

A study on the phytochemistry and antioxidant effect of methanolic extract of *Citrullus lanatus* seed

Jayavelu Sathya and Francis Gricilda Shoba*

PG & Research Department of Zoology, Voorhees College, Vellore, Tamilnadu, India

ABSTRACT

Antioxidants of natural origin are safe for the prevention and progression of various diseases. *Citrullus lanatus* seeds that are traditionally in use can be employed as a natural antioxidant protecting from various ailments. The present study was performed to evaluate the *in vitro* antioxidant potential of *C. lanatus* seed methanol extract (CLS) and to determine the phytochemicals responsible for its activity. The antioxidant activity of CLS was analyzed by DPPH and LPO radical scavenging assays and conventional methods were adopted for quantifying the primary and secondary metabolites in the extract. Qualitative analysis revealed the presence of bioactive compounds like phenols, tannins, flavonoids, glycosides, saponins and steroids. Phenolic content of the extract was 6.45 ± 0.05 GE/g, followed by tannins 5.92 ± 0.15 GE/g and flavonoids 1.30 ± 0.09 QE/g. IC_{50} values for the antioxidant activity were 28.77 μ g/ml and 123.8 μ g/ml for DPPH and lipid peroxidation assay respectively. The study hence confirms *C. lanatus* seed as a potential source of natural antioxidant and therefore an evidence for its ethnomedicinal usage.

INTRODUCTION

Reactive Oxygen Species (ROS) are free radicals, generated from oxygen and this causes damage to other molecules by extracting electrons from them to attain stability [1]. ROS including superoxide radicals, hydroxyl radicals, singlet oxygen and hydrogen peroxide are often generated as byproducts of biological reactions or from exogenous factors [2]. ROS readily attack and induce damage to various bio-molecules including proteins, lipids, lipoproteins and DNA [3], eventually leading to many chronic human diseases, such as cancer, diabetes, aging, atherosclerosis, arthritis, neurodegenerative and cardiovascular diseases [4]. Sources of free radicals include metabolism byproducts, neutrophils, radiations, pollutants, fatty foods, hazardous chemicals and cigarette smoke [5]. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reactions can produce free radicals which start a chain reaction that damages cells [6].

Medicinal properties of plants have been investigated recently throughout the world, due to their potent antioxidant activities, no side effects and economic viability [7]. One of the areas, which attracted a great treaty of attention in recent years, is antioxidant in the control of degenerative diseases in which oxidative dent have been implicated. Several plant extracts have shown antioxidant activity [8].

Citrullus lanatus (Family: Cucurbitaceae) is commonly known as water melon and locally as “Tharpooshini”. The ripe fruits are edible and largely used for making confectionery. Its nutritive values are also useful to the human health. Fruit is used in cooling, strengthening, aphrodisiac, astringent to the bowels, indigestible, expectorant, diuretic and stomachic, purifies the blood, allays thirst, cures biliousness, good for sore ears, scabies, itches, and as brain tonic [9]. The fruits are reported for laxative activity [10], and antioxidant activity [11].

Thus the present study focuses on evaluating the antioxidant activity of methanolic extract of *Citrullus lanatus* seed along with qualitative and quantitative phytochemical analysis.

MATERIALS AND METHODS

Plant collection and extraction

Citrullus lanatus seeds were collected from Vellore District, Tamilnadu. The plant was authenticated by Prof. Jayaraman, Plant Anatomy Research Centre, Chennai and a voucher specimen was deposited in the herbarium (PARC/2012/1195). It was shade-dried at room temperature and coarsely-powdered and subjected to soxhlet extraction with methanol for 24 hours. The extract was filtered and solvent was completely removed by using rotary evaporator. The residue, *C. lanatus* methanol seed (CLS) extract was stored at 4°C until required for use.

Preliminary phytochemical analysis

CLS extract was qualitatively tested for the presence or absence of chemical constituents like phenols, reducing sugars, flavones, glycosides, saponins, alkaloids, steroids, anthroquinones, quinones and tannins [12].

Quantification of primary metabolites

Total protein content

Protein content of CLS was estimated by Lowry method [13]. To 0.1 ml of CLS, 5 ml of alkaline copper sulphate was added, mixed well and allowed to stand for 10 min. To this 0.5 ml of Folin-ciocalteu reagent was added and incubated at room temperature in the dark for 30 min. The absorbance of the reaction mixture was measured at 660 nm. A standard graph of protein was plotted, from which the protein content of the extract was determined.

Total carbohydrate content

Carbohydrate content of CLS was measured by taking 0.5 ml CLS and 4 ml of anthrone reagent. The mixture was heated for 8 min in water bath and cooled. The absorbance of the reaction mixture was measured at 630 nm. A standard graph of glucose was plotted from which the carbohydrate content of CLS was determined [14].

Total lipid content

Lipid content of CLS was measured by taking 0.1 ml of CLS with 4 ml of ferric chloride acetic acid reagent and kept at room temperature for 10 minutes. To this 3 ml of 85% concentrated sulphuric acid was added. The mixture was kept at ice cold condition for 20 minutes. The colour intensity was read at 540 nm. A standard graph of cholesterol was plotted, from which the lipid content of CLS was determined [15].

Quantification of secondary metabolites

Determination of total phenol content

Total phenolic content was measured using Folin-ciocalteu phenol reagent [16]. To 0.1 ml CLS, 5 ml of Folin-ciocalteu phenol reagent and 4 ml of 7.5% sodium carbonate was added. The mixture was allowed to stand for 15 min under room temperature. The absorbance was measured at 765 nm. Total phenol was calculated from a standard graph of gallic acid and the results were expressed as gallic acid equivalents (GE/g).

Determination of total flavonoid content

Total flavonoid content was determined by using aluminium chloride method [17]. To 0.5 ml CLS, 4.5 ml of methanol, 0.1 ml 10% aluminium chloride and 0.1 ml of 1M sodium acetate was added. The mixture was allowed to stand for 30 min under room temperature. The absorbance was measured at 415 nm. Total flavonoid was calculated from a standard graph of quercetin and the results were expressed as quercetin equivalents (QE/g).

Determination of total tannin content

Total tannin content was measured using Folin-phenol reagent [18]. To 0.1 ml CLS, 0.5 ml of Folin-phenol reagent and 5 ml of 35% sodium carbonate was added. The mixture was allowed to stand for 5 min under room temperature. The absorbance was measured at 640 nm. Total tannin was calculated from a standard graph of gallic acid and the results were expressed as gallic acid equivalents (GE/g).

Determination of vitamin C content

The reducing capacity of CLS was expressed as vitamin C content [19]. To 1 ml CLS, a drop of thiourea and 0.25 ml of 2% dinitro phenylhydrazine were added and incubated at 37°C for 3 hours. Then 1.25 ml of 85% sulphuric acid was added under ice-cold condition and kept at room temperature for 30 minutes. The absorbance was measured at 540 nm.

Determination of vitamin E content

Total antioxidant capacity of CLS was expressed as vitamin E content [20]. 1 ml CLS was combined with vitamin E reagent solution and incubated in boiling water bath at 95°C for 90 min. The mixture was cooled to room temperature and absorbance was measured at 695 nm.

In vitro antioxidant assays**DPPH radical scavenging activity**

The DPPH scavenging activity of CLS was measured by adding 10 µl of CLS to 190 µl DPPH in ethanol [21]. After vortexing, the mixture was incubated for 20 minutes at 37°C. Decolourization of DPPH was determined by measuring the absorbance at 517 nm. The percentage inhibition of DPPH radicals by CLS was determined by comparing the absorbance values of the control. IC₅₀ value was also determined. The inhibitory activity was calculated as:

$$\% \text{ Inhibition} = \frac{\text{OD of control} - \text{OD of test}}{\text{OD of control}} \times 100$$

Lipid peroxidation inhibition assay

Lipid peroxide (LPO) inhibitory activity of CLS was performed using different concentration of the extract (1.95-1000 µg/ml) [22]. To 0.1 ml of tissue homogenate, lipid peroxidation was initiated by the addition of 0.1 ml of FeSO₄, 0.1 ml of ascorbate and 0.1 ml of KH₂PO₄. The mixture was made to 3 ml with distilled water and incubated at 37°C for 1 hr. 1 ml of TCA and 1 ml of TBA was added to this reaction mixture and the tubes were boiled for 30 min in water bath. This was centrifuged at 3500 rpm for 10 minutes. The extent of inhibition of lipid peroxidation was evaluated by the estimation of thiobarbituric acid reactive substances (TBARS) level by measuring the absorbance at 532 nm. The IC₅₀ value was determined. The percentage of the lipid peroxidation inhibition was calculated as:

$$\% \text{ Inhibition} = \frac{\text{OD of control} - \text{OD of test}}{\text{OD of control}} \times 100$$

Statistical analysis

Absorbance was read using UV/visible spectrophotometer (Perkin Elmer, Lambda 25, USA). Data were expressed as Mean ± SEM of triplicates. IC₅₀ value was calculated using Graph Pad Prism 5.0.3.

RESULTS AND DISCUSSION

Recently, much attention has been directed toward extracts and biologically active compounds isolated from popular plant species. The use of medicinal plants plays a vital role in covering the basic health needs in developing countries, and these plants may offer a new source antioxidant activity. The use of traditional medicine is widespread and plants still represent a large source of natural antioxidants that might serve as leads for the development of novel drugs. Several anti-inflammatory, digestive, anti-necrotic, neuroprotective, and hepatoprotective drugs have recently been shown to have an antioxidant and/or radical scavenging mechanism as part of their activity [23]. Antioxidants have the ability to protect organisms from damage caused by free radical-induced oxidative stress. A lot of research is being carried out worldwide directed toward finding natural antioxidants of plant origin. The antioxidant activity of the methanolic extract of *Citrullus lanatus* seeds is reported along with the screening of phytochemical constituents.

Table 1: Preliminary phytochemical analysis

S. No.	Phytochemicals	Present / absent
1	Phenols	+++
2	Tannins	+++
3	Flavonoids	++
4	Glycosides	++
5	Saponins	+
6	Steroids	+
7	Carbohydrates	+++
8	Alkaloids	-
9	Anthroquinones	-
10	Quinones	-
11	Proteins	+++
12	Amino acids	++
13	Reducing sugar	+++

‘-’ absence; ‘+’ presence

Citrullus lanatus methanolic seed extract revealed the presence of tannins, saponins, flavonoids proteins and steroids (Table 1). This result indicates that the seed contain an appreciable amount of bioactive compounds [24]. Thus

chemical constituents of plants are desirable because such information will be valuable for synthesis of complex chemical substances and to screen for biological activities [25]. The secondary metabolites are directly responsible for different activity such as antioxidant, antimicrobial, antifungal, and anticancer [26].

Among the primary metabolites, protein content was the highest 20.77%, followed by carbohydrates and lipids. Vitamin C and E content of CLS were also moderate. The secondary metabolites were quantified, and the total phenol was 6.45 ± 0.05 GE/g followed by tannin and flavonoid content (Table 2).

Table 2: Quantitative analysis of primary metabolites

Phytochemicals	Content
Protein	20.77 ± 1.24 % w/w
Carbohydrate	18.30 ± 0.01 % w/w
Lipid	4.80 ± 0.01 % w/w
Phenol	6.45 ± 0.05 GE/g
Tannin	5.92 ± 0.15 GE/g
Flavonoid	1.30 ± 0.09 QE/g
Vitamin E	3.18 ± 0.04 mg/g
Vitamin C	2.65 ± 0.16 mg/g

Values are expressed as Mean \pm SEM (n=3)

In DPPH assay, CLS scavenged the stable DPPH free radical with increasing concentrations. The extract exhibited a significant dose-dependent inhibition of DPPH radical with minimum inhibition of 2.10% at 1.95 μ g/ml and maximum inhibition of 95.91% at 500 μ g/ml. The concentration of CLS extract showing 50% inhibition was at 28.77 μ g/ml. The DPPH is a relatively stable free radical. The assay is based on the measurement of the scavenging ability of antioxidants towards the stable radical DPPH [27]. DPPH is a stable free radical. On addition of antioxidant compounds, the DPPH gets decolorized which can be quantitatively measured from the changes in absorbance [28]. DPPH scavenging activities of CLS methanolic extract increased with the increase in concentration. In LPO inhibitory assay, CLS inhibited lipid peroxidation with increasing concentrations. The extract exhibited a maximum inhibition of 96.79% at 1000 μ g/ml and minimum inhibition of 48.66% 1.95 μ g/ml of CLS. The IC_{50} value of CLS was 123.8 μ g/ml (Figure 1). Assay of TBARS measures malondialdehyde present in the sample, as well as malondialdehyde generated from lipid peroxides by the hydrolytic conditions of the reaction [29]. *C. lanatus* methanolic seed extract inhibited lipid peroxidation significantly in a concentration-dependant manner.

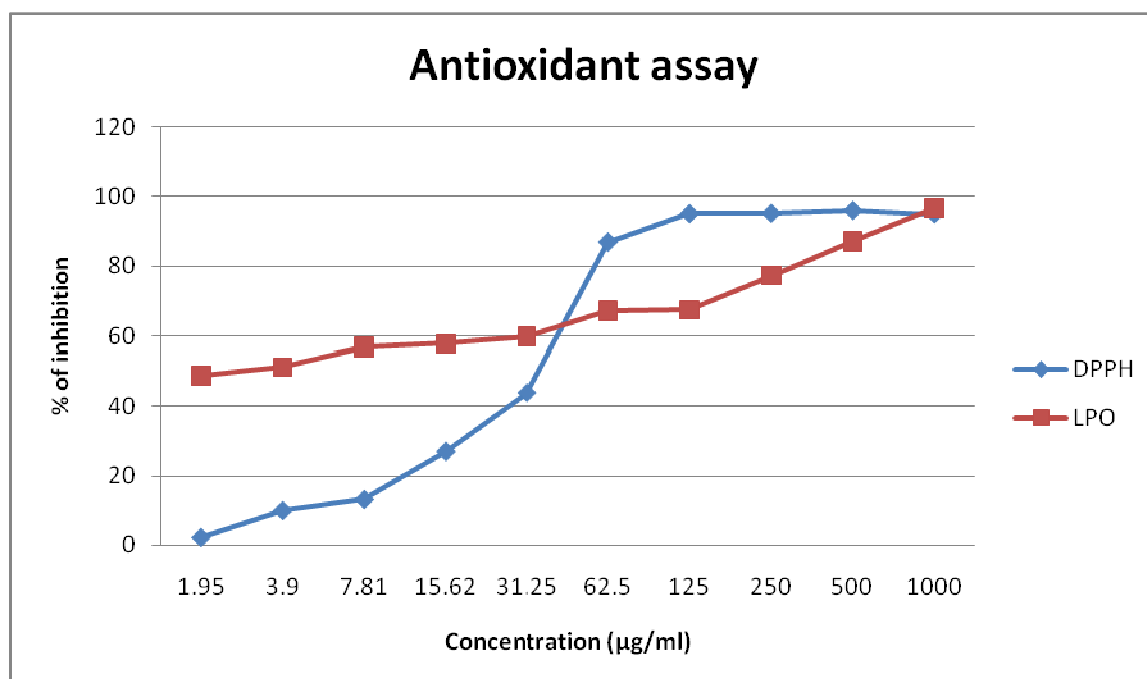


Figure 1: Antioxidant activity of *Citrullus lanatus* seed methanol extract

CLS extract showed a promising result of antioxidant activity which is similar to the previously reported works on *C. lanatus* seed where the n-hexane extract had the highest antioxidant activity due to the presence of high phenolic content followed by ethanolic extract due to the presence of high flavonoid content [30]. The antioxidant activity of the extract increased with the raw material used in the assay. In the present study, the maximum inhibition was in the range of 95% in the presence of 500 µg/ml extract. With the addition of a larger amount of extract to the assay mixture, the degree of inhibition decreased, indicating a pro-oxidant effect. The quality of the antioxidants in the extracts was determined by the IC₅₀ values, denoting the concentration of the sample required to scavenge 50% of the free radicals. The explanation for the higher IC₅₀ value found in the experiment was because the sample used was a crude extract. Our results show that the extract had high effect of scavenging DPPH. The antioxidant effect may be due to the presence of phenolic compounds such as flavonoids.

The tested extract had higher phenol content and hence its greater antioxidant activity. The quality of the obtained extracts was affected mainly by the solvent used for extraction. Methanol is the best solvent for preparing herbal infusions, yielding the strongest antioxidant activity in the extract. The more the polar capacity of the extract, the greater is its antioxidant activity [31].

All plants produce an amazing diversity of secondary metabolites. One of the most important groups of these metabolites is the phenolic compounds. Phenolic antioxidants inhibit lipid peroxidation by trapping the lipid alkoxyl radical. This activity depends on the structure of the molecules, and the number and position of the hydroxyl group in the molecules [32]. It has been shown that phenolics (especially flavonoids) are able to alter peroxidation kinetics by modifying the lipid packing order. They stabilize membranes by decreasing membrane fluidity (in a concentration-dependent manner) and hinder the diffusion of free radicals and restrict peroxidative reaction [33]. Flavonoids are oxidized by radicals, resulting in a more stable, less-reactive radical. In other words, flavonoids stabilize the reactive oxygen species by reacting with the reactive compound of the radical. Because of the high reactivity of the hydroxyl group of the flavonoids, radicals are made inactive [34]. Thus the study confirmed a strong antioxidant activity of *Citrullus lanatus* seed due to the content of phenolic compounds in the extracts.

CONCLUSION

Citrullus lanatus seed methanolic extract showed a strong antioxidant activity by scavenging DPPH and inhibiting lipid peroxidation. In addition, the extract was found to contain noticeable amount of total phenols and flavonoids, which play a major role in controlling oxidation generated by free radicals. The results of this study show that the methanolic extract of *Citrullus lanatus* seed can be used as an easily accessible source of natural antioxidant. However, the phytoconstituents responsible for the antioxidant activity of the extract are not much clear. Therefore, a further study is needed to determine the mechanism behind the antioxidant activity of this plant.

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