International Journal of Botany Studies
www.botanyjournals.com
ISSN: 2455-541X
Received: 30-06-2021, Accepted: 16-07-2021, Published: 31-07-2021
Volume 6, Issue 4, 2021, Page No. 511-515

In vitro study of antiobesity and antioxidant potentials of ethyl acetate extract from the leaves of *Crataeva religiosa*

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Abstract
The present study was evaluated the antioxidant and pancreatic lipase inhibition activities of ethyl acetate extract from the leaves of *Crataeva religiosa*. The antioxidant activity was measured using the free-radical scavenging capacity (DPPH), inhibition of Lipid peroxidation, and nitric oxide scavenging activity and reducing power assays. The pancreatic lipase inhibition assay was used to determine the ethyl acetate extract from the leaves of *Crataeva religiosa*. The EC_{50} value of Lipase inhibition (61.32), DPPH (55.32), inhibition of Lipid peroxidation (50.32), and nitric oxide scavenging activity (72.31). The antioxidant activity of ethyl acetate extract from the leaves of *Crataeva religiosa* assays reveals comparable results. Our results suggest natural resources that possess strong antioxidant and pancreatic lipase inhibitory activities with potential applications in the treatment and prevention of obesity and overweight. The ethyl acetate extract from the leaves of *Crataeva religiosa* were proved to have a great potential as antioxidants and anti-obesity agents.

Keywords: antioxidant; anti-obesity; *Crataeva religiosa*; ethyl acetate extract

Introduction

Phytochemicals derivative from plants are non-nutritional natural molecules that are vital for many body functions in humans. Countless of these moleculesare found in edible products are known to have antioxidant potential due to the occurrence of OH group (Koleva *et al*., 2002) [7]. The antioxidants prohibit the oxidative damage to various macromolecules like nucleic acids, proteins, and lipids and scavenge free radicals generated from biochemical reactions (Kampa and Castanas, 2008) [6]. A reaction of these free radicals with macromolecules has been reported to stimulate apoptosis that may cause various physiological, cardiovascular, and neurological disorders. Numerous types of phytochemicals such as phenolic acids, tannins, anthroquinones and bioflavonoids having antioxidant characteristics have been used to treat many diseases (Floyd and Hensley, 2002) [4].

Pancreatic lipase plays a key role in the efficient digestion of triglycerides. Lipases are involved in the hydrolysis of glycerides to glycerol and free fatty acids. The enzyme inhibition is one of the approaches used to treat obesity due to the fact that 50-70 % of total dietary fat hydrolysis was performed by pancreatic lipase. The mechanism involves inhibition of dietary triglyceride absorption, as this is the main source of excess calories. Besides, pancreatic lipase inhibition does not alter any central mechanism which makes it an ideal approach for obesity treatment. This enzyme has been widely used for the determination of the potential efficacy of natural products as antiobesity agents. Orlistat is the synthetic clinically approved drug used for obesity management. This molecule acts by the inhibition of PL activity and reduction of triglyceride absorption, and its long-term administration accompanying an energy restricted diet results in weight loss.

*Crataeva religiosa*is a moderate sized deciduous tree, 15m (50ft) tall and 9m (30ft) wide. The leaves are clustered at the ends of branchlets, with a common petiole 5 to 10 centimeters long, at the summit of which are tree leaflets. The flowers occur in terminal corymbs, are about 5 centimeters in diameter, greenish-yellow, and the stamens are purplish. The fruits are rounded or ovoid shaped fruit, and are 3 to 5 centimeters in diameters. The fruit has hard and rough rind and has unpleasant smell and burning taste. The *Crataeva religiosa*used traditionally various diseases immunity, restless leg syndrome, weight loss, astringent, cholagogue, strengthens bones, urination and excretion, lower risk of heart problems. The objective of this research is to determine the anti-obesity and antioxidant activity of ethyl acetate extract of *Crataeva religiosa* by investigating the inhibition of lipase and DPPH radical scavenging activity and nitric oxide radical inhibition assay.

Materials and Methods

Plant collection

Crataeva religiosa leaves was obtained from Herbal garden of Government Siddha Medical College, Arumbakkam, Chennai, Tamilnadu, India. A plant taxonomist authenticated the plant and samples were kept in the Medicinal Botany herbarium with voucher specimen numbers MB/GSMC-278/2021.

Extraction

*Crataeva religiosa*preparation, 100 g of the dried plant was crushed using blender to a paste-like state for 1 min. The homogenised sample was firstly freeze dried in order to reduce moisture content of the sample for a more efficient extraction process. The powder was then soaked in n-Hexane to defat for 24 h. It was then soaked in methanol for 72 h to obtain methanol crude extract, which was

concentrated using a rotatory evaporator at 40 °C. The sticky residues were partitioned with chloroform to give chloroform soluble fractions. This was evaporated under reduced pressure and dried using an oven to obtain an anthocynin rich fraction by ethyl acetate (Dasgupta *et al.,* 2014).

Phytochemical Screening

The aqueous extract of from the leaves of *Crataeva religiosa*were subjected to phytochemical screening to determine the presence of secondary metabolites such as alkaloids, flavonoids, terpenoids, tannins, glycosides, saponins and polyphenols using standard procedures (Trease and Evans 1983) [12].

Lipase Inhibition

The ability of the ethyl acetate extract from the leaves of *Crataeva religiosa*to inhibit lipase was measured by Bustanji *et al*. (2011) [1]. The lipase activity was determined by measuring the hydrolysis of p-nitrophenol butyrate (pNPB) to p-nitrophenol at 410 nm using UV-VIS spectrophotometer. Lipase assays were performed by incubating 200 µl of plant extract (5mg/ml ethanol) with 100 µl of PPL solution for 5 min at 37°C; then 10µL of the pNPB substrate (100 mM in acetonitrile) was added. The volume was completed to 1 mL using the buffer. The release of pNPB is estimated as the increment increase in absorbance against blank. The percentage of residual activity of PL was determined by comparing the lipase activity in the presence and the absence of the tested inhibitors. Orlistat (100 µg/ml) was used as a positive standard inhibitor control, whereas plant extract was replaced by ethanol to be used as negative control. All experiments were repeated twice.

DPPH (*A*, *A*-Diphenyl-*B*-Picrylhydrazyl) Assay for Scavenging Activity

DPPH radical scavenging activity was determined according to the method described by Choi *et al*. (2000) [2]. Briefly, 1 ml of the ethyl acetate extract from the leaves of *Crataeva religiosa* (25-100 µ/ml) or standards (BHT and ascorbic acid) were mixed with 1.5 ml (0.02 %) of DPPH solution in methanol. The mixture was incubated in the dark for 30 min, and the absorbance was measured at 517 nm in the spectrophotometer (6800 UV-VIS spectrophotometer) using methanol as blank. The percentage of scavenging of DPPH radicals was calculated by using the following formula: where *AS* is the absorbance of the sample; *AC* is the absorbance of the negative control (ethyl acetate without the sample).

Reducing Power Assay

The reducing power was determined according to the Oyaizu *et al*. (1986) [9]. Aliquot of 0.2 mL of various concentrations of the ethyl acetate extract from the leaves of *Crataeva religiosa* (25-100 µg/mL) were mixed separately with 0.5 mL of phosphate buffer (0.2 M, pH 6.6) and 0.5 mL of 1% potassium ferricyanide. The mixture was incubated in a water bath at 50°C for 20 min. After cooling at room temperature, 0.5 mL of 10% trichloroacetic acid was added to it followed by centrifugation at 3,000 rpm for 10 min. Supernatant (0.5 mL) was collected and mixed with 0.5 mL of distilled water. Ferric chloride (0.1 mL of 0.1%) was added to it and the mixture was left at room temperature for 10 min. The absorbance was measured at 700 nm. Ascorbic acid was used as positive control.

Inhibition of Lipid Peroxidation Activity

Lipid peroxidation induced by Fe^{2+}ascarbate system in egg yolk was assessed as thiobarbituric acid reacting substances (TBARS) by the method of Ohkawa *et al*. (1979). The experimental mixture contained 0.1 ml of egg yolk (25% w/v) in Tris-HCl buffer (20 mM, pH 7.0); KCl (30 mM); $FeSO_4$ $(NH_4)_2SO_4.7H_2O$ (0.06 mM); and different concentrations of ethyl acetate extract from the leaves of *Crataeva religiosa*in a final volume of 0.5 ml. The experimental mixture was incubated at 37°C for 1 h. After the incubation period, 0.4 ml was collected and treated with 0.2 ml sodium dodecyl sulphate (SDS) (1.1%); 1.5 ml thiobarbituric acid (TBA) (0.8%); and 1.5 ml acetic acid (20%, pH 3.5). The final volume was made up to 4.0 ml with distilled water and then kept in a water bath at 95 to 100 °C for 1 hour. After cooling, 1.0 ml of distilled water and 5.0 ml of n-butanol and pyridine mixture (15:1 v/v) were added to the reaction mixture, shaken vigorously and centrifuged at 4000 rpm for 10 min. The absorbance of butanol-pyridine layer was recorded at 532 nm in Deep Vision (1371) UV–Vis Spectrophotometer) to quantify TBARS. Inhibition of lipid peroxidation was determined by comparing the optical density (OD) of test sample with control. Ascorbic acid was used as standard.

Inhibition of lipid peroxidation (%) by the each extracts was calculated according to $1-(E/C) \times 100$, where C is the absorbance value of the fully oxidized control and E is absorbance of the test sample.

Nitric Oxide Radical Scavenging Assay

The nitric oxide radical scavenging assay followed by Panda *et al*. (2009) [10]. The extracts were prepared from a 10 mg/mL ethanol crude extract. These were then serially diluted with distilled water to make concentrations from 25-100 µg/mL of ethyl acetate extract from the leaves of *Crataeva religiosa* and standard. These were stored at 4°C for later use. Griess reagent was prepared by mixing equal amounts of 1% sulphanilamide in 2.5% phosphoric acid and 0.1% naphthylethylenediaminedihydrochloride in 2.5% phosphoric acid immediately before use. A volume of 0.5 mL of 10 mM sodium nitroprusside in phosphate buffered saline was mixed with 1 mL of the different concentrations of the ethanol extracts (10−100 µg/mL) and incubated at 25°C for 180 mins. The extract was mixed with an equal volume of freshly prepared Griess reagent. Control samples without the extracts but with an equal volume of buffer were prepared in a similar manner as was done for the test samples.

Statistical Analysis

All results were expressed as mean ± standard deviation (n=3). Significance of differences from the control was determined by Duncan's test and a p value < 0.05 was considered significant.

Result and Discussion

Phytochemical screening

The phytochemical screening of aqueous extract of *Crataeva religiosa* were studied presently showed the presence of alkaloids, flavonoids, polyphenol, terpenoids, and absence of glycosides and tannin (Table-1).

Table 1: Phytochemical screening of *Crataeva religiosa*

Sl. No.	Phytochemical Constituents	Observation	Aqueous extract of *Crataeva religiosa*
1	Alkaloids -Dragendorff's Test-Mayers test	Orange /red precipitate Yellow or white precipitate	+ +
2.	Flavonoids -Alkalai Reagent -Lead acetate test	Intense yellow colour Precipitate formed	+ +
3.	Glycosides Keller-Killiani test	Reddish brown colour ring formed	-
4.	Tannin -$FeCl_3$ test	Blue black coloration	-
5.	Saponins-Frothing test	Foam	+
6.	Terpenoids-*Salkowski test*	Dark reddish brown color in interface	-
7.	Polyphenols-Ferrozine test	Raddish blue	+
8.	Anthocyanin test Ammonia	Ammonia layer yellow in color	+

The Partial Characterization of by TLC

The ethyl acetate extract of *Crataeva religiosa* loaded on Pre-coated TLC plates (60 F_2 54 Merck) and developed with a solvent system of petroleum ether, chloroform and methanol in the ratio of 1:0.5:0.1 were efficient to extract the antioxidant and anti-obesity compound it is used for further studies. The developed plate was viewed under UV 240nm and 360nm (Table-2 and Fig-1).

Table 2: Partial characterization of ethyl acetate extract of *Crataeva religiosa* by TLC

S.No	Ethyl acetate extract of *Crataeva religiosa*	
	UV 240 nm Rf value	UV 360 nm Rf value
1	0.75	0.75
2	0.63	0.63
3	0.54	0.64
4	0.38	-

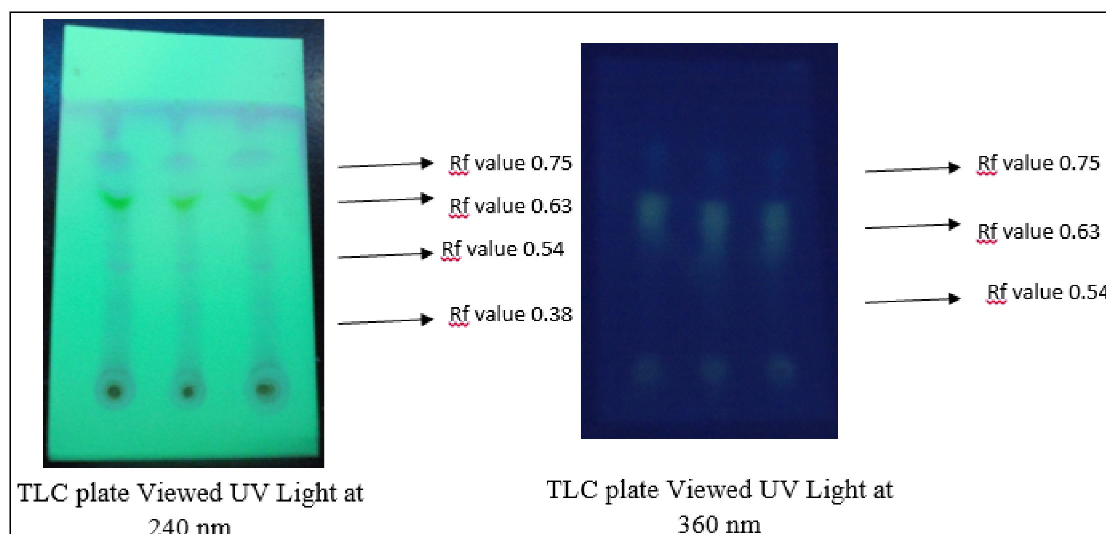

Fig 1: TLC profile of *Crataeva religiosa*

Anti-Obesity Activity Inhibition of Lipase

Result of % lipase inhibition activity of ethyl acetate extract of *Crataeva religiosa* was presented in Table 3. Ethy l acetate extract of *Crataeva religiosa* showed significantly ($p < 0.05$) higher % lipase inhibition and EC_{50} as compared to standard orli state, whereas, ethyl acetate extract of *Crataeva religiosa* showed significantly ($p < 0.05$) lower % lipase inhibition and EC_{50}61.23. Ethyl acetate extract of *Crataeva religiosa* inhibits the conversion of dietary lipid into fatty acid by hydrolysis. Ethyl acetate extract of *Crataeva religiosa* it was reported that flavonoid and alkaloids reduced the triglyceride breakdown and work as a bioactive phytoconstituents.

Table 3: Anti-obesity activity inhibition of lipase by ethyl acetate extract of *Crataeva religiosa*

Different concentration of extract	Ethyl acetate extract of *Crataeva religiosa*	Standard orli state
25 µl/ml	15.32±2.36	13.23±2.89
50 µl/ml	31.45±0.78	28.34±1.89
75 µl/ml	49.32±0.89	47.32±3.21
100 µl/ml	72.34±2.35	68.32±2.56
EC_{50} Value	61.32±0.7	70.32±0.7

Results are expressed as percentage inhibited Lipase formation with respect to control. Each value represents the mean+SD of three experiments

Radical Scavenging Activity DPPH

The antioxidant activity of ethyl acetate extract of *Crataeva religiosa* was primarily assessed by 2, 2-diphenyl-1-picrylhydrazyl (DPPH), which is based on the ability of DPPH to react with proton donors such as phenols. However, ethyl acetate extract of *Crataeva religiosa* free radical scavenging ability remains unknown. The present study showed that ethyl acetate extract of *Crataeva religiosa* exhibits significant free radical scavenging potential (EC_{50}: 55.32 ± 2.45µg/mL, Table-4). The percentages of free radical scavenging are given in Table-4. This property has been widely used to evaluate the free radical scavenging effect of natural antioxidants (Jao and Ko, 2002).

Table 4: Radical scavenging activity DPPH by ethyl acetate extract of *Crataeva religiosa*

Different concentration of extract	Ethyl acetate extract of *Crataeva religiosa*	Standard vitamin-C
25 µl/ml	18.34±0.87	16.32±1.73
50 µl/ml	36.32±3.56	34.32±2.89
75 µl/ml	52.34±2.89	49.32±1.78
100 µl/ml	77.32±1.23	73.21±1.56
EC_{50} Value	55.32±2.45	58.32±1.23

Results are expressed as percentage inhibited Lipase formation with respect to control. Each value represents the mean+SD of three experiments.

Reducing Power Activity

Reducing power assay method is based on the principle that substances, which have reduction potential, react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride to form ferric-ferrous complex that has an absorption maximum at 700 nm. The reducing power of the ethyl acetate extract of *Crataeva religiosa* and standard increases with the increase in amount of sample and standard concentrations. The reducing power shows good linear relation in both standard (R2 = 0.981) and sample extract (R2 = 0.970) (Ferreira *et al*., 2007) [3].

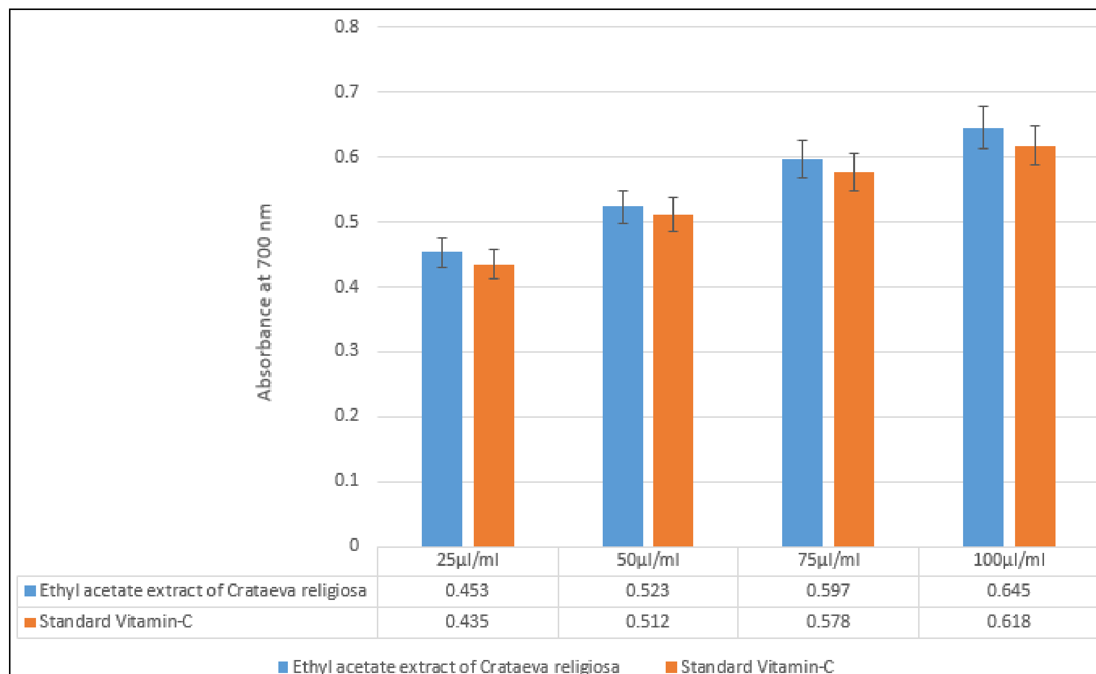

Fig 2: The reducing power activity of the ethyl acetate extractof *Craeva religiosa*

Inhibition OF Lipid Peroxidation

In the present study, egg yolk was used as substrate for free radical mediated lipid peroxidation, which is a non-enzymatic method. Ethyl acetate extract of *Crataeva religiosa* also inhibited the lipid peroxidation induced by ferrous sulfate in egg yolk homogenates. Maximum inhibition was recorded in ethyl acetate extract of *Crataeva religiosa*79.34% with EC_{50} value 50.32 µl/ml and lowest inhibition percentage ascorbic acid 74.56% with EC_{50} 56.31*(Table-5)*. As it is identified that lipid peroxidation is the net result of any free radical attack on membrane and other lipid components present in the system, the lipid peroxidation may be enzymatic (Fe/NADPH) or non-enzymatic (Fe/ascorbic acid). Normally, the mechanism of flavonoid compounds for neutralizing lipid free radicals and preventing decomposition of hydroperoxides into free radicals (Miller, 1996) [8].

Table 5: Inhibition of lipid peroxidation activity of ethyl acetate extract of *Crataeva religiosa religiosa*

Different concentration of extract	Inhibition percentage of lipid peroxidation	
	Ethyl acetate extract of *Crataeva religiosa*	Standard vitamin-C
25 µl/ml	20.31±1.59	17.32±0.78
50 µl/ml	38.64±2.45	36.24±2.14
75 µl/ml	56.34±1.23	54.89±1.45
100 µl/ml	79.34±0.78	74.56±1.78
EC_{50} value	50.32	56.31

[a] Results are expressed as percentage inhibit of lipid peroxidation with respect to control. Each value represents the mean+SD of three experiments.

Nitric Oxide Radical Scavenging

Ethyl acetate extract of *Crataeva religiosa*showed a strong nitric oxide scavenging activity which was comparable to the standards ascorbic acid and rutin. The EC_{50} value 72.31 of ethyl acetate extract of *Crataeva religiosa*was less than ascorbic acid 79.63. Percentage of Nitric oxide radical scavenging activityflavonoid rich fraction of *Cassia alata* flower and standards were presented in Table-8 and Fig-9. In the current study, nitrite was produced by incubation of sodium nitroprusside in standard phosphate saline buffer at

25°C was reduced by ethyl acetate extract of *Crataeva religiosa*. Significant scavenging activity may be due to the antioxidant property of flavonoid, compounds present in ethyl acetate extract of *Crataeva religiosa,* which compete with oxygen to react with nitric oxide, leading to less production of nitric oxide.Since, nitric oxide has an important role in various inflammatory processes. Continualstages of production of this radical are directly toxic to tissues and contribute to the vascular collapse associated with septic shock (Shah and Billar, 1998) [11].

Table 6: Nitric oxide radical scavenging assay of ethyl acetate extract of *Crataeva religiosa*

Different concentration of extract	Percentage of nitric oxide radical scavenging activity	
	Ethyl acetate extract of *Crataeva religiosa*	Standard vitamin-C
25 µl/ml	17.56±1.36	15.64±1.21
50 µl/ml	33.64±1.69	26.34±1.47
75 µl/ml	48.67±1.48	45.63±1.56
100 µl/ml	64.23±2.47	60.32±2.16
EC_{50} value	72.31	79.63

[a] Results are expressed as percentage of Nitric oxide radical activity with respect to control. Each value represents the mean+SD of three experiments.

Conclusions

Our results suggest natural resources that possess strong antioxidant and pancreatic lipase inhibitory activities with potential applications in the treatment and prevention of obesity and overweight problem. The ethyl acetate extract from the leaves of *Crataeva religiosa* have shown possessing strong antioxidants and lipase inhibition potentials. However, future studies are needed for screening in-depth phytochemical, clinical, and possible studies on molecular mechanism of action and identification of the constituents responsible for the antioxidant and lipase inhibition activities.

Acknowledgement

We thank Mr. Sai Prakash Leomuthu, CEO Sairam Institutions, Mr. Sathish Kumar CBO Sairam Institutions. Dr. S. Mathu kumar M. D. (S), Principal Sri Sairam Siddha Medical College West Tambaram for Support and Encouragement to carry out the study.

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