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In-Vitro Antioxidant Activity of *Cassine albens*

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Abstract: ***Cassinealbens (Celastraceae), often known as Bhutekes among tribal groups in north Maharashtra who practiced this plant in there indigenous medicinal system for a range of illnesses, including some psychosomatic conditions, in this study the bioactive chloroform and ethyl acetate fractions of methanolic extract of Casinealbens leaves was evaluated for in vitro antioxidant study using DPPH, ABTS and Total antioxidant capacity. Ethyl acetate fractions was shows more powerful inhibition of DPPH (IC50=70.15) and ABTS (IC50=69.08). Chloroform fraction shows lower inhibition capacity. Total antioxidant capacity of the Chloroform and Ethyl Acetate Fraction, expressed as the number of gram equivalents of ascorbic acid. Values are expressed in mean ± SD (n=3), the Total Antioxidant Capacity of Chloroform and Ethyl Acetate Extract was Found to be 129.25±2.79 (µg/ml) and 100±13.51 (µg/ml) respectively. Thus in the present study, we can conclude that chloroform and ethyl acetate fractions of Cassinealbens possesses antioxidant capability.***

Keywords: *Cassinealbens*, DPPH, ABTS, TAC, Traditionally uses of medicinal plants

1. Introduction

In recent year, there is increase interest in plant derived antioxidants molecule to establish the core treatment protocol, for numerous diseases. The human body uses an antioxidant defence system to neutralize the excessive levels of reactive oxygen species. This system consists of enzymatic and non enzymatic antioxidants (Shalaby and Shasnab, 2013). Antioxidants are the compounds which act against oxidative stress and normalize the imbalance between oxidative stress and natural antioxidant defence system of body (Singh et. al 2019, Patel, 2016). In brain reactive oxygen species (ROS) develops due to higher metabolism rate and more availability of oxygen (Patel, 2016). This leads to generate oxidative stress in neuronal cells and this serves as major pathophysiological factors in neuronal disorders such as, anxiety, depression, alzheimer's disease, parkinson's disease and many more (Franzoni F. et al, 2021). Naturally occurring antioxidants exert actions in variety of ways likes reducing inflammation, promoting apoptosis, activations of glial cells, maintaining nerve cell structure etc (Ceu et al 2020), Thus the compounds which alleviate ROS made a valuable therapeutic approach not just in neuronal disorders but also in cardiovascular diseases, diabetic complications, muscular and skeletal disorders and so on (Singh et. al.2019).

Cassine albens (R.) Kosterm belongs to family celastraceae, locally known as Bhutekes or Bhutyapala, is a plant used in traditional medicine by many tribal communities in India. Its noted traidional uses as its leaves and stem bark used in psychosomatic disorder (Patil and Bhaskar, 2006), sternulatory and fumingatory in hysterical attack (Kirtikar and Basu's book), curing of eye disease with its watery exudates (Narayanan, 2011), fumingatory for hysterical syncope or as a snuff to relieve a headache. Also the leaf paste is used to treat eruptions, wounds, and cuts on the skin; It has the potential to exhibit antimicrobial and wound-healing properties (Korpenwar AN, 2012).

With this valuable background of antioxidant in therapeutics, in this work we evaluated in vitro antioxidant potential of *Cassine albens*, in a DPPH scavenging activity, hydrogen peroxide scavenging activity, total antioxidant capacity and thiobarbituric acid (TBA) method.

2. Material and Methods

1. Extraction and Fractionation Method:

The leaf of *Cassine albens* was collected from the viscinity of Leghapani village in Toranmal forest region. The collected material was kept for shed dry for about a week and coarse powder was made with the help of puvurizer. The coarse powder was extracted with methanol using continuous extraction method by soxhlet apparatus. The dried solid methanolic extract of leaf was suspended in water with the help of mortar and pestle. The suspension then filtered through whatmann filter paper so as to any residual component removed.

The water soluble methanolic extract then subjected for fractionation with Chloroform in separating funnel. After three days aqueous and organic layer was separated and chloroform fraction was obtained by evaporating organic solvent. The dried chloroform fraction was stored in air tight containers for future use. The remaining aqueous phase subjected for fractionation with ethyl acetate in separating funnel. The funnel was shaken occasionally. After three days aqueous and organic layer was separated and Ethyl Acetate fraction was obtained by evaporating organic solvent. The dried Ethyl acetate fraction was stored in air tight containers for future use.

2. Preliminary Phytochemical Test:

The chloroform and ethyl acetate fractions were dissolved in methanol and filtered; the resultant solution was used for preliminary phytochemical investigations.

2.1 Tests for ALKALOID:

a) Dragendroff's Test: Filtrates were treated with Dragendorff's reagent (solution of Potassium Bismuth Iodide). Formation of red precipitate indicates the presence of alkaloids.

b) Mayer's Test: Filtrates were treated with Mayer's reagent (Potassium Mercuric Iodide). Formation of a yellow colored precipitate indicates the presence of alkaloids.

c) Hager's Test: Filtrates were treated with Hager's reagent (saturated picric acid solution). Presence of alkaloids confirmed by the formation of yellow colored precipitate.

2.2 Tests for PHYTOSTEROLS

a) Salkowski's Test: Fractions were treated with chloroform and filtered. The filtrates were treated with few drops of Conc. Sulphuric acid, shaken and allowed to stand. Appearance of golden yellow colour indicates the presence of triterpenes.

b) Libermann Burchard's test: Fractions were treated with chloroform and filtered. The filtrates were treated with few drops of acetic anhydride, boiled and cooled. Conc. Sulphuric acid was added. Formation of brown ring at the junction indicates the presence of phytosterols.

2.3 Tests for FLAVONOIDS:

a) Alkaline Reagent Test: Fractions were treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which becomes colorless on addition of dilute acid, indicates the presence of flavonoids.

b) Lead acetate Test: Fractions were treated with few drops of lead acetate solution. Formation of yellow colour precipitate indicates the presence of flavonoids.

2.4 Tests for DITERPENS:

a) Copper acetate Test: Fractions were dissolved in water and treated with 3-4 drops of copper acetate solution. Formation of emerald green colour indicates the presence of diterpenes.

3. In Vitro Anti-Oxidant Activity

3.1 DPPH scavenging assay:

Procedure:

The free radical scavenging capacity of chloroform and ethyl acetate fractions was determined by using DPPH assay. The stock solution of 1 M DPPH was prepared in methanol and stocked at 20^0C until analysis. Fresh 0.1 mM DPPH working solution was prepared by diluting 10 µl stock solution with 90 µl methanol. Ascorbic acid was used as reference standard and a series of standard and samples. Briefly, The assay was carried out in a 96 well microtiter plate, to 250µl of DPPH solution40 µl of each of the test sample or the standard solutions was added separately in wells of the microtiter plate. The plate was wrapped in aluminum foil and kept in dark chamber for 30 min at room temperature and their absorbance was measured at 517 nm. The % inhibition of both standard and samples was calculated for each concentration and graphs were plotted (% inhibition against concentration). The experiment was carried out in triplicate. From these graphs EC50values were calculated for standard and extracts. (Kalaskar, 2018, Sen et al, 2013, Bhatiya et al, 2011, Palsamy and Karthika, 2014, G. Prakash et al, 2011).

DPPH Scavenging activity (%)=[(Abs $_{control}$-Abs $_{test)]}$ / (Abs $_{control}$) × 100
Where-

Abs $_{control}$ is the absorbance of DPPH + methanol;

Abs $_{sample}$ is the absorbance of DPPH radical + sample (i. e. fractions or standard).

3.2 Total Antioxidant Capacity:

Phosphomolybdate assay system was used to determine the total antioxidant activity of the Chloroform and Ethyl Acetate Fractions to a reagent solution, The phosphomolybdenum method was based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound and the formation of a green phosphate/Mo (V) complex with a maximal absorption at 695 nm., Briefly sulphuric acid (0.6 M), sodium phosphate (28 mM) and ammonium molybdate (4 mM); 100µl of each sample was added and incubated at 95°C in a water bath for 90 min. After cooling to room temperature; absorbance was recorded at 765 nm against reagent blank on micro plate reader using 96 well plate. Total antioxidant capacity of the fractions was calculated using ascorbic acid as standard. The calibrations curve was used to calculate total antioxidant capacity for the test fractions. (Kalaskar and Surana, 2014, Das et al 2014, Phatak and Hendre 2014).

3.3 ABTS

ABTS radical cation scavenging activity was performed by previously reported method with slight modifications. In brief, 7 mM ABTS solution was made to reacted with 2.45 mM potassium persulfate solution and was kept overnight in the dark condition to produce a colored solution containing ABTS·+ radical cation. Before the use, the ABTS radical cation solution was diluted with 50% methanol for an initial absorbance at 734 nm. After the addition of 1.0 ml of diluted ABTS·+ to 10 µl of sample, the absorbance was measured after 5 min of initial mixing. The percentage inhibition was calculated according to the formula (de Vargas 2015, Kalaskar and Surana 2014,)

Scavenging effect (%) = [(control absorbance-sample absorbance) / (control absorbance) X 100.

Result:

1. Preliminary Phytochemical Evaluation:

Preliminary Phytochemical test reveals that numerous Phytochemical classes ranges from alkaloid, steroid and terpenoids, flavonoids etc was found to be present in chloroform and ethyl acetate extract of *c. albens.*

Sr. No	Preliminary Phytoconstituents	Chloroform Fractions	Ethyl Acetate Fractions
1	Alkaloid	Present	Present
2	Sterols and Terpenoids	Present	Present
3	Flavonoids	Present	Present

2. Antioxidant activity of Chloroform and Ethyl acetate fractions of C. albens by the DPPH and ABTS assay

Sr. no	Concentration (µg/mL.)	DPPH radical (% inhibition)			ABTS radical (% inhivition)		
		Chloroform Fraction	Ethyl Acetate Fraction	Standard Ascorbic acid	Chloroform Fraction	Ethyl Acetate Fraction	Standard Ascorbic acid
1	20	13.00	22.66	44	20.14	28.08	37.79
2	40	16.66	46.66	68	32.50	37.35	44.41
3	60	34.00	48.00	88	42.79	47.20	62.20
4	80	45.33	50.66	96	49.41	54.11	71.17
5	100	58.33	61.33	96	52.94	63.38	79.85
IC50		**79.88**	**70.15**	**16.96**	**85.31**	**69.08**	**43.60**

The antioxidant capacity was evaluated by the means of DPPH and ABTS radical scavenging ability (percentage inhibition), in our study the significant antioxidant activity was observed in terms of IC50 value in the Ethyl acetate fraction of c. albens in DPPH assay (70.15) and in ABTS assay (69.08) as compared to reference standard ascorbic acid having IC50 in DPPH assay (16.96) and in ABTS assay (43.60).

3. Total antioxidant Capacity:

Total antioxidant capacity of the Chloroform and Ethyl Acetate Fraction, expressed as the number of gram equivalents of ascorbic acid. Values are expressed in mean ± SD (n=3),

The Total Antioxidant Capacity of Chloroform and Ethyl Acetate Extract was Found to be 129.25±2.79 (µg/ml) and 100±13.51 (µg/ml) respectively.

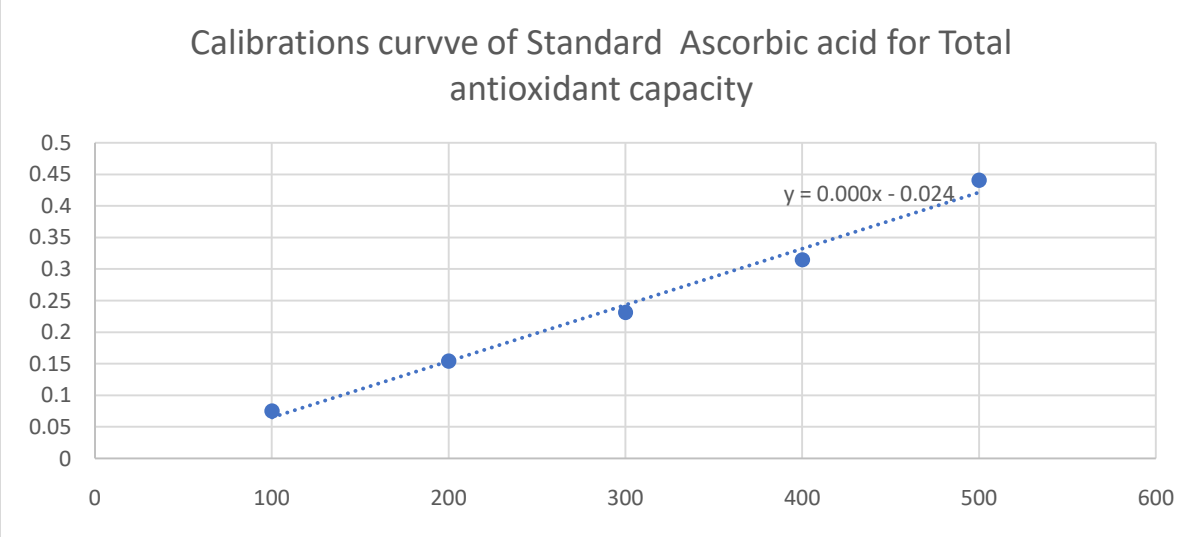

Figure: Standard Ascorbic acid calibration curve for total antioxidant capacity

Total antioxidant Capacity	
Chloroform fraction of *Cassinealbens*	129.25±2.79µl/ml of AAS
Ethyl acetate fraction of *Cassinealbens*	100±13.51µl/ml of AAS

4. Discussion

In the present study we evaluate the antioxidant activity of different fractions of Cassine albens (R.) Kosterm using In -vitro DPPH, ABTS and TAC assay. *Cassine albens* was popular plant used traditionally among the tribal communities residing near Satpura foothill (Bhasker and Patil 2005).

The preliminary Phytochemical test revealed that the presence of steroids and terpenoids, flavonoid, tannins, alkaloid etc. the presence of many such Phytochemical in the leaf of this plant create interest in searching of antioxidant potential compounds.

The DPPH and ABTS radicals enabling the determination of antioxidant capacity of both hydrophilic and lipophilic compounds. Scavenging of antioxidant by 1, 1-diphenyl-2-picrylhydrazine (DPPH) radical method was one the most widely used method in evaluations of antioxidant compounds. DPPH radical reacts with compounds and donate a hydrogen atom. This method is based on the scavenging of DPPH through an antioxidant that decolourizes the DPPH solution. The antioxidant activity is measured by the decrease in absorption at517 nm. (Krishnaiah et al, 2011,) Scavenging activity was expressed as IC50 (inhibitory concentration in µg /mL of samples or positive control that reduces the absorbance of DPPH by 50% when compared with negative control. (REF). The result shows the significant antioxidant capacity of ethyl lacetate fractions (IC50=70.15) as companied to standard ascorbic acid (IC50=16.97).

ABTS assay method involves the reaction of ABTS with potassium persulphate to produce the ABTS radical cation (ABTS. +) a blue green in color. In the ABTS assay antioxidant compounds converts this blue green colour into colourless ABTS at wavelength 7340nm (Boligon et al 2014). In this study Ethyl acetate fractions of C. albens shows better inhibitory capacity (IC50=69.08) as compared to Chloroform fractions (IC50=85.31) against the standard ascorbic acid (IC50=43.60).

The total antioxidant capacity (TAC) assay is a quantitative method to evaluate water-soluble and fat-soluble antioxidant capacity (total antioxidant capacity), in which transforming of relative free radical species MO (VI) into more stable MO (V) non-reactive products occurs. (Alieu et al 2013). The ethyl acetate fractions show highest capacity (100±13.51µl/ml of AAS) as compared to Chloroform fractions (129.25±2.79µl/ml of AAS).

5. Conclusion

In the present study, we can conclude that chloroform and ethyl acetate fractions of *Cassinealbens* possesses antioxidant capability. This activity refers to some of the compounds have a great potential in the antioxidant based treatment of Neurological disorders. In future more rigorous work is needed to ensure the mechanistic pathway of antioxidant activity for this plant.

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