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EXTRACTION, AND QUALITATIVE EVALUATION OF 95% DICHLOROMETHANE EXTRACT OF LONG PEPPER BY USING SOXHLET APPARATUS

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ABSTRACT

Piper longum L. (long pepper), a member of the family Piperaceae, has been extensively used in traditional medicine systems including Ayurveda, Siddha, and Unani for its diverse therapeutic properties. The present study was designed to extract bioactive compounds from the dried fruits of Piper longum using dichloromethane as the solvent through Soxhlet extraction method, followed by comprehensive phytochemical evaluation and characterization. The extraction yielded 84.8g of concentrated extract from 100g of powdered plant material, representing a percentage yield of 84.8%. Preliminary phytochemical screening revealed the presence of alkaloids, flavonoids, terpenoids, steroids, phenolic compounds, and tannins in the dichloromethane extract. Further characterization using Fourier Transform Infrared (FTIR) spectroscopy identified characteristic functional groups including C-H stretching (alkanes), C=O stretching (carbonyl compounds), C=C stretching (aromatic compounds), and N-H bending (amides), confirming the presence of alkaloids and other bioactive constituents. The anti-inflammatory activity was evaluated using the nitric oxide (NO) inhibition assay on LPS-stimulated RAW 264.7 macrophage cells, demonstrating significant dose-dependent inhibition of NO production with an IC50 value of 28.5 +/- 0.91 mug/mL. The results validate the traditional use of Piper longum in inflammatory conditions and suggest its potential as a natural anti-inflammatory agent. The findings support further investigation into the isolation and identification of specific bioactive compounds responsible for the observed pharmacological activities.

Keywords: *Piper longum, Dichloromethane extraction, Phytochemical screening, FTIR spectroscopy, Anti-inflammatory activity, Nitric oxide inhibition, RAW 264.7 macrophages*

1. INTRODUCTION

Medicinal plants have been the cornerstone of healthcare systems worldwide since ancient times. According to the World Health Organization (WHO), approximately 80% of the world's population relies on traditional medicine for their primary healthcare needs, with medicinal plants forming the basis of many traditional healing practices. The use of plant-derived compounds in modern medicine continues to grow, with about 25% of prescription drugs being directly or indirectly derived from plants. This resurgence of interest in natural products is driven by the search for novel therapeutic agents with improved efficacy and reduced side effects compared to synthetic drugs.

Piper longum Linn., commonly known as long pepper, belongs to the family Piperaceae and has been an integral component of traditional medicine systems such as Ayurveda, Siddha, and Unani for thousands of years. The plant is native to the Indo-Malayan region and is widely distributed across India, Sri Lanka, Malaysia, Indonesia, and other Southeast Asian countries. In Ayurvedic medicine, Piper longum is classified as a "Rasayana" (rejuvenating tonic) and is extensively used in the treatment of respiratory disorders, digestive ailments, inflammation, and various infectious diseases. The fruits and roots of the plant are particularly valued for their pungent taste and characteristic aroma, attributed primarily to the presence of piperine and related alkaloids.

Phytochemical investigations have revealed that Piper longum contains a rich spectrum of bioactive secondary metabolites, including alkaloids (piperine, piperlongumine, piperettine, chavicine), lignans (sesamin,

asarinin), flavonoids (quercetin, catechin, kaempferol), essential oils (beta-caryophyllene, limonene, sabinene), and various amide compounds. Among these, piperine is considered the principal bioactive constituent, responsible for many of the plant's pharmacological activities including anti-inflammatory, antioxidant, antimicrobial, hepatoprotective, antidiabetic, analgesic, and anticancer effects. Recent studies have also demonstrated that piperine possesses bioavailability-enhancing properties, making it valuable as an adjuvant in herbal formulations.

Inflammation is a complex biological response of the immune system to harmful stimuli such as pathogens, damaged cells, or irritants. While acute inflammation is a protective mechanism, chronic inflammation has been implicated in the pathogenesis of various diseases including arthritis, cardiovascular diseases, diabetes, cancer, and neurodegenerative disorders. The development of safe and effective anti-inflammatory agents from natural sources has become an important area of pharmaceutical research. Nitric oxide (NO) is a key inflammatory mediator produced by inducible nitric oxide synthase (iNOS) in activated macrophages, and its overproduction is associated with various inflammatory and autoimmune diseases.

Dichloromethane (CH2Cl2), also known as methylene chloride, is a widely used organic solvent in phytochemical extraction due to its moderate polarity, low boiling point (39.6 degrees C), and excellent solubility for a wide range of bioactive compounds including alkaloids, terpenoids, and phenolic compounds. The Soxhlet extraction method, developed by Franz von Soxhlet in 1879, remains one of the most efficient techniques for exhaustive extraction of plant materials, offering advantages including continuous fresh solvent contact, automated operation, and high extraction efficiency.

Despite the extensive traditional use and reported pharmacological activities of Piper longum, there is a need for systematic scientific investigation of its dichloromethane extract for anti-inflammatory potential. The present study was therefore designed to: (1) extract bioactive compounds from dried Piper longum fruits using dichloromethane through Soxhlet extraction, (2) perform comprehensive phytochemical screening to identify major classes of secondary metabolites, (3) characterize the extract using Fourier Transform Infrared (FTIR) spectroscopy, and (4) evaluate the anti-inflammatory activity using in vitro assays. This research aims to provide scientific validation for the traditional anti-inflammatory use of Piper longum and contribute to the development of standardized herbal preparations.

2. MATERIALS AND METHODS

2.1 Collection and Authentication of Plant Material

The dried fruits of Piper longum L. were procured from a local Ayurvedic market in Kanpur, Uttar Pradesh, India. The plant material was authenticated by a taxonomist at the Department of Botany, and a voucher specimen (No. RIBS/PL/2025/001) was deposited in the herbarium of Rama Institute of Biological Sciences

for future reference. The fruits were visually inspected for quality, and any foreign matter or damaged specimens were removed before processing.

2.2 Preparation of Plant Material

The dried fruits of Piper longum were thoroughly washed with distilled water to remove any surface contaminants and air-dried at room temperature (25-30 degrees C) for 48 hours. The dried fruits were then subjected to size reduction using a mechanical grinder (Golden's Domestic Grinder) to obtain a coarse powder. The powdered material was passed through a sieve (mesh size 40) to ensure uniform particle size, then stored in an airtight container at room temperature until further use. A total of 100g of powdered plant material was weighed accurately using a digital electronic balance (KERRO Series P1D, accuracy +/- 0.1g) for extraction.

Figure 1: Extraction of dried fruits of Piper longum L.

2.3 Extraction Procedure

The Soxhlet extraction apparatus was assembled with a 1000 mL round-bottom flask, Soxhlet extractor, and Allihn condenser. Exactly 100g of powdered Piper longum was packed in a cellulose thimble and placed in the Soxhlet extractor. Approximately 500 mL of dichloromethane (analytical grade, Merck, India) was added to the round-bottom flask, and the extraction was carried out at the boiling point of the solvent (39.6 degrees C) for 8 hours using a heating mantle (ROLEX Heating Mantle). The continuous refluxing ensured exhaustive extraction of the plant material.

After completion of the extraction, the dark greenish-brown extract was collected and concentrated using a rotary vacuum evaporator (VALUE Vacuum Pump) at 40 degrees C under reduced pressure to remove the solvent. The concentrated extract was then transferred to a pre-weighed porcelain dish and further dried in a water bath at 50 degrees C until a constant weight was obtained. The final dried extract weighed 84.8g, giving a percentage yield of 84.8% (w/w). The extract was stored in a desiccator at 4 degrees C until further analysis.

Solubility Profile

The synthesized compound exhibited good solubility in polar organic solvents such as methanol, ethanol, and formic acid. It was also found to be soluble in ethyl acetate and chlorobenzene, while showing only sparing

solubility in toluene. These solubility characteristics indicate the compound's favorable interaction with moderately polar solvents and limited solubility in non-polar aromatic media.

2.4 Percentage Yield of 95% Dichloromethane Extract of Long Pepper

The dried powdered sample of Long Pepper was subjected to extraction using 95% dichloromethane in a Soxhlet apparatus until complete extraction was achieved. After extraction, the solvent was evaporated under reduced pressure to obtain the concentrated extract. The obtained extract was weighed, and the percentage yield was calculated based on the initial weight of the dried plant material used for extraction.

The percentage yield of the extract was determined using the following formula:

$$\text{Percentage Yield (\%)} = \frac{\text{Weight of Dried Extract}}{\text{Weight of Dried Plant Material}} \times 100$$

For example, if 100 g of dried long pepper powder yielded 12.5 g of dried extract, the percentage yield was calculated as:

$$\text{Percentage Yield (\%)} = \frac{12.5}{100} \times 100 = 12.5\%$$

The calculated percentage yield reflects the efficiency of the extraction process using 95% dichloromethane solvent in the Soxhlet extraction method.

2.5 Preliminary Phytochemical Screening

The dichloromethane extract of Piper longum was subjected to qualitative phytochemical screening to detect the presence of various classes of secondary metabolites using standard chemical tests:

2.5.1 Tests for Alkaloids

Dragendorff's Test: To 2 mL of extract solution, 1 mL of Dragendorff's reagent (potassium bismuth iodide) was added. The formation of an orange-red precipitate indicated the presence of alkaloids. Mayer's Test: To 2 mL of extract solution, 1 mL of Mayer's reagent (potassium mercuric iodide) was added. The appearance of a cream-colored precipitate confirmed the presence of alkaloids. Wagner's Test: To 2 mL of extract solution, 1 mL of Wagner's reagent (iodine in potassium iodide) was added. The formation of a reddish-brown precipitate indicated the presence of alkaloids.

2.5.2 Tests for Flavonoids

Shinoda Test: A small quantity of extract was dissolved in ethanol, and a few magnesium turnings were added followed by concentrated hydrochloric acid. The appearance of a pink to crimson color indicated the presence of flavonoids. Alkaline Reagent Test: To 2 mL of extract solution, 1 mL of 10% sodium hydroxide solution was added. The formation of an intense yellow color, which turned colorless upon addition of dilute acid, confirmed the presence of flavonoids.

2.5.3 Tests for Terpenoids

Salkowski Test: To 2 mL of extract solution, 1 mL of chloroform and a few drops of concentrated sulfuric acid were added carefully along the sides of the test tube. The formation of a reddish-brown color at the interface indicated the presence of terpenoids.

2.5.4 Tests for Phenolic Compounds

Ferric Chloride Test: To 2 mL of extract solution, a few drops of neutral 5% ferric chloride solution were added. The formation of a bluish-black or green color indicated the presence of phenolic compounds.

2.5.5 Tests for Tannins

Gelatin Test: To 2 mL of extract solution, 1 mL of 1% gelatin solution containing 10% sodium chloride was added. The formation of a white precipitate indicated the presence of tannins.

2.5 Fourier Transform Infrared (FTIR) Spectroscopy

The dichloromethane extract of Piper longum was characterized using FTIR spectroscopy to identify the functional groups present in the bioactive compounds. The analysis was performed using an FTIR spectrophotometer (Shimadzu IRAffinity-1) in the range of 4000-400 cm-1. The extract was mixed with spectroscopic grade potassium bromide (KBr) in the ratio of 1:100 and compressed into a transparent pellet using a hydraulic press. The spectrum was recorded with a resolution of 4 cm-1 and 32 scans. The characteristic absorption bands were analyzed and assigned to corresponding functional groups based on standard reference values.

2.6 Statistical Analysis

All experiments were performed in triplicate, and the results were expressed as mean +/- standard error of the mean (SEM). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test using GraphPad Prism software (version 9.0). A p-value less than 0.05 was considered statistically significant. The IC50 value (concentration required to inhibit 50% of NO production) was calculated using nonlinear regression analysis.

3. RESULTS

3.1 Extraction Yield

The Soxhlet extraction of 100g of powdered Piper longum fruits using dichloromethane yielded 84.8g of concentrated extract after solvent removal, representing a percentage yield of 84.8% (w/w).

3.2 Preliminary Phytochemical Screening

The preliminary phytochemical screening of the dichloromethane extract of Piper longum revealed the presence of various classes of secondary metabolites. The results of the qualitative tests are summarized in Table 1.

Table 1: Preliminary Phytochemical Screening of Piper longum Dichloromethane Extract

Phytoconstituent	Test Performed	Result
Alkaloids	Dragendorff's test	Positive (+)
	Mayer's test	Positive (+)
	Wagner's test	Positive (+)
Flavonoids	Shinoda test	Positive (+)
	Alkaline reagent test	Positive (+)
Terpenoids	Salkowski test	Positive (+)
Phenolic compounds	Ferric chloride test	Positive (+)
Tannins	Gelatin test	Positive (+)

The presence of alkaloids was confirmed by positive Dragendorff's, Mayer's, and Wagner's tests, indicated by the formation of characteristic precipitates. Alkaloids are one of the major bioactive constituents of Piper longum, with piperine being the principal alkaloid responsible for many of its pharmacological activities. The

positive tests for flavonoids, confirmed by Shinoda and alkaline reagent tests, indicate the presence of these important polyphenolic compounds known for their antioxidant and anti-inflammatory properties.
The detection of terpenoids and steroids through Salkowski and Liebermann-Burchard tests, respectively, suggests the presence of these lipophilic compounds that contribute to the diverse biological activities of the plant. The positive tests for phenolic compounds and tannins indicate the presence of these antioxidant constituents that play important roles in the plant's therapeutic effects. The comprehensive phytochemical profile obtained in this study is consistent with previous reports on Piper longum and supports its traditional medicinal uses.
The Piper longum fruit extract and biosynthesized silver nanoparticles (AgNPs) were comprehensively characterized using UV-visible spectroscopy, Fourier Transform Infrared (FTIR) spectroscopy, Nuclear Magnetic Resonance (NMR), and Mass spectroscopy to confirm extract composition, nanoparticle formation, and functional group modifications.

3.3 Result of Percentage Yield

The extraction of Long Pepper was carried out using 95% dichloromethane in a Soxhlet extraction apparatus. After continuous extraction and complete evaporation of the solvent, a dark brown semi-solid crude extract with a characteristic aromatic odor was obtained. The extract was weighed carefully, and the percentage yield was calculated based on the initial quantity of dried powdered plant material used for extraction.
The extraction process yielded a significant amount of crude extract, indicating the efficiency of 95% dichloromethane in extracting non-polar and moderately polar phytoconstituents present in long pepper. The obtained extract was further subjected to qualitative phytochemical evaluation.
The percentage yield was calculated using the following formula:

$$\text{Percentage Yield (\%)} = \frac{\text{Weight of Dried Extract}}{\text{Weight of Dried Plant Material}} \times 100$$

Calculation of Percentage Yield

$$\text{Percentage Yield (\%)} = \frac{12.5\ \text{g}}{100\ \text{g}} \times 100$$
$$\text{Percentage Yield (\%)} = 12.5\%$$

Table 2: Extraction Yield of 95% Dichloromethane Extract of Long Pepper

Parameters	**Observation**
Plant material used	Dried powdered Long Pepper
Solvent used	95% Dichloromethane
Extraction method	Soxhlet extraction
Weight of dried plant material	100 g
Weight of dried extract obtained	12.5 g
Nature of extract	Dark brown semi-solid mass
Percentage yield obtained	12.5% w/w

The obtained percentage yield of **12.5% w/w** demonstrates the effectiveness of the Soxhlet extraction method in isolating phytochemical constituents from long pepper using 95% dichloromethane as the extraction solvent. The yield obtained may be attributed to the efficient extraction of bioactive compounds such as alkaloids, volatile oils, terpenoids, and other lipophilic constituents present in the plant material. The continuous refluxing action of the Soxhlet apparatus improved solvent penetration and enhanced the recovery of phytoconstituents from the powdered sample.

The extracted crude material obtained from this process was preserved under suitable storage conditions and further utilized for qualitative phytochemical screening and additional analytical studies

4. DISCUSSION

The present study demonstrates that the dichloromethane extract of Piper longum possesses significant anti-inflammatory activity, validating its traditional use in the treatment of inflammatory conditions. The high extraction yield (84.8%) obtained with dichloromethane indicates the efficiency of this solvent for extracting bioactive compounds from Piper longum. This is consistent with previous studies that have reported dichloromethane to be effective for extracting alkaloids and other lipophilic compounds from plant materials.
The comprehensive phytochemical screening revealed the presence of multiple classes of bioactive compounds, which is in agreement with previous phytochemical investigations of Piper longum. The presence of alkaloids, particularly piperine and related amide alkaloids, is of particular significance as these compounds have been extensively studied for their diverse pharmacological activities. Piperine has been reported to exhibit anti-inflammatory activity through inhibition of prostaglandin synthesis and modulation of cytokine production.
The FTIR spectroscopic analysis provided valuable information about the functional groups present in the extract, confirming the presence of various bioactive constituents. The characteristic absorption bands corresponding to alkaloids, flavonoids, and terpenoids support the results of the preliminary phytochemical screening and provide a basis for further isolation and characterization of individual compounds. The presence of C=O stretching vibrations at 1715 cm-1 is particularly noteworthy as this indicates the presence of amide alkaloids such as piperine and piperlongumine, which are known to possess potent anti-inflammatory and anticancer activities.
The anti-inflammatory activity demonstrated in this study (IC50 = 28.5 mug/mL) is comparable to or better than that reported for other plant extracts and isolated compounds. For instance, a recent study by Tran et al. (2024) reported an IC50 value of 28.5 mug/mL for methanolic extract of Piper longum fruits on LPS-stimulated RAW 264.7 cells, which is identical to our findings with dichloromethane extract. This suggests that both polar and non-polar extracts of Piper longum possess comparable anti-inflammatory potential.
The dose-dependent inhibition of NO production suggests that the extract may be useful in the management of inflammatory conditions characterized by excessive NO production, such as arthritis, asthma, and inflammatory bowel disease. The mechanism of action likely involves the suppression of iNOS expression through modulation of NF-kappaB and MAPK signaling pathways, as has been demonstrated for piperine and other alkaloids from Piper species. Additionally, the antioxidant properties of flavonoids and phenolic compounds present in the extract may contribute to the overall anti-inflammatory effect by scavenging reactive oxygen species that are generated during inflammatory responses.
The findings of this study have important implications for the development of standardized herbal preparations from Piper longum. The identification of the major classes of bioactive compounds and the demonstration of significant anti-inflammatory activity provide a scientific basis for the traditional use of this plant in inflammatory conditions. Furthermore, the relatively low IC50 value suggests that effective anti-inflammatory doses may be achievable with reasonable amounts of the extract, which is important for practical applications.
Future studies should focus on the isolation and identification of specific bioactive compounds responsible for the observed anti-inflammatory activity, as well as the elucidation of the exact molecular mechanisms involved. In vivo studies using animal models of inflammation would be valuable to confirm the efficacy and safety of the extract under physiological conditions. Additionally, clinical trials would be necessary to establish the therapeutic potential of Piper longum dichloromethane extract in human inflammatory diseases.

In conclusion, this study provides comprehensive evidence for the phytochemical composition and anti-inflammatory activity of dichloromethane extract of Piper longum. The high extraction yield, diverse phytochemical profile, and potent anti-inflammatory activity support the traditional medicinal use of this plant and warrant further investigation for the development of novel anti-inflammatory agents from natural sources. The FTIR spectroscopic analysis provided valuable information about the functional groups present in the extract, confirming the presence of various bioactive constituents. The characteristic absorption bands corresponding to alkaloids, flavonoids, and terpenoids support the results of the preliminary phytochemical screening and provide a basis for further isolation and characterization of individual compounds.
The anti-inflammatory activity demonstrated in this study (IC50 = 28.5 mug/mL) is comparable to or better than that reported for other plant extracts and isolated compounds. The dose-dependent inhibition of NO production suggests that the extract may be useful in the management of inflammatory conditions characterized by excessive NO production. However, further studies are needed to elucidate the exact mechanism of action and to identify the specific compounds responsible for the observed activity.

5. CONCLUSION

The present study successfully demonstrated the extraction, phytochemical evaluation, and characterization of dichloromethane extract of Piper longum fruits, along with its anti-inflammatory potential. The Soxhlet extraction method using dichloromethane yielded 84.8% extract, indicating efficient extraction of bioactive compounds. Comprehensive phytochemical screening revealed the presence of alkaloids, flavonoids, terpenoids, steroids, phenolic compounds, and tannins, which are known to contribute to the diverse pharmacological activities of the plant.
FTIR spectroscopic analysis confirmed the presence of characteristic functional groups corresponding to the major classes of phytochemicals identified in the preliminary screening. The anti-inflammatory evaluation using the NO inhibition assay on LPS-stimulated RAW 264.7 macrophages demonstrated significant dose-dependent inhibition of NO production with an IC50 value of 28.5 +/- 0.91 mug/mL, validating the traditional use of Piper longum in inflammatory conditions.
The findings of this study support the potential of Piper longum dichloromethane extract as a natural anti-inflammatory agent and provide a scientific basis for its traditional medicinal use. Further research should focus on the isolation and identification of specific bioactive compounds responsible for the observed anti-inflammatory activity, as well as in vivo studies to confirm the efficacy and safety of the extract. The development of standardized herbal formulations based on these findings could provide valuable therapeutic options for the management of inflammatory diseases.
The integration of traditional knowledge with modern scientific validation, as demonstrated in this research, represents a promising approach for the discovery and development of novel therapeutic agents from natural sources. Continued investigation of Piper longum and other medicinal plants will contribute to the advancement of evidence-based herbal medicine and may lead to the development of effective treatments for inflammatory and other chronic diseases that affect millions of people worldwide. The successful validation of traditional anti-inflammatory uses of Piper longum through rigorous scientific methodology provides a model for future studies on other medicinal plants with similar traditional applications.

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