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An Evaluation of Haemolytic and Antioxidant Potential of *Kalanchoe pinnata* Leaves

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The present study was carried out to determine the haemolytic and antioxidant potential of *Kalanchoe pinnata* leaves. Antioxidant activity was determined by measuring reducing potential, DPPH radical scavenging (IC_{50}), total phenolic contents and total flavonoid contents. The % yield of leaves extracts were found to be in the range of 2.2 to 3.8 %. The plant leaves contained appreciable levels of total phenolic contents (2.28-6.24 GAE mg/g of dry extract) and total flavonoid contents (2.3-4.5 CE mg/g of dry extract). DPPH radical scavenging was found to be in the range of 35.4-70.12 μg/mL. The haemolytic effect of the plant leaves was found in range of 0.82-4.96 %. The antioxidant activity of plant extracts was also studied using sunflower oil as an oxidative substrate and found that it stabilized the oil. The results of the present investigation demonstrated significant ($p < 0.05$) variations in the results. The correlation between the results of different antioxidant assays and oxidation parameters of oil indicated that leaves extracts, exhibited considerable TPC, TFC and scavenging power, were also more potent for enhancing the oxidative stability of sunflower oil.

Key Words: *Kalanchoe pinnata*, Antioxidant activity, Stabilization, Sunflower oil.

INTRODUCTION

Kalanchoe pinnata is a succulent perennial flowering plants which belongs to the Family Crassulaceae. It is commonly known as air plant[1]. Leaves of this plant are eaten for diabetes control, used as diuretic and for dissolving of kidney stones and taken for respiratory tract infections, headache, cough, chest pain, sores, boils, ulcers and other skin diseases, to cure fever, improving menstrual irregularities and treat hypertension, rheumatism as well as applied to wounds, boils and insect bites. The leaves of the plant are used by traditional medicinal practitioners' for diabetes and the bark as a purgative[2]. In traditional medicine the leaves of this plant have been used for antimicrobial, antifungal, antiulcer, antiinflammatory, analgesic, potent antihistamine and antiallergic activity[3]. Ali and co-workers[4] reported the presence of various medicinally important compounds such as alkaloids, tritepenes, glycosides, flavonides, steroids lipids and bufadienolides and also evaluated the antibacterical activity of leaves extracts of *K. pinnata*. This plant has a high phytotherapeutic potential, as shown by its antiinflammatory, antiulcer, hepatoprotective, antileishmania, immunomodulatory activities, tocolytic effectiveness[5] and used for cardiovascular treatment[6]. This herb is a good source of mineral elements such as Na, Ca, K, P, Mg, Mn, Cu, Zn. Sugar contents includes raffinose, sucrose, glucose, galactose and fructose[7]. As our efforts to explore the medicinal importance of various plants[8-12], *Kalanchoe pinnata* has been subjected to investigate the *in vitro* antioxidant and haemolytic potential of various organic extracts.

EXPERIMENTAL

The plant leaves (*Kalanchoe pinnata*) were collected in April 2011 from botanical garden, University of Agriculture, Faisalabad, Pakistan and further identified by a Taxonomist, Department of Botany, University of Agriculture, Faisalabad, Pakistan.

Extraction: Leaves of the plant were washed with distilled water. Then they were shade dried and grinded to powder form. The powdered plant leaves were extracted with different polarity based solvents (petroleum ether, chloroform, ethyl acetate, *n*-butanol, Absolute methanol, methanol:water (9.5:0.5) and methanol:water (9:1) (Fig. 1). The obtained extracts were concentrated with vacuum rotary evaporator and then stored at -4 ºC for further analysis.

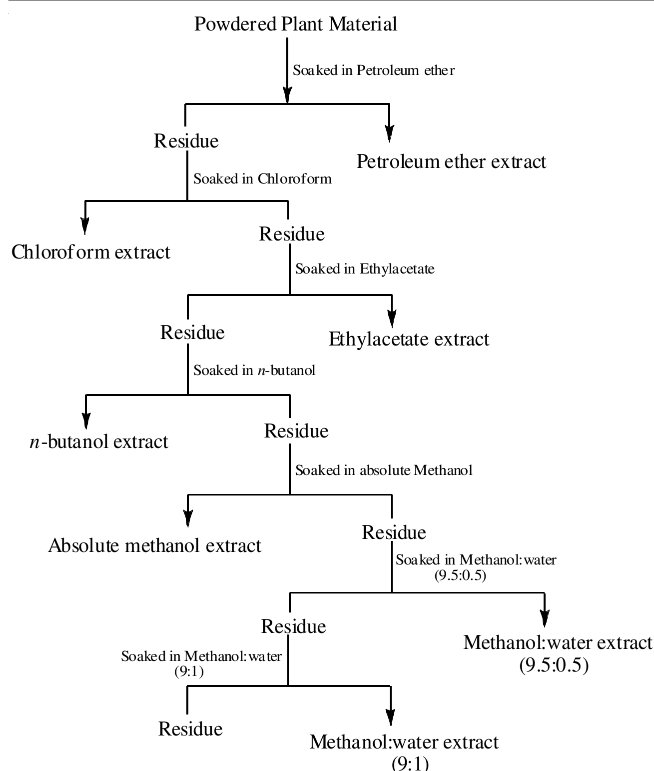

Fig. 1. Schematic diagram for the preparation of *K. pinnata* leaves extracts

Determination of total phenolic contents: Amount of total phenolic contents were assessed using Folin-Ciocalteu reagent procedure[8]. Briefly, 1 mg of dry mass of crude extracts was mixed with 0.5 mL of Folin-Ciocalteu reagent and 7.5 mL deionized water. The mixture was kept at room temperature for 10 min and then 1.5 mL of 20 % sodium carbonate (w/v) was added. The mixture was heated in a water bath at 40 ºC for 20 min and then cooled in an ice bath; finally absorbance at 755 nm was measured (Hitachi U-2001 spectrophotometer). Amounts of TP were calculated using a calibration curve for gallic acid (10-100 ppm) (R^2 = 0.9986). The results were expressed as gallic acid equivalents (GAE) of dry plant matter.

Determination of total flavonoid contents: The total flavonoid content in plant extract and fractions was determined following the procedure as described by Rasool and co-workers[8]. Plant extract/fractions of each material (1 mL containing 0.1 g/mL) was placed in a 10 mL volumetric flask, then added distilled water 5 mL and 0.3 mL of 5 % $NaNO_2$ was added to each volumetric flask initially; after 5 min, 0.6 mL of 10 % $AlCl_3$ was added. After another 5 min, 2 mL of 1 M NaOH was added and volume made up with distilled water. Then solution was mixed. At 510 nm absorbance of the reaction mixture was taken using a spectrophotometer. Total flavonoid content were evaluated as catechin equivalents (g/100 g of dry plant matter).

DPPH radical scavenging assay: The 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) assay was carried out spectrophotometrically as described by Tepe *et al.*[13]. Aliquots (50 µL) of various concentrations (10-100 µg/mL) of the essential oil and extract samples was added to 5 mL of a 0.004 % methanolic solution of DPPH. After 0.5 h incubation period at room temperature, the absorbance was read against a blank at 517 nm:

$$\text{Inhibition (\%)} = 100 \times (A_{\text{blank}} - A_{\text{sample}}/A_{\text{blank}})$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound) and A_{sample} is the absorbance of the test compound. Extract concentration providing 50 % inhibition (IC_{50}) was calculated from a graph plotting percentage inhibition against extract concentration.

Determination of reducing power: The reducing power of the extracts was determined according to the procedure described by Yen *et al.*[14] with little modification. Equivalent volume of leaves crude extracts containing 2.5-10 mg of dry matter was mixed with sodium phosphate buffer (5.0 mL, 0.2 M, pH 6.6) and potassium ferricyanide (5.0 mL, 1.0 %); the mixture was incubated at 50 ºC for 20 min. Then 5 mL of 10 % trichloroacetic acid was added and centrifuged at 980 xg for 10 min at 5 ºC in a refrigerated centrifuge. The upper layer of the solution (5.0 mL) was diluted with 5.0 mL of distilled water and ferric chloride (1.0 mL, 0.1 %) and absorbance noted at 700 nm (Hitachi U-2001 spectrophotometer). The measurement was run in triplicate and results averaged.

***In vitro* haemolytic activity:** Haemolytic activity of the plant was checked by the reported method of Powell *et al.*[15]. 3 mL of freshly obtained heparinized human blood was gently mixed, poured into a sterile 15 mL polystyrene screw-cap tube and centrifuged 5 min, 850 xg. The supernatant was poured off and the viscous pellet washed three additional times with 5 mL of chilled (4 ºC) sterile isotonic phosphate-buffered saline solution, adjusted to pH = 7.4, to stabilize the pH it was mixed for almost 0.5 h at room temperature. The washed cells were suspended in a final volume of 20 mL chilled, sterile phosphate-buffered saline and the cells counted on a haemacytometer.The blood cell suspension was maintained on wet ice and diluted with sterile phosphate-buffered saline to 7.068×10^8 cells mL^{-1} for each assay. Aliquots of 20 µL of plant extract/fractions were aseptically placed into 2.0 mL microfuge tubes. For each assay, 0.1 % Triton X-100 was the positive, 100 % lytic control and phosphate-buffered saline was the negative, background (0 % lysis) control. Aliquots of 180 µL diluted blood cell suspension were aseptically placed into each 2 mL tube and gently mixed three times with a wide mouth pipette tip. Tubes were incubated for 35 min at 37 ºC. Immediately following incubation, the tubes were placed on ice for 5 min then centrifuged for 5 min at 1310 xg. Aliquots of 100 µL of supernatant were carefully collected, placed into a sterile 1.5 mL microfuge tube and diluted with 900 µL chilled, sterile phosphate-buffered saline. All tubes were maintained on wet ice after dilution. Then 200 µL into 96 well plates and three replicates was taken in well plate which contain one positive control (100 % of blood lysis) and other negative control (0 % of blood lysis). After this Absorbance at 576 nm was measured on a microquant. The experiment was done in triplicate. % haemolysis was calculated by following formula:

$$\text{\% Haemolysis} = [\text{As (sample absorbance)}/\text{Ac (control absorbance)}] \times 100$$

Determination of antioxidant efficacy using sunflower oil as oxidation substrate: The crude concentrated various extracts of the plant were separately added into the preheated (50 ºC) refined, bleached and deodorizer sunflower oil at concentration of 400 ppm (w/w). The oil samples were stirred

for 0.5 h at 50 ºC for uniform dispersion. All oil samples were separately stabilized and stored in 100 mL airtight bottle. A control sample was also prepared (without extract) under the same set of analytical conditions. Samples were stored at room temperature. Synthetic antioxidant (BHT) was employed at its legal limit of 200 ppm to compare the efficacy of extracts. Stabilized and control oil samples (100 mL) were placed in dark brown airtight glass bottles with narrow necks and subjected to accelerated storage in an electric hot air oven (IM-30, Irmeco, Germany) at 60 ºC for 28 days. All oil samples were prepared in triplicate. Oil samples were taken after every 7 days intervals.

Measurement of oxidation parameters of sunflower oil: The oxidative deterioration level was assessed by the measurement of peroxide value, free fatty acids conjugate dienes, conjugate trienes and *p*-anisidine values. Determination of the free fatty acids and peroxide value of stabilized and control sunflower oil samples were made following the AOCS official methods Cd 8-53 and F 9a-44 respectively[16]. The oxidation products such as conjugated dienes and conjugated trienes were analyzed by following the IUPAC method II D.23[17]. The absorbance was noted at 232 and 268 nm respectively. The determination of the *p*-anisidine value was made following an IUPAC method II. D. 26[17].

Statistical analysis: Each sample was analyzed individually in triplicate and data was reported as mean ($n = 3 \times 3 \times 1$) ± standard deviation ($n = 3 \times 3 \times 1$). Data were analyzed by analysis of variance (ANOVA) using costat statistical software.

RESULTS AND DISCUSSION

Antioxidant analyses: The yield of plant leaves extracts were found within the range of 2.2-3.8 g/100 g. The maximum yield of extract was obtained with absolute methanol (3.8 %) followed by chloroform (3.6 %), petroleum ether (3.5 %), M:W (9.5:0.5) (2.8 %), M:W (9:1) (2.6 %), ethyl acetate (2.3 %), *n*-butanol (2.2 %). The amount of total phenolic contents determined in plant leaves ranged from 2.28 to 6.24 GAE mg/g of dry leaves extracts (Table-1). Total phenolic contents obtained from absolute methanolic extract was found to be highest (6.24 GAE mg/g) while the lowest total phenolic contents was determined in the petroleum ether extract (2.28 GAE mg/g). The decreasing order of total phenolic contents found in different extracts of plant given as: Absolute methanol (6.24 mg/g) > chloroform (5.55 mg/g) > *n*-butanol (5.14 mg/g) > M:W (9.5:0.5) (4.38 mg/g) > M:W (9:1) (3.58 mg/g) > ethyl acetate (3.38 mg/g) > petroleum ether (2.28 mg/g). Thes phenolic compounds may contribute directly to antioxidative action. The antioxidant activity of phenolic compounds is mainly due to their redox properties which can play an important role in absorbing and neutralizing free radicals.

Total flavonoid contents in plant ranged from 2.3 to 4.5 CE mg/g of dry leaves extracts. However the total flavonoid content obtained in absolute methanol was found to be highest (4.5mg/g) and lowest total flavonoid content values was observed in petroleum ether extract (2.3 mg/g). Total flavonoid content found in different extracts of plant decreased in the following manner: Absolute methanol (4.5 mg/g) > chloroform (3.9 mg/g) > ethyl acetate (3.5 mg/g) > *n*-butanol (2.8 mg/g) > M:W (9:1) (2.6 mg/g) > M:W (9.5:0.5) (3.1 mg/g) > petroleum ether (2.3 mg/g). These differences in the amount of total phenolic contents and total Flavonoid content may be due to varied efficiency of the extracting solvents to dissolve endogenous compounds.

Stankovic and co-workers[18] reported the concentration of highest flavonoid compounds in methanolic extracts (5.2 mg/100 g) of *Teucrium montanum* leaves. While *n*-hexane extract (2.7 g/100 g) showed lowest concentration of flavonoids compounds. These results are in agreement with present results where methanol extracted highest total flavonoid content. The leaves extracts exhibited different radical scavenging activity having IC_{50} value 35.4-70.12 µg/mL. In DPPH free radical scavenging the maximum IC_{50} value was observed with petroleum ether (70.12 µg/mL) and absolute methanol showed the minimum IC_{50} value (35.4 µg/mL) (Table-1). The free radical scavenging activity of absolute methanol extract was superior to that of other solvent extracts. However, all extracts offered slightly less scavenging activity as compared to the synthetic antioxidant BHT (19.2 µg/mL). The nature and amount of secondary metabolites of the plant causes the variation in free radical scavenging ability. The least value of IC_{50} represents the better antioxidant and greater value for less antioxidant activity. The reducing potential of the leaves extracts measured for different concentration showed general increase in activity when concentration increased. Reducing potential of different extracts at 1 mg/mL ranged from 0.34 to 0.37 mg/mL (Fig. 2). The presence of phenolic compounds might be the reason for reducing power. From the literature reports[19,20] it's evident that the reducing power of bioactive compounds is associated with antioxidant activity. The results of this assay indicated that the plant part is a good source of antioxidants with high reducing power. Results of antioxidant analyses showed that the plant leaves containd good amounts of total phenolic contents and total flavonoid content and it also exhibited good scavenging IC_{50} and reducing potential. So it can be used as a source of antioxidants.

TABLE-1
ANTIOXIDANT ANALYSES OF *K. pinnata* LEAVES DIFFERENT EXTRACTS

Parameters	Yield (%)	TPC (GAE mg/g)	TFC (CE mg/g)	DPPH, IC_{50} (µg/mL)
Leaves extracts				
Petroleum ether	3.5 ± 0.03	2.28 ± 0.04	2.3 ± 0.03	70.1 ± 0.15
Chloroform	3.6 ± 0.04	5.55 ± 0.06	3.9 ± 0.04	63.5 ± 0.09
Ethyl acetate	2.3 ± 0.02	3.38 ± 0.05	3.5 ± 0.03	59.6 ± 0.10
n-butanol	2.2 ± 0.04	5.14 ± 0.05	2.8 ± 0.04	40.2 ± 0.07
Absolute methanol	3.8 ± 0.06	6.24 ± 0.10	4.5 ± 0.10	35.4 ± 0.06
M:W (9.5:0.5)	2.8 ± 0.05	4.38 ± 0.05	3.1 ± 0.05	40.6 ± 0.06
M:W (9:1)	2.6 ± 0.04	3.58 ± 0.06	2.6 ± 0.04	45.8 ± 0.05
BHT	-	-	-	19.2 ± 0.04

Values are mean ± SD of three separate experiments

Antioxidant potential of *K. pinnata* leaves extracts for stabilization of sunflower oil: The peroxide value of sunflower oil (SFO) stabilized with different extracts of plant at ambient storage at the concentration of 400 ppm was noted under accelerated storage. Peroxide value is usually used to evaluate the extent of primary oxidation products in oils. A typical

pattern in the rise of peroxide value was observed for almost all sunflower oil treatments (Fig. 3). The control showed the highest peroxide value of all the oil treatments, showing a highest degree of oxidation. A significantly lower peroxide value of solvent extracts in petroleum ether, chloroform, ethyl acetate, *n*-butanol, Absolute methanol, M:W (9.5:0.5) and M:W (9:1) compared with of control clearly indicates good antioxidant activity of *K. pinnata* leaves extracts. Absolute Methanolic extract showed the minimum peroxide value value indicated by the slow rice in formation of hydroperoxide products. Petroleum ether showed maximum value after control. The range of peroxide value value lies between 3.35 to 14.98 meq/kg. The BHT had lowest peroxide value value. All used extracts of the plant controlled peroxide value appreciably; revealing good antioxidant efficacy of extracts in stabilization of oil.

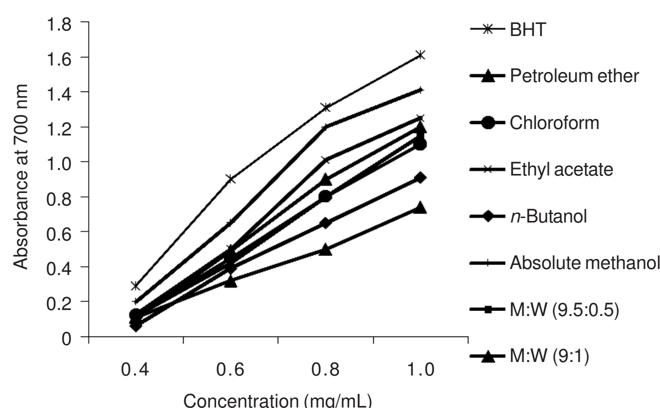

Fig. 2. Reducing power of *K. pinnata* leaves extracts

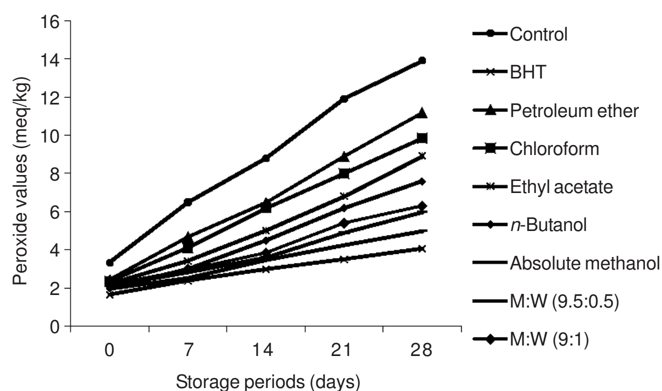

Fig. 3. Peroxide values of sunflower oil stabilized with *K. pinnata* leaves extracts

Formation of free fatty acids might be an important measure of rancidity of foods. Free fatty acids are formed due to hydrolysis of triglycerides and may get promoted by reaction of oil with moisture[21]. The range of free fatty acids value lies between 0.06 to 1.12 (Fig. 4). The amounts of free fatty acids in methanolic extract ranged from 0.02 to 0.30 and in petroleum ether extract value ranged from 0.04 to 0.85. Control exhibited the highest free fatty acids, while Sunflower oil stabilized with BHT exhibited least (Fig. 4). Initially, there was no increase in free fatty acid of stabilized oil samples but, after 7 days of storage, an increase was observed showed the free fatty acid contents of oil samples stabilized with leaves extract of *K. pinnata* under ambient storage conditions. All the oil samples stabilized with plant extracts were found to show a slow, followed by a gradual increase in free fatty acid contents. The lower values of free fatty acid contents of stabilized oil sample than control indicated the effectiveness of leaves extracts as natural antioxidant in retarding the free fatty acid contents. The formation of conjugated dienes (Fig. 5) and trienes (Fig. 6) analyzed for the control and stabilized sunflower oil respectively. Highest contents were observed for control, indicating greater intensity of oxidation. The determination of conjugate dienes and conjugated trienes is a good measure of the oxidative state of oils[22] and thus a good indicator of effectiveness of antioxidants. Conjugate dienes and conjugate trienes contents went on increasing with the increase in storage time. A slow increase in conjugate dienes and conjugate trienes of the stabilized sunflower oil as compared with those of the control indicated the antioxidant potential of the *K. pinnata* leaves. Stabilized sunflower oil conjugated diene and triene contens values were found to be in range of 2.95 to 14.85, 1.9 to 4.65 (ε1cm 232 nm) respectively. Absolute methanol extract showed the lowest values and petroleum ether extract exhibited highest.

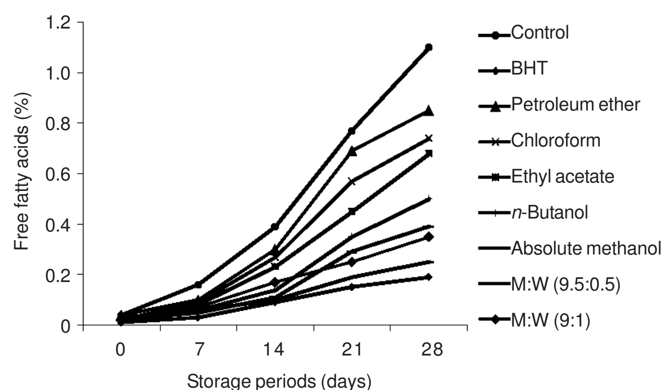

Fig. 4. Free fatty acid contents (%) of sunflower oil stabilized with *K. pinnata* leaves extracts

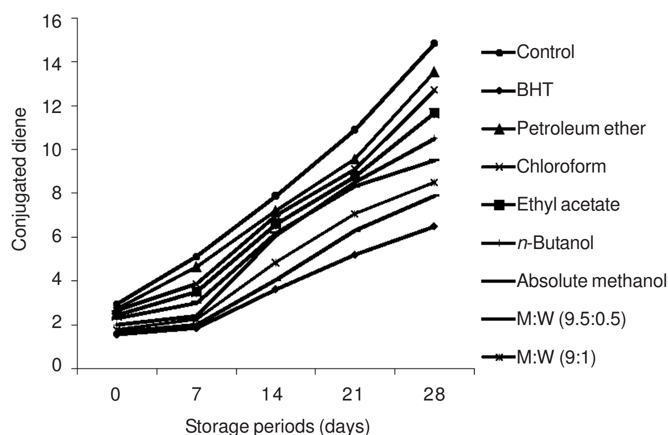

Fig. 5. Relative increase in conjugated dienes content of sunflower oil stabilized with *K. pinnata* leaves extracts

The results for *p*-anisidine values which usually determines the amount of aldehyde in oils presented in Fig. 7. The control sample showed the maximum increase in *p*-anisidine values indicating a higher rate of secondary product formation. A slow increase in PAV of stabilized sunflower oil as compared

with the control indicating the antioxidant potential of the plant leaves. A decreasing order of stability of oil treated with different extracts of plant regarding *p*-anisidine values was found to be: BHT > absolute methanol > M:W (9:1) > M:W (9.5:0.5) > *n*-butanol > ethyl acetate > chloroform > petroleum ether > control. All extracts played a prominent role for stabilization of sunflower oil but after standard BHT, methanol extract was most efficient to stabilize the oil.

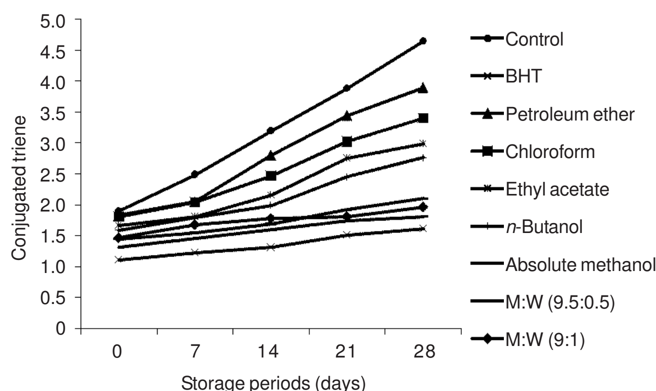

Fig. 6. Relative increase in conjugated trienes content of sunflower oil stabilized with *K. pinnata* leaves extracts

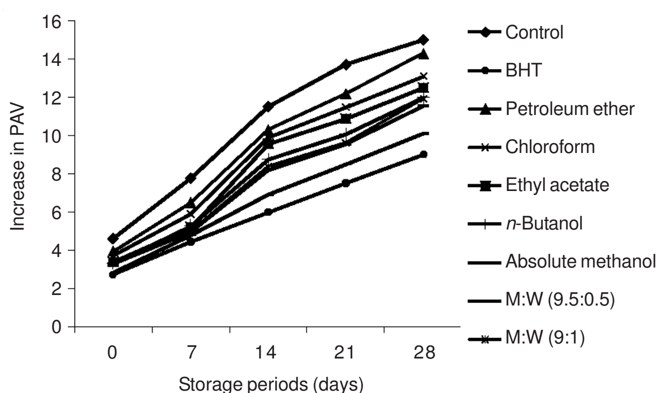

Fig. 7. Relative increase in *p*-ansidine values of sunflower oil stabilized with *K. pinnata* leaves extracts

Haemolytic activity of *K. pinnata* leaves: Haemolytic activity was analyzed against human red blood cells using Triton X-100 as positive control. The % lysis of red blood cells caused by the plant extracts was observed (Table-2). M:W (9.5:0.5) extract showed highest haemolytic effect (4.96 %) followed by chloroform (3.07 %), petroleum ether (2.60 %), Absolute methanol (2.57 %), *n*-butanol (2.41 %), ethylacetate (1.29 %), M:W (9:1) (0.82 %), extracts respectively. The mechanical stability of the erythrocytic membrane is a good indicator of the effect of various *in vitro* studies by various compounds for the screening of cytotoxicity. The percentage lysis of human erythrocytes was below 5.0 % for all samples. All these results were in safe range. Though it can be expected that the plant extracts have a minor cytotoxicity[15,23]. So pharmacologically this plant may be safe to use for human beings as a source of potential drug.

TABLE-2
HAEMOLYSIS (%) CAUSED BY *K. pinnata* LEAVES DIFFERENT EXTRACTS[a]

Leaves Extracts	Haemolysis (%)
Petroleum ether	2.60 ± 0.01
Chloroform	3.07 ± 0.03
Ethyl acetate	1.29 ± 0.01
n-Butanol	2.41 ± 0.02
Absolute Methanol	2.57 ± 0.01
M:W (9.5:0.5)	4.96 ± 0.03
M:W (9:1)	0.82 ± 0.01
Phosphate Buffer Saline (PBS)	0.00
Triton X-100	99.8 ± 1.01

[a]Values are mean ± SD of three separate experiments

Conclusion

The results of the present study concluded that the plant possessed considerable antioxidant potential and it may also be used to stabilize the edible sunflower oil. Hence, the plant leaves investigated can be explored as a potential antioxidant source of natural origin. Haemolytic activity of plant extracts against human erythrocytes was checked and it was in safe range so the investigated plant may be safe use for pharmaceutical and natural therapies.

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