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Investigation of Ethyl Acetate Extract/Fractions of *Acacia nilotica* willd. Ex Del as Potent Antioxidant

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Abstract: This study was planned to evaluate the antioxidant activity of ethyl acetate extract/fractions of *Acacia nilotica* Willd. Ex. Del extracted with different solvents of increasing and decreasing order of solvent polarity. The antioxidative activities, including the 1'-1' diphenylpicryl-hydrazyl (DPPH) radical-scavenging effects, hydroxyl radical scavenging potential, chelating ability, reducing power and lipid peroxidation inhibition in rat tissue homogenate were studied *in vitro*. It was found that the antioxidative effect provided by extract/fractions was strongly concentration dependent and increased on fractionating the extract into water and ethyl acetate fractions. In general, the antioxidative activity increased with increasing extract/fractions concentration to a certain extent, and then leveled off with further increase in antioxidant activity. From a comparison of the antioxidant potential and IC_{50} values for different antioxidative reactions, it seemed that extract/fractions were more effective in scavenging DPPH and hydroxyl radicals than reducing, chelating heavy metals and lipid peroxidation inhibitory potential.

Keywords: *Acacia nilotica*; free radicals; phytochemicals; antioxidant assays; DPPH; lipid peroxidation.

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1. Introduction

Uncontrolled generation of free radicals together with reduced level of antioxidative vitamins and enzymes is considered to be the main contributor to oxidative stress [1]. Free radicals attack membrane lipids, protein and DNA, which is believed to be involved in many health disorders such as diabetes mellitus, cancer, neurodegenerative and inflammatory diseases [2].

Growing knowledge about the health promoting impact of antioxidants in everyday foods, combined with the assumption that a number of common synthetic preservatives may have hazardous effects has led to multiple investigations in the field of natural antioxidants [3]. They are known to have beneficial effects on the prevention or progression of diseases related to oxidative stress on account of their high antioxidant activity. Keeping this in mind the present work was designed to investigate the antioxidant potential of ethyl acetate extract/fractions of *Acacia nilotica* Willd. Ex Del. employing DPPH radical, deoxyribose (site specific and non site specific), reducing power, chelating power ability and lipid peroxidation *in vitro* assays.

2. Materials and Methods

2.1 Chemicals

1'-1' diphenylpicryl-hydrazyl (DPPH), 2-Thiobarbituric acid were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and Deoxyribose was obtained from Lancaster Synthesis Inc. USA. All other chemicals like potassium ferricyanide, trichloroacetic acid, ferric chloride, EDTA, hydrogen peroxide, L-ascorbic acid, Sodium hydroxide, BHA, Folin-Ciocalteu reagent, sodium carbonate and other solvents were procured from CDH and were of analytical grade.

2.2 Preparation of Extract

The bark of *Acacia nilotica* (Family Mimosaceae) was collected washed and dried at room temperature. After grinding to the fine powder it was extracted with different solvents in increasing order of solvent polarity viz. hexane, chloroform, ethyl acetate, acetone, methanol and water and in reverse order. After filtering respective solvents from the supernatant were evaporated in Vacuum Rotary Evaporator to obtain the Crude Extract (CE) and this process was repeated thrice with each solvent. All the extracts were partitioned with the ethyl acetate and water to have the respective fractions (Flow Chart 1 and 2).

2.3 Determination of Total Phenolics

The Total Phenolic Content (TPC) of the ethyl acetate extract/fractions of *Acacia nilotica* was determined by the method of Folin-Ciocalteu reaction using gallic acid as standard [4]. To 100 µL of extract/fractions (20µg/ml) added 500- µL of (50%) Folin-Ciocalteu reagent followed by the addition of 1 ml of 20% Na2CO3 solution. A mixture was incubated at room temperature for 20 min. and the absorbance was measured at 730 nm. The total phenolic content was expressed as gallic acid equivalents (GAE) in milligrams per gram samples.

2.4 Antioxidant testing assays

The antioxidant activity of the ethyl acetate extract/fractions was addressed by employing the following methods:

2.4.1 DPPH scavenging assay

The extracts/fractions were measured in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH following the method given by Blois [5] with little modification. To 2 ml of DPPH (0.1 mM in methanolic solution), added 300µL of extract/fraction concentrations (1 - 100µg/ml). The color change of the reaction mixture was then read at 517 nm against the blank, which did not contain the extract/ fraction. The L-ascorbic acid was used as the positive control. The percent DPPH decolourization of the sample was calculated as:

$$\% \text{ Inhibition} = B0 - B1 / B0 \chi 100$$

Where Bo is the absorbance of the control reaction and B1 is the absorbance in the presence of the sample of plant extracts.

2.4.2 Reducing power assay

The reducing power of ethyl acetate extract/fractions was determined by the method of Oyaizu [6], with modifications. Different concentrations of extract (1 ml) were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [K3Fe(CN)6] (2.5 ml, 1%). The mixture was incubated at 50ºC for 20 min. Aliquots (2.5 ml) of trichloroacetic acid (10%) were added to the mixture, which was then centrifuged for 10 min at 1036χg. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and FeCl3 (2.5 ml, 0.1%), and the absorbance was measured at 700 nm in a spectrophotometer. Increased absorbance of the reaction mixture indicated the increased reducing power.

2.4.3 Deoxyribose degradation assay

The non site and site-specific deoxyribose assays were performed following the method of Halliwell [7] and Arouma [8] with slight modifications. In non site-specific deoxyribose assay; Briefly, the extracts (from 1-100µg/mL) were mixed with a Haber-Weiss reaction buffer [10mM FeCl3, 1mM EDTA (pH 7.4), 10 mM H2O2, 10 mM deoxyribose, and 1mM L-ascorbic acid] and the final volume of all mixtures was made to 1.0 mL. The mixture was then incubated at 37ºC for 1 hr and heated at 80ºC for 30 min with 1 mL of 2-TBA (0.5% 2-TBA in 0.025 M NaOH, 0.02% BHA) and 1 mL of 10% trichloroacetic acid (TCA) in water bath for 45 min. After cooling, absorbance of mixture was measured at 532 nm. A site-specific assay was performed following slight modifications where the EDTA was replaced with a same volume of phosphate buffer. The percentage inhibition was calculated employing the formula as given for DPPH scavenging assay.

2.4.4 Chelating effects on ferrous ions

The chelating effect on ferrous ions was determined according to the method of Dinis [9], with some modifications. The extracts/fractions (0.25 mL) were mixed with 1.75 mL of methanol and 0.25 mL of 250 mM FeCl2. This was followed by the addition of 0.25 mL of 2 mM ferrozine, which was

left to react at room temperature for 10 min before determining the absorbance of the mixture at 562 nm. The chelating effect (%) was calculated from the formula as given for DPPH scavenging assay.

2.4.5 Lipid Peroxidation by Thiobarbituric Acid (TBA) Assay

TBA reacts with malondialdehyde (MDA) to form a diadduct, a pink chromogen, which can be detected spectrophotometrically at 532 nm [10]. Normal male rats (250g) were used for the preparation of liver homogenate. The perfused liver was isolated, and 10% (w/v) homogenate was prepared with homogenizer at 0-4 °C with 0.15 M KCl. The homogenate was centrifuged at 800*g* for 15 min and clear cell-free supernatant was used for the study of *in vitro* lipid peroxidation 1 mL of 0.15 M KCl and 0.5 mL of rat liver homogenates and different concentrations of extract/fractions (50-400µg/ml) were mixed and the peroxidation reaction was initiated by adding 100 µL of 0.2 mM ferric chloride. After incubation at 37 °C for 30 min, the reaction was stopped by adding 2 mL of icecold HCl (0.25 N) containing 15% trichloroacetic acid (TCA), 0.38% TBA, and 0.5% BHT. The reaction mixtures were heated at 80 °C for 60 min. The samples were cooled, centrifuged and the absorbance of the supernatants was measured at 532 nm and the percentage of inhibition was calculated by the formula: -

Inhibition of lipid peroxidation (%) = 1 – (sample OD/blank OD) X 100

2.5 Statistical Analysis

All experiments were repeated at least three times. Results are reported as Mean ± SE. The IC_{50} values (concentration of sample for 50% inhibition) were also calculated for the extract/fractions.

3. Results and Discussion

Many studies carried out over the past few years have shown that polyphenols found in dietary and medicinal plants inhibit oxidative stress [11]. The preventive role of these foods is due to their constituent chemicals, especially the polyphenolic flavonoids, anthocyanins and anthocyanidins [12]. Consequently, we studied its antioxidant activity by a series of *in vitro* protocols using some biologically relevant models. Figures 1-6 depicts the results of ethyl acetate extract/fractions of *Acacia nilotica* obtained in increasing and decreasing order of solvent polarity. It is clear from the results that there was not much difference in the antioxidant potential of the extracts prepared by increasing and decreasing order of solvent polarity. We studied the respective antioxidant activities of different extracts/fractions and attempted to correlate the results in terms of their total phenolic and the antioxidant activity further compared with standard polyphenols.

3.1 DPPH radical scavenging assay

In 1'-1' diphenylpicryl-hydrazyl radical scavenging assay, ethyl acetate extract/fractions of *Acacia nilotica* showed potent antioxidant activity. As shown in figure 1, Among the water, ethyl acetate fractions and the crude ethyl acetate extract, the water fraction obtained by increasing and decreasing order of solvent polarity showed effect of 68.39% and 59.18% respectively at 100µg/ml concentration.

DPPH, a protonated radical, has characteristic absorbance maxima at 517 nm, which decreases with the scavenging of the proton radical which has been widely used to evaluate the free radical scavenging effect of natural antioxidants [13].Many researchers have reported positive correlation between free radical scavenging activity and total phenolic compound. Oki et al., [14] observed that the radical scavenging activity increased with the increase of phenolic compound content. The two

studies conducted by Lu and Foo [15] and Siriwardhana et al., [16] also reported a high correlation between DPPH radical scavenging potential and total phenolic content.

3.2 Reducing power

Figure 2 show that the water fraction reduced the Fe^{3+} to ferrous ions (Fe^{2+}) more effectively (1.626 and 1.423) as compared to the ethyl acetate fraction (1.258 and 1.094) and crude extract (0.674 and 0.522) for increasing and decreasing order of solvent polarity respectively at 150µg/ml concentration. This result was in agreement with that of Yen and Duh [17] who reported that the reducing power of peanut hull extract increased with increase in concentration and correlated (r2 = 0.9793) well with the extent of antioxidant activity. Similarly, Duh [18] found that the antioxidant properties of mung bean hull and burdock extracts were shown to be concomitant with the development of reducing power. In the present study both the fractions (water and ethyl acetate) and crude extract of *Acacia nilotica* exhibited good reducing potential.

It is believed that antioxidant activity and reducing power are related as reductones inhibit LPO by donating a hydrogen atom and thereby terminating the free radical chain reaction [19].

3.3 Hydroxyl radical scavenging assay

The hydroxyl radical scavenging activity of the ethyl acetate extract/fractions of *Acacia nilotica* by site and non-site specific assay is shown in figure 3 and 4 respectively. On comparing both the assays, it was observed that the antioxidant effect was more in site-specific assay for increasing polarity and more in non site-specific assay for decreasing order of solvent polarity. In decreasing order of solvent polarity the trend is same as in increasing solvent polarity but inhibitory potential was less in the former. In nutshell, the hydroxyl radical scavenging potency of extract/fractions of *Acacia nilotica* (increasing order of solvent polarity) was in the order: Water fraction (93.82 and 83.19%) > Ethyl acetate fraction (88.45 and 79.26%) > Crude extract (42.63 and 44.41%) for site and non-site specific assay respectively. On the contrary for decreasing order of solvent polarity was: Water fraction (79.65 and 86.70%) > Ethyl acetate fraction (76.42 and 83.73%) > Crude extract (42.15 and 40.23%) for site and non-site specific assay respectively.

Hydroxyl radical is an extremely reactive free radical formed in biological systems and has the capacity to cause DNA strand breakage, which contributes to carcinogenesis, mutagenesis and cytotoxicity [20]. In addition, this radical species is considered as one of the quick initiators of the LPO process, abstracting hydrogen atoms from unsaturated fatty acids. Reducing agents, such as ascorbic acid, can accelerate $^{\cdot}OH$ formation by reducing Fe^{3+} ions to Fe^{2+} [21]. Deoxyribose is degraded into malonaldehyde on exposure to hydroxyl radicals generated by Fenton systems. If the resulting mixture is heated under acid conditions, malonaldehyde may be detected by its ability to react with thiobarbituric acid to form a pink chromogen [7].

3.4 Metal ion chelating activity

The chelating effect of ferrous ions by the fractions and extract of *Acacia nilotica* and standards is shown in figure 5. Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of chelating agents, the complex formation is disrupted, resulting in decrease of the red colored complex. Measurement of color reduction is the measure of the metal chelating activity. In this assay, both fractions, crude extract and standard compounds interfered with the formation of ferrous and ferrozine complex, suggesting that they have chelating activity and are able to capture ferrous ion before the formation of ferrozine. Figure 5 describes the effect of the water fraction (74.16%) which was found to be more than the ethyl acetate fraction (67.86%) and crude extract (33.23%) obtained by increasing order of solvent polarity. While in decreasing order of solvent polarity the trend was slight

different, in this case ethyl acetate fraction (76.87%) showed more chelating potential than the water fraction (69.13%) and crude ethyl acetate extract (35.58%) at 400µg/ml concentration.

Iron is known to generate free radicals through the Fenton and Haber–Weiss reaction. Metal ion chelating activity of an antioxidant molecule prevents oxyradical generation and the consequent oxidative damage. Metal ion chelating capacity plays a significant role in antioxidant mechanism since it reduces the concentration of the catalysing transition metal in LPO [19]. It is reported that chelating agents, which form *s*-bonds with a metal, are effective as secondary antioxidants since they reduce the redox potential thereby stabilizing the oxidized form of the metal ion [20].

3.5 Lipid peroxidation assay

In Lipid peroxidation, the potential of ethyl acetate extract/fractions of *Acacia nilotica* to inhibit lipid peroxidation in rat liver homogenate, induced by the $FeCl_2$–H_2O_2 system was measured. Figure 6 shows that the water fraction (74.24%) in increasing order of solvent polarity exhibited more LPX inhibition as compared to the ethyl acetate fraction (68.91%) and crude extract (29.24%). In decreasing order of solvent polarity the fashion of antioxidant potential is similar i.e. Water fraction (81.17%) > Ethyl acetate fraction (72.26%) > Crude extract (38.17%) at 700 µg/ml concentration. This assay is less sensitive as compared to the DPPH because in this assay 81.17% inhibition was seen at 700µg/ml concentration whereas in case of DPPH assay, 68.39% inhibition was there only at 100µg/ml concentration. The hydroxyl radicals generated in the Fenton reaction are known to be get scavenged if the rat liver homogenate is co incubated with extract/fractions. In this assay we have noticed that as the concentrations of the extract was increased the inhibition of lipid peroxidation was also increased.

LPO has been broadly defined as the oxidative deterioration of polyunsaturated lipids [22]. Initiation of a peroxidation sequence in a membrane or polyunsaturated fatty acid is due to abstraction of a hydrogen atom from the double bond in the fatty acid. The free radical tends to stabilize by a molecular rearrangement to produce a conjugated diene, which then readily reacts with oxygen molecule to give a peroxy radical. Peroxy radicals can abstract a hydrogen atom from another molecule to give lipid hydroperoxide, R-OOH.

3.6 Total phenolic content

Antioxidant activity of the plant extract is often associated with the phenolic compounds present in them. Hydrogen donating property of the polyphenolic compounds is responsible for the inhibition of free radical induced LPO [23]. Table 1 demonstrates the total phenolic content of the ethyl acetate extract/fractions of *Acacia nilotica*. It was noticed that the water and ethyl acetate fractions showed higher phenolic content than the crude acetone extract in µg/mg as gallic acid equivalent i.e. Water fraction (610) > ethyl acetate fraction (445) > crude acetone extract (165).

In our study, there seemed to be good correlation between the phenolic content and antioxidant activity of the extracts since water fraction with higher phenolic content showed higher antioxidant activity. However, it is known that nonphenolic antioxidants could also contribute to the antioxidant activity of an extract. As in other studies evaluating antioxidant activity of medicinal plants and fruits found a direct linear relationship between the total phenolic content and antioxidant activity indicating that the phenolic compounds might be the major contributors to the antioxidant activities of these extracts [24]. In order to characterize antioxidant activity of a plant extract, it is desirable to subject it to a battery of tests that evaluates the range of activities such as scavenging of the reactive oxygen species, inhibition of membrane LPO and metal ion chelation.

The broad range of antioxidant activity of the extracts indicates the potential of the plant as a source of natural antioxidants or nutraceuticals with potential application to reduce oxidative stress with consequent health benefits. It is pertinent to mention that the results obtained in the present study are in conformity with our previous results on antimutagenic activity against genotoxic injury by NPD,

sodium azide and 2-Aminofluoerene in the Ames *Salmonella* histidine reversion assay and antioxidant activity employing different *in vitro* methods for *Acacia* plants [25-28]. The work further reveals that the *Acacia* species, can be an interesting source of antioxidants with their potential use in different fields viz. food, cosmetics, and pharmaceuticals. Our efforts are underway to isolate and identify the antioxidant molecules in the ethyl acetate extract/fractions of *Acacia nilotica* and study their health promoting potential and mammalian safety.

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