

IN VITRO ANTIOXIDANT ACTIVITY OF EXTRACTS FROM THE LEAVES OF *ABIES PINDROW* ROYLE

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Abstract

Traditionally, the leaves of *Abies pindrow* Royle are employed as an ayurvedic remedy for fever, hypoglycaemic, respiratory and inflammatory conditions. In this study, dichloromethane, methanol and acetone extracts of *A. pindrow* leaves were analysed for their phytochemical content and *in vitro* antioxidant activities. The methanol extract exhibited highest antioxidant activity while acetone extract showed presence of relatively high total phenol and flavonoids contents. The present study provides evidence that extracts of *Abies pindrow* leaves are a potential source of natural antioxidants and could serve as a base for future drugs.

Key words: *Abies pindrow*, antioxidant, free radicals, radical scavenging activity, metal chelating activity, reducing activity.

List of abbreviations: DPPH = 1,1-diphenyl-2-picryl-hydrazyl, ABTS = 2,2 azinobis-3-ethylbenzothiazoline-6-sulfonic acid, NBT = nitroblue tetrazolium, NADH = nicotinamide adenine dinucleotide, PMS = phenazine methosulfate, TPTZ = 2,4,6-tri-(2-pyridyl)-1,3,5-triazine.

Introduction

Plants contain a number of compounds known as phytochemicals which have been found to be responsible for pharmacological effects and include carotenoids, alkaloids, vitamins, minerals and polyphenols. Research shows that phenolic compounds such as flavonoids and phenolic acids exhibit antioxidant properties (Rice-Evans and Miller, 1998; Wang and Lin, 2000). Oxidation or the loss of an electron sometimes results in the production of reactive substances known as free radicals that can cause oxidative stress or damage to the cells. Research suggests that the resulting free radicals are involved in a number of degenerative diseases such as cancer, cardiovascular disease, cognitive impairment, Alzheimer's disease, immune dysfunction, cataracts, and macular degeneration (Aslan and Ozben, 2004; Harman, 1982; Halliwell, 1999; McCall and Frei, 1999). Antioxidants have the ability to capture, deactivate or repair the damage caused by free radicals (Alonso et al., 2002) and non-radical species such as hydrogen peroxide (H₂O₂), hypochlorous acid (HOCl), and peroxynitrite (ONOO⁻) that are associated with many diseases and the process of ageing. In view of the growing interest in natural antioxidants, there is a need to identify and quantify these compounds in various plants with ethnopharmacological importance and to evaluate their potential health benefits.

Abies pindrow Royle is a large evergreen tree belonging to family Pinaceae and is commonly known as West Himalayan Fir. *A. pindrow* leaves are referred as "Taalisa" and "Zarnab" in Aurvedic and Unani system of medicine respectively and are used as a remedy for fever, hypoglycaemia, bronchitis, asthma, haemoptysis and inflammatory conditions (Bhakuni et al., 1971; Chuneekar and Pandey, 1960; Chatterjee and Pakrashi, 1991). *A. pindrow* has also been regarded as carminative, stomachic, astringent, expectorant, tonic, antispasmodic and antiperiodic (Burdi et al., 2007). Leaves of *A. pindrow* have demonstrated anti-inflammatory, analgesic, antiulcerogenic and hypnotic effects in rats, attenuated swim stress in mice and hypotension in dogs (Singh et al., 1998, 2001). Various extracts of leaves of *A. pindrow* were also found to show mast cell stabilizing action, protection against aspirin-induced ulcers in rats and bronchoprotective activity against histamine challenge in guinea-pigs (Singh et al., 2000). The biological activities exhibited by genus *Abies* include antitumor, antimicrobial, anti-ulcerogenic, antiinflammatory, antihypertensive, antitussive, and central nervous system activities (Yang et al., 2008).

Yang et al. (2008) have reviewed that up to now, 277 compounds have been isolated from 19 plants of *Abies* species. Most of the chemical constituents are terpenoids, flavonoids, and lignans, together with minor constituents of phenols, steroids, and others. Glucopyranoside, hydroxyl- flavanone and chalcone glycoside have been reported from ethanol extract of dried stem of *A. pindrow* (Tiwari and Minocha, 1980). Leaves of *A. pindrow* were reported to possess bioflavonoids (Chatterjee et al., 1984), flavonoids, pindrolactone (Tripathi et al., 1996a, b), and maltol (Samejo et al., 2009). Delving deep into the study of phytochemistry and antioxidant activity can lead to a better understanding and appreciation of the pharmaceutical and medicinal value of *A. pindrow*. Thus, quantification of various phytochemicals and evaluation of antioxidant activity of leaves of *A. pindrow* was the aim of the present study.

Materials and Methods

Plant material

A. pindrow leaves were purchased from wholesale supplier of traditional Unani medicine, Ballimaran, Delhi, India, in August 2008, and authenticated at National Institute of Science Communication And Information Resources (NISCAIR), New Delhi, India. A voucher specimen (NISCAIR/RHMD/Consult/2008-09/1069/100) has been deposited at NISCAIR, New Delhi, India.

Extract preparation

A. pindrow leaves were air dried and powdered. 100 g of dried powder was extracted with solvents dichloromethane, methanol and acetone using Soxhlet apparatus for 6 h for each solvent. Extracts were filtered, concentrated under vacuum in a rotary evaporator and stored at 4°C.

Chemicals

All chemicals and reagents used were of analytical grade and obtained mostly from Sigma. Solvents used for extraction of plants were purchased from Fisher Scientific. The polyphenolic standards, gallic acid, quercetin and rutin were obtained from Sigma.

Determination of total phenolics

The amount of total phenolic compounds in the extracts was determined colorimetrically with the Folin–Ciocalteu reagent, using a slightly modified method of Yu et al. (2002). The reaction mixture contained, 0.1 ml sample extract diluted to 1 ml with distilled water, 0.5 ml of Folin–Ciocalteu reagent (1 N) and 1.5 ml of 20% sodium carbonate solution and was incubated for 2 h at room temperature. The volume was raised to 5 ml with distilled water and the absorbance of blue coloured mixture was measured at 765 nm (Spectronic 2202 UV–Vis Spectrophotometer). The concentration of total phenolic compounds was expressed as mg of gallic acid equivalents (GAE) per g of dried extract, using a standard curve of gallic acid described by the equation $y = 0.0265x$ ($R^2 = 0.9977$). Here, y = absorbance and x = concentration.

Determination of total flavonoids

Measurement of total flavonoid content in the investigated extracts was determined spectrophotometrically according to Zhishen et al. (1999), using a method based on the formation of complex flavonoid-aluminium with the absorption maxima at 510 nm. The reaction mixture contained 0.5 ml of extract in DMSO or standard solutions of quercetin, diluted with 2 ml distilled water and 0.15 ml of 5% NaNO_2 . After 5 min, 0.3 ml of 10% AlCl_3 was added. After 6 min, 1 ml of 1 M NaOH was added and the total volume was made up to 5 ml with water. The solution was mixed well and the absorbance was measured against a prepared reagent blank at 510 nm. The flavonoids content was expressed as mg of quercetin equivalents (QE) per g of dried extract, by using a standard graph ($y = 0.0025x$, $R^2 = 0.9974$).

Determination of total flavonols

Total flavonols of extracts were estimated as mg rutin equivalents (RE) /g extract, from the rutin calibration curve ($y = 0.0172x$, $R^2 = 0.9979$). The reaction mixture was prepared by mixing 0.5 ml of extract solutions with 0.5 ml (20 g/l) AlCl_3 and 1.5 ml (50 g/l) of CH_3COONa . The absorbance of reaction mixture was read at 440 nm after incubation at 20°C for 2.5 h (Miliauskas et al., 2004).

Antioxidant activity determination

DPPH radical scavenging assay

The stable DPPH was used for determination of free radical scavenging activity of the extracts (Blois, 1958). 100 μl of the sample was added to 1 ml of 0.1 mM DPPH in methanol and incubated the mixture for 45 min in dark at 37°C. The absorbance was measured at 517 nm using methanol as blank. Trolox was used as positive control. The antioxidant activity of the extract was calculated as % radical scavenging activity using the formula:

$$\text{Scavenging activity (\%)} = (1 - \text{absorbance}_{\text{sample}} / \text{absorbance}_{\text{control}}) \times 100$$

ABTS radical scavenging assay

The ABTS radicals are generated through a chemical oxidation reaction with potassium persulfate (Re et al., 1999). 10 μl of sample was added to 990 μl of ABTS radical solution to get a total reaction volume of 1 ml and incubated at room temperature for 5 min. The absorbance was measured at 734 nm immediately. Trolox was used as positive control. The percentage radical scavenging activity was calculated as follows:

$$\text{Scavenging activity (\%)} = (1 - \text{absorbance}_{\text{sample}} / \text{absorbance}_{\text{control}}) \times 100$$

Superoxide radical scavenging assay

The superoxide radical scavenging activity was determined by method of Robak and Gryglewski (1988). The reaction mixture consisting of 250 µl of NBT (150 µM), 250 µl of NADH (468 µM) and 250 µl of sample was mixed in sodium phosphate buffer (100 mM, pH 7.4). The reaction was initiated by adding 250 µl of PMS (60 µM) to the mixture. The reaction mixture was incubated at 25°C for 5 min, and the absorbance was measured against the corresponding blank solution. Gallic acid was used as positive control. The superoxide radical scavenging activity was calculated using the formula:

$$\text{Scavenging activity (\%)} = (1 - \text{absorbance}_{\text{sample}} / \text{absorbance}_{\text{control}}) \times 100$$

Ferric reducing antioxidant power (FRAP)

The assay was based upon the methodology of Benzie and Strain (1996). FRAP reagent consisted of TPTZ, CH₃COONa buffer (pH 3.6), and FeCl₃ solution at the ratio of 1:10:1 (v/v/v) respectively. 100 µl of the sample was added into 900 µl of FRAP reagent and incubated at room temperature for 6 min, and the absorbance at 593 nm was measured immediately. Trolox was used as calibration standard ($y = 0.160x$, $R^2 = 0.981$). FRAP values were calculated as mg of trolox equivalents (TE)/g extract from three determinations and are averaged.

Reducing power activity

The reducing power of the extracts of *A. pindrow* was determined as per the method of Oyaizu (1986). 1 ml of sample extract was mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml potassium ferrocyanide (1%). After incubating the mixture at 50°C for 20 min., 2.5 ml of 10% trichloroacetic acid was added, and then mixture was centrifuged at 3000 rpm for 10 min. 2.5 ml of supernatant was mixed with 2.5 ml of distilled water and 0.5 ml FeCl₃ (0.1%) and the absorbance was measured at 700 nm and compared with standard trolox.

Metal ion chelating activity

The chelating activity of extract on Fe⁺² was measured according to the method of Dinis et al. (1994). 1 ml of extract was incubated with 50 µl of 2 mM ferrous chloride. The reaction was started by the addition of 200 µl ferrozine (5 mM). After 10 min, the absorbance of ferrous ion-ferrozine complex at 562 nm was read. Na₂EDTA served as the positive control. Triplicate samples were run for each set and averaged. The ability of extracts to chelate ferrous ion was calculated using the following equation:

$$\text{Chelating activity (\%)} = (1 - \text{absorbance}_{\text{sample}} / \text{absorbance}_{\text{control}}) \times 100$$

Results

Total phenolic, flavonoid, and flavonol contents

The total phenolic, flavonoid and flavonol content of acetone extract was found to be highest among the three extracts and was in order of acetone > methanol > dichloromethane (Table 1).

Antioxidant activity evaluation of extracts of *A. pindrow* leaves

DPPH free radical scavenging activity

Methanol extract demonstrated highest activity (IC₅₀ 0.163±0.006 mg/ml) as compared to acetone extract (IC₅₀ 0.194±0.013 mg/ml) and dichloromethane extract (IC₅₀ 3.41±0.331 mg/ml). However, these activities were less than that of standard trolox (Table 2).

ABTS free radical scavenging assay

Trolox equivalent antioxidant capacity (TEAC) of the *A. pindrow* extracts was evaluated as percent inhibition of ABTS free radicals. Methanol extract of leaves of *A. pindrow* (IC₅₀ 0.008±0.001 mg/ml) showed greater activity compared to acetone (IC₅₀ 0.099±0.001 mg/ml) and dichloromethane extract (IC₅₀ 0.016±0.001 mg/ml). These extracts however, showed less scavenging activity in comparison to standard trolox (Table 2).

Superoxide radical scavenging assay

Acetone extract was most active in scavenging superoxide radicals with 68.383±2.529 % inhibition, while dichloromethane and methanol extracts showed 51.794±5.183 % and 43.729±0.417 % inhibition respectively at 0.5 mg/ml. The superoxide radical scavenging activity of the extracts and standard was found to be in order of acetone > dichloromethane > gallic acid > methanol (Table 2).

Metal ion chelating activity

All the extracts of *A. pindrow* leaves exhibited chelating activity by interfering ferrous-ferrozine complex in a dose dependent manner. Among the extracts, methanol extract was most potent (IC_{50} 0.183±0.008 mg/ml). Dichloromethane and acetone extracts possessed almost similar chelating activity (IC_{50} 0.259±0.002 and 0.310±0.033 mg/ml respectively). Standard Na_2EDTA showed highest metal chelating activity (Table 2).

Table 1: Total phenol, flavonoid, and flavonol contents of the extracts from *A. pindrow* leaves

Extracts	Total phenol (mg GAE/g extract)	Total flavonoids (mg QE/g extract)	Total flavonol (mg RE/g extract)
Dichloromethane	62.956±3.027 ^a	261±8.896 ^a	79.07±2.664 ^a
Methanol	170.126±12.546 ^b	316.167±4.726 ^b	108.915±3.336 ^b
Acetone	471.698±3.774 ^c	642.5±4.33 ^c	408.933±24.872 ^c

GAE, Gallic acid equivalents; QE, Quercetin equivalents; RE, Rutin equivalents. Results are expressed as mean± SD of three parallel measurements. Values within a column followed by different letters (a,b,c) are significantly different ($P<0.05$)

Table 2: IC_{50} values of extracts of *A. pindrow* leaves and standards for different antioxidant assays

Activity Sample	DPPH scavenging (mg/ml)	ABTS scavenging (mg/ml)	Superoxide scavenging (mg/ml)	Metal chelation (mg/ml)
Dichloromethane extract	3.41±0.331 ^a	0.016±0.001 ^a	0.434±0.033 ^a	0.259±0.002 ^a
Methanol extract	0.163±0.006 ^b	0.008±0.000 ^b	0.681±0.016 ^b	0.183±0.008 ^b
Acetone extract	0.194±0.013 ^c	0.099±0.004 ^c	0.294±0.006 ^c	0.310±0.033 ^a
Trolox	0.005±0.000 ^d	0.002±0.000 ^d	-	-
Gallic acid	-	-	0.574±0.009 ^d	-
Na_2EDTA	-	-	-	0.015±0.000 ^c

Results are expressed as mean± SD of three parallel measurements. Values within a column followed by different letters (a,b,c,d) are significantly different ($P<0.05$)

FRAP assay

The ability of the plant extracts to reduce ferric ions was determined using the FRAP assay. FRAP values for dichloromethane, methanol and acetone extracts are 19.413±0.562, 73.876±0.780 and 26.236±1.126 mg TE/ g extract respectively.

Reducing power assay

In the reducing power assay, the antioxidant compounds convert the oxidation form of iron (Fe^{+3}) to ferrous (Fe^{+2}). The reducing activities of extracts are presented in Figure 1 and compared to standard trolox. Reducing activity of the standard trolox was found to be highest and could be defined by linear relationship $y = 2.9121x$, $R^2 = 0.9997$. Among the three extracts, reducing activities of methanol and acetone extracts increased in linear relation with concentration as defined by the equations $y = 1.1219x$, $R^2 = 0.9983$ and $y = 0.8759x$, $R^2 = 0.9943$, respectively. Dichloromethane extract showed very less reducing activity and defined by the equation $y = 0.108x$, $R^2 = 0.9509$.

Discussion

Phytochemicals, especially phenolics are suggested to be the major bioactive compounds for health benefits. Phenolic compounds protect plants from oxidative damage and perform the same function for humans (Duthie et al., 1997; Skaper et al., 1997). Several types of polyphenols (phenolic acids, hydrolysable tannins, and flavonoids) show anticarcinogenic and antimutagenic effects (Urquiaga and Leighton, 2000). Flavonoids are considered to be very beneficial compounds due to their potent nature as antioxidants. Flavonols are a class of flavonoids and their consumption has been associated with a variety of beneficial effects including increased activity of erythrocyte superoxide dismutase, a decrease in lymphocyte DNA damage, a decrease in urinary 8-hydroxy-2'-deoxyguanosine (a marker of oxidative damage), and an increase in plasma antioxidant capacity (Williamson and Manach, 2005). In this study, acetone extract of leaves of *A. pindrow* was the richest in total phenolic, total flavonoid and flavonol content. Thus, acetone is the best solvent for obtaining high yield of phenolic compounds in *A. pindrow* leaves.

The DPPH method is an easy, rapid, stable and sensitive way to determine the antioxidant activity of a specific compound or plant extracts (Koleva et al., 2002). In this assay, DPPH free radical accepts hydrogen and gets reduced by an antioxidant. Methanol extract exhibited highest DPPH scavenging among all the extracts. Since, DPPH scavenging assay is a measurement of antioxidant activity of water soluble phenolic compounds (Chun et al., 2005), this study revealed that methanol extract was richer in water soluble phenolics.

ABTS radical is a blue chromophore produced by the reaction between ABTS and potassium persulfate. Addition of the plant extract to this radical cation reduced it to ABTS in a dose dependent manner. Methanol extract was also most active in scavenging ABTS radicals among all extracts of *A. pindrow* leaves. The scavenging activity of the extracts towards ABTS radicals was found to be more as compared to that towards DPPH radicals.

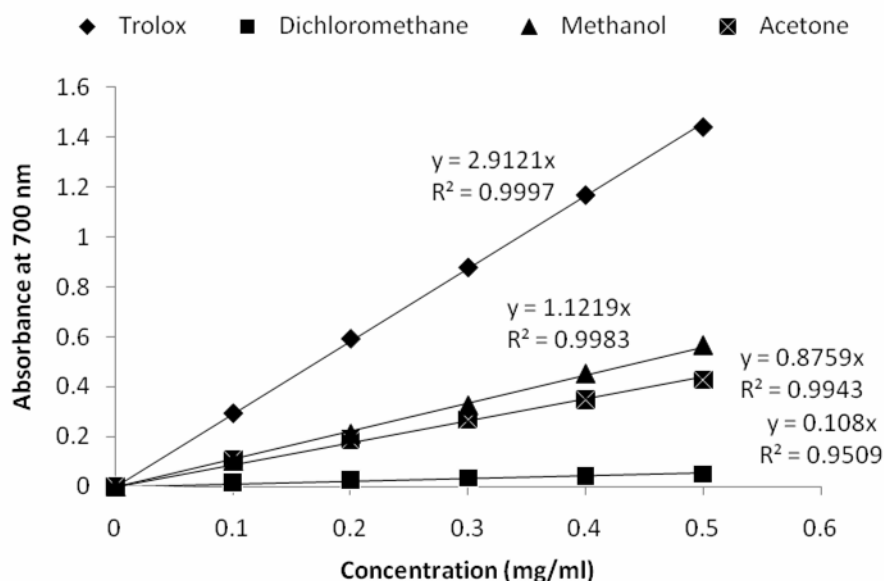


Figure 1: Reducing power activities of various extracts of *A. pindrow* leaves and standard trolox

The superoxide radicals generated from dissolved oxygen by PMS-NADH coupling can be measured by their ability to reduce NBT. The decrease in absorbance at 560 nm with the plant extract indicates their abilities to quench superoxide radicals in the reaction mixture. All extracts of *A. pindrow* leaves were effective in scavenging superoxide radicals with a greater ability than standard gallic acid except methanol extract. Acetone extract was most active in quenching superoxide radicals which could be explained on the basis of its high flavonoid content. Robak and Glyglewski (1988) have reported that flavonoids are effective antioxidants mainly because they scavenge superoxide anions.

The transition metal ion Fe^{+2} possess the ability to move single electrons thus allowing the formation and propagation of many radical reactions, even starting with relatively nonreactive radicals (Halliwell, 1994). The mechanism to avoid reactive oxygen species formation, associated with redox active metal catalysis is through chelation of the metal ions. Presence of chelating agents disrupts the formation of Ferrozine- Fe^{+2} complex monitored by measuring the decrease in the red color of the complex at 562 nm and thus, estimating the chelating activity of the coexisting chelator. The results of our study demonstrate that all extracts have an effective capacity for iron binding, suggesting their antioxidant potential.

In FRAP assay, reduction of the ferric-tripyridyltriazine to the ferrous complex forms an intense blue colour which can be measured at a wavelength of 593 nm. The intensity of the colour is related to the amount of antioxidant reductants in the samples. Methanol extract had the highest FRAP value indicating it to be richer in antioxidant reductants.

Fe^{+3} reduction, an indicator of electron donating activity, is considered to be an important mechanism of antioxidant activity of phenolics (Yildirim et al., 2001). In the reducing power assay, the antioxidants in the samples reduce Fe^{+3} to Fe^{+2} by donating an electron. Amount of Fe^{+2} complex can then be measured spectrophotometrically at 700 nm. Increasing absorbance at 700 nm indicated an increase in the reductive ability of all extracts of *A. pindrow* leaves with increase in concentration.

Acetone extract despite being richer in polyphenols showed smaller antioxidant activity than methanol extract. It could be because several factors like stereoselectivity of the radicals or the solubility of the extract in different test systems have been reported to affect the ability of extracts to react and quench different radicals (Yu et al., 2002).

Based on these results it can be concluded that all the extracts of leaves of *A. pindrow* showed strong antioxidant activity when compared to the standards. However, the difference in the antioxidant activities of the various extracts may be due to their different phytochemical composition. The results obtained emphasize that extracts mainly exhibit their antioxidant potential via free radical scavenging and electron donation. Therefore, it seems reasonable to consider these leaf extracts of *A. pindrow* as new valuable ingredients for pharmaceuticals in the promotion of health as commercial drugs. However, further studies on the bioavailability of the plant extracts and their antioxidant status in animal models are needed to evaluate their potential health benefits.

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