Simultaneous quantification of flavonoids in blood plasma by a high-performance liquid chromatography method after oral administration of *Blumea balsamifera* leaf extracts in rats

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Abstract: The leaves of *Blumea balsamifera* are used as a folk medicine in kidney stone diseases in South-East Asia. Phytochemical investigation revealed leaves contained a number of flavonoids. In view of these, the present work was aimed to quantify and preliminary pharmacokinetic investigation of five flavonoids *viz*. dihydroquercetin-7,4′-dimethyl ether (I), dihydroquercetin-4′-methyl ether (II), 5,7,3′,5′-tetrahydroxyflavanone (III), blumeatin (IV) and quercetin (V) in rat plasma following oral administration (0.5g/Kg) of *B. balsamifera* leaf extract in rats. Quantification was achieved by using a validated, reproducible high-performance liquid chromatographic method. The mean recoveries of I, II, III, IV and V were 90.6, 93.4, 93.5, 91.2 and 90.3% respectively. The limit of quantification was 25 ng/mL for I and IV, 10 ng/mL for II and III and 100 ng/mL for V respectively. The within day and day-to-day precision for all the compounds were < 10%. The validated HPLC method herein was applied for pharmacokinetic studies and the main pharmacokinetic parameters were: $t_{1/2}$ (hr) 5.8, 4.3, 2.9, 5.7 and 7.3, C_{max} (ng/mL) 594.9, 1542.9 1659.9, 208.9 and 3040.4; T_{max} (hr) 4.7, 1.0, 1.0, 3.5 and 2.3; $AUC_{0-\infty}$ (ng hr/mL) 5040, 5893, 9260, 1064 and 27233 for I, II, III, IV and V respectively. The developed method was suitable for pharmacokinetic studies and this preliminary study also revealed significant absorption after oral dosing in rats.

Keywords: *Blumea balsamifera;* flavonoids; RP-HPLC; pharmacokinetic.

INTRODUCTION

Blumea balsamifera DC is a medicinal herb and is widely distributed in South-east Asia. The leaves of this plant are commonly uses as antispasmodic, expectorant stomachic, diaphoretic, in skin diseases, wounds, liver cirrhosis and in kidney stone related diseases (Burkill, 1966; Ismail *et al*., 1999). Furthermore, the Chinese have used preparations of this plant as a carminative, a mild stimulant and as a preventive medicament in times of epidemics and as an abortifacient (Ruangrungsi *et al.*, 1998).

Phytochemical investigation on the leaves of *B. balsamifera* resulted in the isolation and characterization of 11 flavonoids viz. velutin, dihydroquercetin-7,4′-dimethyl ether, blumeatin, ombuine, tamarixetin, rhamnetin, luteolin-7-methyl ether, luteolin, quercetin, 5,7,3′,5′-tetrahydroxyflavanone and dihydroquercetin-4′-methyl ether (Lin *et al*., 1988; Nessa *et al*., 2004a). Flavonoids are an ubiquitous group of polyphenolic substances, which are present in most plants and they have beneficial biological activities (Bast *et al*., 1991; Hertog *et al*., 1994; Knekt *et al*., 1997; Ito *et al*., 1999). Moreover the methanol extracts of the leaves of this plant exhibited antibacterial (Nessa *et al*., 2004b), free radical scavenging activities (Nessa *et al*., 2004a), lipid peroxidation inhibitory activities (Nessa *et al*., 2003), xanthine oxidase inhibitory and superoxide scavenging activities (Nessa *et al*., 2010). These activities were attributed due to the presence of several flavonoids in the leaves extract of this plant. Based on these activities, the leaves of this plant are being sold as nutraceuticals nowadays in South-east Asia. Though a number of bioactivities reported on this plant, but there is no report available on pharmacokinetic studies of this extract. These studies are important to understand the bioavailability of the flavonoids of this extracts in our body. In respect of this a simple, reproducible assay procedure is required for the quantification of extracts rich flavonoids in blood plasma. A number of literatures reported on the pharmacokinetic studies on quercetin as it is widely distributed in many medicinal plants (Hollman *et al*., 1995; 1996). But no reports appear in the literature on the pharmacokinetic studies on dihydroquercetin-7,4′-dimethyl ether, blumeatin, 5,7,3′,5′-tetrahydroxyflavanone and dihydroquercetin-4′-methyl ether. Moreover these flavonoids are only found in the leaves of this plant and percent yields were quite higher than other isolated flavonoids (Nessa *et al*., 2004a). In view of this, we developed a simple HPLC method for the quantification of selected five major flavonoids of *B. balsamifera* viz. dihydroquercetin-7,4′-dimethyl ether (I), dihydroquercetin -4′-methyl ether (II), 5,7,3′,5′-tetrahydr oxyflavanone (III), blumeatin (IV) and quercetin (V) (fig. 1) in rat

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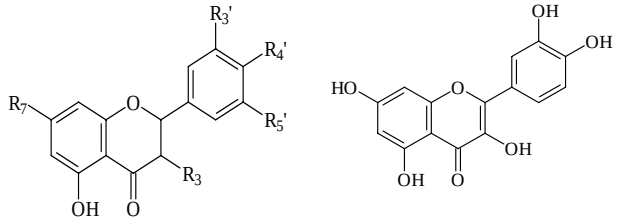
plasma. In our previous work we have reported a reversed phase high performance liquid chromatography (RP-HPLC) method for the quantification of these flavonoids from several leaf samples collected from different places of Malaysia (Nessa *et al*., 2005). However, no reports are available on the quantification of these flavonoids in blood plasma. Therefore, this paper describes their simultaneous quantification in rat blood plasma by using a validated, simple RP-HPLC method and the application of the method in pharmacokinetic study of methanol extracts of leaves of the plant.



Structure A (**1-IV**) Quercetin (**V**)

Fig. 1: Chemical structures of isolated flavonoids of *B. balsamifera* and internal standard (IS) naringenin.

Structure A	R_3	R_7	$R_{3'}$	$R_{4'}$	$R_{5'}$
Dihydroquercetin-7, 4'-dimethyl ether (I)	OH	OCH_3	OH	OCH_3	H
Dihydroquercetin-4'-methyl ether (II)	OH	OH	OH	OCH_3	H
5,7,3',5'-Tetrahydroxy-flavanone (III)	H	OH	OH	H	OH
Blumeatin (IV)	H	OCH_3	OH	H	OH
Naringenin (IS)	H	OH	H	OH	H

MATERIALS AND METHODS

Reagents and materials

Dihydroquercetin-7,4′-dimethyl ether (I), dihydroquer cetin-4′-methyl ether (II), 5,7,3′,5′-tetrahydroxyflavanone (III), blumeatin (IV) and quercetin (V) were isolated from the leaves of *B. balsamifera* and characterized in our laboratory by UV, IR, NMR (1D and 2D), MS (EI/ESI) and elemental analyses (Nessa *et al*., 2004a). The isolated flavonoids were used as standards for the present pharmacokinetic studies. The purity of standards was determined by RP-HPLC analysis and it was 98% for V and 99% for I, II, III and IV respectively. (±)-Naringenin (used as internal standard) was obtained from Sigma Chemical Co. (USA). Methanol (HPLC grade and analytical grade), perchloric acid (70%) and phosphoric acid (85%) were obtained from Merck (Darmstadt, Germany).

HPLC analysis

The profile of five flavonoids in rat plasma was determined using a HPLC system consisting of Gilson Model-302 pump (France) coupled to a variable ultraviolet absorbance detector (Model SPD-10A, Shimadzu, Japan) and an electronic integrator (Chromato-Integrator, Model Hitachi D-2500, Tokyo, Japan). Quantification of flavonoids was carried out employing a Phenomenex (250 x 4.6 mm, 5 μm, Nucleosil) C18 column. The chromatogram was recorded at 285 nm. The mobile phase consisted of methanol- 0.5% phosphoric acid in water (50:50 v/v). The flow rate was kept constant at 0.9 mL/min.

Preparation of sample and standard solutions

About 500 g of dried leaves of *B. balsamifera* (voucher specimen: FRI 57083, The Forest Research Institute of Malaysia) was extracted with analytical grade methanol (2.5 L) using a soxhlet extractor. After filtration the extract was evaporated in vacuum to dryness at 60°C. The samples were freeze dried to remove traces of solvent by using the Cole-Parmer benchtop freeze dryer (Model LD-53, Kingston, NY) and the residue was stored in a refrigerator (4°C) for pharmacokinetic studies. The presences of flavonoids in the extract were examined by conducting HPLC analysis using corresponding standards. The mixed standards solution was prepared by dissolving of dihydroquercetin-7,4′-dimethyl ether (I), dihydroq uercetin-4′-methyl ether (II), 5,7,3′,5 ′-tetrahydroxyflavan one (III), blumeatin (IV) and quercetin (V) (0.1 mg/mL), and internal standard naringenin (1.25 μg/mL) in methanol. Stock solutions were further diluted with methanol to prepare different concentrations of flavonoid standard solutions. All standards were kept at 4 °C before use.

Plasma sample extraction procedure

Extraction procedure was carried out in glass culture tubes (10 mL capacity) pretreated with 5% dichlorodimethylsilane in toluene to minimize drug adsorption. A 200 μL plasma aliquot was vortex-mixed with the internal standard, naringenin (40 μL, 50 ng) and various concentrations of flavonoid working standards solution. For protein precipitation 400 μL of acidified methanol is added to each sample and vortex-mixed for 3 min (Sergey *et al*., 1992). Acidified methanol was prepared by adding 1 mL volume perchloric acid (35%) to 10 mL of methanol and it was used for deproteinization. Following vortex-mixing the sample was centrifuged at 8800*g* for 10 min. The supernatant was transferred to a 1.5 mL microtube and evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 400 μL methanol and vortex-mixed. Finally, a 20 μL aliquot of each sample was injected into the HPLC system for analysis.

Detector linearity

The detector linearity was assessed by injecting aliquots of standards solution in methanol in the range of 25 – 2500 ng/mL for II and III; 50 – 2500 ng/mL for I and IV; and 100 – 2500 ng/mL for V respectively. The peak height

versus the amount of the flavonoids injected was plotted to determine the linearity.

Calibration curves

The calibration curves were prepared by spiking drug-free plasma with standard solutions of flavonoids (I, II, III, IV and V) and a fixed amount of internal standard (40 μL; 50 ng) to give a concentration range of 25-2500 ng/mL. The samples were extracted as described in extraction procedure and the peak height ratio of the flavonoids was plotted against the corresponding concentration of the flavonoids. Linear regression of the peak height ratio of the compounds of interests versus the compound concentration was performed in order to estimate the slope, intercept (peak height for zero concentration) and correlation coefficient of each calibration curve.

Analytical recovery, within-day and day-to-day precision

The absolute recoveries of the extraction procedures for these compounds (I, II, III, IV and V) were determined by comparing the peak height ratio from spiked plasma samples with the peak height ratio of the standard solution of the corresponding concentrations. The within-day precision was assessed by five replicate analyses of plasma samples at four different standard concentrations. The day-to-day assay variation was evaluated by analyzing corresponding plasma samples over a period of five days. A 6-point calibration curve was prepared and two standard solution samples (blind) were run with each assay run.

Pharmacokinetic study

Male, Wistar rats (number of rats: 56), weighing 170-200g were given an oral dose of 0.5 g/kg *B. balsamifera* leaves extract in water. Each animal was used only once and the animals were allowed food and water *ad libitium*. Food was withdrawn from the animal 12 hr prior to the experiment. Both groups only received plain water. The study protocol was approved by the University Animal Ethical Committee, USM, Penang, Malaysia.

Blood samples were drawn by cardiac puncture from both drug treated and control animals into lithium heparin vacutainer. The rats were anaesthetized with sodium phenobarbital prior to blood sampling. Blood samples (3 mL) from drug treated animals were drawn at 0 hr and thereafter 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 16 and 24 hour post dosing. For control group, blood samples were collected at selective time point from respective rats. For each time point 4 rats were sacrificed, 3 for samples and the other for control. All samples were kept at –70°C until analysis by HPLC.

RESULTS

HPLC method development

The detector linearity of five flavonoids of *B. balsamifera* namely dihydroquercetin-7,4′-dimethyl ether (I), blumeatin (IV), quercetin (V), 5,7,3′,5′-tetrahydroxy flavanone (III) and dihydroquercetin-4′-methyl ether (II) was linear over the concentration range of 25 to 2500 ng/mL. Samples with values falls above the calibration curves were diluted prior to extraction. Using 200 μL plasma, the minimum quantification limits for I, II, III, IV and V with the signal to noise ratio 10:1 were 25, 10, 10, 25 and 100 ng/mL respectively.

The calibration curves for flavonoids were linear in the range of 25 – 2500 ng/mL for II, III; and 50 – 2500 ng/mL for I, IV; and 100 – 2500 ng/mL for V respectively. Linear regression analysis was using the equation $y = bx + a$, where a denotes the intercept and b is the slope. The equation of the calibration plots (n = 6) and the correlation coefficient values are shown in Table 1.

Fig. 2 presents the typical chromatograms of blank plasma (A) and spiked standards (1μg/mL) of I, II, III, IV and V in rat plasma (B). Naringenin was used as an internal standard (IS). The method yields a clean chromatogram with baseline separation of II, III, V, I and IV, and IS (naringenin) at the retention time of 10.8 (± 0.2), 13.5 (± 0.3), 26.3 (± 0.8), 30.3 (± 0.8), 34.1 (± 0.9) and 22.1 (± 0.6) min, respectively.

Table 1: Relationships between flavonoid levels and peak height ratio of flavonoids investigated using isocratic reversed-phase HPLC in rat plasma.

Peak No.	Common name	Concentration ranges (ng/mL)	*b	*a	*r
1	Dihydroquercetin-4'-methyl ether (II)	25 - 2500	0.006	- 0.0443	0.9999
2	5,7,3',5'-Tetrahydroxyflavanone (III)	25 - 2500	0.0059	- 0.0273	0.9999
3	Quercetin (V)	100 - 2500	0.0013	- 0.063	0.9981
4	Dihydroquercetin7,4'-dimethyl ether (I)	50 - 2500	0.0039	- 0.0737	0.9998
5	Blumeatin (IV)	50 - 2500	0.0036	- 0.0252	0.9995

*Regression analysis was performed according to the equation y (peak height ratio) = b (slope) x (concentration ng/mL) + a (intercept). r = Correlation coefficients of the regression equation.

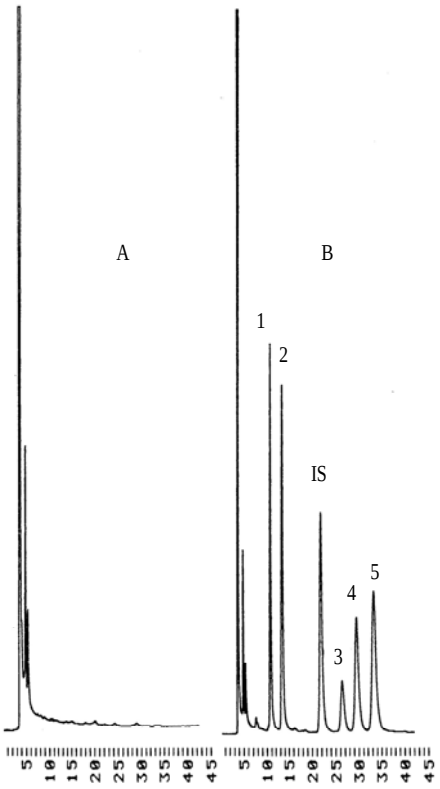


Fig. 2: Representative chromatograms of (A) extracted blank plasma, (B) spiked standards (1 µg/mL) of compound I, II, III, IV, V and internal standard (IS) in rat plasma. Peak identification: 1 = dihydroquercetin-4′-methyl ether (II); 2 = 5,7,3′,5′-tetrahydroxyflavanone (III); IS = naringenin; 3 = quercetin (V); 4 = dihydroquercetin-7,4′-dimethyl ether (I); 5 = blumeatin (IV).

Mean recoveries from plasma were 90.6 ± 2.0% for I, 93.4 ± 2.1% for II, 93.5± 2.2% for III, 91.2 ± 1.3% for IV and 90.3 ± 1.9% for V respectively are shown in table 2.

The within-day variations ranged from 3.9 – 5.9% for I, 3.5 – 6.1% II, 3.7 – 6.3% for III, 4.7 – 6.4% for IV, 5.0 – 6.4% for V respectively. The day-to-day variations for five consecutive days were ranged from 4.5 – 6.2% for I, 4.0 – 6.9% for II, 4.0 – 6.7% for III, 5.3 – 7.6% for IV and 4.9 – 7.9% for V respectively. The values are presented in table 3.

Pharmacokinetic study

The five flavonoids studied were easily absorbed and readily detected in rat plasma after oral administration of the extracts of *B. balsamifera* to the rat. The mean blood levels versus time curve of the five flavonoids are presented in fig. 3. Maximum blood concentration (C_{max}) and the time to achieve the maximum concentration (T_{max}) were obtained from visual inspection of the blood concentration versus time curve. The area under concentration curve (AUC) from zero to infinity was determined by linear trapezoidal rule until the last detectable time point and extrapolated to infinity.

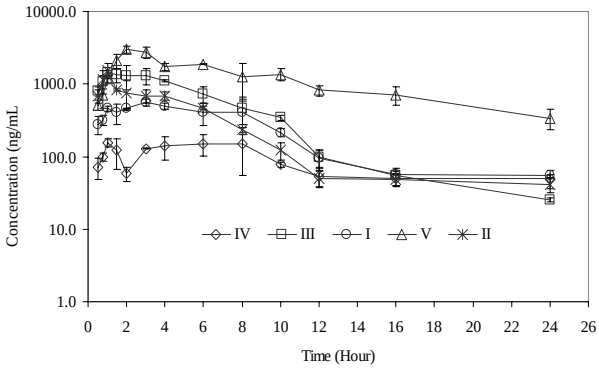


Fig. 3: Plasma concentration time profiles for compound I, II, III, IV and V in rats following oral administration of 0.5g/kg of *B. balsamifera* leaf extract.

Table 2: Mean recoveries of flavonoids of *B. balsamifera* (n = 5) in rat plasma

Flavonoids	Amount spiked (ng/mL)	Mean Recovery (%)	C.V. (%)
I	50	88.7	5.5
	250	89.2	3.6
	500	91.1	4.9
	1000	93.2	5.9
II	50	90.5	3.1
	250	93.2	3.2
	500	94.7	6.7
	1000	95.5	6.4
III	50	90.8	2.7
	250	92.8	3.1
	500	94.5	4.2
	1000	96.0	5.4
IV	50	89.9	3.1
	250	90.7	5.3
	500	91.6	4.5
	1000	92.8	7.2
V	250	88.5	3.2
	500	90.3	5.7
	1000	92.2	7.6

For plasma concentration versus time curve which do not decline over time period, AUC was calculated from time zero to the last detectable time point. Elimination half-life ($t_{1/2}$) was calculated by regression analysis of the log linear portion of the plasma concentration versus time curve. Clearence (Cl) and apparent volume of distribution (V_d) were estimated using standard model independent formula (Rowland and Tozer 1989). Means and S.D

Table 3: Within-day and day-to-day precisions of the method for determination of flavonoids of *B. balsamifera* in rat plasma

Flavonoids	Plasma concentration (ng/mL)	*Within-day precision		*Day-to-day precision	
		Concentration determined (ng/mL)	C.V. (%)	Concentration determined (ng/mL)	C.V. (%)
I	50	49.9	5.1	48.2	6.2
	250	251.0	4.5	249.9	5.3
	500	500.0	5.9	498.6	4.5
	1000	1000.0	3.9	998.5	5.7
II	50	50.7	4.0	48.9	5.1
	250	253.1	3.5	251.3	4.0
	500	504.5	6.1	500.4	6.9
	1000	1001.6	4.6	1000.3	6.3
III	50	51.2	3.7	49.1	4.0
	250	250.6	5.3	249.1	4.9
	500	502.5	4.9	500.1	6.7
	1000	1005.8	6.3	999.9	6.2
IV	50	48.9	4.7	47.3	5.3
	250	249.9	6.4	258.7	7.6
	500	501.3	5.3	499.3	6.3
	1000	1002.1	6.1	1000.0	5.8
V	250	248.3	5.2	247.9	4.9
	500	498.2	5.0	497.5	5.6
	1000	999.5	6.4	997.7	7.9

*n = 5 for each concentration for within-day precision; one concentration line each for 5 days for day-to-day precision

Table 4: Pharmacokinetic parameters of flavonoids in rats following oral administration of 0.5g/kg of *B. balsamifera* leaves extract

Pharmacokinetic parameters	I	II	III	IV	V
C_{max} (ng/mL)	594.9 ± 161.9	1542.9 ± 631.9	1659.9 ± 518.4	208.9 ± 69.0	3040.4 ± 610.9
T_{max} (hr)	4.7 ± 2.8	1.0 ± 0.0	1.0 ± 0.5	3.5 ± 3.9	2.3 ± 0.6
$AUC_{0-\infty}$ (ng hr/mL)	5040 ± 1590	5893 ± 1786	9260 ± 3680	1064 ± 534	27233 ± 9196
$t_{1/2}$ (hr)	5.8 ± 1.2	4.3 ± 0.5	2.9 ± 0.6	5.7 ± 0.8	7.3 ± 0.7
Cl (mL/min/kg)	55.7 ± 14.4	116.6 ± 33.7	54.2 ± 22.3	52.3 ± 24.1	4.7 ± 1.7
V_d (L/kg)	29.3 ± 13.2	42.1 ± 11.5	12.8 ± 2.4	24.1 ± 9.4	2.9 ± 0.8

(Standard Deviation) were determined for the pharmacokinetic parameters. The pharmacokinetic parameters are tabulated in table 4. In the text and tables, results are presented as means ± S.D and as means ± S.E.M. (Standard Error of the Mean) in figures. The data of pharmacokinetic parameters were compared for each flavonoid and was subjected to a one-way analysis of variance (ANOVA). Tukey's test ($p<0.05$) was performed to determine the significance of the difference between means.

DISCUSSION

The extraction procedure of flavonoids from rat plasma was simple and rapid. It demonstrated the isolation of five flavonoids from the plasma. The developed chromatographic conditions showed clear peak without the interference from endogenous substances. The wavelength 285 nm was selected for the analysis and it was the most suitable for simultaneous detection of five flavonoids of *B. balsamifera* namely dihydroquercetin-

7,4′-dimethyl ether (I), blumeatin (IV), quercetin (V), 5,7,3′,5′-tetrahydroxyflavanone (III) and dihydroquercetin -4′-methyl ether (II).

Following oral administration of *B. balsamifera* extract in rats, the compound II and III were rapidly absorbed and reached their maximum blood concentration with mean T_{max} of 1 hr and the overall T_{max} (hr) values were decreased in the order of: II (1.0 ± 0.0) ≈ III (1.0 ± 0.5) > V (2.3 ± 0.6) > IV (3.5 ± 3.9) > I (4.7 ± 2.8). However, the mean values of all compounds were not statistically significant different ($p < 0.05$).

In comparison between maximum blood concentration, compound V exhibited highest blood concentration (C_{max} in ng/mL) amongst the studied compounds and the C_{max} values were decreased in the order of: V (3040.4 ± 610.9) > III (1659.9 ± 518.4) > II (1542.9 ± 631.9) > I (594.9 ± 161.9) > IV (208.9 ± 69.0). The mean values of I and IV; I, II and III were not statistically significant different ($p < 0.05$) but the mean value of compound V was significantly ($p < 0.05$) higher than other compounds.

Compound V was a flavonol, accumulated with time with slow elimination from the blood and showed significantly higher AUC value than the other flavonoids and the mean values of AUC (ng hr/mL) were decreased in the order of: V ((27233 ± 9196) > III (9260 ± 3680) > II (5893 ± 1786) > I (5040 ± 1590) > IV (1064 ± 534). There were not a statistically significant different ($p < 0.05$) between mean values of I, II, III and IV.

The mean values of elimination half life ($t_{1/2}$ in hr) for five compounds were 5.8 ± 1.2, 4.3 ± 0.5, 2.9 ± 0.6, 5.7 ± 0.8 and 7.3 ± 0.7 for I, II, III, IV and V respectively. In comparison between $t_{1/2}$, compound V exhibited significantly higher $t_{1/2}$ and the mean values were decreased as: V > I > IV > II > III. Statistically the difference in the mean values of II and III; I, II and IV; I, IV and V were not significant ($p < 0.05$).

The volume of distribution V_d (L/kg) for compound V was significantly ($p < 0.05$) lower than other studied compounds, and the results were decreased in the order of: II (42.1 ± 11.5) > I (29.3 ± 13.2) > IV (24.1 ± 9.4) > III (12.8 ± 2.4) > V (2.9 ± 0.8). However, the differences between mean values of I, II and IV; I, III and IV; and, III and IV were not significantly ($p < 0.05$) different.

In comparison between mean values of clearance, compound II exhibited significantly ($p < 0.05$) higher clearance Cl (mL/min/kg) than other studied compounds. Overall, the results were decreased in the order of: II (116.6 ± 33.7) > IV (52.3 ± 24.1) > III (54.2 ± 22.3) > I (55.7 ± 14.4) > V (4.7 ± 1.7). However, the compound V showed significantly ($p < 0.05$) lowest Cl and the differences in the mean values of I, III and IV were statistically insignificant.

It appears that molecular structural differences of the compound contributed to their different ranges of pharmacokinetic parameters. To our knowledge no pharmacokinetic data are available for the dihydroquercetin-7,4′-dimethyl ether (I), blumeatin (IV) 5,7,3′,5′-tetrahydroxyflavanone (III) and dihydro-quercetin-4′-methyl ether (II) except for quercetin (V). Most of the pharmacokinetic studies of quercetin were carried out in human (Hollman *et al*., 1995; Hollman *et al*., 1996). However, in our study, it was found that T_{max} (2.3 ± 0.6 hr) for quercetin from *B.* balsamifera leaf extract was similar to the absorption of quercetin from apple (T_{max} 2 hr) (Hollman *et al*., 1995) though the study subject was different.

In principle, a simple, routine, reproducible and accurate HPLC method for the simultaneous determination of five major flavonoids from the leaves of *B. balsamifera* in rat plasma was established. The method was found to be suitable for pharmacokinetic studies and the preliminary studies showed noteworthy absorption after oral dosing in rats. On the whole, knowledge of flavonoids absorption from the *B. balsamifera* leaf extract generated from this preliminary study could be used to devise dose regimens and dosage forms for further screening of this plant extract in human.

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