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Comparative in vitro study on the free radical scavenging capacity of tannin and biflavone fraction from *Ficus racemosa* Linn and *Araucaria bidwilli* Hook

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Abstract : The biflavone and tannin fraction form *Araucaria bidwilli* leaves and *Ficus racemosa* bark extract has various biflavones and tannins has been receiving much attention for its free radical scavenging and antioxidant capacity. Free radical scavenging capacity of enriched biflavone and tannin fractions were investigated by DPPH, hydroxyl and nitrates radicals inhibition properties. The results from DPPH reveals that BFR and TF showed efficient quenching of DPPH* the fractions thus contain free radical quenching compounds, with act as primary radical scavenging that react with DPPH* by providing a hydrogen atom or electron donating ability). The BFR and TF have shown the inhibition of the coupled oxidation of the linoleic acid and β-carotene in emulsified aqueous system. This may be explained by the radical scavenging capacity of fraction and its inhibitory action on nitric oxide and hydroxyl radicals may contribute to some extent to the β-carotene assay. The present data explain the multiple free radical scavenging capacity and antioxidant capacity of biflavone and tannin fractions from the leaves of *Araucaria bidwilli* and bark of *Ficus racemosa* as compared with Quercetin. A further biflavone and tannin fraction has shown good inhibition in scavenging nitrates which is generated in *in-vitro*.
Key words : *Ficus racemosa*, *Araucaria bidwilli*, tannins, biflavone, antioxidant.

1.Introduction :

Antioxidants are exogenous (natural or synthetic) or endogenous compounds acting in several ways including removal of O_2 , scavenging reactive oxygen/nitrogen species or their precursors inhibiting ROS formation and binding metal ions needed for catalysis of ROS generation and up-regulation of endogenous antioxidant defenses [1]. Experimental evidence suggests that free radicals (FR) and reactive oxygen species (ROS) can be involved in a high number of diseases [2,3]. As plants produce a lot of antioxidants to control the oxidative stress caused by sunbeams a,nd oxygen, they can represent a source of new compounds with antioxidant activity. Ayurveda, the Indian traditional health care system (ayus_ life, veda_knowledge, meaning science of life), is the oldest medical system in the world and is being revived in its complete form under the name of Maharishi Ayurved [4]. Araucaria bidwilli hook is commonly known as Bunya-Bunya. Bunya-bunya is native to rainforest communities near the coastal region in south east queensland, Australia. In India this genus is commonly distributed in southern part of Nilgiris hills and northern part of Himalayan regions at

an altitude of 1700 – 2500 M ranges. Araucaria plant is rich in biflavones and also this plant posses wide variety of activities like cytotoxic effect [5].

Ficus racemosa have been extensively used in olden days, Chakara refers to the plants and its medical use in mahakashanya and prescribes it for heart burn and excessive appetite, Yagnyanga suggests the use of the plant in ritual sacrifice .A large decedents tree distributed all over India, found through out the year, grows in evergreen forests most localities along the sides of ravines and banks and streams.[6] The major chemical constituent of Ficus plant is tannins.This genus has high tannin content and few reports have shown anti-cancer, gasteroprotective, anti-inflammatory, free radical scavenging effect of extracts of Ficus [7,8,9]. Therefore the present study has been undertaken to compare the antioxidant potential of the biflavone and tannin fraction separated from *Araucaria bidwilli* Hook and *Ficus racemosa* Linn respectively in various *in-vitro* methods.

2.Materials and methods:

2.1 Collection of Plant material and Authentication

The fresh leaves of *Araucaria bidwillii* Hook were collected from Government Botanical Garden, Udhagamandalam, India and authenticated by Botanical survey of India, Coimbatore, Tamil Nadu. A voucher specimen (JUNPSL 2007- 008) of this plant material has been retained in Department of Pharmacology, Vel's College of Pharmacy, Chennai .

Ficus racemosa bark were collected from Chennai. Tamilnadu. They were identified and autthenticated by Dr. P. Jayaraman, Director, Plant Anatomy Rresearch Centre (PARC), Tambaram, Chennai, Tamil Nadu, the voucher specimen no:Parc/2008/229 has been deposited at the herbarium unit of the Deparment of Pharmacognosy, VEl's College of Pharmacy, Pallavaram, Chennai.

2.2 Separation of biflavones from the leaf extracts of *Araucaria bidwilli* Hook

Dired and powered leaves (2 Kg) collected at Ooty, Tamilnadu. India were completely extracted with petroleum ether (b.p. 40-60^{o}). The treated leaves were dired and again extracted with boiling acetone until the extract was almost colourless. The combined acetone extracts were concentrated at atmospheric pressure to give a dark viscous mass. This was extracted successively with refluxing petroleum ether, benzene, and $CHCL_3$ untill the solvent in each case was almost colourless. The residue was then treated with boiling water. The insoluble mass was dissolved in EtOH and dired under reduced pressure. Solid green extracts (5 Kg) were obtained which responded to the usual colour test for flavonoids.[10]

2.3 Separation of Tannin Fraction (TF) from the bark extract of *Ficus racemosa* Linn

The barks of *Ficus racemosa* were shade dired and coarsely powdered. Powdered material (1kg) was extracted with acetone in water (70%v/v) (2500ml) by cold maceration. Total tannins were separated from crude acetone (70%) extract by the following method. The filtered extract were saturated with Nacl to salt out acetone and the upper solvent phase was removed. This acetone phase was then extracted with three successive 250ml portions of the aqueous containing 0.1% ascorbic acid to prevent auto-oxidation. This acetone was then removed in vacuo at 25^{0}C and equal volume of water was added.

This aqueous solution was then extracted first with three successive portions of petroleum ether (40-60^{0}C) to remove any lipid material and then with three successive portions of ethyl acetate to extract .Finally the remaining aqueous phase containing tannins, then freeze dried.[11]

2.4 Chemicals and drugs

Ferrous sulphate, Ascorbic acid, Deoxyribose, Trichloro acetic acid (TCA) and Thio barbituric acid (TBA) linoleic acid,β-carotene were obtained from Sisco Research Laboratory (SRL), Mumbai, India 1, 1-dipheny1-2-picry1-hydrazy1 (DPPH) was procured from Sigma-Aldrich Co MO, USA. Other chemicals used were of analytical grade. The biflavone and tannin fractions from *Araucaria bidwilli* and *Ficus racemosa*.

2.5 β Carotene – Linoleic acid (Linoleate) assay

The antioxidant capacity is measured by the ability of a compound to minimize the coupled oxidation of linoleic acid and β – Carotene in an emulsified aqueous system. This loses its orange colour when reacting when reacting with radicals[12]. In this method 2ml of β carotene linoleate emulsion (20mg to β carotene/ 0.2ml linoleic acid 5ml of tween 40 and 50ml of distilled water are added with vigorous stirring and saturated with oxygen) is equilibrated at 32p C for 5mts. The oxidation reaction is initiated by adding 10μ APPH (0.9M) (2,2'azobis(2-methyl propionamide) dihydrochloride) with 10min. vortexing the mixture. To this mixture various concentration (0.1mM to 1mM) of test samples are added and the mixture is vortexted again for few minutes. The absorbance of sample is measured at 470nm, immediately after sample preparation (t=0min) and at 15min interval until the end t=120min of the experimental. The percentage inhibition rate of β – carotene bleaching relative to the control was calculated by the formula.

% Antioxidant capacity (AOA)=

$$\frac{[1 - (\text{Abs Sample } t = 0\text{min} - \text{Abs Sample } t = 60 \text{ or } 120\text{min}]}{(\text{Abs control } t = 0 \text{ min} - \text{Abs Control } t = 60 \text{ or } 120 \text{ min}} \times 100$$

2.6 Assay for DPPH radical scavenging capacity

The effect of extract and fraction on DPPH radical was estimated by [13] with minor modification. In brief, 2Ml of DPPH in methanol (3.6×10^{-5} M) were added to 50 µL of various concentrations of extracts, and fractions (0.025Mm – 1mM). The mixture was vortexed for 15 sec and left to stand at 37ºC for 30min. The decrease in the absorbance at 515 nm was continuously recorded in a spectrophotometer for 15 min at room temperature. All determination was performed in triplicate. The DPPH scavenging activity (decrease of absorbance at 151 nm) of extract and fraction were plotted against time and the (%) percentage of DPPH radical scavenging ability of the sample was calculated from the absorbance value at the end of 15 min duration as follows:

$$\% \text{ Inhibition} = \frac{[\text{Abs}_{\text{Control}} - \text{Abs}_{\text{Sample}}]}{\text{Abs Control}} \times 100$$

Where $\text{Abs}_{\text{Control}}$ is absorbance of control at time = 0 and $\text{Abs}_{\text{Sample}}$ is absorbance of test sample at time = 15 min.

2.7 Deoxyribose protection against hydroxyl radicals

Deoxyribose protection against hydroxyl radical (OH*) generated by reacting Fe^{3+} , EDTA, ascorbic acid and H_2O_2[14]. In brief, 1 ml of final reaction solution contained 500 µ1 of various concentration of test materials, $FeC1_3$ (100 µM), EDTA (100 µM), H_2O_2 (1 mM), deoxyibose (3.6 mM) abd ascorbic acid (100 mM) in potassium phosphate buffer (KH_2PO_4-KOH, pH 7.4). Deoxyirbose degradation by hydroxyl radical was measured by using the thiobarbituric acid method. The reaction mixture was incubated for 1 h at 37^0C and further heated in a boiling water bath for 15 min after addition of 1 ml TCA (10% w/v) and 1 ml of TBA (0.5% w/v) and color produced was measured at 532 nm against a blank containing phosphate buffer. Quercetin was used as positive control. The % of OH* radical inhibition was calculated by following formula.

$$\% \text{ Inhibition} = \frac{[\text{Abs}_{\text{Control}} - \text{Abs}_{\text{Sample}}] \times 100}{\text{Abs}_{\text{Control}}}$$

2.8 Nitric oxide scavenging assay

Nitric oxide scavenging assay was measured by spectrophotometric ,method described was mixed with different concentration of extract and fraction dissolved in methanol and incubated at 250c for 30 min. After 30 min 1.5 ml of the incubation solution were removed and diluted with 1.5 ml of modified Griess reagent (sulphanilic acid with naphylethylene diamine dichloride in acetic acid). The absorbance was measured at 546 nm[15].

$$\% \text{ Inhibition} = \frac{[\text{Abs}_{\text{Control}} - \text{Abs}_{\text{Sample}}] \times 100}{\text{Abs control}}$$

2.9 Determination of total phenolic contents

The total phenolic content was determined according to the method described by [16]. Suitable aliquots of the polyherbal formulation was taken in a test tube and made up to the volume of 1 ml with distilled water. Then, 0.5 ml of Folin-Ciocalteu reagent (1:1 with water) and 2.5 ml of sodium carbonate solution (20%) wre added sequently in each test tube. Then the tubes were vortexed, placed in the dark for 40min and absorbance was recorded at 725nm. The amount of total phenolics was caluculated as gallic acid equivalents/mg from the extract.

Figure 1Effect of BFR and TF from the leaves of *Araucaria bidwilli* and bark of *Ficus racemosa* in β-Carotene – Linoleate antioxidant assay method

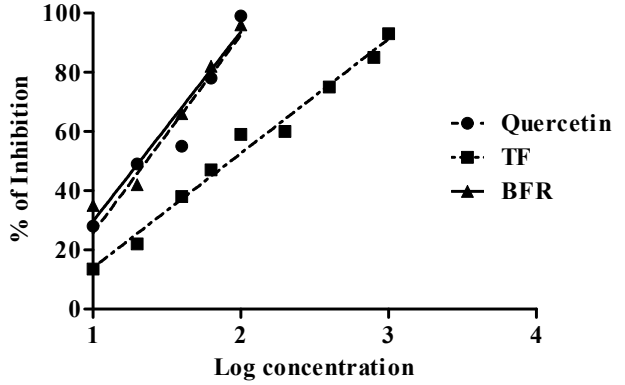


Figure 2 Effect of BFR and TF from the leaves of *Araucaria bidwilli* and bark of *Ficus racemosa* in DPPH assay

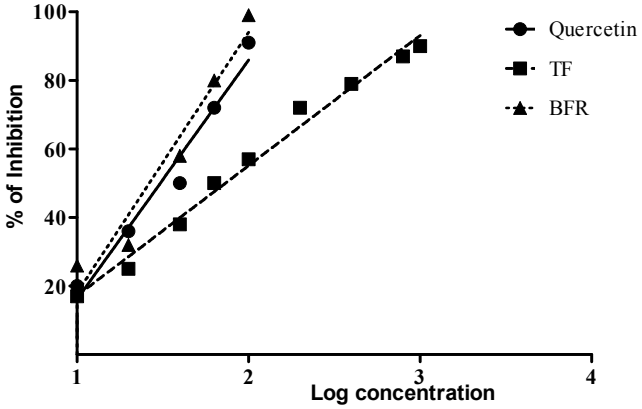


Figure 3 Effect of BFR and TF from the leaves of *Araucaria bidwilli* and bark of *Ficus racemosa* in hydroxyl radical scavenging assay.

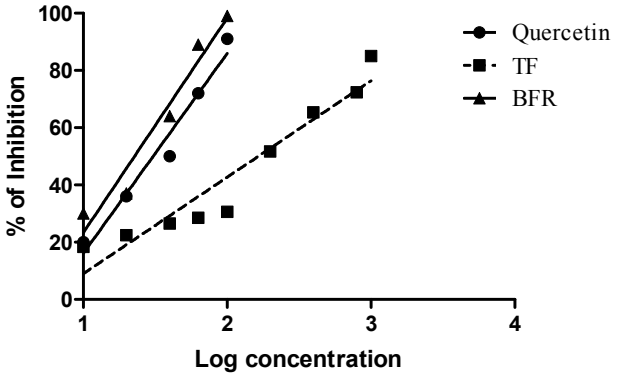
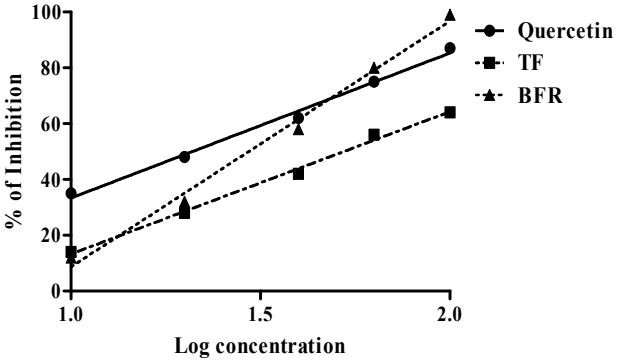


Figure 4 Effect of BFR and TF from the leaves of *Araucaria bidwilli* and bark of *Ficus racemosa* in Nitric oxide method



3. RESULTS

3.1 Effect of biflavone and tannin fractions from Araucaria bidwilli Hook and Ficus racemosa in β-carotene-Linoleic acid (Linoleate) assay

Fig 1 Inhibition of β-carotene results are shown in figure 1**.** Dose dependent inhibition of β-carotene was noted in addition of various concentrations (10µl – 100µl) of biflavone and tannin fraction of *Araucaria bidwilli* and *Ficus racemosa*. The IC_{50} value of biflavone and tannin fraction were 2.18µg/ml and 161.8µg/ml. The R^2 values were 0.9781 and 0.9781 respectively which is comparable to that of IC_{50} and R^2 value of standard Quercetin (13.36µg/ml and 0.9939).

3.2 Effect of biflavone and tannin fractions from *Araucaria bidwilli* Hook and *Ficus racemosa* L in DPPH radical scavenging assay

Figure 2 depicts the free radical scavenging capacity of BFR and TF fraction using DPPH generated radical in *in-vitro*. It was observed that increase in % inhibition of free radicals has observed in increasing concentration of BFR and TF. The IC_{50} values of BFR and TF was found to be 7.7µg/ml and 53.98µg/m and the R^2- linear regression value was found to be 0.9901 and 0.9987. The both the fractions was compared with the standard Quercetin IC_{50}-26.93µg/ml;R^2-0.9987.

3.3 Effect of biflavone and tannin fractions from *Araucaria bidwilli* Hook and *Ficus racemosa* L in deoxyribose protection against hydroxyl radicals.

Figure 3 depicts the ability of biflavone and tannin fractions to quench hydroxyl radicals was tested *in-vitro*. The results indicates that biflavone and tannin fractions of *Araucaria bidwilli* and *Ficus racemesa* exhibited hydroxyl radical quenching with their IC_{50} and R^2 value was found to be 8.07µg/ml and 55.14µg/ml, 0.9910 and 0.9978 respectively. The both fractions was compared with standard Quercetin with IC_{50} value of 13.36mM and R_2 value is 0.9939.

3.4 Effects of biflavone and tannin fractions from *Araucaria bidwilli* Hook and *Ficus racemosa* L in nitric oxide scavenging:

Figure 4 depicts the ability of biflavone and tannin fractions to quench NO radicals was tested *in-vitro*. The results indicates that biflavone and tannin fractions of *Araucaria bidwilli* and *Ficus racemosa* exhibited IC_{50} and R^2 values of 8.07µg/ml and 54.14µg/ml, 0.9910 and 0.9978 respectively compared with standard QuercetinIC_{50} value of 13.36µg/ml and R^2 value of 0.9939.

3.5 Total phenolic content in biflavone and tannin fractions obtained from Araucaria bidwilli Hook and Ficus racemosa L

The total phenolic content of the biflavone and tannin fractions from *Araucaria bidwilli* and *Ficus racemosa* was found to be 40mM and 110mM which is compared with standard gallic acid.

4.Discussions

There is a compelling evidence indicates that increased consumption of antioxidants from fruits and vegetables reduces the risk of various diseases associated with oxidative stress [17]. The present results demonstrate that biflavone and tannin fractions posses free radical scavenging and antioxidant capacity tested in *in-vitro*. The biflavone and tannin fraction form *Araucaria bidwilli* leaves and *Ficus racemosa* bark extract has various biflavones and tannins has been receiving much attention for its free radical scavenging and antioxidant capacity. Free radical scavenging capacity of enriched biflavone and tannin fractions were investigated by DPPH, hydroxyl and nitrates radicals inhibition properties. The results from DPPH reveals that BFR and TF showed efficient quenching of DPPH* the fractions thus contain free radical quenching compounds, with act as primary radical scavenging that react with DPPH* by providing a hydrogen atom or electron donating ability.

It has been reported that mitochondrial metabolism represents a major source of ROS such as nitric oxide and hydroxyl and hydrogen provides. The BFR and TF exhibited inhibition of nitric oxide, hydroxyl radical using of deoxyribose as source, which explain the capacity of BFR and TF to react with one oxidant with either organic radical or redox active compounds. A further biflavone and tannin fraction has shown good inhibition in scavenging nitrates which is generated in *in-vitro*. This putative free radicals are extremely reactive and can be highly vulnerable to DNA, Proteins and lipids [18,19]. The BFR and TF shown the inhibition of the coupled oxidation of the linoleic acid and β-carotene in emulsified aqueous system. This may be explained by the radical scavenging capacity of fraction and its inhibitory action on nitric oxide and hydroxyl radicals may contribute to some extent to the β-carotene assay. The present data explain the multiple free radical scavenging capacity and antioxidant capacity of biflavone and tannin fractions from the leaves of *Araucaria bidwilli* and bark of *Ficus racemosa* as compared with Quercetin.

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