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EXTRACTION OF FLAVONOIDS IN PHALERIA MACROCARPA (GOD'S CROWN PLANT) BY USING MICROWAVE ASSISTED EXTRACTION TECHNIQUE

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Abstract

To develop improve method for extraction of polyphenols is more important as these are associated with health benefits. The aim of present study was to analyse polyphenols in *Phaleria macrocarpa by using* Microvave assisted extraction technique**.** For this experiment leaves, stems and fruits were used. Aqueous methanol and aqueous acetone were used as solvent. Obtained extracts were analyzed for their antioxidant activity using total flavonoid content, FRAP and DPPH assays. Total seven flavanoids were quantitated by HPLC. Results showed in fruit extract antioxidant activity was highest. HPLC analysis showed kaempferol, myricetin, naringin, and rutin were present in abundant amount. From the results obtained *Phaleria macrocarpa* plant can be considered as a natural source due to the presence of good amount of flavonoids compounds.

Key words: Microwave assisted extraction, Flavonoids, Polyphenols, FRAP, DPPH and IC50.

Introduction

God's Crown plant (Mahkota Dewa), with a scientific name of *Phaleria macrocarpa,* is an evergreen tree that belongs to Thymelaeaceae family. This plant is indigenous to Indonesia specifically Papua. Almost all parts of the plant can be consumed: leaves, stem, peel (epicarp) and fruit. God's Crown is a good source of antioxidant, antihistamine, and antitumor. That is why this plant is usually processed into a supplement or directly consumed as a medicine because of the compounds that it contains: flavonoids, saponin, and alkaloid. But, this investigation will only focus on Flavonoids. Flavonoid is water-soluble polyphenolic molecules that contain 15 carbon atoms (2 benzene rings and carbon chain). In plants, flavonoids are responsible for their color, aroma, spore germination, as well as growth and development of seedling (Steffan *et al*., 2005). For humans, flavonoids provide antioxidants to inhibit oxidation in the body and to control the activity of some protein kinases, a biological activity where inactive protein is phosphorylated (addition of phosphate) to become active. Flavonoids are divided into 6 subgroups depending on the position of B ring and the degree of unsaturation and oxidation of the C ring. For example, isoflavones are flavonoids where the B ring is bonded in the third position of the C ring. Each subgroup also has its own subclasses and they come from different sources (Zhang *et al*., 2006).

Free radicals are highly reactive and they attack macromolecules which results in cell damage and homeostatic imbalance (disability to maintain internal environment). As an antioxidant, flavonoids scavenge free radicals by reacting with the reactive compound in the radical which will eventually inactivate the radical because the hydroxyl group in flavonoid is highly reactive. Flavonoids can also chelate metal and stimulate internal antioxidants (Ao *et al*., 2008). These antioxidant properties of flavonoids remove harmful chemicals and enable them to alleviate diseases. One of them is atherosclerosis, a condition when the arteries hardened and flavonoids have the ability to inhibit LDL (low-density lipoprotein) oxidation. In certain conditions, flavonoids don't act as an antioxidant but as pro-oxidants (inhibiting antioxidant property). Nonetheless, it gives benefit like for cell signaling where flavonoids help in cell functions Researches have been done about flavonoids and have gained positive results. A study proposed that flavonoid called epicatechin acts as an insulin receptor activator and help to reduce the detrimental effects of diabetes mellitus. Then, they used molecular dynamics simulation method and found out that flavonoids bind and inhibit the activity of H1N1 influenza virus (Manian *et al*., 2008). These findings have helped experts to develop new drugs for the disease. An Indonesian researcher, Dr. Ir. M. Ahkam Subroto, M.App.Sc also claimed, "The compounds in god's crown plant (*Phaleria macrocarpa*) have two characteristics that may prevent cancer in the human body. Firstly, the high antioxidant content in god's crown plant prevents the growth of cancer cells by throwing away the free radicals out of the body. Secondly, god's crown plant contains compounds that have the ability to hinder the growth of cancer cells. It also encourages those cells to conduct apoptosis (cell suicide). The proteins that bound to the flavonoids also decompose cancer cells into smaller fragments that will perish on its own" (Hakim *et al*., 2004).

Some of the novel methods for extraction of polyphenols include supercritical fluid extraction (SFE), accelerated solvent extraction (ASE), solid-phase micro extraction

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(SPME), ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE). One of the most promising of these methods is MAE. Gao and Liu (*et al*., 2005) compared MAE and UAE to more traditional methods for the extraction of flavonoids from plant tissue. They showed that MAE had the highest extraction efficiency of flavonoids among all tested procedures. Microwave assisted extraction has been widely used as a sample preparation technique in different analytical fields including environment and food & agriculture. The investigation aims to find out the presence of flavonoids in different parts of god's crown plant and determine which part of the plant stem, leaves or fruits, contains the most flavonoids by using microwave assisted extraction technique.

Materials and Method

Sample collection

The fruits, leaves, and stems of the plant were collected in 2018. Stainless steel knife was used manually for cutting off into small pieces. Samples were freezed in liquid nitrogen, vacuumed dried for < 2 days. A powder was obtained by crushing & grinding, then powdered in cyclotech mill using 100-mesh size to get a fine powder (DM), and stored at -70^{0}C for further analyses.

HPLC and AR grade analytical solvents were used in the analysis and purchased from RFCL, Delhi, India). The reference standards were obtained from Sigma- Aldrich (Sigma- Aldrich, St. Louis, MO, USA**)**. Apparatus; Blender (Inter science, Japan), Vortex mixture (Jain Sci. India), Centrifuge, Sigma 2-16 K (SV Instrument, Delhi, India) and rotary evaporator (caterpillar, Prama Inst, India) was used in the evaporation.

Sample preparation

Microwave assisted extraction (MAE)

Microwave assisted extraction was carried out using Microwave reaction system (Multiwave 3000Solv, Anton Paar, Europe). 1 gm of powdered samples were accurately weighed and added 20 ml of solvent aqueous methanol (methanol: water, 80:20 v/v; AME) and aqueous acetone (acetone: water, 80:20 v/v; AAE). Samples were extracted using the following microwave program: 2 min ramp from 100 to 300W, a 3 min hold at 300W, 2 min ramp from 300W to 100W, a 2 min hold 100 W and after cooling the vessels contents. Total seven compounds were used for quantitative analysis of flavanoids. Extracted samples were centrifuged at 4,000 rpm & 4^{0}C filtered through a 0.45µm nylon syringe filter (Millipore) and evaporated with a rotary evaporator (Nutronix, Jain Brothers India) below 50^{0}C under nitrogen before colorimetric and chromatographic analysis.

Antioxidant activities

Polyphenols are known to function as antioxidants through a number of mechanisms including radical scavenging by H-donation, prevention of chain initiation by donating electrons or by binding of transition metal ion catalysts. Hence, we performed antioxidant activity assays, which involved FRAP assay and DPPH radical scavenging.

Total Flavonoid content

Total flavonoid content was determined by colorimetric method (Jia, Tang & Wu, 1999). Briefly 0.1 ml (1mg ml^{-1}) of each extract was diluted with 0.3 ml of distilled water and 0.03 ml of 5 % $NaNO_2$ solution. After 5 min, 0.03 ml of 10 % $AlCl_3$ was added and incubated for 5 min. Then, 0.2 ml of 1M NaOH was added and the total volume was made up to 1 ml with distilled water. The solution was mixed well and the absorbance was measured immediately at 510 nm. The results were expressed as catechin equivalents (mg per gm (DM) as CE).

Ferric reducing activity power (FRAP) assay

The FRAP assay was carried out according to Stratil et al., (2006) using freshly prepared FRAP reagent. Extracts were mixed with FRAP reagent and measured at 593 nm after 40 min. The results were calculated as trolox equivalent (TE µM g^{-1}DW).

DPPH radical scavenging assay

The stable DPPH was used for determination of free radical scavenging of extracts according to the method Chang (2001). Briefly, 10 µl of sample (0.05-10 mg ml^{-1}) were mixed with 90 µl of 50 mM Tris–HCl buffer (pH 7.4) and 200 µl of 0.1 mM DPPH-ethanol solution. After 30 min of incubation at ambient temperature, the absorbance was taken at 517 nm. Catechin was used as a positive control. The inhibition ratio (%) was calculated according to the equation:

% inhibition = [(absorbance of control - absorbance of sample)/absorbance of control] X100

Determination of Flavonoid Compounds by HPLC

The flavonoid compounds of different parts of *P. macrocarpa* were quantitatively measured by a reversed-phase HPLC technique based on the method described by (Kongsuwan *et al*., 2009). Flavonoid compounds standards consisted of quercetin, rutin, myricetin, kaempferol, naringin, apigenin, and luteolin. an aliquot of sample extract was loaded on a high-performance liquid chromatography (HPLC) Agilent-1200 series instrument equipped with a UV-Vis photodiode array (DAD) detector, binary pump, vacuum degasser, auto sampler Analytical separation was carried out on a C18 column (4.6 mm × 100 mm × 5 µm, Agilent Technology) at a flow rate of 0.8 ml min^{-1}, with a two solvent mobile phase (eluent A = 10mM ammonium acetate and 1% Acetic acid in water; eluent B = 1% Acetic acid in methanol). The eluent gradient used for all extracts was described as follows: 0–3 min, 15–40% A; 3-5.5 min, 40-90% A; 5.5–9 min, 90% A; 9–9.5 min, 90-15% A; 9.5-10 min, 15% A.

Statistical analysis

All the data was reported as mean ± SD. Analysis of variance was performed using the ANOVA procedure. Statistical analysis performed according to SAS software. Differences at $P < 0.05$ were considered statistically significant.

Results and Discussion

Antioxidant activity is widely used as a parameter for food and medicinal bioactive components. Different antioxidant compounds may act through different mechanisms and one method alone cannot be utilized to fully evaluate the antioxidant capacity of food so different antioxidant assays with different approach was carried out. The analysis of different antioxidant assays of different extracts observed that results obtained in aqueous methanol extract was slightly higher than acetone and mix solvent. Previously high Total phenolic content of 99 mg GAE/g extract and 125 mg GAE/g extract with water and ethanol extracts, respectively was reported by (Ahmed *et al.*, 2018) in ripened fruit. According to Ahmed fruit is rich in ascorbic acid therefore it shows high antioxidant activity. In this analysis

aqueous methanolic extract shows good antioxidant activity. In fruit FRAP antioxidant activity was 29.5 ± 0.10 as TE mg g^{-1} in DM and IC_{50} of DPPH radical (mg g^{-1} extract) was 9.5 ± 0.10. Methanolic extract of leaves also has a good antioxidant activity. Previously DPPH Mean EC_{50} of water extract of fully ripe fruit was 827 mg/L as reported by (Ahmed *et al*., 2018). Higher DPPH antioxidant activity was found in this analysis in fruit extract. The antioxidant action of polyphenols depends on their free radical scavenging capacity and iron reducing ability. In this study results found by MAE are reasonably high for all antioxidant assays. The antioxidant activities for all samples measured in different solvents are reported in table- 1.

Among the all tested extracts highest amount of all flavanoid compounds was found in aqueous methanolic extract. In fruit extract myrecetine was 72.7± 0.15 µg/g DM followed by kaempferol 62.0 ± 0.14 µg/g DM. Apigenin was not identified in fruit and stem extract but it was 21.4± 0.03 in leaves extract. Kaempferol was also in good amount in fruit aqueous methanoic extract. Previously, the kaempferol content was significantly ($p < 0.05$) higher in the pericarp extract (76 µg/g DW) (Syahida *et al*., 2011). According to Syahida kaempferol, myricetin, naringin, and rutin were the major flavonoids present in the pericarp while naringin and quercetin were found in the mesocarp and seed. In this study results are not really in agreement with previous reported results. The procedural difference of the method, as well as genotopic and environment of the examined, may explain this difference. Polyphenols are not only good antioxidant but also, been shown to be a potent as anticarcinogen and antimutagen. Recent research on antioxidant properties of plant has been accepted that higher intake of natural antioxidants containing polyphenols is associated with long term health benefits. The results presented offer possible avenues towards health promotion and might be useful in the development of raw materials of medicine.

In recent decades, rapidly growing body of literature covers role of flavonoids and their potential effect on human health. Flavonoids are naturally occurring bioactive substances, with various pharmacological actions. Literature serves a huge study that phenolic substances are the main phytochemicals with antioxidant properties found in higher plants. Intake of these substances daily in adequate amount can play an important role in preventing cancer, cardiovascular and various chronic diseases (Belen *et al*., 2010). The fact that one single plant may contain up to several thousand secondary metabolites and polyphenols makes it necessary to develop high performance and rapid extraction methods.

A correct choice of solvent is the fundamental for obtaining an optimal extraction process. It has been established that when solvent polarity is modified by the addition of water, increased yields are obtained. Small amount of water in the extracting solvent can penetrate easily into the cells of plant matrix and thus increases the mass transfer of the active constituents into the extracting solvent (Nagendra *et al*., 2009). This study showed that MAE had the highest extraction efficiency of flavonoids. Microwave assisted extraction has been widely used as a sample preparation technique in different analytical fields including environment and food & agriculture.

The effect of microwave energy is strongly dependent on the dielectric susceptibility of both the solvent and solid plant matrix. Most of the time, the sample is immersed in a single solvent or mixture of solvents that absorb microwave energy strongly. Temperature increases penetration of the solvent into the matrix and constituents are released into the surrounding hot solvent. However in some cases only selective heating of sample matrix is brought about by immersing the sample in a microwave transparent solvent (hexane, chloroform). This approach is particularly useful for thermolabile components to prevent their degradation.

Conclusion

In this study *Phaleria macrocarpa* showed a good antioxidant activity as well as good flavanoid content also. In microwave assisted extraction it can reach higher temperatures than open vessel systems because the increased pressure inside the vessel raises the boiling point of the solvents used. The higher temperatures in turn decrease the time needed for the microwave treatment. Second loss of volatile substances during microwave irradiation is virtually completely avoided. Third, less solvent is required. Because no evaporation occurs, there is no need continually to add solvent to maintain the volume. Also, the risk of contamination is avoided as a result there is little or no risk of airborne contamination and the fumes produced during an acid microwave extraction are contained within the vessel, so no provision for handling potentially hazardous fumes need to be made.

Therefore, decreased extraction times, reduced solvent consumption and increased sample throughput this technique is easy to use and the systems are cheaper compared to other modern techniques. By considering economical and practical aspects, MAE is a strong competitor to other recent sample preparation techniques for extraction of flavonoids and other applications.

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Table 1: Antioxidant activities in *P. macrocarpa*

Commodity	**Antioxidant activities**					
P.macrocarpa	**TFC mg per gm (DM) as CE**		**FRAP as TE mg g $^{-1}$ (DM)**		**IC_{50} of DPPH rad ical (mg g^{-1} extract)**	
	AME	**AAE**	**AME**	**AAE**	**AME**	**AAE**
Fruit	25.5± 0.30	22.8± 0.20	29.5 ± 0.10	25.0± 0.10	9.5 ± 0.10	6.0± 0.10
Stem	19.4± 0.10	18.9± 0.14	20.0 ± 0.31	18.6± 0.17	3.0 ± 0.31	2.6± 0.17
Leaves	24.6± 0.14	22.5± 0.10	27.7 ± 0.40	24.7± 0.20	8.7 ± 0.40	7.2± 0.20

All data are expressed as mean ± SD (n = 3)

Table 2: Contents of flavonoids compounds in fruit, stem and leaf of *P. macrocarpa*

P.macrocarpa	**Flavonoid contents (µg/g DM)**					
	Fruit		Stem		Leaves	
	AME	AAE	AME	AAE	AME	AAE
Apigenin	-	-	-	-	21.4± 0.03	18.6± 0.13
Kaempferol	62.0 ± 0.14	59.6± 0.13	46.6± 0.03	45.6± 0.07	68.6± 0.03	67.2± 0.62
Luteolin	-	-	-	-	-	-
Myricetin	72.7± 0.15	70.2± 0.27	59.90 ± 0.01	58.70± 0.07	69.0± 0.01	68.2± 0.06
Naringin	14.8± 0.07	12.7± 0.07	32.80 ± 0.06	30.80 ± 0.18	43.6± 0.09	44.9± 0.40
Quercetin	45.20 ± 0.003	43.9± 0.06	31.80 ± 0.002	30.2± 0.06	41.9± 0.06	40.7± 0.06
Rutin	17.80 ± 0.001	16.9± 0.06	12.8± 0.06	11.0± 0.06	15.9± 0.06	13.8± 0.06

All data are expressed as mean ± SD (n = 3); - Not detected

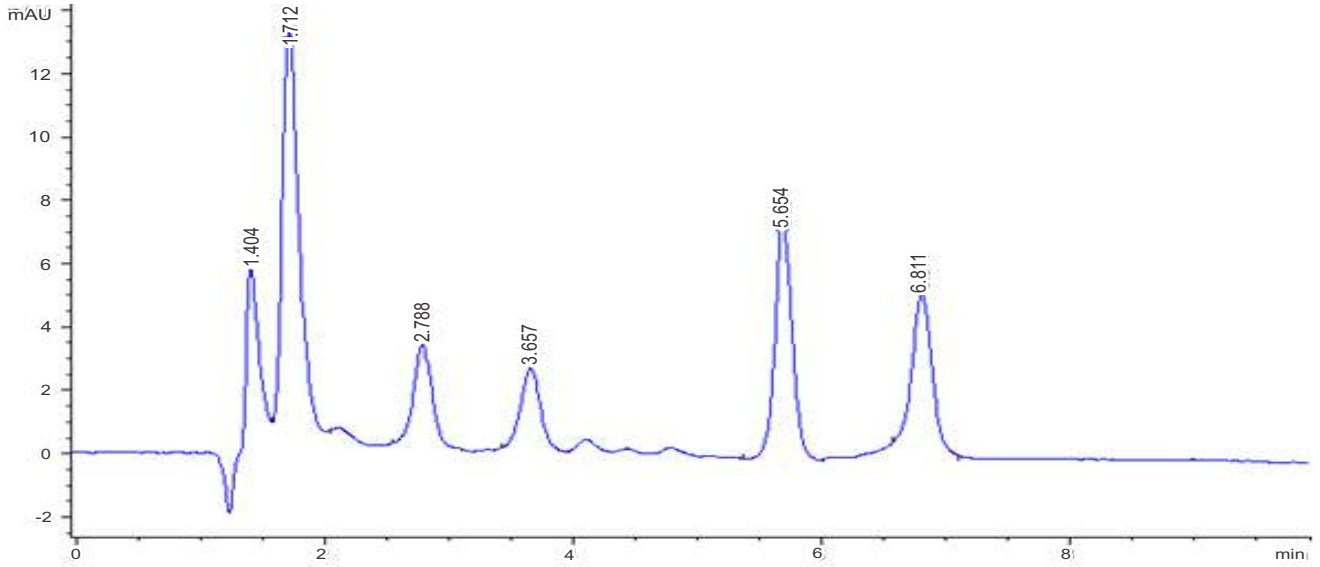

Fig 1: Flavonoids content in fruit extract (*P. macrocarpa)* analysed by HPLC at the wavelength of 350 nm