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Flavonoids in *Rhizophoraceae* mangroves from southwest coast of India

Biochemistry

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ABSTRACT

The observed levels of flavonoid contents confirm the importance of these mangrove plants as excellent sources of plant antioxidants. This study suggests B. gymnorrizha can be a very good source of myricetin. The leaves of K. candel were found to be a rich source of quercetin than the other species under investigation followed by R.mucronata leaves. The presence of luteolin in B. cylindrica alone as well as the variations in different flavonols and flavones in these plants can be helpful for providing chemotaxonomic relationships between different genera of this family. The data presented in this paper offer possible avenues for horticultural approaches towards health promotion by identifying and selecting varieties rich in flavonoids.

KEYWORDS:

Mangroves, Rhizophoraceae, Chemotaxonomy

Introduction

Flavonoids are widely distributed in plant foods and therefore important constituent of human as well as animal food. Their concentration varies from plant to plant or even in different organs of the same plant and geographical location (Anderson and Markham, 2010; Dinelli et al., 2006; Justesen and Knethsen, 2001). Many plants are considered to be excellent sources of flavonoids that could be used, not only to preserve foods, but also to contribute to a healthy diet (Justesen and Knethsen, 2001). Dietary flavonoids are considered to be even more powerful antioxidants than vitamins C and E (Sokol-Letowska et al.,2007).

Food derived flavonoids, especially; flavonols (kaempeferol, quercetin and myricetin) and flavones(apigenin and luteolin) are of particular importance and are reported to exhibit multiple biological functions such as anti-allergenic, anti-artherogenic, anti-inflammatory, antimicrobial, anti-thrombotic, anti-oxidant, cardioprotective and vasodilatory effects (Shahidi et al., 1992; Manach et al., 2005) and have significant vitamin C sparing activity, with myricetin being one of the most active (Miean and Mohamed, 2001). Vegetables, fruits, and beverages are the main dietary sources of these flavonols and flavones (Hertog et al., 1992, 1993).

Although a number of studies on the flavonol and flavone contents of plant sources have been reported from different countries, the compositional data are still insufficient, which necessitates the need to investigate more and more materials for the search of credible and beneficial natural antioxidants as well as to aid the chemical characterisation the plants. There is gap of information regarding the flavonols and flavone contents of mangrove plants. The mangrove plants of the family *Rhizophoraceae* show comparatively higher antioxidant capacity which can be attributed to their higher phenolic content (Arivuselvan et al., 2011; Agooramoorthy et al., 2008). Also, they are source of potent antiviral substances (Premanathan et al., 1999). This chapter is an effort to quantify and qualify five common antioxidant food flavonoids; three major flavonols (kaempferol, quercetin, and myricetin) and two major flavones (luteolin and apigenin) in the leaves and bark of five mangrove plants; *B. cylindrica, B. gymnorrizha, K. candel, R.mucronata and R. apiculata* belonging to *Rhizophoraceae* family using High performance liquid chromatography. All the strategic plant materials have not yet been investigated and quantified for the specific flavonols (kaempeferol, quercetin, myricetin) and flavones (apigenin and luteolin). So, the present work would be informative and novel with regard to the quantification of these specific flavonoids and plant materials. Such study is valuable for researchers in providing a base line data for future detailed characterisation of other phenolics in these and related plants. The total flavonoids from these plants were also assayed.

Materials and Methods

Leaves and bark of five mangrove plants belonging to the Rhizophoraceae family of true mangroves; *Bruguiera cylindrica, B. gymnorrizha, Kandelia candel, Rhizophora apiculata* and R. *mucronata* were collected from three locations in and around Kochi and transported to the laboratory. All the plants were identified by Dr. Khaleel K.M.(Former Director, School of Enviromental Studies, Kannur University), Principal, Sir Sayeed College, Kannur. Voucher specimens (BCP8/2011, BGP8/2011, KCV8/2011, RAA8/2011, RMPP8/2011) were kept in at Inter University Centre for Marine Biotechnology, Cochin University of Science and Technology. The collected leaves and barks of the mangrove plants were washed with water and dried in an incubator at 40°C. Dried samples were ground to produce fine homogenous powders using an electric blender.

The total phenolic content (TPC) of the dried plant parts were determined with the Folin-Ciocalteau assay (Atanassova et al. 2011). Colorimetric aluminum chloride method was used for flavonoid determination (Ghasemi et al. 2009).

The quantitative and qualitative analysis of five food flavonoids, myricetin, quercetin, kaempferol, luteolin and apigenin was carried out using LCUV-MS (Hertog et al., 1992; Chua et al., 2011).

Quantification of flavonoids was performed on a Shimadzu Prominent Liquid Chromatograph. Chromatographic separations were performed on a Supelco C-18 (25cm x 4.6mm, 5μm) column under ambient temperature. The HPLC system was of connected to LC20AD pump. The peaks were detected using the UV-Visible detector SPD-20A at 370nm. The working solutions were injected onto the column which was previously equilibrated with eluent for 60 minutes. The mobile phase consisting of methanol and 0.1% formic acid in low pressure gradient mode (0-5 min, 50% B; 5-11 min, 50-45%B; 11-25 min, 45% B; 25-26 min, 45-50%B, 26-40 min 50% B) with a flow rate of 0.4ml min-1 was used.

Liquid chromatography (LC)- Mass spectrometry (MS) analysis was performed with Shimadzu LCMS 2020 connected to Shimadzu Prominent Liquid Chromatograph equipped with ESI source (nitrogen gas flow rate 2.5 l min-1). The mass spectra were acquired from m/z 200–700. All mass spectrometric data were acquired in negative ionisation mode. The detector voltage was maintained at 1.15KV with an interface temperature of 3500C. The scan speed was 3000 µs-1. Data acquisition and data processing were performed using the software provided by the manufacturer. The scan mode of enhanced mass spectra (EMS) was used to screen the sample profile. Single ion mode was used to determine the characteristic ions and to confirm the presence of agylcon peaks.

The results of reverse phase HPLC chromatogram with detecting at UV- 370 nm showed the released agylcons, namely myricetin, quercetin, luteolin, kaempferol and apigenin in the aqueous methanol mangrove leaves and bark extracts after hydrolysis. The retention time of the agylcons under investigation in the hydrolysed mangrove extracts were identical those of their corresponding standards under the same conditions. Calibration plots of peak area against concentration for all analytes were obtained by linear regression analysis with an average of at least three data points per concentration (five concentration points). Peak identity of the samples was confirmed by comparing the spectrum of each peak with the corresponding standard spectrum and the m/z values. Peaks were only quantified if they matched the above mentioned criteria. Quantification was based on peak area.

Results and discussion

Total flavonoid content (TFC) as determined by Aluminium chloride assay, is reported as quercetin equivalents per gram of dry weight of the sample (mg QE g^{-1}) (Fig I). The highest value for TFC was found to be in the leaves of *K. candel* (10.68±0.27 mg QE g^{-1}) whereas the lowest value was observed for *R.apiculata* bark (2.06±0.03 mg QE g-1). Also *R.apiculata* leaves exhibited lowest TFC (5.51±0.35) among the leaves samples under investigation.

The total flavonoids are found to be more concentrated in the leaves than in the bark. In plants, flavonoids play significant the role in photoprotection , UV protection and to manage heat stress as well as water stress (Anderson and Markham 2006 to be changed to Anderson and Markham 2010). Marinova et al., 2005 has reported that in some of the vegetables such as spring onion, leeks and beans the green parts showed higher phenolic as well as flavonoid content which has been credited to the fact that change in the colouring of the plant is a process associated with the redistribution of phenolics and flavonoids. So the more accumulation of flavonoids in the leaves of mangroves of this study can be attributed to the more exposure of the leaves to solar radiation than the bark.

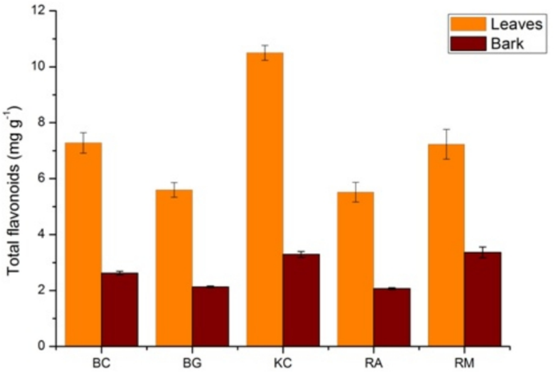

Fig I Variation of total flavonoids in leaves and bark of Rhizophoraceae mangroves

The flavonoid aglycons in the mangrove leaves and bark extracts on dry weight basis are presented in Table I.

Table 1 Results from quantitative and qualitative analysis of flavonoid agylcons (in mg kg^{-1}) of dry weight) from the leaves and bark of *Rhizophoraceae* mangroves.

Plant name	Plant part	Myricetin	Quercetin	Luteolin	Kaempferol	Apigenin
B. cylindrica	Leaves	29±0.78	93.7±2.03	30.5±.89	56±1.09	ND
	Bark	4.8±0.10	13.96±0.02	ND	9±0.14	ND
B. gymnorrizha	Leaves	803±19.09	404±12.12	ND	28±0.84	ND
	Bark	219±5.57	11±0.33	ND	16±0.48	ND
K. candel	Leaves	192±3.76	3570±107.1	ND	1463±39.89	1264±27.92
	Bark	ND	182±5.46	ND	26±0.56	116±3.09
R. apiculata	Leaves	ND	1079±32.37	ND	166±3.98	358±8.74
	Bark	77±1.31	23.7±0.71	ND	ND	ND
R.mucronata	Leaves	59.8±1.59	1960±58.8	ND	585±14.55	ND
	Bark	66±0.2	237±7.11	ND	26±0.49	ND

ND: Not detectable

Flavonoid distribution in the plants reveals its prospects in bioactivity studies. The contents of flavonols, especially quercetin derivates, appear to increase with increased light irradiation, whereas temperature has a less prominent effect on the total flavonol content. But under the same climatic regime, the variations in the flavonoid content can be considered as the genetic nature of the plants (Jakkola and Hohtola, 2010).

Flavonoid distribution in the plants

It is evident from the results that the flavonoid agylcons are more concentrated in the leaves of the mangroves than in the bark. The TFC values of leaves and bark also supports this observation. As the formation of flavonoids is light-dependent, flavonoids occur predominantly in the leaves, and growing plants in glass houses reduces the flavonoid content (Lugasi et al., 2003). The bark of mangroves are reported to be rich sources of tannin (Nurulhuda et al., 1990). So it can be inferred that the bark of mangrove plants are rich in phenolics other than flavonoids. Jaakola et al., 2004 examined the activation of flavonoid biosynthesis by solar radiation in bilberry (Vaccinium myrtillus) leaves. The flavonol quercetin, various anthocyanins glycosides, and the hydroxycinnamic acids were shown collectively to play a predominant role in the defense against high solar radiation; all of these compounds increased markedly with greater exposure to sunlight. In red onions, higher concentrations of quercetin occur in the outermost rings (Smith et al., 2003). So, the increased levels of flavonoids observed in the leaves of mangroves than their barks may be attributed to the increased exposure of the leaves to solar radiation than the barks.

Among the plants of this study, *B. cylindrica* is the species found to possess the least concentration of the food flavonoids. Apigenin was not detected in *B. cylindrica* but this is the only plant of this study in which luteolin is present. The dominance of flavonols over flavones is a noticeable feature of *B. cylindrica*. Various combinations of flavones and flavonols have been shown to exhibit synergism. Kaempferol and luteolin show synergistic effect against herpes simplex virus (HSV) (Amoros et al., 1992). The presence of luteolin along with kaempferol in the leaves of *B. cylindrica* suggests the possibility of such a synergism in its extracts which can impart bioactive properties.

Only the flavonols were found to be present in *B. gymnorrizha*. It is found to be the species having highest myricetin concentration among the plants analysed. The myricetin content in the leaves and bark of *B. gymnorrizha* was found to exceed the quercetin content of these tissues. *B. gymnorrhizha* possessing appreciably high concentration of myricetin than other dietary sources; fruits and vegetables (Lugasi et al., 2003), can be derived as a reliable source of this compound. The bark of *B. gymnorrizha* was found to be the flavonoid rich bark in the present study.

K. candel is the richest source of antioxidant food flavonoids in this study with the highest percentage of the flavonoid agylcons studied. This can be related to the difference in stress responsive mechanism among the plants. The highest levels of quercetin, kaempferol and apigenin express the activation of the oxidative stress as well as the UV protection mechanism in this plant as dihydroxy B ring substituted flavonoids (flavonols) have a greater antioxidant capacity, while their monohydroxy B ring substituted counterparts (apigenin) have greater ability to absorb UV-wavelengths (Kumar and Pandey, 2013). So the extract from this plant may have greater antioxidant capacity as well as high UV- absorbing property. It has also been reported that the flavonoids chrysin, acacetin, and apigenin prevent HIV-1 activation via a mechanism that probably involves inhibition of viral transcription (Critchfield et al., 1996). Also, it has been reported that consumption of onions and/or apples, two major sources of the flavonol quercetin, is inversely associated with the incidence of cancer of the prostate, lung, stomach, and breast. So, the higher contents of these flavonoids in *K. candel* imply its prospective role in prevention of these diseases.

The leaves of the mangrove plant, *R. apiculata* are found to possess a high flavonol content dominated by quercetin and kaempferol while the bark showed the only the presence of myricetin and quercetin. The presence of apigenin is an important feature of this plant. The accumulation of myricetin only in the bark makes *R. apiculata* distinct from other plants of the study. Warm aqueous extract of the bark of *R. apiculata* is used as astringent for diarrhoea, nausea, and vomiting, and as an antiseptic. The extract is also used to stop bleeding in fresh wounds and for the treatment of chronic typhoid fever. So it can be inferred that the potential bioactive character of the bark of *R. apiculata* may have a positive influence of the two flavonols, myricetin and quercetin. *R.mucronata* is a flavonol rich mangrove plant. It exhibited a qualitatively similar flavonoid profile as that of B. gymnorrizha but possess higher concentrations. R.mucronata bark was found to be rich in myricetin content than its leaves. When compared with the other plants the bark of this plant has the highest antioxidant flavonoid content.

Quercetin is the flavonoid found to be present in all the mangroves. Among leaf samples, the leaves of *B. cylindrica* contain lowest quercetin content. The bark of B. gymnorrizha is bark sample having lowest quercetin content. Evidence obtained with an in vitro oxidation model for heart disease has demonstrated that several plant flavonols, such as quercetin, myricetin, and rutin are more powerful antioxidants than the traditional vitamins (Vinson et al., 1995). The flavonol, kaempferol showed its presence in all the Rhizophoraceae mangroves under investigation. It showed highest value in *K. candel* while it was absent in the bark of *R. apiculata*. Quercetin and kaempferol are proved to be the most widespread flavonoids in vegetables and herbs (Lugasi et al., 2003, Justesen and Knuthsen, 2001). The results of the present study are also in favour of this observation. Apigenin is the major flavonoid found in honey and Urtica sp. a herb used frequently for traditional cancer treatment among Turkish people (Karakaya and Nehir, 1998). The presence of apigenin in mangroves *K. candel* and *R. apiculata* indicate the prospects of these mangroves in development of novel therapeutic agents based this compound.

The health influences of flavonoids have yet to be fully established although they have been shown to function in a way similar to antioxidant vitamins and to protect against lipoprotein oxidation in vitro and to have anti-platelet anti-thrombotic actions (Crozier et al., 1997). There are, therefore, grounds for investigating natural sources rich in flavonoids.

Inter-specific variations of flavonoids and their chemotaxon omic significance

Inter-specific variations of flavonoids are significant in chemo taxonomic studies. The variation of the three flavonols myricetin, quercetin and kaempferol among the plant species is shown in Fig II. The flavonol concentration dominates the flavones in the mangrove plant parts. Quercetin is the flavonoid found to be present in greater concentration in the plant tissues. In this study, the genus bruguiera showed low levels of quercetin when compared with the other two; Kandelia and Rhizophora. Next to *K. candel*, rhizophora mangroves possess higher quercetin concentration. Bruguiera plants showed lower quercetin content than the other plants. Previous studies also report quercetin as a major flavonoid present in fruits and vegetables (Hertog et al., 1992; Justesen and Knuthsen, 2001). Eventhough the leaves of *R. apiculata* and the bark of *K. candel* lacked myricetin, it showed its presence all the plants of current investigation. From the taxonomic viewpoint, presence and absence of myricetin is very significant. Its presence is considered as a primitive character in dicots, particularly in woody plants (Joshi et al., 2004; Harborne, 1966). Thus all the mangroves of the family *Rhizophoraceae* can be regarded as primitive in flavonoid patterns because of the presence of myricetin.

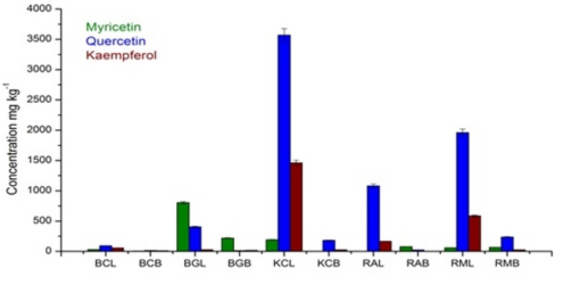

Fig II Variation of myricetin, Quercetin and Kaempferol concent ration among *Rhizophoraceae* mangroves.

The flavonol, kaempferol showed its presence in all the *Rhizophora ceae* mangroves under investigation. It showed highest value in *K. candel* while it was absent in the bark of *R. apiculata.* Next to the Kandelia species, the genus Rhizophora was found be the best contributor of kaempferol. The bruguiera plants showed very low concentration of kaempferol in their tissues.

Flavones, luteolin and apigenin have restricted distribution in the family *Rhizophoraceae* . Luteolin was found in detectable levels only in *B. cylindrica* which is reveals its chemotaxonomic relevance. In all others species this flavone was not detected.The presence of apigenin was detected only in *K. candel* and *R. apiculata.* It was not detected in the genus Bruguiera pointing towards its chem otaxonomic

importance. Comparing the flavonoid composition of the five species reveals greater similarity among *R. mucronata* and *B. gymnorrizha* having only quercetin, myricetin and kaempferol in their leaves and bark. The plants of the same genus, *R. mucronata* show distinct flavonoid composition with respect to the absence of myricetin and apigenin. Myricetin is not detected in the leaves of *R. apiculata* while apigenin was present only in its leaves but not in*R. mucronata* showing the biochemical distinctiveness of the two plants.

The presence of flavonoids in plant foods is largely influenced by genetic factors and environmental conditions and other factors such as germination,degree of ripeness, variety, processing, and storage also influence the content of plant phenolics (Aherne and O'Brien, 2002; Anderson and Markham, 2010). The aglycone results do not completely agree with the existing classifications. Such disagre ement in the aglycon content and classical taxonomy is been previously reported in family *Dipterocarpaceae* in Sri Lanka (Joshi et al., 2004) suggesting the need for a revision of the species and sectional levels in the classical taxonomy based on morphological parameters. Eventhough the present findings are useful in chemical characterisation of *Rhizophoraceae* mangroves more com preh ensive investigation on other areas, such as molecular, cytological, ecological as well as biogeographical aspects are also needed to draw the specific relationships of these plants as the interaction of a number of factors, including species differences, the light regimes under which the plants were grown, as UV-B irradiation is known to induce the accumulation of flavonoids (Li et al., 1993; Lois, 1994; Crozier et al., 1997).

Conclusions

The quantitative analysis of these flavonoids from the plants under investigation is being for the first time. Quercetin was found to be the ubiquitous flavonoid and found to be present in high concentration. It is the flavonoid aglycon which is present in highest concentration in *Rhizophora* plants and *K. candel*. However, further investigations involving more detailed studies are required to establish factors effecting the variations in flavonoid content in order to develop their application for particular chemotaxonomic, food and nutraceutical purposes.

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