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ORIGINAL ARTICLE

A Comparative Study on the Antioxidant Activity of Methanol Extracts of *Acacia nilotica* and *Berberis chitria*

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ABSTRACT

In this study, antioxidant activity of methanol extract of *Acacia nilotica* (AN) and *Berberis chitria* (BC) were evaluated using different *in vitro* methods. ABTS, DPPH, Nitric oxide, hydroxyl radical and hydrogen peroxide scavenging activities were used as standard methods of evaluation of antioxidant properties. Total phenolic content of the selected extracts was estimated by Folin-Ciocalteu method and correlated with antioxidant properties of the extracts. Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and Ascorbic acid (ASC) were used as the reference antioxidant compounds. Both the extracts were found to possess significant antioxidant property. Among the two, *Acacia nioltica* had higher antioxidant property than *Berberis chitria*. The antioxidant property was directly related to the total phenolic content of the extract, which was found to be 9.88 and 2.73 µg/ml, respectively for AN and BC. From the above studies it can be suggested that *Acacia nilotica* could be used as an antioxidant, probably for the treatment of diseases related to free radicals, such as, cancer, diabetes, etc. Further studies are needed on the isolation and identification of antioxidant compounds in AN to prove its worth as antioxidant drug.

Key words: *Acacia nilotica, Berberis chitria*, Antioxidant, Free radical scavenging

Introduction

Reactive oxygen species (ROS) are constantly being generated in the body, as a result of the normal metabolic processes (Halliwell, 1999). Mitochondria, which consume more than 90% of the oxygen in aerobic living organisms, are the main source of ROS and free radicals. Approximately 1-5% of the oxygen consumed by mitochondria is reduced and converted to these reactive oxygen species (Halliwell, 1991). ROS can be classified into oxygen-centered radicals such as superoxide anion (O_2•−), hydroxyl radical (OH•), alkoxyl radical (RO•), peroxyl radical (ROO•) and oxygen-centered nonradical derivatives such as hydrogen peroxide (H_2O_2) and singlet oxygen ($1O_2$). Other common reactive species are nitrogen species such as nitric oxide (NO•), nitric dioxide (NO_2•), and peroxynitrite (OONO –) (Rohrdanz et. al., 2001). ROS cause lipid oxidation, protein oxidation, DNA strand breaks, and modulation of gene expression (Halliwell, 2002**).** They are involved in many diseases such as atherosclerosis, cancer, stroke, asthma, arthritis and other age related diseases (Cross *et. al*., 1987).

Because antioxidant defense in the human body is not completely efficient, increased free radical formation may produce a continuous level of oxidative damage (Gutteridge and Halliwell, 2000; Darley-Usmar and Halliwell, 1996). Oxidative stress refers to a severe disturbance in the prooxidant-antioxidant balance in favor of the prooxidant, leading to potential damage (Sies, 1997). However, they are removed by antioxidant defence mechanisms. Antioxidants are considered as possible protection agents reducing oxidative damage of human body from ROS and retard the progress of many chronic diseases as well as lipid peroxidation (Pryor, 1991).

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Therefore, there is a growing interest in the substances exhibiting antioxidant properties that are supplied to human and animal organisms as food components or as specific pharmaceuticals. Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate and tert-butylhydroquinone are the most commonly used antioxidants at the present time. However, they are suspected of being responsible for liver damage and carcinogenesis (Wichi, 1988). Hence, in recent years, the restriction in the use of synthetic antioxidants, such as BHA and BHT, has caused an increased interest towards natural antioxidant substances (Gülçin, 2005a; Sanchez-Moreno *et. al.*, 1999). Therefore the importance of searching for and exploiting natural antioxidants of plant origin has increased greatly in recent years. There is a growing interest in natural additives as potential antioxidants.

Acacia nilotica tree is the Babbula of Sanskrit writers, who mention the use of young leaves and pods as an astringent. *Acacia nilotica* contains gallic acid, m-digallic acid, (+)-catechin, chlorogenic acid, gallolyated flavan-3, 4-diol, robidandiol (7, 3, 4, 5-tetrahydroxyflavan-3-4-diol), androstene steroid, D-pinitol carbohydrate, catechin-5-galloyl ester (Khalid *et. al.*, 1989). *Acacia nilotica* has anticancer and antimutagenic (Meena et. al., 2006), anti-inflammatory, antiplasmodial (Kirira *et. al.*, 2006), antidiarrhoeal (Agunu *et. al.*, 2005), antihypertensive, antiplatelet aggregatory, molluscicidal, antifungal, antimicrobial activity, inhibitory activity against Hepatitis C and HIV-I (Hussein *et. al.*, 1999). *Berberis chitria* is an erect, spiny shrub up to 3.3 m height with obovate or elliptic-acute, spinere-serrate leaves, deep yellow flowers in loose corymbose panicles and dark red brown oblong-ellipsoid berries. The root and the stem bark are the richest source of alkaloids (5% and 4.2% respectively calculated as berberine and can be used for commercial manufacture of berberine. Plant contains Berberine, jatrorrhizine, o-methylcorydine-N-oxide palmitine, oxyacanthine. Hyperaccumulator and antimicrobial activities of *Berberis chitria* have been exploited (Singh *et. al.*, 2007).

Materials and methods

Chemicals

The chemicals used were Ascorbic acid, 1, 1-diphenyl, 2-picryl hydrazyl (DPPH) . N-(1-naphthyl) ethylene Diamine Dihydrochloride (NEDD), Sodium nitoprusside, sulphanilic acid, sodium chloride (NaCl), Ferric Chloride (FeCl3), disodium hydrogen orthophosphate, potassium dihydrogen phosphate, 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), 1,10-Phenanthroline and Folin-Ciocalteu Reagent. All chemicals and solvents used were of analytical grade and obtained from Hi media Laboratories Pvt. Ltd., Mumbai, Ranbaxy Fine Chemicals Ltd., New Delhi and Central Drug house Pvt. Ltd., New Delhi, India.

Plant materials

Stem Bark of the plant *Acacia nilotica* (family: Mimosaceae) and root of *Berberis chitria* (family: Berberidaceae) were collected in the month of September 2007.

Sample preparation

The plants parts were shade dried, powdered and extracted individually with methanol by hot continuous percolation, using Soxhlet apparatus. The extracts were filtered and concentrated and kept in a vacuum desicator for complete removal of the solvent. Alcohol extract of *Acacia nilotica* (AN) and *Berberis chitria* (BC) were obtained in the yield of 10.6% and 9.2% w/w respectively.

Antioxidant Assays

DPPH radical scavenging

The free radical scavenging activity of AN and BC were measured by 1-diphenyl,2-picryl hydrazyl (DPPH) using the method of Blois, 1958. Solution of DPPH in methanol 0.1 mM was prepared and 1ml of this solution was added to 3 ml of various concentrations of AN, BC and reference compound (10-50 µg). After 30 min, absorbance was measured at 517 nm. Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and Ascorbic acid were used as reference materials. All the tests were performed in triplicate and the graph was plotted with the mean value. The percentage of inhibition was calculated by comparing the absorbance values of control and samples.

ABTS radical scavenging

The spectrophotometric analysis of ABTS scavenging activity was determined according to the method reported by Re *et al.*, 1999. The ABTS was produced by the reaction between 7 mM ABTS in H_2O and 2.45 mM potassium persulfate, stored in the dark at room temperature for 12 h. Before usage, the ABTS solution was diluted to get an absorbance of 0.700 ± 0.025 at 734 nm with phosphate buffer (0.1 M, pH 7.4). For stock solutions of 10 mg of AN and BC was dissolved in 10 ml distillated water. Then, 1 ml of ABTS solution was added 3 ml of AN and BC solution in methanol at different concentrations (25-150 µg/ml). After thirty minutes later, the percentage inhibition of absorbance at 734 nm was calculated for each concentration relative to a blank absorbance (methanol). All determinations were carried out at least three times, and in triplicate. The capability to scavenge the ABTS radical was calculated using the equation:

Inhibition (%) = $[(A_0-A_S) / A_0] \times 100$

Where, A_0 was the absorbance of the control (blank, without extract) and A_S was the absorbance in the presence of the extract.

Nitric oxide radical scavenging

Nitric oxide radicals were generated from sodium nitroprusside solution at physiological pH (Jayaprakasha, 2004). Four milliliters of sodium nitroprusside (10 mM) was mixed with 1 ml of the test extracts / ascorbic acid in phosphate buffer saline (pH 7.4). The test extracts were prepared in different concentrations (10-60 µg/ml). The mixture was incubated at 25 °C for 150 min. To 0.5 ml of the incubated solution, 1 ml of sulphanilic acid reagent was added, mixed well and allowed to stand for 5 min for completion of diazotisation. Then, 1 ml of NEDD was added, mixed and allowed to stand for 30 min in diffused light. A pink colored chromophore was formed. The absorbance of these solutions was measured at 540 nm against the corresponding blank solutions.

Hydrogen peroxide radical scavenging

H_2O_2 scavenging ability of AN and BC was determined according to the method reported by Ruch *et al.*, 1989. A solution of H_2O_2 (40 mM) was prepared in phosphate buffer (pH 7.4). AN and BC in different concentrations (25-75 µg/ml) in 3.4 ml phosphate buffer were added to a H_2O_2 solution (0.6 ml, 40 mM). The absorbance value of the reaction mixture was recorded at 230 nm. Blank solution was containing the phosphate buffer without H_2O_2. The percentage of H_2O_2 scavenging of AN, BC and standard compounds was calculated according to the equation given under ABTS scavenging method.

Hydroxyl radical scavenging

The scavenging activity for hydroxyl radicals was measured with Fenton reaction (Sadasivam and Manikam, 1992). Reaction mixture contained 60 µl of 1.0mM $FeCl_3$, 90 µl of 1mM 1,10- phenanthroline, 2.4 ml of 0.2 M phosphate buffer (pH 7.8), 150 µl of 0.17 M H_2O_2, and 1.5 ml of extract at various concentrations. Adding H_2O_2 started the reaction. After incubation at room temperature for 5 min, the absorbance of the mixture at 560nm was measured with a spectrophotometer. The hydroxyl radicals scavenging activity was calculated according to the equation given under ABTS scavenging method.

Determination of total phenolic content

Total phenol content of the extracts was determined by using the Folin-Ciocalteu method (Roginsky 2003). This test is based on the oxidation of phenolic groups with phosphomolyblic and phosphotungstic acids. After oxidation a green-blue complex formed is measured at 750 nm. In a test tube 200 µl of the extract (10 µg, 20 µg,….50 µg) was mixed with 1ml of Folin-Ciocalteu Reagent and 800 µl of sodium carbonate (0.7 M). After shaking, it was kept for 2 h reaction time. The absorbance was measured at 750 nm. Using gallic acid monohydrate, standard curve was prepared.Using the standard curve the total phenol content was obtained. The total phenol content was expressed as gallic acid equivalent in mg/g of the extract.

Statistical Analysis

All experiments were conducted in triplicate (n=3).Students T-test and ANOVA were used for determination of statistical significance. $p < 0.05$ were regarded as significant.

Results and discussion

In this study, AN and BC exhibited marked DPPH free radical scavenging activity in a concentration-dependent manner. The IC_{50} value of AN was found to be 3.35 µg/ml which is higher than that of BHT (64.28 µg/ml). The scavenging effect of AN, BC and standards on the DPPH radical decreased in the order: BHA > AN >BHT> BC, which were 92.7, 91.1, 12.6 and 12.3%, at the 10 µg/ml, respectively (Fig. 1). The results were found statistically significant ($P<0.01$) at 10 µg/ml concentration.

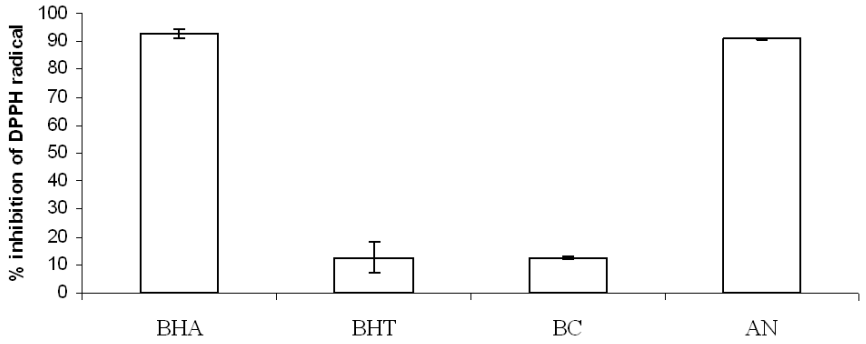

Fig. 1: Comparison of percent inhibition of DPPH radical generation at 10µg/ml conc. of BHA, BHT, BC and AN by the DPPH scavenging method. BHA = Butylated hydroxyl anisole, BHT = Butylated hydroxy toluene, BC = *Berberris chitreya* and AN = *Acacia nilotica.*

AN and BC had effective ABTS radical scavenging activity. The effects of various concentration of AN, BC and standard (ASC) on the ABTS are shown in Fig. 2. There was a significant decrease ($p<0.05$) in the concentration of ABTS due to the scavenging capacity of AN and BC with ASC standard at 25 µg/ml concentration. The IC_{50} value of AN and BC were found to be 19.48 and 58.06 µg/ml.

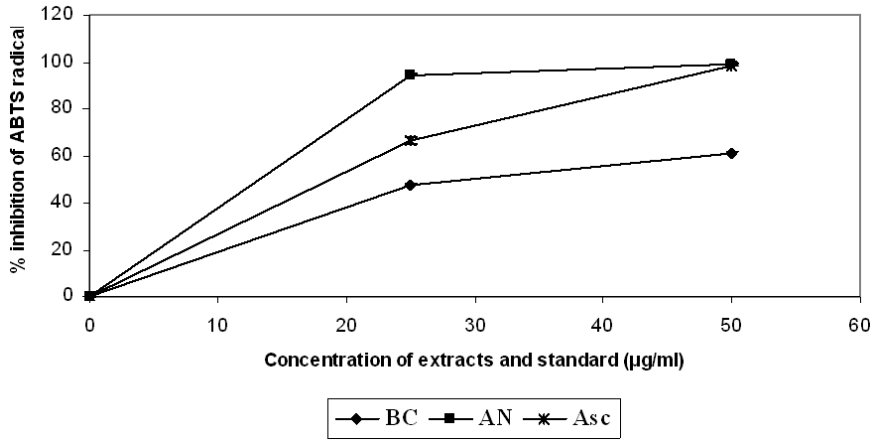

Fig. 2: ABTS radical scavenging activity of methanol extracts of *Acacia nilotica* (AN), *Berberis chitria* (BC) and same doses of Ascorbic acid (Asc).

Fig. 3 depicts the antioxidant activity of the AN, BC and standard (ASC) using the NO-radical scavenging method. The reducing activity of AN, BC and ASC increased with increasing concentration of samples. AN and BC demonstrated an effective reducing activity than control, at different concentrations. These differences were statistically significant ($p<0.05$) at 40 µg/ml concentration. Reducing power of AN, BC and standard compound exhibited the following order: AN > BC >ASC, which were 31.91, 23.95 and 22.77%, at the 40 µg/ml concentration, respectively. The IC_{50} value of AN and BC were found to be 77.24 and 91.64 µg/ml.

The ability of AN and BC to scavenge hydrogen peroxide radical is shown in Fig. 4. AN and BC exhibited 11.64 and 7.63% scavenging effect on H_2O_2 radical hydrogen peroxide, at the 25 µg/ml concentration. On the other hand, BHT, ASC and BHA exhibited 17.78, 16.22 and 7.46% hydrogen peroxide scavenging activity at the same concentration. These results showed that AN and BC have an effective hydrogen peroxide scavenging activity. At the above concentration, the hydrogen peroxide scavenging effect of AN, BC and standards decreased in the order of BHT > ASC > AN > BC > BHA. The IC_{50} value of AN and BC on

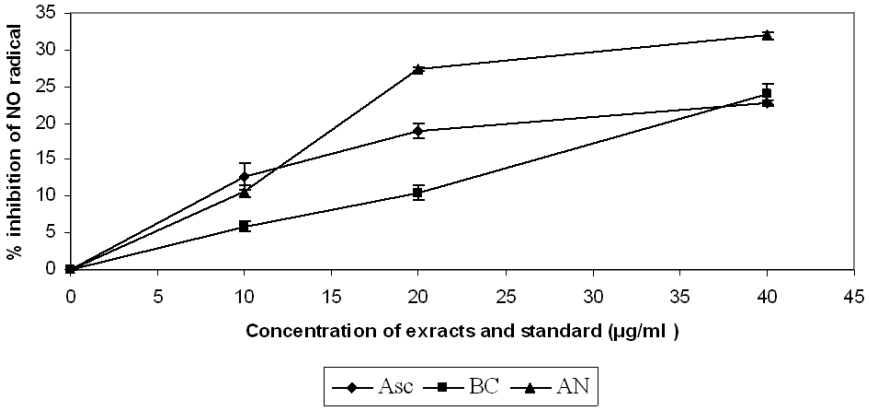

Fig. 3: NO scavenging effect of different concentrations of *Acacia nilotica* (AN), *Berberis chitria* (BC) and Ascorbic acid (Asc).

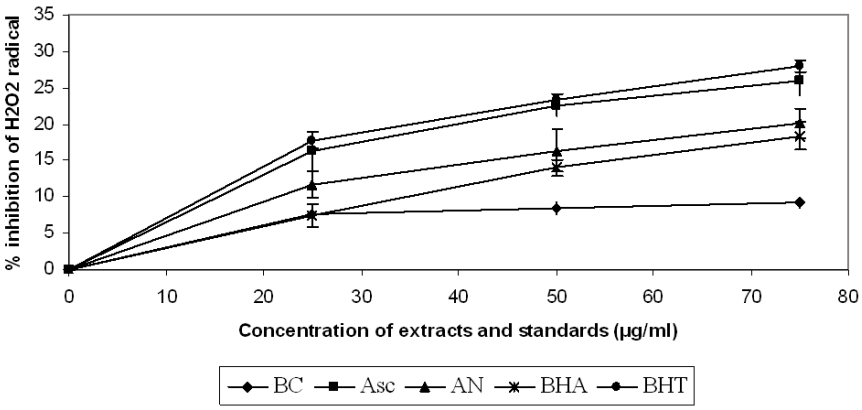

Fig. 4: H_2O_2 scavenging effect of different concentrations of *Acacia nilotica* (AN), *Berberis chitria* (BC), Butylated hydroxyl toluene (BHT), Butylated hydroxyanisole (BHA) and Ascorbic acid (Asc).

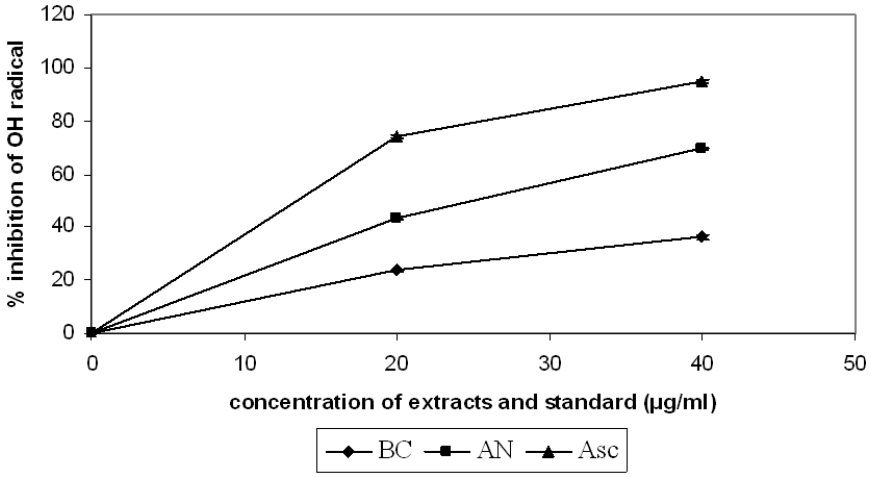

Fig. 5: Hydroxyl radical scavenging effect of different concentrations of *Acacia nilotica* (AN), *Berberis chitria* (BC) and Ascorbic acid (Asc).

scavenging assay were found to be 154.69 and 450.59 µg/ml respectively. Due to the scavenging capacity of AN, BC, there is a significant decrease ($p<0.05$) in the concentration of H_2O_2 with standard ASC at the conc of 25 µg/ml. With BHT and BHA standards, BC and AN are showing significant decrease ($p<0.01$) and ($p<0.05$) respectively at concentrations (25-100 µg/ml).

AN and BC exhibited concentration dependent scavenging activities against hydroxyl radicals generated in a Fenton reaction system (Fig. 5). The IC_{50} of AN and BC were 25.81 and 142.72 μg/ml. AN showed significant decrease (p<0.001) in the concentration of OH, with ASC standard at 40 μg/ml concentration. In addition, the scavenging effect of AN, BC and standard on the OH decreased in the order: ASC > AN > BC which were 74.06, 43.54 and 23.74%, at the 20 μg/ml concentration, respectively. The IC_{50} value of AN and BC on hydroxyl radical scavenging assay were found to be 25.81 and 142.72 μg/ml.

The total phenolic content was measured in terms of gallic acid equivalent for AN and BC, which was found to be 9.88 and 2.73 μg/ml respectively. The potential scavenging abilities of phenolic substances might be due to the active hydrogen donor ability of hydroxyl substitution. Similarly, high molecular weight and the proximity of many aromatic rings and hydroxyl groups are more important for the free radical scavenging by specific functional groups. The methanol extract of *Acacia nilotica* bark exhibited the presence of Alkaloids, glycosides, and tannins, along with phenolics. The high concentrations of these phytochemicals in AN can explain its higher free radical scavenging activity.

Conclusion

From the above studies it can be suggested that *Acacia nilotica* could be used as a easily accessible source of natural antioxidant, which can be used as supplement to aid the therapy of free radical mediated diseases such as cancer, diabetes, inflammation, etc. Further studies are needed on the isolation and identification of antioxidant components in AN. Safety and toxicity studies are also needed for utilization of AN and its components in different antioxidant pharmaceutical formulations.

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