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PHYTOCONSTITUENT ANALYSIS AND IN-VITRO ANTIOXIDANT EFFECT OF *Albizia amara* BARK EXTRACT *Initial*

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Abstract

Ancient peoples used the decoctions prepared by bark of Albizia amara as health drink but lack of scientific scrutiny. In the present study an attempt has been made to explore the antioxidant property of A. amara bark aqueous extract by DPPH and ABTS free radical scavenging assays. Fresh bark are collected from A. amara tree near Tiruppatur regions, and washed and rinsed with distilled water. Then sliced into small pieces and shade dry, crushed into fine powder by homogenizer. Phytoconstituent profile of aqueous bark extract is screened by standard protocols. The free radical potential of bark extract is determined by the various free radical scavenging assays like DPPH, ABTS•+ and nitric oxide. Albizia amara is one such medicinal plant having various pharmacological activities but limited scientific report is available to date to validate their folkloric uses. Results obtained from phytochemical analysis shows the presence various phytochemicals which includes flavonoids, alkaloids, saponins and tannins. Imbalance between the free radical formation and antioxidant system of the body will lead to develop oxidative stress, which damages the cellular macromolecules like DNA, proteins and lipids. The aqueous bark extract of A. amara shows potent antioxidant activity by effectively scavenging the free radicals such as ABTS and DPPH, at dose dependent manner. Ascorbic acid serves as standard for antioxidant assays. Hence, the A. amara bark extract can be considered as a potential candidate for the ailment of various complications caused by oxidative stress.

Keywords: Albizia amara; Free radical; Antioxidant; Oxidative stress; Bark extract and Phytochemicals.

1. Introduction

Mitochondria are the major site for ROS (reactive oxygen species) production through physical and pathological conditions. During cellular respiration in arachidonic acid metabolism, free radical such as singlet oxygen can be generated by couple of oxygenase enzymes such as LOX (lipoxygenases) and COX (cyclooxygenases) in endothelial and inflammatory cells [1]. When compared to other *in-vivo* free radicals, OH• radicals are the most reactive free radical and mainly generated by the reaction between singlet oxygen and hydrogen peroxide radical. The generation of OH• radical reaction is catalyzed by Fe_2^+ or Cu^+. The other potent free radical NO• (radical nitric oxide) is generated during citrulline oxidation from arginine by the enzyme nitric oxide synthase (NOS). Free radicals also generated from some non-enzymatic reactions which includes reaction of oxygen with compounds having organic in nature or by ionizing radiations when cells or tissues were exposed to radiation. During Mitochondrial respiration also free radical can be produced by non-enzymatically [2].

Both exogenous as well endogenous factors contribute the major role in generation of free radicals. Endogenous sources like inflammation, ischemia, cancer, aging, heavy exercise, stress, immune cell activation, infections are responsible for ROS production. When exposure to heavy metals like Cadmium (Cd), Lead (Pb), Mercury (Hg), Iron (Fe) and Arsenic (As), environmental pollutants, smoked meat, used fats and oils, solvents has chemical in nature, radiation exposure, cigarette smoke and side effects of some medications like cyclosporine, gentamycin, tacrolimus and bleomycin are also responsible for exogenous free radical generation. Subsequently, penetration to these exogenous materials in to the body, it should be properly metabolized or degraded. During the degradation process, free radicals can be formed as metabolic by-products [3].

Low to moderate level of reactive oxy radicals in cells can exerts beneficial activity in living organisms. Host organism can use these free radicals as a defensive agent to compete against disease causing pathogens and also they are involved in synthesize of some cellular structures. For destroying invading pathogenic microbes, the free radicals are synthesized and stored in the phagocytes. Due to defective enzyme system of NADPH oxidase in individuals of granulomatous disease are unable to generate $O_2^{\bullet -}$, so they are easily prone to various and tenacious infections. Free radicals are involved in various signaling pathways at cellular level and produced through non-phagocytic isoforms of NADPH oxidase [4].

Intracellular signaling mechanisms of various types of cells like endothelial, fibroblasts, cardiac myocytes, vascular smooth muscle cells and tissues of thyroid are mainly regulated by free radicals. Nitric oxide

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is well-known free radical acts as signaling molecule. NO is a vital cell-to-cell mediator essential for an appropriate modulation in the blood flow, thrombotic involvement and proper activity of neurons, also very crucial for host defense mechanism at non-specific level [5].

The imbalance between ROS generation, accumulation and the ability of the living system to detoxify them by the cells and tissues is termed as oxidative stress. ROS are formed in the biological system as a by-product of various cell metabolisms. Commonly formed reactive oxygen species are $^1O_2$ (singlet oxygen), $O_2^{\bullet-}$ (superoxide radicals), $^\bullet OH$ (hydroxyl radicals) and H_2O_2 (hydrogen peroxide). The metabolic process such as phosphorylation of proteins, apoptosis, factors that activates transcriptional mechanism, differentiation and resistance are mainly depends on suitable ROS generation and low level should be maintained inside the cells. Increased level of ROS can cause some destructive effects on vital cellular arrangements which include membrane damage, changes in protein configuration, lipid arrangements, lipoprotein moiety and nucleic acid structures. Increased oxidative stress leads to onset and development of various diseases such as diabetes, cancer, atherosclerosis and cardiovascular diseases [6]. In order to over from the lethal effects of reactive oxygen species in the living organisms, several antioxidants were formulated and tested. These antioxidants exert some unwanted effects during their action mechanism against oxidants. Hence plant based antioxidants attain major attraction, because of its limited side effects and its various pharmacological benefits. Therefore the present study was aimed to analyze the presence of various pharmacologically active metabolites in the Albizia *amara* aqueous bark extract as well as assess the in-vitro free radical scavenging effect of bark extract against effective free radicals such DPPH, ABTS and superoxide.

2 Experimental Sections

2.1. Chemicals: Griess reagent, DPPH and ABTS were purchased from Himedia, Mumbai. All other chemicals and reagents procured for conducting present study were of analytical grade obtained from SRL, Mumbai.

2.2. Plant material and preparation of bark extract

The fresh bark of A. *amara* was collected from the trees near the Tirupattur, Tirupattur district, Tamil Nadu. The barks were washed thoroughly under running tap water and rinsed in distilled water. They were cut into slices and dried in a shade dry for up to one week, powdered in an electrical grinder which was stored in an airtight container at 5°C until further use.

2.3. Preliminary Phytochemical screening

Preliminary phytochemical analysis was carried out for the extract as per standard methods described by Harbone [7].

1. Detection of Alkaloids

Mayer's test : The extract was treated with Mayer s reagent. Formation of a yellow cream precipitate indicates the presence of alkaloids.

Wagner's test : The extract was treated with Wagner s reagent. Formation of brown/reddish brown precipitate indicates the presence of alkaloids.

2. Detection of Flavonoids

1. Lead acetate test : Extracts were treated with few drops of lead acetate solution. Formation of yellow color precipitate indicates the presence of flavonoids.

2. H_2SO_4 test: Extracts were treated with few drops of H_2SO_4. Formation of orange colour indicates the presence of flavonoids.

3. Detection of Steroids: Two ml of acetic anhydride was added to five ml of the extract and then added each with two ml of H_2SO_4. The color was changed from violet to blue or green indicates the presence of steroids.

4. Salkowski's Test :Five ml of the extract mixed with two ml of chloroform and then added carefully the 3 ml of concentrated H_2SO_4 to form a layer. An appearance of reddish brown colour in the inner face indicates the presence of terpenoids.

5. Detection of Anthraquinones Borntrager's Test: About five ml of the extract was boiled with 10% HCl for few minutes in a water bath. It was filtered and allowed to cool. Equal volume of Chloroform was added to the filtrate. Few drops of 10% ammonia was added to the mixture and heated. Formation of pink colour indicates the presence of anthraquinones.

6. Detection of Phenols

Ferric chloride test :10ml of the extract was treated with few drops of ferric chloride solution. Formation of bluish black colour indicates the presence of phenol.

Lead acetate test: 10 ml of the extract was treated with few drops of lead acetate solution. Formation of yellow colour precipitate indicates the presence of phenol.

7. Detection of Saponins: About 0.5ml of the extracts was shaken with five ml of distilled water. Formation of frothing (appearance of creamy of small bubbles) shows the presence of saponins.

8. Detection of Tannins: A small quantity of extract was mixed with water and heated on a water bath. The mixture was filtered and ferric chloride was added to the filtrate. A dark green colour was formed. It indicates the presence of tannins.

9. Detection of Carbohydrates :0.5ml extracts were dissolved individually in five ml distilled water and filtered. The filtrate was used to test the presence of carbohydrates.

10. Detection of Protein & Amino acids Biuret test : To 0.5 ml of extract equal volume of 40% NaOH solution and two drops of one percent copper sulphate solution was added. The appearance of violet colour indicates the presence of protein.

Ninhydrin test :About 0.5 ml of extract was taken and two drops of freshly prepared 0.2% Ninhydrin reagent was added and heated. The appearance of pink or purple colour indicates that the presence of proteins, peptides or amino acids.

11. Detection of Oils and Resins: The extract was applied on the filter paper. It develops a transparent appearance on the filter paper. It indicates the presence of oils and Resins.

2.4. Free radical scavenging assays

1. DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical scavenging assay

The free radical scavenging capacity of the aqueous bark extract of A. *amara* was determined using DPPH [8]. DPPH (200μM) solution was prepared in 95% methanol. From the stock, bark extract solution prepared in 0.1 mol L^{-1} Tris HCl buffer (pH- 7.9) 200, 400, 600, 800 and 1000 μg/ml were taken in five test tubes. 0.5 ml of freshly prepared DPPH solution was incubated with bark extracts and kept under light protection for 20 min at room temperature. The decrease of absorbance at 517 nm was measured using the spectrophotometer. Deionised water used as a blank experiment and standard ascorbic acid was used as positive control.

2. $ABTS^{\bullet+}$ scavenging assay

$ABTS^{\bullet+}$ radical cation scavenging activity of aqueous bark extract of A. amara was determined according to the method of Re [9]. Briefly, ABTS radical cation ($ABTS^{\bullet+}$) in 20 mmol sodium acetate buffer (pH-4.5) was combined with 2.45 mmol potassium persulfate to generate a stable dark blue-green radical following 12-16 h of incubation at 4°C in the dark. The reaction mixture is suitably diluted to an absorbance of 0.7±0.01 at 734 nm spectrophotometrically to form the test reagent. The reaction mixture containing different concentrations (200-1000 μg/ml) of bark extract and 3.0 ml of test reagent were incubated in a water bath at 30°C for 30 min. The test solution mixture turns colourless and the absorbance is reduced due to the sequestration of unpaired electrons in the test reagent by the antioxidants in the bark extract. Standard ascorbic acid was used as a reference.

3. Assay for nitric oxide (NO) scavenging activity

Sodium nitroprusside (5 mmol) in phosphate buffer pH 7.7 was incubated with 200, 400, 600, 800 and 1000 μg/ml concentrations of bark extract dissolved in a suitable solvent (alcohol) and tubes were incubated at 25°C for 120 min. At intervals, 0.5 ml of incubation solution was removed and diluted with 0.5 ml of Griess reagent. The assay was performed in triplicate and the mean value of absorbance was calculated. The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent N-napthyl ethylenediamine was measured at 546 nm. Standard ascorbic acid was used as a reference [10].

3. Results and Discussion

Presence of biologically active plants secondary metabolites of *Albizia amara*bark extract shown in Table 1. Preliminary phytochemical analysis of aqueous bark extract of A. *amara*plant shows the presence of various important phytochemicals such as alkaloids, flavonoids, phenols, saponins, glycosides, oils and resins. Phytochemicals are derived from ecology as a non-nutrient bioactive compound exerts several pharmacological and beneficial properties to the human health. The qualitative phytochemical screening of aqueous bark extract of A. *amara*evidenced that the bark extract have various important secondary metabolites which readily accounts for the traditional medicinal uses of this A. *amara*plant. The various parts of the A. *amara*plants are widely used for the ailment of several diseases and disorders. Alkaloids play a vital role in human health by possessing potent pharmacological as well as beneficial effects.

Table 1. Phytochemical screening of aqueous *Albizia amara* bark extract

Phytochemicals	Inference
Alkaloids	+
Flavonoids	+
Steroids	-
Terpenoids	-
Anthraquinones	-
Phenols	+
Saponins	+
Tannins	+
Glycosides	+
Proteins	-
Oils and Resins	+

Various synthetic and semisynthetic drugs are mainly derived from alkaloids with slight structural modifications. Alkaloids play an extensive role in human health such as anticancer, antibiotics and various degenerative diseases. Because of their huge pharmacological properties alkaloids contribute great role in the formulation of pharmaceutical drugs especially for cancer as well as inflammatory disorders [11].

Saponins are distributed in wild terrestrial plants warmly involved in our day to day life. It is a structurally and biologically diverse class of triterpenes and steroidal glycosides. Tannins present in the bark extract forms the source for its therapeutic significance. The presence of tannins in the fruits extract forms the basis for its medicinal value. Tannins are considered as important plant secondary metabolite because it possess pharmacological activities like antimutagenic and anticarcinogenic. Tannins in the diet interact with enzymes and proteins and enhance the functions of proteins. Hydroxyl and other functional groups present in the tannins responsible for its significant pharmacological property [12]. Fig.1. represents the *in-vitro* DPPH radical scavenging activity of the A. *amara*bark aqueous extract. The A. *amara*bark extract shows dose dependent scavenging activity against the free radical DPPH.

Bark extract concentration ranges from 200-1000 μg/ml shows 40-72 percentage inhibition of

DPPH radicals. Reduction of DPPH radicals (DPPH*) in methanolic solution under dark condition is the basis of antiradical activity assay. DPPH have odd electron configuration, shows absorption maximum very strong at 517 nm [13]. In the presence of hydrogen donor (antioxidants having free radical scavenging property) the odd electrons in the free radical becomes paired off. After getting paired electron from antioxidants the absorption strength of free radical is decreased and the resulting decolorization is stoichiometric with respect to free radical captured electrons number. The free radical scavenging activities of extracts depend on the capacity of antioxidant compounds to lose hydrogen and the structural conformation of these components. Ascorbic acid serves as standard antioxidant compound.

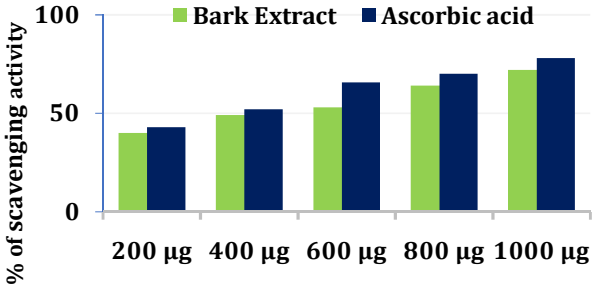


Fig. 1. DPPH radical scavenging assay of aqueous extract of *Albezia amara* bark.

Fig. 2 shows the ABTS radical scavenging activity of the aqueous extract of A. *amara*bark. Plant material effectively scavenges the ABTS free radical at dose dependent manner. 1000 µg/ml shows maximum scavenging activity of 63%. The percentage inhibition ranges from 35-75% with bark extract concentration is 200-1000 µg/ml. When compared to DPPH radicals, $ABTS^{*+}$ produce most powerful free radical and the reactions process with $ABTS^{*+}$ radicals involve a transfer of single electron. The principle of $ABTS^{*+}$ assay is that the preformed radical monocation of $ABTS^{*+}$ is generated by oxidation of $ABTS^{*+}$ with potassium per sulfate and is reduced in the presence of such hydrogen-donating antioxidants. Ascorbic acid serves as reference [14].

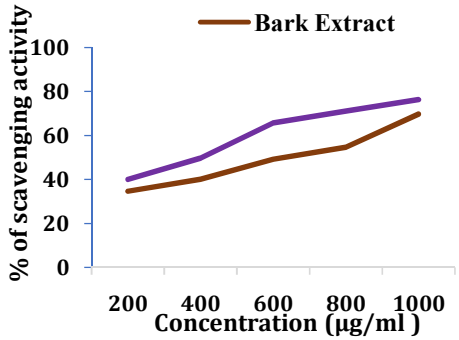
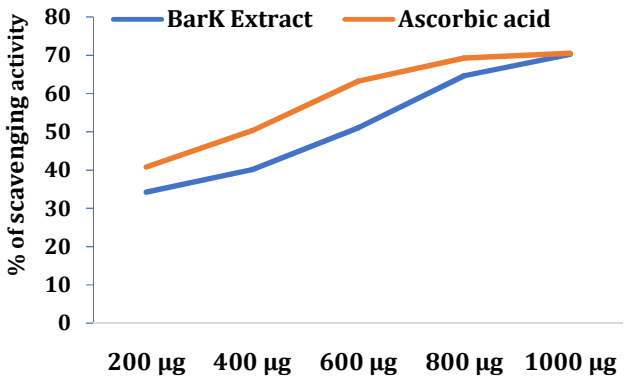


Fig. 2. ABTS radical scavenging assay of aqueous bark extract of *Albiziaamara*

Fig. 3. shows the superoxide radical scavenging activity of A. *amara*bark extract. Aqueous bark extract dose dependently scavenge the superoxide radicals. Superoxide radical scavenging activity of bark extract, ranges from 34 to 70%. The results were compared with ascorbic acid. Superoxides are formed by non-enzymatic auto-oxidation of catecholamines and from molecular oxygen by oxidative enzymes. Superoxides are extremely harmful to various cellular components.

The anionic superoxides play a vital role in the generation of other ROS which includes singlet oxygen, hydroxyl radical and hydrogen peroxides, which leads to oxidative damage in DNA, protein and lipids. Compression of two full negative charges by energetically disfavored diatomic molecules is required for second reduction of superoxide resulted into strong reducing agent than the oxidizing agent and lipid membranes do not allow the superoxide does penetration. Low levels of superoxides are allowed to persistent inside the cells and tissues which indicates limiting superoxide exposure of cell and tissues are very important for survival. The increased concentrations of various phytochemicals such as flavonoids and alkaloids may be responsible for the observed significant *in-vitro* antioxidant property of the *Albizia amara*bark extract [15].



Concentration (µg/ml)

Fig.3. Superoxide radical scavenging assay of aqueous bark extract of A. *amara*

4 Conclusions

Several researchers involving the pharmacological properties of *Albizia amara*plant parts are sparse, which was evidenced from thorough literature survey. The data obtained from the present study reveals the aqueous *Albizia amara*bark extract possess various pharmacologically active phyto-ingredients. The bark extract found to potential antioxidant effect by modulating free radical level at dose dependent manner, which was reveled from various free radical scavenging assays. The presence of secondary metabolites like flavonoids and phenolic compounds in the bark extract readily accounts for its *in-vitro* free radical scavenging property. From the study it can be concluded that the

aqueous extract of *Albizia amara*bark may be considered as a ridiculous cause for the credentials of nutraceuticals with varied medicinal values. After the result, it can be determined that the aqueous extract of *Albizia amara*bark can be considered as a possible source of antioxidants.

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