Annals of Plant Sciences 7.4 (2018) pp. 2204-2207

ANNALS OF PLANT SCIENCES
ISSN: 2287-688X
OPEN ACCESS
www.annalsofplantsciences.com

Research Article

HPLC identification of bioactive flavonoids in the methanolic and aqueous leaf extracts of *Homalium zeylanicum* Benth.

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Received: 3/1/2018; **Accepted:** 3/17/2018

Abstract: Natural products continue to provide unique structural diversity in comparison to standard combinatorial chemistry, which presents opportunities for discovering mainly novel low molecular weight lead compounds. *Homalium zeylanicum* belonging to the family Flacourtiaceae is an important medicinal plant having traditional uses in diabetes, rheumatism and wound healing activities. Chromatographic methods like TLC and HPLC were used for the separation and identification of flavonoids present in methanolic and aqueous leaf extract was studied. The chromatographic methods available for the separation of flavonoids in TLC and HPLC were adopted for the study. Three compounds were identified in TLC study in methanolic leaf extracts. In HPLC analysis, peaks corresponding to flavonoids were obtained and were identified by comparing with literature and confirm that methanolic extract contains Rutin, Quercetin and Myricetin where as in aqueous extract Quercetin, Myricetin and Kaempferol were observed. The anti diabetic activity of isolated compounds was determined by literature and confirms that compounds were found to be having potent anti diabetic activity. This proves that the anti-diabetic activity of *Homalium zeylanicum* was due to the presence of these bio-active compounds.

Keywords: *Homalium zeylanicum*, flavonoids, the anti-diabetic activity, TLC, HPLC.

Introduction

Homalium zeylanicum Benth plant belongs to Flacourtiaceae family and is distributed in evergreen and semi-evergreen forests, native to South India and Srilanka. It is also found in Bangladesh, Laos, Myanmar, Nepal, Thailand and Vietnam (Nambiar, 1996). Common name of the plant includes kalladamba, liyan, mukki. The various parts of plant including bark and leaf having many traditional medicinal uses, mainly in diabetes, rheumatism and wound healing. It has been traditionally used for treating several ailments including rheumatism, anti-inflammatory, hepatoprotective and antidiabetic agent in Rayalaseema region of Andhra Pradesh. In Nigeria, it is used as traditional medicine for the treatment of malaria, ulcer, and inflammatory diseases and as an aphrodisiac. Various studies have been conducted to evaluate the medicinal properties of plant (Bosser and Rabevohitra 1985), (Hansraj, 2009), (Rajkumar and Bagali, 2010), (Rasal, 2009). Though the traditional medicinal uses of both plants are known, but the phytochemical bases for their uses are not known. Hence in the present paper, we are reporting the screening and quantification of phytochemicals present in *Homalium zeylanicum* plants.

Materials and Methods

Collection of Plant Material:

The leaves of *Homalium zeylanicum* were collected during the month of December from plants presents in Tirumala hills and different locations of Chittoor District. Taxonomic identification of the plants was carried out with the help of Sri Venkateswara University botanists.

Instrumentation:

Merck silica gel TLC plates (60 F_{254}) were used for TLC separation of plant extracts. HPLC separation was carried on Peak LC 7000 HPLC with LC software, Rheodyne manual injector with 20µl loop, UV detector and Zorbax ODS column (250×4.5mm; 0.5µ).

Preparation of extracts and preliminary phyto-chemical screening:

The crude extract from leaves of *Homalium zeylanicum*was obtained using soxhlet extraction method using ethyl acetate, methanol and water solvents. The detailed description of preparation of plant extracts, preliminary chemical screening and quantification methodology was discussed in our earlier publication (Anuradha and Mallikarjuna, 2015).

Biological activity study:

The anti microbial zone activity of leaves extracts of *Homalium zeylanicum* were studied by agar well diffusion method. The anti diabetic activity of crude extracts also studied by following *In-vitro* α- amylase inhibition activity by using visible Spectrophotometer. The detailed description was published in our earlier work (Anuradha and Mallikarjuna, 2016).

TLC separation:

Thin Layer Chromatography is a very preliminary analytical method done prior to HPLC and reaction progress can be monitored easily. It can be used for separating compounds from crude extracts and separating impurities from a compound. The Flavonoids present in the methanolic and aqueous extracts of *Homalium zeylanicum* was separated by different TLC separation methods adopted from available literature. Aluminum foil plates that are coated with absorbent silica powder were used as stationery phase and different solvents were

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tested. Extracts were separated using mobile phase of benzene: acetic acid: water in the ratio of 125:72:3 (Shweta and Padma, 2012), ethyl acetate - ethanol – water in the ratio of 5:1:5 (Sathish *et al.,* 2008), Butanol: acetic acid: water in the ratio of 125:72:3 (Krishna and Renu, 2013) and Ethyl acetate: formic acid: water in the ratio of 8:1:1 (El-Olemy *et al.,* 1994). In all the methods visualization was done in Ultraviolet light observation and separated bands were marked for Rf value calculation.

$$Rf = \text{Distance traveled by compound} / \text{Distance traveled by solvent front}$$

Separation of Flavonoids using HPLC:

To determine the known and unknown flavonoids present in the crude extract, RP-HPLC, widely used method for Flavonoids was used. Methanolic and aqueous extracts of *Homalium zeylanicum* was analyzed using different HPLC methods available in literature. Method 1 was used to separate the flavonoids in gradient elution using mobile phase of methanol: water in the ratio of 1:1 (0-10min), 7:30(10-20min) at a flow rate of 1.0ml/min. The separation was achieved on C18 column at run time of 30min (Oliveira *et al.,* 2001). Method 2 consists of the mobile phase in the ratio of (A) methanol and (B) 1% acetic acid/water and the gradient used was 0 min 40%, 10 min 90%, B, 15 min 40%, B until 17 min in gradient elution at flow rate of 0.8ml/min with and UV detection at 360nm (Alvarado *et al.,* 2007). The method 3, consists the separation of flavonoids using Acetonitrile: water (containing 5% TFA) as mobile phase in gradient elution at a flow rate of 1.0ml/min, UV detection at 254nm was carried out on C18 column.

In all three conditions, methanolic and aqueous extracts of *Homalium zeylanicum* was injected a sample volume of 20 µl and chromatograms were recorded. The flavonoids compounds were identified by using retention time of compounds adopted from reference methods.

Results and Discussions

The solvent extraction and the preliminary screening of phytochemicals (secondary metabolites) is an important step in identification and evaluation of bioactive compounds present in plants. This may lead to medicinal plant drug discovery and development of phytomedicine. Hence solvent extraction with soxhlet extraction apparatus using ethyl acetate, methanol and water solvents were used for extracting phyto-constituents from leaves of *Homalium zeylanicum*. Qualitative analysis for phytochemicals of leaves of *Homalium zeylanicum* indicates that Ethyl extract consist of Triterpenoids, Carbohydrates, Flavonoids, Phenols and Methanolic extract consists of Triterpenoids, Alkaloids, Carbohydrates, Flavonoids, and Phenols. Also, aqueous extract consists of Steroids Alkaloids, Carbohydrates, Flavonoids, and Phenols (Anuradha and Mallikarjuna, 2015).

Quantitative estimation confirms the presence of 12.21, 8.223 and 14.83mg /gram extract of flavonoids in ethyl acetate, methanol and water solvent extracts respectively. The phenolic compounds were found to be 4.28, 19.143 and 20.28mg/gram in ethyl acetate, methanol and water extracts respectively and alkaloids were found to be 4.875mg/g and 17.375mg/g in methanol and water solvent extracts respectively (Anuradha and Mallikarjuna, 2015). Anti oxidant activity by DPPH inhibition assay, anti microbial activity by agar plate well diffusion method and anti diabetic activity by α- amylase inhibition activity were studied for methanolic and aqueous extracts of *Homalium zeylanicum* and results confirms that the plant having high radical inhibition, microbial zone inhibition and anti diabetic activity (Anuradha and Mallikarjuna, 2016).

A novel approach was followed with *Homalium zeylanicum* leaf extracts to determine the biological active compounds by using the chromatographic techniques. In order to determine the number of flavanoids present in the methanolic and aqueous extracts of *Homalium zeylanicum,* liquid chromatographic techniques were applied for separation and identification. Plant extracts were applied to a commercially prepared TLC plate with different solvent systems i.e. the aim of this procedure was to identify the number of components in the extract, distinguish the difference between extract, to find out how close components of extract are and to develop solvent systems which can further be used for column chromatography. In *Homalium zeylanicum,* ethyl acetate extract exhibited the presence of two compounds and three compounds in methanolic extract and one compounds in aqueous extract respectively.

The R_f values are obtained by substituting the values in the above said formula. Ethyl acetate extract of *H.zeylanicum* in first mobile phase shown negative response whereas 2 spots were observed in the second mobile phase with R_f values of about 0.74 and 0.62. Methanol extracts shown positive response and observed 2 spots with R_f values of 0.63, 0.57 in the first mobile phase and 0.95 in second mobile phase. Response in water extract was observed only in the first mobile phase with 0.51 R_f value.

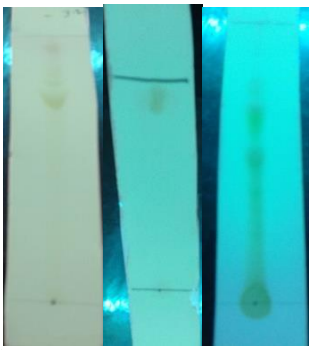

Figure I: TLC results of *Homalium zeylanicum* under UV Light.

Methanolic extract of leaves at condition 1 (figure-II) exhibit the presence of Rutin (7.46min) and Quercetin (14.39min). Methanolic extract of leaves at condition 2 (figure-III) exhibit the presence of Myricetin (7.2min), Quercetin (8.4min). None of the flavonoid compounds in crude extracts were found to have similar retention time with reference condition 3 retention times, hence no compounds was compared. But the peaks found in the extract may considered as unknown flavonoid compounds. Overall compounds obtained in methanolic extract of *Homalium zeylanicum* leaves are Rutin, Quercetin and Myricetin. In aqueous extract of leaves chromatogram at condition 1 (figure-IV) showed the presence of Quercetin (14.7min). With Condition 2, (figure-V) the leaf extract showed the presence of Myricetin (7.38min) and Kaempferol (9.56min). With

condition 3, the leaf extract showed no flavonoid compounds with similar retention time with reference condition 3 retention times, hence no compounds was compared. But the peaks found in the extract may considered as unknown flavonoid compounds. Overall compounds obtained in aqueous extract of *Homalium zeylanicum* leaves are Quercetin, Myricetin and Kaempferol.

All separated compounds indicated the presence of various flavonoids in the different extracts of plant. RP-HPLC results indicate the presence of Rutin, Quercetin and Myricetin in methanolic extract and Quercetin, Myricetin and Kaempferol in aqueous extract of *Homalium zeylanicum*. The anti-diabetic activity of isolated compounds was confirmed by available literature. Niture *et al.,* (2014) confirmed the anti-hyperglycemic activity of Rutin. The anti-diabetic activity of Quercetin and Rutin was confirmed by Jadhav and Puchchakayala *et al.,* (2012), Aguirre *et al.,* (2011) and Vessal *et al.,* (2003) confirmed the anti-diabetic activity of Quercetin. The α-glucosidase inhibitory activity of Myricetin was confirmed by Kang *et al.,* (2015). Anti-diabetic effect of Kaempferol was confirmed by Zang *et al.,* (2011). Our *in-vitro* anti-diabetic experimental results are in agreement with published work. Hence all the isolated compounds in *Homalium zeylanicum* were found to be having anti-diabetic activity. This confirms that the anti-diabetic activity of *Homalium zeylanicum* was due to the presence of these bio-active compounds.

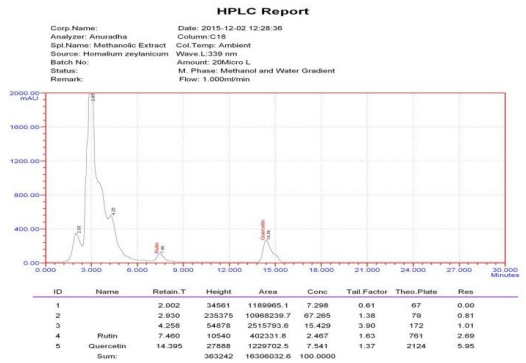

Figure II. Chromatogram obtained for methanolic extract of *H. zeylanicum* in condition 1.

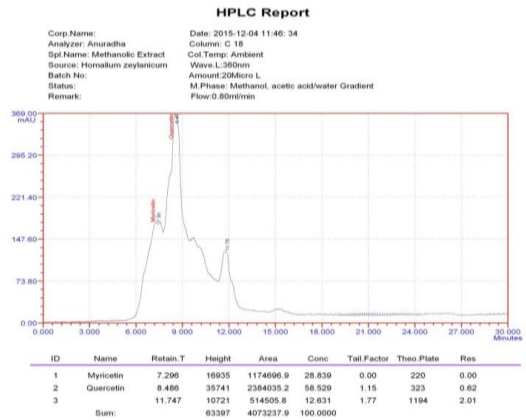

Figure III. Chromatogram obtained for methanolic extract of *H. zeylanicum* in condition 2.

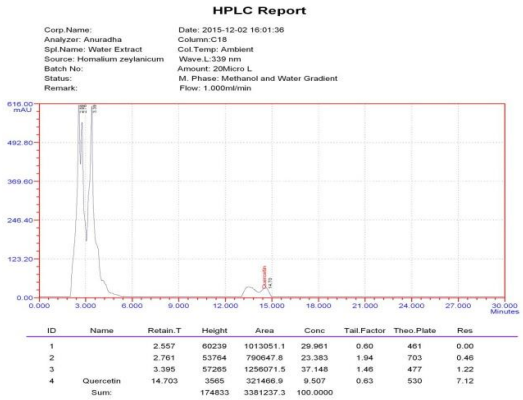

Figure IV. Chromatogram obtained for aqueous extract of *H. zeylanicum*in condition 1.

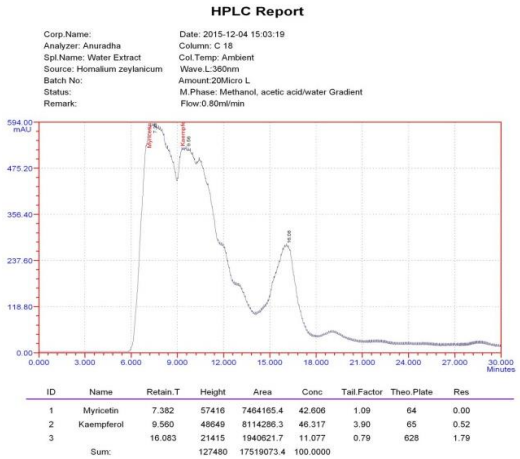

Figure V. Chromatogram obtained for aqueous extract of *H. zeylanicum* in condition 2.

Conclusion

The present study confirms the presence of pharmacologically significant flavonoid compounds in leaf extracts of *Homalium zeylanicum*. Biological activity results of above plant revealed that the plant is having potential anti-diabetic and antioxidant activity and proves traditional use of this plant for medicinal treatment. The plant leaves can be applied for pharmacological treatment. Isolation and purification of the bioactive flavonoids may useful for preparation of drug compounds for pharmacological treatment of specific diseases.

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Cite this article as:

Anuradha Devi V., Veera Kishore I., Raghalatha R., K. Mallikarjuna. HPLC identification of bioactive flavonoids in the methanolic and aqueous leaf extracts of *Homalium zeylanicum* Benth. *Annals of Plant Sciences 7.4 (2018) pp. 2204-2207.*

http://dx.doi.org/10.21746/aps.2018.7.4.5

Source of support: Nil
Conflict of interest: Nil