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Free Radical Scavenging Activity of Leaf Extracts of Indigofera Aspalathoides – An *in vitro* Analysis

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Abstract

The present study was aimed to evaluate the free radical scavenging activity of two fractions from the leaves of Indigofera Aspalathoides. Four invitro models namely- DPPH radical, ABTS radical, Nitric oxide radical and hydroxyl radical scavenging assays were used to analyze the activity. Amount of polyphenolic compounds, major phytochemical with antioxidant activity was also estimated. Both fractions displayed significant antioxidant activity when compared to standard antioxidants. Even though Chloroform fraction contained less amount of polyphenolic compounds, it showed more radical scavenging activity than the Ethanol fraction which indicates the role of structural features of polyphenolic compounds with respect to their Antioxidant potential.

Keywords : Free radical, Antioxidants, Polyphenolic compounds, Indigofera aspalathoides

Introduction

Reactive oxygen species as well as reactive nitrogen species products of normal cellular metabolism are well recognized for playing dual role as beneficial and deleterious [1]. Inability in the maintenance of Redox homeostasis could lead to potential damage – termed as oxidative stress and nitrostative stress [2,3].This occur either when there is an over production of ROS/RNS or a deficiency in enzymatic and nonenzymatic antioxidants which can scavenge these free radicals .Various natural phytochemicals having a chemical structure compatible with putative antioxidants in vivo in particular flavonoids [4,5], isoprenoids and methyl tocol [6] have been considered to be modifier of cellular response to RONS mediated various stimuli 1.By Scavenging RONS 2. Suppressing their generation by inhibiting the enzyme[7] 3.By chelating trace elements involved in free radical production 4. By protecting intracellular antioxidant defense [8] 5. By up regulating intracellular signaling resulting in antioxidant cellular response [9, 10].

In recent years, there has been a worldwide surge towards the identification and use of natural antioxidant principles, which provide enormous scope in correcting redox imbalance and reduce the deleterious effect. Indigofera aspalathoides belonged to Pappiloncea family is a low under shrub widely distributed in South India and Sri Lanka. Sidha physician traditionally uses leaves and flowers of this plant to treat elephantiasis, skin disorder, leprosy and cancer [11]. Studies with stem extract indicates that it has antitumor, antiviral and antibacterial effect [12, 13]. So the possible antioxidant potential of Ethanolic and Chloroform fractions obtained from the leaves of Indigofera aspalathoides have been explored in the present study using invitro experiments.

Materials and methods

Indigofera aspalathoides was collected from Tirunelveli District, Tamil Nadu, in August and September. Leaves were shade dried and powdered. Removal of chlorophyll and dewaxing

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from powered materials was done by treating with Petroleum ether (40-60^{0}C) by hot continuous percolation method in Soxhlet apparatus [14] for 24 hrs. Then the marc was successively subjected to Chloroform (76-78^{0}C) and ethanol extraction for 24 hrs each to obtain the corresponding fraction**.** The extract was concentrated and dried in a desiccator.(Yield: chloroform fraction-2.5%w/w, ethanol fraction – 5.0 %w/w)

Chemicals

DPPH, ABTS, Napthyl ethylene diamine dihydrochloride and sulfanilic acid were purchased from Sigma Aldrich Chemical Pvt. Ltd., Bangalore, India. All other chemicals used were of analytical grade available commercially.

Evaluation of antioxidant activity by invitro methods

1.DPPH(2,2-diphenyl-l-picryl hydrazyl) radical scavenging assay

DPPH assay is based on the measurement of the scavenging ability of antioxidant towards the stable DPPH radical and scavenging activity was determined by the method of Sreejayan M *et al* [15] . The free radical DPPH is purple in color in methanol and is reduced to the corresponding hydrazine, which is yellow in color, when it reacts with hydrogen donor. To 200 µl of 100 µM DPPH solution, 10 µl of various concentrations of the extract or the standard solution(Ascorbic acid) was added separately in wells of the microtitre plate. The plates were incubated at 37 °C for 30 min. Absorbance was measured at 517 nm using ELISA reader.

ABTS [2,29-azinobis-(3-ethyl benzo thiazoline-6-sulfonic acid)] radical cation decolorisation assay .

The experiment was carried out using an improved ABTS decolorisation assay [16].ABTS (54.8 mg) was dissolved in 50 ml of distilled water to 2 mM concentration and potassium persulphate (17 mM, 0.3 ml) was added. The reaction mixture was left to stand at room temperature overnight in dark before use. To 0.2 ml of various concentrations of the extracts or standards, 1.0 ml of distilled DMSO and 0.16 ml of ABTS solution was added to make a final volume of 1.36 ml. Absorbance was measured spectrophotometrically, after 20 min at 734 nm.

Hydroxyl radical scavenging activity assay

This assay was carried out according to the method of Halliwell B et al [17]. To the reaction mixture containing deoxyribose (3 mM, 0.2 ml), ferric chloride (0.1 mM, 0.2 ml), EDTA (0.1 mM, 0.2 ml), ascorbic acid (0.1 mM, 0.2 ml) and hydrogen peroxide (2 mM, 0.2 ml) in phosphate buffer (pH, 7.4, 20 mM), 0.2 ml of various concentrations of extracts or standards in DMSO were added to give a total volume of 1.2 ml. The solutions were then incubated for 30 min at 37 C. After incubation, ice-cold trichloro acetic acid (0.2 ml, 15% w/v) and thiobarbituric acid (0.2 ml, 1% w/v), in 0.25 N HCl were added. The reaction mixture was kept in a boiling water bath for 30 min, cooled and the absorbance was measured at 532 nm

Nitric oxide radical scavenging activity assay

Nitric oxide radical scavenging activity was determined according to the method reported by Garrat [18]. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions, which can be determined by the use of the Griess Illosvoy

reaction. 2 ml of 10mM sodium nitroprusside in 0.5 ml phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of reference compound and extract of various concentrations . The mixture was incubated at 25^{o}C for 150 min. From the incubated mixture 0.5 ml was taken out and added into 1.0 ml sulfanilic acid reagent (33% in 20% glacial acetic acid) and incubated at room temperature for 5 min Finally, 1.0 ml naphthyl ethylene diamine dihydro chloride (0.1% w/v) was mixed and incubated at room temperature for 30 min before measuring the absorbance at 540.

Estimation of total phenolic contents

The amount of total phenolic content in the fractions were determined by the method of Folin–Ciocalteu (1927)[19]. One ml of the both fractions (10g/L) were mixed separately with 5 mL of Folin–Ciocalteu reagent (diluted ten fold) and 4 mL of sodium carbonate (75 g/L). The absorption was read at 765 nm after keeping in RT for 30 minutes. Gallic acid was used as the standard. Total content of phenolic compounds in the fractions were calculated and expressed in Gallic acid equivalent

Estimation of Flavonoids

The content of flavonoids was determined by a pharmacopeia method [20] using rutin as a reference compound. One ml of plant extract in methanol (10 g/l) was mixed with 1 ml aluminum trichloride in ethanol (20 g/l) and diluted with ethanol to 25 ml. The absorption at 415 nm was read after 40 min at 20^{0}C. Blank samples were prepared from1 ml plant extract and 1 drop acetic acid, and diluted to 25 ml. The absorption of rutin solutions was measured under the same conditions. Standard rutin solutions were prepared from 0.05 g rutin. All determinations were carried out in duplicate. The amount of flavonoids in plant extracts was calculated as rutin equivalents (RE) per gram of extract.

Estimation of Flavanol

Content of Flavanol was determined by the method of Yermakov method (1987)[21]. 2 ml of the fractions(10 mg/mL) were treated with 2 mL of Aluminium Chloride (20 g/L) and 6 mL of sodium acetate (50 g/L). Absorption was read at 440 nm after keeping for 2½ hours in room temperature. Rutin was used as the standard. The content of flavanol was calculated as Rutin equivalents per gram of extract

Results and Discussion

In the present study, the antioxidant scavenging activity of chloroform and ethanol fractions of extract from 1A has been done in four different in vitro model. It was observed that free radicals were scavenged by the both fractions in a concentration dependent manner.

DPPH radical is considered to be a model of lipophilic radical. In this mode, scavenging activity is attributed to hydrogen donating ability of antioxidant[22]. This model is used to measure antioxidant activity of different phenolic compounds. Bleaching action of the compounds mainly depends on the number of position of the hydroxyl group present in the phytochemical [23]. Both fractions showed a significant scavenging effect on DPPH radical(Table I).

In the ABTS system(Table II), radical cation is formed prior to the addition of antioxidant test system, rather than generation of radical taking place continually in the presence of antioxidant. This method is used to screen the activity of both lipohilic and

Table I-DPPH radical scavenging Activity of Fractions

Concentration of Fraction used in µg/mL	Ethanol fraction % of inhibition	Chloroform fraction % of inhibition	Standard % of inhibition
1	22.3 ± 1.4	26.8 ±2.3	30.1 ±2.0
2	30.2 ± 2.1	32 .3±1.1	35 .3±1.8
5	35.6 ± 1.6	38.0± 0.9	48 .4±1.1
10	41.9 ± 1.8	49.2 ± 1.4	52 .2±1.5
15	48.4 ± 1.9	55 .1±1.3	59 .0±0.6
20	54.3 ±2.2	62.4 ±0.8	67 .7±0.9
IC_{50}	18.1 µg/mL	10.6 µg/mL	8.8 µg/mL

*All values are expressed as mean ± SEM for three determination

Table II-ABTS Free radical scavenging Activity of Fractions

Concentration of Fraction used in µg/mL	Ethanol fraction % of inhibition	Chloroform fraction % of inhibition	Standard % of inhibition
5	32.7 ±0.6	47.2 ±0.7	38.9 ± 2.1
10	48 .6±1.1	52 .4±0.5	42 .3± 1.3
15	53 .2±.13	65 .6±1.4	51 .2±1.9
20	75.1 ±2.1	78 .8±1.8	65 .9±0.9
IC_{50}	12.1 µg/mL	8.6 µg/mL	14.6 µg/mL

*All values are expressed as mean ± SEM for three determination

hydrophilic antioxidants[24] By the virtue to its excellent spectral properties ,solubility in both organic and aqueous media, stability in wide range of pH, ABTS assay in considered as to be more reliable and accurate. Compounds with one OH group in the aromatic ring which is found in inactive towards the DPPH are significantly active towards ABTS [25,26].

In biochemical system, superoxide radical and H_2O_2 react together to form singlet oxygen and OH radical, which is the most reactive oxygen species among all ROS [27]. It has high ability to reacts with several biological material by hydrogen withdrawal, double bond addition electron transfer and radical formation and initiates auto oxidation, polymerization and fragmentation OH radical can cause sugar fragmentation base loss and leakage of DNA strand[28]. It is the major ROS that cause lipid peroxidation and enormous biological damage[29].In our studies, both fractions exhibit concentration depended scavenging activity against OH radical generated in Fenton system(Table III). This can be due to high active hydrogen donor ability of OH substitution or its chelating power of phenolic group present in the fraction. This in vitro model studies showed that our extract can be used to minimize the adverse effect of OH

Table III-Hydroxy radical scavenging Activity of Fractions

Concentration of Fraction used in µg/mL	Ethanol fraction % of inhibition	Chloroform fraction % of inhibition	Standard % of inhibition
10	35.2 ±1.9	38.3 ±2.1	8.1 ±0.5
20	40.0 ±1.6	49.7 ±1.8	12.2±0.91
30	47.1 ±1.5	58.5±1.3	18.6 ±2.1
40	58.3 ±1.4	65.1 ±0.6	25.3 ±1.8
50	65.7 ±1.2	77.2 ±0.9	33.8 ±1.6
60	78.4 ±1.1	88.8 ±1.1	45.4±0.6
70	84.5 ±0.5	94.8. ±2.0	54.0 ±1.7
IC_{50}	32.5µg/mL	21.85 µg/mL	67.8 µg/mL

*All values are expressed as mean ± SEM for three determination

Table IV-Nitric Oxide radical scavenging Activity of Fractions

Concentration of Fraction used in µg/mL	Ethanol fraction % of inhibition	Chloroform fraction % of inhibition	Standard % of inhibition
10	28.1 ±1.7	33.0 ± 0.9	29.0 ± 1.6
20	37.1 ±2.1	44.1 ±1.0	7..7 ±0.71
30	46.4 ±1.3	52.4 ±2.3	48 .2±0.43
40	54.4 ±1.6	65.9 ± 1.1	57.6 ± 1.5
IC_{50}	35.2 µg/mL	29.95 µg/mL	31.5 µg/mL

*All values are expressed as mean ± SEM for three determination

radical, to prevent lipid peroxidation and DNA fragmentation.

Nitric oxide a gaseous free radical which has some important physiological role, is relatively less reactive. But its metabolic product – peroxynitrite – formed after reacting with O_2 is extremely reactive and directly induce toxic reaction including SH group oxidation, protein tyrosine nitration, lipid peroxidation and DNA modification [30,31]. Our studies showed that both fraction effectively prevents the formation of peroxynitrate and can be used to prevent the adverse effect of metabolities of NO (Table IV).

Content of phenolic compounds

Plant phenolics constitute one of the major groups of compounds that act as primary antioxidants or free radical terminators,. Flavonoids are probably the most important natural phenolics[32] that possess a broad spectrum of chemical and biological activities including radical scavenging properties. Such properties are especially distinct for flavonols[33]. Therefore, the total phenolic content , flavonoids and flavonol in the extracts were also determined. The total phenolic contents in gallic acid equivalents (G AE) for ethanolic and chloroform fractions are 810 ± 9.2 and 476 ± 7.8 mg per gram of

extract respectively, The amount of flavonoids (mg/g), in rutin equivalents for these fractions are 55.08 ± 2 .4 and 15.19 ± 1.5 The concentration of flavonols, in rutin equivalents for both fraction are 23.5 ± 1.4and 6.37± 0.56 mg/g of extract. It is expected that ethanolic fraction which contain significantly high amount of Phenolic compounds than chloroform fraction should exhibit high invitro antioxidant activity than the latter. But in contrary, chloroform fraction displayed significantly high free radical scavenging activity. Rice Evan et *al* [34] has reported that antioxidant property of phenolic compounds depends on three major structural features .1. Number of hydroxyl group attached to the ring structure and its relative position. 2. Presence of unsaturation in the ring which allows electron delocalization across the molecule for the stabilization of the radical [35] . 3.Attachment of groups like carbohydrate to the phenolic hydroxyl groups – Glycosylated flavonoids show less antioxidant activity than aglycon form[36] One of these structural features might have played a major role for chloroform fractions to attain high *in v itro* antioxidant property which need further studies like phytochemical analysis and structural elucidation to corroborate this .

Conclusion

It can be concluded that both fractions have significant free radical scavenging activity. On comparison it was found that chloroform fraction has more free radical scavenging actively than ethanolic fraction. This high Antioxidant potential may be attributed to the presence of polyphenolic compounds with special structural features. These results are encouraging to pursue structural characterization and exploration of their therapeutic use.

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