and alkaloid content were evaluated. Anti-inflammatory activity was assessed using the HRBC membrane stabilization assay, while antibacterial activity was tested against *Bacillus* spp. and *Escherichia coli*. *P. cubeba*-infused coconut oil was prepared and evaluated through a preliminary single-case topical observation. **Conclusion:** The ethanolic extract generally exhibited stronger antioxidant, antimicrobial and in-vitro anti-inflammatory activities, whereas the aqueous extract showed higher total phenolic content. The herbal oil showed encouraging preliminary topical observations for oral ulcer and gingival swelling, but its efficacy cannot be established from a single uncontrolled case. The findings support further optimization and development of standardized *P. cubeba*-based topical formulations with systematic safety, stability and efficacy evaluation.

Keywords: *Piper cubeba*; phytochemicals; antioxidant activity; anti-inflammatory activity; herbal oil; topical formulation.

INTRODUCTION

Piper cubeba L. f., commonly known as cubeb, tailed pepper or Kabab Chini in India, is an aromatic medicinal plant belonging to the family Piperaceae. It is primarily associated with Indonesia and Java and is also distributed in India and other tropical and subtropical regions. The dried fruits, which resemble black pepper but retain a stalk-like attachment, have contributed to its common name "tailed pepper" and have been widely valued as both a spice and medicinal material[1,2]. In India, the plant is known by several vernacular names, including Kabab Chini and Kankola, and it's fruits are extensively used as a spice, flavoring agent and traditional remedy, particularly in Ayurveda and Unani systems of medicine[3]. *P. cubeba* has been used in traditional medicine for centuries for the management of a wide range of disorders. Its fruits have traditionally been employed for digestive complaints, abdominal pain, respiratory disorders, urinary ailments and other conditions, while different preparations, including powders, decoctions and essential oils, have been used for therapeutic purposes[4]. Its fruits are also valued as a spice and flavoring agent, while its aromatic properties support applications in food preservation, cosmetics, and other products[2,3]. Thus, the combined culinary, medicinal and commercial importance of *P. cubeba* has stimulated increasing interest in its phytochemical and pharmacological properties. The diverse biological potential of *P. cubeba* is closely associated with its rich and complex phytochemical composition. The plant contains several classes of secondary metabolites, including lignans, phenolic acids, flavonoids, alkaloids, terpenes, phenylpropanoids, fatty acids, glycosides, tannins and other bioactive constituents[2,4-6]. Lignans represent an important group of constituents in the berries and include cubebin, hinokinin, yatein, cubebininolide, isoyatein and related compounds[7,8]. The

berries also contain the alkaloid piperine and a range of volatile constituents contributing to the characteristic aroma and biological properties of cubeb[4]. Phenolic and flavonoid compounds identified from *P. cubeba* include gallic acid, caffeic acid, ferulic acid, syringic acid, rutin and catechin, while its essential oil contains several volatile compounds, including cubebene and other terpenoid constituents[2]. The presence of these structurally diverse compounds provides a biochemical basis for the broad pharmacological activities reported for the plant. Differences in the chemical profiles of essential oil, oleoresin and solvent extracts have been reported, indicating that extraction conditions can influence the recovery of individual bioactive constituents[2]. Therefore, comparative investigation of extracts prepared using different solvents may provide useful information regarding their phytochemical composition and biological potential.

Various experimental studies have demonstrated that *P. cubeba* possesses a broad spectrum of biological and pharmacological activities, including antimicrobial, antioxidant, anti-inflammatory, antidiabetic, anticancer, hepatoprotective, nephroprotective, immunomodulatory, antiparasitic and wound-healing effects[9-13]. Among these, antimicrobial, antioxidant and anti-inflammatory activities are particularly relevant to the development of plant-based therapeutic preparations. The antimicrobial potential of *P. cubeba* has been demonstrated against a variety of Gram-positive and Gram-negative microorganisms. Extracts prepared from the fruits using different solvents have shown antibacterial activity against organisms including *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and other clinically relevant bacteria, while cubeb essential oil has also demonstrated antimicrobial activity against several bacterial and fungal species[2,3,12].The antioxidant activity of *P. cubeba* has likewise been associated with its abundance of phenolic compounds, flavonoids, lignans and other secondary metabolites. Ethanolic, methanolic and aqueous preparations have demonstrated free-radical-scavenging activity in different experimental systems, supporting the potential contribution of these constituents to the overall antioxidant capacity of the plant[2,14]. Oral ulcers and gingival disorders involve inflammation and tissue irritation. *P. cubeba* exhibits antimicrobial, antioxidant, and anti-inflammatory properties, with studies reporting inhibition of nitric oxide production and NF-κB signalling [2,3,15].

The combined antimicrobial, antioxidant and anti-inflammatory properties of *P. cubeba* are particularly relevant to oral healthcare. Oral ulcerations and gum-related inflammatory conditions involve complex interactions among microbial challenges, inflammatory responses, oxidative stress, and tissue injury[16-18]. Consequently, a natural preparation possessing multiple complementary biological activities may offer advantages over approaches directed toward a single therapeutic mechanism. Despite the considerable pharmacological literature on *P. cubeba*, its potential application specifically in oral healthcare remains comparatively underexplored. Furthermore, although the biological activities of various extracts and isolated compounds have been investigated, differences in extraction methods and phytochemical composition highlight the need for systematic comparative evaluation of different extract types. Considering these observations, *P. cubeba* represents a promising source of bioactive compounds for the development of natural therapeutic preparations. In the present study, ethanolic and aqueous seed extracts of *P. cubeba* will be subjected to qualitative and quantitative phytochemical evaluation, followed by assessment of their antioxidant, anti-inflammatory and antimicrobial activities. In addition, an oil-based topical formulation containing *P. cubeba* seed will be developed and evaluated for its potential application in oral ulcerations and gum-related disorders. The study is intended to investigate whether the combined phytochemical and biological properties of *P. cubeba* can be translated into a practical herbal preparation with potential to alleviate symptoms, support tissue healing and contribute to the development of plant-based oral healthcare products.

METHODOLOGY

1. Preparation of Aqueous and Ethanolic Extracts from *Piper cubeba* Seeds

For preparation of extracts, 3 g of *Piper cubeba* seeds were ground using a sterile mortar and pestle and the powdered material was separately dissolved in 15 mL of distilled water and ethanol in clean glass containers. Each container was covered with aluminum foil and left undisturbed at room temperature overnight to allow extraction. The mixtures were subsequently filtered and the filtrates were examined for volume and color. The resulting aqueous and ethanolic extracts were stored at room temperature and used for all subsequent experimental procedures.

2. Qualitative Phytochemical Analysis of *Piper cubeba* Extracts

A series of qualitative tests were performed to evaluate the phytochemical profile of the extracts[19].

a) Flavonoids - Detection of flavonoids was based on their characteristic color reaction under alkaline conditions. In this test, 2 mL of the crude extract was treated with an equal volume of 40% sodium hydroxide solution. The emergence of a yellow coloration confirmed the presence of flavonoids.

b) Polyphenols- Polyphenols were identified using ferric chloride as the reagent. Equal portions of the extract and 5% ferric chloride solution were combined and gently heated. The formation of a deep blue complex was taken as evidence of polyphenolic compounds.

c) Tannins- Tannins were qualitatively detected using ferric chloride and potassium dichromate tests. For Braymer's test, 1 mL of the extract was mixed with 2 mL of 10% ferric chloride solution, and the development of a dark blue coloration indicated the presence of tannins. For the potassium dichromate test, a few drops of each extract were placed in separate test tubes, and 0.5 mL of 10% potassium dichromate solution was added

gradually, dropwise. The formation of a yellow precipitate was considered a positive indication of tannins.

d) Saponins- Saponins were assessed through their ability to produce stable froth. Approximately 2 mL of the extract was added to 5 mL of distilled water and shaken vigorously. The persistence of foam for at least 30 seconds was considered indicative of saponins.

e)Alkaloids- Alkaloids were detected using Wagner's reagent. In this assay, 1 mL of the extract was treated with a few drops of the reagent (prepared by dissolving 2 g Iodine and 6 g potassium iodide in 100 mL distilled water). The appearance of a brown precipitate was taken as evidence of alkaloid presence.

f) Terpenoids - To test for terpenoids, 1 mL of the extract was combined with 0.5 mL chloroform in a test tube. A few drops of concentrated sulfuric acid were carefully added. The formation of a reddish-brown coloration at the interface of the two phases indicated a positive reaction.

g)Glycosides- Glycosides were qualitatively assessed by mixing 1 mL of extract with 2 mL glacial acetic acid and 1 mL of 5% ferric chloride solution, followed by gentle heating. After cooling, the mixture was layered over 2 mL concentrated sulfuric acid. The development of a reddish-brown ring at the junction confirmed the presence of glycosides.

3. Thin-Layer Chromatographic Separation of Phytochemicals

The phytochemical composition of aqueous and ethanolic extracts of *Piper cubeba* was compared by using Thin layer chromatography (TLC). The stationary phase was prepared by incorporating silica Gel G, calcium sulfate and distilled water in the ratio of 7:1.04:14 respectively, from the obtained slurry, the mixture was uniformly coated onto sterilized glass slides, which was later air-dried and heat-activated. Carefully three drops of each extract were applied along the line of origin. The separation was achieved using chromatographic separation in a separation chamber with chloroform and methanol (15:1, v/v) as mobile phase. After development TLC plates were air dried and viewed under both visible and ultraviolet light. The retention factor (Rf) values of the separated components were calculated to determine their relative mobility[20].

4. Quantitative Determination of Total Phenolic Content (TPC)

Folin-Ciocalteu method was employed for the quantification of total polyphenolic content in the extracts[21]. A gallic acid solution (1 mg/mL) was used as the standard for the preparation of the calibration curve by plotting absorbance values against known volumes (25–125 µL). For sample analysis, 50 µL of each extract was utilized. Each reaction mixture consisted 2.5 mL of Folin-Ciocalteu reagent and 2 mL of sodium carbonate solution. Following incubation at room temperature, the optical density was measured at 670 nm against a blank control. The TPC of the extracts was calculated using the gallic acid calibration curve and expressed as milligrams of gallic acid equivalents (GAE) per gram of sample.

5. Quantitative Determination of Total Flavonoid Content (TFC)

The quantification of flavonoids in the aqueous and ethanolic extracts of *Piper cubeba* was carried out using the aluminum chloride colorimetric method[22]. A quercetin solution (1 mg/mL) served as the standard for preparation of the calibration curve, with volumes ranging from 100 to 500 µL. For sample analysis, 50 µL of each extract was tested following the standard protocol for total flavonoid content (TFC) estimation. The absorbance of the yellow-colored complex formed was measured spectrophotometrically at 415 nm. The flavonoid concentration in the samples was calculated from the quercetin calibration curve and expressed as milligrams of quercetin equivalents (QE) per gram of sample.

6. Assessment of Total Antioxidant Capacity (TAC)

The phosphomolybdate method was employed to determine the total antioxidant capacity (TAC) through spectrophotometric analysis[19]. Ascorbic acid served as the standard, with a calibration curve established from varying concentrations of a 1 mg/mL stock solution. Samples and standards were treated with phosphomolybdate reagent and warmed to 95°C for 15 minutes. Optical density was then measured at 670 nm. The TAC values are reported in terms of milligrams of ascorbic acid equivalents per gram of sample (mg/g).

7. Ferric Reducing Potential Assay (FRPA) for Antioxidant Activity

The ferric reducing potential assay was employed to evaluate the antioxidant activity of water and ethanolic extracts of *Piper cubeba*[21]. Ascorbic acid (1 mg/mL) was used as a standard and a calibration curve was generated using a series of dilutions ranging from 25 to 125 µL. For analysis, 50 µL of each extract was processed separately according to the standard protocol. The optical density of the samples was measured at 670 nm using a spectrophotometer. Antioxidant capacity was calculated from the calibration curve derived from the standard readings and the results were expressed as milligrams of ascorbic acid equivalents (AAE) per gram of sample.

8. Quantitative Estimation of Total Alkaloid Content

The Bromocresol Green (BCG) method was utilised for the quantification of alkaloids present in the extracts. This method is based on the principle that alkaloids exist in their protonated cationic form under acidic conditions, and these positively charged molecules interact electrostatically with the sulfonphthalein group of BCG dye. This results in formation of yellow colored complex, the intensity of which is directly proportional to the concentration of alkaloids in the sample. For preparation of the calibration curve, atropine sulfate (1 mg/mL) was used as the standard, with volumes ranging from 200 to 1000 µL. Each reaction mixture consisted of the standard solution (made up to 2 mL with distilled water), Na_2HPO_4 buffer (pH 4.7), 50 µL of

BCG dye, and 3.5 mL of chloroform. Following incubation for 10 minutes at room temperature, the optical density of the yellow ion-pair complex was measured spectrophotometrically at 430 nm.

9. In Vitro evaluation of Anti-Inflammatory Activities of the extracts

The anti-inflammatory activity of the extracts was assessed using the human red blood cell (HRBC) membrane stabilization method[19]. A 10% erythrocyte suspension was prepared from fresh human blood and diluted in phosphate-buffered saline (PBS, pH 7.4). Test extracts, diluted 200-folds, along with the standard drug aspirin (15 mg/mL), were incubated with the suspension at 37 °C, followed by exposure to 54 °C to induce hemolysis. After centrifugation, the supernatant was collected and the absorbance was measured spectrophotometrically at 540nm. The percentage inhibition of hemolysis was calculated using the following formula:

$$\text{Percent Inhibition} = \frac{\text{Absorbance of Control} - \text{Absorbance of Sample}}{\text{Absorbance of Control}} \text{ x } 100$$

10. Evaluation of Antibacterial Activity Against Gram-Positive and Gram-Negative Bacteria

The antimicrobial activity of the extracts prepared from *Piper cubeba* was evaluated against both Gram-positive and Gram-negative bacteria. *Bacillus* spp. and *Escherichia coli* were used as representative Gram-positive and Gram-negative bacterial strains, respectively. Separate culture plates were prepared for each bacterial strain. Approximately 40 µL of the respective bacterial culture was uniformly spread on the surface of the agar plates. Wells were then prepared in the agar, and 40 µL and 80 µL of each ethanolic and aqueous extract were added separately into the respective wells. The plates were subsequently incubated at room temperature for overnight, and the antimicrobial activity of the extracts was assessed based on the inhibition of bacterial growth around the wells.

11. Preparation of *Piper cubeba*-Infused Herbal Oil

Herbal oil was prepared from Kebab Chini (*Piper cubeba*) powder using coconut oil as the carrier oil. For which, 7.4 g of the dried powder was added to 54 mL of pure coconut oil in an airtight glass container. The mixture was kept under direct sunlight for 4 days without disturbance to facilitate infusion of the bioactive constituents into the oil. After the infusion period, the preparation was filtered through a clean strainer to remove the plant material. The resulting infused oil was collected in sterile, airtight glass jars and stored appropriately until further use.

12. Observational Assessment of Herbal Oil Application for Oral Ulcers and Gingival Swelling

The formulated herbal oil was evaluated through an observational application in a 48-year-old man who reported recurrent oral ulcers and gum swelling. For the treatment of oral ulcers, one drop of the formulated herbal oil was applied directly to the affected area. For gum swelling, a sufficient quantity of the oil was gently applied to the affected gingival area once daily at night before bedtime, following thorough rinsing of the mouth with water. The application was continued until the reported symptoms, including ulceration, gum swelling and associated pain subsided. The response to the herbal oil was observed throughout the application period. The affected areas were photographed daily to document the progression of the lesions and changes in swelling and healing during the observation period. The photographic records were used for comparative assessment of the affected areas before and during the application of the formulated herbal oil.

RESULTS

1. Preparation of Aqueous and Ethanolic Extracts from *Piper cubeba* Seeds

Table 1: Color and extraction yield of *Piper cubeba* extracts prepared using different solvents

Sample Extract	Color	Initial Volume	Final volume	% yield
Ethanolic	Light Green	15 ml	12 ml	80%
Aqueous	Pink	15 ml	11 ml	73.4%

The extracts of *Piper cubeba* were prepared using ethanol and water as solvents (Figure 1). The ethanolic extract exhibited a light green color and showed an extraction yield of 80.0%, whereas the aqueous extract was pink in color and showed an extraction yield of approximately 73.4% (Table 1). Thus, the ethanolic extract of *Piper cubeba* exhibited a comparatively higher extraction yield than the aqueous extract under the experimental conditions employed.



Fig. (1). Ethanolic (right) and aqueous (left) extracts of *Piper cubeba*

2. Qualitative Phytochemical Analysis of *Piper cubeba* Extracts

Table 2: Qualitative Assessment of Phytochemical Presence and Relative Abundance in Ethanolic and Aqueous extracts of *Piper cubeba*.

Phytochemical	Sample	Presence (+) /Absence (-)
Flavonoid	Ethanolic	+
	Aqueous	+
Polyphenol	Ethanolic	+
	Aqueous	+
Tannins	**Braymer's test -**	
	Ethanolic	-
	Aqueous	+
	Dichromate test -	
	Ethanolic	+ +
	Aqueous	-
Saponin	Ethanolic	-
	Aqueous	+
Alkaloids	Ethanolic	+
	Aqueous	-
Terpenoids	Ethanolic	+ + +
	Aqueous	+ + +
Glycosides	Ethanolic	+ + +
	Aqueous	+ +

Qualitative phytochemical screening of the ethanolic and aqueous extracts of *Piper cubeba* revealed the presence of several bioactive constituents (Table 2). Flavonoids and polyphenols were detected in both ethanolic and aqueous extracts. Tannins showed solvent-dependent variation, with the Braymer's test being positive in the aqueous extract but negative in the ethanolic extract, whereas the dichromate test was positive in the ethanolic extract and negative in the aqueous extract. Saponins were detected only in the aqueous extract, while alkaloids were detected only in the ethanolic extract. Terpenoids were abundantly present in both extracts. Glycosides were also detected in both extracts, with a higher relative abundance in the ethanolic extract (+++) compared with the aqueous extract (++). Overall, the results demonstrated variation in the phytochemical profile of *Piper cubeba* depending on the extraction solvent, with both extracts showing the presence of multiple classes of phytochemicals.

3. Thin-Layer Chromatographic Separation of Phytochemicals

Table 3: Retention Factors (Rf) of Separated Components in Crude Kiwi and Strawberry Extracts via Thin Layer Chromatography.

Sample	Observed under	No. of spots	Colour of spots	Rf value
Ethanolic	Visible	1	Brown	1.00
	UV	2	Orange	1.00
			Blue	0.29
Aqueous	Visible	1	Brown	0.56
	UV	1	Bluish-green	0.56

The chromatographic analysis or TLC of *Piper cubeba* extract in ethanol and water showed different chromatograms (Figure 2). The ethanolic extract showed higher chromatographic diversity especially under the UV illumination, which suggests more UV-active constituents in the extract. The aqueous extract, on the other hand, presented a simpler chromatographic profile. The variations in spot patterns and in color of the two extracts show variations in the phytochemical composition and polarity of the extracts obtained from the various solvents. Overall, the TLC profile confirms the presence of several chemically diverse constituents in *Piper cubeba* and demonstrates that the extraction solvent influences the composition of the resulting extracts.

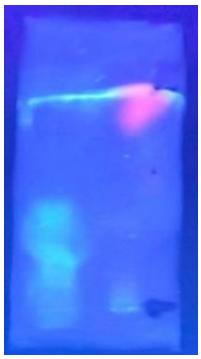


Fig. (2). Thin-layer chromatographic (TLC) profiles of *Piper cubeba* extracts: aqueous extract (left) and ethanolic extract (right).

4. Quantitative Determination of Total Phenolic Content (TPC)

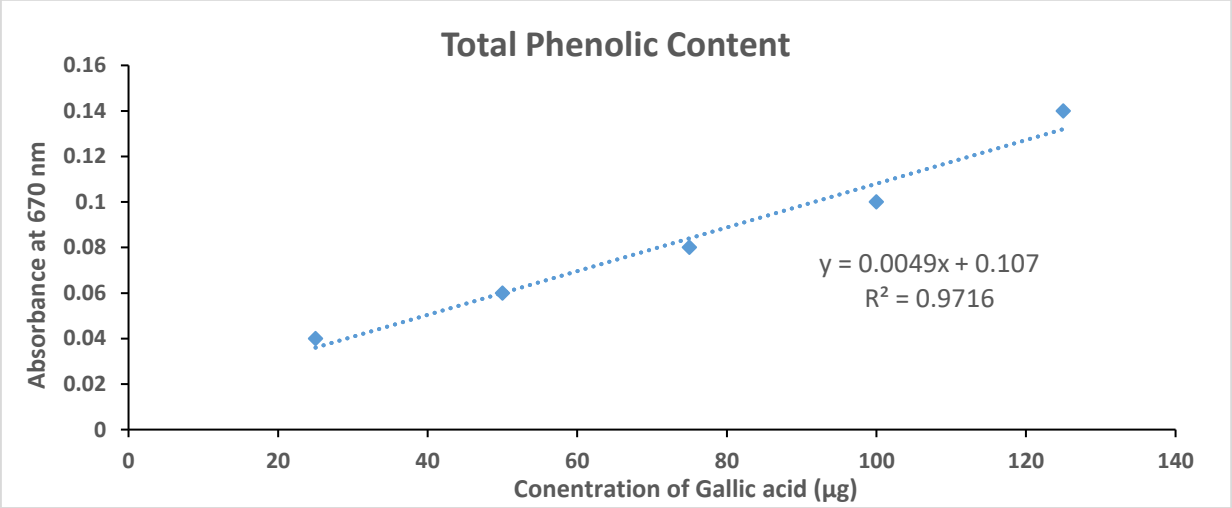


Fig. (3). Standard Calibration Curve for the Estimation of Phenolic Content.

The total phenolic content (TPC) of the ethanolic and aqueous extracts of *Piper cubeba* was quantitatively determined using the Folin-Ciocalteu colorimetric method, with gallic acid employed as the reference standard. A 50 µL aliquot of each extract was subjected to the assay, and the phenolic content was calculated from the linear calibration curve generated using gallic acid. The TPC of the ethanolic extract was estimated to be 1.31 mg gallic acid equivalents (GAE)/g of sample, whereas the aqueous extract exhibited a comparatively higher TPC of 3.032 mg GAE/g of sample. Although the qualitative phytochemical screening showed a relatively similar intensity of color reaction for phenolic compounds in both extracts, the quantitative estimation provided a clear distinction in their phenolic contents. The higher TPC observed in the aqueous extract quantitatively confirms its greater abundance of phenolic constituents compared with the ethanolic extract under the experimental conditions.

5. Quantitative Determination of Total Flavonoid Content (TFC)

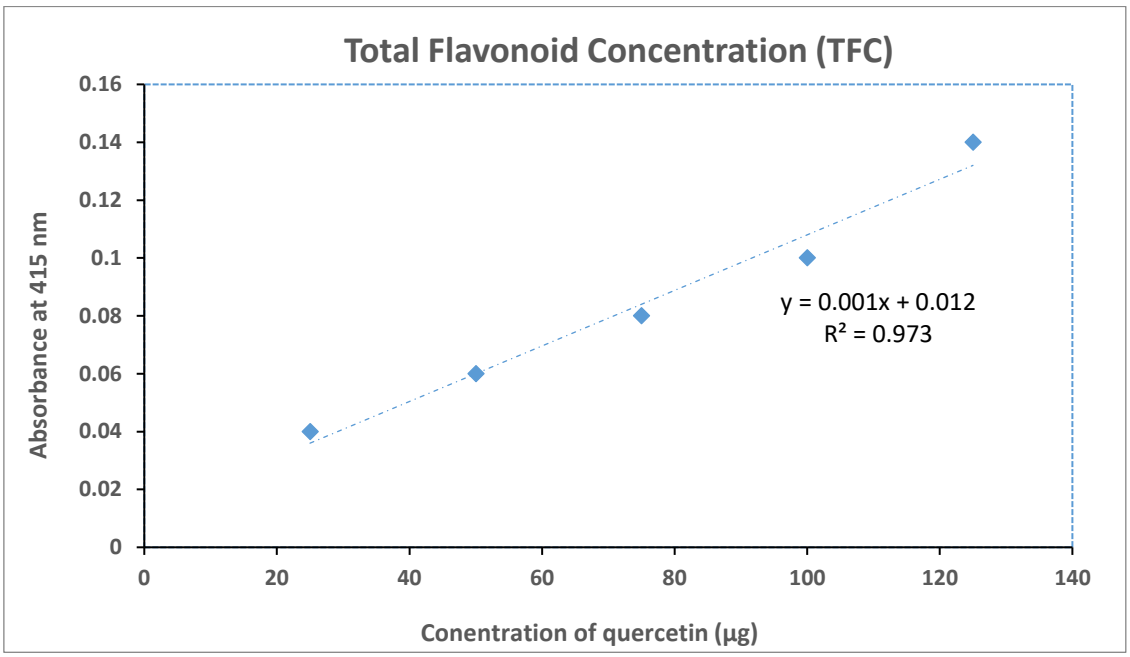


Fig. (4). Standard Calibration Curve for the Estimation of Total Flavonoid Content.

Quantitative estimation of the total flavonoid content (TFC), using quercetin as the standard, revealed a marked variation between the two Piper cubeba extracts. The ethanolic extract contained 0.0208 mg quercetin equivalents (QE)/g, while the aqueous extract contained 0.00306 mg QE/g. The ethanolic extract therefore exhibited a considerably higher flavonoid content than the aqueous extract, indicating that ethanol was more effective than water in extracting flavonoid constituents from Piper cubeba under the conditions employed.

6. Assessment of Total Antioxidant Capacity (TAC)

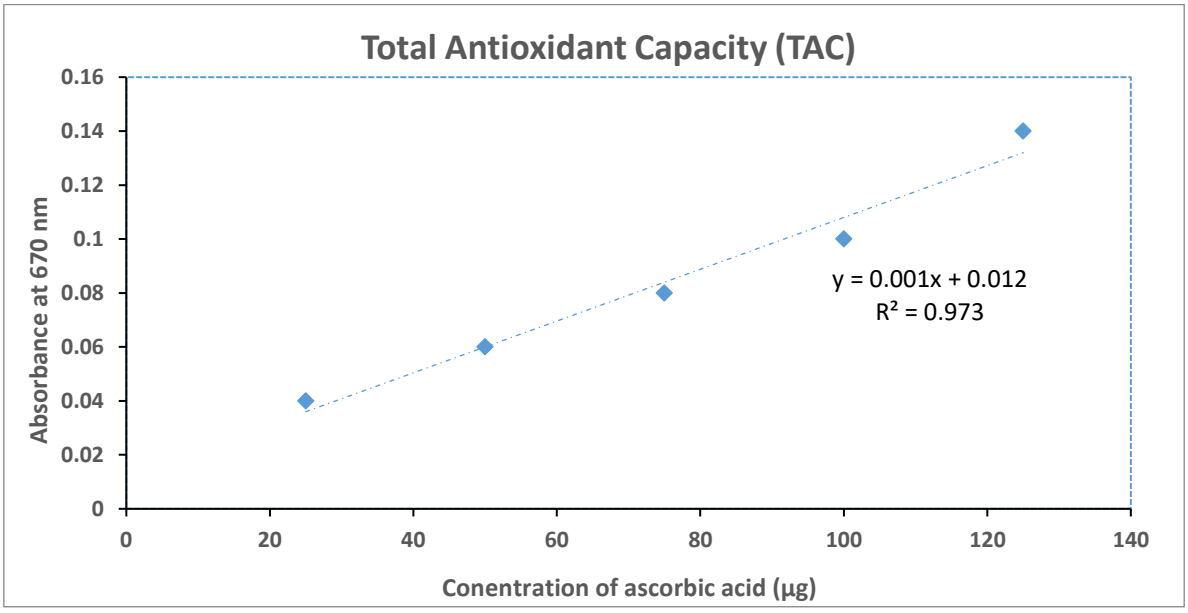


Fig. (5). Standard Calibration Curve for the Estimation of Total Antioxidant Activity (TAC).

Total antioxidant capacity (TAC) of Piper cubeba extracts was determined by the phosphomolybdate assay using ascorbic acid as the reference standard. The calibration curve generated with ascorbic acid was used for quantitative estimation. The ethanolic extract recorded a TAC of 0.73 mg/mL ascorbic acid equivalents (AAE), whereas the aqueous extract showed 0.28 mg/mL AAE, indicating approximately 2.6-fold higher antioxidant capacity in the ethanolic extract.

7. Ferric Reducing Potential Assay (FRPA) for Antioxidant Activity

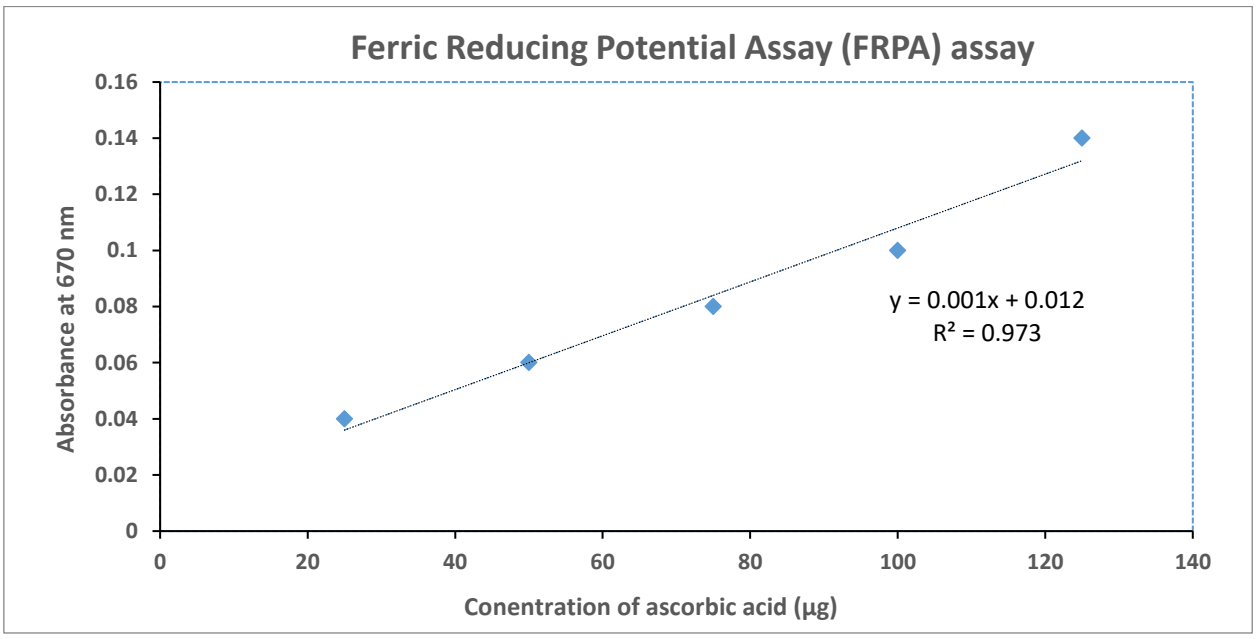


Fig. (6). Standard Calibration Curve for the Ferric Reducing Potential Assay determination.

Ferric reducing potential of Piper cubeba extracts was assessed using the Ferric Reducing Potential Assay (FRPA) to determine the antioxidant potential of the extracts, with ascorbic acid as the reference standard. The ethanolic extract exhibited a reducing potential of 3.64 mg ascorbic acid equivalents (AAE)/mL, compared with 0.98 mg AAE/mL for the aqueous extract. Thus, the ethanolic extract showed a markedly greater ferric-reducing capacity, consistent with the trend observed in the total antioxidant activity assay.

8. Quantitative Estimation of Total Alkaloid Content

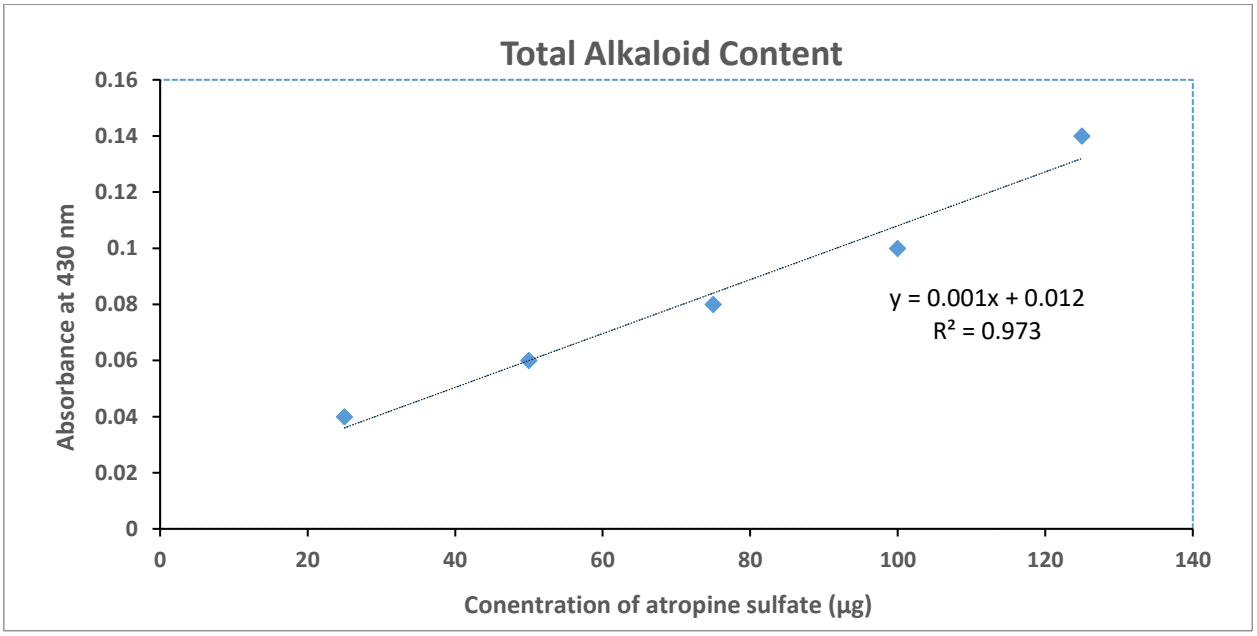


Fig. (7). Standard Calibration Curve for estimating total alkaloid content.

The alkaloid content of *Piper cubeba* extracts was quantified using the Bromocresol Green (BCG) method. The ethanolic extract exhibited an alkaloid content of 0.76 mg/mL, compared with 0.36 mg/mL in the aqueous extract, representing an approximately 2.1-fold higher alkaloid content in the ethanolic extract.

9. In Vitro evaluation of Anti-Inflammatory Activities of the extracts

The anti-inflammatory activity of the extracts was evaluated using the membrane stabilization method. The control showed an optical density (OD) of 0.42,

while the standard drug exhibited an OD of 0.33, corresponding to 21.43% inhibition of hemolysis. The ethanolic extract showed an OD of 0.18 with 57.14% inhibition, whereas the aqueous extract exhibited an OD of 0.22 with 47.62% inhibition. Both ethanolic and aqueous extracts demonstrated higher percentage inhibition than the standard drug, indicating greater membrane-stabilizing and anti-inflammatory activity under the experimental conditions. Among the tested samples, the ethanolic extract exhibited the highest inhibitory activity (57.14%), followed by the aqueous extract (47.62%) and the standard drug (21.43%).

10. Evaluation of Antibacterial Activity Against Gram-Positive and Gram-Negative Bacteria

The antimicrobial activity of the Piper cubeba extracts varied between the tested bacterial strains and extraction solvents. Against the Gram-positive Bacillus spp., a clear zone of inhibition was observed around the wells containing the ethanolic extract, with the more evident inhibition observed at 40 µL. In contrast, no clear zone of inhibition was observed around the wells containing the aqueous extract, indicating the absence of detectable antibacterial activity under the conditions tested. Against the Gram-negative Escherichia coli, the ethanolic extract showed only slight traces of inhibition of microbial growth at both 40 and 80 µL concentrations. However, the observed inhibition was minimal and insufficient to conclusively establish antibacterial activity against E. coli. Overall, the findings indicate that the ethanolic extract demonstrated more evident antimicrobial activity against the tested Gram-positive Bacillus spp., whereas the aqueous extract did not show detectable inhibition under the experimental conditions.

11. Preparation of *Piper cubeba*-Infused Herbal Oil

Following the 4-day infusion period, the Piper cubeba-infused coconut oil developed a green-brownish color with a distinct characteristic fragrance, indicating the transfer of extractable constituents from the plant material into the carrier oil. After filtration, 49.6 mL of infused oil was recovered from the initial 54 mL of coconut oil. The prepared oil was collected in a sterile, airtight glass container and stored until further use. The resulting herbal oil was subsequently made available for topical application in cases of oral ulcers and gum swelling.



Fig. (8). Piper cubeba-Infused Herbal Oil

12. Observational Assessment of Herbal Oil Application for Oral Ulcers and Gingival Swelling

Following topical application of the formulated herbal oil, the affected oral ulcer and gingival swelling were assessed daily under supervision (Figures 9 and 10). A progressive reduction in ulcer size and surrounding redness was visibly observed from the first day of application, accompanied by reported relief from pain after the initial application. The oral ulcer showed complete healing by the third day of treatment. Similarly, the gingival swelling gradually subsided and was markedly reduced within 5 days, with the patient reporting associated relief from pain. These observations indicate a favorable symptomatic response to the topical application of the formulated herbal oil during the observation period.



Fig. 9. Sequential photographic observation of the gingival swelling following topical application of *Piper cubeba* herbal oil over a 5-day period: Day 1 to Day 5.



Fig. 10. Sequential photographic assessment of the oral ulcer following topical application of formulated *Piper cubeba* herbal oil over a 3-day period: Day 1, Day 2, and Day 3.

DISCUSSION

The present study demonstrated that the extraction solvent influenced the phytochemical composition and biological activities of *Piper cubeba* seed extracts. The ethanolic extract showed a higher extraction yield (80%) than the aqueous extract (73.4%), indicating greater extraction of ethanol-soluble constituents. Such differences may be related to the polarity and solubility of individual phytoconstituents in the extraction solvents. Previous studies have reported phenolic compounds, flavonoids, lignans, alkaloids and volatile constituents as important chemical groups in *P. cubeba*[2,4,23,24]. The TLC profiles further indicated differences in the chemical composition of the ethanolic and aqueous extracts, as distinct spots were observed under visible and UV illumination. These findings support the presence of different classes of extractable constituents in the two solvents. Lignans are particularly characteristic of *P. cubeba*[25-27], with cubebin, hinokinin, yatein, isoyatein and related compounds previously isolated from its fruits and seeds[4,7,23].

Quantitative analysis showed that the aqueous extract contained higher total phenolic content, whereas the ethanolic extract contained higher total flavonoid content. In contrast, the ethanolic extract showed greater total antioxidant capacity and reducing power. This suggests that antioxidant activity was not determined solely by the total phenolic or flavonoid concentration but may depend on the specific antioxidant constituents and their interactions within the extract. Antioxidant activity of *P. cubeba* extracts, essential oil and oleoresins has previously been reported, supporting the present findings[2,24-26]. The higher alkaloid content observed in the ethanolic extract further indicates the influence of extraction solvent on the recovery of secondary metabolites. The presence of several phytochemical classes, including alkaloids, phenolics, flavonoids, lignans and terpenoids, may collectively contribute to the biological properties of *P. cubeba*. Previous reviews have similarly emphasized the contribution of these phytoconstituents to the pharmacological activities of the plant[2,24]. The antimicrobial activity varied according to both the extract and bacterial strain. The ethanolic extract showed a clear zone of inhibition against Gram-positive Bacillus spp., whereas no inhibition was observed with the aqueous extract. Against Gram-negative Escherichia coli, only slight traces of inhibition were observed with the ethanolic extract at 40 and 80 µL, which were insufficient to conclusively establish antibacterial activity. The comparatively greater response against the tested Gram-positive organism is consistent with previous reports describing antimicrobial and biocidal properties of P. cubeba essential oil and oleoresins[2,25-27]. The HRBC membrane stabilization assay demonstrated anti-inflammatory potential of both extracts. The ethanolic extract produced 57.14% inhibition of hemolysis, while the aqueous extract produced 47.62%, compared with 21.43% for aspirin under the conditions used in the present study. Thus, both extracts exhibited membrane-stabilizing activity in vitro, with the ethanolic extract showing the greater response. Choi and Hwang (2003) reported inhibition of acute and subacute inflammatory responses, while Yam et al. (2008) demonstrated inhibition of COX-1, COX-2 and 5-LOX activities and attenuation of IL-6 induction. Perazzo et al. (2013) also reported anti-inflammatory and analgesic effects of hydroalcoholic extracts and fractions from P. cubeba seeds. In addition, Qomaladewi et al. (2019) reported anti-inflammatory activity associated with inhibition of Src/Syk and NF-κB signaling[15,28-30]. The formulated *P. cubeba* herbal oil provided a preliminary basis for topical application. In the single observational case, reduction in ulcer size and redness was visibly noted from the first day, with reported pain relief and complete healing of the oral ulcer by the third day. Gum swelling and associated pain were also reduced during the observation period. However, because this was an uncontrolled single-patient observation without a comparator, these findings should be regarded as preliminary and cannot establish therapeutic efficacy. Nevertheless, they provide a useful basis for further investigation of *P. cubeba*-based topical formulations. Previous work has also demonstrated the potential of *P. cubeba* essential-oil formulations in wound-healing applications[11], while recent research has highlighted the antioxidant, antibacterial and other dermacosmeceutical properties of *P. cubeba* preparations[24]. Overall, the findings demonstrate that P. cubeba seed extracts possess diverse phytochemical constituents along with measurable antioxidant, antimicrobial and in vitro anti-inflammatory properties. The differences between ethanolic and aqueous extracts highlight the importance of solvent selection in determining phytochemical composition and biological activity. The topical observation provided encouraging

preliminary evidence for the potential of the prepared *P. cubeba* oil as a botanical topical preparation. The observed response supports further investigation of the oil through systematic evaluation of different concentrations, suitable formulation bases, physicochemical properties, stability, safety, and controlled biological efficacy. Further development of the prepared oil into standardized topical formulations, including ointments and other semisolid preparations, may enhance its potential for practical applications. Overall, the findings highlight the promise of the prepared *P. cubeba* oil as a potential botanical ingredient for the development of standardized topical formulations and warrant further investigation.

CONCLUSION

The present study confirmed the presence of diverse phytochemicals in *Piper cubeba* seeds, with differences between aqueous and ethanolic extracts. The ethanolic extract generally showed stronger antioxidant, antimicrobial, and in-vitro anti-inflammatory activities, particularly against *Bacillus* spp., while activity against *E. coli* was minimal. The preliminary herbal-oil formulation showed encouraging topical observations in a single case of oral ulcer and gum swelling; however, these findings are preliminary and cannot establish therapeutic efficacy. Overall, the study highlights *P. cubeba* as a promising source of bioactive compounds and provides a basis for further isolation and characterization of active constituents, optimization of the herbal-oil formulation, and development of standardized topical preparations. Future studies should evaluate their safety, stability, dose optimization, mechanism of action, and therapeutic efficacy through well-controlled in-vitro and in-vivo studies.

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Ethical Approval: The study involving hemolysis testing of human blood cells and the topical application of herbal oil to a patient suffering from oral ulcers and gum swelling was approved by the Institutional Ethics Committee of IILR Academy, Indore (IEC No. 19/07/21), where the study was conducted. Written informed consent was obtained from the patient for the topical application of herbal oil.

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