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Abstract

The present investigation was conducted to investigate the in-vitro activity of ethanolic extract of roots of Centaurea behens by using DPPH radical scavenging activity, nitric oxide radical scavenging activity, hydrogen peroxide radical scavenging activity, hydroxyl radical. Result suggests that the extract possess significant antioxidant activity as compared to the standard ascorbic acid and thus further in vivo investigation is required to evaluate the medicinal significance of the extract which can be used for assessing the possible therapeutic importance of the drug.

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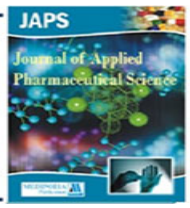

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In-Vitro Antioxidant Activity of Ethanolic Extract of *Centaurea behen*

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ABSTRACT

The present investigation was conducted to investigate the in-vitro activity of ethanolic extract of Roots of *Centaurea behens* by using DPPH radical scavenging activity, nitric oxide radical scavenging activity, hydrogen peroxide radical scavenging activity, hydroxyl radical. Result suggests that the extract possess significant antioxidant activity as compared to the standard ascorbic acid and thus further in vivo investigation is required to evaluate the medicinal significance of the extract which can be used for assessing the possible therapeutic importance of the drug.

Keywords: Antioxidants, Centaurea behen, ethanolic extract, free radicals, reducing ability.

INTRODUCTION

Oxygen is essential for the survival of all on this earth. During the process of oxygen utilization in normal physiological and metabolic processes approximately 5% of oxygen gets univalently reduced to oxygen derived free radicals (Yu 1994 and Halliwell B 1998) like superoxide, hydrogen peroxide, hydroxyl and nitric oxide radicals. All these radicals known as reactive oxygen species (ROS) exert oxidative stress towards the cells of human body rendering each cell to face about 10000 oxidative hits per second (Lata *et al.,* 2003). Various reactive oxygen species (ROSs) are formed in the living organism in different ways i.e. normal aerobic respiration, stimulated polymorphonuclear leukocytes and macrophages and peroxisomes. These appear to be the endogenous source of oxidants. Exogenous sources of free radical include tobacco smoking, ionizing radiation, certain pollutants, organic solvents and pesticides.

(Beris H 1991)All these free radicals are capable of reacting with membrane lipids, nucleic acids, proteins, enzymes and other micro molecules resulting in cellular damage.(Shivprasad HN *et al.,* 2005) Free radicals are involved in the development of degenerative diseases. (Campbell I C *et al.,*1995) They have also been implicated in the pathogenesis of diabetes, liver damage, nephrotoxicity, inflammation, cancer, cardiovascular disorders, neurological disorders, and in the process of aging. (Marx J L 1987) To protect these free radical induced damage, antioxidants are the most popular agents that interactively and synergistically neutralize free radicals. Hence, there has been an increased interest in the food industry as well as in preventive medicine in the development of ''Natural antioxidants'' from plant materials. Therefore, the plants with antioxidant properties are becoming more and more popular all over the world. (Lata H *et al.,* 2003) *Centaurea behan* is commonly called as safed behman and the plant belongs to the family Asteraceae/Compositae. *Centaurea behen* is traditionally used for the treatment of Jaundice and cystis Fibrosis.

MATERIAL AND METHOD

Plant Material

The roots of the *centaurea behen* were collected from Mahavir Ayurvedic Bhandar, Mumbai, India. The plant material were identified and authenticated by Dr Pandit, Department of life Sciences, Khalasa College, Mumbai, India. The material was dried in shade and powdered roots 100g were extracted with Ethanol in a soxhlet apparatus for 72hrs. The extract was dried in the evaporator. The Yield was 16.3w/w.

CHEMICALS

Ascorbic acid, 2,2 diphenhy-1-picryl hydrazyl hydrate (DPPH), Phosphate buffer, Potassium Ferricynide, Trichloro-acetic acid, were obtained from Bharati Vidyapeeth's College of Pharmacy, Navi Mumbai, India. All the chemicals employed in the study were of analytical grade which was purchase from respective suppliers.

PHYTOCHEMICAL SCREENING

Preliminary photochemical screening of the powdered roots performed for the presence of Alkaloids, Glycosides.

IN-VITRO ANTIOXIDANT ACTIVITY

DPPH (2, 2-diphenyl 1, 1-picrylhydrazyl) radical scavenging activity:

1ml of (10-100) of each extract or standard was added to 2ml of DPPH solution in methanol (o.1mM). After incubation for 30 min at 37 the absorbance of each solution was measured at 517 nm using UV spectrophotometer. Corresponding blanks were taken using methanol instead of DPPH solution. A similar procedure was repeated with distilled water instead of extract were performed in triplicate. The decrease in absorbance indicates increase in free radical scavenging activity. (Badami *et al.,* 2005 and Kikuzaki H 1993) The (%) scavenging activity was measures using the following formula.

$$(\%)\ \text{Scavenging activity} = \frac{\text{Control absorbance- Test absorbance x 100}}{\text{Control absorbance}} \quad ..(1)$$

Hydrogen radical scavenging activity:

solution of H2O2 was prepared in phosphate buffer (pH 7.4). 6 ml of 40mM H2O2 solution was mixed with 1.0 ml of different concentration (10-100) of extract or standard substance. After incubation at 37 for 10 minutes absorbance was measured at 230nm. Corresponding blank solution was repeated with distilled water instead of extract which serves as control. Ascorbic acid was used as standard. All the test was performed in Triplicate. The Decrease in absorbance Indicated increase in free radical scavenging activity. The % Scavenging activity was measured by using formula. (Sroks Z *et al.,* 2003)

Reducing Power activity

1ml of extract of various concentrations (10-100) was mixed with phosphate buffer (2.5ml), pH 7.40) and potassium ferricynide (2.5ml, 1%). The mixture was incubated at 50 for 20 minutes. Aliquot of trichloro-acetic acid (2.5ml, 10%) were added to the mixture, then centrifugated at 3000 x g for 10 min. Upper layer of the solution (2.5ml) was mixed with distilled water (2.5ml0 and freshly prepared ferric chloride solution (0.5ml,0.1%). Absorbance was measured at 7000nm. Corresponding blank solution were taken containing same solution without ferric chloride .Ascorbic acid was used as standard. All the tests were performed in triplicate. Increased in the absorbance of the mixture indicates increased reducing power. (Mathiesen L *et al.,*1995)

Nitric oxide radical Scavenging activity

3 ml of 10 mM sodium nitroprusside in phosphate buffer was added to 1 ml of different concentration (10-100 µg/ml) of extract or standard. The resulting solution was then incubated at 25°C for 60 minutes. 5 ml of Griess reagent (1% sulphanilamide, 0.1% naphthyl ethylenediamine dihydrochloride, 2% H3PO4) was then added to incubated sample. The absorbance of chromophore formed during diazotization of nitrite with the sulphanilamide and subsequent coupling with naphthyl ethylenediamine dihydrochloride was measured at 546 nm using UV spectrophotometer. Corresponding blank solutions were taken containing same solution without sodium nitroprusside. A similar procedure was repeated with distilled water instead of extract, which served as control. Ascorbic acid was used as standard. All the tests were performed in triplicate. The % scavenging activity was measured by using formula. (Herencia F 2002 and Sawant HP 2010)

Hydroxyl radical scavenging activity

The deoxyribose assay is based on a Fenton reaction that produces •OH in the presence of H2O2 and Fe2+. Starting from Fe3+, ascorbic acid initiates the reaction by reduction of Fe3+ [Eq.

(1)] and the production of •OH by the Fenton reaction [Eq. (2)] oxidizes deoxyribose and gives rise to malondialdehyde [MDA, Eq. (3)]. This derivative can be determined by the formation of a pink colored chromogen after reaction with thiobarbituric acid (TBA) [Eq. (4)].

Fe3 + + Ascorbic	acid Fe2+ + Ascorbyl radical	…(1)
Fe2+ + H2O2	Fe3+ + •OH + OH-	… (2)
•OH + Deoxyribose	MDA	… (3)
MDA + 2TBA	Chromogen (A ?=532nm)	...(4)

Hydroxyl radical scavenging was measured by assessing the competition between deoxyribose and the test compounds for hydroxyl radicals generated from the FeCl3/EDTA/ascorbate/H2O2 system. The hydroxyl radicals attack deoxyribose, resulting in the formation of thiobarbituric acid-reactive substances. (Lloyd RV *et al.*,1997)

STATISTICAL ANALYSIS

The values were expressed as mean ± SEM. The statistical analysis was carried out by one way analysis of variance (ANOVA) followed by Dunnet's't'-test. P values < 0.05 were considered significant.

RESULTS AND DISCUSSION

Free radicals are known to play a definite role in a wide variety of pathological manifestations. Their broad range effects in biological systems have drawn the attention of many experimental works. It has been proven that these mechanisms may be important in the pathogenesis of certain diseases and aging. There are many reports that support the use of antioxidant supplementation in reducing the level of oxidative stress and in slowing or preventing the development of complications associated with diseases. They exert their action either by scavenging the reactive oxygen species or protecting the antioxidant defense mechanisms. (Umamaheswari M et al.,2008).

DPPH free radical scavenging activity of plant extract and ascorbic acid

Proton radical scavenging action is one mechanism for oxidation. The reduction capability of DPPH radicals was determined by the decrease in its absorbance at 517 nm, suggesting that antioxidant activity of CB extract is due to its proton donating ability (Ganapathy S *et al.*, 2007) and it showed concentration dependent DPPH scavenging activity. CB extract required 2.28 fold the concentration of ascorbic acid to scavenge DPPH.

Table. 1: DPPH free radical scavenging activity of plant extract and ascorbic acid.

Concentration (µg/ml)	(%) Scavanging Effect of Extract	% Scavanging Effect of Ascorbic acid
10	19.21± 0.164	33.87± 0.130
20	22.79± 0.144	43.60 ± 0.210
40	39.56 ± 0.419	66.26 ± 0.281
60	57.20 ± 0.176	75.67 ± 0.103
80	72.40 ± 0.258	79.33 ± 0.194
100	80.12 ± 0.101	82.42 ± 0.325

Values are expressed as mean ± SEM of 3 observations.

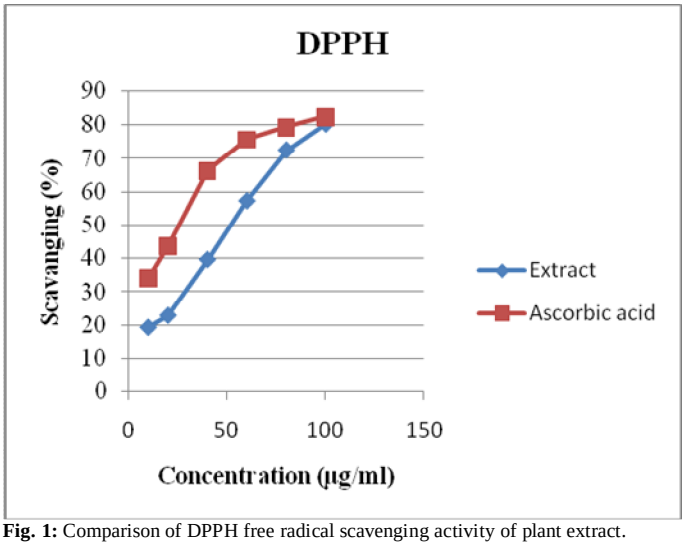

Fig. 1: Comparison of DPPH free radical scavenging activity of plant extract.

H2O2 radical scavenging activity of plant extract and ascorbic acid

Hydrogen peroxide itself is not very particularly reactive with most biologically important molecules, but is an intracellular precursor of hydroxyl radicals which is very toxic to the cell. (Ganapathy S 2007 and Adesegun SA 2009) the decreased absorbance of reaction indicates increased reduction of hydrogen peroxide and hydroxyl radical production. CB Root extract scavenged hydrogen peroxide which may be attributed to the presence of phenolic groups that could donate electrons to hydrogen peroxide, thereby neutralizing it into water. (Jafri MA *et al.*, 1999) CB Root extract required a 1.13 fold dose than ascorbic acid to scavenge H2O2.

Table.2: H2O2 radical scavenging activity of plant extract and ascorbic acid.

Concentartion (µg/ml)	(%)Scavanging Effect of Extract	(%)Scavanging Effect of Ascorbic acid
10	33.74 ± 0.177	38.70 ± 0.218
20	37.63 ± 0.221	42.74 ± 0.198
40	59.23 ± 0.145	63.82 ± 0.151
60	75.36 ± 0.179	76.61 ± 0.170
80	78.73 ± 0.229	82.98 ± 0.172
100	85.94 ± 0.150	89.12 ± 0.224

Values are expressed as mean ± SEM of 3 observations.

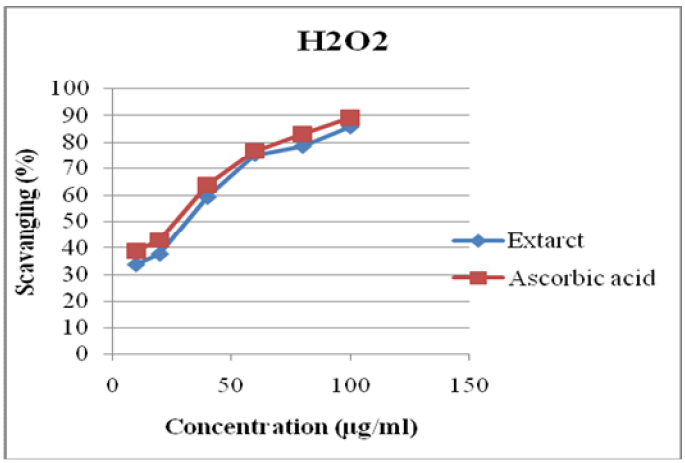

Fig. 2: H2O2 radical scavenging activity of plant extract and ascorbic acid.

Reducing power of plant extract and ascorbic acid

In reducing power assay, the presence of antioxidants in the sample reduced Fe3+/ ferricyanide complex to the ferrous form. This reducing capacity of compounds could serve as an indicator of potential antioxidant properties and increase in absorbance could indicate an increase in reducing power. (Umamaheswari M 2008 Adesegun SA 2009)and CB Root extract required a 2.0 fold dose than ascorbic acid to reach the similar reducing power. Although the greatest reducing power was obtained from ascorbic acid, our results showed that CB Root extract is an electron donor and could react with free radicals, convert them to more stable products and terminate radical chain reaction.

Table. 3: Reducing power of plant extract and ascorbic acid.

Concentartion (µg/ml)	Absorbance (Extract)	Absorbance (Ascorbic acid)
10	0.311 ± 0.0082	0.428 ± 0.0202
20	0.412 ± 0.0073	0.607 ± 0.0081
40	0.627 ± 0.0071	0.736 ± 0.0066
60	0.671 ± 0.0075	0.790 ± 0.0054
80	0.721 ± 0.0047	0.848 ± 0.0156
100	0.773 ± 0.0037	0.876 ± 0.0103

Values are expressed as mean ± SEM of 3 observations

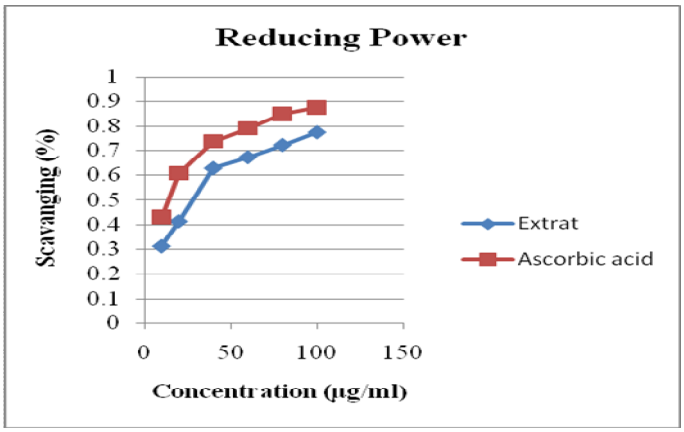

Fig 3: Comparison of reducing power of plant extract and ascorbic acid.

Nitric oxide radical scavenging activity of plant extract and ascorbic acid

Nitric Oxide (NO), a reactive free radical generated from L-arginine by NO synthase, is well documented as a physiological messenger molecule. Excessive amounts of NO, however, are potentially toxic and have been implicated in numerous pathological situations and chronic inflammation.(Yabuki M *et al.*, 1999) Scavenger of nitric oxide competes with oxygen leading to reduced production of nitric oxide suggesting that CB Root extract showed antioxidant activity. CB Root extract required a 1.1 fold dose than ascorbic acid to scavenge nitric oxide radical.

Table. 4: Nitric oxide radical scavenging activity of plant extract and ascorbic acid.

Concentartion (µg/ml)	(%)Scavanging Effect of Extract	(%)Scavanging Effect of Ascorbic acid
10	22.12 ± 0.262	25.36 ± 0.083
20	32.73 ± 0.195	35.23 ± 0.158
40	40.25 ± 0.181	40.93 ± 0.180
60	43.63 ± 0.173	48.80 ± 0.408
80	61.32 ± 0.189	61.78 ± 0.213
100	68.90 ± 0.148	71.30 ± 0.308

Values are expressed as mean ± SEM of 3 observations.

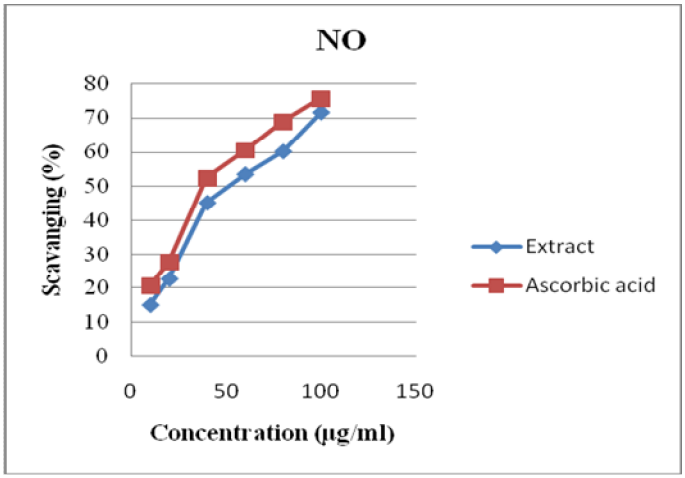

Fig .4: Comparison of nitric oxide radical scavenging activity of plant extract and ascorbic.

Hydroxyl radical scavenging activity of plant extract and ascorbic acid

Hydroxyl radical is the most deleterious and reactive among the reactive oxygen system (ROS) and it bears the shortest half-life compared with other free radicals. The oxygen derived hydroxyl radicals along with the added transition metal ion (Fe2+) causes the degradation of deoxyribose into malondialdehyde which produces a pink chromogen with thiobarbituric acid. (Sundararajan R 2006 and Adesegun SA 2009) The CB Root extract showed concentration dependent hydroxyl radical scavenging activity and CB Root extract required a 1.15 fold dose than ascorbic acid to scavenge hydroxyl radicals.

Table.5. Hydroxyl radical scavenging activity of plant extract and ascorbic acid

Concentration (µg/ml)	(%) Scavenging effect of Extract	(%) Scavenging effect of Ascorbic acid
10	15.17 ± 0.138	20.66 ± 0.127
20	22.74 ± 0.163	27.74 ± 0.244
40	45.25 ± 0.215	52.46 ± 0.160
60	53.58 ± 0.197	60.46 ± 0.170
80	60.18 ± 0.170	68.97 ± 0.237
100	71.59 ± 0.146	75.67 ± 0.216

Values are expressed as mean ± SEM of 3 observations

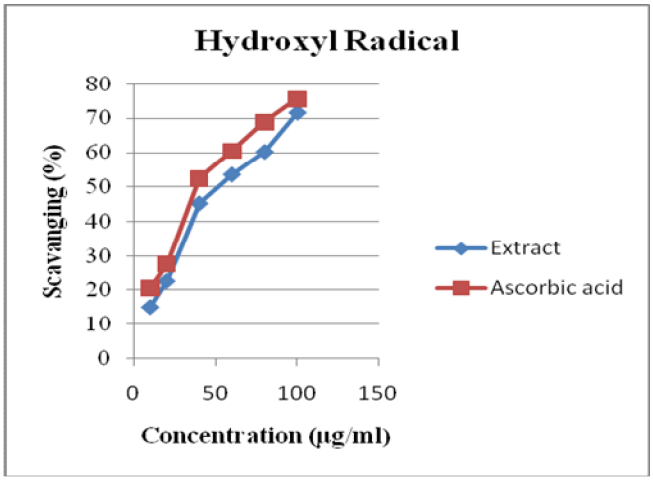

Fig 5: Comparison of Hydroxyl radical scavenging activity of plant extract and ascorbic acid.

CONCLUSION

The results of in vitro antioxidant tests suggested that the hydro-alcoholic extract of *Centaurea behen* possesses strong free radical scavenging activity that is analogous to a well known standard anti-oxidant ascorbic acid, which could exert beneficial action against pathological alterations caused by the presence of hepatotoxins. However, the components responsible for the antioxidative activity are currently unclear. Therefore, further investigations need to be carried out to isolate and identify the antioxidant compounds present in the plant extract. Furthermore, the in vivo antioxidant activity of this extract needs to be assessed prior to clinical use.

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