Antioxidant and anti-inflammatory activities of Aerva pseudotomentosa leaves

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ABSTRACT

Context: Aerva pseudotomentosa Blatt. & Hallb. (Amaranthaceae), commonly called ‘Bui’, is a medicinal plant of the arid region. It is used for the treatment of inflammatory disorders, such as rheumatic pain, and healing of wounds, which are associated with oxidative stress.

Objective: The present study evaluated the antioxidant potential of Aerva pseudotomentosa leaves by in vitro methods and its anti-inflammatory effect in rats.

Material and methods: The aqueous extract (APAE) was analyzed by HPTLC and HPLC. The antioxidant effect of APAE was evaluated by various in vitro methods [DPPH (1, 1-diphenyl-2-picryl-hydrazil) and hydrogen peroxide free radical scavenging, reducing power, and anti-lipid peroxidation assays]. Anti-inflammatory effect was studied in carrageenan and formalin-induced paw oedema models in rats. APAE (200 and 400 mg/kg) and standard drug, indomethacin (10 mg/kg), were administered orally 1 h before carrageenan/formalin administration and inflammation was noted up to 5 h.

Results: HPLC analysis of APAE revealed the presence of rutin. APAE showed significant scavenging effect on DPPH (IC₅₀ 49.37 µg/mL) and peroxide (IC₅₀ 288.2 µg/mL) radicals. The extract exhibited reducing potential and inhibition of lipid peroxidation. APAE treatment significantly attenuated mean increase in paw volume and exhibited inhibition of paw oedema in both in vivo models with inhibition of 45.11% and 49.42%, respectively at 5 h.

Discussion and conclusion: APAE exhibited in vitro antioxidant and anti-inflammatory activities. Anti-inflammatory effect of APAE may be attributed to its antioxidant potential, due to the presence of rutin and other phenolics. This study substantiates folk use of leaves in inflammatory disorders.

Introduction

The Aerva genus (Amaranthaceae), includes about 29 species, which are distributed in Asia, Africa, and Australia. Six species of this genus exist in the dry desert areas of Pakistan (Khan et al. 1970). Plants of Aerva genus are popular as medicinal herb in several traditional systems of medicine. They are used as a valuable remedy for fever, rheumatism, gastric troubles, cough, sore throat, and wounds (Pullaiah 2006; Ahmed et al. 2010; Quattrocchi 2012). Similar to other Aerva species, Aerva javanica var. javanica, which is a variety of Aerva javanica (Burm. f.) Juss. ex Schult., and is traditionally used to treat pain and inflammation in ‘Qadanwari’ area of Nara desert of Pakistan (Bhatti et al. 2001). Previous studies with Aerva javanica var. javanica have demonstrated its anti-inflammatory and antioxidant activity (Al-Fatimi et al. 2007; Elaseed et al. 2015). Preclinical studies have demonstrated that some species of this genus such as Aerva lanata, Aerva sanguinolenta have analgesic and anti-inflammatory effects (Sharma et al. 2011; Mandal et al. 2015). Phytochemical investigations on these plants revealed the presence of various flavonoids, terpenoids, alkaloids, tannins, saponins, carbohydrate, and phenolic compounds (Emam 1999; Kumar et al. 2013).

Aerva pseudotomentosa Blatt. & Hallb. (Synonym: Aerva javanica var. bovei Webb), locally known as ‘Bui’, is a perennial herb or under shrub that grows in Thar desert of India and Nara desert of Pakistan. Aerva pseudotomentosa possesses many ethnomedicinal properties. It is used as folk medicine by the local inhabitants in India and Pakistan both. The decoction of the whole plant is used by the Thari people as a remedy for toothache (Qureshi & Bhatti 2008). Traditionally, A. pseudotomentosa is used for the treatment of various ailments like diuretic, demulcent and lithiastic. Roots of the plant are used for headache, decoction of inflorescence given as a remedy for inflammation, while wooly seeds are used for stuffing pillows, which provides relief from headache and rheumatic pain (Asolkar et al. 1992). Some folklore uses of A. pseudotomentosa were reported first time during ethnobotanical study on Nara desert of Pakistan. Paste made up of leaves and inflorescence is applied externally to treat inflammation of the joints and healing of wounds (Qureshi & Bhatti 2009).

Previous studies with crude ethanol extract of whole plant and inflorescence of A. pseudotomentosa demonstrated anticancer (Dhawan et al. 1980), analgesic, and anti-inflammatory effects (Agarwal et al. 2013). Apart from this A. pseudotomentosa possesses several ethnoveterinary medicinal uses like poultice of inflorescence tied locally to cure muscular injury in any body part of the animal, purgative and anthelmintic (Galav et al. 2010).
In view of wide ethnomedicinal use of *A. pseudotomentosa* in inflammatory conditions, there are no scientific studies carried out to delineate its anti-inflammatory potential. Further free radicals play an important role in initiating pro-inflammatory response and inducing inflammation. Hence, the present study demonstrates the antioxidant and anti-inflammatory potential of *A. pseudotomentosa* leaves.

**Material and methods**

**Chemicals and drugs**

Ascorbic acid, butylated hydroxy toluene (BHT), tannic acid (Merck Chemicals, Mumbai) were procured from the local market while quercetin, 1,1-diphenyl-2-picryl-hydrazil (DPPH) and carrageenan were obtained from Sigma-Aldrich, St. Louis, MO. Indomethacin was obtained as gift sample from M/S Plethico Pharmaceuticals Ltd, Indore, India.

**Collection and authentication of plant material**

The fresh leaves of *A. pseudotomentosa* were collected from Banad area of Jodhpur district, Rajasthan, India in May, 2012, and authenticated by Dr. R.P. Pandey, Senior Scientist, Botanical Survey of India, Jodhpur. A voucher specimen no. BSI/AZC/1/2012/Tech/2012-13 (Pl. Id.) was deposited in the herbarium of the Institute.

**Preparation of extracts**

The leaves of *A. pseudotomentosa* were shade-dried and coarsely powdered. The powdered leaves were extracted with petroleum ether (60–80°C) in Soxhlet apparatus to remove fatty substances. The marc was finally macerated with distilled water to obtain aqueous extract. The extract solution was filtered with Whatman filter paper No. 1 and solvent was removed under reduced pressure at 50–60°C and stored in a desiccator. The yield of *A. pseudotomentosa* aqueous extract (APAE) was 8% w/w.

**Preliminary phytochemical screening**

Preliminary phytochemical screening of the APAE (Khandelwal 2006) were carried out to detect the presence of flavonoids, alkaloids, terpenoids, saponins, fixed oils, steroids and glycosides.

**Quantitative estimation of phytoconstituents**

**Determination of total phenolic content**

The total phenolic content (TPC) of the extract was determined spectrophotometrically (Singleton et al. 1999). Folin-Ciocalteu’s reagent (1 mL), previously diluted (1:20), was added to 1 mL of APAE (1000 μg/mL) and mixed thoroughly. To the mixture, 4 mL of sodium carbonate (75 g/L) and 10 mL of distilled water were added and mixed well. The mixture was allowed to stand for 2 h at room temperature. The contents were then centrifuged at 2000 g for 5 min and the absorbance of the supernatant was read at 760 nm. A standard curve was plotted using various concentrations of tannic acid. Results were expressed as mg of tannic acid equivalents (TAE) per gram of extract.

**Total flavonoid content**

Total flavonoid content (TFC) was measured by aluminum chloride colorimetric assay (Marinova et al. 2005). The extract (1000 μg/mL) (1 mL) or standard solution (1 mL) of different concentrations of quercetin was added to 10 mL volumetric flask containing 4 mL of distilled water. To the above mixture, 0.3 mL of 5% NaNO₂ was added. After 5 min, 0.3 mL of 10% AlCl₃ was added. At 6th min, 2 mL of 1 M NaOH was added and the total volume was made up to 10 mL with distilled water. The solution was mixed well and the absorbance was measured against prepared reagent blank at 510 nm. Total flavonoid content of the extract was expressed as milligram of quercetin equivalents per gram of extract.

**Ascorbic acid content**

The ascorbic acid content was assayed as per the method described by Omaye et al. (1979) with some modifications. APAE (1 g) was ground in a pestle and mortar with 5 mL TCA (10%), and centrifuged at 3500 rpm for 20 min. The pellet was re-extracted twice with 10% TCA and supernatant was collected. To 0.5 mL of the supernatant, DTC reagent (2,4-dinitrophenyl hydrazine-thiourea-CuSO₄ reagent) (1 mL) was added and mixed thoroughly. The tubes were incubated at 37°C for 3 h and 0.75 mL of ice cold 65% H₂SO₄ was added to this solution. The tubes were then allowed to stand at 30°C for 30 min and the resulting color was read at 520 nm in a spectrophotometer. The ascorbic acid content was determined using a standard curve prepared with ascorbic acid and the results were expressed as mg/g of extract.

**TLC and HPTLC (high performance thin layer chromatography) analysis**

A qualitative densitometric TLC and HPTLC analysis was performed in APAE for the development of characteristic fingerprint profile of *A. pseudotomentosa* leaves powder extract. Powder (10 g) was soaked in 100 mL of distilled water and kept over-night. To it 50 mL of water was added again, boiled for 20 min and filtered. The filtrate was concentrated and made up to 3 mL in a graduated test tube. Absolute ethyl alcohol (7 mL) was added and made up to 10 mL in volumetric flask. TLC and HPTLC analysis were performed on (Merck) aluminium plate 60 f254 precoated with silica gel of 0.2 mm thickness. Previously prepared APAE solution (5, 10, 15, 20 μL) was applied on the plate with the help of CAMAG Linomat IV automated spray equipped with a 100 μL of the syringe and the plate was developed with toluene:ethyl acetate:formic acid (5:10:0.5) as a mobile phase. The plate was visualized under UV at 254 nm and 366 nm and photos were captured. The plate was scanned at 254 nm by CAMAG TLC Scanner operated by WINCATS software before it was dipped in vanillin sulfuric acid and kept in oven 105°C till the color of the spots appeared.

**Estimation of rutin in APAE by high performance liquid chromatography (HPLC)**

For the standardization, the estimation of rutin was carried out in APAE by HPLC at Natural Remedies Pvt. Ltd., Bangalore, Karnataka, India. The HPLC system (Shimadzu Corporation, Japan) having Phenomenex-Luna C-18 (2) of size 250 × 4.60 mm and 5 μm internal diameter and photo diode array detector was
used. Standard rutin (Natural Remedies Pvt. Ltd, percent purity ≥95%) (0.1 mg/mL) and APAE (50.0 mg/mL) was prepared in HPLC grade methanol and eluted in mobile phase consisting of potassium dihydrogen orthophosphate (KH$_2$PO$_4$) (A) and acetonitrile (B). The mobile phase gradient (A:B) were 95:5 (0.01 min) to 55:45 (18 min), 20:80 (25 min) to (28 min), 55:45 (35 min) and 95:5 (40 min) to (45 min). The wave length, flow rate, and injection volume were 280 nm, 1.5 mL/min and 20 μL, respectively. The chromatograms were recorded. The amount of rutin was calculated by the following formula:

\[
\text{Amount of rutin} = \frac{\text{[Area of sample/ Area of standard]} \times \text{[Weight of standard (mg)/Standard dilution (mL)]} \times \text{[Sample dilution (mL)/Weight of the sample (mg)]}}{\text{Purity of the standard (}%)}
\]

**Determination of in vitro antioxidant activity**

**DPPH (1,1-diphenyl-2-picryl-hydrazil) free radical scavenging activity**

The free radical scavenging activity of APAE was measured by in vitro DPPH method as previously described (Brand-Williams et al. 1995). Briefly, 0.1 mM solution of DPPH in ethanol was prepared and 3.5 mL of the solution was added to 0.5 mL of different concentrations (20–100 μg/mL) of extract was added in distilled water. The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. The absorbance of the resulting solution was measured at 517 nm. Butylated hydroxyl toluene (BHT) was taken as standard antioxidant. The percent DPPH scavenging effect was calculated using the following equation:

\[
\text{DPPH scavenging effect (}% = \frac{A_0 - A_1}{A_0} \times 100
\]

where, $A_0$ was the absorbance of the control and $A_1$ was the absorbance in the presence of the test.

**Reducing power assay**

The reducing power of the APAE was determined as per previously described method (Oyaizu 1986). Different concentrations (100–1000 μg/mL) of extract were prepared in double distilled water. Each concentration (0.5 mL) was mixed with phosphate buffer (1.5 mL, 0.2 M, pH 6.6) and potassium ferrocyanide (1.5 mL, 1%). The mixture was incubated at 50 °C for 20 min. A portion (2.5 mL) of TCA (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The supernatant of the solution (1.5 mL) was diluted with distilled water (1.5 mL). Finally, FeCl$_3$ (300 μL, 0.1%) was added to it and the absorbance of the resulting solution was measured at 700 nm. The experiments were performed in triplicate.

**Scavenging of hydrogen peroxide**

The ability of APAE to scavenge hydrogen peroxide radicals was determined according to the method described by Ruch et al. (1989) with slight modification. In the present study, a solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer saline (pH 7.4). Hydrogen peroxide concentration was determined spectrophotometrically from absorption at 230 nm. APAE (1.5 mL) in different concentrations (100–500 μg/mL) in distilled water was added to a hydrogen peroxide solution (1.5 mL, 40 mM). The ratio of the amount of extract/standard solution and hydrogen peroxide solution were fixed from preliminary experiments. The absorbance of hydrogen peroxide at 230 nm was determined within 10 min against a blank solution containing in phosphate buffer saline without hydrogen peroxide. Ascorbic acid was used as the standard antioxidant compound. The percentage of scavenging of hydrogen peroxide by APAE and standard (ascorbic acid) was calculated as below.

\[
\text{H}_2\text{O}_2 \text{ scavenging effect (}% = \frac{A_0 - A_1/A_0}{} \times 100
\]

where, $A_0$ is the absorbance of the control, and $A_1$ is the absorbance in the presence of the test.

**Anti-lipid peroxidation assay in flax seeds homogenate**

Lipid peroxidation in flax seeds homogenate was evaluated by the method described earlier (Ohkawa et al. 1979). In this assay, flax seeds were used as source of polyunsaturated fatty acid (PUFA) and their homogenate were prepared in 0.15 M KCl. The reaction mixture containing (1 mL, 10%) flax seeds homogenate and APAE (1 mL) of different concentrations (20–100 μg/mL) was prepared. Lipid peroxidation was initiated by adding 100 μL of 15 mM FeSO$_4$ solution. The reaction mixtures were incubated for 30 min at 37 °C. After incubation, the reaction was stopped by adding (1 mL) of (0.67%) ice-cold thiorbarbituric acid (TBA) solution. The reaction mixtures were heated for 60 min at 80 °C, cooled, and centrifuged at 5000 rpm for 15 min. The absorbance of the supernatant was measured at 532 nm against a blank, which contained all reagents except homogenate and drug. FeSO$_4$ induced control contained homogenate plus FeSO$_4$ while sample contained APAE/BHT in various concentrations in addition. The per se optical density (OD) of the different test/standard was subtracted to get sample OD reading at different concentrations. The percentage of anti-lipid peroxidation effect (ALP %) was calculated by the following formula:

\[
\text{ALP (}% = \frac{\text{FeSO}_4 \text{ Induced OD } - \text{ Sample OD}/\text{FeSO}_4 \text{ Induced OD}}{} \times 100
\]

**Pharmacological assessment**

**Animals**

The experiments were carried out using healthy adult albino Wistar rats (200–250 g) of either sex obtained from animal house of the IPS College of Pharmacy, Gwalior (Registration no.1039/ac/07/CPCSEA). The animals were kept in controlled conditions of temperature (25 ± 2 °C), relative humidity (50 ± 5%) and 12 h light-dark cycle with standard rodent food and water provided ad libitum. The animal experiments were performed according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India and the protocols were approved by Institutional Animal Ethics Committee (Approval no. IPS/COP/IAEC/01).

**Acute toxicity study**

Acute oral toxicity study was carried out as per Organization for Economic Cooperation and Development (OECD) guidelines (OECD 2001). The overnight fasted rats ($n = 3$) were orally treated with 2000 mg/kg of extract and were observed for 24 h and then up to 14 days for any mortality and moribund stage.
Assessment of anti-inflammatory activity

Carrageenan-induced paw oedema

The anti-inflammatory effect of *A. pseudotomentosa* was determined by carrageenan-induced paw oedema test according to method described by Winter et al. (1962). Briefly, rats were divided into four groups (5 rats/group).

- Group I: vehicle treated rats received distilled water (5 mL/kg, p.o.)
- Group II (reference standard): received indomethacin (10 mg/kg, p.o.)
- Group III & IV: received APAE in two doses (200 and 400 mg/kg, p.o.)

Treatments were given 1 h before administration of 0.1 mL 1% carrageenan in the sub plantar region of right hind paw to induce paw oedema. The paw volume was measured at different time points using plethysmometer (Ugo Basile, Italy) before and after 1, 3 and 5 h of administration of carrageenan (Taesotikul et al. 2003). The percent inhibition was calculated by following formula:

\[
\text{% Inhibition of paw edema} = \left(\frac{(V_c - V_t)}{V_c} \times 100\right)
\]

where, \(V_c\) = Increase in paw volume of control group and \(V_t\) = Increase in paw volume of the treated group.

Formalin-induced paw oedema

The rats were divided into four groups (5 rats/group).

- Group I: vehicle treated rats received distilled water (5 mL/kg, p.o.)
- Group II (reference standard): received indomethacin (10 mg/kg, p.o.)
- Group III & IV: received APAE in two doses (200 and 400 mg/kg, p.o.)

Formalin (0.05 mL, 2.5%) was injected into the dorsal surface on the left hind paw of rats of all groups. The paw volume was measured using plethysmometer before (0 min) and at an interval of 1, 3 and 5 h after formalin administration. The percent inhibition was calculated by following formula (Dubuisson & Dennis 1977).

\[
\text{% Inhibition of paw edema} = \left(\frac{\left((P_{V_t} - P_{V_0})\right)\text{control} - \left((P_{V_t} - P_{V_0})\right)\text{treated}}{\left((P_{V_t} - P_{V_0})\right)\text{control}}\right) \times 100
\]

where, \(P_{V_t}\) = Paw volume after formalin injection and \(P_{V_0}\) = Paw volume before formalin injection.

Statistical analysis

The data were analyzed with two-way ANOVA followed by Bonferroni post hoc test using (Prism Pad statistics software ver. 4). A statistical difference of \(p < 0.05\) was considered significant in all the cases.

Results

Preliminary phytochemical screening

Preliminary phytochemical screening of the APAE revealed the presence of flavonoids, alkaloids, terpenoids, saponins, fixed oils, steroids and glycosides.

Quantitative estimation of phytoconstituents

Total flavonoid, phenolic and ascorbic acid content of APAE

The total flavonoid content of APAE was found to be 248.5 mg quercetin equivalents/g of extract while the total phenolic content of APAE was found to be 359.3 mg tannic acid equivalents/g of extract. The ascorbic acid content of the extract was found to be 0.728 mg/g of extract.

TLC and HPTLC analysis

TLC and HPTLC analysis of APAE was performed using a mobile phase (toluene:ethyl acetate:formic acid). Figure 1(a–c) indicates the TLC fingerprint of APAE. TLC profile of APAE revealed that at 254 nm, two spots (Rf values 0.64 and 0.71) exhibited green fluorescence, whereas at 366 nm, 5 spots with Rf values 0.11, 0.21, 0.67, 0.69 and 0.75, respectively, exhibited blue fluorescence. Scanned HPTLC chromatogram of APAE detected the presence of 8 peaks (Figure 2), out of which the prominent peaks were having Rf values 0.64 and 0.71, respectively (Table 1).

Estimation of rutin in APAE by HPLC

HPLC analysis revealed the presence of rutin in APAE. The peak of reference standard rutin and APAE are shown in Figure 3(a) and (b), respectively. The estimated amount of rutin was found to be 0.006 (% w/w).
Antioxidant activity

DPPH scavenging assay
In this study APAE in the concentration range of (20–100 µg/mL) inhibited DPPH radical scavenging as indicated by concentration-dependent decrease in the purple color of the solution. Similar effect was obtained with standard antioxidant, BHT in the concentration range of 2–10 µg/mL. The linear regression coefficient of APAE and BHT were \( r^2 = 0.9465 \) and \( r^2 = 0.9798 \), respectively, suggesting that the DPPH scavenging was concentration dependent. The IC\(_{50}\) value of APAE and BHT, obtained from regression analysis, were 49.37 and 4.22 µg/mL, respectively. The results are expressed in Table 2.

Reducing power assay
APAEE in the concentration range of (100–1000 µg/mL) showed concentration-related reduction of ferricyanide to ferrocyanide as indicated by increase in the green color absorbance measured at 700 nm. However, the efficacy was found to be lower than that of standard antioxidant, ascorbic acid. Linear regression analysis of concentration versus absorbance at 700 nm was carried out which showed that linear regression coefficient of APAE and ascorbic acid were \( r^2 = 0.9816 \) and \( r^2 = 0.9164 \), respectively, suggesting that the reducing potential was concentration dependent. The results are depicted in Figure 4.

\[ \text{Table 1. HPTLC profile of aqueous extract of } Aerva \text{ pseudotomentosa.} \]

| Peak | Rf  | Height | Area     |
|------|-----|--------|----------|
| 1    | 0.02| 20.0   | 184.8    |
| 2    | 0.12| 16.6   | 366.4    |
| 3    | 0.16| 18.0   | 445.3    |
| 4    | 0.29| 28.1   | 1151.5   |
| 5    | 0.50| 18.7   | 506.6    |
| 6    | 0.64| 387.9  | 14465.7  |
| 7    | 0.71| 462.1  | 13911.0  |
| 8    | 0.97| 40.8   | 645.8    |

\[ \text{Figure 2. HPTLC fingerprint of } Aerva \text{ pseudotomentosa aqueous extract.} \]

H\(_2\)O\(_2\) scavenging assay
APAEE in concentration range of (100–500 µg/mL) showed H\(_2\)O\(_2\) scavenging as indicated by concentration-dependent decrease in the absorbance of H\(_2\)O\(_2\) solution. Similar effect was obtained, with standard antioxidant ascorbic acid in the concentration range of 20–100 µg/mL. Linear regression analysis of concentration versus percent H\(_2\)O\(_2\) inhibition was carried out. The linear regression coefficient of APAE and ascorbic acid were \( r^2 = 0.9811 \) and \( r^2 = 0.9798 \), respectively, suggesting that the scavenging of H\(_2\)O\(_2\) was concentration dependent. The IC\(_{50}\) value of APAE and ascorbic acid, obtained from linear regression analysis,
were 288.20 and 52.75 µg/mL, respectively. The results are expressed in Table 3.

**Anti-lipid peroxidation (LPO) assay in flax seed homogenate**

APAЕ in concentration range of (20–100 µg/mL) showed anti-lipid peroxidation effect as indicated by concentration-dependent decrease in the absorbance of thiobarbituric acid reactive substances (TBARS). Similar effect was obtained, with standard antioxidant BHT in the concentration range of 20–100 µg/mL. Linear regression analysis of concentration versus percent anti-LPO was carried out. The linear regression coefficient of APAЕ and ascorbic acid were \( r^2 = 0.9321 \) and \( r^2 = 0.9006 \), respectively, suggesting that the inhibition of LPO was concentration-dependent. The results are expressed in Figure 5.

**Figure 3.** (a) HPLC chromatogram of reference standard rutin (retention time 11.427). (b) HPLC chromatogram of APAЕ (retention time 11.423).
Acute toxicity study

Acute toxicity study revealed no lethality or any toxic reactions or moribund state up to the end of the study period. APAE was safe up to a dose level of 2000 mg/kg of body weight (limit test) and approx. LD50 observed was more than 2500 mg/kg.

Anti-inflammatory activity

Carrageenan-induced paw oedema

The anti-inflammatory effect of APAE on carrageenan-induced rat paw oedema is shown in Table 4. After 3rd h of carrageenan administration, APAE at the dose of 400 mg/kg, exhibited significant \((p < 0.05)\) inhibitory effect on paw oedema, while the lower dose 200 mg/kg was ineffective when compared to control. After 5th h, APAE (200 and 400 mg/kg) showed a significant inhibition of the mean increase in paw volume \((p < 0.01, p < 0.001\), respectively) as compared to control group. Indomethacin also exhibited significant anti-inflammatory effect \((p < 0.001)\). Marked anti-inflammatory effect was noted at 5th h after carrageenan administration.

Formalin-induced paw oedema

Treatment with APAE at dose of 400 mg/kg produced significant reduction in paw oedema at 3rd h and 5th h (49.42%) respectively, while dose of 200 mg/kg exhibited significant inhibitory effect only at 5th h (45.36). The inhibitory effect of APAE at dose of 400 mg/kg (49.42%) was comparable to standard drug, indomethacin (53.47%). The results are shown in Table 5.

Discussion

In the present study, results of in vitro antioxidant methods demonstrated that APAE possess antioxidant and free radical scavenging activity. In the in vivo study, APAE significantly inhibited carrageenan and formalin induced paw oedema in rats.

In the present study, HPTLC fingerprinting of APAE revealed the presence of eight different peaks. Preliminary phytochemical
analysis of extract showed the presence of flavonoids, alkaloids, terpenoids, saponins, fixed oils, steroids and glycosides in APAE. The peaks were observed probably due to the presence of these phytoconstituents in *A. pseudotomentosa* aqueous extract. Therefore, this HPTLC fingerprinting with recorded Rf values can be used as reference standard for research on medicinal properties, authorization, characterization and distinguish the plant from other species of *Aerva* genus. Acute toxicity study of APAE revealed that there was no moribund state, and the drug was safe on oral administration.

Oxidative stress is involved in the pathophysiology of inflammation, degenerative disease, diabetes, cancer, ischemic heart disease and many more. Antioxidant property of a compound depends on its ability to suppress the formation of ROS (reactive oxygen species), free radical scavenging and inhibiting cellular microsomal P-450-linked mixed function oxidation (MFO) reaction (Malhotra et al. 2008). Plant-based natural antioxidants are the substances that react with free radicals to prevent the oxidation of substrate. In the present study, APAE was tested for antioxidant potential in four classical *in vitro* models. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Gülcin et al. 2003). APAE inhibited DPPH free radical formation in concentration-dependent manner in comparison with BHT, a standard antioxidant. It indicates free radical scavenging potential of APAE. 

H₂O₂ is a weaker oxidizing agent and it enters in the human body through the vapor, eye and skin contact. H₂O₂ rapidly decomposes into oxygen and water in the body and initiate lipid peroxidation and causes DNA damage by producing OH radicals. It crosses cell membrane and reacts with ferric and copper ions, which shows toxic effects (Begum et al. 2007). APAE exhibited concentration-dependent H₂O₂ scavenging effect. Reducing power indirectly evaluates the antioxidant activity. It is used to determine the extent of reduction of ferricyanide to ferrocyanide by antioxidants. APAE showed significant reducing potential when compared with the standard drug ascorbic acid. However, reducing power observed was one tenth of that of ascorbic acid.

Lipid peroxidation is the oxidative degeneration of polysaturated fatty acids (PUFA) and involves lipid radicals leading to membrane damage. Free radicals have the ability to induce lipid peroxidation in PUFA rich tissues or biomaterials (Coyle & Puttfarcken et al. 1993). This property of free radicals was exploited to study lipid peroxidation in PUFA rich source-flax seed (*Linum usitatissimum* Linn.) belongs to the family Linaceae (El-Beltagi et al. 2011). Considering the constraints with the use of animals and their tissues in small experiments, use of flax seeds as lipid PUFA source can be a good choice for such lipid peroxidation assays. In the present study, the lipid peroxidation in flax seeds was induced by pro-oxidant substance like ferrous sulfate and anti-lipid peroxidation effect of APAE was assessed by measuring the formation of TBARS. The anti-lipid peroxidation assay revealed that APAE attenuated the lipid peroxidation induced by ferrous sulfate in flax seeds. A similar effect was also observed with standard antioxidant, BHT. The initiation of lipid peroxidation by ferrous sulfate takes place through ferroly-perferryl complex or through hydroxyl radicals by Fenton's reaction thereby initiating a cascade of oxidative reactions. The anti-lipid peroxidation effect shown by APAE may be due to several reasons such as inhibition of ferroly-perferryl complex formation, scavenging of superoxide radical or chelation of iron itself. The anti-lipid peroxidation effect of APAE suggests its antioxidant action. In the above-mentioned models for screening of antioxidant assessment, it is clear that APAE has effective antioxidant potential, although lesser in potency than that of the standard antioxidants. The antioxidant potential of APAE may be attributed to the high flavonoid and phenolic content of the plant as revealed from their quantitative estimation. The total phenolic content of APAE was higher than values for other *Aerva* species reported in the literature (Ragavendra et al. 2012). Polyphenolic compounds like flavonoids and phenolic acids are reported to have multiple biological effects including antioxidant activity (Kähkönen et al. 1999) support the present findings.

The results obtained from anti-inflammatory study showed that APAE treatment showed inhibition of carrageenan-induced inflammation and development of oedema in rat paw. Carrageenan-induced paw oedema is described as a biphasic event; the early phase within 1 h involves the production of histamine, bradykinin, cyclooxygenase products and 5-hydroxytryptamine, while late phase is associated with neutrophil infiltration as well as the production of metabolites of arachidonic acid (Boughton-Smith et al. 1999). Moreover, inflammation is also involves the role of reactive species. APAE inhibited both phases of oedema by possibly reducing the release of histamine, serotonin, bradykinin and prostaglandins.

Another model used to evaluate the anti-inflammatory effect of APAE was the formalin test. It involves moderate and long lasting pain perception. Subcutaneous injection of formalin in the right hind paw of animals evoked behavioral nociceptive response in biphasic pattern; the early phase is short lived or about 3–5 min characterized by c-fiber activation due to peripheral activation, while the late phase caused by local tissue inflammation with longer persistent period (Tjølsen et al. 1992). In this study the results showed that APAE treatment exhibited inhibition of formalin-induced inflammation. Treatment at dose of 400 mg/kg p.o. produced significant reduction in paw oedema at 3rd and 5th h, respectively, while dose of 200 mg/kg p.o. exhibited significant inhibitory effect only at 5th h. It is difficult to elucidate the exact mechanism involved in anti-inflammatory action of APAE. It is probably due to inhibition of inflammatory cascade and synthesis of prostaglandins. Phenolic compounds such as flavonoids, tannins, ascorbic acid exert their effect by inhibition of pro-inflammatory enzymes such as cyclooxygenase and lipoxygenase. Qualitative and quantitative phytochemical investigation (TPC, TFC) revealed fair amount of flavonoids and phenolic compounds in the extract. HPTLC analysis revealed the presence of various phytoconstituents with different Rf values. Standardization of APAE by HPLC revealed the presence of flavonoid, rutin (0.006%) (Figure 3(a) and (b), respectively). Previously, rutin demonstrated anti-inflammatory effect in rats by preventing oedema formation induced by histamine and serotonin as well as found extremely effective in acute and chronic phase of inflammation in adjuvant-induced arthritis by reducing oedema, nodule and ankylosis (Borissova et al. 1994; Guardia et al. 2001) Additionally, it has been reported for strong antioxidant capacity and as a potential candidate for inhibition of cyclooxygenase (COX-2) enzymes and phospholipase A2 activity, the initial enzyme in the arachidonic acid cascade (Lindahl & Tagesson 1997). As neutrophils plays pivotal role in inflammatory process. Rutin was demonstrated significant inhibitory effect on neutrophil chemotaxis and degranulation. It prevented polymorphonuclear migration towards fMet-Leu-Phe (neutrophil chemotactic factor) and partial inhibitory effect on degranulation of fMet-Leu-Phe neutrophils (Selloum et al. 2001).

Phytochemical investigation on other species of *Aerva* genus revealed the presence of kaempferol-3-O-β-D-4″-E-p-coumaroyl-α-L-rhamnosyl(1→6)-glucoside, aervanone, isorhamnetin-3-O-
β-[4″-p-coumaroyl-α-rhamnosyl(1→6)galactoside], ecydysteroids, ascorbic acid, β-sitosterol, gallic acid, alkaloids, and terpenoids (Garg et al. 1980; Saleh et al. 1990; Mussaadig et al. 2013; Saleem et al. 2013). This evidence suggests that anti-inflammatory effect of APAE may be attributed to presence of rutin and other phenolic compounds and further strengthens the present findings. Free radicals attract various inflammatory mediators in the inflammatory process. They cause release of proinflammatory cytokines and initiate the inflammation (Zhang & Jianxiong 2007). It seems that antioxidant effect of APAE might have a contributing role to anti-inflammatory effect of APAE. Flavonoids like rutin are strong antioxidant compound particularly for excellent scavenging activity (Nagasawa et al. 2002). Rutin has remarkable potency to donate electron to reactive free radicals by converting them into more stable species and quenching the free radical chain reaction this may be due to presence of phenolic rings and free hydroxyl groups in the chemical structure (Yang et al. 2008). These free hydroxyl groups could donate hydrogen to prevent further oxidation. So this could be logical to establish relationships that antioxidant effect of APAE may be due to the presence of flavonoids.

Conclusions
In conclusion, the aqueous extract of A. pseudotomentosa leaves exhibited significant anti-oxidant and anti-inflammatory activity. The anti-inflammatory effect could be attributed to antioxidant potential of APAE which may be due to presence of flavonoid and phenolic compounds present in the leaves extract. The findings of the study validate the uses of leaves of A. pseudotomentosa as folklore medicine in treatment of inflammatory conditions. However, the isolation of compounds responsible for anti-inflammatory effect is subject of further investigation.

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Disclosure statement
The authors declare that there is no conflict of interest.

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