SCREENING ON ANTIAGGREGATORY ACTIVITY OF TWO ISOLATED FLAVONOIDS FROM RHIZOMES OF *KAEMPFERIA PARVIFLORA* WALL. (BLACK GINGER)

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Abstract

This paper focused on the health aspects of flavonoids for humans. Among natural compounds, present in everyday diet, flavonoids have shown beneficial effects in prevention of cardiovascular diseases which can be attributed, at least partially to the described antiaggregatory activity i.e. antiplatelet effects of flavonoids. The rhizomes of black ginger were collected from Lashio Township, Northern Shan State and identified by authorized botanist at Botany Department, University of Yangon. Preliminary phytochemical tests were carried out by test tube methods. According to the results, it was found that α-amino acids, flavonoids, glycosides, organic acids, phenolic compounds, starch and terpenoids were found to be present. However, alkaloids, carbohydrates, cyanogenic glycosides, reducing sugars and tannins were not detected. 1.78% of PE, 1.92 % EtOAc, 2.50 % EtOH, 2.87 % MeOH and 4.30 % H_2O crude extracts were prepared by successive Soxhlet extraction method. Silica gel column chromatographic separation of pet-ether extract from rhizomes of black ginger yielded 0.057% of 5-hydroxy -3,7-dimethoxy flavone (m.pt = 149-150 ºC, yellow needles) and 0.035% of 5-hydroxy-3,7,4'-trimethoxy flavone (m.pt = 144- 146 ºC, yellow needles). The isolated compounds were firstly characterized by their physicochemical properties such as R_f values, melting points, solubilities and some colour tests. The isolated compounds were also identified by using modern spectroscopic techniques such as UV-visible, FT-IR, ^{1}HNMR, ^{13}CNMR and 2DNMR spectroscopy. Black ginger contains polymethoxy flavones which are flavonoids that exhibit various bioactivities. Among these, *in vitro* aggregatory activity such as platelet-agglutination, platelet-aggregation and clot retraction were investigated by using Ackroyd's method. It was observed that the isolated compounds possessed antiaggregatory activity.

Keywords: flavonoid, Ackroyd's method, antiaggregatory, platelet-agglutination, platelet-aggregation

Introduction

Kaempferia parvilfora Wall. is with deep purple-colored rhizomes, belonging to Zingiberaceae family and it is known in common name as black ginger, black tumeric, na-nwin net and ga-mone ne (Kress, 2003). It has been mainly used as medicinal resource particularly for treating cardiovascular diseases related to oxidative stress and platelet activation. Black ginger contains abundant amounts of flavonoids and flavonoid glycosides. Especially, it was considered that black ginger is a major source of methoxy flavones with wide range of biological activities such as antiplatelet, antioxidant, anti-inflammatory, antimicrobial and gastroprotective effects (Saokaew *et al*., 2016). The photographs of black ginger are shown in Figure 1.

[1] Dr, Associate Professor, Department of Engineering Chemistry, Technological University (Banmaw)

(a) (b)

Figure 1 Photographs of *Kaempferia parviflora* Wall. (Black Ginger) (a) plant and (b) rhizome

Flavonoids are a group of polyphenolic compounds, which are classified as flavones, flavanones, catechins and anthocyanins. Many have low toxicity in mammals and some of them are widely used in medicine for maintenance of capillary integrity (Cesarone *et al.,* 1992). They have several great advantages over other therapeutic agents because many diets are rich in polyphenolic compounds and are consumed daily having a relatively long half-life with minimum side effects and is easily absorbed in the intestine after ingestion (Agrawal, 2011). In the developed countries, most of the older population is affected by cardiovascular diseases. Platelets are involved in haemostasis, thrombosis and inflammatory processes, hence as a consequence of that physiological role heart stroke and cardiovascular insult can occur. Due to the ever increasing pharmacological interest in antiplatelet agents, a systematic experimental evaluation of large flavonoid series is needed (Sweetman, 2011).

Activated platelets adhering to vascular endolhelium generate lipid peroxides and oxygen free radicals that lead to platelet adhesion and aggregation (Tzeng *et al.,* 1991). Antiaggregatory is preventing the aggregation of platelets. Platelets, the smallest of blood cells, can only be seen under a microscope. They are literally shaped like small plates in their non-active form. A blood vessel will send out a signal when it becomes damaged. When platelets receive that signal, they'll respond by travelling to the area and transforming into their "active" formation. To make contact with the broken blood vessel, platelets grow long tentacle and then resemble a spider or an octopus. A normal platelet count ranges from 150,000 to 450,000 platelets per microliter of blood. Having more than 450,000 platelets is a conduction called thrombocytosis; having less than 150,000 is known as thrombocytopenia. Once in the blood stream, platelets have a life span of 7 to 8 days. About 200 billion platelets are produced every day (Carola, 1990).

This study was undertaken to evaluate the reputation of antiaggregatory property with the isolated flavonoid compounds from the rhizomes of *Kaempferia parviflora* Wall.

Materials and Methods

Collection and Preparation of Samples

The rhizomes of black ginger were collected during the months of October to December in the year of 2002, from Lashio Township, Northern Shan State. A voucher, specimen of the

plant was identified by authorized botanists at Botany Department, Yangon University. After washing with water, the rhizomes were air-dried and ground into powder by a grinding machine. These powder samples were stored in air-tight container.

Preliminary Phytochemical Tests

The dried powdered sample (1 g) was subjected to the tests for alkaloids, α-amino acids, carbohydrates, cyanogenic glycosides, flavonoids, glycosides, organic acids, phenolic compounds, saponins, steroids, tannins and terpenoids as the preliminary phytochemical tests according to the test tube methods (M-Tin Wa, 1970).

Preparation of Various Crude Extracts

Various crude extracts (PE, EtOAc, 95 % EtOH and MeOH) were prepared by successive soxhlet extraction method and then boiled with distilled water. The extracts were concentrated by vacuum rotatory evaporator under reduced pressure to yield PE, EtOAc, 95 % EtOH, MeOH and H_2O extracts. The crude extracts were used to test bioactivities and to isolate some bioactive organic compounds.

Separation and Isolation of Phytochemical Constituents from PE Crude Extract

Compound A and compound B were isolated from column chromatographic separation and purified by washing with PE and EtOAc. After purification, these two compounds were stored for further studies.

Some Physicochemical Properties of Isolated Compounds

The isolated compounds were taken to determine the corresponding physical properties such as R_f values, melting points and solubilities in pet-ether, chloroform, ethyl acetate, ethanol and methanol. As well as some chemical properties were made on TLC chromatogram visualizing with 5 % H_2SO_4, vanillin-H_2SO_4, 1 % $FeCl_3$, Mg/HCl, Liebermann-Burchard reagent followed by heating and also treated with I_2 vapour, NH_3 vapour and NaOH.

Structural Elucidation and Identification

The structures of isolated compounds were elucidated and identified by modern spectroscopic techniques such as UV, FTIR, ^{1}H NMR and ^{13}C NMR.

***In vitro* Screening of Antiaggregatory Activity of Isolated Compounds**

To determine the effects of isolated compounds on platelet functions, *in vitro* tests such as platelet-agglutination, platelet-aggregation and clot-retraction were investigated by Ackroyd's methods at Pathology Department, Department of Medical Research, Yangon, Lower Myanmar.

(a) Blood collection

Blood was obtained from healthy volunteers who did not take any medication for 14 days and were fasting overnight prior to the study. Venous blood was collected into 1/10th volume of Acid-citrate-dectrose (ACD), in a polypropylene or siliconized glass tube. Approximately 10-20 mL of blood are needed a full aggregation study.

(b) Preparation of platelet-rich plasma (PRP)

Whole blood was collected into tubes with the anticoagulant EDTA and centrifuged for 10 min at 1500 rpm to remove the cells from plasma. The resulting supernatant is designated plasma or platelet-rich plasma (PRP). Following centrifugation, it was immediately transferred into a clean polyproplyene tube using a pipette.

(c) Preparation of serum

The whole blood was collected into a covered glass tube and allowed the blood to clot by leaving it undisturbed for 30 min at room temperature. Then remove the clot by centrifuging at 3500 rpm for 10 min. The resulting supernatant is designated as serum and it was immediately transferred into a clean polypropylene tube using a pipette.

(d) Preparation of test sample solutions

Each isolated compound (0.02 g) was dissolved in 1 mL of DMSO. These solutions were passed through a syringe filter PVDE 0.22 μm and then four-fold serial dilutions were made with normal saline to obtain solutions with the concentrations of 0.0312 μg, 0.125 μg, 0.5 μg and 2 μg/mL.

(i) Procedure for platelet-agglutination test

The same volumes of PRP, serum and serial diluted sample solutions were successively added into a respective small plastic tube and followed by incubating the mixture at 37 °C for 1 h. The mixture without serial diluted sample solutions served as a control. Then 30 μL of the mixture for each was dropped onto a slide and viewed under a microscope and the appearances on slide was recorded as well as compared with control (Dacie and Lewis, 1975).

(ii) Procedure for platelet-aggregation test

Only PRP as control and PRP with serial diluted sample solutions in each glass tube were agitated in water-bath at 37 °C and watched every 30 min followed by recording their appearances and compared with control (Dacie and Lewis, 1975).

(iii) Procedure for clot-retraction test

Only normal whole blood and whole blood with normal saline as control and whole blood with serial diluted sample solutions in each tube were warmed in water-bath at 37 °C. They were inspected after 2 h, 4 h, 6 h, 24 h and then their appearances were recorded and compared with control (Dacie and Lewis, 1975).

Results and Discussion

Preliminary Phytochemical Investigations

In order to find out the types of phytochemical constituents present in the rhizomes of black ginger, the preliminary phytochemical investigations were firstly carried out by the standard test tube methods. From these experiments, α-amino acids, flavonoids, glycosides, organic acids, phenolic compounds, saponins, starch, steroids and terpenoids were observed. However, alkaloids, carbohydrates, cyanogenic glycosides, reducing sugars and tannins were absent. These results are summarized in Table 1.

Table 1 Results of Preliminary Phytochemical Investigation on Black Ginger

No.	Types of Compounds	Extracts	Reagents	Observation	Remark
1.	Alkaloids	1% HCl	Mayer	No White ppt.	–
			Dragendorff Wagners	No Orange ppt.	–
				No Brown ppt.	–
2.	α–Amino acids	H_2O	Ninhydrin	Violet spot	+
3.	Carbohydrates	H_2O	10% α-napthol and conc. H_2SO_4	No Red ring	–
4.	Cyanogenic glycosides	H_2O	Sodium picrate	No Deep blue	–
5.	Flavonoids	EtOH	Mg and conc. HCl	Pink colour	+
6.	Glycosides	H_2O	10% lead acetate	White ppt.	+
7.	Organic acids	H_2O	Bromocresol green	Deep blue	+
8.	Phenolic compounds	H_2O	$K_3Fe(CN)_6$ and $FeCl_3$	Bluish black	+
9.	Reducing sugars	H_2O	Benedict's reagent	No brick-red ppt.	–
10.	Saponins	H_2O	Shaking	Frothing	+
11.	Starch	H_2O	Iodine	Bluish black	+
12.	Steroids	PE	H_2SO_4 and acetic anhydride	Colour change	+
13.	Tannins	H_2O	Gelatin and 1% $FeCl_3$	No ppt.	–
14.	Terpenoids	$CHCl_3$	H_2SO_4 and acetic anhydride	Colour change	+

\+ = present
– = absent

Preparation of Some Crude Extracts

After carrying out the preliminary phytochemical tests, to separate and isolate some organic constituents present in the rhizomes of black ginger, some crude extracts were successively prepared by Soxhlet extraction method. Phytochemical content in the rhizomes of black ginger is various so that different solvents were successively used by their polarity order. Data showed that watery extract had the highest yield (4.30 %) and then methanolic, ethanolic, ethyl acetate and petroleum ether extracts had yielded 2.87 %, 2.50 %, 1.92 % and 1.78 % respectively. These results informed that polar compounds can be easier to be extracted compared to non-polar compounds.

Separation, Isolation and Purification of Isolated Compounds

Compounds A and B were isolated from column chromatographic separation of 2.5 g of PE crude extract using silica gel GF_{254} as adsorbent and eluting with PE : EtOAc (20 : 1) as solvent system. Purification of these two compounds was done by washing with PE and EtOAc.

Some Physicochemical Properties of the Isolated Compounds

To identify the structures of isolated compounds, they were firstly characterized by determination of their physical properties such as R_f values, melting points and solubilities, and some chemical properties especially colour tests. To distinguish the steroid and terpenoid compounds, Liebermann-Burchard reagent was used. Steroidal compound gave blue green colour with the Liebermann-Burchard reagent in PE solvent and terpenoids provided pink colour in $CHCl_3$ solvent. Flavonoids were generally identified by treating with Mg in conc. HCl to give pink colour. 1% $FeCl_3$ was used for determining the phenolic compounds, giving deep blue/

green / brown colour. The isolated compounds were also characterized by colour tests with 5 % H_2SO_4, anisaldehyde-H_2SO_4, vanillin-H_2SO_4, Liebermann-Burchard on TLC chromatograms followed by heating, and also treated with I_2 vapour, NH_3 vapour, NaOH etc. The results of some physicochemical properties of the isolated compounds are illustrated in Tables 2 and 3.

Table 2 Some Physical Properties of the Isolated Compounds

No.	Isolated Compound	Physical State	Colour	Melting Point (°C)	R_f Value	Solvent system (PE : EtOAc)	Solubilities				
							PE	$CHCl_3$	EtOAc	EtOH	MeOH
1	A	solid	yellow	149–150	0.55	9 : 1 v/v	–	+,Δ	+, Δ	+, Δ	+, Δ
2	B	solid	yellow	144–146	0.31	9 : 1 v/v	–	+, Δ	+, Δ	+, Δ	+, Δ

\+ = soluble
– = insoluble

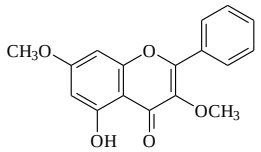
Table 3 Some Chemical Properties of the Isolated Compounds

No.	Isolated Compound	Spraying agents									Remark
		5 % H_2SO_4	vanillin - H_2SO_4	anisaldehyde- H_2SO_4	Liebermann-Burchard, Δ	1% $FeCl_3$	Con. HCl & Mg*	I_2 vapour	1% NaOH	NH_3 vapour	
1	A	yellow	yellow	yellow	yellow	black	yellow	yellow	yellow	yellow	Flavonoid
2	B	yellow	yellow	yellow	yellow	black	yellow	yellow	yellow	yellow	Flavonoid

Identification and Structural Elucidation of the Isolated Compounds

Compound **A** : 5-hydroxy-3,7-dimethoxy flavone (Sutthanut, 2007)

yellow needle



yield 0.057 %

m.pt 149 – 150 °C

TLC R_f = 0.55; PE : EtOAc = 9 : 1 v/v, yellow with 5%H_2SO_4, yellow with vanillin-H_2SO_4, yellow with anisaldehyde– H_2SO_4, Liebermann-Burchard, black with 1% $FeCl_3$, yellow with I_2 vapour, yellow with 1% NaOH, yellow with NH_3 vapour, active under UV

UV(MeOH), λ_{max} (nm) = 268, 346

MeOH / NaOH = 283, 360

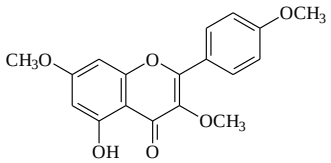
MeOH / NaOAc = 267, 340

MeOH / NaOAc / H_3BO_3 = 267, 342

	MeOH / $AlCl_3$	= 276, 381
	MeOH / $AlCl_3$ / HCl	= 276, 381
IR	(KBr), ν_{max} = 3473, 3414, 2846, 1657, 1602, 1497, 1380, 1343, 1258, 1218, 1176, 1093, 1029, 1000, 815, 604 cm^{-1}	
^{1}HNMR	(400 MHz, $CDCl_3$), δ 12.58 (1H, s), 8.06 (2H, m), 7.54 (2H, m), 6.45 (1H, d), 6.35 (1H, d), 3.86 (6H, s) ppm	
^{13}CNMR	(400 MHz, $CDCl_3$), δ 55.87, 60.31, 92.26, 97.92, 106.16, 128.6, 130.49, 130.91, 139,72, 155.8, 156.90, 161.91, 165.53, 176.87 ppm	

Compound **B** : 5-hydroxy-3,7,4′-trimethoxy flavone (Sutthanut, 2007)

yellow needle



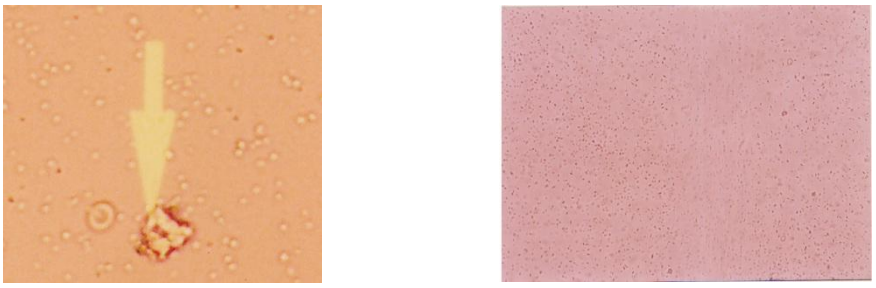
yield	0.035 %	
m.pt	144-146 °C	
TLC	R_f = 0.31; PE : EtOAc = 9 : 1 v/v, yellow spots with 5% H_2SO_4, vanillin-H_2SO_4, anisaldehyde–H_2SO_4, Liebermann- Burchard, I_2 vapour, 1%NaOH and NH_3 vapour, black with 1% $FeCl_3$, active under UV.	
UV, λ_{max} (nm)	MeOH	= 267, 339
	MeOH / NaOH	= 280, 364
	MeOH / NAoaC	= 269, 336
	MeOH / NaOAc / H_3BO_3	= 269, 336
	MeOH / $AlCl_3$	= 268, 342
	MeOH / $AlCl_3$ / HCl	= 274, 338
IR	(KBr), ν_{max} = 3451, 3076,2939,1657, 1603, 1503, 1455, 1433, 1376,1343, 1311,1253, 1226, 1166, 1199,1097, 1073, 999, 942, 895 cm^{-1}	
^{1}HNMR	(400 MHz, $CDCl_3$), δ 3.86 (3H, s), 3.87 (3H, s), 3.89 (3H, s), 6.36 (1H, d, J = 2.2 Hz), 6.44 (1H, d, J = 2.2 Hz), 7.02 (2H, d, J = 9.3 Hz), 8.07 (2H, d, J = 9.3Hz), 12.67 (1H, s, OH) ppm	
^{13}CNMR	(400 MHz, $CDCl_3$), δ 55.4, 55.8, 60.1, 92.2, 97.8, 106.0, 114.0, 122.8, 130.1, 138.8, 155.9, 156.7,161.6, 162.6,165.4, 178.7 ppm	

In vitro Antiaggregatory Activity of the Isolated Compounds

In vitro antiplatelet activity of the isolated compounds were investigated by screening on agglutination, aggregation and clot-retraction tests according to Ackroyd's method.

In agglutination test, only PRP in serum was used as control and the mixture was incubated at 37 °C for 1 h. When 30 μL of the mixture was dropped onto slide and viewed under

microscope, platelet-agglutination was found. This is due to the antibody in the serum activated the platelets to occur agglutination. However, platelets did not agglutinate by the antibody in the serum with serially diluted sample solutions. Therefore the isolated compounds can inhibit the action of agglutinant substances. These appearances are shown in Figure 2.



(a) (b)

Figure 2 Photographs of (a) platelet agglutination and (b) no agglutination under microscope

In aggregation test, only PRP was added into the polypropylene tube and used as control. When it was agitated in water-bath at 37 °C, platelets began suspension and their size increased as granule and then platelets aggregated as now flakes. However, PRP with serially diluted sample solutions in each tube did not show successive steps of aggregation. Therefore, the isolated compounds can prevent the activation process of platelets. This mechanism is illustrated in Figure 3.

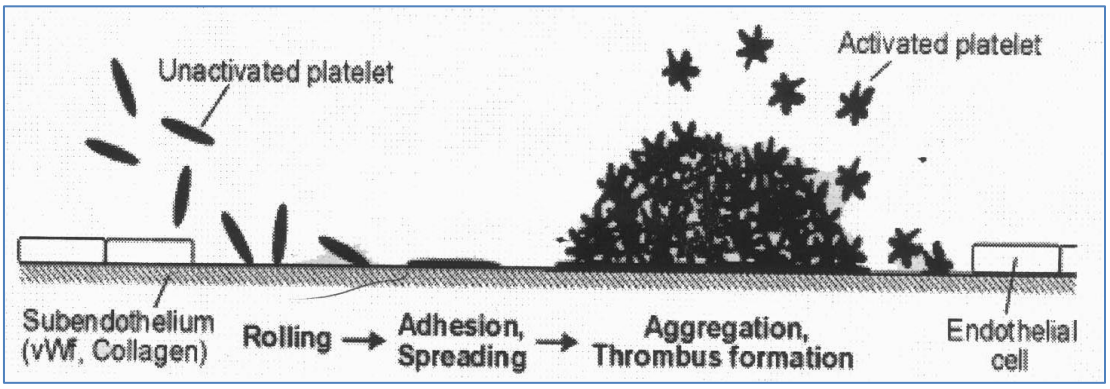


Figure 3 Mechanism of platelet aggregation (Fressinaud *et al*., 1994)

In clot-retraction test, only whole blood and whole blood with normal saline were used as controls. When they were allowed to clot at 37 °C, clotting was formed within a few minutes. Then, the blood clot began to contract and a straw colored fluid called serum was extruded out of the clot. This phenomena is called clot-retraction and it is caused by releasing of multiple coagulation factors from platelets trapped in the fibrin mesh of clot. In the case containing the serial diluted sample solutions, the prolong clot-retraction time was observed by extruding a little serum from the clot. Although normal clot-retraction time is 0-2 h, in this case prolonged nearly 6 h. This highlighted that isolated compounds can produce an inhibitory effect on the platelet functions. This appearance is shown in Figure 4.

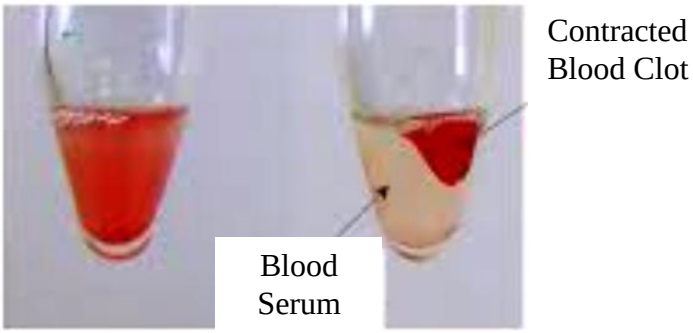


Figure 4 Photograph of clot-retraction (John, 2016)

According to the structure activity relationship (SAR), monohydroxylated flavones are most potent if substituted at position-6- of the A-ring (6-hydroxy flavone). However, most naturally occurring flavonoids are hdyroxylated at position 7, thus having lower antiaggregatory effect. Although increase in number of hydroxyl groups does not influence on antiaggregatory effect, o-methylation increases the activity (Bozina *et al*., 2009). This is probably due to greater volume and higher lipophilicity of the methyl group compared to hydroxyl group. Higher lipophilicity can lead to significant interactions with the platelet's membrane by increasing rigidity and can prevent the aggregation. Based on these observations, structure activity relationship between flavonoids and antiaggregatory activity revealed that two isolated compounds can inhibit the activation of platelet functions. Out of these, 5-hydroxy-3,7,4' -trimethoxy flavone (A) may be more potent than 5-hydroxy-3,7-dimethoxy flavone (B).

Conclusion

From the present investigations on the rhizomes of *Kaempferia parviflora* Wall. (Black Ginger), it could be deduced that the isolated flavonoids possess inhibitory effects on platelet activation and they may be used as platelet activation reducer and they can serve as source of information for further assessment of food or medicine influence on antiaggregation and anticoagulation treatment. Therefore, this structure activity relationship of flavonoids with the modulation of platelet function may guide the design, optimization and development of flavonoid scaffolds as antiplatelet agents.

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