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Estimation of Total Phenols and Flavonoids in Selected Indian Traditional Plants

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Abstract Traditionally, aerial parts of *Abies pindrow* Royle (Himalayan Fir; Pinaceae); *Abies webbiana* Lindl. (Talispatra; Pinaceae); *Cephalandra indica* Naud. (Ivy Gourd; Cucurbitaceae) and roots of *Calotropis gigantea* (L.) Dryand (Giant Milkweed; Asclepiadaceae) have been used in the Indian systems of medicine for the treatment of various ailments. But no systematic phytochemical work has ever been carried out on these potential plants. Thus, it was planned to estimate total phenols and flavonoids content in methanol extract, ethyl acetate fraction (EAF) and remaining methanol extract (RME) of selected plants. Properly identified plants were defatted with petroleum ether, and then separately extracted in a Soxhlet apparatus with methanol. The methanol extract of each plant was partitioned by ethyl acetate solvent to obtain EAF of respective plant. The total phenols and flavonoids contents were estimated using standardized procedures. Quantitative determination of total phenols and total flavonoids was done using standard curve of gallic acid (linearity: 20 to 120 mg/ml; $r^2 = 0.995$) and quercetin (linearity: 30 to 180 mg/ml; $r^2 = 0.997$), respectively. EAF of selected plants contained higher content of phenols and flavonoids, where as lowest content was observed in RME of plants. The content of total phenols and flavonoids in selected plants were found to be in order of *C. indica* > *A. webbiana* > *A. pindrow* > *C. gigantea*. The available literature reveals that polyphenols have been reported to possess varied pharmacological activities. As selected Indian plants contain polyphenols as major class of phytoconstituents, it is suggested that these constituents may be responsible for their medicinal uses.

Keyword: *Abies pindrow*, *Abies webbiana*, *Calotropis gigantea*, *Cephalandra indica*, Flavonoids, Phenols

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1. INTRODUCTION

Nature has bestowed our country with an enormous wealth of medicinal plants. Therefore, India has referred to as the medicinal garden of the world. About 45,000 different plant species and 15,000 medicinal plants are recorded in India. Three of the ten most widely selling herbal medicines in the developed countries, namely preparation of *Allium sativum*, *Aloe barbadensis* and *Panax* spp. are available in India (Dubey *et al*., 2004). Indian pharmaceutical industry's annual turnover is of Rs. 14,500 crores with a growth rate of 15 percent, while annual turnover of Indian herbal medicinal industry is about Rs. 2,300 crore (Sharma *et al*., 2008). Despite a vast flora of medicinal plants, India's share in global export of medicinal plants related trade is just 0.5%. Thus, new strategies are required to develop interest of researchers to exploit our wealth, i.e., natural resources properly.

Most of these medicinal plants are rich sources of polyphenols. But no work has been carried out to estimate total phenols and flavonoids in the plants. *Abies pindrow* Royle, *Abies webbiana* Lindl., *Cephalandra indica* Naud. and *Calotropis gigantea* (L.) Dryand are such plants which contain polyphenols as major class of phytoconstituents, and have long tradition of use in the treatment of various ailments in Indian system of medicines (Chopra *et al*., 1956 and Khare, 2007).

***Abies pindrow* Royle** (Himalayan Fir; Pinaceae) has been traditionally used in the treatment of various ailments especially neurodegenerative disorders (Gupta and Sharma, 2007 and Duke *et al*., 2008). ***Abies webbiana* Lindl.** (Talispatra; Pinaceae) has been traditionally used in the treatment of various ailments such as cough, asthma and chronic bronchitis (Kirtikar and Basu, 1975). ***Cephalandra indica* Naud.** (Ivy Gourd; Cucurbitaceae) has been traditionally used in treatment of diabetes in the Ayurvedic and Unani system of medicines (Chandira *et al*., 2010). Other traditional uses include anti-inflammatory, antipyretic, analgesic, antispasmodic, antimicrobial, cathartic, antibacterial and expectorant (Lee *et al*., 2003). ***Calotropis gigantea* (L.) Dryand** (Giant Milkweed; Asclepiadaceae) has been traditionally used in treatment of asthma, leprosy, epilepsy, convulsions and mental disorders (Jha, 2001; Jain and Tarafder, 1970; Iyengar *et al*., 1986; Katewa *et al*., 2003).

A survey of literature revealed that no systematic work has been undertaken with a view to estimate total phenols and flavonoids in selected plants. Thus, it was considered worthwhile to estimate total phenols and flavonoids in methanol extract, ethyl acetate fraction (EAF) and remaining methanol extract (RME) of selected traditional plants.

2. METHODS AND METHODOLOGY

2.1. Plant materials

A. pindrow aerial parts were collected from Gulaba Kothi, Manali, India at the height of 2000-2100 m in the month of September, 2011. *C. gigantea* roots and *C. indica* aerial parts were procured from Himalaya Herbs Store, Madhav Nagar, Saharanpur, (Uttar Pradesh), India in the month of July, 2011. *A. webbiana* aerial parts were procured from D.G. Ayurvedic Sangrah, Andheri, Mumbai, India in the month of September, 2012. *C. indica* was identified by Botanical Survey of India, Dehra Dun (Reference no. - BSI/NRC/Tech/2011-12/585, dated 22/09/2011). *A. pindrow, A. webbiana* and *C. gigantea* were identified by Dr. Sunita Garg, Chief Scientist and Head, Raw Materials Herbarium and Museum, NISCAIR, New Delhi (Reference no. - NISCAIR/RHMD/Consult/2013/2242/23, dated 21/05/2013).

2.2. Solvents

Methanol (S.D. Fine Chemicals, Mumbai, India), ethyl acetate (E Merck, Delhi, India) and petroleum ether (60-80ºC) (RFCL Ltd., New Delhi, India), all of LR grade, were employed.

2.3. Chemicals and instruments

Gallic acid and quercetin (Hi-media Laboratories Pvt. Ltd) were used as standard drugs. Folin Ciocalteu's reagent (S.D. Fine Chem. Ltd., Mumbai), Sodium carbonate (Central Drug House Pvt. Ltd., Mumbai), Aluminium chloride and Potassium acetate (S.D. Fine Chem. Ltd., Mumbai) reagents were used in estimation of total phenols and flavonoids in test samples. Rotary vacuum evaporator (BUCHI, Switzerland) was used for recovery of solvents under reduced pressure. UV/VIS spectrophotometer (Schimadzu, Japan) was used for taking absorbance of test samples.

2.4. Preparation of extracts

All plant materials were rinsed with normal saline to remove dirt, dried under sunlight and powdered in a grinder. Dried and powdered plant materials (250 g) were separately extracted in a Soxhlet apparatus successively using solvents in order of increasing polarity *viz.,* petroleum ether and methanol. The solvents were recovered under vacuum using rotary vacuum evaporator. Dried methanol extracts were stored in vacuum desiccator and subjected to phytochemical screening (Farnsworth, 1966).

The methanol extract (20 g each) of plant materials was separately suspended uniformly in water, placed in a round bottom flask and partitioned with ethyl acetate by heating at 50ºC for 30 min along with continuous stirring. This procedure of partitioning with ethyl acetate was repeated for ten times. All the separated layers of ethyl acetate were pooled and concentrated. This procedure yielded ethyl acetate (Polyphenol -rich) fractions of different plant materials.

2.5. Estimation of total phenols content

2.5.1. Preparation of standard

Ten mg of gallic acid was dissolved in 100 ml of 50% methanol (100 µg/ml) and then further diluted to 20, 40, 60, 80, 100 or 120 µg/ml (Madaan *et al*., 2011). To one ml aliquot of each dilution, 9 ml distilled water and 1.5 ml Folin Ciocalteu's reagent were added in a test tube. The mixture was allowed to incubate at room temperature for 5 min. Four ml of 20% (w/w) Na_2CO_3 was then added in each test tube and volume was made up to the mark of 25 ml with distilled water. The mixture was agitated and left to stand for 30 min at room temperature. Absorbance of the standard was measured at 765 nm using UV/VIS spectrophotometer against blank, i.e., distilled water.

2.5.2. Preparation of test sample

Two hundred fifty mg of methanol extract was added to 15 ml of methanol (50%) and extracted for three times by maceration for 2 h, then filtered and make up the volume with methanol (50%) in volumetric flask upto 25 ml. To one ml aliquot of sample, 9 ml distilled water and 1.5 ml Folin Ciocalteu's reagent were added in a test tube. The mixture was allowed to incubate at room temperature for 5 min. Four ml of 20% (w/w) Na_2CO_3 was then added in each test tube and volume was made up to the mark of 25 ml with distilled water. The mixture was agitated and left to stand for 30 min at room temperature. Absorbance of the standard was measured at 765 nm using UV/VIS spectrophotometer against blank, i.e., distilled water. Similar procedure was adopted to prepare test sample of ethyl acetate fraction (EAF) and remaining methanol extract (RME), and absorbance of the sample was measured at 765 nm using UV/VIS spectrophotometer against blank, i.e., distilled water.

A standard curve of absorbance against gallic acid concentration was prepared (Figure 1), and used for estimation of total phenols content in test samples. Results were expressed as percentage w/w and calculated using following formula.

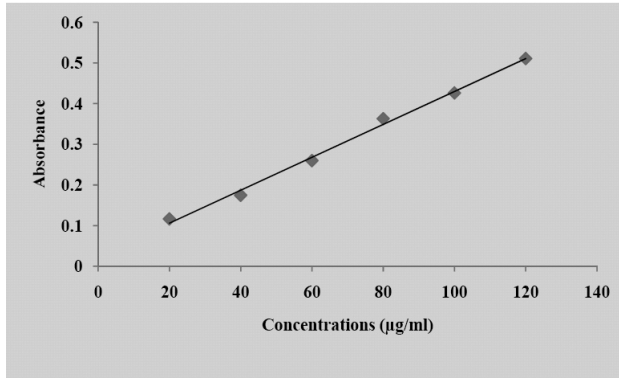

Figure 1: Standard curve of absorbance against gallic acid concentration
$y = 0.004x + 0.025$; $R^2 = 0.995$

Total phenolic content (% w/w) = GAE × V × D × 10^{-6} × 100 / W, GAE - Gallic acid equivalents (µg/ml), V - Total volume of sample (ml), D - Dilution factor, W - Sample weight (g)

2.6. Estimation of total flavonoids content

2.6.1. Preparation of standard

Twenty mg of quercetin was dissolved in 100 ml of methanol (200 µg/ml) and then diluted to 30, 60, 90, 120, 150 or 180 µg/ml (Madaan *et al*., 2011). The standard solutions (0.5 ml) were separately taken in test tubes. To each of test tube, 1.5 ml of methanol (95%), 0.1 ml of aluminium chloride (10%), 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water were added. The reaction mixtures were incubated at room temperature for 30 min and their absorbance were measured at 415 nm with UV/VIS spectrophotometer. The amount of aluminium chloride (10%) was substituted by the same amount of distilled water in blank.

2.6.2. Preparation of test sample

Two hundred fifty mg of methanol extract, EAF and RME were dissolved separately in 25 ml of methanol. Similarly, 0.5 ml of methanol extract, EAF and RME were reacted with aluminium chloride for determination of flavonoids content as described in section '*Preparation of standard*'. The amount of aluminium chloride (10%) was substituted by the same amount of distilled water in blank.

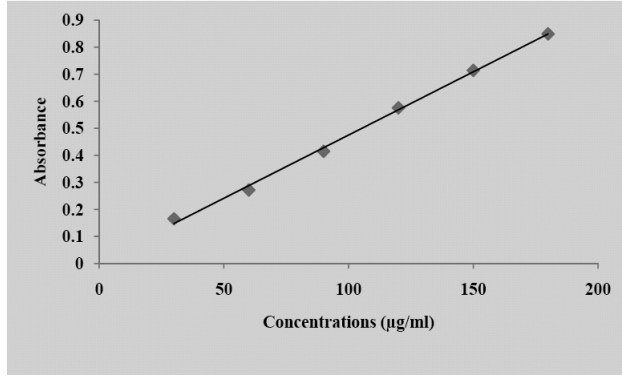

Figure 2: Standard curve of absorbance against quercetin concentration
$y = 0.004x + 0.007$; $R^2 = 0.997$

A standard curve of absorbance against quercetin concentration was prepared (Figure 2), and used for estimation of total phenols content in test samples. Results were expressed as percentage w/w and calculated by following formula.

Flavonoids content (% w/w) = $QE \times V \times D \times 10^{-6} \times 100 / W$, QE - Quercetin equivalents (µg/ml), V - Total volume of sample (ml), D - Dilution factor, W - Sample weight (g)

2.7. Statistics

The results were expressed as mean ± standard deviation (S.D).

3. RESULTS AND DISCUSSION

Aerial parts of *A. pindrow, A. webbiana*, *C. indica* and roots of *C. gigantea* were defatted by extracting with petroleum ether (60-80^{0}C) in Soxhlet apparatus. The marcs of plants were then separately extracted with methanol in a Soxhlet apparatus. The methanol extracts of selected plants were subjected to standard phytochemical screening procedures in order to ascertain various classes of phytoconstituents present therein. The results of phytochemical screening showed presence of flavonoids, steroids, triterpenoids, carbohydrates and proteins in *A. pindrow*; flavonoids, alkaloids, steroids, triterpenoids, tannins, carbohydrates and proteins in *A. webbiana*; alkaloids, flavonoids and tannins in *C. indica* and flavonoids, steroids, triterpenoids, alkaloids, saponins and tannins in *C. gigantea*. As polyphenols are the major class of phytoconstituents present in selected plants, thus it was planned to separate polyphenolic rich fractions from methanol extracts of selected plants by standard procedures

and to estimate contents of total phenols and flavonoids in different extracts/ fractions. Yields of methanol extracts of selected plant and their EAF were depicted in table 1 and 2 respectively.

Table 1: Yields of methanol extracts of selected plants

Plant	Yield (% w/w)
A. pindrow	14.80
A. webbiana	16.21
C. indica	13.24
C. gigantea	14.35

Table 2: Yields of ethyl acetate fractions obtained from methanol extracts of selected plants

Plant	Yield* (% w/w)
A. pindrow	20.45
A. webbiana	18.45
C. indica	16.22
C. gigantea	15.25

*Calculated with respect to methanol extract

Quantitative determination of total phenols and total flavonoids was done on the basis of a standard curve of gallic acid (linearity: 20 to 120 mg/ml; r^2 = 0.995; figure 1) and quercetin (linearity: 30 to 180 mg/ml; r^2 = 0.997; figure 2) respectively. EAF of various plants contained higher content of total phenols and flavonoids than methanol extract and RME (table 3).

It is evident from table 3, methanol extract of *A. webbiana* (15.58% w/w) contained higher content of total phenols followed by methanol extracts of *C. indica* (10.69% w/w), *A. pindrow* (7.61% w/w) and *C. gigantea* (6.66% w/w), whereas EAF of *C. indica* (20.78% w/w) showed presence of slightly higher content of phenols than *A. webbiana* (20.20% w/w). RME of all plants showed presence of about half of the content of total phenols than crude methanol extracts of plants. The methanol extract of *C. indica* was found to be contain highest content of flavonoids (3.89% w/w) followed by methanol extract of *A. webbiana* (1.76% w/w), *A. pindrow* and *C. gigantea* (1.33% w/w each). EAF of plants contained almost double the content of total flavonoids than their

Table 3: Percentage content of total phenols and flavonoids in the methanol extract, ethyl acetate fraction and remaining methanol extract of selected traditional plants

Plant material	Test sample	Total phenols content (% w/w) Mean^n±S.D.	Total flavonoids content (% w/w) Mean^n±S.D.
A. pindrow	Methanol extract	7.61±0.21	1.33±0.22
	Ethyl acetate fraction	11.58±0.11	3.94±0.08
	Remaining methanol extract	3.59±0.19	0.61±0.03
A. webbiana	Methanol extract	15.58±0.42	1.76±0.11
	Ethyl acetate fraction	20.20±0.32	4.74±0.22
	Remaining methanol extract	9.22±0.14	0.63±0.41
C. indica	Methanol extract	10.69 ± 0.95	3.89 ± 0.95
	Ethyl acetate fraction	20.78 ± 3.10	6.78 ± 3.10
	Remaining methanol extract	4.85± 1.20	1.22± 0.03
C. gigantea	Methanol extract	6.66±0.19	1.33±0.07
	Ethyl acetate fraction	9.18±0.06	2.47±0.07
	Remaining methanol extract	3.65±0.31	0.49±0.01

n=3

respective methanol extract where as RME of plants possessed about half the content of total flavonoids of their respective methanol extracts.

The mean percentage of total phenols and flavonoids content in EAF of selected traditional plants was found to be higher than methanol extract and RME. This observation suggests that most of polyphenols in the methanol extract could be extracted using ethyl acetate.

Phenolic compounds are widely distributed natural antioxidants (Skerget *et al*., 2005). Naturally occurring plant phenols and flavonoids possess a broad range of pharmacological activities such as antioxidant, antimutagenic, antimicrobial, antiulcer, antiarthritic, anti cancer and protein kinase inhibition (Marinova *et al*., 2005; Sulaiman and Balachandran, 2012). Free radicals have been found to play a major role in several human diseases, including, brain disorders (Haramoto *et al*., 2008), as brain is more vulnerable to oxidative stress due to imbalance between reactive oxygen radicals and antioxidant defence system of our body (Hotta *et al*., 2002). Exhaustive pharmacological work has been conducted where polyphenols have been reported to possess

strong action on CNS (Fernandez *et al*., 2006; Viola *et al*., 1995; Saaby *et al*., 2009).

The selected plants in present studies have long traditional of use in the treatment of various ailments especially in CNS disorders, and the available literature reveals that polyphenols play a pivotal role in treating CNS diseases. As the selected plants contained polyphenols as major class of phytoconstituents, it is anticipated that CNS activities of these plants may be attributed to these constituents.

4. CONCLUSION

Finally, it is suggested that polyphenols may be responsible for most of activities of selected plants. Detailed neuropharmacological activities studies of polyphenol rich fractions are further needed with a view to validate traditional claims of Indian medicinally promising plants.

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5. DECLARATION OF INTEREST

The authors report no declaration of interest.

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