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IN-VITRO ANTIOXIDANT AND IMMUNOMODULATORY ACTIVITY OF CITRULLUS LANATUS SEED

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ABSTRACT

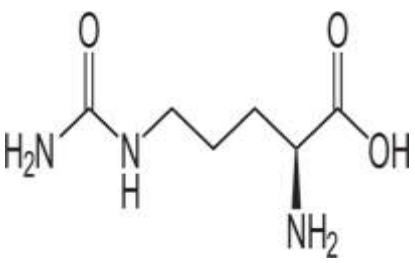
To explore antioxidant, and immunomodulator activities of Citrullus lanatus seed extract by utilizing in-vitro models. Hydroalcoholic extract of Citrullus lanatus seeds was prepared and arranged in the different concentrations to study the antioxidant activity by different methods and immunomodulatory activity also.. The present investigation will be supportive to the scientific documentation related in-vitro studies. The DPPH Free radical scavenging assay results revealed that the hydroalchoholic Citrulus lanatus seed extracts were found to have good antioxidant activity. The IC50 value of hydrogen peroxide scavenging activity of hydroalchoholic extract of Citrullus lanatus seeds was found to be 290µg/ml. The reducing capabilities of hydroalchoholic seed extract and Immunomodulatory activity by NBT assay was found to be in dose dependent manner. In-vitro studies may be helpful to understand the molecular mechanism of oxidation and to reveal phytochemicals of the extract responsible for showing antioxidant and immunomodulator activity. Citrullus lanatus seeds extract exhibited significant in-vitro antioxidant and Immunomodulatory activity.

KEYWORDS: Antioxidant, Immunomodulator, In-vitro.

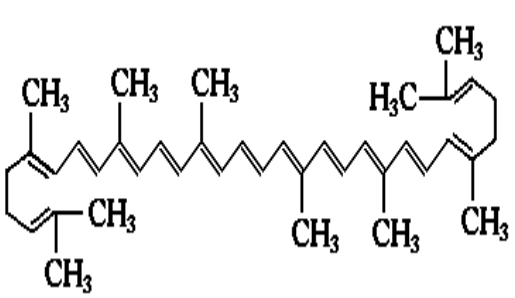
INTRODUCTION

Watermelon (*Citrullus lanatus*), from the family of cucumber (Cucurbitacea), is a large, oval, round or oblong tropical fruit[1]. The skin is smooth, with dark green rind or sometimes pale green stripes that turn yellowish green when ripe. It is a very rich source of vitamins and also serves as a good source of phytochemicals[2].

The therapeutic effects of *C. lanatus* fruit have been reported and attributed to certain phytochemical compounds[3]. For instance, beta carotene and lycopene have been established to play a key role in the treatment of cancer and cardiovascular diseases[4]. The therapeutic effect of watermelon has been reported and has been ascribed to antioxidant compounds[5-6]. Among them, citrulline (It is an α-amino acid) and lycopene (It is an acyclic isomer of ß -carotene. It is a 40 carbon atom, open chain polyisoprenoid with 11conjugated double bonds. Its molecular formula is $C_{40}H_{56}$.) have been demonstrated to play a prominent role in the treatment and management of ailments such as cancer and cardiovascular diseases[7]. It also reported having analgesic and anti-inflammatory of seeds[8], anti-ulcerative activity[9-10], antimicrobial activity[11], laxative activity of fruit[12], antioxidant of fruit [13], and hepatoprotective[14].



Citrulline



Lycopene

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MATERIALS AND METHODS

Chemical and Reagents

DPPH- (1, 1- dipheny l-2- pieryl-hydrazyl) (Sigma chemicals Ltd), Ascorbic Acid (S.D. Fine Chemicals). All the chemicals used in this study were of analytical grade.

Plant Material

The seeds of plant *Citrullus lanatus* L. were collected from the market samples from Maraimalai nagar, Tamil nadu, India in the month of September, 2015. The plant was taxonomically identified by Professor Dr. Jayaraman, Taxonomist, Chennai. The dried seeds (100 gm) were powdered and subjected at maceration using n-Hexane as a de-fatting agent at room temperature. After filtration, the extractive solution was evaporated to dryness by rotary evaporator (40 °C). The marc was extracted with continuous soxhlet extraction for 7 days using ethanol: water (70:30) as solvent. After filtration, the extractive solution was evaporated to dryness by rotary evaporator (40°C). The yield of the defatted hydroalcoholic extract was 7.4%.

Phytochemical Investigations

Chemical tests were carried out on the extract using the standard procedure to identify the constituents as described by Sofowora[15] and Trease and Evans[16].

Determination of Total Flavonoids

Aluminum chloride colorimetric method was used to determine Total Flavonoids contents (TFC) in extracts. [17] briefly, an aliquot of 0.5 ml of 2% AlCl3 was added to 0.5 ml of sample solution. After 1 h at room temperature, the absorbance was measured at 420 nm at the final concentration of 1000 µ/ml). TFC was calculated as mg quercetin equivalent (QE) /100 g sample dry weight. The calibration curve was prepared by quercetin solution (0 - 100µg/ml) in ethanol.

In-vitro Antioxidant Activity

Determination of DPPH Radical Scavenging Activity

The radical scavenging activity of the extract was determined using Schmeda- Hirschmann et al. method[18-19]. Accurately 6 ml of DPPH (20µg/ml) methanolic solution was added to 20 µl of DMSO solution of each extract at room temperature. The mixture was shaken vigorously and absorbance was measured at about 517 nm using spectrophotometer.

Determination of Hydrogen Peroxide Scavenging Activity

Scavenging activity of Hydrogen peroxide (H2O2) by the plant extract was determined by the method (20). Plant extract (4 ml) prepared in distilled water at various concentration (50, 100, 200, 400, 800 µg/ml) was mixed with 0.6 ml of 4 mM H2O2 solution prepared in phosphate buffer (0.1 M pH 7.4) and incubated for 10 min. The absorbance of the solution was taken at 230 nm. Ascorbic acid was used as a positive control compound. The percentage of inhibition was calculated by comparing the absorbance values of the control and test samples using Eq. given below. IC50 values were estimated from the % inhibition versus concentration plot, using a non-linear regression algorithm.

$$\% \text{ inhibition} = \frac{(\text{Absorbance of control} - \text{absorbance of sample})}{\text{Absorbance of control}} \times 100$$

Ferric Reducing Antioxidant Power Assay

1 ml of different concentrations (25 to 900 µg/ml) of the extract fractions was mixed with potassium ferricyanide (2.5 ml, 1%) 2.5 ml of phosphate buffer (pH 6.6). The mixture was incubated at 50°C for 20 min. 2.5 ml TCA (10%) was added to it and centrifuged at 3000 rpm for 10 min. 2.5 ml of supernatant was taken and 2.5 ml water and 0.5 ml FeCl3 (0.1%) were added to it. The absorbance was measured at 700 nm. Higher absorbance of the reaction mixture indicated higher reducing power [21-22].

In-Vitro Immunomodulatory Activity Using Nitroblue Tetrazolium Test (NBT)

The assay mixture consisted of 0.2 ml of 5 × 106/ml of leucocyte suspension and 0.2-ml freshly prepared 0.15% NBT solution. 0.1 ml of test substance at different concentrations was added to the reaction mixture. 0.1 ml of endotoxin-activated plasma was added to the 0.15% NBT solution and leucocytes which served as a positive control (standard). A normal control was maintained in another test tube with leucocytes suspension, distilled water and NBT solution. All the test tubes were incubated separately at 37ºC for 20 min and centrifuged gently

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at 400g for 3-4 min. The supernatant was discarded. A drop of PBS was added and the cells were gently re-suspended at the bottom of the test tube. A film was made by allowing a drop of this fluid to dry on a microscope slide. Slides were dried for 10-15 mins. Methanol fixation was carried out and again slides were kept for drying purpose. Slides were further stained in Carbol Fuschin or Giemsa stain for 15 mins and washed under tap water. After complete drying, the slides were observed under light microscope with oil immersion objective. 200 neutrophils were counted and the % of NBT positive cells containing the blue spots (stimulated) were determined[24-26].

RESULT AND DISCUSSION

Phytochemical Investigations

The defatted hydroalcoholic extract of *Citrullus lanatus* seeds were tested for different phytoconsituents like alkaloids, glycosides, saponinins, tannins, terpinoids, reducing sugars, phenolic compounds, flavanoids, protein, carbohydrates and volatile oils. The Knowledge of the chemical constituents of plants is desirable because such information will be valuable for synthesis of complex chemical substances and to screen for biological activities

Determination of Total Flavonoids

Flavonoids are the largest group of naturally occurring phenolic compounds, which occurs in different plant parts both in free state and as glycosides. They are found to have many biological activities including antimicrobial, mitochondrial adhesion inhibition, antiulcer, antiarthritic, antiangiogenic, anticancer, protein kinase inhibition, etc[27]. The flavonoids have two benzene rings separated by a propane unit. The flavones and flavonols are the most widely distributed of all the phenolics[28]. Flavonoids are particularly beneficial, acting as antioxidants and giving protection against cardiovascular disease, certain forms of cancer and age-related degeneration of cell components. But in total Flavanoid content, it was found defatted hydroalcoholic extract to posses 15 µg/mg equivalent of Quercetin. Flavonoids play some important pharmacological roles against diseases, such as cardiovascular disease, cancer, inflammation and allergy and other oxidative stress related diseases[29].

Antioxidant Activity

In-vitro antioxidant studies are widely carried to screen various plant containing phenolic and flavanoids constituents. Plant derived antioxidant compounds, flavonoids and phenolics have received considerable attention because of their physiological effect like antioxidant, anti-inflammatory, antitumor activities and low toxicity compared with those of synthetic phenolics antioxidant such as BHA (Butylated Hydroxyanisole), BHT (Butylated Hydroxytoluene) and Propyl Gallate(PG)[30-31].

DPPH is a purple colored stable free radical; when reduced it becomes the yellow-colored diphenyl-picryl hydrazine. DPPH radicals react with suitable reducing agents and then electrons become paired-off and the solution loses colour stoichimetrically with the number of electrons taken up. Such reactivity has been widely used to test the ability of compounds/plant extracts to act as free radical scavengers. In this present study, the DPPH radical scavenging activity of defatted hydroalcoholic extract of *Citrullus lanatus* seeds was detected and compared with Ascorbic acid. Though the extracts showed good DPPH scavenging activity but it was less effective than standard Ascorbic acid (Table 1). The IC_{50} values for DPPH assay of defatted hydroalcohlic extract was found 285µg/ml (Table 2). The difference of activity is due to presence of flavonoid or phenolic components in extract. Thus, choosing the appropriate solvent is one of the most important factors for obtaining extracts with a high content of bioactive compounds and antioxidant activity[32].

Table 1: DPPH RADICAL SCAVENGING ACTIVITY FOR ASCORBIC ACID

S.No.	Concentration (µg/ml)	% Inhibition
1.	12.5	29
2.	25	45
3.	50	63
4.	100	80

Table 2: DPPH RADICAL SCAVENGING ACTIVITY FOR SAMPLE EXTRACT

S.No.	Concentration of Extract (µg/ml)	% Inhibition
1.	100	27
2.	200	44
3.	400	62

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4.	800	83
5.	1000	90

In ferric reducing antioxidant assay, a yellow colour of the test solution changes to various shades of green and blue as per the reducing power of phytoconstituents present in sample. The presence of radicals causes the conversion of the Fe 3+ / ferricyanide complex to the ferrous form.The reductive capabilities of defatted hydroalcoholic extract of Citrullus lanatus seeds was detected and compared with Ascorbic acid. The ferric reducing antioxidant activity of defatted hydroalcoholic extract was found maximum at 800µg/ml (Figure 1). The reducing power in the extracts indicated that some components in the extract were electron donors that could react with the free radicals to convert them into more stable products to terminate radical chain reaction. Antioxidants are strong reducing agents and this is principally based on the redox properties of their hydroxyl groups and the structural relationships between different parts of their chemical structure[33-34].

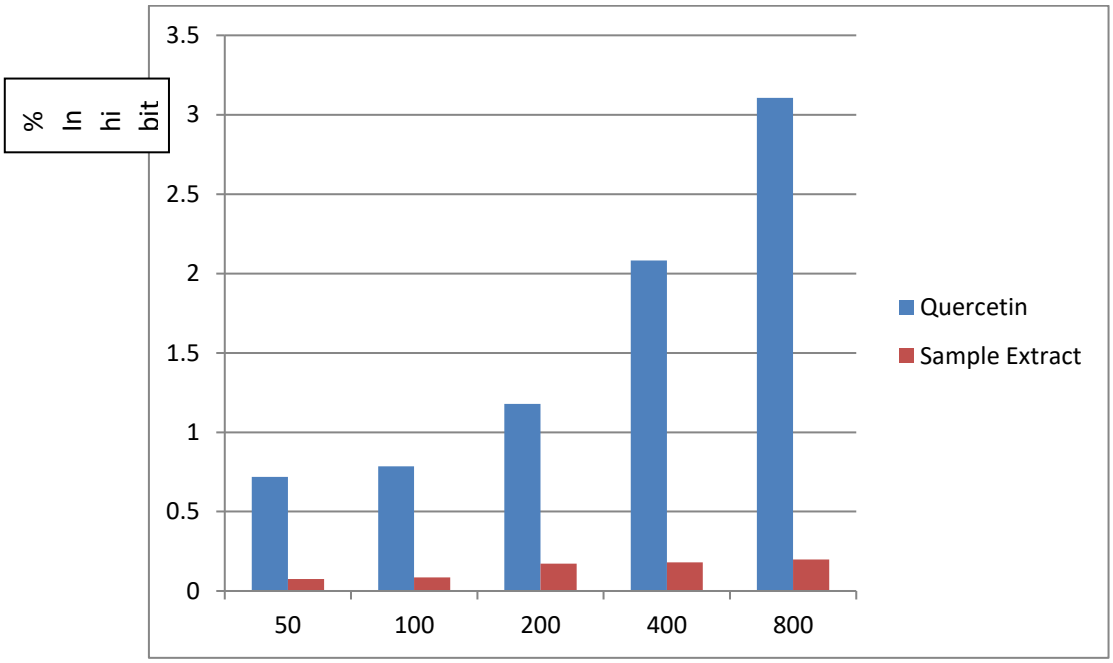

FIGURE1: Reducing Ability Of Hydroalchoholic Extract Of Citrullus lanatus Seeds

Hydrogen peroxide (H2O2), a biologically relevant, non-radical oxidizing species, may be formed in tissues through oxidative processes. Hydrogen peroxide (H2O2) which in turn generate hydroxyl radicals (•OH) resulting in initiation and propagation of lipid peroxidation[35] The hydrogen peroxide scavenging activity of hydroalcohlic extract of *Citrullus lanatus* seeds were detected and compared with Ascorbic acid. The IC_{50} values for hydrogen peroxide scavenging activity of defatted hydroalcoholic extract was found 290µg/ml (Figure 2). Though the extracts showed good hydrogen peroxide scavenging activity but it was less effective than standard Ascorbic acid. The ability of the extracts to quench OH^- seems to be directly related to the prevention of the lipid peroxidation and appears to be moderate scavenger of active oxygen species, thus reducing rate of chain reaction.

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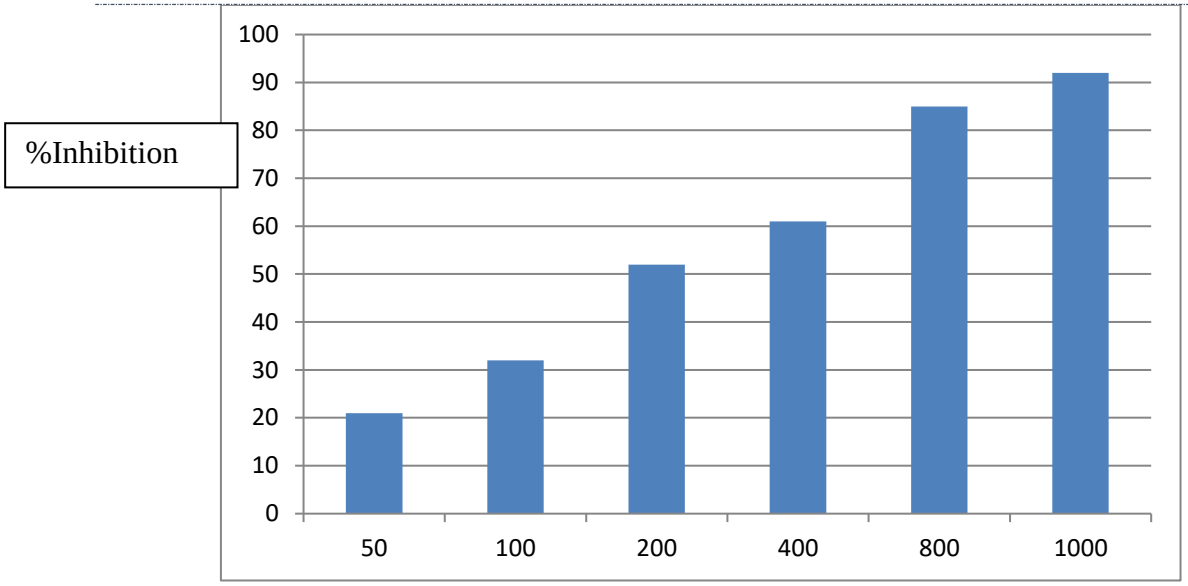

Figure:2: Hydrogen Peroxide Scavenging Activity of Hydroalchoholic Extract Of Citrullus lanatus Seeds

Immunomodulatory Activity

Nitroblue tetrazolium dye test is used to assess the Immunomodulatory activity of the test compound by determining its ability to stimulate the phagocytic activity in leucocytes. Once stimulated, the membrane permeable, water soluble, yellow-colored, nitroblue tetrazolium is reduced to blue NBT formazan crystals by the leucocytes. The *C. lanatus* stimulated phagocytic activity of the leucocytes in a concentration dependent manner as seen by the increased percentage of NBT positive cells. It showed 2.5±0.28 %, 37.6 ±4.03, 57.00±2.82 and 63.00±5.65% of NBT positive cells at the concentration of 100, 200, 400 and 800µg/ml respectively (Figure 3).

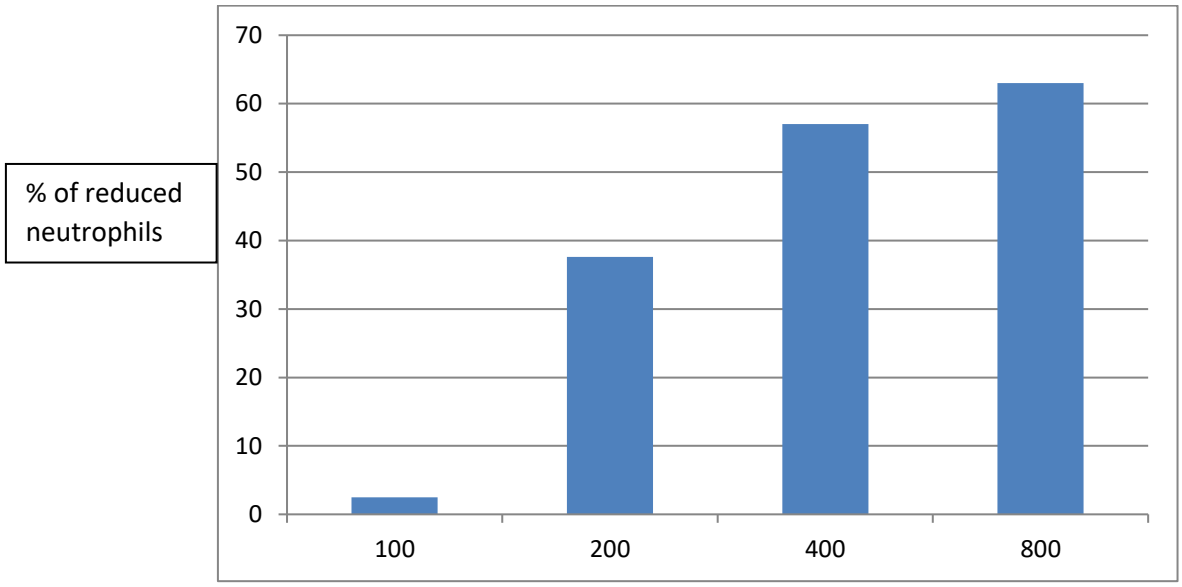

Figure3: Percentage of Reduced Neutrophils after treatment with extracts by Nitroblue Tetrazolium Test (NBT)

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CONCLUSION

In conclusion, on the basis of the results of this investigation, the seeds of *C. lanatus* could be considered as promising candidate from which relatively safe antioxidant, and Immunomodulator constituents might be obtained to prevent the disease induced by oxidative pathways inside body. Thus Hydroalcoholic extract could be further analyzed in vivo and further characterization of its active compound could lead to the discovery of a new candidate drug for the patient with HIV and Cancer as the plant showed immnunomodulatory effect in a dose dependent manner, as a immunotherapeutic agents in the near coming future.

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