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HPLC-DAD ANALYSIS AND FREE RADICAL SCAVENGING POTENTIAL OF *QUERCUS DILATATA* L.

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Abstract

Free radicals are molecules or atoms that have at least one unpaired electron which increases the chemical reactivity of the molecule. The main objectives of the present study was to evaluate the antioxidant potential and HPLC screening of antioxidant compounds (rutin, quercetin and gallic acid) from *Quercus dilatata* L. The antioxidant activity of *Q. dilatata L.* extracts/fractions was determined by using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay. Four partitioned fractions of *Q. dilatata* (n-hexane, ethyl acetate, n-butanol and aqueous) were prepared among which ethyl acetate fraction showed highest activity (IC_{50} 38.02 µg/ml). Furthermore, the screening of rutin, quercetin and gallic acid in the partitioned fractions was done by HPLC-DAD which showed that the most active fraction i.e. ethyl acetate fraction contains all of them while aqueous fraction showed the presence of two i.e., rutin and gallic acid. Butanol fraction showed only rutin content, while n-hexane fraction did not show the presence of any of the above mentioned compounds. Thus it can be concluded that good antioxidant potentials may be due to the presence of these well known antioxidant compounds in *Q. dilatata* in association with other unidentified compounds.

Introduction

It has been estimated that one human cell faces approximately 10,000 oxidative attacks per day from $^{\bullet}OH$ (oxygen free radicals) and other such species (Halliwell & Aruoma, 1991; Dreher & Junod, 1996; Jaruga & Dizdarogluo, 1996; Wang *et al.,* 1998; Marnett, 2000; Dizdarogluo *et al.,* 2002). These agents produce eternal alteration of genetic material by oxidative damage (Fig. 1), which is considered the initial step towards mutagenesis responsible for carcinogenesis (Halliwell & Gutteridge, 1989; Valko *et al.,* 2004).

DNA modification done by free radicals is corrected by specific and non-specific repair mechanisms, which are thought to occur mainly by base excision (Wallace, 1998, Valko *et al.,* 2004). However, escape or failure to complete patch up of DNA damage could result in mutations such as base replacement and deletion (Dreher & Junod, 1996; Marnett, 2000). The chance of mutation is directly proportional to the number of escaped DNA lesions or abrasions produced by oxidative damage. It is well-known that repair mechanisms become weaker with age and thus DNA abrasions are gathered with age and the chance of mutagenesis and cancer development is increased many fold (Haq *et al*., 2012).

To protect the cells against the free radicals attack, the search for new free radicals scavenger from the natural sources is exceedingly required. Among living organisms, the use of plants as medicine is vital part of history and represents most considerable direct ancestor to modern medicine. In recent times, some of the most inspiring clinical proof of the value of the herbs in treating cancer allows us to rebuild the story of these plants and their ultimate uses in these cases (Nobili *et al.,* 2009, Abbassi *et al*., 2011). In the past, the specific plant to be used and the method of application for particular ailment were passed down through oral history. Later on, information regarding medicinal plants was recorded in herbals (Balunas & Kinghorn, 2005). These plants having ethnopharmacological uses have been the primary source of early drug discovery and chronological experiences with these plants as curative tools have helped to isolate and develop single chemical entities in modern medicine (Fabricant & Fransworth, 2001).

Quercus dilatata (Fagaceae) is commonly known as holly oak. Local name is Barungi, Mom. Synonym is *Quercus floribunda* Lindl. Camus. The Fagaceae (beech family) is the small family of monoecious trees and shrubs comprising 6-8 genera and about 800 species (Shah *et al.,* 2005) which are monoecious, evergreen or deciduous trees, rarely shrubs. Over 400 species of *Quercus* are distributed in America, temperate Europe, Asia and sub-tropical Africa. In Pakistan this evergreen tree is found in the Himaliyas mountains specifically in Dir, Chitral, Swat, Hazara, Tirah, Kurram Agency, Murree hills and Azad Kashmir (Nasir & Ali, 1972). Different species of *Quercus* genus have many medicinal properties like, anti-inflammatory, antifungal, antibacterial, and anti tumor properties but this paper provides the first report about antioxidant activity of *Quercus dilatata* and identification of three very important natural antioxidants compounds i.e. rutin, quercetin and gallic acid in it by using HPLC/DAD.

Material and Methods

The chromatographic system consisted of Agilent Chem station Rev.B.02-01-SR1(260), Agilent 1200 series binary gradient pump coupled with diode array detector (DAD; Agilent technologies, Germany), Discovery-C18 analytical column (4.6 x 250mm, 5µm particle size, Supelco, USA). PDA Spectrophotometer (8354 Agilent technologies, Germany) Rotary evaporator (Buchi, Switzerland), incubator IC83 (Yomato, Japan). Transparent 96-well plates, micro plate reader (Biotek, USA), incubator.

Reagents and chemicals: All the solvents i.e. methanol, acetonitrile, acetic acid, dimethylsulfoxide (DMSO) and water were of HPLC grade were purchased from Sigma (Sigma-Aldrich, Germany). All the Chemicals were analytical grade i.e., rutin, quercetin, gallic acid and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were also purchased from Sigma.

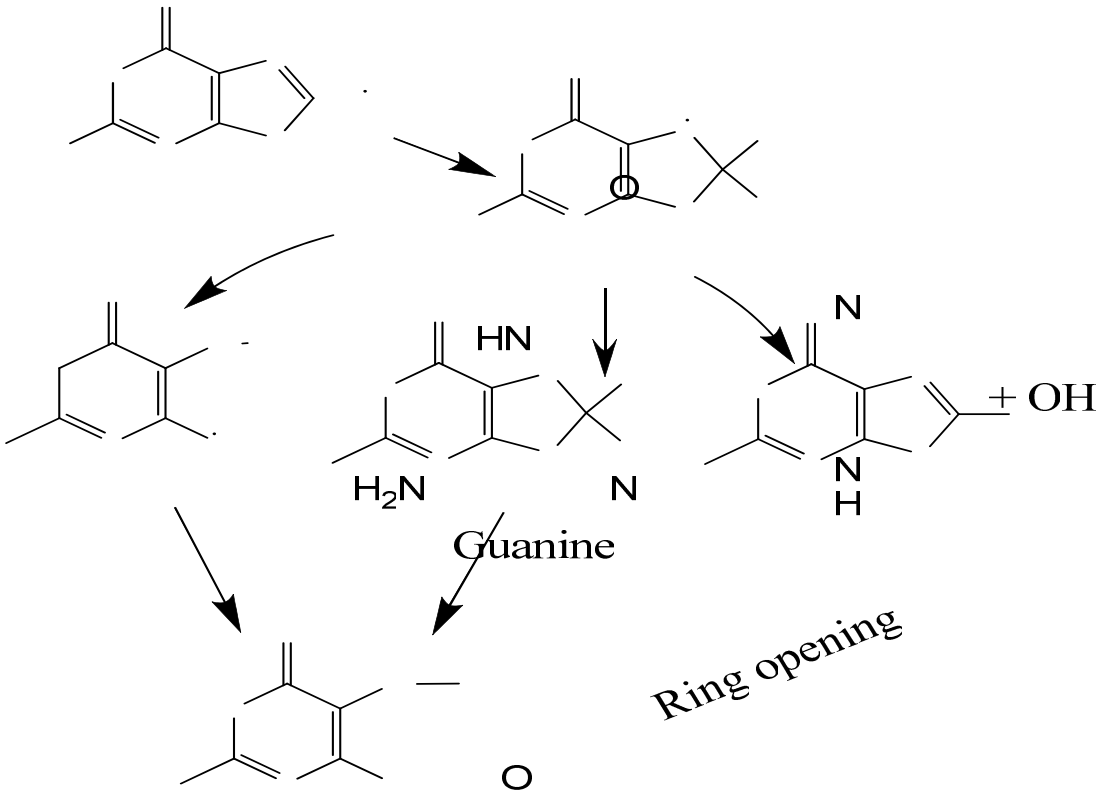

Fig. 1. Attacking pattern of hydroxyl free radical (•OH) on the C8 position of guanine as an example of conversion of a purine base into pyrrimidine (Valko *et al*., 2004).

Plant material: Aerial part of *Quercus dilatata* (11.00 kg) dry weight was collected in September, 2010 from Murree hills, Pakistan. The plant species was identified by Dr. Rizwana Aleem Qureshi (Taxonomist), Department of Plant Sciences, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan and a voucher specimen numbering HMP-460 was deposited in Herbarium of Medicinal Plants of Pakistan, Quaid-i-azam University, Islamabad 45320, Pakistan.

Extraction and fractionation: Fresh plant material was taken and kept under shade till drying. Crude plant extract was prepared by maceration procedure. Plant material was macerated in methanol and chloroform mixture (1:1) for 1 week at room temperature and was shaken well every twelve hourly. It was then filtered, concentrated and an aqueous suspension of crude extract was partitioned with n-hexane, ethyl acetate and n-butanol. These were then dried in rotary evaporator to yield n-hexane, ethyl acetate, n-butanol and aqueous fractions.

DPPH Free radical scavenging assay: Crude extract and all its fractions were subjected to DPPH free radical scavenging assay. DPPH free radical scavenging activity was determined according to the method reported by Co *et al.,* (2012) with some modifications. Gallic acid was used as a positive control. DPPH solution (316 µM) was made in methanol. Test samples solution, 20.0 mg/ml in 100% DMSO, was prepared and 5 µl the test solution was added in respective well of 96-well plate. DPPH solution (95 µl) was added in each well and mix thoroughly to get the final test concentration of 1000 µg/ml. It was incubate at 37°C for 1 h. Absorbance was measured at 515 nm using micro plate reader. Each fraction which showed more than 50% inhibition at 1000 µg/ml was tested at lower concentrations i.e. 500, 250, 125, 62.5, 31.25 and 15.62 µg/ml to find its IC_{50}. Pure DMSO was used as negative control. The test was performed in triplicate. Percentage scavenging was measured according to following formula and IC_{50} value was calculated by Table Curve 2D 4v (AISN software).

$$\% \text{ scavenging} = [(Ac - As)/Ac] * 100$$

Where Ac = Mean absorbance of negative control, As = Mean absorbance of test sample

HPLC-DAD Analysis

Preparation of Standard solution for HPLC analysis: Stock solutions of all three standards were prepared in methanol, at concentration of 1 mg/ml and then were further diluted with methanol to get 10, 20, 50, 100, 150 and 200 µg/ml for the preparation of calibration curve. All the solutions were filtered through 0.2 µm Sartolon Polyamide membrane filter (Sartorius).

Preparation of sample solution for HPLC analysis: Samples for HPLC analysis were prepared at concentration of 10 mg/ml in methanol. Samples were dissolved in methanol with the aid of sonication and were filtered through 0.2 µm Sartolon Polyamide membrane filter (Sartorius). All the samples were prepared freshly and used for analysis at HPLC.

Chromatographic conditions: HPLC analysis was carried out by using the chromatographic system consisted of Agilent Chem station Rev.B.02-01-SR1(260) software and Agilent 1200 series binary gradient pump coupled with diode array detector (DAD; Agilent technologies, Germany) having Discovery-C18 analytical column (4.6 x 250mm, 5µm particle size, Supelco, USA). Method was followed as described by Zu *et al*., 2006 with slight modification according to the system suitability. Briefly mobile phase A was methanol-acetonitrile-water-aectic acid (10:5:85:1) and mobile phase B was methanol-acetonitrile-acetic acid (60:40:1). A gradient of time 0-20 min 0% B-50% B, 20-25 min 50% B-100% B and than isocratic 100% B till 30 min was used. Flow rate was 1 ml/min and injection volume was 20 µl. Rutin and gallic acid were analyzed at 254 nm and quercetin was analyzed at 368nm. Every time column was preconditioned for 10 min before the next run.

Results and Discussion

Complex series of cellular and molecular changes contributing in cancer development are intervened by a diversity of endogenous and exogenous stimuli. One type of endogenous damage arises from reactive oxygen species (ROS), also called as oxygen free radicals (OFR), which is product of normal cellular metabolism. These OFR attack not only nucleic acid bases but also deoxyribosyl backbone of DNA (Valko *et al*., 2004). Endogenous DNA lesions are genotoxic and induce mutations. The most comprehensively studied lesion is the formation of 8-hydroxy aduct radical of guanine (Fig. 1) and its subsequent products (Valko *et al*., 2004). This lesion is imperative because it is comparatively easily formed and is mutagenic and therefore is a potential biomarker for carcinogenesis. Mutation by this lesion involves GC→TA transversion. Therefore OFR are considered as an important class of carcinogens (Valko *et al*., 2004). Non enzymatic and enzymatic antioxidants play their role to balance the effect of OFR. Antioxidants can either directly scavenge or prevent generation of OFR/ROS. The discovery of novel and safer antioxidants from natural products to combat and/or prevent OFR/ROS mediated diseases is a continuous process. For the measurement of potential free radical scavenging activity of the plants extracts/fractions, DPPH free radical scavenging assay was utilized in this study. DPPH is stable nitrogen centered free radical that can accept an electron or hydrogen radical to become a stable diamagnetic molecule. DPPH radicals react with suitable reducing agents, and then losing color stoichometrically with the number of electrons consumed, which is measured spectrophotometrically at 517 nm (Haq *et al.,* 2012). The DPPH test is a non-enzymatic method currently used which provides very accurate information for the identification of potential free radical scavengers. In the present study crude extract and all the partitioned fractions of *Q. dilatata* exhibited significant DPPH radical scavenging activity except n-hexane fraction which showed lowest antioxidant activity (IC_{50} 1000 µg/ml). Ethyl acetate fraction showed maximum antioxidant activity with IC_{50} 38.02 µg/ml, while n-butanol fraction showed second highest activity with IC_{50} 53.87 µg/ml followed by aqueous fraction having IC_{50} 57.27 µg/ml. Crude extract itself also showed significant antioxidant potential with IC_{50} 200.6 µg/ml (Table 1). The purpose of the fractionation was to estimate the distribution of the antioxidant compounds on the basis of polarity and portioning coefficient among different groups. So the significant antioxidant activity of different fractions of *the Q. dilatata* shows that this plant can be a good source of different antioxidant compounds which are distributed in the wide range of polarity.

Table 1. Percentage scavenging and IC_{50} values of crude extract of *Q.dilatata* and its fractions.

Sr.No.	Samples	% scavenging at (µg/mL)							IC_{50} (µg/mL)
		15.25	31.25	62.50	125	250	500	1000	
1.	n-Hexane	1.91±1.5	3.45±1.9	7.60±1	23.80±0.8	35.76±0.9	25.65±1.1	19.89±2.00	>1000
2.	Ethyl acetate	25±0.3	45.37±0.8	65.6±0.5	71.60±0.6	78.17±1.1	95±0.1	96±0.2	38.02
3.	Aqueous	12±1.4	25±1.6	52±1.9	79±1.6	85±0.9	92.5±0.6	90±1.3	57.27
4.	n-Butanol	15±1.00	28±1.8	55±1.1	78±0.6	83±0.3	90±0.9	92.14±1.2	53.87
5.	Crude extract	11±0.9	23±0.4	29±1.1	30±1.9	58±1.3	65±0.7	75±0.4	200.6

± = Standard error

IC_{50} = Concentration at which 50% inhibition obtained

Gallic acid was used as positive control and its IC_{50} value was 3.2µg/mL

Reversed phase HPLC with C18 column is the most popular technique for the analysis of flavonoids and phenols in different plants and it is also used to distinguish the different species of plants on the basis of variation in flavonoids and phenols contents (Kerhoas *et al*., 2006). The present modified method is simple and easy to use and effective enough for the identification and quantification of rutin, quercetin and gallic acid compounds in plants extracts. Standards were selected on the basis of their medicinal properties mentioned in literature; Rutin and quercetin act as antiangiogenesis (Igura *et al*., 1997), anti-inflammatory, antitumour (Calabro *et al*.,2005), gallic acid act as antioxidant and free radical scavenger (Yilmaz & Toledo, 2004). In the present investigation the partitioned solvent fractions (n-hexane, ethyl acetate, n-butanol and aqueous fractions) were analyzed for the presence of rutin, quercitin and gallic acid by comparing their absorption spectra on C-8

RP-HPLC system with that of the standard (Fig. 2). Rutin and gallic acid were analyzed and quantified at 254nm while, quercetin was quantified at 368 nm using peak area and calibration curve. The HPLC-DAD chromatograms showed that ethyl acetate fraction contain all of these compounds in it (Fig. 2A), n-butanol fraction contained only rutin (Fig. 2B), aqueous fraction contain both the rutin and gallic acid (Fig. 2C), while n-hexane fraction did not obtained any of them during fractionation.

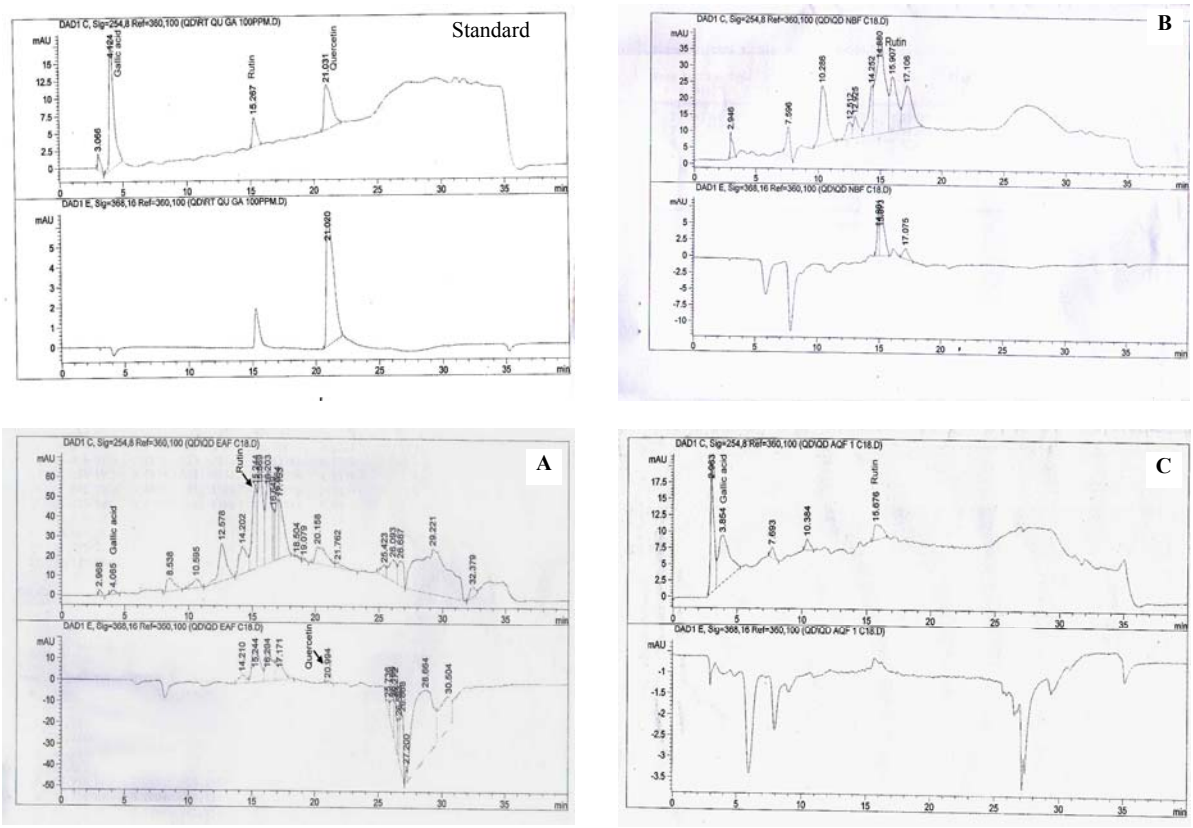

Fig. 2. HPLC-DAD chromatograms indicating the retention time and peak area of Gallic acid, Rutin and Quercetin: Chromatogram in the background = standards, A = Ethyl acetate fraction (EAF), B = n-butanol fraction (NBF) and C = Aqueous fraction (AQF).

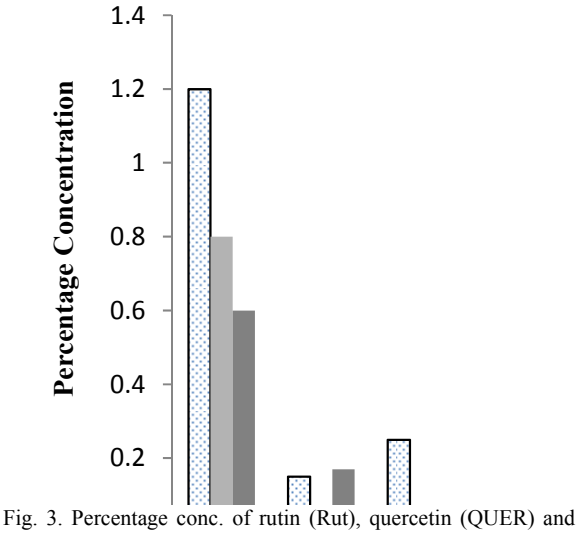

Fig. 3. Percentage conc. of rutin (Rut), quercetin (QUER) and gallic acid (GA)(g/100g) in ethyl acetate (QDE), aqueous(QDA), n- butanol (QDB), n-hexane fractions (QDN) and crude extract (QDC) of *Quercus dilatata*.

In the crude extract the percentage of the rutin, quercetin and gallic acid is calculated as 0.25%, 0.12% and 0.11% respectively (Fig. 3). Among the fractions, ethyl acetate fraction showed the highest amount of rutin, quercetin and gallic acid i.e., 1.2%, 0.8% and 0.6% respectively. The others fractions, aqueous fractions and n-butanol fractions had rutin 0.15% and 0.25% and aqueous fraction also has gallic acid 0.17%. In hexane fractions none of the compound was detected. In this study, a direct correlation between antioxidant potential (lower IC_{50}) and the amount of rutin, quercetin (flavonoids) and gallic acid (phenol) is found in the extracts/fractions. Other studies have revealed that phenols and flavonoid play vital role in antioxidation as well as biological function of plants (HO *et al*., 2003) and the antioxidative potential of these flavonoids and phenols may help to prevent diseases (Burns *et al*., 2000). Therefore, the results suggest that *Q. dilatata* possesses compounds with the antioxidant properties like rutin, quercetin and gallic acid which could be isolated and then used as antioxidants for the prevention and treatment of free radical induced disorders. Previously, antioxidant activity of Quercus genus was reported (Al Mustafa &

Thunibat, 2008; Chevolleau, 1992; McCune & Johns, 2002) and gallic acid, rutin and quercitin were isolated from genus Quercus, yet this is the first report of identification of antioxidant compounds i.e., gallic acid, rutin and quercetin from *Q. dilatata.* This study also provides a new scientific investigation that *Q. dilatata* can be good candidate for the activity guided isolation of other antioxidant compounds which are distributed in different fractions and have wide range of polarity.

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