

ORIGINAL ARTICLE

Free Radical Scavenging Activity and HPLC Analysis of *Araucaria cunninghamii* Aiton ex D. Don Leaf Extract

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Received April 10, 2014

Background: Several diseases are caused in the body due to oxidative stress of free radicals. The objectives of the present study were to investigate the antioxidant activity of *Araucaria cunninghamii* Aiton ex D. Don (Araucariaceae) leaf extract. The dried leaves of *Araucaria cunninghamii* Aiton ex D. Don (Araucariaceae) were extracted with 80% methanol. The antioxidant activity of the extract was predicted through *in vitro* model systems such as 1,1-diphenyl-2-picryl hydrazyl (DPPH), reducing power and nitric oxide scavenging assays. The total phenolic, flavonoid and tannin content of the extract were also estimated in order to draw the correlation with their bioactivities. The extract was also investigated for several polyphenolic compounds using High-performance liquid chromatography (HPLC).

Results: The antioxidant activity of extract was found to be excellent in all the three assays. A positive correlation between the antioxidant activity and phenolic content was observed. HPLC analysis identified the presence of Gallic acid, Catechin, Chlorogenic acid, Epicatechin, Caffeic acid, Umbelliferone, Ellagic acid, Quercetin and Kaempferol in the methanol leaf extract of *Araucaria cunninghamii* Aiton ex D. Don.

Conclusions: The results of the present study point towards the fact that *Araucaria cunninghamii* Aiton ex D. Don leaves possess good antioxidant potential. The strong antioxidant activity can be correlated with the polyphenolic compounds present in the leaves.

Key words: Antioxidant activity, *Araucaria cunninghamii* Aiton ex D. Don, HPLC.

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It has been long proposed that oxidative stress is known to cause extreme harm and breakdown of cellular and extracellular macromolecules (Halliwell *et al.*, 1999). This stress leads to certain effects which

are associated with many diseases like cancer, cardiovascular diseases and inflammatory diseases. It's of significance to mention that at low concentrations ROS and free radicals activate the host cell's defence mechanism against any kind of biotic as well as abiotic stress. However there is a natural antioxidant defence system in our body to protect us from oxidative stress by means of different methods. But most of the humans do not have sufficient safeguard. Therefore, there is always a demand for antioxidants from natural sources. It is richly supported in various ancient scriptures of medicinal importance and strongly supported by modern science that the oxidative damage is countered in living beings by consumption of antioxidant rich phytochemicals such as anthocyanins, carotenoids, flavonoids, isoflavones, flavones, polyphenols, tannins, catechins, isocatechins, xanthophylls and many other terpenoid compounds. It is impossible to underestimate the therapeutic and pharmaceutical impact of naturally extracted plant products. However, it is of immense importance to understand the feasibility of the extraction and purification process of these phytochemicals. Antioxidant activity of plants is measured through various *in vitro* models like total antioxidant capacity estimation, DPPH, reducing power and Nitric Oxide scavenging assays.

Araucaria cunninghamii Aiton ex D. Don is commonly known as colonial pine, arakaria and dorriigo pine. It is a coniferous tree which belongs to family Araucariaceae. This plant can even survive in adverse environmental conditions. It was an important

source of timber for masts and spars of sailing ships in historic times. Its seeds are edible. The resin from stem of *Araucaria cunninghamii* Aiton ex D. Don is dissolved in alcohol to treat kidney ailments. This plant has not been explored for its antioxidant activities. Therefore, present study was carried out to investigate the antioxidant activities of this plant.

MATERIALS AND METHODS

Plant material

The leaves of *Araucaria cunninghamii* Aiton Ex D. Don were the study material for the present study. Those were collected from Botanical garden of Guru Nanak Dev University, Amritsar and authenticated at department of botanical and environmental sciences Guru Nanak Dev University, Amritsar.

Preparation of extract

Washed and air dried leaves were finely chopped and extracted thrice with 80 % methanol for 24 hours. Then the extract was filtered with whattman no. 1 filter paper. Filtrate was dried in Rotary vacuum evaporator (Buchi R-210, Switzerland) at 40°C temperature. The dried methanol crude extract was stored for performing various assays at 4°C.

Evaluation of antioxidant activity

DPPH radical scavenging activity

Decolorization of purple colour of methanol solution of DPPH by different plant extracts gives a measure of their hydrogen donating ability. This assay was performed according to the method given by Blois in 1958. Plant extract was mixed with DPPH (0.1mM) in methanol solution. After 20 min incubation at room temperature the absorbance was read at 517 nm. The inhibitory percentage of DPPH was

calculated according to the following equation:

$$\% \text{ Inhibition} = \frac{\text{Ac-As}}{\text{Ac}} \times 100$$

Ac = Absorbance of control

As = Absorbance of sample

Reducing power assay

Reducing power assay was performed on different concentrations of extract according to Oyaizu 1986. In this assay, the oxidation state of iron (Fe^{+3}) in ferric chloride is converted to ferrous (Fe^{+2}) by the antioxidant compounds. 1 ml of different concentrations (0-1000 $\mu\text{g}/\mu\text{l}$) of methanol crude extract, made by serial dilutions, was mixed with 2.5 ml phosphate buffer and 2.5 ml of 1% potassium ferricyanide and incubated at 50°C for 20 minutes. 2.5 ml of 10% TCA was added to 2.5 ml of supernatant. Then 2.5 ml distilled water and 0.5 ml FeCl_3 was added. The absorbance was noted at 700 nm. The intensity in absorbance could be the measurement of antioxidant activity of the extract. Rutin was used as standard. The reducing potential (%) was calculated.

Nitric Oxide scavenging assay

Nitric oxide is a significant physiological messenger molecule in immunological, neuronal and cardiovascular tissues. At physiological pH, aqueous solution of Sodium nitropruside spontaneously releases nitric oxide. This nitric oxide reacts with oxygen to generate nitrite ions. This diazotization of nitrate with sulphanilamide is followed by union with N- (1-naphthyl) ethylenediamine and chromophore formation takes place. Absorbance of this chromatophore is read at 546 nm. Nitric oxide inhibition activity of leaves extract of *A. cunninghamii*

was performed according to the method reported by Acharyya *et al.*, (2010) with minor modifications. Different concentrations of the sample (0-1000 $\mu\text{g}/\text{ml}$) were prepared in methanol and mixed with 8.1 ml (10 mM) sodium nitropruside in 0.5 M phosphate buffer (pH 7.4) and incubated at 25°C for 15 min. Greiss reagent (1% sulphanilamide in 5% H_3PO_4 and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride in water) was added to the above solution and the absorbance was noted at 546 nm. In the control the similar amount of 0.5 M phosphate buffer was used in place of plant extract with same reaction mixture. Ascorbic acid was used as standard. The percent scavenging of nitric oxide was calculated according to the following equation:

$$\% \text{ Scavenging} = \frac{\text{Ac-As}}{\text{Ac}} \times 100$$

Ac = Absorbance of control

As = Absorbance of sample

Total antioxidant capacity

The assay is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acidic pH. The antioxidant capacity is expressed as ascorbic acid equivalent (AAE). Minor modifications were applied on method suggested by Prieto *et al.*, (1999). 0.3 ml of sample solution (100 $\mu\text{g}/\text{ml}$) was mixed with 3ml of reagent solution consisting of 0.6M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate. The above solution was incubated for 90 minutes at 95°C and then cooled to room temperature. The absorbance was read at 695 nm against a blank. Total antioxidant capacity of plant extract/fractions

was measured from the regression equation prepared from the concentration versus optical density of ascorbic acid.

Determination of total phenolic compounds

Assessment of total phenolic content was carried out spectrophotometrically using Folin-Ciocalteu (FC) reagent according to Yu *et al.* (2002). A complex redox reaction was carried out by phenolic compounds with phosphotungstic and phosphomolybdic acids present in the FC reagent. The total phenolic compounds of the methanol crude extract were expressed as gallic acid equivalents (GAE) which indicated the phenolic content equal to the gallic acid (mg) in one gram of dry material. 100 µl extract was taken in a test tube and diluted with 900 µl distilled water. Then 500 µl FC reagent and 1.5 ml of 20% Na₂CO₃ was added to this solution and the volume was raised to 5 ml with distilled water. The mixture was incubated at room temperature for 2 hours and the absorbance of the solution was taken at 765 nm on UV-VIS spectrophotometer. Total phenol content was estimated from standard curve.

Determination of total flavonoid content

The total flavonoid content was estimated using the method given by Kim *et al.*, (2003). 4 ml of distilled water was added to 1 ml extract of 100µg/ml concentration. Then, added 0.3 ml NaNO₂ and 0.3 ml AlCl₃. Incubated the mixture for 5 minutes at room temperature. 2 ml of NaOH and 2.4 ml distilled water was added to the incubated solution and the absorbance was taken at 510 nm using UV-VIS spectrophotometer. Total flavonoid content was estimated from standard curve. Rutin was used as

standard and total flavonoid content was expressed as rutin equivalents (RE) in mg/g of dry sample.

Total tannin content

Method given by Polshettiwar *et al.*, (2007) was used with minimal modifications. 100 µl sample solution was mixed with 7.5 ml distilled water, 500 µl FC reagent (1:1), 1 ml of 35% Na₂CO₃ and the volume was raised to 10 ml with distilled water. Above mixture was incubated for 30 min. at room temperature. Then the absorbance was observed at 725 nm. Tannic acid was used as standard and the results were expressed as tannic acid equivalents (TAE).

Phytochemical analysis

Phytochemical testing was performed on the extract using standard procedures described by Anyasor *et al.*, (2010) to identify the presence and absence of chemical constituents like anthraquinones, cardenolides, cardiac glycosides and saponin.

Test for anthraquinones

0.5 g of the extract was shaken with 10 ml of benzene and filtered. 10% of ammonia solution was added to filtrate and the mixture was shaken. The formation of a pink, red or violet colour on the ammoniac phase indicates the presence of anthraquinones.

Test for cardenolides

1 ml of the sample extract was added to 2 ml of benzene. The formation of a turbid brown colour is a sign of the presence of cardenolides.

Test for cardiac glycosides

2 ml glacial acetic acid was mixed with 1 drop of ferric chloride solution and 0.5 g of the extract was dissolved in it. Above solution was under layered with

2 ml of concentrated sulphuric acid. A brown ring formation at the inter phase indicates the presence of deoxy sugar characteristics of cardiac glycosides.

Test for saponins

1 ml of the extract was mixed with one ml of distilled water and shaken briskly. A stable continuous lather specifies the presence of saponins.

HPLC analysis for polyphenols

The polyphenolic compounds were analyzed by HPLC study of the extract. The mixture of 11 polyphenolic standards namely, Gallic acid (C₇H₆O₅), Catechin (C₁₅H₁₄O₆), Chlorogenic acid (C₁₆H₁₈O₉), Epicatechin (C₁₅H₁₄O₆), Caffeic acid (C₉H₈O₄), Umbelliferone (C₉H₆O₃), Coumaric acid (C₉H₈O₃), Rutin (C₂₇H₃₀O₁₆), Ellagic acid (C₁₄H₆O₈), Quercetin (C₁₅H₁₀O₇) and Kaempferol (C₁₅H₁₀O₆) was thinned with methanol at different concentrations by serial dilution for quantitative analysis. The chromatography was carried out through a 150x4.6 mm C-18 column with a pore size of 5µm at room temperature with a flow rate of 1ml/min at λ 280 nm. The solvent system included solvent A (0.01% acetic acid in water) and solvent B (methanol). The gradient runs as 70% A and 30% B, reaching 45% B at 12 min, 75% B at 13.5 min, 75% B at 15 min, 50% B at 16.6 min, 25% B at 18 min, 25% B at 20 min, 30% B at 21 min, and stopped at 22 min with elution of 4 min. The calibration curves were generated for by plotting concentrations versus peak areas. The detection of every compound was based on a combination of retention time and spectral similarity. The detection and quantification limit for all the detected compounds were calculated on the basis of signal-to-noise ratio (S/N) of 3 and 10 with

corresponding standard solution, respectively.

Statistical analysis

All experimental measurements were carried out in triplicate. The data was analyzed statistically by one-way analysis of variance (ANOVA) as described by Bailey (1995) and comparisons with *P*-values ≤ 0.05 were considered significantly different. Mean value, standard deviation and standard error were also calculated.

RESULTS AND DISCUSSION

In the present study, methanol crude extract was found to possess significant DPPH radical scavenging activity with an IC₅₀ of 181.897. It was observed that with increase in concentration of extract, an increase in DPPH radical scavenging activity took place. The effect of the extract in DPPH assay is shown in Fig. 1.

The straight relationship among antioxidant activity and reducing power of plant extracts were reported. However, with the increasing concentration of plant extract, the reducing power increased but as compared to the standard (Rutin), a little reducing power activity was shown by extract of *Araucaria cunninghamii* Aiton ex D. Don. The IC₅₀ value was found to be 1384.417. The effect of leaf extract in reducing power assay is shown in Fig. 2.

Methanol crude extract of *Araucaria cunninghamii* Aiton ex D. Don exhibited a strong effect in prevention of nitric oxide radical with IC₅₀ 1026.512. The effect of leaf extract in nitric oxide scavenging assay is shown in Fig. 3.

Total antioxidant capacity in leaf extract was observed to be 322.500 mg/g of Ascorbic Acid. Standard curve of ascorbic acid for total antioxidant

capacity is given in Fig. 4. It is possible that the phytochemicals present in the extract/fractions provide the essential factor as a radical scavenger and are responsible for the high scavenging activity.

The above assays confirmed the antioxidant potential of the plant. According to some previous reports, the phenolic compounds are responsible for the antioxidant activity (Masteikova *et al.*, 2007). For that reason the total phenolic, flavonoid and tannin contents in the leaf extract were estimated. Total phenolic, flavonoid and tannin contents (mg/g) in methanol crude extract of *Araucaria cunninghamii* Aiton ex D. Don were determined by using regression equation of calibration curve. Total phenols were expressed as Gallic acid equivalents (GAE), flavonoids were expressed as rutin equivalents (RE) and tannins were expressed as tannic acid equivalents (TAE). It is clear on the basis of results that the leaf extract of *Araucaria cunninghamii* Aiton ex D. Don has phenolic compounds. The amount of phenolic compounds in the extract was 36.111 mg/g. The flavonoids content in the extract was 42.9 mg/g.

Very less tannin content i.e. 0.007mg/g was present in the extract.

The antioxidant activity is determined not only by phenol compounds, but also by a complex of other components of medicinal raw material (Masteikova *et al.*, 2007). Therefore tests for the presence of some other phytochemicals were also performed. Results of phytochemical investigation are shown in Table. 1. Anthraquinones, cardiac glycosides and saponins were found to be present in the leaf extract whereas the extract was lacking cardenolides.

In the leaf extract the phenolic compounds were found to be present in significant amounts. Those polyphenolic compounds were identified qualitatively by HPLC analysis. HPLC analysis identified the presence of Gallic acid, Catechin, Chlorogenic acid, Epicatechin, Caffeic acid, Umbelliferone, Ellagic acid, Quercetin and Kaempferol in the methanol leaf extract of *Araucaria cunninghamii* Aiton ex D. Don. Chromatogram of methanol crude extract of *Araucaria cunninghamii* Aiton ex D. Don leaf in HPLC analysis is given in Fig. 5.

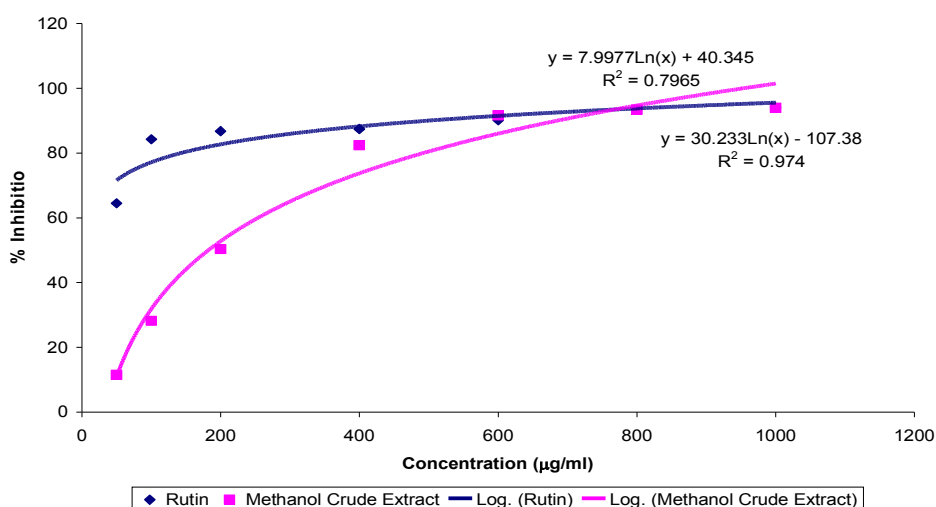


Figure 1. The hydrogen donating ability of methanol crude extract of *Araucaria cunninghamii* Aiton ex D. Don Leaf in DPPH assay.

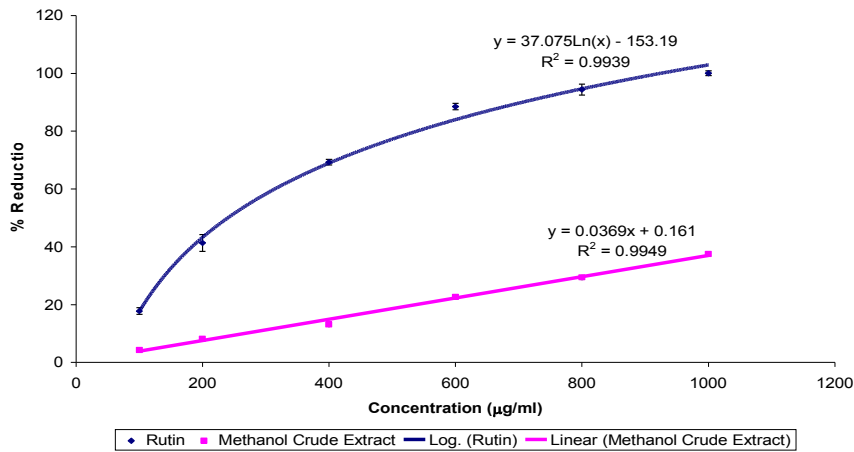


Figure 2. The effect of methanol crude extract of *Araucaria cunninghamii* Aiton ex D. Don Leaf in reducing power assay

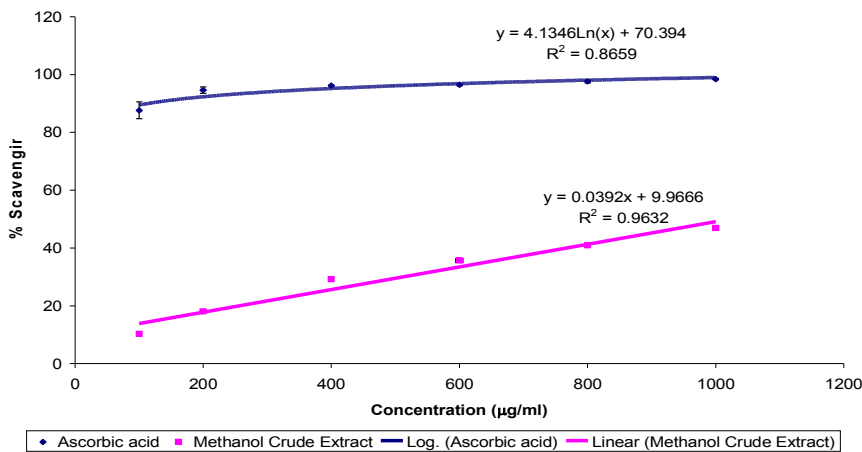


Figure 3. The effect of methanol crude extract of *Araucaria cunninghamii* Aiton ex D. Don Leaf in nitric oxide scavenging assay

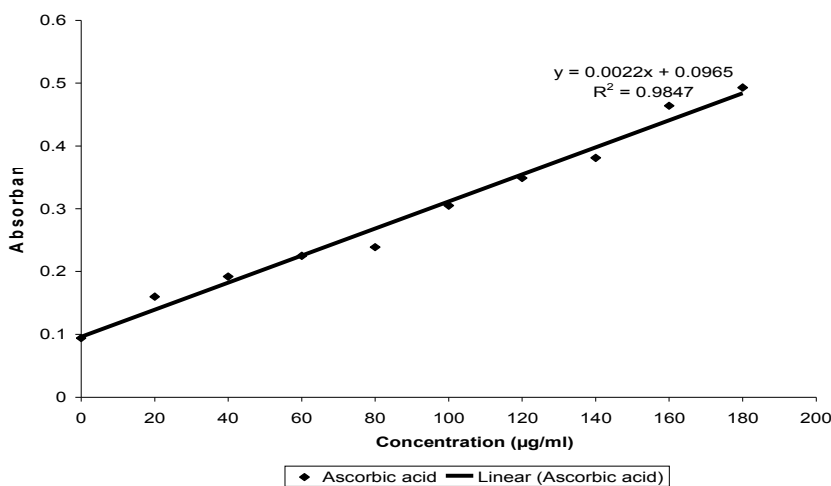


Figure 4. Standard curve of ascorbic acid for total antioxidant capacity

Table. 1: Phytochemical screening of methanol crude extract of *Araucaria cunninghamii* Aiton ex D. Don leaf.

| | Anthraquinones | Cardenolides | Cardiac Glycosides | Saponin |
|------------------------|----------------|--------------|--------------------|---------|
| Methanol Crude Extract | + | - | + | + |

+ Present - Absent

CONCLUSIONS

The study evidently points out that the leaf extract of *Araucaria cunninghamii* Aiton ex D. Don possess antioxidant potential. This is also evident that the antioxidant activity of leaves is due to the polyphenolic compounds identified by the HPLC analysis. In the future studies, the compounds isolated from *Araucaria cunninghamii* Aiton ex D. Don may serve as the model compounds for designing the drugs to treat various diseases associated with free radicals induced tissue damage like cancer, diabetes etc. Further research and investigation will be required for making clear the active principles and for substantiating the claim as presented in this research work. However, it can be said that the secondary metabolite intermediates in the form of tannins, polyphenols and various terpenoid fractions extracted from these gymnosperms offer unique and specific antioxidant capabilities that need more research. These antioxidant fractions are stress busters and of extreme therapeutic value for treatment of diabetes, cardio-vascular disorders and cancers.

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