Quantification of polyphenolic compounds and flavonoids in *Achillea millefolium* and *Equisetum arvense*

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Abstract: Flavonoids have been of considerable importance and interest because of their medicinal activity. Responding to their numerous health benefits, a comparative study on the quantitative determination of total polyphenolic compounds and flavonoids was carried out in *Achillea millefolium* and *Equisetum arvense*. Total polyphenolic compounds were quantified by Folin-Ciocalteau method using different solvents in order to prove their extraction efficiency. Focus within total polyphenolic quantification study was placed on the traditional reflux and solvents used were: water, 100% acetone, 100% ethanol, 80% ethanol, 50% methanol and 70% methanol. In order to make flavonoids free from glycosidic moiety for quantification, hydrolysis was performed in 50% MeOH at 90°C using 6 M HCl concentration. Reverse phase high performance liquid chromatography (RP-HPLC) in gradient elution mode at 50°C using Hypersil BDS (RP-18) column was employed for the separation of flavonoids. Mobile phase used consisted of different combinations of water-methanol-tetrahydrofuran-phosphoric acid. Flavonoids quantified were luteolin, quercetin, apigenin, isorhamnetin and kaempferol.

Keywords: Medicinal plants, Polyphenolic compounds, Flavonoids, Hydrolysis, HPLC-PDA.

INTRODUCTION

Medicinal herbs and herbal products have been of considerable vitality for their various biologically and pharmacologically important chemicals (Qureshi *et al.*, 2012a; Qureshi *et al.*, 2011a; Qureshi *et al.*, 2013; Qureshi *et al.*, 2014a; Qureshi *et al.*, 2014b; Qureshi *et al.*, 2012b; Qureshi *et al.*, 2011b). Flavonoids are products of secondary metabolism and are polyphenolic in their chemical structure. They are found in high amount in medicinal plants, fruits, beverages and vegetables in the form of sugars conjugates mainly of glucose, rhamnose and rutinose (Herrmann, 1988) or as aglycones (Markham, 1982). A lot of research is going on in this field because of their health benefits. Flavonoids have been suggested to be called as vitamin C2 or vitamin p (Rusznyak and Szent-Györgyi, 1936). Studies revealed that they are antioxidants (Burns *et al.*, 2000; Kaneko and Baba, 1999). In vivo and epidemiological studies showed that they are associated with reduced risk of cardiovascular diseases and certain cancers (Avila *et al.*, 1994; Hertog *et al.*, 1993; Khan *et al.*, 2003; Knekt *et al.*, 1996; Piantelli *et al.*, 1995) (Cross *et al.*, 1996; Gaspar *et al.*, 1993; Qureshi *et al.*, 2014a).

Flavonoids are mainly present in the leaves and outer parts of the plants. Some times they occur in plants as aglycon, but most commonly as *O*-glycosides (Stobiecki, 2000) generally located at 3- or 7-position. In *C*-glycosides the sugar group is attached directly to the carbon atoms in ring A of the aglycon at C-6 or C-8 postion (Scalbert and Williamson, 2000; Stobiecki, 2000). More than 50 different glycosides of the most common flavonoids have been described (Hermann, 1976). The flavonoid group is sub divided into many sub groups. Flavonols, flavones, flavanols, isoflavones, flavanones and anthocyanidins are the main sub groups. They are largely planar molecules and their structural variation comes in part from the pattern of substitution: hydroxylation, methoxylation, prenylation,or glycosylation (Prasain *et al.,* 2004). Fig. 1 shows the structure of different flavonoids under study.

In flavonols, glycosylation mainly occur at 3-O and 7-O position. While 5-O glycosides are very rare for flavonoids with a carbonyl group at C-4 position; as OH group at C-5 position involve in forming a hydrogen bond with the adjacent carbonyl group at C-4 position (Prasain *et al.,* 2004). The major flavonol aglycon found are quercetin, myricetin, kaempferol and isorhamnetin while some fruits and vegetables contain flavones such as apigenin and luteolin. There is a large number of flavonol glycosides present in plants. More than 200 glycosides of quercetin alone have been described (Harborne, 1965). Flavones also occur as glycosides but the range of different glycosides is less than in the case of the flavonols as they are lacking a 3-OH group. Glycosylation in this group mainly occurs at 7-O position. Unlike flavonols, they are also present in the form of C-glycoside in which the sugar molecule is bound to C-C bond. The presence of large number of glycosidic forms limits their quantification in plants. To overcome this problem the

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samples were hydrolyzed through acid removing the glycon moieties freeing the aglycones, which were then analysed by reversed phase HPLC-PDA (Häkkinen *et al.,* 1998; Hertog *et al.,* 1992; Nuutila *et al.,* 2002). In hydrolysis the most problematic factor is the completeness of hydrolysis as identical optimum conditions cannot be achieved for all the flavonoid glycosides present in the same plant (Hertog *et al.,* 1992). Time required for the complete hydrolysis depends on the binding sites of the sugar on the flavonoid nucleus. C-glycosides are very resistant to acid hydrolysis, while O-glycosids are easily hydrolyzed. In O-glycosidic flavonoids, 7-O- glycosides need more time than 4′-O-glycosides which in turn need more time than 3-O-glycosides (Harborne, 1965). The aim of this study is the quantitative analysis of total polyphenolic compounds and flavonoids in herbs: *Achillea millefolium* and *Equisetum arvense*. The study specifically focuses on the optimization of extraction conditions for polyphenolic compounds, quantification of total polyphenolic compounds, optimization of chromatographic conditions, and quantification of flavonoids by HPLC using photodiode array detector (PDA).

MATERIALS AND METHODS

Chemicals and reagents

Methanol, acetone, ethanol absolute, THF, acetonitrile, formic acid were procured from Merck KGaA (Darmstadt, Germany). Diethyl ether, HCl (37%), Folin-Ciocalteau's phenol reagent 2N and phosphoric acid (85%) were purchased from Sigma Aldrich (Steinheim, Germany). Sodium carbonate and tetra-butylhydroxyanisole (t-BHA) were obtained from Fluka Biochemika (Buchs, Switzerland). Water purified by a Nano Pure-unit (Branstead, Boston, MA, USA) was used. All these chemicals and reagents were of analytical grade and used without further purification. Standards gallic acid (≥97%), quercetin dihydrate (min. 98%) were purchased from Sigma-Aldrich. Apigenin (≥95%), kaempferol (≥96%) and isorhamnetin (HPLC grade), luteolin (HPLC grade) were obtained from Extra Synthese (Genay, France). All plants materials were provided by Bionorica AG a phytoneering company GmbH (Neumarkt, Germany).

Extraction and determination of total polyphenolic compounds

Extraction for polyphenolic compounds by classical extractions of *Achillea millefolium* and *Equisetum arvense* in different solvents were performed. These solvents were water, 100% acetone, 100% ethanol, 80% ethanol, 50% methanol and 70% methanol.

Classical extraction

About one gram of each powdered plant material was extracted in 20ml of each solvent by refluxing for 2 hours with continuous stirring using six-place heating carousel reaction station. Extracts were allowed to cool at room temperature and then centrifuged for 10minutes at 14×1000g using eppendorf centrifuge (Eppendorf 5415 D, Hamburg, Germany). They were preserved at -20°C temperature till further work.

Preparation of Standards

Stock solution of gallic acid was prepared with a concentration of 0.25mg/ml in double distilled water. Further, working standard solutions were made for the standard calibration curve diluting stock solution with double distilled water to the desired concentrations (0.01 mg/ml -0.2mg/ml). Six calibration standards were made; each of which was analyzed thrice a time.

Folin-Ciocalteau method

Polyphenolic compounds were determined by Folin-Ciocalteau method (Singleton and Rossi, 1965) using gallic acid as the reference standard. 5ml of diluted FC reagent (1:10 FC reagent to water) were added to one milliliter of each of standards, extract and blank (water) in test tubes. These were mixed thoroughly through vortex mixing. 4ml of Na_2CO_3 solution (7.5%) were added to each mixture after 8 minutes and mixed thoroughly through vortex mixing. These test tubes were covered and stored for 2 hours at room temperature and away from strong light. Absorbance of these test solutions were read against the prepared blank at 740nm using UV-visible spectrophotometer (UV 2000, Hitachi).

Quantification of flavonoids

Preparation of calibration standards

Stock solutions of the six standards apigenin, isorhamnetin, kaempferol, luteolin, myricetin and quercetin were prepared in methanol/water (50:50, v/v) in the concentration range 0.255mg/ml -2.02mg/ml and stored at -20°C until use. Working standard solutions for calibration curve were made by diluting stock solutions with methanol/water (50:50, v/v) to the desired concentrations (apigenin 0.053mg/ml -0.315 mg/ml, isorhamnetin 0.01mg/ml -0.061mg/ml, kaempferol 0.05 mg/ml -0.302mg/ml, luteolin 0.026mg/ml -0.154mg/ml and quercetin 0.01mg/ml -0.505mg/ml). Six calibration standards were made, each of which was analyzed thrice a time.

Extraction and hydrolysis

Quantification of flavonoids aglycon in plant extracts was performed after subjected them to simultaneous extraction and hydrolysis. Simultaneous extraction and hydrolysis of the herbs was performed according to hydrolysis procedure as published by Hertog *et al*. with some changes (Hertog *et al.,* 1992). 20ml of 62.5% of aqueous methanol containing 2g/L of tert-butyl hydroxyanisole (t-BHA) was added to about one gram of powdered plant material in a round bottom flask. 5ml of 6M HCl was added and mixed carefully. The mixture was refluxed for 2 hours at 90°C with continuous stirring using six place

Table 1: Quantification of flavonoids in hydrolysed plant extracts

Name of extract	Polyphenolic Compds. (mg/g)	Luteolin (mg/ml)	Quercetin (mg/ml)	Apigenin (mg/ml)	Kaempferol (mg/ml)	Isorhamnetin (mg/ml)
Achillea millefolium	68.84	0.03831	0.01202	0.04833	0.00046	0.00117
Equisetum arvense	25.86	0.00074	0.08734	0.00476	0.01095	Not detected

heating carousel reaction station. Extracts were let to cool at room temperature and centrifuged for 10 minutes at room temperature.

Chromatographic parameters

Shimadzu HPLC (LC-10) equipped with photo diode array detector PDA (SPD-M10 Avp) was used for the quantification of flavonoids. LCMS-Solution, version 3 and LCMS-Post run, version 3-H2 software was used for online control and post run analysis. Hypersil BDS (RP-18) 125mm × 4mm column of 3µm particle size and 130Å pore size was used for chromatographic separation. Gradient elution was carried out using mobile phase A: 900ml water with 100ml methanol and 10ml phosphoric acid (85%) and B: 600ml water with 300ml tetrahydrofuran (THF), 100ml methanol and 10ml phosphoric acid (85%). Column temperature was 50°C and a flow rate of 0.5ml per minute was used. Gradient was started at 10% B and raised in 10 minutes to 60% B linearly and was hold at this concentration for 35 minutes. After that, a linear gradient to 100% B up to 53minutes was applied. The column was equilibrated at 100% B for 3 minutes and then changed to the initial conditions in 4 minutes. 50% methanol was used as washing solution for the auto injector and the sample injection loop. The whole analysis took 70 minutes. Detection was made in the wavelength range of 200 to 600nm.

RESULTS

Qualitative and quantitative assessment of flavonoids in medicinal plants were carried out focusing on optimization of extraction conditions for polyphenolic compounds, quantification of total polyphenolic compounds, optimization of chromatographic conditions, and quantification of flavonoids by HPLC-PDA. Results of total poly phenolic compounds have been given in table 1. Total polyphenolic compounds obtained in *Achilla mellifolium* was 68.84mg/g while in *Equisetium arvense* 25.86mg/g amount yielded.

Fig. 2 shows the HPLC-PDA chromatograms of individual plants obtained at 370nm. From the results (table 1) it appeared that quercetin, leutolin, apigenin and kaempferol were quantified in both the plant extracts. Isorhamnetin was only found in very small amounts in *Achillea*. In *Achillea* apigenin was high in concentration among the five flavonoids while in *Equisetum* highest amount of quercetin was obtained. Amounts (mg/ml) of leutolin, quercetin, apigenin, kaempferol and Isorhamnetin resulted in *Achillea* were 0.03831, 0.01202, 0.04833, 0.00046 and 0.00117 respectively. In *Equisetum arvense* the concentrations of leutolin, quercetin, apigenin and kaempferol were 0.00074mg/ml, 0.08734mg/ml, 0.00476mg/ml and 0.01095mg/ml respectively.

DISCUSSION

Extraction and determination of total polyphenolic compounds

To develop an extraction method that is suitable for all polyphenolic compounds or even a specific class of phenolic compounds, is a significant challenge for the researchers because of their structural diversity. Secondly, non-uniform distribution of these compounds in plants at the tissue, cellular or sub cellular level make it more complicated to achieve a satisfactory extraction procedure. Furthermore, these compounds are present in plants in free, conjugated and polymeric forms or may coexist in complex form with carbohydrates, protein or other plant components. All these factors directly affect the solubility of these compounds in various solvents. To evaluate the best suitable solvent for the extraction of polyphenolic compounds from powdered plants, six different solvents that is 100% acetone, 100% ethanol, 80% ethanol, 50% methanol and 70% methanol and water were used.

Compound	R_1	R_2	R_3	R_4	R_5
Apigenin	H	OH	H	H	OH
Isorhamnetin	OMe	OH	H	OH	OH
Luteolin	H	OH	OH	H	OH
Myricetin	OH	OH	OH	OH	OH
Quercetin	OH	OH	H	OH	OH
Kaempferol	H	OH	H	OH	OH

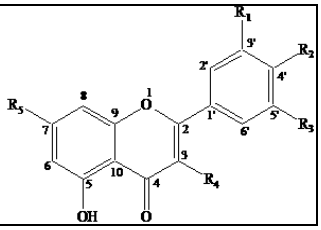

Fig. 1: Structure of different flavonoids

For the extraction of polyphenolic compounds from the plants method of refluxing was chosen. Time duration for the extraction under reflux in all media was two hours and temperature varies depending upon the boiling point of solvent. Extraction under reflux was carried out in each solvent (Hong *et al.,* 2001; Sultana *et al.,* 2008).

In order to determine the efficiency of the extracting solvents and to optimize the extracting media, Folin-Ciocalteau method for the determination of polyphenolic acid was used (Singleton and Rossi, 1965). Gallic acid was used as standard and six calibration standards of the concentrations 0.01mg/ml 0.2mg/ml were made in water. Absorbance was measured at wavelength maximum 740nm. A six-point calibration curve with R^2 value of

0.9975 was obtained. Results showed that *Achillea* and *Equisetum* gave better yield in 50% methanol using traditional reflux extraction.

Quantification of flavonoids

Because of the presence of large number of flavonoid glycosides, quantification of individual flavonoid glycosides are difficult. Additionally the availability and the cost of standards for these glycosidic forms of flavonoids also make it difficult to quantify these in complex plant extracts. These points make a strong base for the need of hydrolysis to make the aglycon moiety free from glycosidic part.

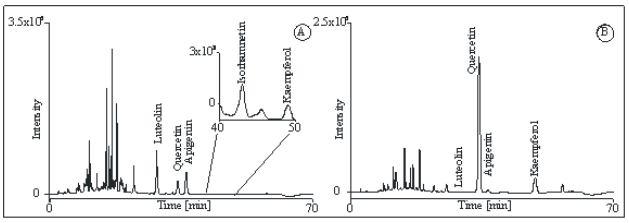

Fig. 2: HPLC-PDA chromatograms for quantification of flavonoids in hydrolysed plant extracts, A: *Achillea millefolium*, B: *Equisetum arvense*. Chromatographic conditions: Mobile phase A: 900ml H_2O + 100ml MeOH +10ml H_3PO_4 85% and B: 600ml H_2O + 300ml THF + 100ml MeOH + 10ml H_3PO_4 85%, Inj. Vol: 10µL, Flow rate: 0.5ml/min, Column Temp: 50°C.

Extraction and hydrolysis was performed simultaneously for each plant material. As flavonoid glycosides are more soluble in water and flavonoid aglycon in methanol (Hennig, 1980). Therefore, efficiency of the extraction depends on the water/methanol ratio. We used 50% methanol and the antioxidant tertiary butyl hydroxyanisole (t-BHA). Concentration of the HCl in the final extracting media was 1.2M.

HPLC-PDA analysis for quantification of flavonoids

RP-HPLC with photo diode detector (PDA) was used for the separation of the flavonoids aglycon. Preliminary separations of the flavonoids were performed using water and acetonitrile with 0.05% formic acid with different gradient timing. Prontosil C-18 column with dimensions 250mm × 4.6 mm and 5µm particle size was used. However, this did not enable base line separations between the flavonoids. Especially resolution between the two pairs that is the quercetin-luteolin and apigenin-kaempferol was not acceptable because of their similar polarity.

Because of these disadvantages, a water methanol tetrahydrofurane with phosphoric acid as modifier was used as mobile phase as established by Stecher *et al* (Stecher *et al.,* 2001). Hypersil BDS (RP-18) column with dimensions 125mm × 4mm with particle size 3µm (pore size 130 **Å**), column temperature of 50°C and a flow rate of 500µL/min was used. This system gave the best base line separation as well as peak purity, peak shape and minimum peak tailing. The peak tailing depends upon the use of different modifiers. When formic acid was used instead of phosphoric acid, the peak tailing increased. The order of elution was from most polar luteolin to least polar kaempferol that is luteolin, quercetin, apigenin, isorhamnetin and kaempferol respectively.

Wavelength maxima for all the analytes investigated were determined as 351nm, 373nm, 340nm, 373nm and 368nm of luteolin, quercetin, apigenin, isorhamnetin and kaempferol respectively.

Quantification of the four flavonoids quercetin, isorhamnetin and kaempferol were made by measuring their absorbance in plant extracts at 370nm, for luteolin at 350nm and for apigenin at 340nm. Six points calibration curve for each standard were made and the R^2 values obtained were 0.9946, 0.9995, 0.9981, 0.9894, 0.9981 for luteolin, quercetin, apigenin, isorhamnetin and kaempferol respectively.

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