



IN-VITRO ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITY OF METHANOL EXTRACT OF *OXALIS CORNICULATA* LINN

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Received: 22 Oct, Revised and Accepted 28 Nov 2009

ABSTRACT

Methanol extract of whole plant of *Oxalis corniculata* Linn (Family: Oxalidaceae) was assessed for its antioxidant and anti-inflammatory activity by *in-vitro* methods. Antioxidant activity was studied using 1, 1-Diphenyl-2-Picrylhydrazyl (DPPH) and nitric oxide radical scavenging activity. Inhibition of lipid peroxidation was studied by thiobarbituric acid reactive substances (TBARS) method on isolated rat liver tissues. Quantitative analysis of antioxidative components like total amount of phenolics, flavonoids and flavonols were estimated using spectrophotometric method. *In-vitro* anti-inflammatory activity was evaluated using albumin denaturation assay, membrane stabilization assay and proteinase inhibitory activity at different concentrations. Aspirin was used as a standard drug for the study of anti-inflammatory activity. Linear regression analysis was used to calculate IC₅₀ value. Results showed that, the extract exhibited significant DPPH and nitric oxide radical scavenging activity with IC₅₀ value of 302.93±4.17 and 73.07±8.28µg/ml respectively. Lipid peroxidation induced by the Fe²⁺, was inhibited by the extract with IC₅₀ value 58.71±2.55µg/ml. Total phenol content was estimated as 25.62±0.10mg of gallic acid equivalents of dry extract. Total flavonoids and flavonols were found to be 150.88±12.61 and 150.16±2.16 mg of rutin equivalents per gram of dry extract respectively. Extract also showed *in-vitro* anti-inflammatory activity by inhibiting the heat induced albumin denaturation and Red Blood Cells membrane stabilization with the IC₅₀ values of 288.04±2.78 and 467.14±9.56µg/ml respectively. Proteinase activity was also significantly inhibited by the extract (IC₅₀=435.28±5.82µg/ml). From the results, it is concluded that flavonoids and related polyphenols present in the *O. corniculata* extract may be responsible for the activity.

Keywords: *Oxalis corniculata*, radicals, antioxidant, anti-inflammatory

INTRODUCTION

It is commonly accepted that, in a situation of oxidative stress, reactive oxygen species, such as superoxide (O₂⁻), hydroxyl (·OH) and peroxy (·OOH, ROO·) radicals are generated. The reactive oxygen species play an important role related to the degenerative or pathological processes of various serious diseases such as ageing¹, cancer, coronary heart disease, Alzheimer's disease^{2,3}, neurodegenerative disorders, atherosclerosis, cataracts and inflammation⁴.

In many inflammatory disorders there is excessive activation of phagocytes, production of O₂⁻, ·OH radicals as well as non-free radical species (H₂O₂)⁵ which

can harm surrounding tissue either by powerful direct oxidizing action or indirectly with hydrogen peroxide (H₂O₂) and ·OH radicals formed from O₂⁻, which initiates lipid peroxidation resulting in membrane destruction. Tissue damage then provokes inflammatory response by production of mediators and chemotactic factors⁶. The reactive oxygen species are also known to activate matrix metalloproteinase (e.g. collagenase) causing increased destruction of tissues e.g. collagenase damage seen in various arthritic reactions⁷.

Hence, the agents that can scavenge these reactive oxygen species can be beneficial in the treatment of inflammatory disorders. *Oxalis*

corniculata Linn is a small procumbent herb, with stems rooting and pubescent with appressed hairs, leaves palmately 3-foliolate. It is commonly known as 'Indian sorrel' in English and 'Tinpatiya' in Hindi. This plant is well known for its medicinal value as a good appetiser and as a remover of kapha, vata and piles. In Indian traditional medicine, it is used as anti-inflammatory, digestive, diuretics, antibacterial, antiseptic, cardiopathy and hepatopathy. It is also known to cure dysentery, diarrhea and skin diseases^{8,9}.

It showed the presence of glyoxylic acid, oxalic acid, pyruvic acid, vitexin and isovitexin, vitexin-2-O-beta-D-glucopyranoside, neutral lipids, glycolipids; vitamin C; phospholipids; fatty acids, 18:2, 18:3, 16:0; saturated (C10-C14) acids; alpha- and beta-tocopherols; 2-heptenal; 2-pentylfuran; trans-phyto (3,7,11,15-tetramethyl-2-hexadecene-1-ol)¹⁰⁻¹².

It is reported to exhibit hypoglycemic, antihypertensive, uterine relaxant, antipsychotic, CNS-stimulant, antiyeast, smooth muscle relaxant, chronotropic and inotropic effect¹³⁻¹⁵ and antibacterial activity¹⁶.

Although *O. corniculata* is widely used in ethnomedicine for the treatment of inflammatory and related disorders, its antioxidant and anti-inflammatory properties have not yet been pharmacologically evaluated. Hence, the present study was undertaken to evaluate antioxidant and anti-inflammatory activity of *O. corniculata* Linn by *in-vitro* methods.

MATERIALS AND METHODS

Plant material

The fresh whole plant of *O. corniculata* Linn was collected from in and around the city of Mumbai, Maharashtra and authenticated by Dr. Ganesh Iyer, Botanist at Ramnarayan Ruia College,

Mumbai. A voucher specimen (2008/11/08) has been kept in our laboratory for future reference.

Preparation of plant extract

The whole plant of *O. corniculata* was shade dried, powdered and defatted using petroleum ether (60-80°C) and successively extracted with methanol. Extract was filtered through vacuum filter and the filtrate was concentrated in vacuum evaporator. Dried extract was used for the further study.

Experimental animals

Male Sprague-Dawley rats (200–250g) were procured from Glenmark Pharmaceuticals, Mhape, Navi Mumbai. Animals were placed in polypropylene cages in a controlled room temperature 22±1°C and relative humidity of 60–70% in registered animal house (87/1999/CPCSEA). They were maintained with standard pellet diet (Amrut brand, Sangli, India) and water *ad libitum*. Animals were acclimatized to laboratory conditions for seven days before commencement of experiment. Ethical clearance was obtained from Institutional Animal Ethical Committee.

Chemicals and reagents

1, 1-Diphenyl-2-Picrylhydrazyl (DPPH) was procured from Sigma Aldrich (St. Louis, MO, USA). Sodium nitroprusside (SNP) was purchased from Merck Ltd. India, Mumbai. Orthophosphoric acid (H₃PO₄), pyridine, perchloric acid, ferrous sulfate, sodium dodecyl sulfate (SDS), sodium nitrite (NaNO₂), aluminium chloride (AlCl₃) and Folin Ciocalteu's reagents were purchased from S D fine Chem. Ltd, Mumbai. Thiobarbituric acid (TBA) and bovine albumin fraction were procured from Central Drug House Pvt. Ltd, New Delhi. Casein and trypsin were procured from Hi media Lab. Ltd, Mumbai. N-1 Naphylethylenediamine dihydrochloride was obtained from LOBA CHEME Pvt.

Ltd. Mumbai. All the other chemicals and reagents were of pure analytical grade and obtained from local supplier.

Phytochemical evaluation

Methanol extract of *O. corniculata* was studied for its phytoconstituents such as alkaloids, steroid and/or triterpenoids and their glycosides, tannins, flavonoids and their glycosides, carbohydrates and cardiac glycosides using different phytochemical tests¹⁷.

Assessment of *in-vitro* antioxidant activity

1, 1-Diphenyl-2-picrylhydrazyl radical scavenging activity

The ability of the extract to scavenge DPPH radicals were determined by the method of *Gyamfi et al*¹⁸ with minor modifications. A 0.5 ml of aliquot of test extract at different concentrations in methanol was mixed with 0.5 ml of 100 mM methanolic solution of DPPH. After 30 min incubation in darkness and at ambient temperature, the resultant absorbance was recorded at 517 nm. The percentage inhibition was calculated using the following formula.

$$\text{Percentage inhibition} = \frac{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})}{\text{Abs}_{\text{control}}} \times 100$$

IC₅₀ values were calculated as the average of triplicate analyses.

Determination of nitric oxide radical scavenging activity

The compound SNP is known to decompose in aqueous solution at physiological pH (7.2) producing nitric oxide radicals (NO·). Under aerobic conditions, NO· reacts with oxygen to produce stable products (nitrate and nitrite). The quantities of which can be determined using Griess reagent. The scavenging effect of the plant extract on nitric oxide was measured according to the modified method of *Marcocci et al*¹⁹. 1 ml of extract solution at different

concentrations was added in the test tubes to 1 ml of SNP solution (100 mM) and the tubes were incubated at 29°C for 2.5 h. An aliquot of 1 ml of the incubation solution was removed and diluted with 1 ml of Griess reagent (1% Sulfanilamide in 2% H₃PO₄ and 0.1 % N-1-Naphthylethylenediamine dihydrochloride). The absorbance of the chromophore that formed with Naphthylethylenediamine dihydrochloride was immediately read at 540 nm. The percentage inhibition was calculated using the formula mentioned above.

Inhibition of lipid peroxidation by TBARS method

Lipid peroxide formation was measured (lipid peroxidation assay) by the modified method of *Ohkawa et al*²⁰ and *Masao et al*²¹. Male Sprague-Dawley rat (weighing 200-250 g) was sacrificed by dislocation of the neck. The abdomen was opened; the liver was removed and then homogenized in phosphate buffer saline (pH 7.0). 1 ml of liver homogenate (10%, w/v) was added to the test extract of different concentrations. The lipid peroxidation was initiated by adding 100 µl of 15 mM FeSO₄ solution. After 30 min of incubation at room temperature, 0.1 ml of reaction mixture (liver homogenate + test drug) was taken in a tube containing 0.1 ml of SDS (8.1%w/v), 0.75 ml of 20% acetic acid and 0.75 ml of 0.8% TBA solution. The volume in each tube was made upto 2 ml with distilled water and then heated on water bath at 95°C for 60 minutes. After 60 minutes, the volume in each tube was made upto 2.5 ml and then 2.5 ml of N-butanol: pyridine (5:1) was added in each tube. The reaction mixture was vortexed and centrifuged at 4000 rpm for 10 minutes. The organic layer was removed and absorbance was read at

532 nm in a UV spectrophotometer. The experiment was performed in triplicate. The percentage inhibition was calculated using the formula mentioned above.

Quantitative analysis of antioxidative components

Determination of total phenolics, flavonoids and flavonols

Total phenolics content was determined according to the method of *Hammerschmidt et al* ²². Briefly, 0.2 ml of the test solution was mixed with 1 ml of 10% Folin-Ciocalteu solution and 0.8 ml of 7.5% sodium carbonate solution. The mixture was incubated for 1 h at room temperature. The absorbance at 760 nm was measured and converted to phenolic contents according to the calibration curve of gallic acid.

Total flavonoids content was estimated colorimetrically based on the method modified by *Zhishen et al* ²³. To 0.1 ml of test extract (10 mg/ml) in a 10 ml volumetric flask, distilled water was added to make the volume to 5 ml and 0.3 ml 5% NaNO₂ was added to this. 3 ml of 10% AlCl₃ was added 5 minutes later. After 6 minutes, 2 ml of 1M sodium hydroxide was added and the absorbance was measured at 510 nm. Rutin was used as a standard for constructing a calibration curve.

Total flavonols were estimated as rutin equivalents, expressed as mg of rutin per gram of dry extract by the method of *Miliauskas et al* ²⁴. The rutin calibration curve was prepared by mixing rutin solution with 2 ml (20 gm/l) AlCl₃ and 6 ml (50 gm/l) sodium acetate. The absorption at 440 nm was read after 2.5 h at 20°C. The same procedure was carried out with 2 ml of plant extract

(10 gm/l) instead of rutin solution. All determinations were carried out in triplicate and the mean values were used.

Assessment of *in-vitro* anti-inflammatory activity

Inhibition of albumin denaturation

Method of *Mizushima et al* ²⁵ was followed with minor modifications. The reaction mixture was consisting of test extract at different concentrations and 1% aqueous solution of bovine albumin fraction. pH of the reaction mixture was adjusted using small amount of 1N HCl. The samples were incubated at 37°C for 20 min and then heated at 57°C for 20 min. After cooling the samples, the turbidity was measured spectrophotometrically at 660 nm. The experiment was performed in triplicate. Percent inhibition of protein denaturation was calculated as follows:

$$\text{Percentage inhibition} = (\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}) \times 100 / \text{Abs}_{\text{control}}$$

Membrane stabilization test

Preparation of Red Blood cells (RBCs) suspension

Fresh whole human blood (10 ml) was collected and transferred to the heparinized centrifuged tubes. The tubes were centrifuged at 3000 rpm for 10 min and were washed three times with equal volume of normal saline. The volume of the blood was measured and reconstituted as 10% v/v suspension with normal saline ²⁶.

Heat induced hemolysis

The reaction mixture (2 ml) consisted of 1 ml of test drug solution and 1 ml of 10% RBCs suspension, instead of drug only saline was added to the control test tube. Aspirin was taken as a standard drug. All the centrifuge tubes containing reaction mixture were incubated in a

water bath at 56°C for 30 min. At the end of the incubation, the tubes were cooled under running tap water. The reaction mixture was centrifuged at 2500 rpm for 5 min and the absorbance of the supernatants was taken at 560 nm. The experiment was performed in triplicates. Percent membrane stabilization activity was calculated by the formula mentioned above ²⁷.

Proteinase inhibitory action

The test was performed according to the modified method of *Oyedepo et al* ²⁸. The reaction mixture (2 ml) was containing 0.06 mg trypsin, 1 ml 20 mM Tris HCl buffer (pH 7.4) and 1 ml test sample of different concentrations. The mixture was incubated at 37°C for 5 min and then 1 ml of 0.8% (w/v) casein was added. The mixture was incubated for an additional 20 min. 2 ml of 70% perchloric acid was added to terminate the reaction. Cloudy suspension was centrifuged and the absorbance of the supernatant was read at 210 nm against buffer as blank. The experiment was performed in triplicate. The percentage inhibition of proteinase inhibitory activity was calculated.

Statistical analysis

The results are expressed as the mean±SD for three replicates. Linear regression analysis was used to calculate IC₅₀ value.

RESULTS AND DISCUSSION

Phytochemical analysis

The yield of the methanol extract of *O. corniculata* was found to be 10.16%. It showed the presence of carbohydrates and glycosides, phytosterols, phenolic compounds/tannins, flavonoids, proteins, amino acids and volatile oils are shown in Table 1.

In-vitro antioxidant activity

1,1-Diphenyl-2-picrylhydrazyl radical scavenging activity

1, 1-Diphenyl-2-picrylhydrazyl radical scavenging assay is the most widely used method for screening antioxidant activity, since it can accommodate many samples in a short period and detect active ingredients at low concentration ^{29, 30}. The decrease in absorbance of the DPPH radical caused by antioxidant was due to the scavenging of the radical by hydrogen donation. It is visually noticeable as a color change from purple to yellow. The methanol extract of *O. corniculata* showed DPPH radical scavenging activity in a concentration-dependent manner (Fig 1), with the correlation coefficient values (r) of 0.949 and IC₅₀ value of 302.93±4.17µg/ml.

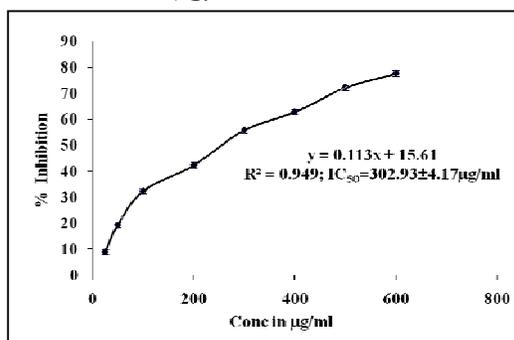


Fig. 1: Effect of methanol extract of *O. corniculata* on 1, 1-Diphenyl-2-picrylhydrazyl radical scavenging activity

Values represent in the figure are mean±SD of three replicates; linear regression analysis was used to calculate IC₅₀ value

Determination of nitric oxide radical scavenging activity

The effect of methanol extract of *O. corniculata* on nitric oxide radical scavenging activity is shown in Fig 2. The compound, SNP is known to decompose in aqueous solution at physiological pH (7.2) producing NO·. Under aerobic conditions, NO· reacts

with oxygen to produce stable products (nitrate and nitrite). This leads to reduction of nitrite concentration in the assay media ¹⁹. Here, methanol extract of *O. corniculata* exhibited potent nitric oxide radical scavenging activity with correlation coefficient values (r) of 0.909 and IC₅₀ value of 73.07±8.28µg/ml.

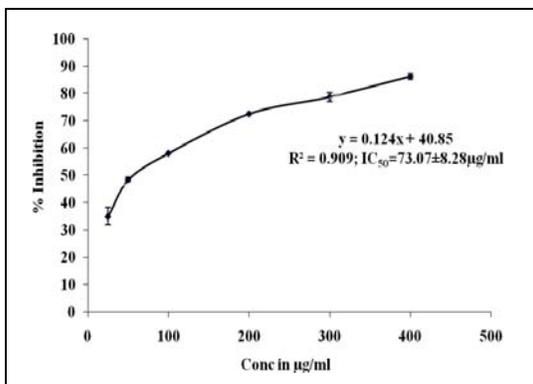


Fig. 2: Effect of methanol extract of *O. corniculata* on nitric oxide radical scavenging activity

Values represent in the figure are mean±SD of three replicates; linear regression analysis was used to calculate IC₅₀ value.

Inhibition of lipid peroxidation by TBARS method

Lipid peroxidation is a very important process in free radical pathology as it is damaging to cells. The liver of rat was used as a source of polyunsaturated fatty acids for determining the extent of lipid peroxidation. Malondialdehyde, a lipid peroxidation product, is an indicator of reactive oxygen species (ROS) generation in the tissue ³¹. The inhibition of lipid peroxide formation by methanol extract of *O. corniculata* is shown in Fig 3. It showed the maximum inhibition of peroxide formation with low IC₅₀ value of 58.71±2.55µg/ml at correlation coefficient value (r) of 0.968.

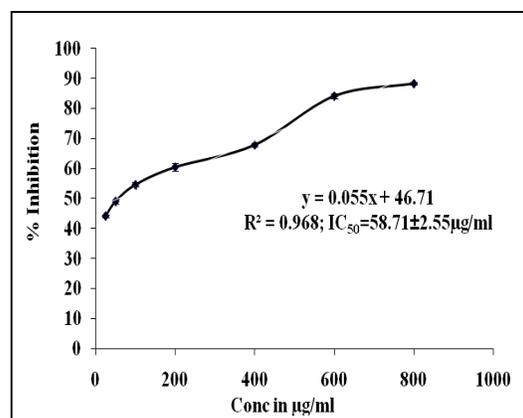


Fig. 3: Effect of methanol extract of *O. corniculata* on lipid peroxidation by TBARS method

Values represent in the figure are mean±SD of three replicates; linear regression analysis was used to calculate IC₅₀ value.

Quantitative analysis of antioxidative components

Determination of total phenolics, flavonoids and flavonols

The phenolic substances are known to possess the ability to reduce oxidative damage and act as antioxidants ³². They can trap the free radicals directly or scavenge them through a series of coupled reactions with antioxidant enzymes ³³. In addition, it was reported that phenolic substances were associated with antioxidant activity and played important role in stabilizing lipid peroxidation ³⁴. This activity is believed to be mainly due to their redox properties, which play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides ³⁵. Methanol extract of *O. corniculata* showed the total phenol contents of 25.62±0.10 mg of gallic acid equivalents per gram of dry extract. Total flavonoids and total flavonols were found to be 150.88±12.61 and 150.16±2.16 mg of rutin equivalents per gram of dry extract respectively (Table 1).

Table 1: Phytochemical and antioxidative components of the methanol extract of *O. corniculata*

Parameters studied	Methanol extract of <i>O. corniculata</i>
Extractive yield	10.16 %
Phytoconstituents	Carbohydrates and glycosides, phytosterols, phenolic compounds/tannins, flavonoids, proteins, amino acids and volatile oils
Total phenol content	25.62±0.10 ^a
Total flavonoids content	150.88±12.61 ^b
Total flavonols content	150.16±2.16 ^b

^a mg of Gallic acid equivalent per gram of extract, ^b mg of Rutin equivalent per gram of extract. Values represent in the results are mean±SD of three replicates

***In-vitro* anti-inflammatory activity**

Inhibition of albumin denaturation

Denaturation of proteins is a well-documented cause of inflammation. Phenylbutazone, salicylic acid, flufenamic acid (anti-inflammatory drugs) etc, have shown dose dependent ability to thermally induced protein denaturation ³⁶. As a part of the investigation on the mechanism of the anti-inflammatory activity, ability of extract to inhibit protein denaturation was studied. It was effective in inhibiting heat induced albumin denaturation at different concentrations as shown in Table 2. Maximum inhibition, 85.92±1.48% was observed at 800µg/ml. IC₅₀ value was found to be 288.04±2.78µg/ml at correlation coefficient value (r) of 0.946. Aspirin, a standard anti-inflammatory drug showed the maximum inhibition, 75.89±0.56% at the concentration of 200µg/ml.

Membrane stabilization test

Stabilization of the RBCs membrane was studied to further establish the mechanism of anti-inflammatory action of *O. corniculata*. The extract was effective in inhibiting the heat induced

hemolysis at different concentrations. These provide evidence for membrane stabilization as an additional mechanism of their anti-inflammatory effect. This extract may possibly inhibit the release of lysosomal content of neutrophils at the site of inflammation. These neutrophil lysosomal constituents include bactericidal enzymes and protease, which upon extracellular release cause further tissue inflammation and damage ³⁷. Test extract (200-800µg/ml) inhibited the heat induced hemolysis of RBCs to varying degree as shown in Table 2. It showed the maximum inhibition 75.79±0.55% at 800µg/ml. IC₅₀ was observed at 467.14±9.56µg/ml at correlation coefficient value (r) of 0.998. Aspirin, standard drug showed the maximum inhibition, 85.92±0.75% at 200µg/ml. Although the precise mechanism of this membrane stabilization is yet to be elucidated, it is possible that the *O. corniculata* produced this effect surface area/volume ratio of the cells, which could be brought about by an expansion of membrane or the shrinkage of the cells and an interaction with membrane proteins ²⁷.

Table 2: Effect of methanol extract of *O. corniculata* on albumin denaturation, membrane stabilization and proteinase inhibitory activity

Test Sample	Conc. (µg/ml)	% Inhibition		
		Albumin denaturation	Membrane stabilization	Proteinase inhibition
Methanol extract of <i>O. corniculata</i>	50	23.70±1.48	19.84±0.60	20.29±1.33
	100	33.82±1.13	22.60±0.68	22.79±0.67
	200	48.88±0.74	29.49±0.90	28.33±1.63
	400	65.43±1.13	43.69±1.32	42.70±0.82
	600	76.54±0.85	59.74±1.08	65.72±1.35
	800	85.92±1.48	75.79±0.55	81.49±0.40
Correlation coefficient value (r)	-----	0.946	0.998	0.988
IC ₅₀ value	-----	288.04±2.78	467.14±9.56	435.28±5.82
Aspirin	100	67.45±0.64	72.56±0.58	56.45±0.45
	200	75.89±0.56	85.92±0.75	92.87±0.76

Values represent in the results are mean±SD of three replicates; linear regression analysis was used to calculate IC₅₀ value

Proteinase inhibitory activity

Proteinases have been implicated in arthritic reactions. Neutrophils are known to be a rich source of proteinase which carries in their lysosomal granules many serine proteinases. It was previously reported that leukocytes proteinase play an important role in the development of tissue damage during inflammatory reactions and significant level of protection was provided by proteinase inhibitors³⁸. *O. corniculata* methanol extract exhibited significant antiproteinase activity at different concentrations as shown in Table 2. It showed maximum inhibition 81.49±0.40% at 800µg/ml. IC₅₀ value was found to be 435.28±5.82µg/ml at correlation coefficient value (r) of 0.988. Aspirin showed the maximum inhibition 92.87±0.76% at 200µg/ml.

Recent studies have shown that many flavonoids and related polyphenols contribute significantly to the antioxidant and anti-inflammatory

activity of many plants^{39, 40}. Hence, the presence of flavonoids in the methanol extract of *O. corniculata* may be contributed to its antioxidant and anti-inflammatory activity.

CONCLUSION

In conclusion, present study revealed the *in-vitro* antioxidant and anti-inflammatory activity of methanol extract of *Oxalis corniculata* Linn. The presence of flavonoids and related polyphenols may be responsible for the activity. Further investigations are required to find active component of the extract and to confirm the mechanism of action.

ACKNOWLEDGEMENTS

Authors are thankful to University Grants Commission, Govt. of India, New Delhi for financial support and Dr. Ganesh Iyer, Botanist, Ramnarayan Ruia College, Mumbai for authentication of the fresh whole plant of *Oxalis corniculata* Linn.

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