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Research Article

Evaluation of the Neuroprotective Potential of Citrullus Lanatus Seeds: An In-Vitro Study

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Neurodegenerative disorders associated with oxidative stress and neuronal damage represent a growing global health concern, necessitating the exploration of novel plant-based therapeutic agents. Citrullus lanatus (watermelon) seeds are known to contain diverse bioactive phytoconstituents such as flavonoids, phenolic compounds, alkaloids, and essential fatty acids that may exhibit neuroprotective properties. The present study aimed to evaluate the in vitro neuroprotective potential of Citrullus lanatus seeds extracts in hexane and methanol. The seeds were processed and extracted using hexane and methanol as solvents, followed by phytochemical screening to identify major secondary metabolites. Neuroprotective activity was assessed through in vitro antioxidant assays, including free radical scavenging assays (DPPH and ABTS) and reducing power assays (FRAP), along with evaluation of enzyme inhibitory activity relevant to central nervous system (CNS) disorders. Among the tested extracts, the methanol extract exhibited the highest antioxidant and neuroprotective activity. The results demonstrated significant antioxidant activity in a concentration-dependent manner, indicating the ability of the extracts to neutralize oxidative stress, a key factor in neuronal degeneration. The presence of phenolic and flavonoid compounds is suggested to contribute to the observed neuroprotective effects. The findings indicate that Citrullus lanatus seeds possess promising neuroprotective potential and may serve as a natural source for the development of therapeutic agents targeting CNS disorders. Further in vivo and clinical studies are recommended to validate these effects and elucidate the underlying mechanisms of action.

Keywords: *Citrullus lanatus*, neuroprotection, in-vitro study, antioxidant activity, CNS disorders, phytochemicals.

INTRODUCTION

Neurodegenerative disorders affecting the central nervous system (CNS), including Alzheimer's disease, Parkinson's disease, epilepsy, and other cognitive impairments, have emerged as major global health challenges due to their increasing prevalence and limited therapeutic options. These disorders are commonly associated with oxidative stress, neuroinflammation, mitochondrial dysfunction, and progressive neuronal damage. Excessive production of reactive oxygen species (ROS) plays a crucial role in neuronal degeneration by damaging cellular proteins, lipids, and DNA, ultimately leading to impaired neuronal function and neuronal cell death [Uttara et al., 2009 and Barnham et al., 2004]. Therefore, the search for natural neuroprotective agents possessing antioxidant potential has gained considerable scientific attention. Medicinal plants represent an important source of bioactive compounds with therapeutic properties and relatively fewer adverse effects compared to synthetic drugs. Plant-derived phytochemicals such as flavonoids, phenolic acids, alkaloids, and terpenoids have been widely reported to exhibit antioxidant, anti-inflammatory, and neuroprotective activities [Edeoga et al., 2005].

These compounds can scavenge free radicals, modulate neurotransmitter systems, and protect neurons against oxidative injury and neurotoxicity [Spencer JPE 2009]. *Citrullus lanatus* (watermelon), belonging to the family Cucurbitaceae, is widely cultivated in tropical and subtropical regions and is primarily consumed as a nutritious fruit. Apart from its dietary importance, different parts of the plant, including seeds, rind, and pulp, have been traditionally used for various medicinal purposes. The seeds of *Citrullus lanatus* are rich in proteins, essential fatty acids, vitamins, minerals, and biologically active phytoconstituents such as phenolics and flavonoids, which are known to possess antioxidant and pharmacological properties [Perkins-Veazie et al., 2006]. Previous studies have indicated that these bioactive constituents may contribute to protective effects against oxidative stress–mediated diseases and metabolic disorders [Naz et al., 2014]. Despite the nutritional and medicinal significance of *Citrullus lanatus* seeds, their neuroprotective potential remains insufficiently explored. Investigation of their biological activity may provide valuable insights into the development of plant-based therapeutic agents for CNS disorders. In-vitro experimental models serve as reliable preliminary tools for evaluating antioxidant capacity and neuroprotective effects prior to in-vivo investigations [Szymańska et al., 2016]. Therefore, the present study aims to evaluate the neuroprotective potential of methanol and hexane extracts of *Citrullus lanatus* seeds through in-vitro assays, focusing on antioxidant activity and their possible protective role against oxidative stress associated with neuronal damage. The findings of this study may contribute to the identification of natural neuroprotective compounds and support future research toward the development of safer and more effective CNS therapeutics.

MATERIAL AND METHODS

2.1. Preparation of Extracts

Seeds of Citrullus lanatus were purchased from the Gwalior (India) market and air-dried, then powdered using a grinder. The powdered plant seed material was extracted with polar solvents such as methanol and non-polar solvent, i.e., n-hexane using the Soxhlet method.

2.2. Qualitative phytochemical analysis

Tests for flavonoids, tannins, carbohydrates/ glycosides, proteins, saponins, resins, steroids, terpenoids and alkaloids were carried out using standard methods (Harborne, 1973; Trease and Evans, 1989).

2.2.1. Quantification of Total Phenolic Content (TPC)

Total phenolic content (TPC) was estimated using the method described by [Attar et al. 2017] with slight modifications. Briefly, 40 mL of the sample solution (5 mg/mL stock) and standard tannic acid were added to a 5 mL test tube. Then, 1 mL of pre-diluted (1:10) Folin–Ciocalteu reagent and 0.8 mL of sodium carbonate solution (7.5% w/v) were added to the mixture. The reaction mixture was thoroughly mixed and allowed to incubate at room temperature for 60 min. Following incubation, the absorbance of the mixture was recorded at 765 nm using a Double beam UV–Vis spectrophotometer (Lab Tronics, Model LT-2203). The total phenolic content was calculated and expressed as milligrams of tannic acid equivalents (mg TAE) per gram of extract.

2.2.2. Determination of tannin contents

The tannin content of each sample was estimated using insoluble polyvinyl-polypyrrolidone (PVPP), a tannin-binding agent, according to the method reported by Makkar et al. (1993).

2.2.3. Determination of flavonoids and flavanols

The flavonoid content was determined according to the method described by Kumaran and Karunakaran (2006) with slight modifications. The method was based on the formation of a flavonoid–aluminum complex, which shows maximum absorbance at 415 nm. The absorbance of the standard rutin solution (0.5 mg/mL) prepared in methanol was measured under the same conditions. The amount of flavonoids present in both solvent seed extracts, expressed as rutin equivalents (RE), was calculated using the following formula:

Flavonoid content = $(A \times m°) / (A° \times m)$

where A represented the absorbance of the seed extract solution, A° represented the absorbance of the standard rutin solution, m represented the weight of the seed extract (mg), and m° represented the weight of rutin in the solution (mg). The flavonoid content was expressed as mg rutin equivalents per mg of plant extract.

2.2.4. The flavanol

content was also determined according to the method described by Kumaran and Karunakaran (2006) with slight modifications. The amount of flavanols present in the plant extracts, expressed as rutin equivalents (RE), was calculated using the same formula as used for flavonoid determination.

2.2.5. Determination of Total Alkaloid Content (TAC)

Total alkaloid content (TAC) was determined according to the method reported by Ghane et al. (2018). Briefly, 69.8 mg of bromocresol green was dissolved in 3 mL of 2 N NaOH and 5 mL of distilled water. The solution was heated and further diluted to 1000 mL with distilled water. Subsequently, 100 mL of plant extract (prepared from 5 mg/mL stock solution) was mixed with 1 mL of bromocresol green solution and 1 mL of sodium phosphate buffer. The reaction mixture was then extracted with 2 mL of chloroform. The absorbance of the chloroform layer was measured at 470 nm using a UV–Vis spectrophotometer. Galanthamine (20–120 mL) was used as the standard for calibration, and the total alkaloid content was expressed as milligrams of galanthamine equivalents (mg GE) per gram of extract.

2.3. Assessment of Acetylcholinesterase Inhibition using microplate

Acetylcholinesterase (AChE) inhibitory activity was determined using Ellman's colorimetric method, as described by [Ellman et al.1961] and modified by Eldeen et al. In a 96-well microplate, 25 µL of 15 mmol/L acetylthiocholine iodide (ATCI) prepared in water, 125 µL of 3 mmol/L DTNB in Buffer A (50 mmol/L Tris–HCl, pH 8, containing 0.1 mol/L NaCl and 0.02 mol/L $MgCl_2 \cdot 6H_2O$), 50 µL of Buffer B (50 mmol/L, pH 8, containing 0.1% bovine serum albumin), and 25 µL of plant extract at different concentrations (0.007, 0.016, 0.031, 0.063, and 0.125 mg/mL) were added. The absorbance was measured spectrophotometrically using a microplate reader (Labtronics Model LT-2203) at 405 nm at 45-second intervals for three consecutive readings. Subsequently, acetylcholinesterase enzyme (0.2 U/mL) was added to each well, and absorbance was recorded five times consecutively at 45-second intervals. Galantamine was used as a positive control. Any increase in absorbance due to spontaneous hydrolysis of the substrate was corrected by subtracting the absorbance recorded before enzyme addition from that recorded after enzyme addition. The percentage inhibition of acetylcholinesterase activity was calculated using the following equation:

$$\text{Inhibition (\%)} = 1 - (A_sample / A_control) \times 100$$

where A_ sample represents the absorbance of the sample extracts and A_control represents the absorbance of the blank solution (methanol/ethyl acetate in 50 mmol/L Tris–HCl buffer, pH 8). The extract concentration required to achieve 50% inhibition (IC_{50}) was determined by plotting the percentage inhibition against the corresponding extract concentrations.

2.4. Antioxidant assay

2.4.1. DPPH radical scavenging activity

DPPH radical scavenging activity was determined following the method described by (Brand-Williams et al. 1995). A methanolic solution of DPPH (0.025 g/L) was freshly prepared. An aliquot of plant extract (1 ml) at different concentrations (50–300 µg/ml) was mixed with 3 ml of DPPH solution. The reaction mixtures were incubated in the dark for 30 minutes, after which the decrease in absorbance was recorded at 515 nm. Antioxidant activity was calculated using a standard calibration curve prepared with different concentrations of ascorbic acid (20–100 µg/ml). The results were expressed as milligrams of ascorbic acid equivalent per gram of extract (mg AAE/g extract), with a correlation coefficient of $R^2 = 0.990$.

2.4.2. ABTS Radical Scavenging Activity

$ABTS^{\cdot+}$ radical scavenging activity was determined using the method described by Re et al. [18]. The ABTS radical cation was generated by reacting 7 mM aqueous ABTS solution with 2.45 mM potassium persulfate solution. The reaction mixture was allowed to stand at room temperature for 12–16 h in the dark. The resulting $ABTS^{\cdot+}$ solution was diluted with ethanol (1:89, v/v) and equilibrated at 30 °C to obtain an absorbance of 0.70 ± 0.02 at 734 nm. Different concentrations of plant extract (30–120 µg/ml) were mixed with 3 ml of the diluted $ABTS^{\cdot+}$ solution. After incubation for 30 min, the absorbance was measured at 734 nm. A calibration curve was prepared using Trolox standard solutions (20–120 µg/ml), and the antioxidant activity was expressed as milligrams of Trolox equivalents per gram of extract (mg TE/g extract). The correlation coefficient for the calibration curve was $R^2 = 0.996$.

2.4.3. Ferric Reducing Antioxidant Power (FRAP) Assay

The ferric reducing antioxidant power (FRAP) assay was performed according to the method described by (Patel et al., 2020). The FRAP reagent was prepared by mixing acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM HCl, and 20 mM $FeCl_3$ in the ratio of 10:1:1 (v/v/v). For the assay, 20 µL of plant extract (prepared from 5 mg/mL stock solution) was mixed with 1 mL of FRAP reagent. The reaction mixture was incubated at 37 °C in a water bath for 30 min. After incubation, the absorbance was measured at 593 nm using a UV–Vis spectrophotometer. Standard $FeSO_4 \cdot 7H_2O$ (40–160 µg/mL) was used to construct the calibration curve. The results were expressed as milligrams of Fe (II) equivalents per gram of extract. For α-glucosidase inhibitory activity, the absorbance was measured at 405 nm. The control was prepared without any extract, and acarbose was used as a positive control. The inhibition of α-glucosidase activity was calculated and expressed as percentage inhibition.

2.4.4. Metal Chelating Activity (MC)

The metal chelating activity of Citrullus lanatus seeds extracts was determined to evaluate the total antioxidant capacity, following the method previously reported by (Attar and Ghane 2019). Briefly, 50 µL of 2 mM $FeCl_2$ solution was added to 40 µL of plant extract (5 mg/mL stock) or standard EDTA solution (25–100 µg/mL). The reaction was initiated by adding 100 µL of 5 mM ferrozine solution, followed by the addition of 650 µL of distilled water to make up the final volume. The reaction mixture was vigorously shaken and allowed to stand at room temperature for 10 min. Subsequently, the absorbance of the solution was measured at 562 nm using a UV–Vis spectrophotometer. Na_2-EDTA was used as a positive control. The metal chelating activity was expressed as milligrams of EDTA equivalents (mg EE) per gram of extract.

2.4.5. Phosphomolybdate Assay (PMA)

Total antioxidant capacity was evaluated using the phosphomolybdate assay following the method described by (Prieto et al. 1999) with minor modifications. In this assay, 20 µL of plant extract (5 mg/mL stock) or standard ascorbic acid (25–100 µg/mL) was mixed with phosphomolybdate reagent containing 0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate. The reaction mixture was then incubated in a water bath at 90 °C for 90 min. After incubation, the mixture was cooled to room temperature, and the absorbance was measured at 695 nm using a UV–Vis spectrophotometer. The total antioxidant capacity was expressed as milligrams of ascorbic acid equivalents (mg AAE) per gram of extract.

RESULTS AND DISCUSSION

3.1. Preliminary phytochemical screening of methanol and hexane extracts

3.1.1. Total Phenolics

Phenolic compounds are a natural group of secondary metabolites that are known to effectively scavenge free radicals in biological systems. A higher phenolic content has been reported to be linearly correlated with enhanced antioxidant activity [Roya et al., 2013]. Due to their diverse health benefits, phenolics have gained considerable attention among researchers and consumers. In addition, phenolic compounds have been reported to possess anticarcinogenic, antiatherogenic, anti-inflammatory, and antimicrobial properties [Scalbert et al., 2005]. In the present investigation, higher phenolic content was observed

in the methanol extracts of seeds, with values of 146.39 ± 1.9mg, respectively (Table 1). Moderate phenolic content was exhibited by the hexane extracts of seeds.

3.1.2. Total tannins

Tannins are high molecular weight phenolic compounds that exhibit multiple biological activities, including metal ion chelation and protein precipitation, and are considered natural antioxidants. It has been reported that tannins demonstrate stronger antioxidant activity compared to low molecular weight phenolics (Yokozawa et al., 1998). In the present study, a higher tannin content was observed in the methanol extract of seeds compared to the hexane extract (Table 1). The total tannin content of the methanol seed extract was recorded as 12.19 ± 1.8 mg, whereas a comparatively lower (3.43±2.0) tannin content was observed in the hexane seed extract. These findings indicate that methanol was more effective in extracting tannin compounds from the seeds.

3.1.3 Total flavonoids

Flavonoids are one of the most common groups of natural antioxidants. Due to their high redox potential, they act as singlet oxygen quenchers, reducing agents, hydrogen donors, and metal chelators (Cao et al., 2009). In the present analysis, a higher flavonoid content was observed in the hexane extract compared to the methanol extract (Table 1). The flavonoid content of the seed extracts was recorded as 14.90 ± 0.9 mg in the methanol extract and 15.05 ± 0.3 mg in the hexane extract, indicating that the hexane extract exhibited slightly higher flavonoid content.

3.1.4. Total Alkaloids

Alkaloids are important secondary metabolites that were discovered and utilized as early as 4000 years ago and are well recognized for their rich therapeutic potential. Alkaloids have been reported to exhibit antiproliferative, antibacterial, and antioxidant activities, making them valuable candidates for drug development (Amirkia and Heinrich, 2014). In the present study, the alkaloid content of the seed extracts was determined, and a higher amount was observed in the hexane extract compared to the methanol extract. The alkaloid content was recorded as 8.90 ± 0.5 mg in the methanol extract and 82.07 ± 0.9 mg in the hexane extract, indicating that the hexane extract exhibited higher alkaloid content.

3.2. Antioxidant assays

3.2.1. DPPH radical scavenging activity

The method is based on the reduction of DPPH radicals by antioxidants, resulting in the formation of a more stable DPPH molecule (Patel et al., 2020). The methanolic solution of DPPH exhibits a deep violet color with strong absorption at 515 nm. This absorption decreases due to a color change from violet to yellow, which occurs when the DPPH radicals are scavenged by plant antioxidants. In the present study, among the seed extracts, higher radical scavenging activity was observed in the methanol seed extract (25.02 ± 0.08 mg AAE/g extract). In contrast, the hexane extract showed comparatively lower radical scavenging activity (20.05 ± 0.16 mg AAE/g extract) (Fig. 1). These results are in good agreement with the findings of Kikuchi et al. (2015), who reported that the methanol extract of Citrullus lanatus seeds exhibited significant DPPH radical scavenging activity, with an IC_{50} value of 78.56 µg/ml.

3.2.2. ABTS radical scavenging activity

In this assay, the green color of the ABTS radical solution gradually faded and became colorless due to the reduction of the ABTS radical by antioxidants (Patel et al., 2018). In the present study, the hexane and methanol seed extracts exhibited higher radical scavenging capacity (8.60 ± 0.38 and 15.12 ± 0.08 mg TE/g extract, respectively) (Fig. 2). These results are consistent with the findings of D. Neglo, C. O. Tettey, E. K. Essuman et al. (2021), who reported that the methanol extract of Citrullus lanatus seeds showed higher ABTS radical scavenging activity compared to the hexane extract.

3.2.3. FRAP Assay

The FRAP assay is based on the ability of antioxidant compounds to reduce the TPTZ-Fe (III) complex to an intensely blue-colored TPTZ-Fe (II) complex in an acidic medium, with maximum absorption observed at 593 nm. The reducing properties are generally

associated with the presence of reductant compounds, which inactivate oxidants by donating electrons (Patel et al., 2020). In the present study, significantly higher FRAP values were exhibited by the methanolic extract of *Citrullus lanatus* seeds (140.74±9.89 mg Fe (II) equivalent/g extract) compared to the hexane extract (60.12±5.90 mg Fe (II) equivalent/g extract) (Fig. 3).

3.2.4. Metal chelating activity

Metal ions are considered natural constituents of biological systems; however, excessive levels or exposure to certain metal ions are known to induce the generation of reactive oxygen species (ROS). These ROS are responsible for oxidative deterioration of biological macromolecules and an increased risk of various diseases. Therefore, protection of biological systems is provided by antioxidants either through the scavenging of free radicals or by chelation of metal ions (Attar and Ghane, 2019). In the present study, the methanolic seed extract exhibited higher metal chelating activity (0.25 ± 0.001 mg EE/g extract), whereas the hexane extract showed moderate activity (0.18 ± 0.008 mg EE/g extract) (Fig. 4). Similar findings were reported by Rahman et al., where methanolic extracts of Cucurbitaceae species exhibited higher chelating activity (12.4 mg EDTA equivalent/g extract).

3.2.5. Phosphomolybdenum Assay

The antioxidant capacities of different extracts were evaluated, and the results are presented in Fig. 5. The phosphomolybdenum assay is based on the reduction of Mo (VI) to Mo (V) by antioxidant compounds, followed by the formation of a green-colored phosphate–Mo (V) complex, which exhibits maximum absorption at 695 nm (Prieto et al. 1999). In the present study, the methanol seed extract exhibited significantly higher antioxidant activity (233.61±2.99 mg AAE/g extract) compared to hexane solvent extracts (70.22±1.9 mg).

3.2.6. Determination of Acetylcholinesterase Inhibition

Symptoms of Alzheimer's disease (AD) and other forms of dementia can be managed using agents that restore acetylcholine levels through inhibition of the two major cholinesterases, acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). In the late stages of AD, AChE activity declines by up to 85%, while BChE becomes the predominant cholinesterase in the brain. BChE, mainly associated with glial cells and specific neuronal pathways, hydrolyzes acetylcholine in a manner like AChE, thereby terminating its physiological action. Inhibition of AChE has also been reported to enhance cholinergic transmission and reduce β-amyloid aggregation and neurotoxic fibril formation associated with AD (Hodges, 2006). In the present study, methanol and hexane extracts of Citrullus lanatus seeds were evaluated for acetylcholinesterase inhibitory activity at different concentrations. The percentage inhibition data are presented in Fig. 6 and expressed as mean ± standard deviation of triplicate experiments. The IC_{50} value was defined as the concentration required to achieve 50% inhibition. The results demonstrated moderate inhibitory activity for the methanol extract of Citrullus lanatus seeds, which inhibited acetylthiocholine iodide hydrolysis in a dose-dependent manner, reaching maximum inhibition of 83.54% at 0.125 mg/ml. The calculated IC_{50} value for the methanol extract was approximately 0.29 mg/ml, indicating moderate acetylcholinesterase inhibitory potential. In contrast, the hexane extract of Citrullus lanatus seeds exhibited weak inhibitory activity, showing less than 50% inhibition across the tested concentrations. A gradual increase in inhibition percentage with increasing extract concentration indicated a clear dose-dependent relationship for the methanol extract, while the hexane extract displayed comparatively lower effectiveness. Currently, selective cholinesterase inhibitors with minimal dose-limiting side effects remain limited, and existing compounds may not sufficiently modulate acetylcholine levels to achieve full therapeutic efficacy (Felder et al., 2000). Therefore, the search for safer and more effective acetylcholinesterase inhibitors from natural sources continues. The moderate inhibitory activity observed in the present study suggests that Citrullus lanatus seed extracts, particularly the methanol extract, may serve as potential candidates for further investigation in the management of neurodegenerative disorders, including Alzheimer's disease.

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Table.1 Total phenolic content, total flavonoid content, total tannin content, total alkaloid content and acetylcholine esterase inhibitory activities from Citrullus lanatus seed extracts.

Citrullus lanatus seeds extracts	Phytochemicals				
	Total phenolic	Total flavonoids	Total tannins	Total Alkaloids	Acetylcholine esterase
Methanol	146.39 ± 1.9[a]	14.90 ± 0.9[a]	12.19 ± 1.8[c]	8.90 ± 0.5[d]	49.04 ± 0.6[d]
Hexane	70.89 ± 1.6[b]	15.05 ± 0.3[c]	3.43 ± 2.0[a]	16.67 ± 0.1[e]	82.07 ± 0.9[b]

Values were the means of three replicates ± Standard Error (SE). Mean value with different alphabets in column were showed statistically significant differences ($p < 0.05$) according to Duncan multiple range test.

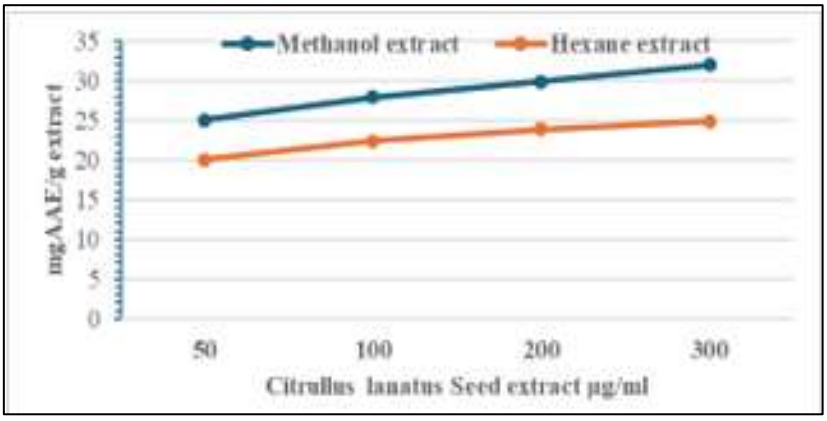

Fig. 1: Antioxidant activity determined by DPPH radical scavenging assay for seed extracts of Citrullus lanatus. Values are expressed as mean ± standard error of three replicates.

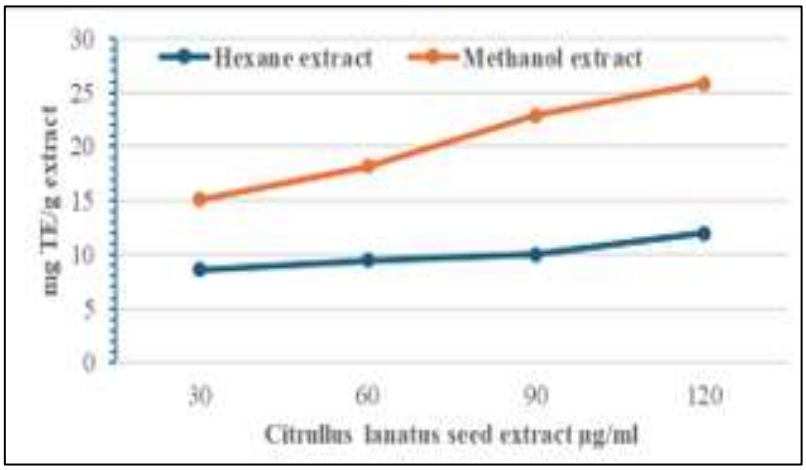

Fig. 2: ABTS$^{\cdot+}$ radical scavenging activity of Citrullus lanatus seed extracts. Results are presented as mean ± SE (n = 3).

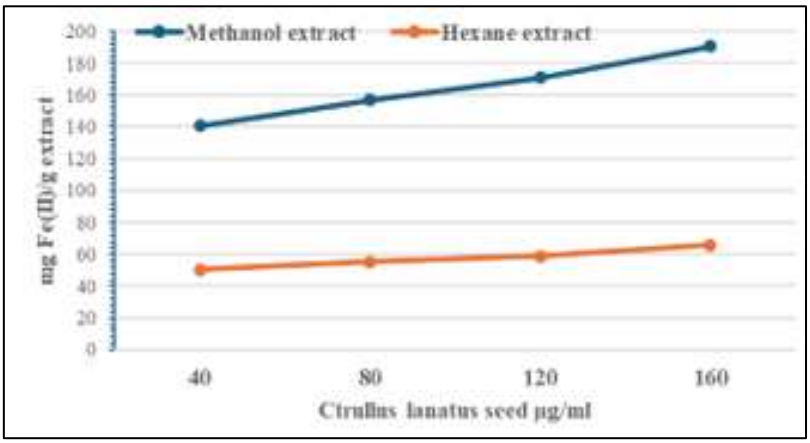

Fig 3: FRAP activity of seed extracts of Citrullus lanatus. Values are expressed as mean ± SE of three replicates.

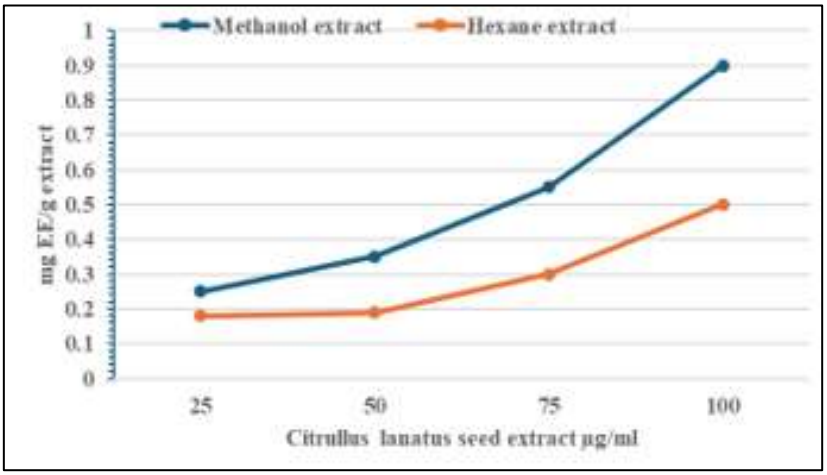

Fig. 4: Metal chelating activity of Citrullus lanatus seed extracts. Values represent mean ± standard error of three replicates.

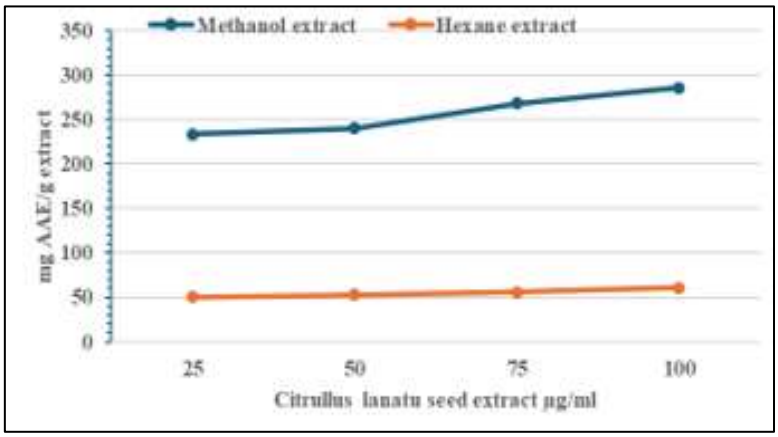

Fig. 5: Phosphomolybdenum reduction assay of Citrullus lanatus seed extracts (mean ± SE, n = 3).

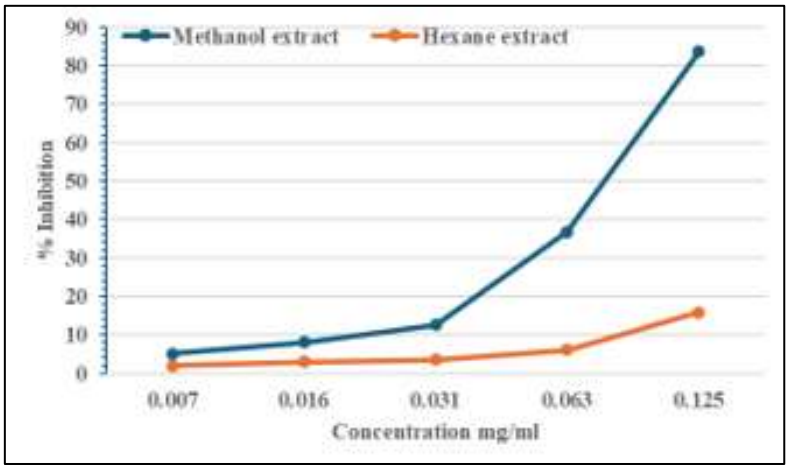

Fig. 6: AChE inhibitory activity of methanol and hexane extracts of Citrullus lanatus seed. Values are means of three replicate determinations ±SE (N=3).