

Antioxidant Properties of the Oleanolic Acid Isolated from *Cassia auriculata* (Linn)

*Senthilkumar P K¹, M. Kandhavelu¹, D. Reetha²

1. Division of Microbiology, Faculty of Science, Annamalai University, Chidambaram, Tamilnadu-608002, India.

2. Department of Microbiology, Faculty of Agriculture, Annamalai University, Chidambaram, Tamilnadu-608002, India.

ABSTRACT

Medicinal plants are sources of great economic value all over the world medicinal herbs have a long history in improving human health and curing various diseases. A wide interest has been made for researchers using herbal material in identification of the active components and verification of their efficiency. Many researches are working seriously to find out substitutes for antibiotics as they cause side effects on the functioning of different parts of the body, organs and systems over the last twenty years. To screen the bioactive compound and to analyze the antioxidant properties of selected medicinal plant. The bioactive compound was isolated from the medicinal plant *Cassia auriculata* and it was identified as oleanolic acid by different spectral studies viz., FT-IR (Fourier Transform Infrared (spectroscopy), ¹H NMR and ¹³C NMR (Nuclear Magnetic Resonance). The leaf extract of the *Cassia auriculata* significantly shows the antioxidant properties in higher level against the entire test. To understand the pharmacological role of the isolated bioactive compound oleanolic acid, it has become important to study. This research can be considered as an important preliminary study on the isolation and identification of bioactive compound from Indian medicinal plants that may be useful in the treatment of microbial diseases in the future.

Keywords: Antioxidant, *Cassia auriculata*, medicinal plants, oleanolic acid

Received 16 Dec 2013

Received in revised form 09 Jan 2014

Accepted 11 Jan 2014

*Address for correspondence:

Dr. Senthilkumar P K,

Assistant Professor, Division of Microbiology, Faculty of Science, Annamalai University, Chidambaram-608002, Tamilnadu, India.

E-mail: mailtopks@yahoo.com

INTRODUCTION

Plants have been an essential part of human society since the start of civilization. Around 400 plants were mentioned having therapeutic values were mentioned by Rigveda, Yajurveda, Atharvanaveda [1]. Herbal medicines are being used by about 80 per cent of the world Population, primarily in the developing countries for their health care. Ancient literature states that herbal medicines are used for age-related diseases namely memory loss, disorders like diabetes, etc. [2]. Various types of organic and inorganic materials leached out from plant which inhibits the growth of microbes. The use of bearberry and cranberry juice to treat urinary tract infections were reported in different manuals of phytotherapy, while the plant species such as lemon, garlic and

the neem were described as broad spectrum of antimicrobial agents [3].

The therapeutic activity of these plants is ascribed that the quantitative measures of these active chemical compounds indicate the level of the therapeutic activity of the plant material [4]. Plants have been the basis of traditional medicines throughout the world for thousands of years and continue to provide new remedies to humankind; a great deal of effort has therefore focused on using available experimental techniques to identify natural antioxidants from plants. Medicinal plants and herbs are promising and diverse sources of natural antioxidants. Therefore, a great number of different spices and aromatic herbs have been investigated for their antioxidant activity [5]. It is an erect

shrub found throughout India in open areas of forest.

The leaves of *Cassia auriculata* are used for ulcer, leprosy and skin diseases. Flowers are useful in diabetes and throat troubles [6]. The leaf of this plant has been used in the traditional system of Indian medicine for the treatment of jaundice, liver disease [7, 8]. *Cassia auriculata* is effective remedy for diabetes. The powder of the herb, mixed with honey is equally efficacious in such a case. Its seeds can be used in similar manner as flowers. The seeds of *Cassia auriculata* find their application in purulent ophthalmic that is inflammation of the eye or conjunctiva. They should be finely powdered and blown in to the affected eyes. An ointment prepared from them and oil can be applied to the affected eye to cure the disease. The bark has astringent properties and useful in regulating menstrual cycle. The bark is also used in treatment of rheumatism and eye disease.

MATERIALS AND METHODS

Collection of plant

Healthy and well grown leaves of selected plant *cassia auriculata* (**Fig. 1**) were collected from the area in and around Chidambaram, Cuddalore district, Tamilnadu, India. The leaves were immediately brought to the laboratory using separate polythene bags. First they were washed with tap water, then surface sterilized in 10 per cent sodium hypochlorite solution to prevent the contamination from the microbes, then rinsed with sterile distilled water and air dried in shade at room temperature and the samples were ground into a fine powder (**Fig. 1**).



Preparation of plant extract

Forty grams of the powdered leaves were loaded in soxhlet apparatus and fractionated in 125 ml of methanol solvent. The fraction was evaporated at rotary evaporator at 40°C [9].

Separation of bioactive compound

After the conformation of the antimicrobial activity, 5 kg of air-dried and powdered plant material of *cassia auriculata* was exhaustively extracted with methanol using soxhlet apparatus. Removal of the solvent from the combined methanol extracts under reduced pressure at 40°C gave a residue (40g). This residue was used for column chromatography to separate the bioactive principle and it was analysed for the identification of the compound using spectral analysis namely FT-IR, ¹H NMR, ¹³C NMR, conformed as oleanolic acid [10].

In vitro Antioxidant activity of Oleanolic acid

Chemicals

Nitro blue tetrazolium (NBT), ethylene diamine tetra acetic acid (EDTA), sodium nitroprusside (SNP), trichloro acetic acid (TCA), thiobarbituric acid (TBA), 1,1-diphenyl-2-picrylhydrazyl (DPPH), potassium hexacyano ferrate [K₃Fe(CN)₆] and L-ascorbic acid were purchased from Sisco Research Laboratories Pvt. Ltd., India. All other chemicals and solvents used were of analytical grade available commercially.

Preparation of Sample

The isolated oleanolic acid was stored in refrigerator until used. The oleanolic acid of different concentration was dissolved in distilled water for *in vitro* activity.

DPPH radical-scavenging activity

DPPH radical-scavenging activity was determined by the method [11]. A 2 ml aliquot of DPPH methanol solution (25 mg/ml) was added to 0.5 ml sample solution at different concentrations (20, 40, 60, 80 and 100 µg/ml respectively). The mixture was shaken vigorously and allowed to stand at room temperature in the dark for 30 min. Then the absorbance was measured at 517 nm in a spectrophotometer. Lower absorbance of the reaction mixture indicated higher free-radical scavenging activity. The scavenging activity of sample was expressed as 50% effective concentration (EC₅₀), which represented the

concentration of sample having 50% of DPPH radical scavenging effect.

Superoxide anion scavenging activity assay

The scavenging activity of the oleanolic acid towards superoxide anion radicals was measured by the method [12]. Superoxide anions were generated in a non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS-NADH) system through the reaction of PMS, NADH and oxygen. It was assayed by the reduction of nitrobluetetrazolium (NBT). In these experiments the superoxide anion was generated in 3 ml of Tris-HCl buffer (100 mmol, pH 7.4) containing 0.75 ml of NBT (300 μ mol) solution, 0.75 ml of NADH (936 μ mol) solution and 0.3 ml of different concentrations of the oleanolic acid. The reaction was initiated by adding 0.75 ml of PMS (120 μ mol) to the mixture. After 5 min of incubation at room temperature, the absorbance at 560 nm was measured in spectrophotometer.

Hydroxyl radical scavenging activity assay

The scavenging activity for hydroxyl radicals was measured with Fenton reaction [13]. Reaction mixture contained 60 μ l of 1 mmol of FeCl_2 , 90 μ l of 1 mmol of 1.10-phenanthroline, 2.4 ml of 0.2 mol of phosphate buffer (pH 7.8), 150 μ l of 0.17 mol of H_2O_2 , and 1.5 ml of oleanolic acid at various concentrations. Adding H_2O_2 started the reaction. After incubation at room temperature for 5 min, the absorbance of the mixture at 560 nm was measured with a spectrophotometer.

Nitric oxide scavenging activity assay

Nitric oxide radical scavenging activity was determined according to the method reported by [14]. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions, which can be determined by the use of the Griess-Illsovoy reaction. 2 ml of 10 mM sodium nitroprusside in 0.5 ml phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of oleanolic acid at various concentrations and the mixture incubated at 25°C for 150 min. From the incubated mixture 0.5 ml was taken out and added into 1.0 ml of sulfanilic

acid reagent (33% in 20% glacial acetic acid) and incubated at room temperature for 5 min. Finally, 1.0 ml of naphthylethylenediaminedihydrochloride (0.1% w/v) was mixed and incubated at room temperature for 30 min. The absorbance at 540 nm was measured with a spectrophotometer.

Hydrogen peroxide scavenging activity assay

Hydrogen peroxide scavenging activity of the oleanolic acid was estimated by replacement titration [15]. Aliquot of 1.0 ml of 0.1 mmol of H_2O_2 and 1.0 ml of various concentrations of oleanolic acid were mixed, followed by 2 drops of 3% ammonium molybdate, 10 ml of 2 mol of H_2SO_4 and 7.0 ml of 1.8 mol KI. The mixed solution was titrated with 5.09 mmol of $\text{Na}_2\text{S}_2\text{O}_3$ until yellow color disappeared.

Fe^{2+} chelating activity assay

The chelating activity of the oleanolic acid for ferrous ions Fe^{2+} was measured according to the method of [16]. To 0.5 ml of oleanolic acid, 1.6 ml of deionized water and 0.05 ml of FeCl_2 (2 mmol) was added. After 30s, 0.1 ml of ferrozine (5 mmol) was added. Ferrozine reacted with the divalent iron to form stable magenta complex species that were very soluble in water. After 10 min at room temperature, the absorbance of the Fe^{2+} Ferrozine complex was measured at 562 nm.

Iron reducing power assay

The Fe^{3+} reducing power of the oleanolic acid was determined by the method of [17] with slight modifications. The oleanolic acid (0.75 ml) at various concentrations was mixed with 0.75 ml of phosphate buffer (0.2 mol, pH 6.6) and 0.75 ml of potassium hexacyanoferrate [$\text{K}_3\text{Fe}(\text{CN})_6$] (1%, w/v), followed by incubating at 50°C in a water bath for 20 min. The reaction was stopped by adding 0.75 ml of trichloro acetic acid (TCA) solution (10%) and then centrifuged at 3000r/min for 10 min. 1.5 ml of the supernatant was mixed with 1.5 ml of distilled water and 0.1 ml of ferric chloride (FeCl_3) solution (0.1%, w/v) for 10 min. The absorbance at 700 nm was measured as the reducing power. Higher absorbance of the reaction mixture indicated greater reducing power.

IC₅₀ Value

Inhibition Concentration (IC₅₀) was introduced by Brand-Williams and his colleagues [18] for the interpretation of the results from DPPH method. The discoloration of sample was plotted against the sample concentration in order to calculate the IC₅₀ value. It is defined as the amount of sample necessary to decrease the absorbance of DPPH by 50%.

RESULTS**ANTIOXIDANT ACTIVITY OF OLEANOLIC ACID**

To understand the pharmacological role of the isolated bioactive compound, oleanolic acid, it has become important to study its antioxidant properties. The *in vitro* technologies have been used for the determination of antioxidant activity, based on the ability of the oleanolic acid to scavenge various free radicals and the results are presented in (Table 1).

Table 1: Radical Scavenging Activity of Oleanolic Acid at Different Concentrations

Concentration (µg/ml)	Superoxide radical scavenging (%)	Hydroxyl radical scavenging (%)	Nitric oxide radical scavenging (%)
10	30.25 ± 2.46	25.45 ± 3.15	11.25 ± 0.78
50	54.68 ± 2.78	45.56 ± 2.86	17.36 ± 2.12
100	86.96 ± 3.01	58.18 ± 2.69	21.53 ± 2.56
250	-	69.09 ± 3.15	29.12 ± 2.14
500	-	81.82 ± 2.87	35.68 ± 2.74
750	-	92.73 ± 3.22	44.36 ± 2.32
1000	-	94.55 ± 2.98	52.36 ± 2.41
IC ₅₀ (µg/ml)	37.69	4.469	1.36

Values are means ± SD (n = 3).

DPPH Radical scavenging activity

The oleanolic acid exhibited a significant dose dependent inhibition of DPPH activity. The IC₅₀ value of oleanolic acid was found to be at 32.46 µg/ml.

Superoxide anion radical scavenging activity

The superoxide anion radical scavenging activity of oleanolic acid was assayed by the PMS-NADH system and is shown in (Table 1). The superoxide scavenging activity of oleanolic acid was increased markedly with the increase of concentrations. The half inhibition concentration (IC₅₀) of oleanolic acid was 37.69 µg/ml.

Hydroxyl radical scavenging activity

Hydroxyl radical is very reactive and can be generated in biological cells through the Fenton reaction. (Table 1) showed the oleanolic acid exhibited concentration dependent scavenging activities against hydroxyl radicals generated in a Fenton reaction system. The IC₅₀ of oleanolic acid was 4.46 µg/ml.

Nitric oxide radical scavenging activity

Oleanolic acid is also moderately inhibited nitric oxide in dose dependent manner (Table 1) with the IC₅₀ value being 1.36 µg/ml.

Hydrogen peroxide scavenging activity

Oleanolic acid demonstrated hydrogen peroxide scavenging activity in a concentration dependent manner with an IC₅₀ of 0.984 mg ml⁻¹.

The ferrous ion chelating activity

The formation of the ferrozine-Fe²⁺ complex is interrupted in the presence of oleanolic acid, indicating that have chelating activity with an IC₅₀ of 0.241 mg ml⁻¹.

DISCUSSION

Traditional medicine is an important source of potentially useful new compounds for the development of chemotherapeutic agents.

The identification of antioxidant activity is beneficial to biological system against ROS damage. Recently, importance has been given for *in vitro* antioxidant study to understand the pharmacological role of

medicinal plant and its isolates. *In vitro* techniques have been used for the detection of antioxidants, which are based on the ability of compounds to scavenge free radicals [19]. Free radicals are harmful by-products generated during normal cellular metabolism, which could initiate oxidative damage to body [20]. Antioxidants are believed to play a significant role in the body's defense system against free radicals. Recently, numerous reports have described antioxidants and compounds with radical-scavenging activity present in fruits, vegetables, herbs and cereals extracts [21]. The DPPH radical was widely used to evaluate the free-radical scavenging capacity of antioxidants [22].

The DPPH antioxidant assay is based on the ability of DPPH a stable free radical, to decolorize in the presence of antioxidants. The DPPH radical contains an odd electron, which is responsible for the absorbance at 517 nm and also for visible deep purple color. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized which can be quantitatively measured from the changes in absorbance. The antioxidant activity of oleanolic acid is showed in the oleanolic acid exhibited a significant dose dependent inhibition of DPPH activity. The IC₅₀ value of oleanolic acid was found to be at 32.46 µg/ml.

Superoxide is biologically important since it can be decomposed to form stronger oxidative species such as singlet oxygen and hydroxyl radicals, are very harmful to the cellular components in a biological system [23]. The superoxide scavenging activity of oleanolic acid was increased markedly with the increase of concentrations. The half inhibition concentration (IC₅₀) of oleanolic acid was 37.69 µg/ml. These results suggested that oleanolic acid had superior superoxide radical scavenging effect.

Hydroxyl radical is very reactive and can be generated in biological cells through the Fenton reaction. The IC₅₀ of oleanolic acid was 4.46 µg/ml. The potential scavenging abilities of phenolic substances might be due to the active hydrogen donor ability of hydroxyl substitution. Similarly, high molecular weight and the proximity of

many aromatic rings and hydroxyl groups are more important for the free radical scavenging by specific functional groups [24].

Nitric oxide (NO) is a potent pleiotropic mediator of physiological processes such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical, which plays many roles as an effector molecule in diverse biological systems including neuronal messenger, vasodilation, antimicrobial and antitumor activities [25]. Oleanolic acid also moderately inhibited nitric oxide in dose dependent manner with the IC₅₀ being 1.36 µg/ml.

Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. Hydrogen peroxide can cross cell membranes rapidly, once inside the cell, H₂O₂ can probably react with Fe²⁺ and possibly Cu²⁺ ions to form hydroxyl radical and this may be the origin of many of its toxic effects [26]. It is therefore biologically advantageous for cells to control the amount of hydrogen peroxide that is allowed to accumulate. Oleanolic acid demonstrated hydrogen peroxide scavenging activity in a concentration dependent manner with an IC₅₀ of 0.984 mg/ml.

Ferrozine can make complexes with ferrous ions. In the presence of chelating agents, complex (red colored) formation is interrupted and as a result, the red color of the complex is decreased. Thus, the chelating effect of the coexisting chelator can be determined by measuring the rate of color reduction. The formation of the ferrozine-Fe²⁺ complex is interrupted in the presence of oleanolic acid, indicating that have chelating activity with an IC₅₀ of 0.241 mg/ml. Ferrous iron can initiate lipid peroxidation by the Fenton reaction as well as accelerating peroxidation by decomposing lipid hydroperoxides into peroxy and alkoxy radicals [27]. Metal chelating activity can contribute in reducing the concentration of the catalyzing transition metal in lipid peroxidation. Furthermore, chelating agents that forms bonds with a metal are effective as

secondary antioxidants because they reduce the redox potential, and thereby stabilize the oxidized form of the metal ion [28]. Thus, oleanolic acid possessed a marked capacity for iron binding, suggesting their ability as iron chelator proved to be an antioxidant.

For the measurements of the reducing ability, the Fe^{3+} - Fe^{2+} transformation was investigated in the presence of oleanolic acid. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. However, the activity of antioxidants has been assigned to various mechanisms such as prevention of chain initiation, binding of transition-metal ion catalysts, decomposition of peroxides, reductive capacity and radical scavenging [29,30]. In the present study, the reducing power of oleanolic acid increased with increasing dosage. All the doses showed significantly higher activities than the control exhibited greater reducing power, indicating that oleanolic acid consists of triterpenoids compounds that cause the greater reducing power.

CONCLUSION

The antioxidant properties of oleanolic acid were evaluated by estimating the free radical scavenging activities such as superoxide anion scavenging activity, hydroxyl radical scavenging activity, nitric oxide scavenging activity and hydrogen peroxide scavenging activity. The increase in the ferrous iron chelating activity, iron reducing power activity with oleanolic acid was observed and indicate that oleanolic acid is used as an antioxidant compound.

REFERENCES

- Rajasekara Pandiyan M, Sharmaila Banu G, Kumar G. Antimicrobial activities of natural honey from medicinal plants on antibiotic resistant strains of bacteria. Asian J. Microbiol. Biotechnol. Environ. Sci., 2007; 9: 219-224.
- Park SJ, Lee SG, Shin SC, Lee BY, Ahn YJ. Larvicidal and ntfeeding activities of oriental medicinal plant extracts against four species of forest insect pests. Appl. Entomol. Zool., 1997; 32: 601-608.
- Heinrich M, Barnes J, Gibbons S, Williamson EM. Fundamental of pharmacognosy and phytotherapy. Churchill Livingstone, Edinburgh. 2004.
- Scalbert A, Menon AR, Gopal GV. Antimicrobial properties of tannins. Phytochemistry, 1991; 30: 3875-3883.
- Couladis M, Tzakou O, Verykokidou E, Harvala C. Screening of some Greek aromatic plants for antioxidant activity. Phytother Res., 2003; 17: 194-195.
- Kirtikar KR, Basu BD. Indian Medicinal Plants, Second edition (Published by Lalit Mohan Basu, Allahabad, India). 1935; 1492.
- Rajagopal S, Manickam P, Periyasamy V, Namasivayam N. Effect of *Cassia auriculata* leaf extract on lipids in rats with alcoholic liver injury. Asia Pac. J. Clin. Nutr., 2002; 11: 157-163.
- Rajagopal J, Anderson WJ, Kume S, Martinez OI, Melton DA. Insulin staining of ES cell progeny from insulin uptake. Science, 2003; 299: 363.
- Vogel H. Textbook of Practical Organic Chemistry. The English Language Book Society and Longman, London, 1978; 1368.
- Senthilkumar PK, Reetha D. Isolation and identification of antibacterial compound from the leaves of *Cassia auriculata*. Europe Review Medical Pharmacology Science, 2011; 15: 1034-1038.
- Shimada K, Fujikawa K, Yahara K, Nakamura T. Antioxidative properties of xanthum on the autoxidation of soybean oil in cyclodextrin emulsion. J. Agrl. Food Chem., 1992; 40: 945-948.
- Liu F, Ooi VEC, Chang ST. Free radical scavenging activity of mushroom polysaccharide extracts. Life Sci., 1997; 60: 763-771.
- Yu W, Zhao Y, Shu B. The radical scavenging activities of radix puerariae flavonoids: A chemiluminescence study. Food Chem., 2004; 86: 525-529.
- Garrat DC. The quantitative analysis of drugs Japan: Chapman and Hall. 1964; 3: 456-458.
- Zhang XY. Principles of Chemical Analysis. Beijing: China Science Press. 2000; 275-276.
- Dinis TCP, Madeira VMC, Almeida LM. Action of phenolic derivatives (acetaminophen, salicylate, and 5-aminosalicylate) as inhibitors of membrane lipid peroxidation and peroxyl radicals scavengers. Arch. Biochem. Biophys., 1994; 315: 161-169.
- Oyaizu M. Studies on products of browning reactions: antioxidant activities of products of browning reaction prepared from glucose amine. Jpn. J. Nut., 1986; 44: 307-315.
- Brand-Williams W, Cuvelier ME, Berset C. Use of a free radical method to evaluate

- antioxidant activity, Lebensmittel-Wissenschaftund-Technologie. Food Science and Technoogy, 1995; 28: 25-30.
19. Acker CI, Brandao R, Rosario AR, Nogueir CW. Antioxidant effect of alkynylselenoalcohol compounds on liver and brain of rats in vitro. Environmental Toxicology and Pharmacology, 2009;28: 280-287.
 20. Halliwell B, Gutteridge JMC. Free radicals in biology and medicine (3rded.). Oxford: Oxford University Press. 1999.
 21. Hou WC, Lin RD, Lee TH, Huang YH, Hsu FL, Lee MH. The phenolic constituents and free radical scavenging activities of *Gynuraformosana*Kiamnra. J. Sci. Food Agri.,2005; 85: 615-621.
 22. Nuutila AM, Pimia RP, AarniM, Caldenty KMO. Comparison of antioxidant activities of onion and garlic extracts by inhibition of lipid peroxidation and radical scavenging activity. Food Chem., 2003; 81: 485-493.
 23. Korycka-Dahl M, Richardson M. Photo generation of superoxide anion in serum of bovine milk and in model systems containing riboflavin and amino acids. J. Dairy Sci.,1978;61: 400-407.
 24. Hagerman AE, Riedl KM, Jones GA, Sovik KN, Ritchard NT, Hartzfeld PW. High molecular weight plant polyphenolics (tannins) as biological antioxidants. J. Agril. Food Chem. 1998; 46: 1887-1892..
 25. Miller MJ, Sadowska-krowicka H, Chotinaruemol S, Kakkis JL, Clark DJ. Amelioration of chronic ileitis by nitric oxide synthase inhibition. J. Pharma. Exp. Therap., 1993; 264: 11-16.
 26. Halliwell B, Gutteridge JMC. Free Radicals in Biology and Medicine Oxford: Clarendon, 1993; 419-422.
 27. Halliwell B. Reactive oxygen species in living systems: Source biochemistry and role in human disease. Am. J. Med., 1991;91: 14s-22s.
 28. Gordon MH. The mechanism of the antioxidant action in vitro. In B.J.F. Hudson (Eds.), Food Antioxidants. London: Elsevier. 1990; 1-18.
 29. Diplock, AT. Will the 'good fairies' please prove to us that vitamin E lessens human degenerative disease. Free Radical Res., 1997; 27: 511-532.
 30. Yildirim A, Mavi A, Oktay Q, Kara AA, Algur OF, Bilaloglu SD. Comparison of antioxidant and antimicrobial activities of *Tilia* (*Tiliaargentea*Desf Ex DC), Sage (*Salvia triloba* L.), and Black Tea (*Camellia sinensis*) extracts. J. Agril. Food Chem., 2000;48: 5030-5034.