

## Original Research Article

# Evaluation of *In vitro* Antioxidant and *In vivo* Antihyperlipidemic Activities of Methanol Extract of Aerial Part of *Crassocephalum crepidioides* (Asteraceae) Benth S Moore

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### Abstract

**Purpose:** To evaluate the antioxidant and antihyperlipidemic activities of the methanol extract of aerial part of *Crassocephalum crepidioides* (Asteraceae) Benth. S. Moore.

**Methods:** The methanol extract of *Crassocephalum crepidioides* was prepared by organic solvent extraction (Maceration) and assessed for *in vitro* antioxidant activity using various methods, namely, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, total phenol and flavonoid content and reducing capacity. The extract was also evaluated for antihyperlipidemic activity in high-fat diet and triton WR-100 (iso-octyl polyoxyethylene phenol)-induced hyperlipidemic albino rats by evaluating serum total cholesterol (TC), high-density lipoprotein cholesterol (HDL-c), triglycerides (TG), very low density lipoprotein cholesterol (VLDL-c), low-density lipoprotein cholesterol (LDL-c) level and atherogenic index (AI).

**Results:** The plant extract showed significant antioxidant activity in a dose-dependent manner. In high-fat diet hyperlipidemic rats, the extract significantly reduced ( $p < 0.01$  or  $0.001$ ) serum TC, TG, LDL-c and VLDL-c levels, but significantly increased ( $p < 0.05$ ) serum HDL-c level. In triton-induced hyperlipidemic rats, the extract significantly reduced ( $p < 0.05$ ,  $p < 0.01$ ,  $p < 0.001$ ) serum TC, TG, LDL-c, VLDL-c levels but significantly increased ( $p < 0.05$ ) serum HDL-c level. The extract showed significant reduction ( $p < 0.01$ ) in AI at 300 mg /kg/day dose in both high fat-induced ( $1.70 \pm 0.25$ ) and triton-induced ( $1.5 \pm 0.17$ ) hyperlipidemic albino rats.

**Conclusion:** Based on these findings, the extract of *Crassocephalum crepidioides* aids in lowering hyperlipidemia in rats. This is probably due to its antioxidant activity.

**Keywords:** Antioxidant, Antihyperlipidemic, *Crassocephalum crepidioides*, Triton WR-100, Atherogenic index

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## INTRODUCTION

Reactive oxygen species (ROS), such as superoxide anions, hydrogen peroxide, and hydroxyl, nitric oxide and peroxy nitrite radicals, play an important role in oxidative stress related

to pathogenesis of various important diseases [1]. The body possesses several defense systems comprising enzymes and radical scavengers [2]. Some of them constitute the repair systems for biomolecules that are damaged by the attack of free radicals [3].

Antioxidants from plant materials terminate the action of free radicals, thereby protecting the body from various diseases [4].

Endothelial cells, smooth muscle cells, and macrophages are the sources of oxidants for the oxidative modification of phospholipids. Oxidized-LDL can damage endothelial cells and induce the expression of adhesion molecules such as P-selectin and chemotactic factors such as monocyte chemoattractant protein-1 and macrophage colony stimulating factor. These processes lead to the tethering, activation, and attachment of monocytes and T- lymphocytes to the endothelial cells [5,6]. There is evidence that macrophages in atherosclerotic lesions express myeloperoxidase that yields a unique pattern of protein oxidation products. Myeloperoxidase is also pinpointed as a pathway that promotes LDL oxidation [7]. Natural products with antioxidant potential have been proposed as effective in the treatment of metabolic syndrome like insulin resistance, diabetes, obesity, altered lipid profile, and hypertension [8-10]

The genus *Crassocephalum* is in the family Asteraceae (Compositae) in the major group Angiosperms (Flowering plants). Its fleshy, mucilaginous leaves and stems are commonly eaten, and many parts of the plant have medicinal uses [11]. Moreover, *Crassocephalum crepidioides* has been reported as effective in the treatment of indigestion, stomach ache, epilepsy, sleeping sickness and swollen lips, and also possesses antitumor activity associated with nitric oxide production [12]. It was also found that it is a potent antioxidant, and protects against hepatotoxicity [13]. Despite the long traditional use of this plant in the treatment of various pathological conditions, no systemic phytochemical and pharmacological works have been carried out on this potential medicinal plant. Therefore, the aim of the present study is to identify antioxidants and antihyperlipidemic effects of methanol extract of *Crassocephalum crepidioides*.

## EXPERIMENTAL

### Plant material

*Crassocephalum crepidioides* was collected from Fatickchari, Chittagong in October 2013. Taxonomic identification of this plant was made by Dr. Shaikh Bokhtear Uddin, Associate Professor, Department of Botany, University of Chittagong (CU), Bangladesh. A voucher specimen (no. SUS 5396) was deposited in the

herbarium of Department of Botany, University of Chittagong for preservation.

### Preparation of extracts

The plant was washed, shade dried and powdered in a heavy-duty Willy mill (Bells India Ltd.), and then 500 g of dried powder was soaked in 2500 mL of methanol. After 15 days, the whole mixture was filtered through cotton wool and the filtrate was concentrated under reduced pressure using a rotatory evaporator method. The yield of extract was 43 %. The extract was stored in the refrigerator at 4 °C until further use. The extract was dissolved in 1 % carboxyl-methyl cellulose used for the animal studies [14].

### Preliminary phytochemical screening of methanolic plant extract

The methanol extract of *Crassocephalum crepidioides* was subjected to preliminary phytochemical screening for the presence of alkaloids, glycosides, cardiac glycosides, steroids, coumarin, tannins, flavonoids, saponins and reducing sugar [15,16]. The color intensity or the precipitate formation was used as analytical responses to these tests.

### Experimental animal

Wistar albino rats of either sex, aged 8 - 10 weeks, weighing 120 - 250 g each obtained from the animal house of Jahangirnagar University were used for the experiment. They were kept in clean and dry polypropylene cages with 12 h light, dark cycle at 25 ± 2 °C and 45 - 55 % relative humidity in the animal house. The rats were fed with a standard laboratory diet and water *ad libitum*. Food was withdrawn 12 h prior to and during the experiment. As these animals are very sensitive to environmental changes, they are kept before the test for at least 3 to 4 days in the environment where the experiment will take place [17]. The protocol used in this study in the rat as an animal model for diabetic and antihyperlipidemic research was carried out based on the guidelines of the Institutional Animal Ethics Committee (IAEC).

### Acute toxicity studies

*Crassocephalum crepidioides* in the dose range of 100 - 2500 mg/kg was dissolved in 1 % carboxyl-methyl cellulose and administered orally, each group comprised of five rats. Mortality was observed after 72 h. Acute toxicity was determined according to the method of Litchfield and Wilcoxon [18].

### Determination of DPPH scavenging activity

The assay was conducted on the basis of scavenging activity of the stable DPPH free radical following a previously described method with some minor modifications [19,20]. To 1 ml of the sample (5, 10, 20, 30, 40, and 50 µg/mL) was added 3 ml of a 0.1 mmol/L methanol solution of DPPH. The absorbance of all the samples was determined at 517 nm (Shimadzu-1800, Japan) after an incubation period of 30 min. Inhibition of DPPH (D) was determined according to Eq 1.

$$D (\%) = \{(Ac - As)/Ac\}100 \dots\dots\dots (1)$$

where Ac and As are the absorbance of control and test samples, respectively.

### Evaluation of total phenolic content

The total phenolic content of the extract of *Crassocephalum crepidioides* was determined using the Folin-Ciocalteu method [21]. The extract was oxidized with Folin-Ciocalteu reagent and was neutralized with sodium carbonate. The absorbance of the resulting blue color solution was measured at 760 nm after 60 min using gallic acid (GA) as standard. Total phenolic content was expressed as mg GA equivalent/gm of extract.

### Assessment of total flavonoid content

The flavonoid content was determined using a method described by Karunakaran *et al* with quercetin as reference standard [22]. The extract (1 mg) was mixed with 1 ml of aluminium trichloride in ethanol (20 mg/mL), and then one drop of acetic acid was added. The mixture was then diluted up to 25 mL with ethanol. The absorbance at 415 nm was read after 40 min. The absorbance of blank samples and standard quercetin solution (0.5 mg/mL) in methanol was measured under the same condition.

### Reducing power assay

The assay was conducted according to a previously reported method [23,24]. According to this method, the reduction of Fe<sup>3+</sup> to Fe<sup>2+</sup> was determined by measuring absorbance of Pearl's Prussian blue complex. This method is based on the reduction of (Fe<sup>3+</sup>) ferricyanide in stoichiometric excess relative to the antioxidants. To different concentrations of the extracts (125, 250, 500 and 1000 µg/mL) were added 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1 % potassium ferricyanide [K<sub>3</sub>Fe(CN)<sub>6</sub>]. The mixture was incubated at 50 °C for 20 min and 2.5 mL of 10 % trichloroacetic acid was added to

the mixture and centrifuged at 3000 rpm for 10 min. The upper layer (2.5 mL) was added to (2.5 mL) distilled water and FeCl<sub>3</sub> (0.5 mL, 0.1 %), and the absorbance was measured at 700 nm.

### High fat induced hyperlipidemia model

The method of Blank *et al* [25] with modification was used to produce high fat diet induced hyperlipidemia. Normal food pellets were crushed into small pieces using a mortar and pestle and then ground into a fine powder in a mixer grinder (Philips HL 7720). The other ingredients i.e. cholesterol 2 %, cholic acid 1 %, sucrose 40 %, and coconut oil 10 % were added in the mixer grinder to make small balls of feed and later this was stored in self-sealing plastic cover in refrigerator at 2 to 8 °C.

The feed for normal group was prepared similarly by grinding only the normal food pellets and then mixing with water without the other excipients [26-28]. This of feed was done once in three days for all the animals. The animals were fed with the high fat diet for 30 days and the serum blood cholesterol level was routinely checked.

### Experimental design for high fat induced hyperlipidemic rats

Wistar rats weighing 150-180 g were divided into 5 groups of 5 animals each.

*Group 1:* Served as normal control and were given only vehicle (distilled water)

*Group 2:* Received high fat diet served as hyperlipidemic control (positive control)

*Group 3:* Received 10 mg/kg/day atorvastatin served as standard

*Group 4:* Received 150 mg/kg/day plant extract

*Group 5:* Received 300 mg/kg/day plant extract

After treatment for 14 days with the test sample, on the 15th day rats were kept fasting and blood was collected by retro orbital sinus puncture, under mild ether anesthesia. The collected samples were centrifuged for 30 min at 2000 rpm and serum sample so collected were used for various biochemical tests.

### Triton X-100-induced hyperlipidemia model

Twenty five Wistar rats were randomly divided into 5 groups of 5 each. The first group was given a standard pellet diet, water and orally

administered with 5 % carboxy methyl cellulose (CMC). Groups 2, 3, 4 and 5 were injected (i.p.) with 10 % aqueous solution of Triton X-100 mg/kg body weight. After 72 h of triton injection, the second group received a daily dose of 5 % CMC (p.o.) for 7 days, groups 4 and 5 were received a daily dose of plant extract 150 mg/kg and 300 mg/kg in 5 % CMC (p.o) for 7 days and group 3 was administered with the standard atorvastatin 10 mg/kg (p.o) for 7 days. Food was withdraw 10 h prior to blood sampling.

### Experimental design for triton X-100 induced hyperlipidemic rats

*Group 1:* Administered vehicle and serve as normal control.

*Group 2:* Administered Triton X (TR) and served as hyperlipidemic control (positive control)

*Group 3:* Administered atorvastatin (10 mg/kg/day, p.o.)

*Group 4:* Administered plant extract (150 mg/kg/day, p.o.)

*Group 5:* Administered plant extract (300 mg/kg/day, p.o.)

On the 8th day, blood was collected from rats by retro-orbital sinus puncture, under ether anesthesia. The collected samples were centrifuged for 15 min at 2500 rpm. Thereafter, serum samples were collected and determined TC, TG and HDL-c with the appropriate kits (Randox Laboratories Limited, Crumlin, UK). VLDL-c and LDL-c were calculated as in Friedewald's relationships (Eqs 2 and 3) [30].

$$\text{VLDL-c} = (\text{TG})/5 \dots\dots\dots (2)$$

$$\text{LDL-c} = \text{TC} - (\text{HDL-c} + \text{VLDL-c}) \dots\dots\dots (3)$$

Atherogenic index was calculated as in Eq 4 (Schulpis' equation) [31].

$$\text{Atherogenic index (AI)} = (\text{TC} - \text{HDL-c})/\text{HDL-c} \dots\dots (4)$$

### Statistical data analysis

All the data are expressed as mean  $\pm$  standard deviation (SD) and were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's test using SPSS software (version16).  $p < 0.05$  was considered statistically significant.

## RESULTS

### Phytochemical profile

The qualitative phytochemical profile of the extract shows the presence of alkaloids, glycosides, tannins, flavonoids, saponins and reducing sugar (Table 1).

### Acute toxicity

The plant extract was safe up to a dose of 2500 mg/kg of body weight. The behavior of the animals was observed for the first 8 h, then at an interval of every 8 h during next 72 h. The extract did not produce a significant change in animal behavior or mortality.

### DPPH scavenging activity

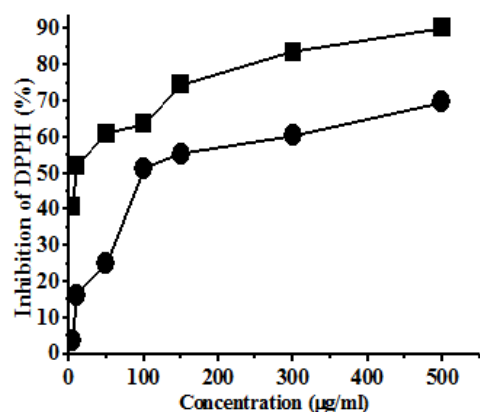
The DPPH radical scavenging activity of methanol extract of *Crassocephalum crepidioides* was found to increase with increasing concentration of the extracts (Figure 1). The DPPH radical contains an odd electron, which is responsible for absorbance at 515-517 nm and also for a visible deep purple color.

The IC<sub>50</sub> value of the extract was 130.320  $\mu\text{g/mL}$  while the IC<sub>50</sub> of ascorbic acid was 11.24  $\mu\text{g/mL}$ . These results indicate significant antioxidant activity of methanol extract of *Crassocephalum crepidioides*.

**Table 1:** Qualitative phytochemical profile of methanol extract of *Crassocephalum crepidioides*

Examination	Test performed	Inference
Alkaloids	Wagner's test	+++
Glycosides	General test	+++
Cardiac glycosides	Keller-killiani test	$\pm$
Steroids	Salkowski's test	$\pm$
Coumarin	General	++
Tannins	Ferric chloride test	++
Flavonoids	Conc. HCl & alcoholic test	++
Saponins	Shake test (aq. solution)	+
Reducing sugar	Fehling's test	++

**Key:** + = weak intensity reaction, ++ = medium intensity reaction, +++ = strong intensity reaction,  $\pm$  = present/absent, Conc. = concentrated, aq = Aqueous

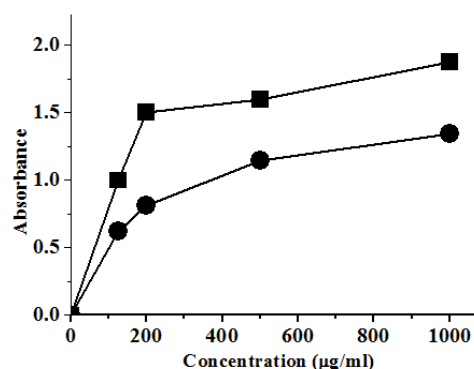


**Figure 1:** DPPH Scavenging activity of methanol extract of *Crassocephalum crepidioides* compared with ascorbic acid as standard. **Key:** ■ = Ascorbic acid, ● = Methanol extract

#### Total phenol and flavonoid content

Total phenol and flavonoid content of methanol extract of the aerial part of *Crassocephalum crepidioides* were expressed in gallic acid quercetin equivalents respectively. The phenolic content of the extract under this investigation correlates with their antioxidant activity ( $128.016 \pm 0.056$  mg GAE/g). Flavonoid content of the extract was also found to be significant compared to standard ( $168.33 \pm 0.061$  mg QE/g).

#### Reducing power assay



**Figure 2:** Reducing power of methanol extract of *Crassocephalum crepidioides* compared with ascorbic acid as standard. **Key:** ■ = Ascorbic acid, ● = Methanol extract

The methanol extract of *Crassocephalum crepidioides* showed reductive capabilities compared to ascorbic acid, which was determined using the potassium ferricyanide reduction method. The reducing power of the extracts was found to increase with increasing concentration (Fig 2).

#### Effect of *Crassocephalum crepidioides* extract on lipid profile

In the high fat diet-induced hyperlipidemia model, oral administration of methanol extract of *Crassocephalum crepidioides* (150 mg/kg and 300 mg/kg, p.o.) significantly reduced the serum TC, TG, LDL-c, VLDL-c levels but significantly increased serum HDL-c level compared with a positive control group (Table 2).

Serum lipid parameters were significantly reduced ( $p < 0.05$ ) by fourteen days treatment with *Crassocephalum crepidioides* at dose levels of 150 and 300 mg/kg, when compared with the positive control group. The 300 mg/kg showed significant ( $p < 0.01$ ) antihyperlipidemic activity compared with the positive control group and all treated groups showed significant reduction ( $p < 0.05$ ) in AI compared to positive control group.

#### Effect of methanol extract of *Crassocephalum crepidioides* on lipid profile in Triton X-100-induced hyperlipidemia model

In triton X-100 induced hyperlipidemia model, oral administration of methanol extract of *Crassocephalum crepidioides* (150 mg/kg and 300 mg/kg, p.o.) significantly reduced the serum TC, TG, LDL-c, VLDL-c levels but significantly increased serum HDL-c level as compared with a positive control group (Table 3).

This study showed serum lipid in rats were significantly reduced ( $p < 0.05$ ) by fourteen days treatment with *Crassocephalum crepidioides* at dose levels 150 mg/kg and 300 mg/kg, when compared with the positive control group. 300 mg group animals have shown very significant ( $p < 0.01$ ) compared with the positive control group. Decrease in the AI was observed in all treated groups with *Crassocephalum crepidioides* and atorvastatin when compared to triton X-100 treated group.

## DISCUSSION

Antioxidant activity can be attributed to various mechanisms like prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, reductive capacity, and radical scavenging activity [4]. In the present study, four different antioxidant methods for evaluation of antioxidant activity have been used. Methanol extract of *Crassocephalum crepidioides* produced significant antioxidant activity. This can be attributed to the flavonoids

**Table 2:** Effect of methanol extract of *Crassocephalum crepidioides* on serum lipid profile in high-fat diet-induced hyperlipidemic rats

Group	TC (mg/dl)	HDL-c (mg/dl)	TG (mg/dl)	VLDL-c (mg/dl)	LDL-c (mg/dl)	AI (mg/dl)
Control	83.13 ±0.87	42.93±1.19	66.35 ±1.64	13.27 ±0.33	26.92 ±0.59	0.94 ±0.04
Positive control	185.89±2.06	35.12±2.59	126.56 ±1.03	25.31 ±0.21	125.46±4.33	4.32 ±0.43
Atorvastatin 10 mg/kg	86.01 ±2.08***	44.29±1.98***	75.87 ±1.47***	15.17 ±0.29***	26.54 ±1.59***	0.94 ±0.06***
Plant extract 150 mg/kg	127.49±3.03***	40.04±1.25*	115.64 ±2.47**	23.13 ±0.49**	64.32 ±3.39***	2.19 ±0.15*
Plant extract 300 mg/kg	108.47±4.92***	40.38±4.10*	105.64 ±2.47**	21.13 ±0.49**	46.96 ±3.95***	1.70 ±0.25**

Values were expressed as mean ±SD (n=5). Values were statistically significant at \*P<0.05 and more significant at \*\*P<0.01, \*\*\*P<0.001 vs positive control using one way ANOVA followed by Dunnet's test

**Table 3:** Effect of methanol extract of *Crassocephalum crepidioides* on serum lipid parameter (mg/dl) levels in Triton X-100-induced hyperlipidemic rats

Group	TC (mg/dl)	HDL-c (mg/dl)	TG (mg/dl)	VLDL-c (mg/dl)	LDL-c (mg/dl)	AI (mg/dl)
Control	84.03 ±0.74	42.79 ±1.48	66.41 ±1.59	13.28 ±0.32	27.95 ±2.09	0.96 ±0.08
Positive control	187.51 ±2.07	34.97 ±2.27	127.05 ±3.09	25.41 ±0.62	127.13 ±1.11	4.38 ±0.29
Atorvastatin 10 mg/kg	86.15 ±2.45***	45.54 ±3.71***	73.77 ±2.26***	14.75 ±0.45***	25.85 ±4.89***	0.90 ±0.17***
Plant extract 150 mg/kg	125.86 ±3.53***	35.37 ±2.96	115.44 ±2.53**	23.08 ±0.51*	67.39 ±6.27***	2.58 ±0.38*
Plant extract 300 mg/kg	108.68 ±2.81***	43.49 ±1.98***	105.77 ±5.58*	21.15 ±1.12**	44.03 ±5.55***	1.5 ±0.17**

Values were expressed as mean ± SD (n = 5). Values are statistically significant at \*p < 0.05 and more significant at \*\*p < 0.01, \*\*\*p < 0.001 vs positive control using one way ANOVA followed by Dunnet's test

and other phytoconstituents present in the extract.

Hyperlipidemia is one of the important risk factors involved in the development of cardiovascular diseases. Atherosclerosis, ischemic heart disease and myocardial infarction are strongly associated with disorders of lipid metabolism and plasma lipoproteins [32]. It was considered that the Triton treated rats were useful as an acute hyperlipidemia model associated with inactive lipoprotein lipase [33]. Triton acts as a surfactant to block the uptake of lipoprotein from the circulation [34]. AI is believed to be an important risk factor for diagnosis of atherosclerosis. The methanol extract of our *Crassocephalum crepidioides* reduced atherogenic index, which is one of the most important risk factors of atherosclerotic plaques. This indicates that *Crassocephalum crepidioides* not only reduces the synthesis of cholesterol, but may also reduce its metabolism. The extract showed the presence of flavonoids and it is reported that flavonoids can inhibit hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase activity [33]. However, further studies are required to clarify the possible mechanism.

## CONCLUSION

The methanol extract of *Crassocephalum crepidioides* possesses significant antioxidant activity as well as promising antihyperlipidemic activity. There is, however, need for further studies to determine the exact mechanism of antihyperlipidemic activity and also to isolate the active constituents of the plant.

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