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PHENOLIC ACID AND FLAVONOL CONTENTS OF GEMMO-MODIFIED AND NATIVE EXTRACTS OF SOME INDIGENOUS MEDICINAL PLANTS

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Abstract

In the present study the amount of three important flavonols (kaempferol, quercetin, myricetin) and five phenolic acids (gallic acid, ferulic acid, cinnamic acid, vanillic acid and caffeic acid) were determined from four medicinally important indigenous plants like *Euphorbia tirucalli, Trigonella foenum-graecum, Cyperus rotundus and Rheum emodi*. Methanolic extracts of native parts and gemmo-modified extracts from fresh germinating parts of medicinal plants were prepared and investigated by reverse phase high-performance liquid chromatographic (RP-HPLC). The total flavonols contents varied significantly among medicinal plants. *Rheum emodi* exhibited the highest amount of total flavonols (881.5±1.3 mg kg^{-1}) but among gemmo-modified extracts, higher flavonols were detected in gemmo-modified extract of *Trigonella foenum-graecum* as compared to its seed extracts. However the other gemmo-modified extract of *Cyperus rotundus* showed relatively less total flavonol than rhizome extract. Gallic and ferulic acid were the most abundant phenolic acids identified in all plants. The chlorogenic acid, *p*- coumaric acid and ferulic acids are main phenolic acids present in these plants. Caffeic acid was only detected in gemmo-modified extract of *Trigonella foenum-graecum*. The significant amount of a variety of flavonol and phenolic acids that may impart their medicinal potential for alleviation of any disorder were available in the investigated plants.

Introduction

Polyphenols are widely distributed and important class of plant secondary metabolites, which possess aromatic ring with one or more hydroxyl substituents. Phenolic compounds are including flavonoids and phenolic acids are most frequently occurring in combination with sugars as glycosides (Harborne, 1998). Flavonoids are divided into many categories, including flavonols, flavones, catechins, pro-anthocyanidins, anthocyanidins and iso-flavonoids (Cartea *et al.,* 2011*).* Phenolic acids present in plants are hydroxylated derivatives of benzoic and cinnamic acids. Hydroxybenzoic acids subdivided into gallic, vanillic, and synergic acid while hydroxycinnamic acids include caffeic, *P*-coumaric and ferulic acids (Cartea *et al.,* 2011). Flavonoids and phenolic acids have many functions in plants. They are very important for growth development and play key role in defense against microbial activities, and infections. They provide oxidative stabilities to the plants in case of injuries (Cetkovic *et al*., 2007).

Currently, polyphenols have attracted great attention and get a high importance due to their antioxidant activity (Khasawneh *et al.,* 2011; Jaafar *et al*., 2012). Pharmacological activities of many plants, fruits and vegetables are closely related to the presence of natural antioxidants especially, phenolic acids and flavonoids. These compounds have great importance for their ability to prevent oxidation and used as major ingredients in foods for preservation (Chlopcikova *et al*., 2004; Slusarczyk *et al*., 2009). Antioxidants significantly decrease the adverse effect of reactive species and at the same time antioxidant therapy has great impact in the treatment of many other diseases (Kumar *et al*., 2007; Mardu *et al*., 2012). Flavonoids have great importance and application in pharmaceutical and food industries. Many flavonoids have been tested as medicinal agents against human diseases including microbial infections, cancer, heart diseases and AIDS (Rice-Evans & Packer, 1998; Carlo *et al*., 1999; Yao *et al*., 2004; Noriaki *et al*., 2005; Jiangrong & Jiang, 2007).

From the last few years interest in studying and quantifying the polyphenolic components of fruits, vegetable and medicinal plants has been increased due to their potential health benefits. Gemmo-therapy is a new and less studied field, in which fresh germinating parts of medicinal plants are used for therapeutic action. Gemmo-therapeutically treated preparations contain more bioactive components than other herbal products.

Although many reports on the flavonols and phenolic acid contents of plant sources have been presented from deferent countries, the quantitative and qualitative information are still insouciant, which entails the exploration of more and more medicinal plants for the search of credible and beneficial natural anti-oxidants. Moreover there is less information about the phenolic acids and flavonols contents of indigenous plants of Pakistan. The present study was therefore, undertaken with the main objective to quantify the levels of less studied, but important, flavonols (kaempferol, quercetin, myricetin), phenolic acids (gallic acid, ferulic acid, cinnamic acid, vanillic acid and caffeic acid) in gemmo-therapeutically treated and native part of selected medicinal plants. To the best of our knowledge, under study gemmo-therapeutically treated plants have not yet been investigated and quantified for the specific flavonols (kaempferol, quercetin, myricetin) and phenolic acids. Four medicinally important plant materials *Euphorbia tirucalli, Trigonella foenum-graecum, Cyperus rotundus and Rheum emodi* has been the subject of our present study. So, the present work would be informative and novel with regard to the quantification of specific phenolic acids and flavonols in gemmo-therapeutically treated and native medicinal plants. Such study is valuable for researchers and alternative and complementary medical practitioners and thus a step towards their potential commercialization as nutraceuticals and anti-oxidant applications in the marketplace.

Materials and Methods

Plant material: Four medicinally important indigenous plants including *Euphorbia tirucalli, Trigonella foenum-graecum Cyperus rotundus* and *Rheum emodi* were selected for this study. Medicinal plants were collected from botanical garden, University of Agriculture, Faisalabad. Fresh growing parts (buds, shoots and young leave) of plants were washed thoroughly and ground into paste for the preparation of gemmo-modified extract. Other parts of plants were dried under shade and changed into fine powder form and kept into air tight containers.

Preparation of native extract: Rhizomes *of Rheum emodi,* whole plant of *Euphorbia tirucalli,* rhizomes *of Cyperus rotundus* and seeds of *Trigonella foenum-graecum* were used for the preparation of native extracts. Selected plant parts (30g) were subjected to 12h extraction with methanol. The extract was then filtered and solvent was completely evaporated with rotary evaporator (Rota vapor R-II, Buchi) under reduced pressure approximately at 40ºC.

Preparation of gemmo-modified extract: Gemmo-modified extracts of *Cyperus rotundus* and *Trigonella foenum-graecum* were prepared from buds/shoots and young leaves of plants. Paste of plant material (30g), which was freshly harvested from plants during their growing stage was macerated with 300mL mixture of glycerin and methanol in a ratio of 1:2 and shake strongly. After 1 month, the macerate was filtered and solvent was removed with rotary evaporator and crude extract was stored in refrigerator till further analysis.

Hydrolysis of extracts: Both methanolic and gemmo-modified extracts of all five plants were analyzed thorough HPLC. Acid hydrolysis of the extract was carried out following the method of Sultana and Anwar 2008. Plant extract (5 g) was refluxed for 1 h with methanol (50 mL) and HCl (5mL, 2 M), to obtain free phenolic acids from their bound forms. After reflux the extracts were filtered through filter paper (Whatman No.1) and then again filtered through 0.45 µm cellulose acetate (Millipore) membrane filters.

HPLC conditions for polyphenols analysis: The analytical HPLC system (model LC-10A, Shimadzu, Kyoto, Japan) used in analysis was comprised of SCL-10A system control unit, UV-visible detector (SPD-10AUV λ max 360 nm), Rheodyne injector, CTO-10A column oven and LC-10 AS pumps. The separation of flavonols was achieved on Hypersil-ODS column (4.6 x 250 mm, I.d., 5-*u*m) at ambient temperature. The mobile phase consisted of solvent A (3% aq. trifluoroacetic acid) and solvent B (acetonitrile and methanol (80:20 v/v), and the mobile phase was run with isocratic elution at flow rate of 1mL/min. The column and detector (λ max 280 nm) used for phenolic acid separation were similar to that used in flavonol analysis. The mobile phase consisted of solvent A (methanol and 1% acetic acid) and Solvent B (water and 1% acetic acid). The mobile phase was run with isocratic elution at flow rate of 1mL/min and wave length of 360 nm was used for detection of phenolic acids (Sultana & Anwar 2008).

Identification of flavonols (kaempferol, quercetin, myricetin) and phenolic acid in plant extracts was made by comparing their retention times with standards (Sigma Chemicals Co., St Louis, MO, USA). Quantitative determination was carried by using calibration curves of the standards.

Results

Reverse Phase High Performance Liquid Chromatography (RP-HPLC) was used for the identification and quantification of important phenolic acids and flavonol from medicinal plants (Table 1). Myricetin was the most abundantly present flavonol in all medicinal plants. Highest level of myricetin was found in gemmo modified extract of *Trigonella foenum-graecum* (830±1.00 µg/g) followed by *Euphorbia tirucalli (*821±0.45 µg/g), *Rheum emodi* (708.7±1.2 µg/g), rhizomes of *Cyperus rotundus* (702±0.23 µg/g) and seed extract of *Trigonella foenum-graecum* (547±0.9µg/g). Gemmo-modified extracts of *Cyperus rotundus* contained 104±0.5 µg/g myricetin. Quercetin was another very important widely distributed flavonol. The highest contents were found in rhizomes of *Cyperus rotundus* (110.6±0.56 µg/g) followed by *Rheum emodi* (67.5± 0.9 µg/g) and *Europhobia tirucalli* (1.3±0.2 µg/g). Quercetin was not detected in *Trigonella foenum-graecum* and gemmo-modified extract of *Cyperus rotundus.*

Kaempferol is also very important flavonol, and the concentration varied extensively among the different medicinal plants. *Rheum emodi* showed highest content (106 ± 1.3 µg/g) followed by *Cyperus rotundus* rhizome (32 ±0.5 µg/g) and gemmo--modified extract of *Trigonella foenum-graecum* (1.13±0.8 µg/g). Kaempferol was not detected in the extract of *Europhobia tirucalli*, gemmo-modified extract of and *Cyperus rotundus* and *Trigonella foenum-graecum* seed.

Table 1. Flavonol contents of different medicinal plants (µg/g of dry weight of plant) quantified by HPLC.

	Myricetin	Quercetin	Kaempferol	Total flavonols
Rheum emodi	708.7 ± 1.2	67.5 ± 1.5	106 ± 1.3	881.5 ± 1.3
Euphorbia tirucalli	821 ± 0.45	1.31 ± 0.2	N.D	822.31 ± 0.35
Cyperus rotundus (gemmo-modified)	104 ± 0.5	N.D	N.D	104 ± 0.5
Cyperus rotundus (Rhizome*)*	702 ± 0.23	110.6 ± 0.56	32 ± 0.5	744.6 ± 0.83
Trigonella foenum-graecum (gemmo modified)	830 ± 0.9	N.D	1.13 ± 0.8	831.3 ± 1.0
Trigonella foenum-graecum (Seed)	547 ± 1.5	N.D	N.D	547 ± 1.5

Values are mean ± S.D of triplicate analysis, N.D = not detected

Table 2. Phenolic acids and flavanol contents (µg/g of dry weight) in different medicinal plants quantified by HPLC.

	Gallic acid	Catechin	Chlorogenic acid	Caffeic acid	*p*-coumaric acid	Ferulic acid
Rheum emodi	90.9 ± 0.05	5.5 ± 0.5	62.8 ± 0.9	N.D	112 ± 0.9	102.5 ± 0.8
Euphorbia tirucalli	11.9 ± 1.2	4.59 ± 0.9	42.6 ± 1.3	N.D	97.3 ± 0.9	N.D
Cyperus rotundus (Rhizome)	68.0 ± 1.3	1.6.8 ± 1.3	29.1 ± 0.7	N.D	116.74 ± 1.2	41.17 ± 0.55
Trigonella foenum-graecum (Seed)	10.24 ± 0.25	3.4 ± 0.2	41.90 ± 1.3	N.D	81 ± 0.9	104.5 ± 1.3
Trigonella foenum-graecum (gemmo-modified)	15.76 ± 0.8	0.63 ± 0.01	29.81 ± 0.09	9.2 ± 0.02	70.20 ± 0.02	114.25 ± 0.0.9

Values are mean ± SD of triplicate analysis, N.D = not detected

Five phenolic acids including, gallic acid, chlorogenic acid, *p*-coumaric acid, ferulic acid, caffeic acid and one flavanol (catechin) were determined by HPLC The phenolic acids identified by HPLC method varied widely in medicinal plants. The amount of polyphenols identified in different medicinal plants has been shown in Table 2. Gallic acid was the most abundant phenolic acid identified in all plants. The chlorogenic acid, *p*- coumaric acid, ferulic acid, caffeic acids are main phenolic acids present in these plants. Rhizomes of *R. emodi* contained *p*-coumaric acid (112.5±0.9 µg/gm) in the highest concentration followed by ferulic acid (102.8±0.8 µg/gm), gallic acid (90.9±0.05 µg /g), chlorogenic acid (62.8±0.9 µg/g), and catechin (5.56±0.5 µg /g).

Euphorbia tirucalli was found to be highest in *p*-coumaric acid (97.3±0.9 µg/g), followed by chlorogenic acid (42.67±1.3 µg/g), gallic acid (11.9±1.2 µg/g) and catechin (4.59±0.9 µg /g). Ferulic acid and caffeic acid were not detected in extract of *Euphorbia tirucalli.* Gallic acid (68.0±0.8 µg /g), *p*-coumaric acid (116.74±1.2 µg/g), ferulic acid (41.17 ± 1.5 µg/g), chlorogenic (29.11 ± 0.7 µg/g), and catechin (1.68 ± 1.3 µg/g) was found in *Cyperus rotundus* rhizomes. Caffeic acid was not found in rhizomes of *Cyperus rotundus.* In comparison, *Trigonella foenum-graecum* seeds contained considerable amount of ferulic acid (104.8±1.3 µg/g) followed by *p-coumaric* acid (81.79 ± 0.9 µg /g), chlorogenic acid (41.90±1.3 µg/g), gallic acid (10.24±1.6 µg/g) and catechin (3.4±0.2 µg/g). Gemmo modified extract of *Trigonella foenum-graecum* contained gallic acid (15.79 ±0.8 µg/g), catechin (0.63±0.01 µg/g), chlorogenic acid (29.81±0.09 µg/g), caffeic acid (9.21±0.02 µg/gm), *p*- coumaric acid (70.20±0.02 µg/g) and ferulic acid (114.25±0.9 µg/g).

Discussion

Plant polyphenolics have attained remarkable attention in the present scenario as potential source of antioxidants (Katalinic *et al*., 2007, Wajdylo *et al*., 2007) because they protect human beings against heart disease, cancer, and infectious diseases (Cai *et al*., 2004; Yao *et al*., 2004; Jiangrong & Jiang, 2007; Khan *et al*., 2012). Phenolic acids are big class of compounds widely present in plants. The number of hydroxyl groups on phenol ring is actually essential part for antiradical activity. Antioxidant activity increases with an increase in number of hydroxyl groups. All types of phenolic acids have shown their potential in antioxidant activity. For examples, *p*-coumaric acid has ability to stop oxidative damage and was found to inhibit the lipid peroxidation (Niwa *et al*., 2001; Tapia *et al*., 2004). Important phenolic acids like caffeic acid (Gulçin, 2006), ferulic acid (Karamac *et al.,* 2005, Balasobashini *et al*., 2004), gallic acid and chlorogenic acid (Lan, 2007,) are also well reported antioxidants and exhibit strong pharmacological actions (Cai *et al*., 1997).

Though Many pervious reports are available for HPLC analysis of phenolic acid (Ozkan *et al*., 2006; Oslzewskai, 2007; Amber *et al*., 2012), there are very limited literature about the plants under study. HPLC analysis showed high flavonoid contents in all medicinal plants but varying in concentration. HPLC analysis revealed the presence of quercetin, myricetin and catechin in high and kaempferol in less quantity. Flavonoids are widely distributed polyphenolic compounds and acts as free radical scavengers by fast donation of hydrogen atoms to free radicals. Antioxidant activity of medicinal plant is mainly attributed to flavonoids content of medicinal plants. Antioxidant activity of flavonoids is largely depend on the molecular structure (availability of phenolic hydrogen atom) and substitution pattern of hydroxyl groups, which effects on the stability of resulting phenoxyl radical by hydrogen bond or delocalization of free electron (Amic *et al*., 2003). Quercetin, catechin and myricetin are potent free radical scavengers (Bouchet *et al*., 1998; Middleton, 2000; Levites, 2001; Silva *et al*., 2002; Materska & Perucka, 2005; Dukic *et al*., 2008).

Considerable variations have been observed in phenolic compounds of all tested medicinal plants. The *Rheum emodi* contained appreciable amount of myricetin, quercetin catechin and phenolic acids (gallic acid, *P*-coumaric acid, ferulic acid and chlorogenic acids). High polyphenolics and flavonols content are responsible for the excellent antioxidant potential of *R. emodi*. In literature antioxidant potential of different rhubarb species has been reported (Ozturk *et al*., 2007).

Cyperus rotundus rhizomes has highest amount of quercetin. Myricetin and kaempferol are also present in good quantities. It contains gallic acid, *P*-coumaric acid, ferulic acid and chlorogenic acids. *Cyperus rotundus* rhizomes have good combination of phenolic acids and flavonols, which contributes collectively to the strong antioxidant action of rhizomes. These results are in agreements with earlier reports which confirm the antioxidant activity of rhizomes of *C. rotundus* (Nagulendran *et al*., 2007; Yazanparast & Ardestani, 2007; Jahan *et al*., 2011) and may be the reason of its strong cardioprotective potential (Jahan *et al*., 2012). Gemmo modified extract of *Cyperus rotundus* showed significantly ($p<0.05$) fewer amount of total phenolics than the methanolic extract of rhizome. Quercetin and kaempferol were not detected into gemmo extract.

Euphorbia tirucalli has good quantity of antioxidant polyphenolics and demonstrated strong action towards free radicals. Myricetin was predominate flavonol in *Euphorbia tirucalli*. Phenolic acids including *p*-coumaric acid, gallic acid chlorogenic acids and catechin are identified by HPLC as major antioxidant constituents. In some previous reports plants of Euphorbiaceae family such as *Euphorbia hirta* (Sharma *et al*., 2007) and *Euphorbia tirucalli* (Jyothi *et al*., 2008) showed high phenolic contents and antioxidant activity. No significant ($p<0.05$) difference in polyphenolic contents was observed between gemmo-modified and methanolic extracts of dry plant.

Trigonella foenum-graecum is a commonly used herb having a lot of medicinally important properties (Hussain *et al*., 2011). HPLC analysis confirmed high concentration of phenolic acid in seed and gemmo-modified extracts. Myricetin was detected in high concentration but quercetin was not detected in both seed and gemmo-modified extract. Among the both extracts gemmo-modified extract demonstrated a high phenolic contents than seed extracts. Fresh geminating leaves contained high polyphenolic contents and antioxidant enzyme activity which contributes in its improved antioxidant activity compared to seed extracts (Jahan *et al*., 2012). Gemmo therapy; is the less studied research field, which is based on the use of embryonic and germinating parts of plants. The fact is that at this stage metabolic activity is at its peak and activity of enzymes and hormones is high. Some other studies have been suggested that phenolics are natural defense compound for plants. During early stage of germination when biological activity is on peak free radical are generated frequently and increased oxidation stress. A variety of secondary metabolites are synthesized due to photosynthesis, the phenolic contents are higher in young leaves (Randhir *et al*., 2004; Shahidi & Nackz 2004; Bhakta *et al*., 2009). Results of *Trigonella foenum-graecum* are also not in agreement with investigations of Wajdylo *et al*., (2007). The difference may be explained by the difference in extraction conditions, and climatic variations. These findings are in agreement with the results of Randhir *et al*., (2004) who reported higher phenolic contents and greater antioxidant activity in germinating parts. The antioxidant activity and phenolic content of *Trigonella foenum-graecum* seed has been earlier reported by Souri *et al*., (2008), but limited studies have been available on gemmo-modified extract.

Conclusion

The significant amount and good combinations of a variety of flavonols and phenolic acids that may impart their medicinal potential for alleviation of any disorder were available in the investigated plants. Gemmo-modified extract of *Trigonella foenum-graecum* could be an additional and superior source of natural antioxidants.

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