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ANTIOXIDANT AND ANTI-INFLAMMATORY PROPERTIES OF TERPENOID FRACTION ISOLATED FROM THE SHOOT OF *Plectranthus hadiensis*

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ABSTRACT

In the present study, antioxidant and anti-inflammatory activities of terpenoid fraction isolated from shoot of *P. hadiensis* was investigated. The terpenoid fraction was obtained from the aqueous ethanolic extract by liquid-liquid partitioning using Ethyl acetate as solvent. The terpenoid fraction thus obtained was concentrated and taken for further analysis and bioassays. The terpenoid fraction (IC_{50} 22.76 ± 0.787 µg/mL) exhibited excellent free radical scavenging capacity towards 1, 1 –diphenyl – 2- picrylhydrazyl stable free radical (DPPH·). The reducing power of the fraction and BHT, which was taken as the standard increased with increasing concentration. The nitric oxide radical scavenging capacity of the fraction (IC_{50} 79.84 ± 0.519 µg/mL) of *P. hadiensis* is comparable to that of the standard Ascorbic acid (IC_{50} 75.81 ± 0.754 µg/mL). The results showed that the terpenoid fraction isolated from the shoot of *P. hadiensis* had a strong reductive capacity. The *in vitro* anti inflammatory assays included BSA denaturation inhibition, HRBC membrane stabilization and Platelet aggregation inhibition assays. The fraction showed IC_{50} values of 56.18±0.766 µg/mL, 57.17±0.890 µg/mL and 54.26±0.744 µg/mL for BSA denaturation inhibition, HRBC membrane stabilization and Platelet aggregation inhibition assays respectively, which was compared with the standard non-steroidal drug, viz., Diclofenac sodium. The study demonstrated that the terpenoid fraction efficiently scavenged DPPH, $OH^{\bullet}$ and NO radicals. The anti-inflammatory activity of the fraction showed that it can be positively compared to the standard drug used for treating inflammation-related ailments. The results of the present study revealed that the terpenoid fraction obtained from the shoot of *P. hadiensis* has potent antioxidant and anti-inflammatory activity.

KEYWORDS: ***P. hadiensis*, Terpenoid fraction, Antioxidant activity, Anti-inflammatory activity.**

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INTRODUCTION

Interest in search of new natural antioxidants has grown over the past years as reactive oxygen species (ROS) production and oxidative stress have been shown to be linked to chronic diseases such as Cancer, Cardiovascular disease, Osteoporosis, and Neurodegenerative diseases. Such natural antioxidant substances are believed to play a potential role in interfering with the oxidation process by reacting with free radicals, chelating catalytic metals and scavenging oxygen in biological systems[1]. Although inflammation acts as an adaptive host defense against infection or injury and is primarily a self limiting process, inadequate resolution of inflammatory responses often leads to various chronic ailments including cancer[2]. Although the present treatment regimens for both inflammation related ailments and cancer are effective, their adverse side effects are far more detrimental. Hence, there is a need to develop new drugs with novel modes of action that do not produce considerable side effects. Plants used in Ayurvedic formulations are a source of compounds which have high potential as therapeutic molecules. *P. hadiensis (Hribera)* belongs to the family of *Lamiaceae*, which is a shrub with slightly rough brownish corky bark. Leaves are fleshy, ovate or orbicular, acute, retuse or emarginated at apex[3]. Flowers are violet or lavender, in long racemes. The genus *Plectranthus* is an important source of medicinal natural products particularly terpenoids, flavonoids and iridoids. Traditionally, this genus has been reported to possess a number of medicinal properties. The methanolic extract of *P. hadiensis* has already been reported to have antioxidant and anti-inflammatory activities[4]. The objective of this study was to explore antioxidant activity and anti-inflammatory activity of the terpenoid fraction isolated from the shoot of *P. hadiensis* which is used in the Ayurvedic formulations used for treating inflammation and related ailments

MATERIALS AND METHODS

Collection and Extraction

The shoot part of the plant was collected from Kerala, India and authenticated by Dr. Kunhikannan, Scientist E, IFGTB, Coimbatore, India (Voucher specimen no: IFGTB-27/07/11-001). The sample (100 g) was shade dried and mechanically powdered separately to obtain a course powder, which was subjected to extraction in a reflux tube with 5 times its volume of aqueous ethanol (80%). The terpenoid fraction was obtained from the (80%) aqueous ethanolic extract by liquid-liquid partitioning using ethyl acetate as solvent. The terpenoid fraction thus obtained was concentrated *in vacuo* at 40 ± 1^0 C by rotary flash evaporator (Buchi type rotavapor, Switzerland) under reduced pressure and was used for the assays.

(a) Antioxidant activity

(i) Scavenging Capacity towards DPPH· Stable Radical

The determination of DPPH· scavenging activity of the terpenoid fraction was based on the method as described by Blois (1984)[5,14]. Briefly, 1 mL of aliquots of the fraction and standards (25, 50, 75, 100 and 250 µg/mL) was added to the Methanol solution of DPPH· (5 mL, 0.1 mM) and vortexed. After 20 min reaction at 25°C in dark, the absorbance was measured at 517 nm against a blank in a UV-Vis spectrophotometer (Elico, India). Ascorbic acid was used for comparison. The percentage quenching of DPPH· was calculated as follows: Inhibition of DPPH· (%) = $(Control_{517nm}-Sample_{517nm})/Control_{517nm}x100$, where, $Sample_{517nm}$ was absorbance of the sample and $Control_{517nm}$ was absorbance of control. The result was expressed as IC_{50}, which means the concentration at which DPPH· radicals were quenched by 50%.

(ii) Reductive Capacity (RC)

The reducing capacity of the fraction was measured using the potassium ferricyanide reduction method[6]. Various concentrations of the fraction and standards (25, 50, 75, 100 and 250 µg/mL) were added to 2.5mL of (0.2 M) sodium phosphate buffer (pH 6.6) and 2.5 mL of potassium ferricyanide [K_3Fe_3 (CN) $_6$] (1%) solution and vortexed. After incubation at 50°C for 20 min, 2.5 ml of TCA (10%, w/v) was added to all the tubes and centrifuged (Remi, India) at 3000 x *g* for 10 min. Afterwards, upper layer of the solution (5 mL) was mixed with deionized water (5 mL). To this, 1 mL of $FeCl_3$ (1%) was added to each test tube and incubated at 35°C for 10 min. Increased absorbance of the reaction mixture indicated increasing reducing power. BHT was used for comparison.

(iii) Scavenging Capacity towards Nitric Oxide Radical (NO)

Nitric oxide (NO) generated from sodium nitroprusside (SNP) in aqueous solution at physiological pH was estimated by the use of Griess reaction with minor changes[7]. The reaction mixture (3 mL) containing SNP (10mM, 2 mL), phosphate buffer saline (0.5 mL) and the terpenoid fraction at different concentrations and standards (25, 50, 75, 100 and 250 µg/mL) were incubated at 25º C for 150 min. After incubation, 0.5 mL of the incubated solution containing nitrite was pipetted and mixed with 1 ml of sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min for completing diazotization. Then, 1 mL of N-1-naphthyl ethylene diamine dihydrochloride was added, mixed and allowed to stand for 30 min at 25º C. The absorbance of pink coloured chromophore formed during diazotization was immediately measured at 540 nm in a UV-Vis spectrophotometer. Ascorbic acid was used for comparison. The percentage scavenging of NO was calculated as follows: Inhibition of NO (%)**=(**$Control_{540nm}$-$Sample_{540nm}$) / $Control_{540nm}$x100, where, $Sample_{540nm}$ was absorbance of the sample and $Control_{540nm}$ was absorbance of control.

(b) Anti-inflammatory activity

(i) Bovine Serum Albumin (BSA) denaturation inhibition assay[8]

The terpenoid fraction obtained was dried in a vacuum oven and redissolved in isosaline. Different concentrations of the fraction of *P. hadiensis* (25, 50, 75, 100, 250 µg/mL) were made from the above stock solution and added to 1.8 mL of 1 % of BSA solution. The pH was adjusted to 6.5 using 1N HCl and the solution was incubated at 37^0 C for 20 minutes and heated to 57^0 C for 10 minutes. After cooling, absorbance was measured at 660 nm. Diclofenac sodium was used as the standard and the control was taken without the fraction. The activity was calculated using the formula: **(**$Control_{660nm}$-$Sample_{660nm}$)/$Control_{660nm}$x100.

(ii) Human Red Blood Cell (HRBC) membrane stabilization assay[9]

Blood was collected freshly and mixed with equal volume of Alsever's solution. Then, it was centrifuged at 3000 rpm for 15 minutes. The packed cells were washed with isosaline and a 10 % suspension was made with isosaline. Different concentrations of the terpenoid fraction (25, 50, 75, 100, 250 µg/mL) were prepared in isosaline. To 0.5 mL of the prepared sample, 1 mL phosphate buffer, 2 mL hyposaline and 0.5 mL HRBC suspension was added and incubated for 30 minutes at 37^0C and then centrifuged at 3000 rpm for 20 minutes. Absorbance was measured at 560 nm. Diclofenac sodium was used as the standard and control was taken without the fraction. The activity was calculated using the formula: **(**$Control_{560nm}$-$Sample_{560nm}$)/$Control_{560nm}$x100.

(iii) Platelet aggregation inhibition assay[10]

The platelet rich plasma with 1.2 x 10^7 platelet cells for each assay was re-suspended in pH 7.4 Tris buffer. The platelet aggregation was recorded as absorbance values of spectrophotometer measurement. To determine the *in vitro* inhibition of platelet aggregation, different concentrations of the terpenoid fraction (25, 50, 75, 100, 250

µg/mL) in isosaline were used. The platelet aggregation was induced with ADP at a concentration of 1mM. Diclofenac sodium was used as the standard. The absorbance was recorded after 5 minutes at 660nm. Control was taken without the fraction. The activity was calculated using the formula: **(**$Control_{660nm}$-$Sample_{660nm}$)/$Control_{660nm}$x100.

Statistical analysis of data

The data obtained from antioxidant and anti-inflammatory assays were presented as means of 3 replicate determinations ± standard deviation (SD) using Graph pad prism software version 4.

RESULTS

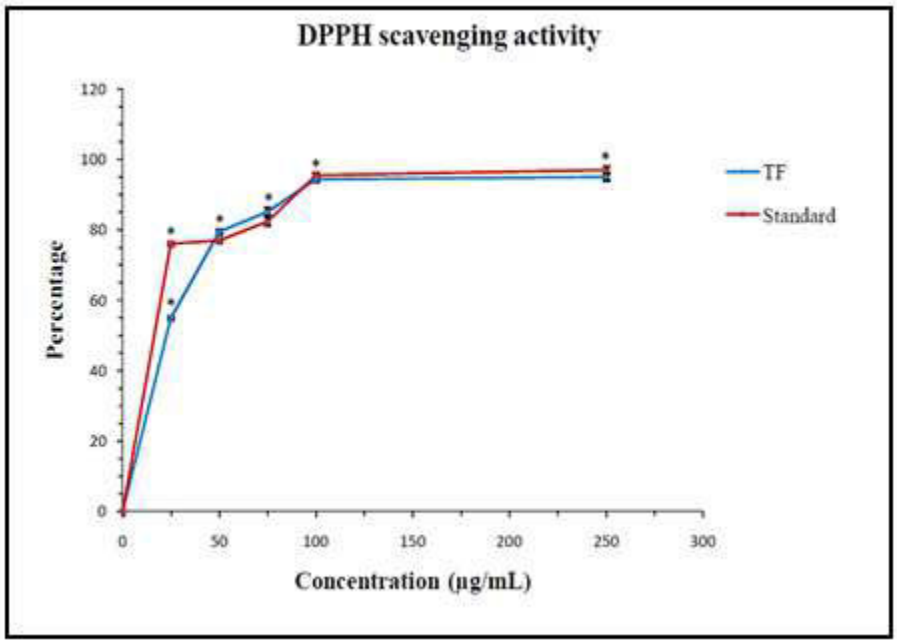
(a) Antioxidant activity

(i) DPPH· Quenching Capacity

The DPPH radical scavenging effect of the terpenoid fraction isolated from *P. hadiensis* was tested and compared with the standard Ascorbic acid. The terpenoid fraction showed significant differences in the scavenging activity towards DPPH radicals in a dose-dependent manner which is depicted in the figure 1. The scavenging effect of the fraction on the DPPH radical increased with increase in the concentration. The IC_{50} of the terpenoid fraction was found to be 22.76 ± 0.787 µg/mL which can be positively compared with the IC_{50} of the Ascorbic acid (16.44 ± 0.567 µg/mL), which was taken as the standard.

Figure 1

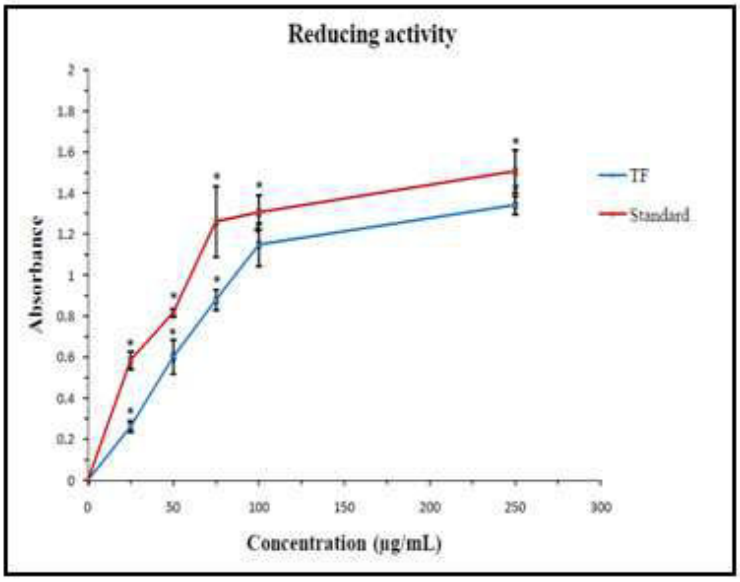
DPPH radical scavenging activity of the terpenoid fraction



(ii) Reducing Capacity

Figure 2 shows the reducing power of the terpenoid fraction of *P. hadiensis* and the standard BHT using the potassium ferricyanide reduction method. The reducing power of the fraction and BHT, which was taken as the standard, increased with increasing concentration. At 50µg/mL concentration, the absorbance of both fraction and BHT were found to be 0.602 and 0.817 respectively. Reducing power of the fraction components serves as a significant indicator of its potential antioxidant activity.

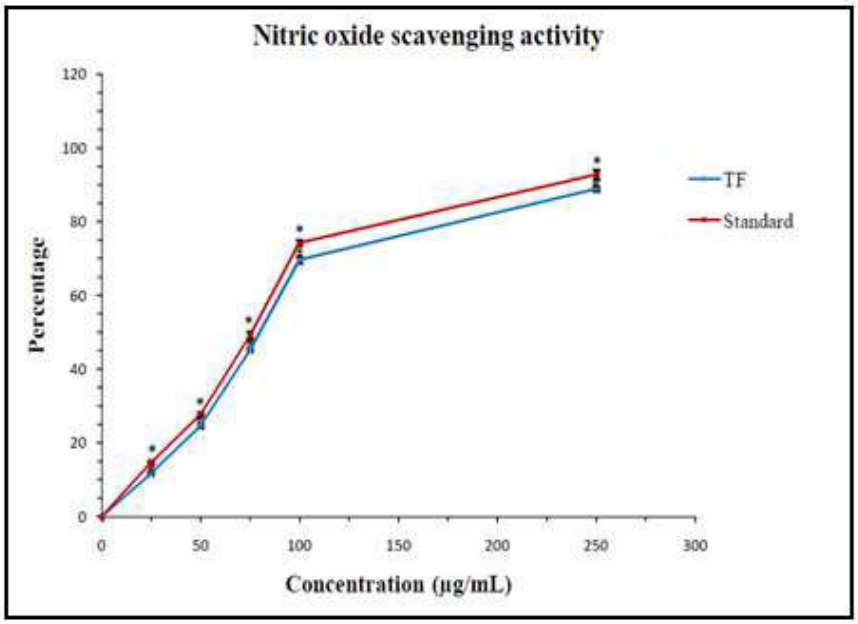
Figure 2
Reducing activity of the terpenoid fraction



(iii) NO scavenging capacity

The scavenging of the Nitric oxide radicals by the terpenoid fraction increased in a dose-dependent manner. A significant decrease in the NO radical due to the scavenging ability of the fraction and Ascorbic acid can be observed in the figure 3. The IC_{50} of the fraction and Ascorbic acid, the standard, were found to be 79.84 ± 0.519 µg/mL and 75.81 ± 0.754 µg/mL respectively. Suppression of released NO may be partially attributed to direct NO scavenging, as the terpenoid fraction decreased the amount of nitrite generated from the decomposition of SNP *in vitro*.

Figure 3
Nitric oxide scavenging activity of the terpenoid fraction



(b) Anti-inflammatory activity

(i) BSA denaturation inhibition assay

The inhibitory action of the terpenoid fraction on protein (BSA) denaturation inhibition is shown in table 1. The percentage inhibition of BSA aggregation by fraction and the standard Diclofenac sodium followed a similar pattern, increasing with increase in respective concentrations. The IC_{50} of the fraction and Diclofenac sodium is found to be 56.18 ± 0.766 µg/mL and 26.06 ± 0.956 µg/mL respectively.

Table 1

BSA denaturation inhibition by the terpenoid fraction

Concentration (µg/mL)	Percentage BSA denaturation inhibition	
	Terpenoid fraction	Standard (Diclofenac sodium)
0	0	0
25	38.81±0.581	48.73±0.716
50	45.73±0.759	75.27±0.782
75	62.99±0.271	85.54±1.074
100	81.04±1.035	90.46±0.947
250	86.10±1.183	92.93±1.260
IC_{50}	56.18 ± 0.766	26.06 ± 0.956

(ii) HRBC membrane stabilization assay

Various concentrations of the terpenoid fraction was assessed for its membrane stabilizing property and it was observed that with increase in concentration of both fraction and Diclofenac sodium, the membrane stabilizing activity also increased (Table. 2). At 250 µg/mL concentrations, both the fraction and Diclofenac sodium showed similar membrane stabilizing effect, 87.26 % and 88.78 % respectively.

Table 2

HRBC membrane stabilization by the terpenoid fraction

Concentration (µg/mL)	Percentage HRBC membrane stabilization	
	Terpenoid fraction	Standard (Diclofenac sodium)
0	0	0
25	33.16±0.682	48.36±0.562
50	41.68±0.576	74.13±0.767
75	71.06±0.343	82.26±1.365
100	84.59±1.498	88.41±1.122
250	87.26±1.354	88.78±1.458

(iii) Platelet aggregation inhibition assay

The platelet aggregation inhibition activity of the fraction was tested in the concentration ranges of 25 µg/mL, 50 µg/mL, 75 µg/mL, 100 µg/mL and 250 µg/mL for the study. There was 27.36 % aggregation inhibition of platelet against ADP with a dose of 25 µg/mL fraction which gradually increased (87.49 % for 250 µg/mL) with an increase in the dose (Table. 3). There was no appreciable change on further increase of the dose. The IC_{50} of fraction and Diclofenac were found to be 54.26 ± 0.960 µg/mL and 35.16 ± 0.774 µg/mL respectively.

Table 3
Platelet aggregation inhibition by the terpenoid fraction

Concentration (µg/mL)	Percentage Platelet aggregation inhibition	
	Terpenoid fraction	Standard (Diclofenac sodium)
0	0	0
25	27.36±0.215	30.72±0.287
50	44.50±0.680	78.14±0.557
75	76.77±0.987	89.36±0.319
100	85.93±1.340	91.47±1.144
250	87.49±1.578	92.31±1.561
IC_{50}	54.26 ± 0.960	35.16 ± 0.774

DISCUSSION

P. hadiensis (Forssk.) Schweinf. ex Spreng belongs to the order and family of Lamiales and Lamiaceae respectively. The family Lamiaceae contains several genera such as sage (*Salvia*), basil (*Ocimum*) and mint (*Mentha*), with a rich diversity of ethnobotanical uses. The Lamiaceae family in general is found to be rich in antioxidant phytochemicals especially terpenes and phenolic compounds[11]. Terpenoids have been found to be very good antioxidant agents and some terpenes have been found to be promising candidates for use in cancer therapy as well[12]. The terpenoids isolated included mainly Geraniol and related monoterpenoids. These terpenoids have been found to possess antioxidant and anti-inflammatory properties[12,13]. The term "free radicals" designates a family of compounds characterized by great reactivity to the impaired electron in the outer orbital. It belongs to reactive oxygen species (ROS), as well as reactive nitrogen species (RNS) which include nitric oxide and peroxynitrite. Although structurally different, free radicals share similar mechanisms to harm body's cells and tissues through damage on DNA, proteins and lipids[13]. DPPH assay evaluates the ability of antioxidants to scavenge free radicals. 2, 2-diphenyl-1-picrylhydrazyl (DDPH) is a free radical, stable at room temperature, which produces a violet colour in methanol. It gets reduced in the presence of an antioxidant molecule and gives rise to uncolored methanol solutions. The reduction capability of DPPH was determined by the decrease in its absorbance at 517 nm, which is induced by antioxidants. The results of the study infer that the terpenoid fraction reduces the radical to the corresponding hydrazine when it reacts with the hydrogen ions released from the samples, which contain antioxidant principles[14]. In the reducing power assay, the presence of antioxidants in the fractions results in the reduction of the Fe^{3+}-ferricyanide complex to its ferrous form which is measured by the intensity of the resultant Prussian blue colour complex. The higher absorbance at high concentration indicates the strong reducing capacity. Hydroxyl radical ($^{\cdot}OH$), the most reactive free radical, has the capacity to conjugate with nucleotides in DNA, cause strand breakage, and lead to carcinogenesis, mutagenesis and cytotoxicity[15]. In this study, the terpenoid fraction isolated from the shoot of *P. hadiensis* showed its ability to scavenge $^{\cdot}OH$ radicals which was compared to the standard BHT used.

Nitric oxide (NO) is highly reactive molecule that participates in the signal transduction in cardiovascular and immune systems. It is often characterized by contrasting actions as it can exhibit antioxidant and pro-oxidant functions as well as anti-apoptotic and proapoptotic effects[16]. NO is a potent pleiotropic inhibitor of physiological processes such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical that plays many roles as an effector molecule in diverse biological systems including neuronal

messenger, vasodilation and antimicrobial and antitumor activities[17]. In our study, we noted that the scavenging of NO by the terpenoid fraction increased in a dose dependent manner. Denaturation of proteins is well-documented cause of inflammation and rheumatoid arthritis. Several anti-inflammatory drugs have shown dose dependent ability to inhibit thermally induced protein denaturation[18]. When BSA is heated, it undergoes denaturation and expresses antigens associated with type III hypersensitive reaction and which are related to diseases such as serum sickness, glomerulonephritis, rheumatoid arthritis and systemic lupus erythematosus. Thus, this assay was applied to detect the activity of the fraction, which can stabilize the protein from denaturation process. The terpenoid fraction exhibited relatively higher stabilization effect by inhibiting the hypotonicity-induced lyses of erythrocyte membrane. The inflammatory responses get limited by preventing the release of lysosomal constituents of activated neutrophils thereby the damage to the tissue is reduced[4]. The exact mechanism of stabilization of the membrane by the fraction is not known, but it can be observed that the osmotic loss of intracellular electrolytes and fluid components was inhibited under induced hemolysis. Since inflammation responses amplify any disease conditions, it can be suggested that the terpenoid fraction can also be used to reduce the inflammatory responses that is activated in most disease conditions. Since platelet aggregation is a vital pathogenic marker of inflammation, the activity of the fraction to inhibit platelet aggregation was assessed and compared with the standard NSAID. During the formation of the primary haemostatic plug ADP is released from the platelet and induces further platelet aggregation[19,20]. The results on platelet aggregation reflects that the fraction could either inhibit PGs synthesis pathway or 5-HT release.

CONCLUSION

In conclusion, this study focused on antioxidant and anti-inflammatory properties of the terpenoid fraction isolated from *P. hadiensis*. The study demonstrated that the terpenoid fraction efficiently scavenged DPPH, $OH^{\bullet}$ and NO radicals. This infers that the terpenoid fraction seems to be good sources of natural antioxidants. The anti-inflammatory activity of the fraction showed that it can be positively compared to the standard drug used for treating inflammation-related ailments thus substantiating the health promoting properties of *P. hadiensis* and its use in Ayurvedic formulations as well. Further studies are underway to determine the mechanism by which the fraction exerts the antioxidant and anti-inflammatory activities at molecular levels.

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