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Comparative Evaluation of Antioxidant and Anti-inflammatory Properties from *Piper peepuloides* Roxb and *Piper schmidtii* Hook. F. – An *in vitro* Approach

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Abstract

In this article, the antioxidant and anti-inflammatory properties of *Piper peepuloides* and *Piper schmidtii* are discussed. The study was focused on the secondary metabolites, antioxidant, anti-inflammatory, and antibacterial activities of the extracts from the successive solvent extractions. The ethanol extract of *P. peepuloides* was the most active in the total phenolic (783.91 mg gallic acid equivalent [GAE]/100 g extract), tannin (597.81 mg GAE/100 g extract), and flavonoid (52.23 mg RE/100 g extract). Ethanol extract of leaves responded well against diphenyl-1-picrylhydrazyl (IC_{50}10.16 µg/mL), ABTS (55763.89 µg TE/g extract), superoxide (42.94%), and phosphomolybdenum (751.46 mg AAE/100 g extract) antioxidant assays. The *P. schmidtii* leaf extract was found to be good at getting rid of free radicals. The ethanol extract of *P. peepuloides* leaves also had a lot of antioxidant power. In the anti-inflammatory assay, the ethanol extract of *P. peepuloides* showed a high level of inhibition (69.59%). *P. peepuloides* and *P. schmidtii* leaf extracts have a lot of antioxidant power and could be used as a good source of natural antioxidants and anti-inflammation.

Keywords: Antioxidant and anti-inflammatory, *Piper peepuloides*, *Piper schmidtii*

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Introduction

The *Piper* L. genus belongs to *Piperaceae* family which is good to human health and important commercially. More than 600 species of *Piper* have been identified in the Southeast Asia region, about 1500 species that have been reported throughout the pantropical and neotropical regions of the world.[1] Analgesic, anti-inflammatory, anti-diarrheal, antispasmodic, hepatoprotective, insecticidal, and larvicidal effects that have been reported in *Piper nigrum* L. and its active ingredient "Piperine" demonstrate have high pharmacological property.[2] Wild edible pepper is found distributed in shola forests user *Piper peepuloides* and *Piper schmidtii*, which are known as tippili and kattu kurumilagu.

Antioxidants play a significant role in scavenging the free radicals and reactive oxygen species (ROS) produced in the body and are obtained from the natural product. Plant kingdoms are the store house of natural antioxidant which plays a major role in the food system, they scavenge the free radicals and quench the singlet-oxygen formation.[3] Antioxidant compounds reduce the action of ROS in tissues and cells reducing the damage of body parts.[4] Natural products with antioxidant capacity are used as an assistant in the protection of the endogenous system and these have an increase in search of antioxidant and nutraceutical properties.[5] In the present scenario, synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole play a significant role in food industry as it acts very much to hold the role of natural antioxidant. In spite of the major drawback, such products have been analyzed to fail off due to instability of the molecule. Researches have proved that free radicals act as promoters of carcinogenesis.[6,7]

Inflammation is the first line of defense mechanism in the body as this response holds on with ROS which play a dual role, namely, a signaling molecule and acts as a mediator of inflammation.[8] ROS are created by cells and are engaged in the host defense mechanism, such as polymorphonuclear neutrophils, which cause endothelial dysfunction through the oxidation of important cellular signaling proteins like tyrosine phosphatases.[9] Pattern-recognition receptors, which are germline-encoded receptors, help the innate immune system recognize pathogens including viruses, bacteria, and fungi. This triggers the inflammatory responses such as systemic vasodilation, vascular leakage, and leukocyte emigration.[10] Separately, both plants were investigated scientifically for their diverse medicinal properties. There is currently no published scientific research on the antioxidant and anti-inflammatory properties of polyherbal formulations containing these two plants. (*P. peepuloides*+*P. schmidtii*) Therefore, the present study is focused to explore the efficacy of an inflammatory formulation against the effects of *P. peepuloides* and *P. schmidtii* as well as to offer the necessary scientific support.

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Materials and Methods

Plant Sample

A sample of *P. peepuloides* and *P. schmidtii* leaves was gathered in Coonoor in September 2022 and it is certified by the Botanical

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Survey of India's Southern Regional Centre in Coimbatore, Tamil Nadu. (Ref. Nos.: BSI/SRC/5/23/2022/Tech./403 and 404). To prevent the decomposition of some bioactive chemicals, the samples were promptly packed in plastic bags.

Preparation of Samples

To remove dust and extraneous contaminants, the leaf samples were gently cleaned with running water and dried for 7 days, the leaf samples were dried in the shade at a temperature of (25°C). Following drying, the leaf samples were ground into a powder using a grinder for 30 s.

Extraction Procedure for Dry Leaf Samples

A Soxhlet device was used to prepare extractions after mixing about 70 g of leaf powder with 500 mL of several solvents, including petroleum ether, chloroform, ethyl acetate, ethanol, and hot water. The extractions were dried before being used for more investigation.

Phytochemical Screening

P. peepuloides and *P. schmidtii* leaves were experimented using the standard method for determining phytochemicals (such as quinones, carbohydrates, alkaloids, reducing sugar, polyphenols, phenols, flavonoids, resins, glycoside, phlobatannins, xanthoproteins, triterpenoids, coumarins, cardiac glycoside, and cholesterols) for a preliminary qualitative phytochemical analysis.[11]

Quantification of Bioactive Secondary Metabolites

Total phenolic contents

The following approach, which uses gallic acid as the standard, was used to determine the total phenolic content of *P. peepuloides* and *P. schmidtii* leaves.[12] Each test tube received 100 μL of the extract solution and 100 μL of the reference solution, each at a different concentration. Each test tube received 2.5 mL of solutions containing various concentrations of 5% of sodium carbonate and 500 μL of diluted Folin-Ciocalteau phenol regent. To speed up the reaction, the test tubes were kept at 25°C for 20 min. The UV apparatus was used to position the test tubes and the absorbance at 725 nm was measured. The total phenolic content was determined using a standard curve that has been constructed based on the gallic acid equivalent (GAE).

Determination of tannin contents

The plant extract was treated with polyvinylpolypyrrolidone and the amount of tannin in the extract was determined using the Folin-Ciocalteu method. Three tests were conducted and the outcomes were expressed as GAEs. The tannic acid equivalents were used to calculate the amount of tannins.[13]

Total flavonoid contents

To evaluate the test content of the total flavonoid content in *P. peepuloides* and *P. schmidtii*, Rutin was utilized as a reference.[14] 800 μL of the extract solution and 100 μL of the reference solution, both in variable concentrations, were poured into the various test tubes. Each test tube received 150 μL of NaNo3, 150 μL of Alcl3 (10%), 2 mL of NAOH (4%), and 200 μL of distilled water to speed up the reaction. The sample, a standard, and a blank was utilized, along with a UV equipment to detect absorbance at 420 nm. The amount of rutin in a dried extract that contained all flavonoids was shown as mg/g.

In vitro **Antioxidant Activity**

Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

P. peepuloides and *P. schmidtii* leaf extracts radical scavenging ability against stable DPPH was assessed using a slightly modified version method of[15] 2.9 mL of methanolic DPPH and 100 μL of sample at a variable concentration were included in the reaction mixture (0.3 mM). After 20 min of resting at room temperature, the final solutions were tested for absorbance at 517 nm. The IC_{50} values (concentration providing 50% inhibition) were calculated from the graph of scavenging activity versus sample concentrations.

ABTS radical scavenging assay

The technique was used for the ABTS assay.[16] By reacting an aqueous solution of potassium persulfate with an aqueous solution of ABTS, radical cations (ABTS+) were formed (2.4 mM). Before usage, the combination was kept at room temperature and in the dark for 12 to 16 h. To attain an absorbance of 0.700 ± 0.05 at 734 nm, the solution was then diluted with distilled water. At room temperature, 30 L of the sample was permitted to be added to 30 μL of the ABTS solution. The absorbance at 734 nm was measured immediately after 6 min. Using a Trolox solution as the reference curve, the total antioxidant content of *P. peepuloides* and *P. schmidtii* was calculated as mM of Trolox equivalents per 100 g of dry weight.

Superoxide radical scavenging activity

The experiment relied on the ability of different extracts to scavenge the superoxide radicals produced by the riboflavin-light-NBT combination, hence inhibiting formazan production.[17] 100 μL of sample solution, BHT, and rutin were added to 3 mL of reaction mixture containing 50 mM sodium phosphate buffer (pH-7.6), 20 μg riboflavin, 12 mM EDTA, and 0.1 mg NBT. After exposing the reaction mixture with samples to light for 90 s, the reaction began. As a comparison, we employed a darkened reaction mixture devoid of sample to light up. Absorbance reading was taken at 590 nm, referenced it to a blank, and found that it was highest immediately after illumination (unilluminated reaction mixture without sample). This formula was used to determine the scavenging activity on superoxide anion generation: Scavenging activity (%) = [(Control OD – Sample OD)/Control OD] × 100.

Phosphomolybdenum assay

Antioxidant levels were evaluated using the phosphomolybdenum technique. This experiment was carried out using a reagent solution containing 0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate. In a water bath preheated to 95°C, the plant extracts (0.5 mL) and the phosphomolybdenum reagent solution (4.5 mL) were combined for 90 min. Absorbance

was measured at 695 nm once the solution had cooled to room temperature. The positive reference standard was ascorbic acid.[18]

In vitro Anti-inflammatory Activity

Membrane stabilization method

Healthy goat was selected for the study and it was confirmed that it did not received any medication in 2 weeks. A sample of goat blood was collected and it was centrifuged for 10 min at 3000 rpm. The sample and regular saline were combined to create a 10% of red blood cells (RBC) solution. To study the impact on heat-induced hemolysis, 1 mL of sample was added to this suspension and incubated at 56°C for 30 min. The mixture was chilled after incubation and centrifuged at 2500 rpm for 5 min. The supernatant's absorbance was then determined at 560 nm using a spectrophotometer (Thermo Scientific genesys 50 UV-Vis spectrophotometer, USA). Equation was used to determine the proportion of hemolysis that was inhibited.

Result and Discussion

Quantification of Bioactive Secondary Metabolites

Determination of total phenolics, tannin, and flavonoid contents of P. peepuloides and P. schmidtii leaves

Leaf extracts of *P. peepuloides and P. schmidtii* were analyzed for total phenolic content and the results are presented in Table 1. Total phenolics were found to be greater in the ethanol extracts of *P. peepuloides* when compared to *P. schmidtii* leaf extracts (783.91 and 750 mg GAE/100 g extract, respectively). Ethanol extract of *P. schmidtii* (597.81 mg GAE/g extract) was found to have lower tannin concentrations than ethanol extract of *P. peepuloides* [Table 1]. The ethanol extracts of both the samples had the highest flavonoid concentration (52.23 mg RE/100 g and 46.78 mg RE/100 g extract, respectively) of any of the extracts tested. In most extracts, the flavonoid concentration of *P. peepuloides* leaf was found to be greater than that of *P. schmidtii*. Both the plant sample *P. peepuloides* and *P. schmidtii* have slight variation in presence of Phenolics, Tannins, and Flavonoid content when compared to other *Piper* varieties. The total phenolic content was analyzed for six varieties of *P. nigrum* (36.71–58.90 mg GAE/g) and was found to be higher than that of *Piper longum* (29.53 mg GAE/g) and *Piper retrofractum* (32.60 mg GAE/g), according to research conducted by Luca *et al*. (2021) on phytochemicals in methanolic fruit extracts of *P. nigrum*, *P. longum*, and *P. retrofractum*. The total flavonoid concentration was highest in *P. retrofractum* (19.70 mg RE/g), the total phenolic content was lowest in *P. nigrum* (1.44 mg RE/g), When compared to *P. peepuloides* and *P. schmidtii* leaves extract. Therefore, it has been acknowledged that a greater number of secondary metabolites in plant samples are an excellent sign of its superior antioxidant capacity.

In vitro Antioxidant Assays

DPPH$^{\cdot}$ scavenging activity

Standardized reduction potential of dihydroxyphenyl phosphate radical scavenging assay is one of the most widely used assays for measuring antioxidant activity of a sample extract. It is believed that this is the most time-efficient, trouble-free, and helpful approach. The method is popular because it can effectively screen large numbers of samples and is unaffected by the polarity of sample extracts.[19,20] As shown in Figure 1, both *P. peepuloides* and *P. schmidtii* leaves have the ability to quench DPPH radicals. In this analysis, both natural antioxidant Rutin and synthetic antioxidant BHT commercially serve as golden standard. The antioxidants BHT and rutin, both of which are the concentration of the sample used in the experiment that resulted in a 50% decrease in the starting concentration of DPPH (IC_{50}) was determined. Higher IC_{50} values for DPPH radical scavenging capacities were observed for the ethanol extracts of *P. peepuloides* (10.16 μg/mL) and *P. schmidtii* (18.98 μg/mL) leaves compared to those of other solvent extracts. The IC_{50} values for the naturally occurring antioxidant rutin and the synthetic antioxidant BHT were determined to be 8.35 g/mL and 9.93 g/mL, respectively. The IC_{50} value for *Piper guineense* was reported by[21] to be in the range of 69.05–74.0 μg/mL. Both *P. nigrum* and *P. refractum* have been shown to have powerful DPPH radical scavenging action, according to research published.[22]

ABTS$^{\cdot+}$ scavenging activity

Plants are the warehouse of chemical compounds with high antioxidant capacity. In this assay, potassium persulfate is used to detect the hydrogen-donating ability of the selected sample. Which is than spectrophotometrically examined for discoloration by Samec *et al.* (2010) and Keser *et al.* (2012).[23,24] Table 2 displays the findings of a study comparing the cation radical scavenging capabilities of the selected samples *P. schmidtii* (55763.89 μg TE/g extract) and *P. peepuloides* (39409.72 μg TE/g extract) had greater radical scavenging capabilities in their respective ethanol extracts. Extracts of natural antioxidant rutin (138541.7 μg TE/g sample) and synthetic antioxidant BHT (139722.2 μg TE/g extract) were analyzed. Similarly, reported the *P. longum* ethyl acetate extract has high radical scavenging activity-based concentration dependence.

Table 1: Phenolic, tannin, and flavonoids content of *Piper peepuloides and Piper schmidtii* leaves extract

Sample	*Extracts*	*Phenolic GAE/g extract*	*Tannin GAE/g extract*	*Flavonoids RE/100 g*
Piper peepuloides	Petroleum ether	28.94±0.87	28.45±1.06	13.59±1.76
	Chloroform	59.35±1.82	55.05±1.77	10.32±1.06
	Ethyl acetate	135.67±1.01^{c}	112.00±2.05^{d}	25.36±1.04^{c}
	Ethanol	783.91±1.82^{a}	409.74±1.6^{d}	52.23±1.38^{a}
	Hot Water	194.73±0.87^{b}	180.80±2.54	11.41±0.11
Piper schmidtii	Petroleum ether	28.94±1.75	8.58±1.45	10.87±0.58
	Chloroform	21.05±1.75	19.37±1.90	8.62±0.92
	Ethyl acetate	664.62±1.33^{d}	539.10±1.36^{b}	21.95±1.74^{d}
	Ethanol	750±0.87^{b}	597.81±1.48^{a}	46.78±1.04^{b}
	Hot Water	392.39±1.82	316.98±2.68^{c}	9.03±0.47

Values are mean of triplicate determination (n=3) ± standard deviation, statistically significant at $P<0.05$ where $^{a}>^{b}>^{c}>^{d}$ in each column

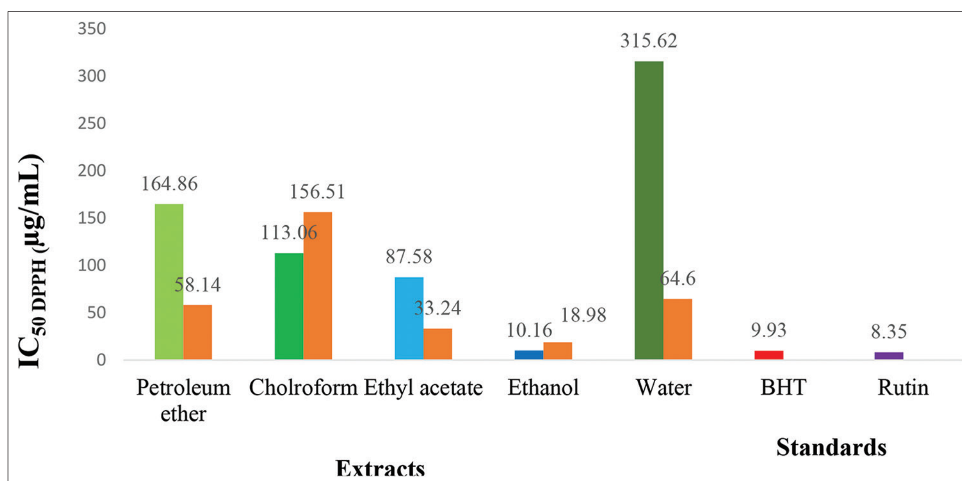

Figure 1: Diphenyl-1-picrylhydrazyl radical scavenging activity of *Piper peepuloides and Piper schmidtii* leaves extract

Table 2: ABTS scavenging activity and Superoxide radical of *Piper peepuloides and Piper schmidtii* leaves extract

Sample	*Extracts*	*ABTS scavenging activity (µM TE/g extract)*	*Superoxide radical scavenging activity percentage of inhibition (%)*
Piper peepuloides	Petroleum ether	32222.22±910.09	26.32±0.78
	Chloroform	38854.17±1513.10	18.15±0.27
	Ethyl acetate	37812.5±993.68	31.59±1.76
	Ethanol	39409.72±1027.68	42.94±0.45[b]
	Hot Water	38958.33±1705.68	35.99±0.64[c]
Piper schmidtii	Petroleum ether	28715.28±262.14	14.31±0.78
	Chloroform	44201.39±420.98[d]	12.15±0.27
	Ethyl acetate	49861.11±1517.88[c]	25.58±1.76
	Ethanol	55763.89±1327.18[b]	36.93±0.45[d]
	Hot water	44027.78±781.82[d]	26.78±1.11
Standard	Rutin	138541.7±416.66[a]	74.7±0.25[a]
	BHT	139722.2±636.46[a]	74.2±0.1[a]

Values are mean of triplicate determination (n=3) ± standard deviation, TE- Trolox Equivalents, statistically significant at *P*<0.05 whereas [a]>[b]>[c]>[d] in each column. BHT: Butylated hydroxytoluene

[25] The ethanolic extract of *P. longum* and P. nigrum reported that both plant samples have high radical scavenging activity.[26] *P. nigrum* fruit was subjected to an ABTS radical scavenging activity with results ranging from 24813 to 54212 µM TE/g.[27]

Superoxide radical scavenging activity

Superoxide radical is recognized to be damaging to cellular components since it is a precursor of more ROS. There is convincing evidence that the extracts are effective radical scavengers when exposed to superoxide radicals produced *in vitro* with the riboflavin-NBT-light system. Table 2 displays the outcomes of comparison of superoxide anion scavenging activities of *P. peepuloides* and *P. schmidtii* leaves. Scavenging activity appears to be highest in ethanol extracts of both the samples *P. peepuloides* (42.94%) and *P. schmidtii* (36.93%). When compared to the plant extract, the standards (ascorbic acid and BHT) showed much higher radical scavenging activity in this assay.[28] *P. longum* possessing high radical scavenging activity and also revealed the several amino acids such as valine, proline, isoleucine and leucine which highly contribute to the scavenging of free radicals against superoxide ions.[28] These components have been identified to have a high antioxidant capacity as that of the selected plant sample *P. peepuloides* and *P. schmidtii.*

Phosphomolybdenum assay

Antioxidant activity of *P. peepuloides* and *P. schmidtii* leaves solvent extracts was measured using the phosphomolybdenum assay [Figure 2]. The two leaf ethanol extracts demonstrated the highest antioxidant activity (751.46 and 535.53 mg AAE/g extract) when compared to other solvent extracts. This was measured by the decrease of the phosphomolybdenum complex.[29,30] The ethanolic fractions of *P. cubeba* and has reported to have high antioxidant activity.[29,30] The radical scavenging assay in *P. nigrum* seed which ranged from 0.76-79.12 µg/ml).[31] When compared to other Piper the results of other *Piperaceae* members have been identified to have low scavenging property than the selected plant sample *P. peepuloides and P. schmidtii*.

In vitro **Anti-inflammatory Activity**

Membrane stabilization assay

The ability of various extracts from the leaves of *P. peepuloides* and *P. schmidtii* to stabilize membranes after heat-induced rupture of RBC membranes was investigated in this work. The data are shown in Figure 3. It reveals that both *P. peepuloides* (69.59) and *P. schmidtii* ethanolic extracts showed the best protection against heat-induced hemolysis (65.85). In addition, the ethanolic extract effectively warded off heat-related damage

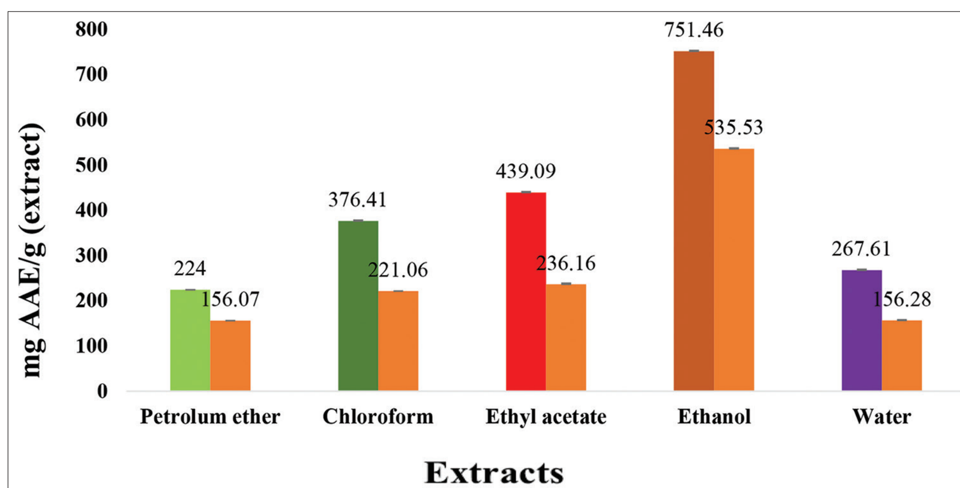

Figure 2: Phosphomolybdenum Assay of *Piper peepuloides* and *Piper schmidtii* leaves extract. Values are mean of triplicate determination (*n*=3) ± standard deviation, statistically significant at $P<0.05$ where $^a>^b>^c>^d$

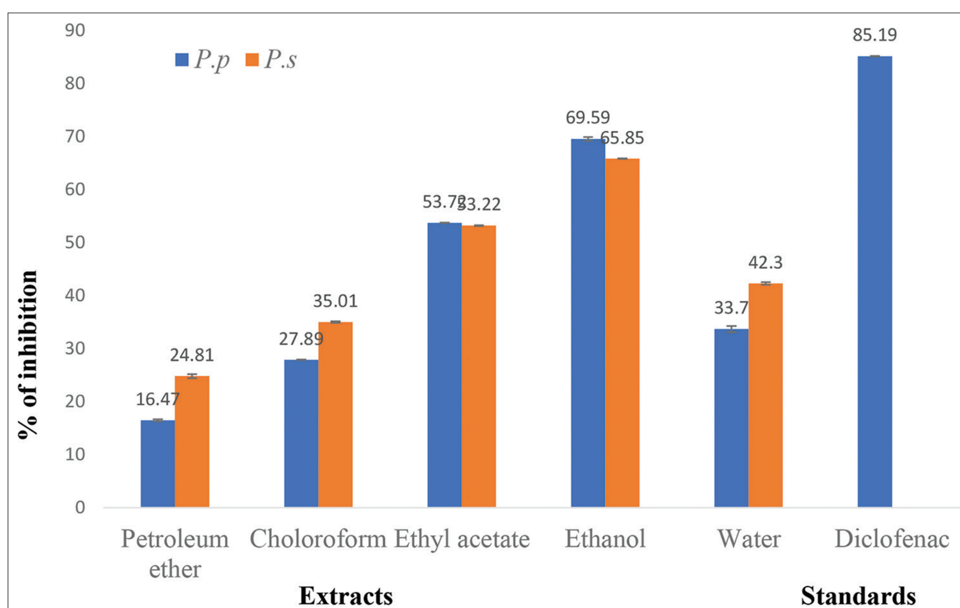

Figure 3: *In vitro* anti-inflammatory activity of *Piper peepuloides* and *Piper schmidtii* leaves extract

to the RBCs' membrane. In contrast to the other extracts, comparing aspirin to the *P. peepuloides* and *P. schmidtii* extracts at a dosage of 1 mg/mL, 85.19% of aspirin provided protection. These findings imply that *P. peepuloides* extracts might protect the cell membrane, hence reducing inflammation. The lysosomal enzyme in RBC is ruptured by a hypotonic solution's excessive fluid collection, making these cells more vulnerable to subsequent damage from lipid peroxidation caused by free radicals.[32] Following extracellular release, lysosomal component leakage results in tissue inflammation and injury. Stabilization of the lysosomal membrane plays a crucial role in the regulation of the inflammatory response.[33]

Conclusion

In conclusion, the antioxidant and anti-inflammation activity of *P. peepuloides* and *P. schmidtii* has shown to be promising as a food additive to replace synthetic antioxidants. Thus, the study ascertains the value of plants used traditionally, which could be of considerable interest to the development of new drugs. Further studies are warranted for the isolation and identification of individual bioactive compounds and also *in vivo* studies are needed for understanding their mechanism of action as an antioxidant and anti-inflammation drug which can be a cost effective and reliable source of medicine for the human welfare.

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