Antiplatelet Activity and Quantification of Polyphenols Content of Methanol Extracts of *Ocimum basilicum* and *Mentha spicata*.

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ABSTRACT

Ocimum basilicum and *Mentha spicata* are beneficial materials for various applications in the food, nutraceutical and pharmaceutical. The *in vitro* antiplatelet activity of *Ocimum basilicum* and *Mentha spicata* extracts were determined in the present study. HPLC analysis of the methanolic extracts was carried out to determine phenolic compounds. The *in vitro* antiplatelet activity was determined by platelet aggregation assay using platelet rich plasma (PRP). HPLC analysis indicated the presence of polyphenols such as quercetin, rutin, kaempferol and caffeic acid in the methanol extracts of the investigated plants. The percentage inhibition of platelet aggregation was found to be 63.79 ±1.2 and 73.2 ±0.6 for *Ocimum basilicum* and *Mentha spicata* respectively. The IC_{50} values for inhibition of platelet aggregation were found to be 8.433 and 8.586 mg/ml for *Ocimum basilicum* and *Mentha spicata*. The presence of the bio-active compounds especially, polyphenols in the crude extracts of the investigated plants may be responsible for the platelet inhibitory function. Hence the present study warrants further investigations on *Ocimum basilicum* and *Mentha spicata* extracts to understand the underlying mechanism of antiplatelet activity.

Keywords: *Ocimum basilicum*, *Mentha spicata,* antiplatelet activity, platelet rich plasma, polyphenols.

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INTRODUCTION

Platelets are important players in the process responsible for the hemostasis and thrombosis. Recognition of the role of pathological thrombosis in cardiovascular and cerebrovascular events and a better understanding of the procoagulant states has spurred the development of new anticoagulants and new indications for their use. Since there is a high incidence of thromboembolic diseases there is a continuous research of new antithrombotic agents with limited adverse side effects and the recognition of herbal remedies as another therapeutic alternative begins to be considered an important goal for the pharmaceutical industries. Several studies have documented the anticoagulant/antiplatelet activities of natural products over last two decades [1]. Vegetarian dietary practices have been associated with a reduction in many diseases, including arterial thrombosis and ischemic heart disease through the antithrombotic effect associated with many vegetables. Based on these records, many studies worldwide have focused on the anticoagulating activity of many medicinal plants with the hope of finding new and more effective agents. In particular, among botanicals, some essential oils were reported to affect hemostasis in various experimental models [2]. Formulations of garlic containing alk(en)yl thiosulfates in particular, emerged as effective compounds in inhibiting platelet aggregation in human volunteers [3]. Flavonoids, polyphenols, terpenoids and isothiocyanates are among these compounds with proven cardioprotective effects [4]. A comparative screening of the antiplatelet effects of *Ocimum basilicum and Mentha spicata* was attempted in the present study. Reverse phase HPLC analysis was also carried out to identify the polyphenols such as quercetin, rutin, kaempferol, caffeic acid.

MATERIALS AND METHODS

Extraction of the plant material

Ocimum basilicum and *Mentha spicata,* were collected from the small farms around the Bedong and Semeling regions of Sungai Petani, Kedah, Malaysia. The plants were identified by Mr. V. Shanmugam (Botanist, USM) and voucher specimens were submitted to the herbarium unit, USM and the accession number (11292, 11295) was obtained. The aerial parts of the procured plant samples were washed thoroughly under running tap water, dried at room temperature (30°C) for a week and the dried plant material were grounded to a fine powder. 200 g of the finely powdered plant material of the selected plants were macerated and soaked in 80% methanol for 4 days. The extracts were then clarified by filtration through filter paper (Whatman No.1) and is then concentrated *in vacuo* using a rotary evaporator at 40°C to give the respective crude extracts.

HPLC determination of polyphenols (quercetin, rutin, kaempferol, caffeic acid)

HPLC-determination was carried out on a Waters 2695 separation module which consists of an integrated quaternary solvent delivery system and sample management platform. 0.1 g of the solvent free crude extracts were re-dissolved in 5 ml of 100% methanol. The extracts were filtered through 0.20 μm microfilter. 20 μl was injected into the HPLC. 1 mg of the standards (quercetin, rutin, kaempferol, caffeic acid) were diluted serially in HPLC grade acetonitrile and filtered through 0.2 μm microfilter. 20 μl was injected into the HPLC. The column used was a reverse-phase C_{18} Novapak column (4.6 x 250 mm I.D; 5μm). A two solvent gradient system was used. The optimized mobile phase was (A) water: formic acid (99:1) and (B) 49% water, 50% methanol, 1% formic acid. The sample injection volume was 20 μl. Gradient elution was performed at a flow rate of 1 ml / minute for 30 minutes. The detector monitored the sample at 254.9 nm for quercetin and 256.1 nm for rutin and 230.1 nm for kaempferol and caffeic acid. The identification of the compounds in the samples were achieved by comparison of both retention time (t_R) values and absorption spectra obtained for each eluted peak of the samples with those obtained for external standards quercetin, rutin, kaempferol and caffeic acid purchased from Sigma chemicals [5].

In vitro platelet aggregation assay

The antiplatelet activities of the crude extracts were determined by i*n vitro* platelet aggregation assay. Platelet-rich plasma (PRP) and tyrode buffer were used for the assay. PRP was prepared by centrifugation of citrated blood at 22˚C for 6 min, at 400 g. Platelets were adjusted to 3.0×10^8 cell/ml with sterile saline. The platelet-rich plasma (0.13×10^{-7} for each assay was resuspended in tyrode buffer (pH adjusted to 7.4 with 0.25M HCL). Plant extracts were dissolved in 2% (v/v) DMSO in saline at concentrations 0.38-12.5 mg/ml. 40 μl

of the plant extracts was added to the platelet suspension for 1min exposure at 37 ˚C. Aggregation of the platelet was induced by the addition of 2µM calcium chloride. Platelet aggregation was recorded by measuring the increase in transmittance value in a spectrophotometer. Aspirin was used as standard. The extent of platelet aggregation is expressed as % Inhibition (X) using the following equation: X = (A-B/A) x 100, where A is the maximal aggregation rate of control, B- maximal aggregation rate of sample [6].

Statistical analysis

One way ANOVA and Tukey and LSD tests and Paired T test were carried out   by using IBM SPSS 20 software. Regression analysis was done determine the relationship between inhibition of platelet aggregation of the plant extracts tested and the different doses employed. The significance was confirmed by Tukey and LSD test ($p<0.05$).

<div align="center">

RESULTS

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HPLC determination of polyphenols (quercetin, rutin, kaempferol, caffeic acid)

The HPLC profile indicated the presence of the flavonoids, quercetin and rutin, in *Ocimum basilicum* and *Mentha spicata.* Kaempferol was found to be present in *Ocimum basilicum* and *Mentha spicata.* Caffeic acid was present in *Ocimum basilicum, Mentha spicata*. Table 1 represents the retention time and amount of quercetin, rutin, kaempferol, caffeic acid in the investigated plant extracts. Figure 1-3 indicates the HPLC profile of the standards and plant extracts.

***In vitro* platelet aggregation assay**

The percentage inhibition of platelet aggregation was found to be 63.79 ±1.2, 73.2±0.6 for *Ocimum basilicum* and *Mentha spicata* extracts. The IC_{50} value for the percentage inhibition of platelet aggregation were determined for a concentration range 2-10 mg/ml. *Ocimum basilicum* and *Mentha spicata* indicated IC_{50} values of 8.433 and 8.586 for the percentage inhibition of platelet aggregation.

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DISCUSSION

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HPLC analysis of the crude extracts of the plants was done to confirm the presence polyphenols, including flavonoids such as quercetin, rutin, kaempferol and caffeic acid. For the HPLC determination, a rapid and simple reverse-phase HPLC method was developed by using a C18 Novapak column (4.6 x 250 mm I.D; 5µm) with a PDA detector.

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**Table 1: Retention time and concentrations of poly phenols in OB, MS**

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Plant	Phenols	Retention Time (min)	Concentration of phenols (µg/g)
Standards	Quercetin	20.944	
	Rutin	17.889	
	Kaempferol	73.785	
	Caffeic acid	14.741	
Mentha spicata	Quercetin	21.006	386.95
	Rutin	18.417	8766.3
	Kaempferol	74.800	234.15
	Caffeic acid	14.525	78.11
Allium cepa	Quercetin	21.769	107.82
	Rutin	18.361	1663.73
	Kaempferol	nd	nd
	Caffeic acid	14.478	4.16
Ocimum basilicum	Quercetin	21.106	1026.43
	Rutin	17.927	19642.43
	Kaempferol	74.242	88.37
	Caffeic acid	14.552	107.17

Table 2: Percentage inhibition of platelet aggregation

Plant	% Inhibition of platelet aggregation (5min) -	IC_{50} (mg/ml)
Ocimum basilicum	63.79 ±1.2*	8.433
Mentha spicata	73.2 ±0.6*	8.586
Aspirin	85.56±2.0	-

The percentage inhibition of platelet aggregation was calculated from the maximum optical density reading at 5 min by using the formula 1- (O.D of sample- O.D of blank/ O.D of negative control - O.D of blank). Values represent mean±sd, n=3 replicates.

 * indicates values significantly ($p < 0.05$) higher than control.
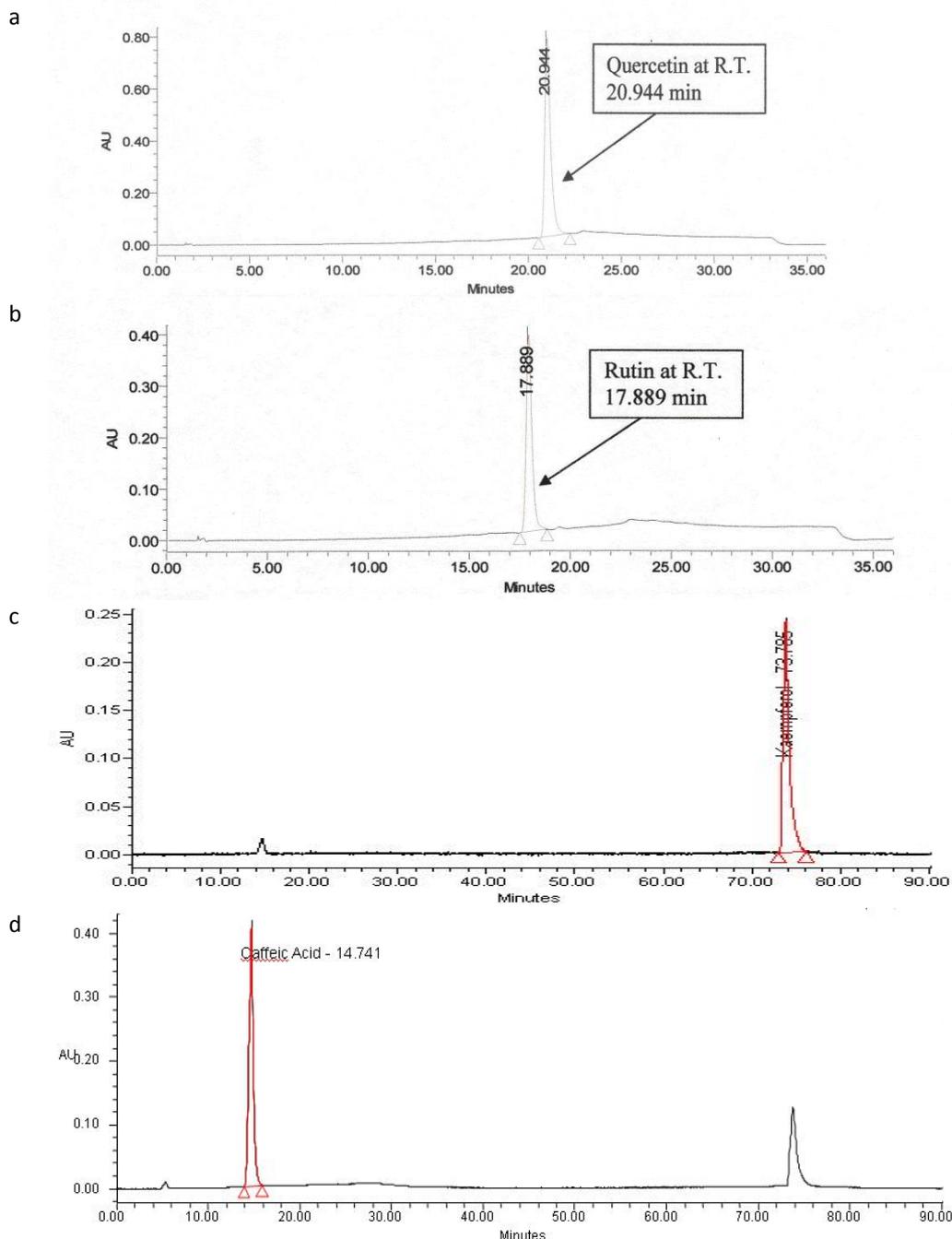
Figure 1: HPLC chromatogram of standards: a: 254.9 nm for quercetin, b: 256.1 nm for rutin, c: 230.1 nm for kaempferol, d: 230.1 nm for caffeic acid
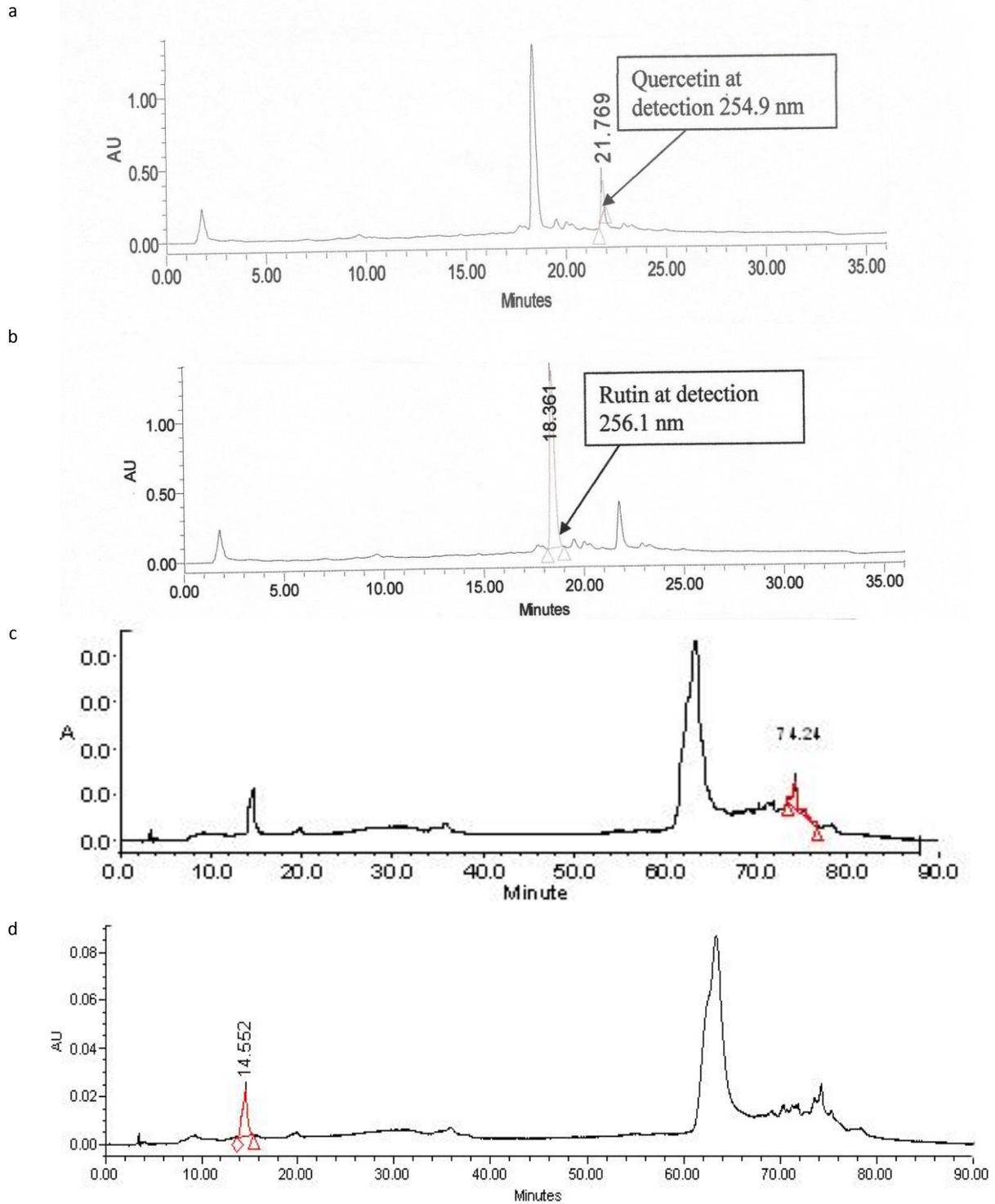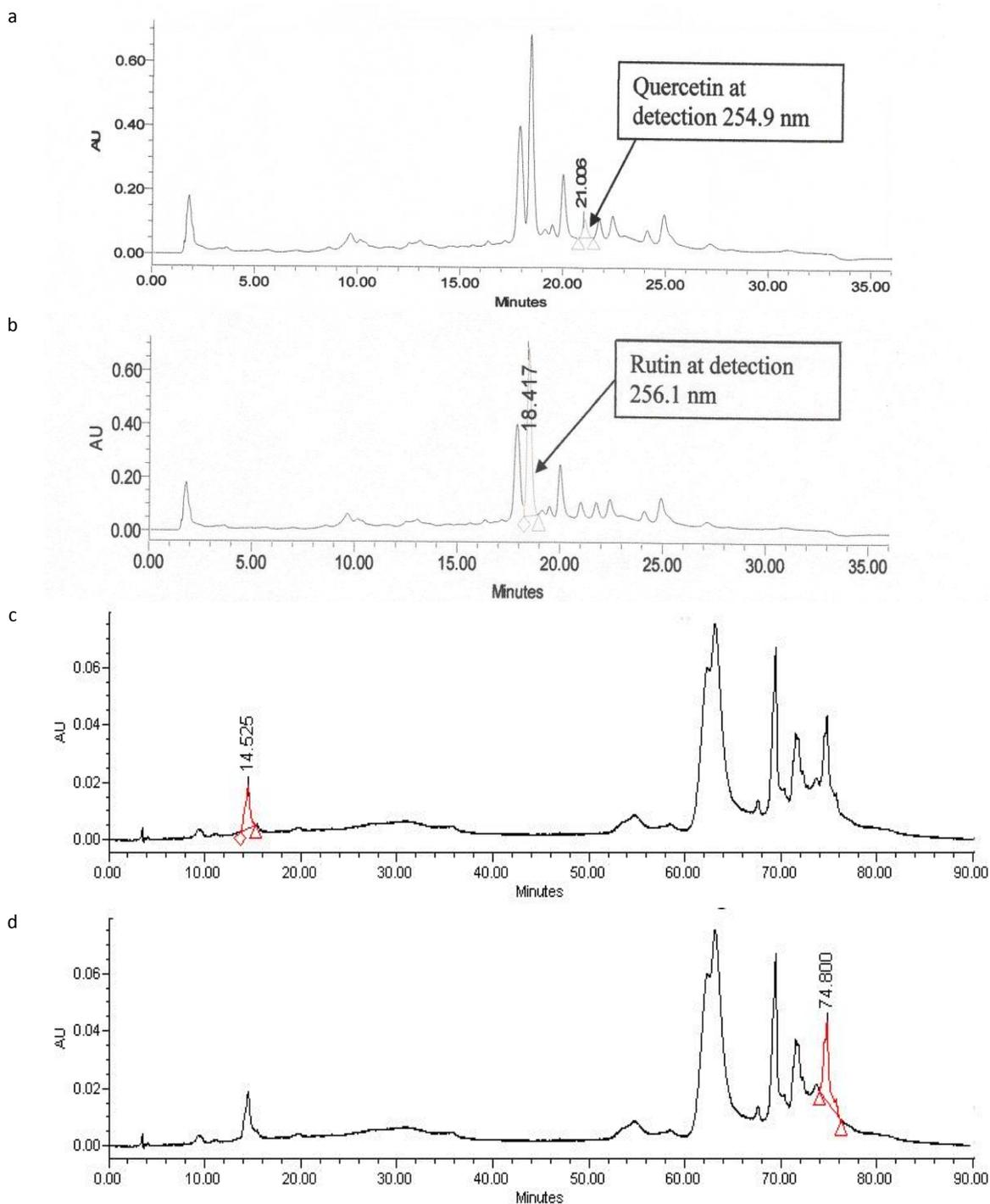
Figure 2: HPLC chromatogram of *Ocimum basilicum*: a: 254.9 nm for quercetin, b: 256.1 nm for rutin, c: 230.1 nm for kaempferol, d: 230.1 nm for caffeic acid

Figure 3: HPLC Chromatogram of *Mentha spicata* a: 254.9 nm for quercetin, b: 256.1 nm for rutin, c: 230.1 nm for kaempferol, d: 230.1 nm for caffeic acid

The detector monitored the sample at 254.9 nm for quercetin, 256.1 nm for rutin and 230.1nm for kaempferol and caffeic acid. Similarly a recent study has reported the identification of quercetin glycosides in human plasma by reverse-phase HPLC-UV-MS method at 254 nm using a PDA detection system [7]. The identification of the compounds in the samples were achieved by comparison of both retention time (t_R) values and absorption spectra obtained for each eluted peak of the samples with those obtained for external standards quercetin, rutin, kaempferol and caffeic acid purchased from Sigma chemicals [9]. A reverse-phase HPLC has been used in a number of occasions for the analysis of flavonoids in plants, it is used to distinguish species based on the quantitative variation of flavonoids among them [8, 9]. HPLC coupled with diode-array

detection was used to identify and quantify the phenolic compounds such as rosmarinic acid, quercetin, and kaempferol in selected culinary herbs and medicinal herbs [10]. To accomplish the selectivity and specificity detection required to identify quercetin and its metabolites at trace levels in complex biological matrices, preliminary experiments had proved that HPLC-MS are superior when compared to UV or electrochemical detection.

In the present study, the HPLC profile indicated the presence of quercetin, rutin and caffeic acid in *Mentha spicata* and *Ocimum basilicum* extracts. Tannins, phenolic acids (caffeic acid, coumeric acid derivatives), flavone glycoside (apigenin and luteolin derivatives) have been reported in the essential oil fraction of *cymbopogan citratus* [11]. The concentration of quercetin was found to be 1026.43 and 386.95 µg/g in the crude extracts of *Ocimum basilicum* and *Mentha spicata.* The amount of rutin was found to be 19642.43, 8766.3, µg/g in *Ocimum basilicum* and *Mentha spicata* extracts. *Ocimum basilicum* was found to contain the highest concentration of rutin.

Both species of the plant showed significant amount of the flavonoids, quercetin, rutin and kaempferol, The major flavonoids that was found in these plants was found to be rutin, quercetin followed by kaempferol. In the present study the flavonoids, rutin, quercetin and kaempferol were reported in both of the plants investigated unlike previous studies which have reported either one of the flavonoids in local vegetables [12]. The highest total flavonoid content as found to be in *Ocimum basilicum* followed by *Mentha spicata.* Studies have reported highest total flavonoid content in onion leaves and trace quantities in *Mentha spp.* Allium vegetables (onion leaves, chinese chive leaves and garlic) contained quite high flavonoid content. The flavonoid content reported in the present study was found to be higher than previously reported studies [13]. Flavonols in the edible portion of the *Allium* vegetables (leeks, shallots, green onions, garlic and onions) range from less than 0.03 to 1 g/kg, white onions contained no detectable flavonols when compared to yellow and red onion which contained 60-1000 mg/kg [14]. A report on the locally consumed vegetables including pegaga, semambu, papaya shoot, cekur manis, belimbi leaves, cashew shoot, kesom leaves indicated high flavonoid content. Among flavonoids, one of the major subgroups ubiquitously occurring in vegetables is flavanol-type flavonoids including kaempferol, quercetin and myrecitin and their glycosides. Quercetin glucosides and rutin are present in onion and common vegetables. In vegetables quercetin glycosides predominant, but glucosides of kaempferol, luteolin and apigenin are also present. The difference in the reported flavonoid content may be attributed to the difference on the method employed, lack of precision and accuracy, parts of the plants used, cultivars or varieties used. The concentration of secondary metabolites in plants is dependent on certain factors such as growing condition, size, degree of ripeness and variety. Phenolic compounds are usually affected by temperature changes and acidic conditions during extraction process. Drying below 50˚C yields highest phenolic compounds, increasing temperature above 60˚C lowers the phenolic content [15].
Quercetin is a potent anticancer agent and antioxidant that can contribute to the prevention of atheroscelorosis. Quercetin is a chemopreventive and chemotherapeutic agent with anti-inflammatory potential and anti-tumor potential that can reduce mutagenicity of cooked foods and enhance the action of anticancer agents and inhibit the growth of tumorigenic cells [14]. Kaempferol and its derivatives have been identified in various vegetables and fruits including onions, French beans and has antioxidant, antitumor, anti-inflammatory and anti-ulcer activity [16]. Caffeic acid, both esterified and free form is generally the most abundant phenolic acid and represents 75% of the total hydroxy cinnamic acid content of fruits [17]. Over the past decade, researchers and food manufacturers have become increasingly interested in polyphenols due to their abundance in our diet and their probable role in the prevention of various diseases associated with oxidative stress, such as cancer and cardiovascular and neurodegenerative disease.

Laboratory evaluation of platelet aggregation permits the measurement of qualitative and semi-quantitative temporal parameters of platelet function in the presence of several aggregation provoking agents such as ADP, collagen, adrenalin, thrombin and arachidonic acid. Quantifications can be performed on platelet-rich fractions or whole blood samples. Platelet aggregation was recorded by measuring the increase in transmittance value in a spectrophotometer. Variation in light transmittance is measured over time. Inhibition of platelet aggregation was measured by the increase in % transmittance value. The % transmittance value of the crude extracts of the selected plants which was found to be increased with increasing concentrations. There was a significant increase in the % transmittance value for the extracts of *Ocimum basilicum, Mentha spicata.* Aspirin was used as a positive control which indicated a maximum transmittance of 73.9%. The IC_{50} for the percentage inhibition of platelet aggregation of the selected plant extracts were determined for a concentration range 2-10 mg/ml. *Ocimum basilicum* and *Mentha spicata* indicated IC_{50} values of 8.433 and

8.586 for percentage inhibition of platelet aggregation. Many *in vitro* and *in vivo* studies have been done to evaluate the antiaggregant action of natural products as a means to drug lead discovery. Medicinal plant formulations from *Panax ginseng, Allium sativum, Ginkgo bilbo, Camellia sinensis* and *Zingiber officinale* are most studied because they can inhibit or increase the anticoagulant or antiplatelet aggregation effects. Antithrombotic activity has been investigated by using *in vitro* platelet aggregation tests in tomato and onion varieties A recent study has reported thrombolytic activity in common thyme, lemon balm, wasabi root, lemon verbena, chervil. Spearmint and lemon grass was found to exhibit antiplatelet activity [18]. *O. basilicum* was found to contain eugenol-containing oil which inhibited thromboxane A_2 formation and calcium mobilization in platelets [19]. Antiplatelet activity of can be ascribed to the presence of phenols or phenyl propanoids for *O. basilicum.* In the case of *M. spicata,* cyclooxygenase inhibition can be the mechanism for antiplatelet activity. Recent research has proven the role of platelets in atherosclerosis and the compounds that inhibit platelet function are of great interest. Bio-active compounds such as flavonoids and polyphenols may be responsible for the platelet inhibitory function. Flavoniods prevents adhesion and aggregation of platelets and studies have proven that flavonoids isolated from *Salidago* species inhibited aggregation of platelets [6]. The mechanism of action of the platelet inhibitory action may be blocking the major chemical mediators, prostaglandins (PG) and seratonin (5-HT). The lipid soluble flavonoids of low polarity may be responsible for the effect and the action of mechanism may have correlation with increasing activity of the anticoagulant and fibrinolytic system. Flavonoids are more important class of compounds with antiaggregant activity. Flavonoids are capable of inhibiting lipid peroxidation and platelet aggregation by acting on enzyme systems such as cyclooxygenase and lipoxygenase and interfering in the production of metabolites of AA [20]. The platelet inhibitory effects of the crude extracts of the selected plants *Ocimum basilicum* and *Allium cepa* may be due to the presence of the bioactive polyphenols, quercetin, rutin, kaempferol and caffeic acid which could have exerted antithrombotic property because of their chemical nature.

CONCLUSIONS

Ocimum basilicum and *Mentha spicata* extracts inhibited platelet aggregation and the presence of the bio-active compounds especially, polyphenols may be responsible for the reported activity. Hence the present study warrants further investigations on the plant extracts to understand the underlying mechanism of antiplatelet activity and for the isolation and purification of the bioactive principles.

ACKNOWLEDGEMENTS

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