9-Hexadecenoic Acid Rich HPLC Fraction of *Pithecellobium dulce* Methanolic Seed Extract Exhibits Potential Anti-inflammatory Activity by Inhibiting IL-8, IL-6, and PGE2: Phytochemical Characterization, *in-vitro* and *in-vivo* Evaluation

Babanna YELUGUDARI [1] [iD], Nageshwar MESRAM [1] [iD] Pratap Reddy KARNATI [1*] [iD]

1 Department of Zoology, Faculty of Neurobiology, Osmania University, Hyderabad, India.
* Corresponding Author. E-mail: pratapkreddyou@gmail.com (P.R.K); Tel. +00-000-000 00 00.

ABSTRACT: Pithecellobium dulce (Roxb.) Benth. has been used as a folk medicine due to its wide range of pharmacological applications. In light of the lack of significant studies examining the anti-inflammatory potential of seeds and their phytochemical constituents, we investigated the molecular basis behind the therapeutic efficacy of methanolic extract both in-vitro and in-vivo. The phytochemical investigation of methanolic seed extract using GC-MS analysis revealed the presence of 30 molecules of diverse classes. Further solvent fractionation followed by RP-HPLC separation demonstrated the presence of 9-Hexadecenoic acid and 8,11,14 (Z, Z, Z)-Eicosatrienoic acid and were further structurally characterized by 1H-NMR and HRMS. 9-Hexadecenoic acid-rich HPLC fraction exhibited the highest % inhibition of albumin denaturation and % reduction of IL-8 and IL-6 in HaCaT cells as well as NO and PGE-2 production in RAW267.4 cells among other test samples. In-vivo anti-inflammatory findings revealed that 9-Hexadecenoic acid-rich fraction potentially reduced rat paw edema by 84.62% at 150 µg/mL, which is superior to the diclofenac. The results of the present study revealed that the 9-hexadecenoic acid-rich fraction of methanolic seed extract of P. dulce displayed potential anti-inflammatory activity both in-vitro and in-vivo, which suggests an efficient alternative in treating a variety of ailments related to inflammation and pain.

KEYWORDS: *Pithecellobium dulce*; Methanolic seed extract; Anti-inflammatory activity; 9-hexadecenoic acid; GC-MS; HPLC.

1. INTRODUCTION

Medicinal plants are valuable sources of chemical entities with biological and pharmacological properties and are always used as an identifying resource for new drug leads [1]. The attention towards great scientific interest in herbal medicines lies in preventing and treating several ailments and preventing and minimizing the adverse effects of conventional treatments. Therefore, looking for the role of plant secondary metabolites as excellent drug candidates for developing novel phytopharmaceuticals with different pharmacological activities is pertinent [2-4]. The application of secondary metabolites is not limited to human therapy but extends to various fields like veterinary, agriculture, scientific research, and numerous other areas [6]. The World Health Organization (WHO) encourages, promotes, and facilitates the use of herbal medicine in developing countries for various health programs.

Inflammation is a complex physiological response that integrates multiple vascular and cellular events. The acute state of inflammation is identified by high vascular permeability that leads to exudation of high-level neutrophils containing fluid and results in stimulation of pro-inflammatory regulators, such as serotonin, histamine, bradykinin, and prostaglandins [7-10]. Application of non-steroidal anti-inflammatory therapeutic molecules (NSAIDs) and corticosteroids are used as part of standard line therapy to alleviate the symptoms of inflammation, such as rheumatoid arthritis, severe pain, asthma, etc. Nonetheless, the prolonged application of corticosteroids and NSAIDs is related to serious adverse effects, such as intestinal or gastric ulceration, kidney dysfunction, sodium retention, edema, and arterial hypertension [11]. Therefore, much attention has been paid in recent years in search of new anti-inflammatory lead candidates having plant origin.

Thistes the development of novel therapeutic molecules used to counteract the inflammation and be used in various ailments where the inflammation response amplifies the disease process. In support of that, many anti-inflammatory molecules identified over the last decade are explored mainly from natural sources [12-13].

In recent years, the pharmaceutical and food industries have shown a growing interest in studying the metabolic profiles of several therapeutic plants. Plants produce secondary metabolites as a means of defending themselves against biotic and abiotic stresses, and some of these compounds have positive effects on the organisms that eat them as well. *Pithecellobium dulce* (Family: Mimosaceae) is among one of them, commonly known as "Jungle Jalebi" in Hindi, and is an evergreen, spiny tree widely distributed throughout India and also found in South Africa, Australia, and Asia. Fruits of *Pithecellobium dulce* (*P. dulce*) have been used as a food additive for their excellent nutritional and medicinal importance. In the traditional system of medicine, it is used to treat diabetes, peptic ulcer, toothache, leprosy, and earache and is also used as an astringent, emollient, and abortifacient [14, 15]. Research has recently focused on the chemical composition of various plant parts, including arils, leaves, fruits, etc., and screening them for anti-oxidant activity, which has led to identifying potential phytotherapeutic candidates. The strong anti-oxidant activity of some of these compounds suggests they may play a role in warding off the onset of persistent illnesses [16, 17]. Even though the anti-inflammatory properties of various parts of *P. dulce* have been studied [18-21]. No significant study has been established regarding anti-inflammatory activities related to the phytochemical constituents. Given the above fact, the present study is designed and executed to characterize the methanolic seed extract of *P. dulce* and evaluate its molecular basis for anti-inflammatory potential via *in-vitro* and *in-vivo* methods.

2. RESULTS AND DISCUSSION

2.1 Phytochemical analysis of methanolic seed extract of *P. dulce*

Initially, the methanolic seed extract of *P. dulce* was qualitatively analyzed to determine the distribution of secondary metabolites that included terpenoids, flavonoids, fatty acids, tannins, phenols, alkaloids, saponins, glycosides, and steroids (Table 1). The relative abundance of fatty acids & oils, flavonoids, and steroids was rich in methanolic extract. A minimal amount of alkaloids was found in the methanolic extract. Saponins and glycosides were found to be absent. It has been identified from the qualitative analysis that the phytochemical composition of seeds was quite different from other plant parts, such as leaves and bark, which were reported through various studies [22]. In addition, *P. dulce* methanolic seed isolate was exposed to quantitative phytochemical examination to determine total phenolic, flavonoids, triterpenoid, and fat content, and the results are presented in Table 2. The study revealed that the total fat content of methanolic extract (83.15 ± 7.14 mg/g of a mixture of standard fatty acids) was higher than the phenolic content (13.51 ± 3.12 mg; gallic acid equivalent/g dried extract), and total flavonoid content (10.66 ± 2.17 mg rutin equivalent/g dried extract). In addition, the total triterpenoid content (3.16 ± 1.07 mg oleanolic acid equivalent/g dried extract) was the lowest among all other chemical classes.

Table 1. Qualitative phytochemical screening of *P. dulce* seeds.

Chemical Classes	Methanolic extract
Alkaloids	0.1 -2 %
Saponin	< 0.1 %
Triterpenes & Terpenoids	2.1 - 5 %
Fatty acids	> 5%
Flavonoids	21-50
Tannins	0.1 – 2 %
Steroids	0.1 – 2 %
Glycosides	< 0.1 %
Phenols	2.1 - 5 %

Table 2. Total phenolic, flavonoid, triterpenoids, and fat content in *P. dulce* seed extract.

Quantitative phytochemical analysis	The methanolic seed extract of *P. dulce*
Total Phenolic content (mg/g Gallic acid equivalent)	13.51 ± 3.12
Total Flavanoid Content (mg/g Rutin equivalent)	10.66 ± 2.17
Total triterpenoids content (mg/g Oleanolic acid equivalent)	3.16 ± 1.07
Total fat content (mg/g of fatty acid mixture)	83.15 ± 7.14

The results are represented as mean ± standard deviation of triplicate readings (n=3).

2.2 Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

The existence of chemical constituents in the methanolic extract of *P. dulce* seeds was investigated by GC-MS analysis (Fig. 1). The results of active chemical components and their corresponding retention times (R_t), % peak areas, molecular formula, and molecular weight are shown in table 3. A total of 30 molecular components representing 99.34% of the methanolic extract of *P. dulce* seeds by GC-MS were identified. The major chemical entities identified were 9-Hexadecenoic acid (54.17%), n-Hexadecanoic acid (13.57%), Formic acid of ethenyl ester (7.55%), Eicosanoic acid (5.53%), and Hexadecanoic acid of methyl ester (3.43%). GC results also showed that twenty-five molecules were found in very low quantities, with peak areas ranging from 0.23- 2.01% (Table 3). It has been previously reported that the methanolic leaf extract of *P. dulce* contains a different molecular composition than seeds [22, 23]. The GC-MS analysis of a column-purified methanolic extract of *P. dulce* leaves revealed the presence of three critical secondary metabolites: Squalene, 9-Octdecenoic acid (Z)-2-hydroxy-13-propanediyl ester, and 9-Octdecenoic acid 1,2,3-propanediol ester. Based on these findings, it was concluded that the leaves and seeds of *P. dulce* are highly fatty acid rich. These results are not entirely consistent with the phytoconstituents of the bark identified in a previous study, in which 29 distinct compounds were identified, the bulk of which were proanthocyanidins and phenolic acids [24].
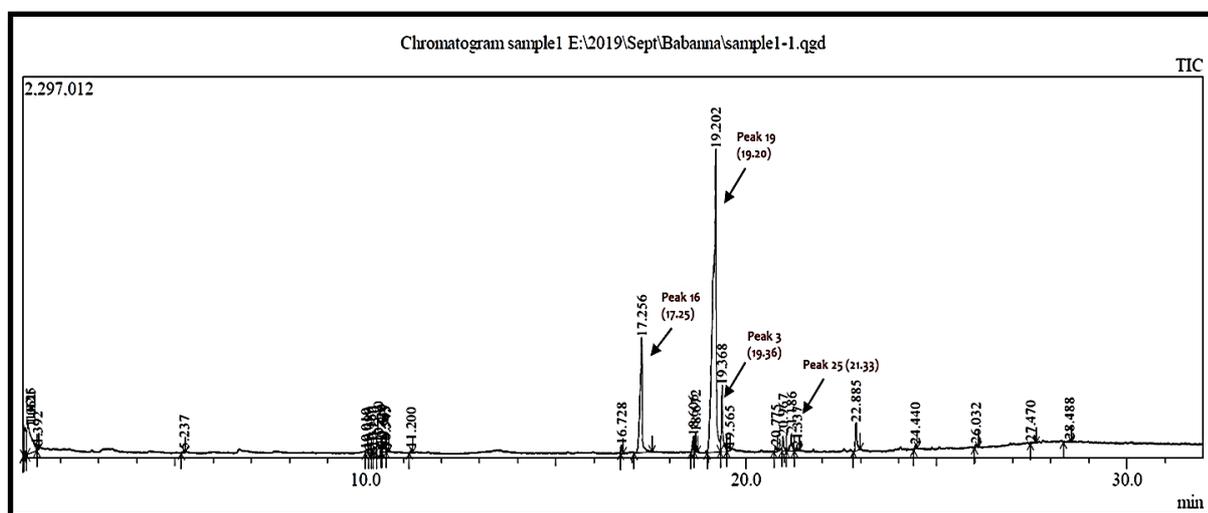

Figure 1. GC-MS chromatogram of the methanolic seed extract of *P. dulce* and molecular peaks were identified with the NIST17 database.

Table 3. GC-MS chemical composition of methanolic extract of *P. dulce* seeds.

Peak	Name	R. time (RT)	RI (R.index)	Peak (%)	M F	MW
1	2-Ethenoxypropane	1.02	34.49	0.71	$C_5H_{10}O$	86.13
2	Ethenyl formate	1.06	35.85	3.29	$C_3H_4O_2$	72.06
3	Pyridine	1.12	37.88	4.77	C_5H_5N	79.09
4	2-Methylpyrazine	1.39	47.01	0.68	$C_5H_6N_2$	94.11
5	3,5-Dihydroxy-6-methyl-2,3-dihydropyran-4-one	5.23	1070.49	0.28	$C_6H_8O_4$	144.12
6	3-Methyloctan-3-yl *N*-(1-phenylethyl)carbamate	10.01	1327.09	0.32	$C_9H_{11}NO_2$	165.19
7	Non-2-enoic acid	10.09	1331.64	0.45	$C_9H_{16}O_2$	156.22
8	4-(1-Aminoethyl)-3,3-dimethylazetidin-2-one	10.16	1335.63	0.35	$C_7H_{14}N_2O$	142.21
9	9-Oxabicyclo[3.3.1]nonane-1,4-diol	10.22	1339.04	0.49	$C_8H_{14}O_3$	158.19
10	2,6-Dimethylocta-1,7-dien-3-ol	10.34	1345.87	1.45	$C_{10}H_{18}O$	154.24
11	6-Methoxy-2-phenyl-4,4*a*,6,7,8,8*a*-hexahydropyrano[3,2-d][1,3]dioxine-7,8-diol	10.42	1350.43	0.37	$C_{13}H_{24}O_6$	276.32
12	2-*Tert*-butyl-4-methyl-5-oxo-1,3-dioxolane-4-carboxylic acid	10.50	1354.98	0.78	$C_9H_{14}O_5$	202.20
13	2-[(2-Methylpropan-2-yl)oxycarbonylamino]-3-[2-(trifluoromethyl)-1*H*-imidazol-5-yl]propanoic acid	10.54	1357.26	0.21	$C_{12}H_{16}F_3N_3O_4$	323.27
14	Diethyl 2-methoxybenzene-1,4-dicarboxylate	11.20	1394.82	0.25	$C_{12}H_{14}O_4$	222.23
15	Methyl hexadecanoate	16.72	1794.81	0.47	$C_{17}H_{34}O_2$	270.45
16	n-Hexadecanoic acid	17.25	1840.61	12.99	$C_{16}H_{32}O_2$	256.42
17	Methyl, 9,12-octadecadienoate	18.60	1961.36	0.87	$C_{19}H_{34}O_2$	294.47
18	Methyl, octadec-10-enoate	18.67	1967.80	1.02	$C_{19}H_{36}O_2$	296.48
19	9-Hexadecenoic acid	19.20	1999.05	51.06	$C_{16}H_{30}O_2$	254.40
20	Ethyl icosanoate	19.36	1998.21	5.01	$C_{20}H_{40}O_2$	312.53
21	1,2-Epoxytetradecane	19.56	1997.16	0.48	$C_{14}H_{28}O$	212.37
22	12-Methyloctadeca-2,13-dien-1-ol	20.77	1990.80	0.38	$C_{19}H_{36}O$	280.48
23	Docos-13-enoic acid	20.96	1989.80	1.02	$C_{22}H_{42}O_2$	338.56
24	Icosanoic acid	21.18	1988.64	1.01	$C_{20}H_{40}O_2$	312.53
25	Icosa-8,11,14-trienoic acid	21.33	1987.85	0.22	$C_{20}H_{34}O_2$	306.48
26	Docosanoic acid	22.88	1979.71	1.95	$C_{22}H_{44}O_2$	340.58
27	Methyl 11-oxoicosanoate	24.44	1971.50	0.24	$C_{21}H_{42}O_2$	326.55
28	2,2,4,4,6,6,8,8,10,10,12,12,14,14,16,16,18,18-Octadecamethyl-1,3,5,7,9,11,13,15,17-nonaoxa-2,4,6,8,10,12,14,16,18-nonasilacyclooctadecane.	26.03	1963.14	0.22	$C_{18}H_{54}O_9Si_9$	667.38
29	2-Methyl-5-prop-1-en-2-ylcyclohex-2-en-1-yl]propanoate	27.47	1955.57	0.18	$C_{13}H_{20}O_2$	208.29
30	17, 6-Hydroxy-5,6-dimethylheptan-2-yl]-10,13-dimethyl-1,2,3,4,6,7,8,9,11,12,14,15,16,17-tetradecahydrocyclopenta[a]phenanthrene-3,5,6-triol	28.48	1950.26	0.26	$C_{28}H_{48}O_4$	448.67

2.3 Solvent fractionation and HPLC analysis of Chloroform fraction

As a first step in separating bioactive compounds, methanolic seed extract was fractionated successively using ethyl acetate, diethyl ether, and chloroform. *In-vitro* anti-inflammatory studies revealed that the chloroform fraction exhibited the potential activity among other solvent fractions, including methanolic seed extract. High-performance liquid chromatographic separation was used to identify bioactive compounds responsible for the anti-inflammatory activity displayed by the chloroform fraction *in-vitro*. The chloroform fraction of methanolic isolate of *P. dulce* seeds was exposed to reverse phase HPLC using a gradient elution of two solvents, acetonitrile and water. The important biomolecules were identified by comparing the retention time profiles of reference standard HPLC runs of 8,11,14, (*Z,Z,Z*)-Eicosatrienoic acid (Fig. 2a), 9-Hexadecenoic acid (Fig. 2b). This revealed that peaks corresponding to 8,11,14 (*Z,Z,Z*)-Eicosatrienoic acid and 9-Hexadecenoic acid of chloroform fraction eluted at retention times of 33.87 and 25.48 minutes respectively are concurrently mapped with the corresponding reference standards.
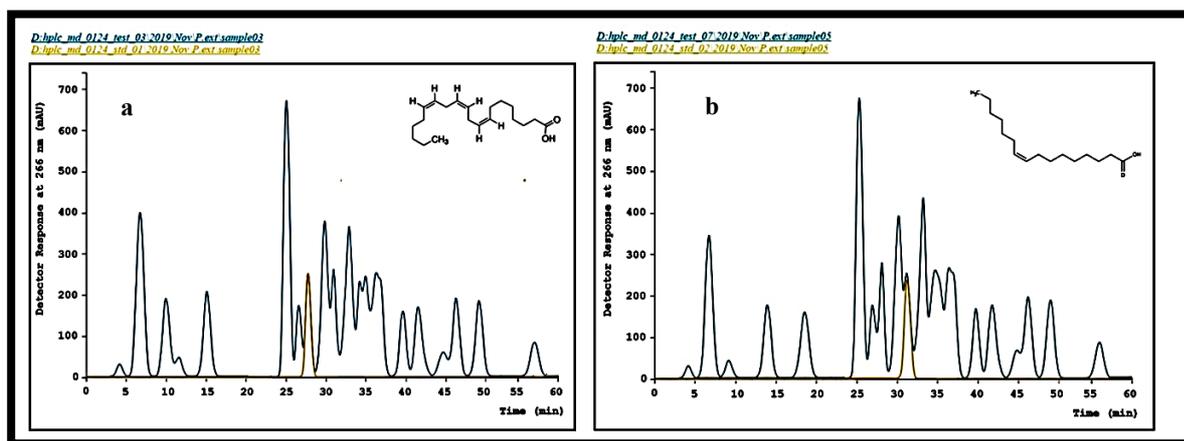
Figure 2. HPLC chromatograms of chloroform fraction of methanolic extract of *P. dulce* compared with reference standard chromatograms of 8,11,14, (*Z,Z,Z*)-Eicosatrienoic acids (a), 9-Hexadecenoic acid (b) using a gradient elution technique.

2.4 Structural characterization

HPLC peak fractions were successfully purified using SPE analytical cartridges, and 8,11,14, (*Z,Z,Z*)-Eicosatrienoic acid (Peak fraction 1) and 9-Hexadecenoic acid (Peak fraction 2) were structurally characterized by ^{1}H-NMR and HRMS spectra (Fig. 3a and 3b).

Spectroscopic data of 8,11,14, (*Z,Z,Z*)-Eicosatrienoic acid*:* Pale yellow powder: ^{1}H-NMR (*400MHz, DMSO-d6*) δ; 11.97 (*s*, 1H), 5.39 (*dt, J* = 14.94, 4.61 Hz, 1H), 5.34 (*dt, J* = 14.94, 4.61 Hz, 5H), 2.80 (*t, J*= 4.11 Hz, 4H), 2.16 (*t, J* = 7.06 Hz, 2H), 2.04 (*dt, J* = 6.59, 4.61 Hz, 2H), 1.55 (*tt, J* = 7.06, 6.81 Hz, 2H), 1.47 (*tt, J* = 6.81, 6.59 Hz, 2H), 1.43 (*tt, J* = 6.81, 6.59 Hz, 2H), 1.30 (*tq, J* = 6.94, 6.78 Hz, 2H), 1.30 (*tt, J* = 6.81, 6.78 Hz, 2H), 1.28 (*tt, J* = 6.81 Hz 4H); (M+H)[+] of $C_{16}H_{30}O_2$ is 255.2318 (Calc.) 255.2309 (found).

Spectroscopic data of 9-Hexadecenoic acid*:* White amorphous powder. ^{1}H-NMR (*400MHz, DMSO-d6*) δ; 11.90 (*s*, 1H), 5.42 (*dt, J* = 14.94, 4.61 Hz, 2H), 2.17 (*t, J* = 7.66 Hz, 2H), 2.05 (*dt, J* = 6.59, 4.61, 1.00 Hz, 4H), 1.56 (*tt, J* = 7.06, 6.81 Hz, 2H), 1.53 (*tt, J* = 6.81, 6.59 Hz, 2H), 1.45 (*tt, J* = 6.81, 6.59 Hz, 2H), 1.32 (*m, J* = 6.81 Hz, 4H), 1.30 (*tq, J* = 6.94, 6.78 Hz, 2H), 1.29 (*m, J* = 6.94, 6.78 Hz, 4H); (M+H)[+] of $C_{21}H_{36}O_2$ is 321.2788 (Calc.) 321.2753 (found).
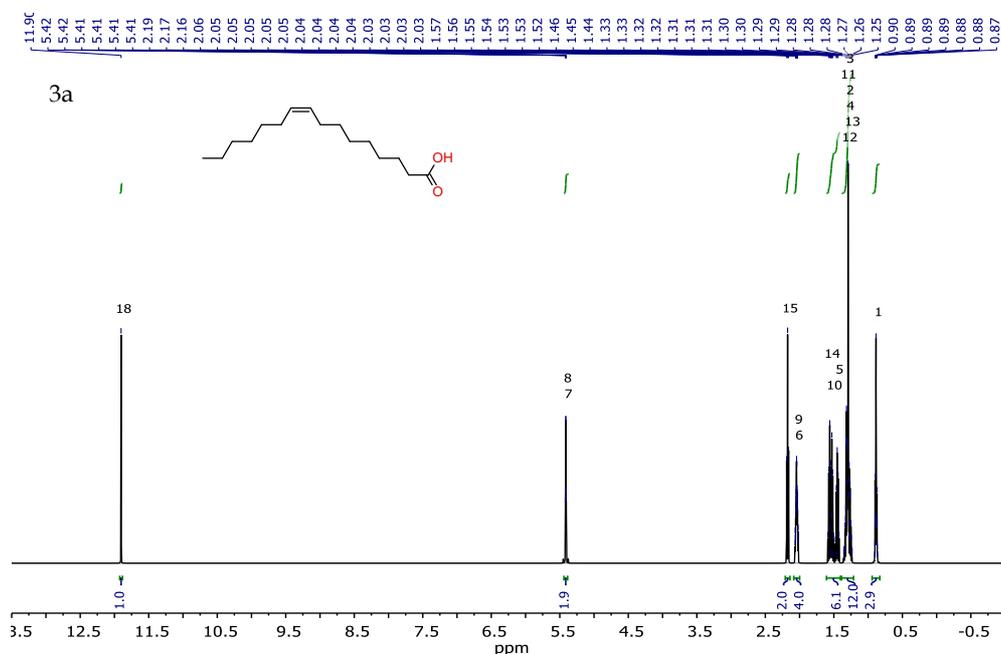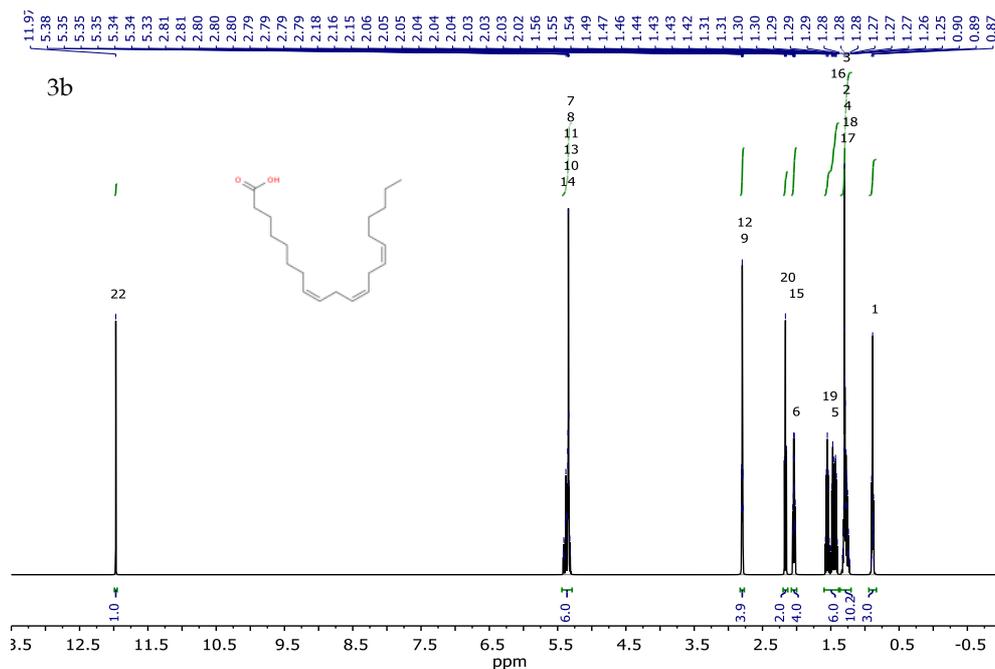
Figure 3. ^{1}H NMR structural characterization of 9-Hexadecenoic acid (a); 8,11,14, (Z,Z,Z)-Eicosatrienoic acids (b).

2.5 *In-vitro* Anti-inflammatory activity

2.5.1 Albumin denaturation

The present results showed that the capacity to inhibit albumin denaturation by methanolic seed extract (250 µg/mL), chloroform fraction (250 µg/mL), and its corresponding HPLC peak fractions of 8,11,14, (Z,Z,Z)-Eicosatrienoic acid (PF1) and 9-Hexadecenoic acid (PF2) at 50, and 100 µg/mL concentrations (Fig. 4). Diclofenac sodium at 100 µg/mL was used as a positive control. Results showed that the HPLC peak fraction 9-Hexadecenoic acid (PF2) at 100 µg/mL had a higher percentage of albumin denaturation and a higher anti-inflammatory activity (94.64 ± 3.58) than the positive control (90.37 ± 4.61). As a further observation, the chloroform fraction also demonstrated good anti-inflammatory activity (71.48 ± 5.33), higher than the methanolic extract (37.69 ± 3.12). It was also observed that the anti-inflammatory activity of PF2 was found to be concentration-dependent over the range of 50 to 100 µg/mL. This was further confirmed by examining the functional investigation of pro-inflammatory cytokines IL-8 and IL-6 Levels in HaCaT cells.
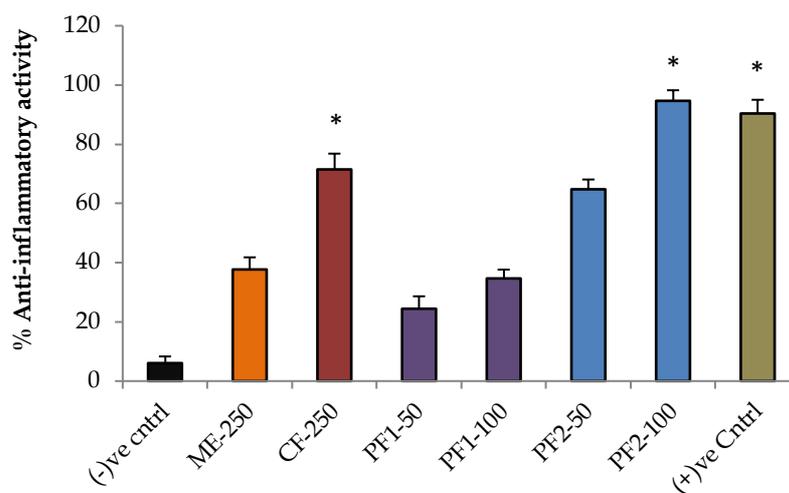

Figure 4. *In-vitro* Anti-inflammatory activity of various test solutions of *P. dulce* based on albumin denaturation; All values were expressed as Mean ± SD. The differences between negative control and treatment groups were tested for significance using ANOVA followed by Dunnet's t-test. * $P < 0.05$ were considered significant

2.5.2 Anti-inflammatory potential of PF2 based on IL-8 and IL-6 expression in HaCaT cells

To further confirm the anti-inflammatory properties of 9-Hexadecenoic acid (PF2), and chloroform fraction of methanolic extract, we used the HaCaT cellular inflammation model to investigate the status of pro-inflammatory cytokines interleukin-8 (IL-8) and interleukin-6 (IL-6), after treatment. When the HaCaT cells are stimulated with TNF- α, they produce high levels of IL-8 and IL-6, confirming the cells' inflammatory behaviour. According to the results shown in figures 5a and 5b, the PF2 at 100 µg/mL significantly reduced the expression levels of IL-8 and IL-6 in HaCaT cells, which agrees with the results from albumin denaturation experiments. Interestingly, PF2 reduced the expression of IL-8 and IL-6 in HaCaT cells by 31.05 ± 4.55 and 37.14 ± 4.47 %, respectively, which is superior to the positive control budesonide (35.24 ± 6.58 and 41.73 ± 7.81 for IL-8 and IL-6 respectively). Chloroform fraction at 250 µg/mL also showed considerable reduction of IL-8 and IL-6 levels by 56.47 ± 7.54 and 61.48 ± 7.51 %, respectively, over the treatment, which is superior to both crude methanolic extract (78.56 ± 5.24 and 81.41 ± 5.87 for IL-8 and IL-6 respectively) and PF1 treatment (71.66 ± 5.21 and 71.59 ± 5.46 for IL-8 and IL-6 respectively). These findings demonstrated that methanolic extracts at 250 µg/mL and PF1 treatment at 100 µg/mL showed a nonsignificant effect in reducing IL-8 and IL-6 expression levels in HaCaT cells (Figures 5a, 5b, and 5c). This assay revealed that the HPLC-purified fraction of 9-Hexadecenoic acid (PF2) caused a significant reduction of both IL-8 and IL-6 levels in TNF-α stimulated HaCaT cells when compared with the effect shown by the positive control budesonide. These results confirmed the 9-Hexadecenoic acid-rich HPLC fraction's promising anti-inflammatory behaviour, as shown in figures 5a, 5b, and 5c.
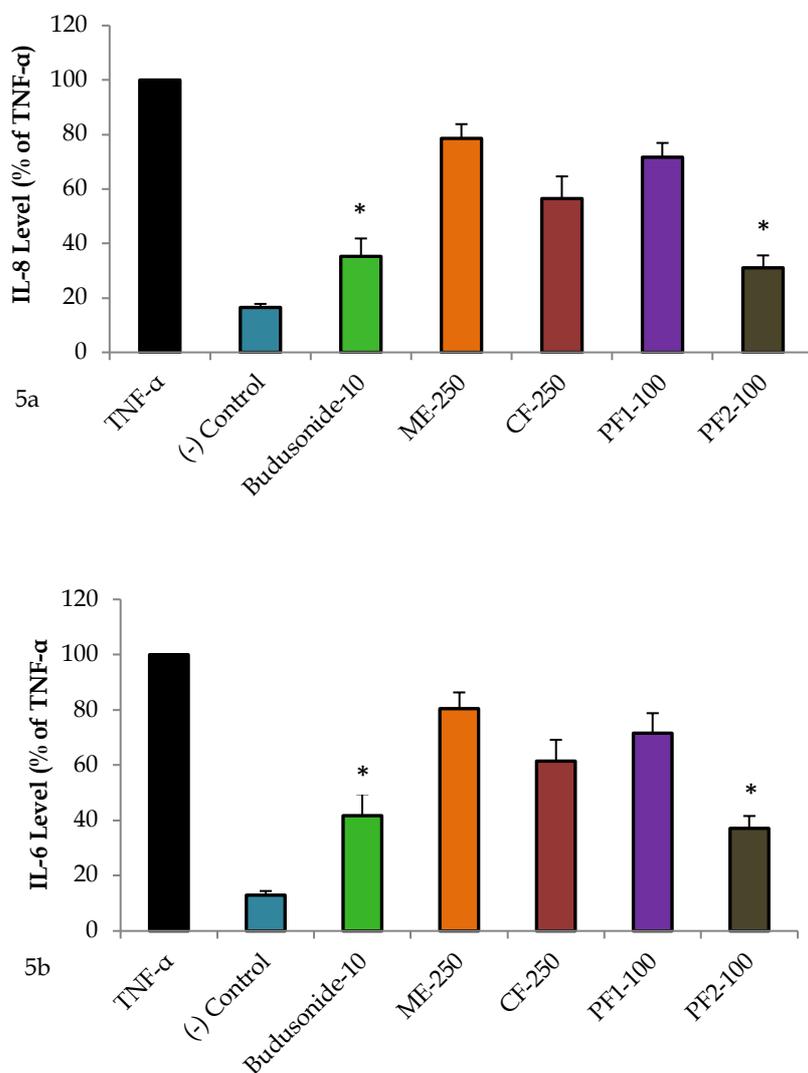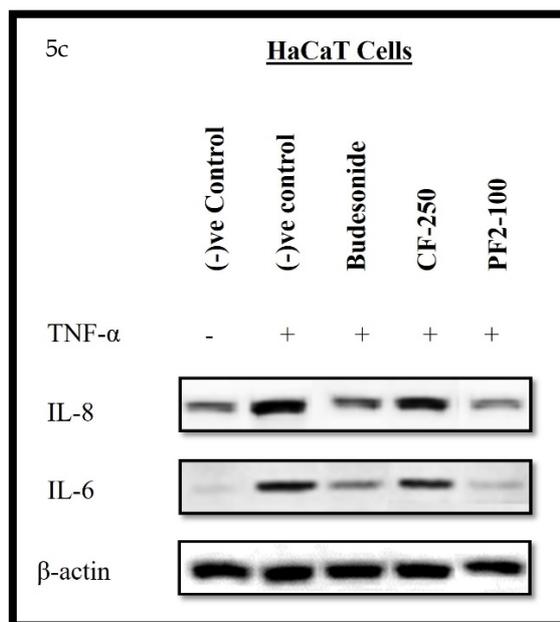
Figure 5. Effect of various test samples of *P. dulce* seed extract on IL-8 (5a) and IL-6- (5b) levels released by HaCaT cells, after stimulation with TNF-α (20 ng/mL); western blot analysis of IL-8 and IL-6 (5c) over the treatment of various test samples; All values were expressed as Mean ± SD. The differences between TNF-α and other groups were tested for significance using ANOVA followed by Dunnet's t-test. * P<0.05 were considered significant.

2.5.3 Effect of PF2 and Chloroform fraction on the production of NO and PGE2

Further investigations were conducted to determine whether PF2 and chloroform fraction were able to control inflammation through inflammatory mediators such as nitric oxide (NO) and prostaglandin-2 (PGE-2). To this end, the effect of PF2 and chloroform fraction on the production of NO and PGE2 in LPS-treated RAW264.7 cells was determined. The results demonstrated that PF2 at 150 µg/mL significantly (p<0.01) inhibited LPS-mediated NO production (22.05 ± 1.53 %) and PGE-2 (18.63 ± 1.94 %) in RAW264.7 cells. 2 It is interesting to note that the reduction of NO and PGE-2 production by PF2 was superior to the standards Isothiourea and aspirin, which is consistent with those of previous anti-inflammatory studies. Moreover, this effect was dose-dependent, as shown in Fig. 6a and 6b. PF2 demonstrated a reduction in NO of 61.33 to 22.05 % and PGE-2 of 57.69 to 18.62 % over a concentration range of 50 to 150 µg/mL, demonstrating the dose dependant anti-inflammatory responses. Chloroform fraction showed a negligible effect on NO and PGE-2 suppression in RAW264.7 cells (Fig. 6c).
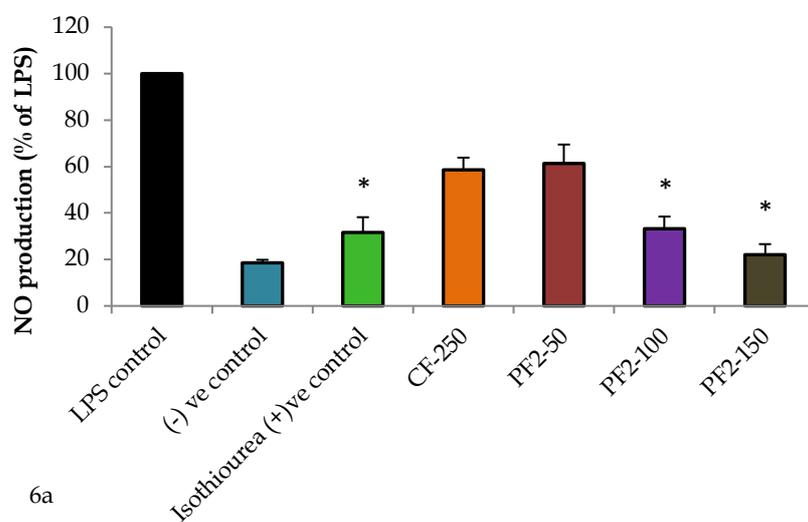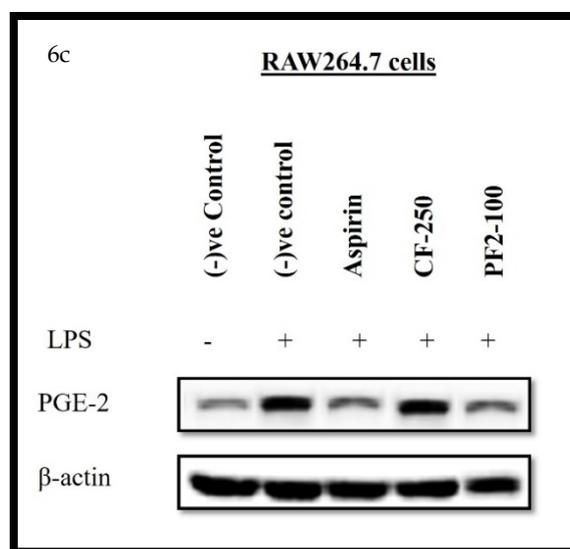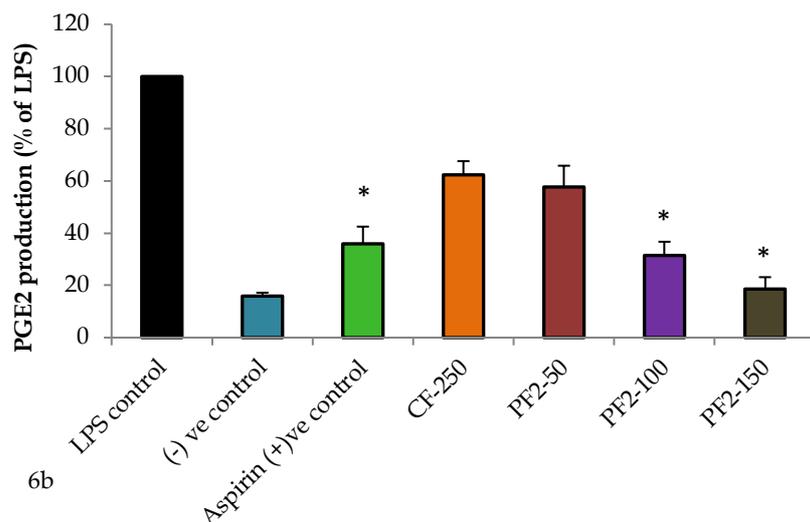
Figure 6. Effect of PF2 and Chloroform fraction of methanolic seed extract of *P. dulce* on NO (6a) and PGE-2 (6b) levels released by RAW264.7 cells, after stimulation with LPS (1 µg/mL); western blot analysis of IL-8 and IL-6 (6c) over the treatment of various test samples; All values were expressed as Mean ± SD. The differences between LPS and other treatment groups were tested for significance using ANOVA followed by Dunnet's t-test. * P<0.05 were considered significant.

2.5.4 Cell viability

The cytotoxic properties of methanolic extract, chloroform fraction of methanolic extract, and HPLC peak fractions PF1 and PF2 were examined against HaCaT and Raw267.4 cells at 24 h of treatment in order to examine the toxic effects. According to the results in figure 7, all test samples exhibited negligible cytotoxicity against both HaCaT and Raw267.4 cells. Cell viability of all test samples at the highest concentration of 500 µg/mL against both the cells was between 95% and 97%. At the lowest concentration of 5 µg/mL, the viability of all test samples against HaCaT and Raw267.4 cells was around 99%. In comparison with control cells, these values indicated similar viable status. These results demonstrated that the percent cell viability of all the extracts and peak fractions at all concentrations showed negligible cytotoxicity at 24 h incubation, and the differences were insignificant.
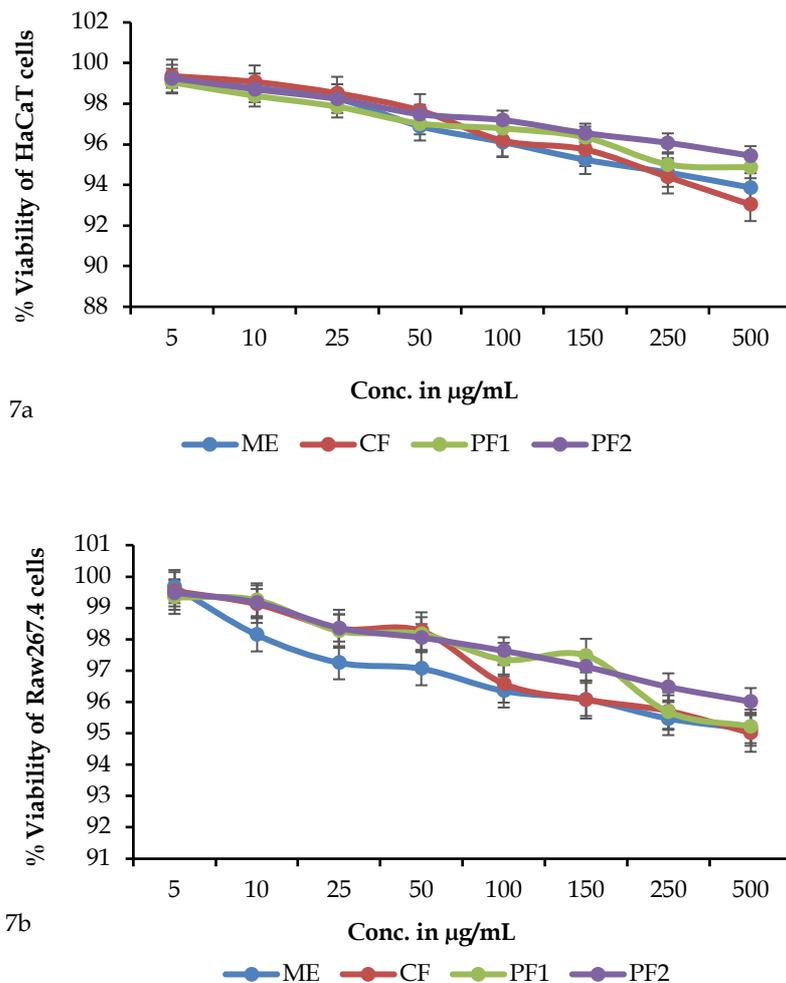
7a

7b

Figure 7. Effects of various test samples of *P. dulce* seed extract on HaCaT and cell viability; Three independent experiments were conducted to obtain the results. All values were expressed as Mean ± SD.

2.6 *In-vivo* **Anti-inflammatory activity**

The *in-vivo* anti-inflammatory effect of 9-hexadecenoic acid-rich HPLC fraction and chloroform fraction of methanolic seed extracts of *P. dulce* on carrageenan-induced rat paw edema was shown in Figures 8a and 8b. The results demonstrated that the 9-hexadecenoic acid-rich fraction exhibited significant time-dependent anti-inflammatory activity similar to that of the positive control at a dose of 100 mg/kg body weight. The results revealed that the 9-hexadecenoic acid-rich fraction showed a similar pattern of edema inhibition compared to the positive control throughout our study. In particular, PF2 and diclofenac showed the same effect of edema suppression after 75 minutes of treatment. As a function of edema inhibition, diclofenac and the 9-hexadecenoic acid-rich fraction have demonstrated 63 to 82% and 65 to 84% anti-inflammatory activity, respectively, after 90 minutes of treatment. In contrast to the considerable *in-vitro* potential, the chloroform fraction failed to exhibit a significant % anti-inflammatory activity in the edema rat model, as shown in figures 8a and 8b. These results demonstrated the significant *in-vivo* anti-inflammatory activity of PF2 in a carrageenan-induced rat paw model.
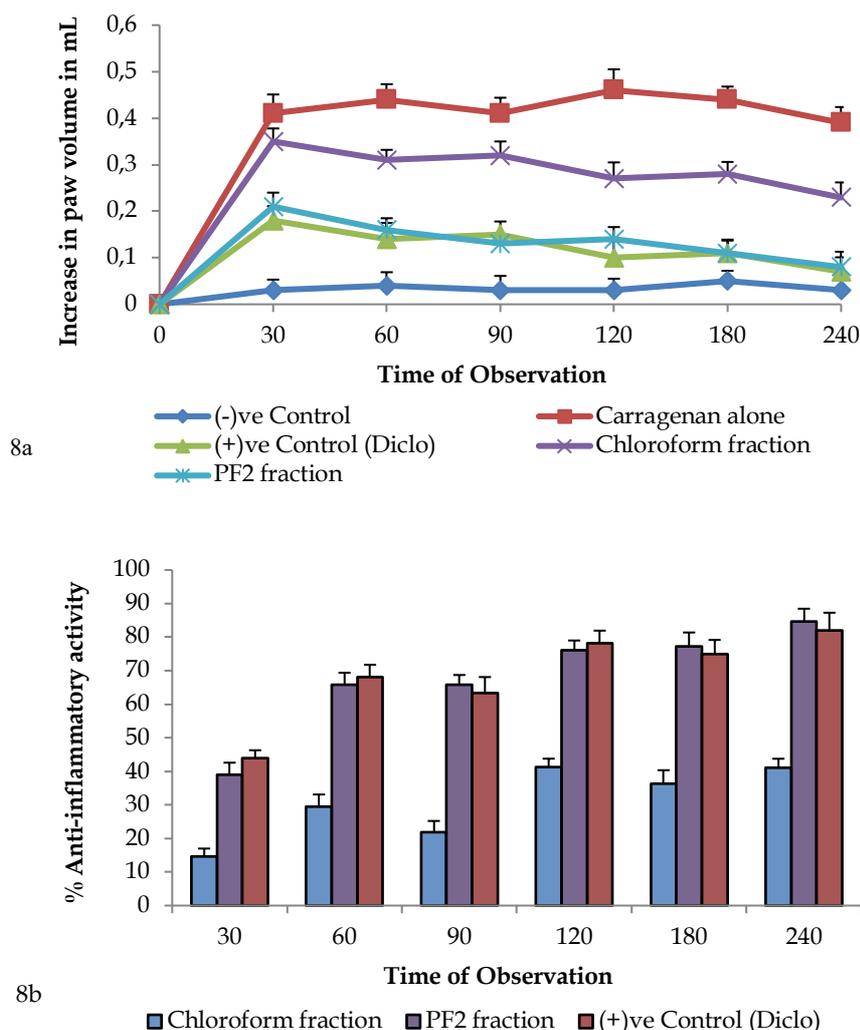
Figure 8. Evaluation of the anti-inflammatory effect of chloroform fraction and 9- hexadecenoic acid-rich HPLC fraction (PF2) of *P. dulce* seeds in the carrageenan-induced paw edema model. (a) Increase paw volume (mL) of rats upon treatment in various groups during 4 h of observation. (b) % anti-inflammatory activity of CF, PF2, and positive control during 4 h of observation over carrageenan induction.

3. CONCLUSION

In the present study, the methanolic seed extract of *Pithecellobium dulce* was phytochemically investigated and characterized using GC-MS analysis. Chromatographic analysis revealed the presence of 9-Hexadecenoic acid, n-Hexadecanoic acid, and Eicosatrienoic acid as major components. Further fraction using chloroform followed by HPLC analysis led to the isolation of 8,11,14, (Z,Z,Z)-Eicosatrienoic acid and 9-Hexadecenoic acid peak fractions. The *in-vitro* anti-inflammatory evaluation was assessed using albumin denaturation, examining IL-8 and IL-6 status in HaCaT and production levels of NO and PGE-2 in Raw267.4 cell-based inflammation models. The results of the 9-hexadecenoic acid-rich HPLC fraction showed potent anti-inflammatory effects by reducing the expression levels of IL-8, IL-6, NO, and PGE-2 production in cell models. In addition, no significant cytotoxicity was found by any extract or fraction of *P. dulce* examined via cell viability studies. An *in-vivo* study of the 9-hexadecenoic acid-rich HPLC fraction showed a significant reduction of paw edema caused by carrageenan, demonstrating a potential anti-inflammatory effect. This study suggested that the 9-hexadecenoic acid-rich HPLC fraction has superior anti-inflammatory potential both *in-vitro* and *in-vivo*, supporting the conventional use of this plant against inflammatory responses. Such activity seems to be attributed to the 9-hexadecenoic acid, the molecule isolated and identified from the chloroform fraction of methanolic extract of *Pithecellobium dulce* seeds. Furthermore, profound studies on 9-hexadecenoic acid are essential to understand pharmacokinetic behaviour such as bioavailability, toxicological parameters, how various processes regulate the metabolic pathways, etc.

4. MATERIALS AND METHODS

4.1 Plant material

The seeds of *Pithecellobium dulce* (Roxb.) Benth. were collected from Osmania University, Hyderabad, India, in August 2017. The plant material was authenticated with voucher no.119 and deposited at the Dept. of Botany, Osmania University, and Hyderabad. The seeds were examined carefully after separation from the fruits, and then old, deformed and damaged seeds were removed. Healthy seeds were spread out and dried at 25 °C at room temperature for about seven days and grounded in coarse powder using an electric blender, and it was further used for vehicle extraction.

4.2 Preparation of extract

The seed extract preparation was carried out in the same manner as that described by S. Chandra Sekhar et al. [25] with the aforementioned improvements. The air-dried pulverized seed content of *P. dulce* was sieved to mesh size 40. The powdered material (243 g) was extracted with methanol (4x500 mL) using a soxhlet extractor for 24 h. The methanolic isolate was concentrated under reduced pressure at 50 °C (Heidolph Hei-VAP rotary evaporator, Germany) and lyophilized to afford the crude methanolic extract (27.3g; yield: 11.23% w/w). The isolate was stored under cold conditions for further analysis.

4.3 Phytochemical investigation

4.3.1 Qualitative Analysis: Qualitative examination was performed as per standard protocols to identify the existence of various phytoconstituents like alkaloids, terpenoids, fats, oils, steroids, saponins, flavonoids, glycosides, phenols, and tannins present in the methanolic extract of *P. dulce* seeds [26, 27].

4.3.2 Quantitative analysis

Total Phenolic Content (TPC): The presence of overall phenolic content in the methanolic crude isolate of *P. dulce* seeds was determined by the Folin-Ciocalteu reagent method with few suitable changes. Briefly, 1 mL of isolate (1 mg/mL) was taken and placed with 2.5 mL of Folin-Ciocalteu reagent (10 %), followed by the introduction of 2 mL 2% sodium carbonate (Na_2CO_3). The reaction mixture was kept aside for 15 min in a tightly enclosed container, and the absorbance was measured in a 96-well microtitre plate at 765 nm using an absorbance microtitre plate reader (Tecan's Sunrise 11831, Mannedorf, Switzerland). Different dilutions of gallic acid (1 mg/mL) that are at 1, 0.5, 0.25, 0.10, 0.05, 0.02, 0.01 and 0 mg/mL were used to construct a calibration curve to report the findings as Gallic acid equivalent (GAE) mg/g of extract dry weight [28]. The analytical procedure was carried out in triplicate.

Total Flavonoid Content (TFC): The entire flavonoid content of the methanolic seed extract of *P. dulce* was determined by a colorimetric method involving aluminium chloride ($AlCl_3$). In brief, 0.5 mL of seed isolate was placed into a solution containing 1.5 mL MeOH, 0.1 mL of 10% Aluminium chloride ($AlCl_3$), and 0.1 mL of 1M potassium acetate (CH_3COOK), and subsequently added 2.8 mL of water to make it a suitable reaction mixture. Then the contents were placed in optimum conditions for 30 minutes in a tight container to avoid light exposure, and then absorbance was determined at 420 nm (UV-1650, Shimadzu, Kyoto, Japan). Quercetin was selected at different dilutions of 1, 0.50, 0.25, 0.10, 0.05, 0.02, 0.01, and 0 mg/mL to plot the calibration curve. The experiment was performed in triplicate, and the results are expressed as quercetin equivalent (QE) mg/g of extract dry weight.

Total triterpenoid content: The entire triterpenoid content was quantified using the earlier procedure [29]. In brief, 1 mL of the extract was taken with 1 mL of 5% w/v vanillin-glacial acetic acid and 2 mL of perchloric acid added to the above solution. The mixture of reaction components was placed at suitable conditions at 60 °C for 15 minutes, followed by cooling in an ice container for 20 minutes. Finally, 10mL of acetic acid was introduced, and all contents were mixed thoroughly. Later 10 mins of incubation, the absorbance reading was determined at 538 nm. A calibration curve was plotted using a standard oleanolic acid, and the total triterpenoid content was indicated as oleanolic acid equivalents (OAE, mg/g isolate dry weight).

Total Fat content: In this study, 0.1 mL of methanolic seed extract was pipetted out and evaporated under the flow of nitrogen gas. Next, the sample was re-suspended in 0.1 mL of acetone followed by 0.9 mL of 1.5% sulfosalicylic solution. It was shaken vigorously for 30 minutes, followed by 30 minutes standing. Spectrometric analysis (UV-1650, Shimadzu, Kyoto, Japan) measures the sample absorbance at 440 nm. Based on the lipid identification provided by GC-MS/MS analysis, the lipid quantification is calculated based on a calibration curve of standard fatty acids (lipid concentration vs. absorption reading) as described earlier [30].

4.4 GC-MS analysis of methanolic seed extract

GC-MS study was carried out to analyze the bio-molecular composition of methanolic seed extract of *P. dulce* as described by JA Anthony et al., [31] using a gas chromatographic system Agilent 6890A (Agilent Technologies, USA), set up with TG-5MS polydimethylsiloxane, capillary column (30 m x 250 µm diameter x 0.25 µm film thickness) coupled with MS-5975 inert MSD and triple axis mass selective ion detector. The initial temperature was set at 90 °C (0 min), and the final temperature was raised to 250 °C with a final time of 10 °C/min, while 300 °C was adjusted as inlet temperature. 1 µL of the sample was injected with split mode and the ratio was 10:1 without derivatization, as described by JA Anthony and his co-workers [31]. Thermal aux temperature and MS quadruple temperatures were set at 285 °C and 150 °C, respectively. The MS scan range was adjusted between 35-520 units, and helium was selected as the carrier gas at a flow rate of 1.0 mL/min. The spectral data were examined by MSD Chem-station edition F.01.01.2317 (Agilent Technologies, Germany). The details of chromatographic peaks of MS data were assessed by critical search using the NIST17 database (https://www.sisweb.com/software/nist-msms.htm). The relative outcome of substances was determined based on gas chromatography (GC) regions with an FID correction factor.

4.5 Fractionation of methanolic seed extract

In order to separate the bio-active components from the methanolic seed extract of *P. dulce*, 7.3 g of lyophilized powder of methanolic extract was then suspended in milli-Q water (100 mL) and partitioned successively with calculated quantities of ethyl acetate (3x25 mL), diethyl ether (3x25 mL), and chloroform (3x25 mL). All the fractions were dried under vacuum to a constant weight, and the resulting isolates were stored in cold conditions for further studies.

4.6 High-performance liquid chromatographic separation of chloroform fraction

The chloroform fraction of methanolic *P. dulce* seed extract was separated and characterized using HPLC by comparing chromatographic runs of selected standards identified by GC-MS/MS. The HPLC system consists of a dual solvent delivery system (Perkin-Elmer Series 200 LC pumps connected to a high-pressure mixer device), with a Perkin-Elmer Series 200 autosampler (Norwalk, CT) fitted with a 10µL sample loop and Luna C8(2) column (150 x 4.6mm; 5µm) was used, purchased from Phenomenex, USA. Degassed solvents were used for all experiments. Typical HPLC conditions maintained were reported below. The column conditioning was performed by passing a mixture of ACN (Solvent A): water (Solvent B) at a proportion of 90:10 v/v for 10 minutes at a flow rate of 500 µL/min. In order to analyze the fingerprint profile of the chloroform fraction of methanolic seed extract of *P. dulce*, 5 mg/mL of sample solution was prepared in a mixture of isopropyl alcohol: water (80:20 v/v) solution. Finally, the solution was centrifuged at 7000 rpm for 10 min to eliminate undissolved particles. After that, a 10 µL sample was introduced into a chromatographic system, and a phase-wise gradient protocol was applied to move from solvent A to B (A/B (70:30) to A/B (90:10) in 40 min, A/B (90:10) to A/B (70:30) in 20 min) for 60 minutes of run time. The flow rate of the chromatographic system was fixed at 500 µL/min. The presence of individual compounds was confirmed using the reference standards of 8,11,14, (Z,Z,Z)-Eicosatrienoic acid and 9-Hexadecenoic acid by dissolving in a mixture of isopropyl alcohol: water (80:20 v/v) solution. Similar chromatographic conditions were maintained for the analysis of standard runs. Data processing and analysis were performed using Chromeleon 6.8 (Thermo Scientific).

4.7 Purification and structural characterization

Purification of the selected HPLC peak fractions was accomplished using aminopropyl SPE cartridges suitable for the isolation of free fatty acids [32]. In order to elute the desired fatty acids, chloroform-isopropanol (2:1, v/v) was used first, followed by quantitative recovery with a mixture of diethyl ether: acetic acid (98:2, v/v). Further structural analysis of 8,11,14, (Z,Z,Z)-Eicosatrienoic acid and 9-Hexadecenoic acid was confirmed by Proton-NMR and mass spectra. Nuclear magnetic resonance spectra (^{1}H) were recorded on a Bruker 500 MHz NMR spectrometer in *DMSO-d6*. In addition, data processing and analysis were done in MestReNova-11.0.3. HR-MS was performed using a JEOL-HX 110 spectrometer, and spectroscopic data were reported as m/z values.

4.8 *In-vitro* Anti-Inflammatory activity

4.8.1 Albumin denaturation inhibitory activity

The assay was performed by the methods described earlier [33, 34] with some modifications. Methanolic extract (250 µg/mL), chloroform fraction of methanolic extract (250 µg/mL), and its corresponding HPLC

peak fractions of 9-Hexadecenoic acid (PF1) at 50 & 100 µg/mL and 8,11,14, (Z,Z,Z)-Eicosatrienoic acid (PF2) at 50, and 100, µg/mL concentrations, and positive standard (diclofenac) which was prepared at 100 µg/mL were selected for the experiment. A reaction mixture for each test sample consisted of 500 µL of bovine serum albumin (5%), 2.5 mL of phosphate-buffered saline, and 2 mL of the test extract. Methanol alone was used as a negative control. The mixtures were then nurtured at 37 °C for 20 minutes, followed by 5 minutes of heating at 70 °C. After chilling, each sample was measured at 660 nm, and the data were analyzed by the spectral manager. The percentage of protein denaturation was calculated using the following formula:

% Denaturation inhibition = (Abs of control-Abs of test/Abs of control) x 100

Cell Culture: Well-established inflammatory HaCaT (Immortalized human keratinocytes) cell model and RAW264.7 (Human macrophages) cells were obtained from the American Type Culture Collection (ATCC). HaCaT and RAW264.7 cells were grown in DMEM and RPMI, respectively accomplished with 1% antibiotic solution Pen Strep (Biowest), 10% fetal bovine serum (FBS, Biowest) at an incubation temperature of 37°C in a humidified conditions of 5% CO_2 in cell culture flasks (Thermo Fisher Scientific, USA). A cell confluence of 80-90% was used for the experiments [35].

4.8.2 Estimation of IL-6 and IL-8 from HaCaT Cells

The experiments were performed according to a method developed earlier [36] with few changes. First, HaCaT cells were cultured in a 24-well plate at a density of 5 x 10^4 cells per well, followed by 24 h incubation under similar harvesting conditions, as indicated above. After 24 h of the incubation period, the medium was replaced with a fresh medium containing either methanolic extract (ME at 250 µg/mL) or chloroform fraction of methanolic extract (CF at 250 µg/mL) and HPLC Peak fraction of 8,11,14-(Z,Z,Z)-Eicosatrienoic acid (PF1 at 100 µg/mL), and 9-Hexadecenoic acid (PF2 at 100 µg/mL) or 10 µM budesonide (Positive control) or the vehicle control (0.12% methanol) with the introduction of 20 ng/mL TNF-α (Humanzyme, Chicago, USA). Later 24 h post-incubation with TNF-α, cell-free supernatants were separated and analyzed. IL-6 and IL-8 were measured in the supernatants using ELISA kits (BD Biosciences, San Diego, USA) as per the manufacturer's instructions. The detection limits for IL-6 and IL-8 were 1.3 to 1.8 and 25.0 pg/mL, respectively, and there is no cross-reactivity with any other interleukin. The absorbance of each sample was determined at 450 nm using a microplate reader (Tecan's Sunrise 11831, Mannedorf, Switzerland).

4.8.3 NO and PGE2 production from RAW264.7 cells

RAW264.7 macrophage cells (1 x 10^6 cells/mL) were cultured for 24 h, pretreated with methanolic extract (ME at 250 µg/mL) or chloroform fraction of methanolic extract (CF at 250 µg/mL) and HPLC Peak fraction of 8,11,14-(Z,Z,Z)-Eicosatrienoic acid (PF1 at 100 µg/mL), and 9-Hexadecenoic acid (PF2 at 100 µg/mL) or 10 µM budesonide (Positive control) or the vehicle control (0.12% methanol), for 30 min, and further incubated with LPS (1 µg/mL) for 24 h. The effect of sample extracts on NO and PGE2 production was determined by the Griess assay and enzyme immunoassays (EIAs), respectively, as previously described [37].

4.8.4 Cell Viability Assay

In order to know the viable conditions of HaCaT and RAW264.7 cells over the treatment with different samples, during anti-inflammatory studies, cytotoxic assays were performed using the MTT assay. The basic principle of the MTT assay was calculating the relative number of viable cells to convert MTT dye (Sigma-Aldrich, Germany) into purple formazan. In brief, after stimulation of HaCaT cells with TNF-α and RAW264.7 with LPS, the cells were treated with saline (-ve control), methanolic extract (ME), chloroform fraction (CF), and HPLC peak fractions PF1 and PF2 for 24 h. After incubation, the supernatant was separated, and leftover bounded cells were washed with PBS and finally exposed to 500 µL MTT (1 mg/mL in PBS) per well and then again incubated the cells for 2 h. The resulting purple formazan was quantified spectrophotometrically using a microplate reader (Tecan's Sunrise 11831, Mannedorf, Switzerland) at 570 nm. The results were represented in percent cell viability based on MTT depletion, as calculated using the following equation.

% MTT reduction = (absorbance of sample/absorbance of control) x 100.

4.9 *In-vivo* anti-inflammatory activity

Animals: Prior to the experimental setup, male Wistar rats weighing about 180-200 g were obtained from Pharmacology Laboratory, PGP Life Sciences, Hyderabad. The study was approved by the Institutional animal ethics committee of PGP Life Sciences, Hyderabad, with approved protocol No: PGPLS/IEAC/SP/RP-081/2020. The animals were housed in a darkroom (12/12 h light/dark cycle) at a temperature of 25 ± 2 °C.

Rats were fed and drank *ad libitum*. Five groups were selected (A, B, C, D, and E) fo*r in-vivo* anti-inflammatory evaluation, and each group consisted of six animals (n=6).

4.9.1 Carrageenan-induced Paw edema model

In order to evaluate the *in-vivo* anti-inflammatory potential of the 9-hexadecenoic acid-rich HPLC fraction (PF2), a model of carrageenan-induced inflammation in a rat model was used, and the volume of paw edema was measured with the help of a plethysmometer, as previously described, with slight modifications [38]. Group A animals were treated orally with the only vehicle (Saline solution) and were considered a negative control. Induction of edema was performed by injecting 100 µL of 1% freshly prepared carrageenan in saline solution into the right-hind paw of each rat of 4 groups (Groups B, C, D, and E). Group B animals were considered diseased models. Groups C, D, and E animals were treated with diclofenac sodium (20 mg/kg bw), considered positive control group, chloroform fraction (CF at 100 mg/kg bw), and purified HPLC peak fraction of 9-hexadecenoic acid (PF2 at 100 mg/kg bw) respectively after 30 mins of carrageenan injection. The paw thickness of each animal in the group was measured just before the carrageenan injection, at "0 min" and then at 30, 60, 90-, 120-, 180-, and 240-min post carrageenan injection. The difference in paw thickness was measured by subtracting paw thickness at "0" min from paw thickness at respective time points.

The percentage (%) inhibition of edema is calculated using the following formula.

% inhibition = T_o - T_t / T_0 x 100

T_t is the thickness of the paw of rats given test extract at the corresponding time, and T_o is the paw thickness of rats of the control group simultaneously.

4.10 Statistical analysis

All the experimental findings were represented as Mean ± Standard deviation (SD). Results were analyzed using one-way ANOVA followed by Dunnett's t-test. P values <0.05 were considered statistically significant.

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Conflict of interest: The authors declared no conflict of interest.

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