Indian J. Applied & Pure Bio. Vol. 41(3), 1543-1550 (2026). **ISSN: 0970-2091**

Study of antioxidant and anticholinesterase activity of whole plant extract of *Protulacaria afra* Jacq.

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Abstract

The objective of this work was to assess the *in vitro* antioxidant potential and *in vitro* anticholinesterase potential of the methanolic extract of *Portulacaria afra* Jacq. The extract of plant was found to be brown in color and was oleo-resinous in nature. The dry weight (%) of the extract with reference to the weight of dry leaf powder was found to be 17.1%. The findings of preliminary phytochemical analysis suggest the presence of alkaloids, phenolics, terpenoids, flavonoids and carbohydrates in the methanolic extract of *Protulacaria afra.* The total phenolic content of MEPA was found to be 47.25 ± 0.151 GAE/mg. The methanolic extract was assessed for its ability to inhibit the DPPH radical, indicated by reduction in absorbance and the purple color of the solution. The IC_{50} of DPPH radical inhibition by the extract of *Portulacaria afra* was found to be 436.20µg/mL. The extract able to inhibit AChE activity dose dependently from 17.02 to 60.04%. The IC_{50} was calculated from inhibition percentage and was found to be 415.00 µg/mL.

Key words : *Portulacaria afra* Jacq. anti-oxidant, anti-cholinesterase, extraction, DPPH.

Alzheimer's disease (AD) is a progressive neurodegenerative disorder, and the most predominant cause of dementia in the elderly, associated with gradual memory loss, cognitive deficit and behavioral abnormalities[18]. Reduced cholinergic activity and oxidative stress has been recognized as major contributing factor in the pathogenesis of AD. The loss of cholinergic neurons and the associated decrease in levels of acetylcholine (ACh) has been found to correlate well with the cognitive impairment seen in AD patients[11]. Therefore, inhibition of acetylcholinesterase (AChE) as well as butyrylcholinesterase (BChE), which are involved in the breakdown of acetylcholine, has been the main strategy followed for the treatment of AD. Oxidative stress is deûned as an imbalance between oxidants and antioxidants that causes a rise in oxidant levels[16]. It is well recognized as one of the clinical markers of AD; nevertheless, it is still unclear whether oxidative stress is a cause or

a consequence of the process that occurs in AD patients' brains[3].

Portulacaria afra is a household ornamental plant grown for prosperity and well-being. Though the plant has been found to contain antioxidant compounds and secondary metabolites[2], not much pharmacological and biological potential of the plant has been scientifically explored. A few reports of anti-nociceptive, anti-inflammatory, hemolytic, anti-bacterial, anti-oxidant and cytotoxicity studies have been found in literature[19,12,1,20,7,15]. The present study aimed to perform methanolic extraction of *P. afra* and assess it anti-oxidant and anti-cholinesterase potential.

Extraction of plant material :

The extraction was carried out using methanol as the solvent by hot continuous extraction method using a soxhlet apparatus. Briefly, 100 g of powder was packed in a thimble and the thimble was placed in the extractor of the soxhlet apparatus. 200 mL of pure methanol was flown down the extractor in to the round bottom flask. The flask was heated at 75°C to carry out the extraction process. The extraction was carried out for 11 hours (complete extraction of contents was confirmed by the clear solution in the siphon tube of the extractor). The extract was filtered hot through Whatman filter paper in order to remove any impurity. The extract was then concentrated on boiling water bath to obtain the oleo-resinous residue. The oleo-resinous extract was collected and placed in desiccators to remove the excessive moisture**.** The dried extract was weighed and stored in desiccator for further analysis[8,10,4,17].

Phytochemical Screening of the extract :

The methanolic extract was tested for the presence of various phytochemicals using the reported methods[10,6].

Test for alkaloids :

The extract was mixed with 2 mL of 1% HCl solution, warmed on water bath and filtered. The filtrate is used for various test procedures for alkaloids as follows.

1. ***Mayer's test:*** To 1 mL of filtrate, two drops of Mayer's reagent was added along the sides of test tube.
2. ***Wagner's test:*** A few drop of Wagner's reagent was added to 1 mL of the filtrate along the sides of test tube.
3. ***Hager's test:*** A few drops of Hager's reagent were added to 1 mL of filtrate along the sides of test tube.
4. ***Dragendroff's Test:*** A few drops of Dragendroff's reagent were added to 1 ml of the filtrate.

Test for glycosides :

1. ***Bontrager's test:*** The extract was boiled with 1.0 ml of dilute sulphuric acid in a test tube for 5 min and filtered hot. The filtrate was cooled and shaken with an equal volume of dichloromethane and the lower layer (dichlormethane) was separated and shaken with half its volume of dilute ammonia.
2. ***Froth test:*** 1 ml solution of the extract in water was placed in a test tube and shaken vigourously.
3. ***Keller killiani test (Test for deoxy sugars):*** The extract is extracted with chloroform

and evaporated to dryness. To the residue was added 0.4 mL of glacial acetic acid containing a trace amount of ferric chloride. The solution was transferred to a test tube and 0.5 mL of concentrated sulphuric acid is added along the wall of the test tube.

Test for triterpenoids :

1. ***Salkowski test:*** The extract was dissolved in chloroform and a few drops of conc. sulphuric acid were added to it. The mixture was shaken well and allowed to settle for few minutes.

Test for tannins and phenolics :

1. ***Gelatin test:*** To the extract was added 1% gelatin solution containing 10% sodium chloride.
2. ***Ferric chloride test:*** To the extract was added a freshly prepared solution of ferric chloride.
3. ***Vanillin hydrochloride test****:* Test solution of the extract was treated with few drops of vanillin hydrochloride reagent.
4. ***Alkaline reagent test:*** Test solution of the extract was treated with sodium hydroxide solution.
5. ***Lead acetate test:*** To the test solution of the extract was added a few drops of 10% lead acetate solution.

Test for flavonoids :

1. ***Shinoda test:*** To the test solution of the extract, a few fragments of magnesium ribbon were added and concentrated hydrochloric acid was mixed drop wise to it.
2. ***Zinc hydrochloride reduction test:*** To the test solution a mixture of zinc dust and concentrated hydrochloric acid was added.
3. ***Alkaline reagent test:*** To the test solution a few drops of sodium hydroxide solution was added. Later if color appeared, a few drops of concentrated hydrochloric acid were added to it.

Test for proteins and amino acids :

1. ***Millons test*****:** Test solution of the extract was allowed to react with 2 ml of Millon's reagent (mercuric nitrate in nitric acid containing traces of nitrous acid).
2. ***Ninhydrin test:*** The solution of extract was boiled with 0.2% solution of ninhydrin.

Test for carbohydrates :

1. ***Molisch's test:*** The extract was boiled with 10 mL of distilled water for 10 min, cooled and filtered. To 2 mL of the filtrate was added 2 drops of Molisch's reagent and the mixture was shaken well. 1 mL of concentrated sulphuric acid was flown down the sides of the test tube and allowed to settle for a few minutes.

Total Phenolic Content :

The total phenolic content in the leaf extract was determined quantitatively using Folin-Ciocalteu reagent method, using gallic acid as the reference standard. For total phenolic content determination, 200 µL of sample was mixed with 1.4 mL purified water and 100 µL of Folin-Ciocalteu reagent. After incubating at room temperature for 15 min, 300 µL of 20% Na_2CO_3 aqueous solution was added and the mixture was allowed to incubate

at room temperature for 2 h. The absorbance of the solution was measured at 760 nm with a UV-Vis spectrophotometer[13]. Standard solutions of gallic acid (10-100 ppm) were similarly treated to plot the analytical curve. The control solution contained 200 µL of methanol and suitable reagents, and it was prepared and incubated under the same conditions as the rest of the samples. Results were expressed as milligrams of gallic acid equivalent (GAE) per 100 g of the dry sample.

In vitro antioxidant activity of extract by DPPH assay :

The antioxidant action of the extract was determined using 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. The free radical scavenging activity of the synthesized molecules was measured in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH. The test samples (100 µL, 100-500 µg/mL) were prepared in DMSO and were mixed with 1.0 mL of DPPH solution and filled up with methanol to a final volume of 4 mL. Absorbance of the resulting solution was measured at 517 nm in a visible spectrophotometer[5]. Ascorbic acid was used as the reference compound. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. Radical scavenging activity was expressed as the inhibition percentage of free radical by the sample and was calculated.

Evaluation of Anti-cholinesterase Activity:

The extract suspended in required volume of dimethylsulfoxide (DMSO) and diluted using phosphate buffer solution (PBS) (pH = 7.8) for the final range of concentrations (100-500 µg/mL). A common chromogenic agent, Ellman's reagent was used for AChE catalyzed hydrolysis. In fresh 96-well microplates, each well was filled with 40 µL of 50 mM Tris-HCl buffer pH 8.0, 20 µL of test sample solution, 100 µL of Ellman's reagent (prepared by dissolving 18.5 mg reagent in 10 mL of buffer), and 20 µL of AChE solution (prepared by dissolving 0.26 U/mL enzyme in 15 mL buffer). The contents were mixed and incubated at 25°C for 15 min. The absorbance was measured at 412 nm. Now, 20 µL of 15 mM acetyl thiocholine (ACTh) (prepared by dissolving in water) was added as a substrate to the wells and the plate was incubated for 20 min at 37°C. Blank determination was done without test samples to obtain 100% AChE activity[14,10]. The absorbance was measured at 412 nm and the percentage AChE inhibition was calculated.

Extraction yield and phytochemical screening:

The extract of leaf was found to be dark brown in color and was oleo-resinous in nature. The dry weight (%) of the extract with reference to the weight of dry leaf powder was found to be 17.2%. The results of phytochemical screening are presented in table-1.

Total Phenolic Content :

MEPA was evaluated for quantification of the total phenolic content concentration in extract. Standard curve of gallic acid was plotted in distilled water for determining absorption data The total phenolic content of MEPA was found to be 47.25 ± 0.151 GAE/ mg.

Table-1. Phytochemical screening of MEPA

Phytochemical	Test	Observation	Inference
Alkaloid	Mayer's reagent	cream colour precipitate	Alkaloid Present
	Hager's reagent	yellow colour precipitate	
	Wagner's reagent	reddish brown precipitate	
	Dragendorff's reagent	reddish brown precipitate	
Flavonoid	Shinoda test	red color	Flavonoid Present
	Alkaline reagent test	Yellow color that turns red on acidification	
	Zinc HCl reduction test	red color	
Glycoside	Froth Test	No Frothing	Glycoside absent
	Bontrager's Test	Rose pink or red color in the ammonical layer not found	
	Keller-Kiliani Test	No color in acetic acid layer	
Phenolic and Tannins	Ferric chloride	Blue green color	Phenolics and Tannins present
	Gelatin Solution	White precipitate	
	Alkaline reagent test	Yellow to red precipitate	
	Vanillin HCl test	Purplish red color	
Proteins	Millon's Test	no precipitate	Protein absent
	Ninhydrin Test	No coloration	
Carbohydrates	Molisch's Test	Purple ring	Carbohydrate present
Triterpenoids	Salkwoski Test	Yellow color in lower layer	Triterpenes present

Antioxidant activity :

The methanolic extract was assessed for its ability to inhibit the DPPH radical, indicated by reduction in absorbance and the purple color of the solution (Table-2).

Table-2. % Inhibition of DPPH radical

Conc (µg/mL)	Absorbance	% DPPH Inhibition
Control	1.823	
MEPA 100	1.647	9.65
MEPA 200	1.328	27.15
MEPA 300	1.115	38.84
MEPA 400	0.972	46.68
MEPA 500	0.839	53.98

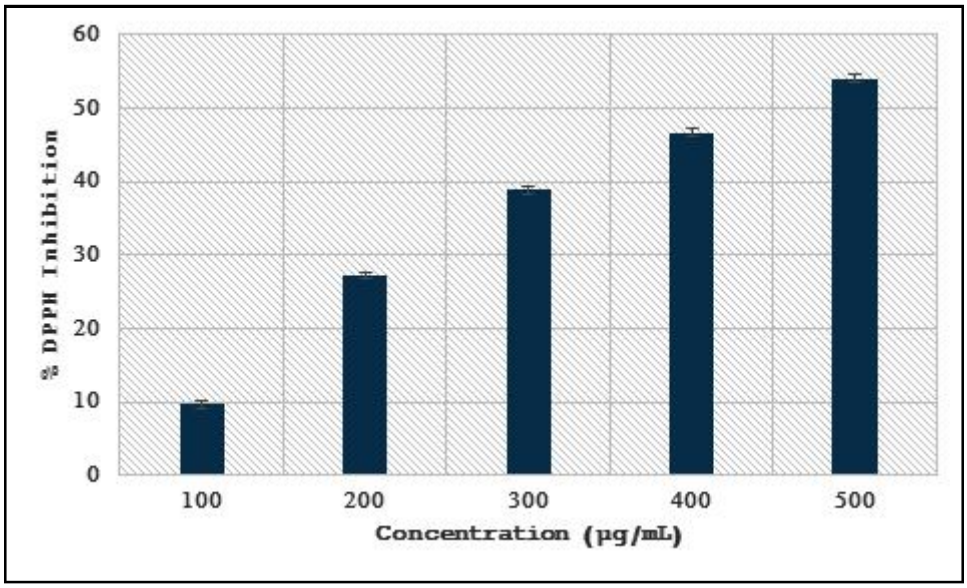

Figure 1. % DPPH radical inhibition by extract

The IC_{50} of DPPH radical inhibition by the extract of *Portulacaria afra* was found to be 436.20µg/mL.

Anticholinesterase activity :

The anticholinesterase activity was evaluated by Ellman's method and the results of inhibition of cholinesterase activity was calculated from the absorbance of blank and the test solutions (Table-3).

Table-3. %Inhibition of cholinesterase action

Concentration	Absorbance		% AChE Inhibition
	Blank	Extract	
	0.423	-	-
100 µg/mL	-	0.651	17.02
200 µg/mL	-	0.301	28.84
300 µg/mL	-	0.269	36.4
400 µg/mL	-	0.222	47.51
500 µg/mL	-	0.169	60.04

The extract able to inhibit AChE activity dose dependently from 17.02 to 60.04%. The IC_{50} was calculated from inhibition percentage and was found to be 415.00 µg/mL.

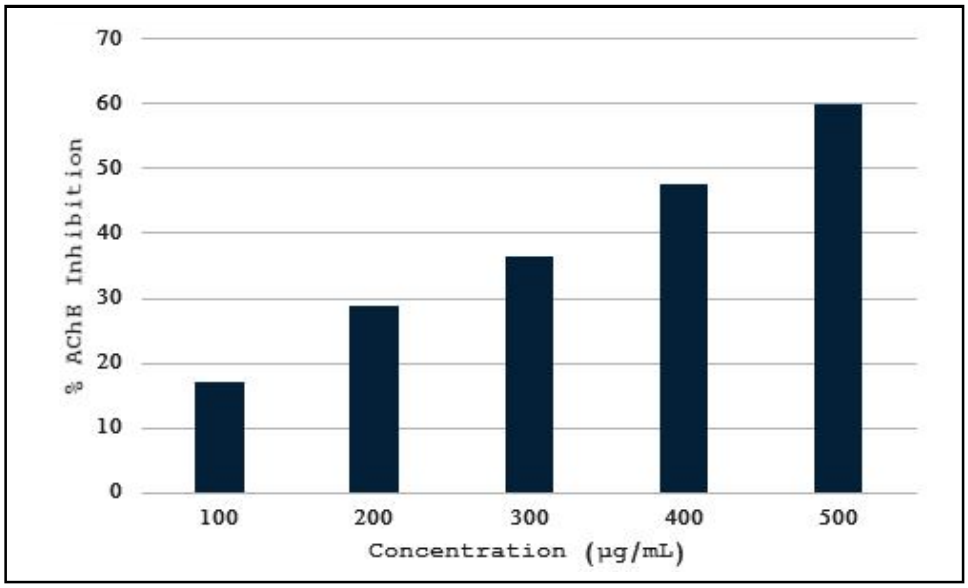

Figure 2. Percent AChE Inhibition

The objective of the present study was to assess the anti-oxidant and anti-acetylcholine esterase potential of methanolic extract of the whole plant of *Protulacaria afra* using the DPPH radical scavenging assay method and Ellman's method. The methanolic extract of the plant was found possess anti-oxidant and anticholinesterase action. Further investigations need to be carried out for determining the active principle in extract responsible of its actions and assessment of the extract in treatment of Alzheimer's disease.

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