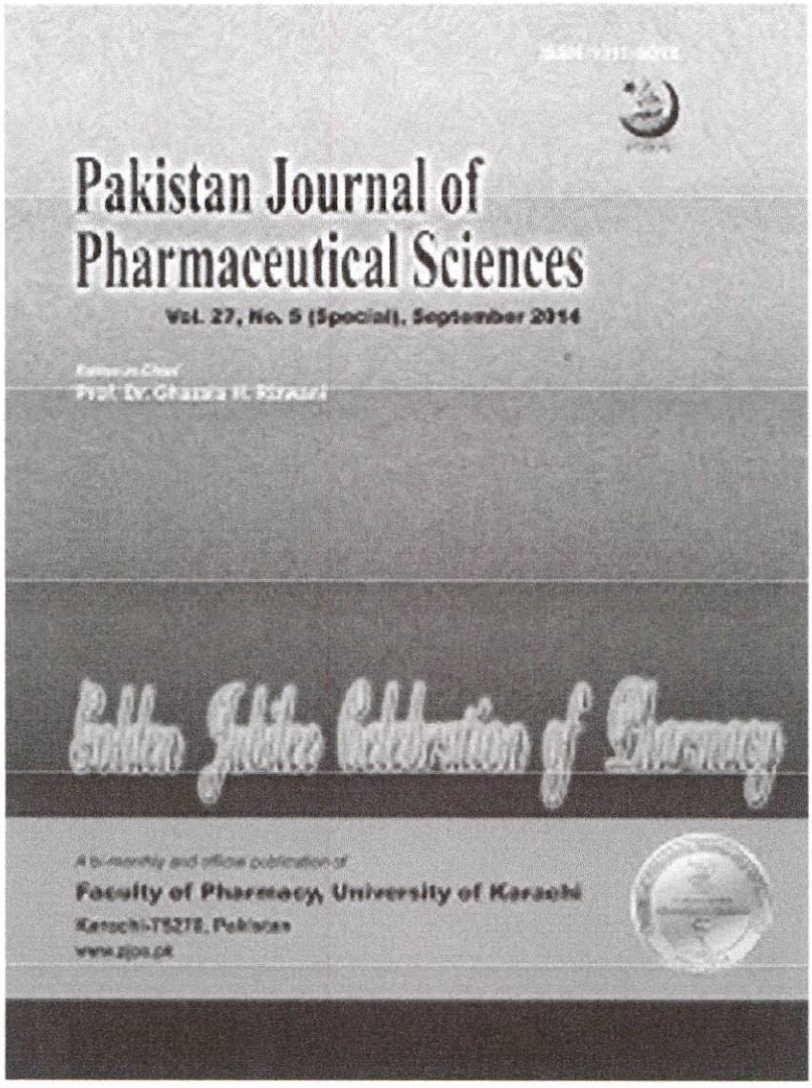
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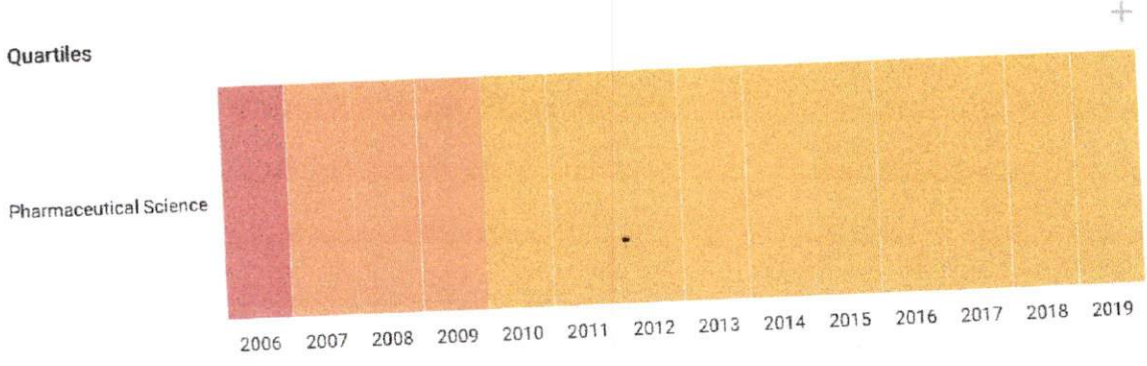

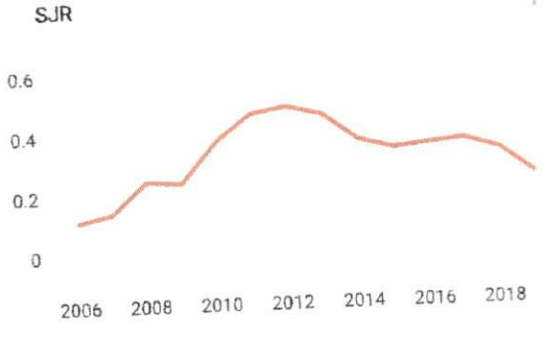

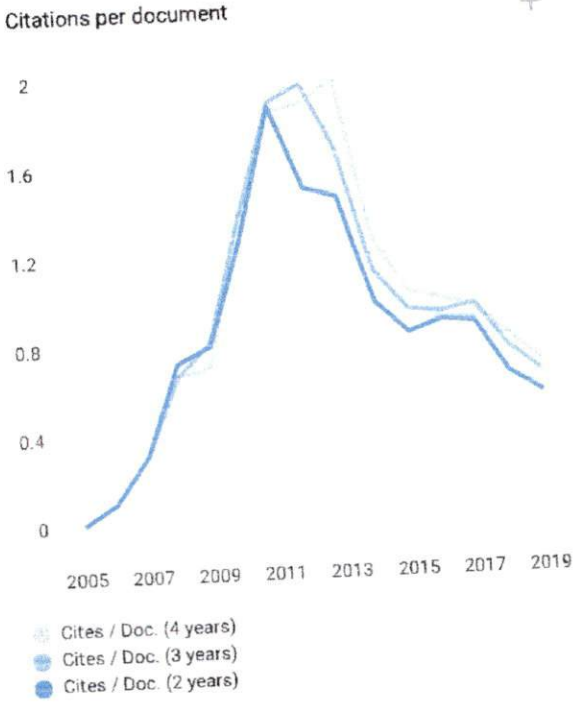

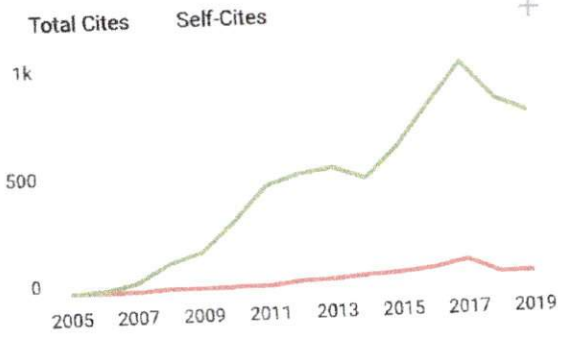

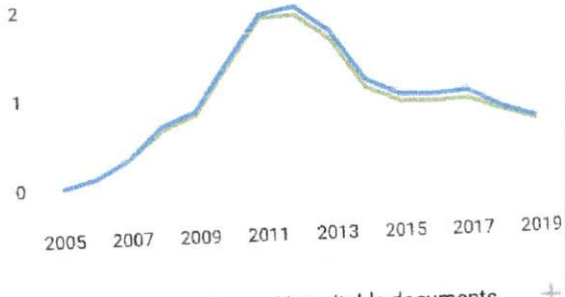

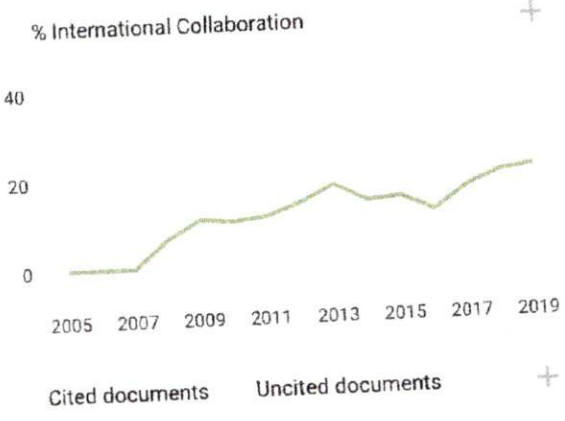

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Bioanalytical method development and validation for quantification of morachalcone A in rabbit plasma using high performance liquid chromatography

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Abstract: *Artocarpus champeden* (*A. champeden*) ethanol extract has been reported as antimalarial activity and prospective to be developed as phytomedicine products. The active marker compound was identical with known prenylated chalcone compound, Morachalcone A. To further develop phytomedicine products from *A. champeden* especially in aspects of bioavailability and pharmacokinetic, a valid, selective and sensitive analytical method becomes important to determine morachalcone A in plasma. The aim of study was to develop and validate selectivity and sensitivity of High Performance Liquid Chromatography (HPLC) method to determine morachalcone A in rabbit plasma. This method was used a RP-18 Column (250 x 4.6 mm i.d, 5 µm), under isocratic elution and acetonitrile:water (50:50 v/v) was used as mobile phase with flow rate of 1.0ml/min. Detection was carried out at 368 nm, 4-hydroxychalcone and methanol were used as internal standard and precipitant. Results showed that this HPLC method was selective with good linearity in range of 3096.774 to 154.839ng/ml. LOD and LLOQ were 89.384 and 154.839ng/ml, respectively. The mean %different was found between 2.79 to 14.33%. Intra and inter-day precision were ≤15% and recovery from this extraction method of morachalcone A and Internal Standard were 80-120%.

Keywords: HPLC, Morachalcone A, bioavailability, pharmacokinetic, 4-hydroxychalcone, rabbit plasma.

INTRODUCTION

Artocarpus champeden (*A. champeden*) is one of plant species in family of Moraceae and locally known as "cempedak". It is widely spread in Indonesia and has been traditionally used for treatment of malaria (Hakim *et al.*, 2006). In previous studies, *A. champeden* extract have been reported for its antimalarial activity against *Plasmodium falciparum* 3D7 strain and several prenylated flavonoids isolated from *A. champeden* exhibited in vitro antimalarial activity against *P. falciparum* 3D7 strain as well (Widyawaruyanti *et al.*, 2007). One of prenylated flavonoids from *A. champeden* is morachalcone A which was isolated from the ethanolic extract of *A. champeden* stem bark. It was exhibited antimalarial activity against *P. falciparum* 3D7 strain with IC_{50} value of 0.18µg/ml. Regarding to its antimalarial activity, the ethanolic extract of *A. champeden* stem bark is prospective to be developed as antimalarial phytomedicine product with morachalcone A as an active marker compound (Hafid *et al.*, 2012).

Method development and validation plays a significant role in evaluation and interpretation of bioavailability, bioequivalence, pharmacokinetic and toxicokinetic studies. These studies were important in the development of antimalarial phytomedicine product from *A. champeden* ethanolic extract (Ma *et al.*, 2015). Such study is required to obtain a reliable analytical and sensitive method to analyze marker compound of *A. champeden* ethanolic extract, especially at a number of tracer (Harahap *et al.*, 2016). One of these methods is High Performance Liquid Chromatography (HPLC).

Despite morachalcone A can be quantified in the ethanol extract of *A. champeden* stem bark using High Performance Liquid Chromatography Photo Diode Array (HPLC-PDA) (Hafid *et al.*, 2012). However, there are still no reported methods for determination of morachalcone A in plasma. Therefore, in this present work, we wanted to develop an accurate, selective and validated analytical method to determine morachalcone A in rabbit plasma. In this work, we report a development and validation of HPLC method to determine morachalcone A in rabbit plasma.

MATERIALS AND METHOD

Chemicals and reagents

Morachalcone A (98% on assay) was purchased from Chemfaces (China). The internal standard 4-Hydroxychalcone (97% on assay) was obtained from Organic Chemistry Laboratory, Yogyakarta State

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University (Indonesia). Acetonitrile, methanol and water were HPLC grade. All solvent was purchased from Merck (Singapore). Lithium heparin was purchased from BD (USA). Six rabbits (New Zealand rabbit, male, 4-5 months old and with 2-3 kg body weight) were used for the study and were purchased from rabbit farms in Malang (Indonesia).

Apparatus

Bath sonicator (Sonica EP series), microbalance (Radwag), centrifuge (Thermo Legend Micro 17 e), micropipettes (Soccorex), vortex mixture (Maxi mix II Barnstead Thermolyne) were used for the study.

HPLC system and conditions

System consisted of a pump (LC-06 AD) equipped with SPD-M20A and connected to CBM-20A Shimadzu (Japan) with computer (HP). Manual Rheodyne injector model 7725i (with 20 µl loop size). The software used was LC Workstation. Chromatographic separations were performed on Lichrosphere 100 RP18 5µm LichoCART @ 250-4 (250 x 4,6 mm i.d.) column and attached to a guard column (octadecylsilane guard column) and at ambient temperature. Mobile phase consists of mixture acetonitrile and water (50:50 v/v), which was pumped 1.0ml/min and in isocratic mode. Before use, the mobile phase was filtered with 0.22µm cellulose membrane (Whatman) and degassed. Wavelength to analysis was detected at 368 nm.

Blank rabbit plasma sample preparation

Six male healthy New Zealand rabbits weighing 2-3 kg body were acclimatized for one weeks in order to observe their good health and suitability. After one week, approximately 5ml rabbit blood samples were taken and collected into heparinized tubes via vena auricuralis then centrifuged (3,000 rpm, 10 min) to separate the plasma. The plasma samples were stored at -20^{0}C prior to analysis. This research protocol was approved by Animal Care and Use Committee (ACUC), Faculty of Veterinary Medicine, Universitas Airlangga with reference number 660-KE for notice of approval.

Preparation of stock, calibrations solutions and quality control samples

Stock solution of morachalcone A (0.96mg/ml) and 4-hydroxychalcone as Internal Standard (0.96mg/ml) were diluted in methanol. Calibration and controls was made by dilute stock solution with methanol until certain concentration. Solution of Internal Standard (IS) (300.387ng/ml) was made by dilute the IS stock solution. All solutions were kept at 4^{0}C and brought to room temperature before use. For calibrations, rabbit plasma contain morachalcone A was prepared in concentration range of 3096.774; 1548.387; 774.194; 387.097; 193.548 ng/mL; and 154.839ng/ml. After that, into the calibrations plasma was added 10.0µl a certain of morachalcone A working solution into 90.0µl of blank rabbit plasma and shortly vortexed. Quality control (QC) samples were made by the same method as making calibration standards using three concentrations: low (464.516ng/ml), medium (1470.968ng/ml) and high (2477.419ng/ml).

Sample preparation

5.0µl of IS solution (300.387ng/ml) was added into 50.0µl aliquot of rabbit plasma containing certain concentrations of morachalcone A. The solution then vortexed (30 seconds) and 100µl of methanol was added to precipitate protein in plasma. Samples were vortexed (1 minute), sonicated (3 minutes) then centrifuged at 14,000 rpm (10 min) at 4^{0}C, 20.0µl of supernatant was taken and inject to the HPLC system.

System suitability test

System suitability test performed on morachalcone A (619.355ng/mL) and IS (300.387ng/ml) in rabbit plasma then extracted using protein precipitation method. 20.0µl of supernatant injected into the HPLC equipment in optimum condition, done six times a repetition then record the number of theoretical plate, selectivity factor and asymmetry factor. The coefficient of variation of retention time and area ratio chromatogram also calculated too.

Method validation

Selectivity

Selectivity was determined by analyzing blank plasma from six different rabbits, blank plasma was spiking with morachalcone A (at LLOQ concentration) and the IS. The %different of morachalcone A also was calculated and its value should be less than 20% (Harahap *et al.*, 2016).

Linearity, limit of detection (LOD) and lower limit of quantification (LLOQ)

Linearity was determined by plotting peak area ratio (y) of morachalcone A to internal standard (morachalcone A/IS) versus the concentration (x) of morachalcone A. Linearity was made by analyzing spiked samples on five different days. The corresponding slope (b) and residual standard deviation (Sy) values were used to calculate limit of detection (LOD) using following equation (Hafid *et al.*, 2015):

$$LOD = \frac{3 \times Sy}{\text{slope (b)}}$$

The LLOQ was defined as lowest concentration with acoefficient variation (%CV) and accuracy (%indifferent) should be-≤20%. The response signal of the LLOQ sample should be at least 5 times response signal of blank sample (FDA, 2013).

Precision and accuracy

Intra-day precision and accuracy were performed by analyzing samples (n=5) in replicates at three concentrations (464.516ng/ml, 1470.968ng/ml and 2477.419ng/ml) on one day and the time gap was eight hours. Inter-day precision and accuracy were analyzed by

analyzing same QC samples on three consecutive days in samples replicates (n=5). Intra and inter-day precision were estimated by calculate the coefficients of variation (% CV) from QC samples." %different" or "%bias" was used to calculate intra and inter-day accuracy, which can be calculated using the following equation (Kumar *et al.*, 2006):

$$\% \text{ different} = \frac{\text{Observed concentration} - \text{nominal concentration}}{\text{nominal concentration}}$$

Extraction recovery

The extraction recovery from clean up sample from plasma was determined at 464.516, 1470.968 and 2477.419ng/ml by spiking morachalcone A into drug-free plasma. Morachalcone A extraction recovery from plasma sample was determined by comparing response from peak area ratio of QC samples with peak area response from non-extracted control samples which prepared at the same concentration level (Singh *et al.*, 2012). This step was repeated to determine the extraction recovery from IS.

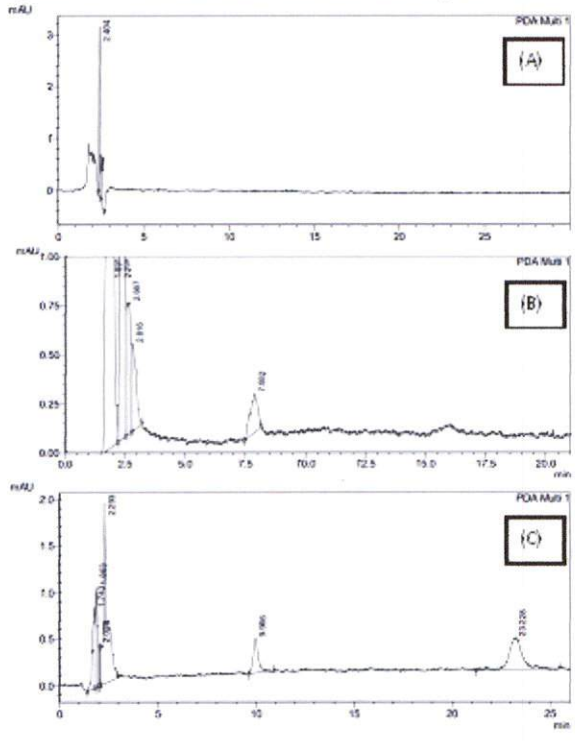

Fig. 1: Chromatogram of (A) Blank plasma, (B) blank plasma spiked with IS, (C) blank plasma spiked with morachalcone A (154.839ng/ml) and IS (300.387ng/ml).

Stability

Stability of morachalcone A in rabbit plasma was determined by analyze the QC plasma samples containing morachalcone A at 464.516 and 2477.419ng/ml (n=3). These results were compared with freshly prepared plasma samples. Freeze/thawstability was determined after 3 complete cycles (-20^{0}C) on three days. Short-term stability was determined following incubation of samples had been spiking with morachalcone A at room temperature (24h). Long-term stability was determined following keep of spiked plasma samples (30 days) at -20°C.

RESULTS

System suitability test

The results of system suitability test repeatability demonstrated by injection, indicating that the analytical methods used have met the system suitability criteria. Coefficient variation value of retention time was 0.25 and 0.27% for morachalcone A and IS, respectively, the ratio of the chromatogram area was 6.01% which was <10% for the analysis of biological fluid samples (Harmita, 2006). The value of number of theoretical plate from morachalcone A 2167.445 and for IS was 2531.573 which were more than 2000, selectivity factor from morachalcone A was 2.650 and 11.2275 for IS which were more than 1.0 and asymmetry factor morachalcone A 0.940 and 0.984 for IS which less or equal than 2.0 (USP, 2015).

Selectivity

The aim of selectivity test was to ensure originality of the peak in sample analysis. The selectivity was determined by analyze blank rabbit plasma sample, blank rabbit plasma that was spiked with internal standard (4-hydroxychalcone) and both of morachalcone A and internal standard (4-hydroxychalcone). fig. 1 showed that there was no interference of endogenous compounds from blank plasma from the six different rabbits. %different of morachalcone A was 13.66% which was <20% (Harahap *et al.*, 2016). Retention time of morachalcone A and IS were 23.228 and 9.996 minutes, respectively.

Linearity, LOD and LLOQ

The correlation between morachalcone A standard concentration versus response area showed linier correlation at concentration range of 3096.774 to 154.839ng/mL with R^2 = 0.9994 (r = 0.9997). LOD and LLOQ for morachalcone A in plasma were 89.384 and 154.839ng/ml, respectively. The precision (%CV) and accuracy (%different) from LLOQ were 7.59 and 10.87%, respectively which was <20% (FDA, 2013).

Precision and accuracy

QC samples plasma contain morachalcone A at 3 concentration levels of 464.516, 1470.968 and 2477.419ng/ml were analyzed for precision and accuracy. The inter- and intra-day precision and accuracy values of this method are presented in table 1. The range for intra-day precision (%CV) was 3.88-7.08% and range for inter-day precision (%CV) was 4.98-10.80%. The range for intra-day accuracy (%different) for morachalcone A was 3.35 to 14.33%, and range for inter-day accuracy (%different) was 2.79 to 10.07%. These results showed that this method fulfilled acceptance criteria of FDA because these values were <15% (FDA, 2013).

Table 1: Intra and inter-day precision and accuracy of morachalcone A in rabbit plasma (n=5)

Assay	Concentration (ng/ml)		Precision	Accuracy
	Nominal	Observed (mean ±SD)	CV (%)	Different (%)
Intra-day	464.516	397.947 ± 25.935	6.52	14.33
	1470.968	1265.608 ± 49.166	3.88	13.96
	2477.419	2560.525 ± 181.292	7.08	3.35
Inter-day	464.516	417.741 ± 20.819	4.98	10.07
	1470.968	1429.971 ± 154.417	10.80	2.79
	2477.419	2305.863 ± 221.884	9.62	6.92

Table 2: Stability of morachalcone A in rabbit plasma at different storage conditions (n=3)

Storage condition	Concentration (ng/ml)		Different (%)
	Nominal	Observed	
Three freeze-thaw cycles at -20^0 C	2477.419	2088.691±272.723	8.03
	464.516	452.351±41.641	7.09
Room temperature for 24 hours	2477.419	2609.952±358.123	8.30
	464.516	500.505±60.746	14.23
Frozen (-20^0 C) for 30 days	2477.419	2482.221±1462.859	2.05
	464.516	461.880±23.564	6.77

Extraction recovery

The aim of extraction recovery was to ensure pertains to the extraction efficiency of an analytical method within the limits of variability. Extraction recovery from morachalcone A in plasma at 464.516, 1470.968, and 2477.419ng/ml were found to be 95.38±1.60; 84.45±3.25; and 80.291±1.55%, respectively. Extraction recovery from IS was 99.54±1.31%. Extraction recovery for both morachalcone A and IS were within 80-120% and fulfilled the acceptance criteria (Caufield and Steward, 2012).

Stability

Stability of morachalcone A in plasma (464.516 and 2477.419ng/ml) under different temperature and storage condition is showed in table 2. The result of stability test showed that morachalcone A in plasma were stable for all conditions, i.e. 24h stored at room temperature; 3 freeze/thaw cycles and 30 days stored at -20°C. The %different value was <15% and fulfilled the acceptance criteria from FDA (FDA 2013).

DISCUSSION

The developed method provided a specific assay and sensitive for morachalcone A in rabbit plasma. 4-hydroxychalcone was selected as internal standard because its core structure is similar with morachalcone A, and it can be separated from morachalcone A. Another study also used 4-hydroxychalcone as internal standard to develop analytical method to determine pharmacokinetic parameter of ezetimibe in human plasma (Bae *et al.*, 2012). Morachalcone A was detected at 368 nm using PDA detector to increase the signal of compound and reduce the signal of plasma interferents. Ratio and flow rate of mobile phases were optimized by several trials to obtain good resolution and symmetric peak for morachalcone A. This method was optimized by observe 4 chromatographic parameters: retention time, resolution, number of theoretical plate (N) and tailing factor of various compositions. Acetonitrile-water (50:50 v/v) and flow rate 1.0ml/min were selected as mobile phase with isocratic eluation because it is simple, easy to use and produces parameter which fulfilled the chromatography acceptance criteria (Kumar and Sunandamma, 2012). Method for sample clean up (to remove protein and interference) before sample was injected into HPLC was important also in the development of this method. A simple protein precipitation using methanol was employed in this study (Singh *et al.*, 2012). Methanol was used as solvent to precipitate protein in plasma because its efficiency in precipitating protein in human plasma has been reported >90% and morachalcone A was dissolve in methanol as well (Bueno *et al.*, 2011). This method also fulfills the acceptance criteria from FDA for bioanalytical method validation (FDA, 2013).

CONCLUSION

The HPLC method was valid for determination of morachalcone A in rabbit plasma (in vitro) and showed good chromatographic parameters including selectivity, linearity, sensitivity, accuracy, precision, stability and %recovery of extraction. The developed method also can be used to determine morachalcone A in rabbit plasma, thereby enabling to determine the bioavailability and pharmacokinetic parameter of *A. champeden* ethanolic extract using morachalcone A as active marker compound.

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