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LCMS/MS Metabolite Profiling and Analysis of Acute Toxicity Effect of the Ethanolic Extract of *Centella asiatica* on Zebrafish Model

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ABSTRACT

Centella asiatica or known as 'pegaga' in Malaysia, is a popular medicinal herb, which is being used as main ingredient or incorporated into various herbal products. Apart from efficacy, the chemical profile and potential toxic effect of the plant are two important aspects of concern towards ensuring product satisfaction and safety of consumers. This paper reports the qualitative and quantitative chemical analysis of the leaf ethanolic extract of *C. asiatica* using LCMS/MS. The acute toxiciy effect of the extract and selected marker chemical constituents were further analysed using a zebrafish model. Twenty constituents, were identified and the main chemical marker constituents of the plant viz asiaticoside, asiatic acid, and madecassic acid were further quantified. Asiaticoside was found to be present in higher concentration than the other marker constituents. Meanwhile in the acute toxicity test, the LD_{50} of the extract on the zebrafish model was determined to be 1250 mg/L while 100% mortality was observed at the highest test concentration of 2500 mg/L. However, acute toxicity

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evaluation on four marker triterpenoids of the herb, i.e asiatic acid, madecassic acid, asiaticoside and madecassoside, indicated them to be quite safe on the zebrafish model, with no mortality shown for test concentrations between 10 to 500 mg/kg BW.

Keywords: Acute toxicity, *Centella asiatica*, HPLC-DAD-LCMS/MS, metabolite profile, zebrafish model

INTRODUCTION

Plant secondary metabolites have numerous properties with potential applications as pharmaceuticals, agrochemicals, industrial chemicals, flavours and fragrances. Profiling these secondary metabolites, especially in cases where the metabolites are used in their original metabolome such as, extracts, resin or exudates, is an important element in ensuring productivity, efficacy and quality of the botanical product. There is already a growing commercial interest that is fostering the application of metabolite profiling technologies and development of metabolite profiles of various botanical products (Verpoorte et al., 2007). Meanwhile, drug discovery research is very intensive in terms of both costs and the time from discovery to development (Nguyen et al., 2014). Among the reasons is the fact that preclinical researches have been using mainly rodents as the translational animal model which have several limitations such as high costs, low throughput and time-consuming, among others (Stewart et al., 2014). A complementary model to the rodent model would certainly be a beneficial addition towards obtaining a better insight into the pathology of diseases.

The zebrafish is a new promising model in drug discovery research (Howe et al., 2013).This is due to several positive points about its use, including high physiological homology to human (70% of human gene have at least one obvious zebrafish orthologue), high throughput value, genetic tractability, low cost, sensitive to various drugs and pharmacological agents (such as ethanol) and have a short reproductive cycle (Stewart et al., 2013). The pennywort (*Centella asiatica* Linn), is a small, perennial, herbaceous creeper from the plant family Apiaceae. The genus *Centella* itself consists of 50 species which is well-distributed in tropical and subtropical regions of the world (Azerad, 2016). The species is native to tropical countries such as India, Sri Lanka, China, Indonesia, Malaysia, South Africa and Madagascar (Orhan, 2012). The plant is also known by several synonyms such as *C. coriacea* Nannfd*., Hydrocotyle asiatica* L*., H. lunata* Lam*.,* and *Trisanthus cochinchinensis* Lour. Meanwhile, the vernacular names of the plant are also many, such as Indian pennywort, brahma-manduki, brahmi-buti (India), tsubokusa (Japanese), tungchian, luei gong gen (China), gotu kola (Western), pegaga (Malaysia), *Hydrocotyle asiatique* (French), asiatischer wassernabci (German), idrocotile (Itali), bavilacqua (Mauritius) and blasteostimulina (Spain) (Gray et al., 2018). The evergreen herb have been reported to

grow well, mostly in moist, sandy or clay soils (Roy et al., 2009). The small, fan-shaped or round leaf, of about 1.4 to 1.7 cm in size, is the main vegetative part, used as vegetable and traditional medicine in various communities of the world. Based on the available literature, *C. asiatica* is a popular medicinal plant with reported uses ranging from treatment of skin problems (Azis et al., 2017), asthma and digestive disorders (Choudhury et al., 2015), hepatitis and syphilis (Xing et al., 2009), stomach ulcers, mental fatigue, diarrhoea, epilepsy (Kumar & Gupta, 2002), anxiety (Wijeweera et al., 2006) and for memory enhancement (Subathra et al., 2005; Soumyanath et al., 2012). Its dermatological use is the basis for its wide acceptance as an active ingredient in various commercially available cosmetic products (Schaneberg et al., 2003). In Malaysia, an ethnomedicinal survey of plants used by Orang Asli communities in the state of Perak, reported that the leaves were boiled to prepare an infusion used for postpartum treatment (Samuel et al., 2010). The plant is also a popular 'ulam' or raw vegetable side dish consumed as part of a healthy diet among the locals. *Centella asiatica* is also widely used in the treatment for epilepsy, for healing wounds and promoted as a healthy tonic drink in ancient times (Brinkhaus et al., 2000). The plant has also been prescribed in other traditional medicine systems such as Ayurveda (Kumar & Gupta, 2002), Traditional Chinese Medicine, Kampo Medicine and African traditional medicine for various related uses (Long et al., 2012). In Traditional Chinese Medicine, the plant is prescribed for dermal problems, heatstroke, diarrhoea, leprosy, hepatitis, jaundice, acute glomerulonephritis, diabetes and cerebrospinal meningitis (Luo et al., 2015; Hsu et al., 2015; Xia et al., 2015). In fact, *C. asiatica* is listed in the Indian Herbal Pharmacopoeia, and the Pharmacopoeia of the People's Republic of China, as well as in the European Pharmacopoeia (Schaneberg et al., 2003). The main chemical class of compounds found in *C. asiatica* is the pentacyclic triterpenoids present either in their free or glycosidic forms, asiatic acid, madecassic acid, asiaticoside and madecassoside (Figure 1), being a few examples (Shao et al., 2014). Apart from triterpenoids, flavonoids, phenolic acids and sterols (Orhan, 2012) as well as essential oils (Francis & Thomas, 2016) have also been reported.

Botanical products containing *C. asiatica*, either on its own or in combination with other herbs or medicinal plants, are now commonly found in the herbal and neutraceutical products market. Product quality, efficacy and toxicity are relevant issues that require regular and close monitoring so as to ensure the safety of consumers. This paper reports the LCMS/MS metabolite profile and the acute toxicity effect of the alcoholic extract of a locally grown *C. asiatica*, which are vital ground work for the ensuing research on standardization and pharmacological evaluation of the plant extract.

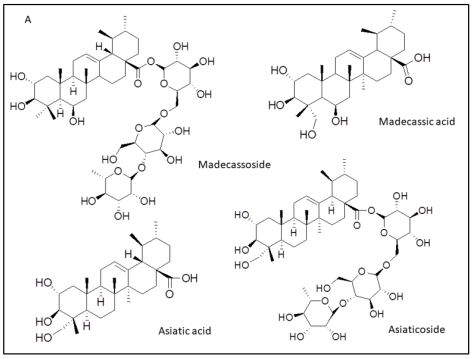

Figure 1. Triterpenoids found in *Centella asiatica*

MATERIALS AND METHOD

General Instrumentation

High performance liquid chromatography (HPLC) was performed on Agilent HPLC (1200 series, USA) system equipped with binary gradient pump and a UV detector. The system was controlled by a personal computer using Agilent Chemstation software. Liquid chromatography mass spectrometry (LCMS/MS) analysis was obtained using an ion-trap mass spectrometer equipped with an electrospray ionization (ESI) source, coupled to an Agilent HPLC equipped with a UV detector, binary gradient pumps, C-18 (4.0 x 250 mm I.D, 1.8 µm particle size, Agilent Technology), column oven, sample auto-injector, and controlled with Chemstation software.

Chemicals and Reagents. Analytical grade solvents (methanol and ethanol) were purchased from Fischer Scientific, Malaysia and HPLC grade acetonitrile was supplied by Merck, Germany. Ultrapure water with a resistivity greater than 18m, obtained from a certified Milli-Q system (Millipore, Bedford, MA, USA) was used for HPLC and LCMS/MS analysis. Asiaticoside, asiatic acid and madecassic acid (purity: 98%) were purchased from Sigma Aldrich (St. Louis, USA).

Plant material. The ethanolic leaf extract of *C. asiatica* was provided by Atta-ur-Rahman Institute for Natural Product Discovery (AuRINS), Universiti Teknologi MARA (UiTM) Puncak Alam, Selangor. A reference voucher specimen (CA-K017X) has been deposited at the Herbarium of AuRINS. The extract was prepared by maceration of powdered, air-dried leaves of *C. asiatica* in 70% aqueous ethanolic solution, filtered (Whatman filter paper No 1), dried in vacuo and further lyophilized. The final extract was kept at -20°C prior to use.

Zebrafish. Wild type, shortfin adult zebrafishes (*Danio rerio*), weighing 0.5±0.02 g, were used for all fish experiments. Zebrafish supply was obtained from 3B Aquatics (B.B.

Bangi, Selangor, Malaysia). Each time, upon delivery, the zebrafishes (mixed sex) were maintained at 26 ± 2°C, in holding water (aerated, dechlorinated tap water, pH 7.0 ± 0.5, total ammonia < 0.01 mg/l), on a 14:10 hours (light/dark) cycle. The zebrafishes were acclimatized for at least 7 days before utilized in the toxicity test. All zebrafishes used in the experiment were experimentally naive. They were fed with Tetramin Tropical fish flakes, daily (twice a day) except 24 hours prior to and until the end of experiment time. All fish experimental protocols were approved by Institutional Animal Care and Use Committee (IACUC) Universiti Putra Malaysia (Approval no: AUP-R013/2015). All experiments were conducted in a specially designated room for fish experiment where unwanted disturbances were kept to the minimum.

Qualitative and Quantitative HPLC Analysis

Chromatographic Conditions. Chromatographic separation was performed on an Agilent column (RP-18, 4.0 mm x 250 mm, 1.8 µm) by gradient elution with mobile phase system made up of water (solvent A) and HPLC grade acetonitrile (solvent B). The mobile phase composition (A:B) was held at 5:95 for the first 5 mins, gradually increased to 15:85 over the next 15 min, was again held constant at 15:85 for the ensuing 5 mins, and then changed to 85:5 in the final 5 mins. The injection volume and temperature were 20 µL and ambient temperature, respectively. The flow rate was kept at 1 mL/min throughout the analysis which was carried out at ambient temperature. The wavelength for peak detection was set to 210 nm. The gradient system was used for both qualitative and quantitative analysis.

Preparation of Standard and Sample Solutions. Standard stock solutions of asiaticoside, asiatic acid, and madecassic acid were prepared by accurately weighing and dissolving 2 mg of each standard in 1 mL HPLC grade methanol to give a concentration of 1 mg/mL (1000 ppm). Serial dilutions of 500 ppm, 250 ppm, 125 ppm and 62.5 ppm were then made using HPLC grade methanol solutions. A 1 mg/mL solution of the sample was also prepared in HPLC grade methanol. The prepared solutions were filtered through 0.22 µm filter membrane and the resultant filtrates submitted to HPLC analysis.

HPLC Method Development and Calibration. For each of the prepared standard solutions, triplicate HPLC runs were performed and the calibration curves for each was constructed by plotting mean area under curve versus concentration of the standards (µg/µl). The relative standard deviation of the content of each standard was obtained from repeated injections (n=3) of the sample solution. The recovery rate and accuracy of the method was determined by adding 62.5 ppm of each standard to 2000 ppm of the extract. The quantitative determination was carried out twice. Before the calibration curve was determined, the noise level of the system was first determined by running a blank sample

dissolved in methanol. The HPLC method was then validated for its specificity, linearity, accuracy, and sensitivity according to guidelines published by the International Conference on Harmonisation (ICH), 2009.

For assessment of linearity of the developed method, six concentrations of the standard solutions were prepared i.e 2000 ppm, 1000 ppm, 500 ppm, 250 ppm, 125 ppm and 62.5 ppm. Three individually prepared replicate of each concentration was analyzed. The linearity of the method was evaluated by visual inspection of the plot of peak areas as a function of anayte concentration i.e via determination of the regression equation, y = mx + c (where y = peak area, x + concentration of standard, c + y intercept) and coefficients (*R2*) corresponding to the standard compounds. The mean, standard deviation and relative standard deviation (RSD) values for each test concentration was calculated.

Limit of detection (LOD) was measured by conducting serial dilutions of the sample. The lowest concentration of the standard solution was determined by sequentially diluting the sample. The chromatogram was observed and the lowest detectable concentration and RSD was recorded. Six replicates for the lowest detectable concentration was prepared. From this replicates, the standard deviation of the signal obtained was calculated as below equation. Meanwhile the limit of quantitation (LOQ) is the concentration that gives a signal-to-noise ration of 10:1 (a peak with height at least 10 times as high as baseline noise level). The LOD and LOQ values were calculated based on the standard deviation of the response and the slope obtained from the linearity plot of each standard compound, as represented by the following equations:

$$LOD = \frac{3.3\,\delta}{S} \qquad LOQ = \frac{10\delta}{S}$$

where S is slope of calibration curve (x axis) and δ is standard deviation (y intercept from regression line) and S is slope of calibration curve.

Accuracy of the method was assessed from sample recoveries which was evaluated by spiking a known concentration of the mixed standards into the extract. Basically, to a prepared the sample solution, 62.5 ppm of each standards were added to 2000 ppm of the extract. The mixture was then subjected to HPLC analysis and the percent recovery calculated according to the following equation:

$$\text{Recovery} = \frac{detected\ amount}{added\ amount} = \frac{(A-B)}{C} * 100\%$$

where A is the amount of sample solution with spiked standards, B is the amount of sample solution, and C is the added amount of the standards.

Conditions for LCMS/MS Analysis

The LC column used for LC-MS/MS analysis was an Agilent C18 reverse phase column with dimensions 4.0 mm (id) x 250 mm (length) and 1.8 µm particle size. Column

temperature was maintained at 50°C. Sample elution was performed in a gradient manner using mobile phase comprising of water containing 0.1% acetic acid (solvent A) and HPLC grade acetonitrile containing 0.1% acetic acid (solvent B). The mobile phase composition (A:B) was gradually increased from 5:95 to 15:85 over 25 min and returned to initial condition (95%) for 5 min for solvent A, and 5% to 85% for 25 min and then decreased to initial condition (5:95) over the next 5 min, for a total LC run time of 30 min The injection volume was 20 µL and elution was with a constant flow rate of 1.00 mL/min.

For the mass analysis, the source conditions were: nebulizer pressure was 40 psi drying gas flow was set at 12 L/min and drying gas temperature was 350°C. Data acquisition was performed by Agilent MassHunter workstation Data Acquisition, while data processing was carried out with Masshunter Qualitative Analysis software. The MS acquisitions were performed in the positive and negative electrospray ionization mode, for the mass range of 50 to 1000 *m/z*. Additionally, MS/MS experiments were carried out in the automatic and multiple reaction monitoring (MRM) mode. Automatic MS/MS low-energy collision dissociation (CID) was performed at 5-8 eV collision energy. Peak identification was carried out based on comparison with literature values.

Zebrafish Acute Toxicity Study

Acute toxicity was evaluated in accordance with the Organization for Economic Cooperation and Development (OECD) guidelines for the Testing of Chemicals (Rufli, 2014).

Preparation of Test Solutions. Test solutions were prepared fresh each time. Stock solution of the extract was prepared by dissolving 10 g in 4 L distilled water to form a 2500 mg/L solution. From the stock solution of the extract, serial dilutions were made to prepare 1250, 625, 312.5, and 156.5 mg/L test concentrations. Since pure standard compounds were insoluble in water, test solutions of the compounds were prepared in phosphate-buffered saline (PBS) solution, with vortexing to homogenize the solution. In addition, based on the previous acute toxicity study by Duggina and co-workers (2015) who reported an LD_{50} value of 1000 mg/kg in rats for *C. asiatica* saponin fraction, the test concentrations for the pure compounds in the acute toxicity test were set to 10, 50, 100, and 500 mg/kg.

Acute Toxicity Test Procedure. Fifty adult zebrafishes were divided into five groups of ten fishes (n=10), to represent four test concentration groups and one control (distilled water) group. Plastic fish tanks of dimensions 20 cm (length) x 12.5 cm (width) x 11 cm (height) with a 2L volume capacity were used for the test. The tanks were filled to the brim with the respective test solutions. Each group of adult zebrafishes were then released into the respective tanks. Fish mortality was recorded after 2, 6, 24, 48, 72 and 96 hours. The LC_{50} (test concentration that kills 50% of the fishes) value was then determined from

a plot of number of mortality versus concentration. The test was a static test i.e no water or solution change after throughout the experiment.

Acute Toxicity Test Procedure for Pure Compounds. One hundred and seventy adult zebrafishes were divided into seventeeen groups, with ten fishes per group (n=10), to represent four test concentration groups for each of the four selected pure compounds, and one control (distilled water) group. Test solutions of the pure compounds were intraperitoneally injected to the zebrafishes, with an injection volume of 10 µl as all the fishes have similar body weight (0.5 ±0.02 g). After injection, the fishes were released into test chambers or tanks containing distilled water and their mortality recorded as before. Again, the test was a static test, thus the pH and temperature of the water in the test chambers were monitored daily.

RESULTS AND DISCUSSIONS

LCMS/MS Metabolite Profile of *Centella asiatica* Extract

The LCMS full scan and total ion chromatograms in the positive and negative modes are shown in Figure 2A to 2C. Careful analysis of the base peaks and MS/MS fragments obtained from the LCMS/MS analysis allowed the tentative assignment of twenty chemical constituents comprising several of the marker triterpenoids of *C. asiatica*, several phenolic acids and flavonoids. Tentative identification of major constituents existed in *C. asiatica* ethanolic extract were listed in Table 1. In the LC chromatogram, the peak at retention time (R_T) 2.45 min could be assigned to the presence of any of the three isomers chlorogenic acid, crypto-chlorogenic acid or neo-chlorogenic acid based on the $[M-H]^-$ at m/z 353.09 and fragment ion at m/z 191.06 for the loss of the caffeoyl moiety ($[M-C_9H_7O_3]$) in its MS/MS spectrum (Supplementary information). These isomeric phenolic acids, also known as caffeoylquinic acids (CQAs) have been reported previously as major constituents in *C. asiatica* (Abas et al., 2014; Alqahtani et al., 2015; Long et al., 2012). Meanwhile, dicaffeoylquinic acid (DCQA) was also detected at R_T 4.76 min, based on its $[M-H]^-$ ion at m/z 515.12 and fragment ion at m/z 191.06 ((Supplementary information). Similarly, the peak was assigned to any of the possible isomers of DCQAs, which also have been reported previously as constituents of *C. asiatica* (Roy et al., 2009).

Apart from phenolic acids, flavonoids are another group of compounds reported in *C. asiatica* (Gray et al. 2018; Krishnaiah et al. 2009). Both positive and negative ion modes were utilized to ionize the flavonoid glycosides and their aglycones, but the negative mode ESI was more sensitive for detection of the flavonoid glycosides in the extract. Quercetin and kaempferol were amongst the major flavonoids detected in the extract. The peak at R_T 6.33 min showed $[M-H]^-$ ion at m/z 301.04 (Supplementary information), which suggested it to be that of quercetin. This was supported by the $[^{1,3}B-H]^-$ ion at m/z 151.00 due to loss

of a $C_8H_6O_2$ radical (ring B). Similarly, the peak at R_T 7.39 min with $[M-H]^-$ at 285.04 could be assigned to kaempferol based on the $[^{1,3}B-H]^-$ and $[^{0,4}A-H]^-$ ions at m/z 133.03 and 108.02, respectively, due to losses of $C_8H_6O_2$ (ring B) and $C_4H_4O_2$ (ring A) radicals. Meanwhile, the flavonoid glycoside, rutin was detected by the peak at R_T 3.8 min with $[M-H]^-$ ion of m/z 609.15, with fragment ions at m/z 301.06 due to the $[M-H-Glc-Rha]^-$ ion. Other flavonoids detected were myricetin (R_T 0.55 min), castiliferol (R_T 3.06 min), patuletin (R_T 7.77 min) and naringin (R_T 13.69 min), tentatively identified based on their $[M-H]^-$ and MS/MS fragmentations. All these flavonoids have been reported previously to occur in *C. asiatica.*

It has been reported that the triterpenoid saponins (asiaticoside and madecassoside) and their aglycones, (asiatic acid and madecassic acid) were the most abundant constituents of *C. asiatica* (Gray et al., 2018). In the present study, these pentacyclic triterpenoid compounds were also detected. Asiatic acid was assigned to the peak at R_T 10.52 min, which showed an $[M-H]^-$ ion at ion of m/z 487.34 (Supplementary information). Loss of two molecules of H_2O gave the fragment ion at m/z 451 while a low abundance fragment ion at m/z 441.34 was assigned as a $[M-H-HCOOH]^-$ ion, in accordance with that reported by Xia et al. (2015). However, MS/MS experiment failed to produce any significant fragment ions. The peaks for asiaticoside and madecassic acid were detected at R_T 6.48 and 9.36 min, respectively. Asiaticoside exhibited an $[M-H]^-$ at m/z 487, at fragment ions at m/z 453.34, 407.33, which were due to the ions $[M-H-2Glc-Rha]^-$ and $[M-H-2Glc-Rha-HCOOH]^-$, respectively (Supplementary information). As was the case with asiatic acid, MS/MS experiment on the deprotonated ion of asiaticoside failed to produce any significant fragment ions for asiaticoside.

Madecassic acid, exhibited a pseudomolecular ion $[M+2Na-H]^-$ at m/z 549.34 (Supplementary information). Fragment ion at m/z 487 was attributable to $[M-2Na-H-HCOOH]^-$. MS/MS experiment on the $[M-H]^-$ ion of madecassic acid also failed to produce any significant fragment ions. The peak at R_T 5.91 min in the LCMS spectrum was tentatively identified as madecassosside based on the $[M-H]^-$ ion at m/z 973.50 (Supplementary information). MS/MS fragment ion at m/z 487.34 was due to $[M-H-3Glc]^-$ while the fragment ions at m/z 469.33 and 451.32 suggested a consecutive losses of 2 molecules of H_2O to give a $[M-H-3Glc-H_2O]^-$ and $[M-H-3Glc-2H_2O]^-$ ions, respectively. In addition to these chemical markers for *C. asiatica*, two other triterpenoid saponins were detected, tentatively identified as brahminoside B (R_T 3.54 min) and quadranoside IV (R_T 5.90 min), based on their $[M-H]^-$ and MS/MS fragmentations.

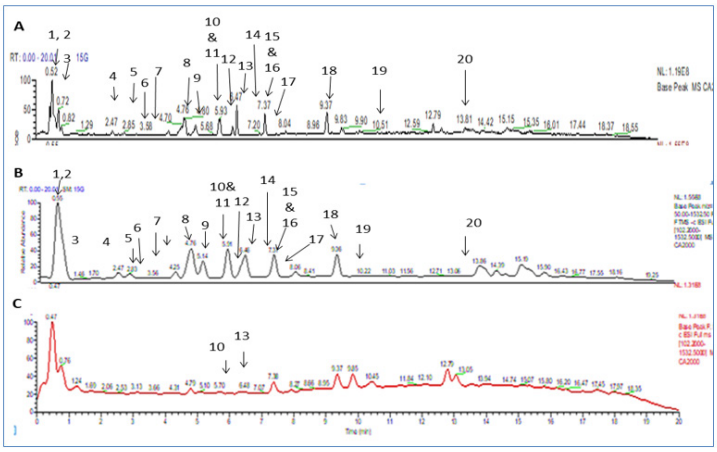

Figure 2. LCMS/MS chromatograms of leaf ethanolic extract of *Centella asiatica* extract, A: Full scan base peak B: Negative ion mode C: Positive ion mode.

Quantitative HPLC Analysis of Marker Triterpenoids of *Centella asiatica*

An HPLC method was developed for the quantification of several triterpenoid saponins in the leaf ethanolic extract of *C. asiatica*. To ensure the methodology used for HPLC quantification was reproducible, method validation was carried out using external calibration. The regression and coefficients (R^2) corresponding to the triterpenoids are given in Table 2. The calibration curves (Supplementary information) were linear over the selected concentration range (62.5 to 1000 ppm) with R^2 equal to or higher than 0.997. According to Shabir 2004, a method may be classified as precise, sensitive and accurate for quantification of compounds, if the precision is around 3% RSD and below, and when the LOD and LOQ is less than 15.

The contents of three *C. asiatica* marker triterpenoids (Table 3) were quantified using the validated HPLC method. The analytical HPLC run was completed in 40 mins. Under the optimized chromatographic conditions, sufficient separation was achieved for the compounds in the *C. asiatica* extract, as shown in Figure 3A. The three marker triterpenoids i.e asiaticoside, madecassic acid, and asiatic acid were detected at R_T 16.01, 20.46 and 22.72 min, respectively. Based on the chromatogram the triter penoids were not the major constituents of the extract. The chromatograms of the reference standards for confirmatory identification of asiaticoside, asiatic acid, and madecassic acid are shown in Figures 3B to 3D.

The contents of three *C. asiatica* marker triterpenoids (Table 3) were quantified using the validated HPLC method. The analytical HPLC run was completed in 40 mins. Under the optimized chromatographic conditions, sufficient separation was achieved for the compounds in the *C. asiatica* extract, as shown in Figure 3A. The three marker triterpenoids i.e asiaticoside, madecassic acid, and asiatic acid were detected at R_T 16.01,

Table 1

Tentative identification of major chemical constituents in leaf ethanolic extract of Centella asiatica.

No.	Tentative assignment	R_T (min)	Molecular Weight	$[M+H]^+$ (m/z)	$[M-H]^-$ (m/z)	Molecular formula	MS/MS fragment ions	References
1	Myricetin	0.55	318.24	-	317.05	$C_{15}H_{10}O_8$	290.05, 215.11	METLIN, ID 3448
2	Bornyl acetate	0.57	196.29	-	195.05	$C_{12}H_{20}O_2$	160.84, 119.80	NIST, No 413981
3	Catechin	0.71	290.27	-	289.02	$C_{15}H_{14}O_6$	133.01	Massbank : BS003015
4	CQA isomer	2.45	354.31	-	353.09	$C_{16}H_{18}O_9$	191.06	Massbank : FIO00626
5	Castilliferol	3.06	432.38	-	431.19	$C_{24}H_{16}O_8$	123.95, 103.92, 87.92	METLIN, ID : 50408
6	Rutin	3.80	610.52	-	609.15	$C_{27}H_{30}O_{16}$	301.06	Massbank: FIO00596
7	DCQA isomer	4.76	516.45	-	515.12	$C_{25}H_{24}O_{12}$	191.06, 179.03, 173.04	METLIN, ID : 87146
8	Ursolic acid	5.22	456.71	-	455.19	$C_{30}H_{48}O_3$	453.34, 407.33	METLIN, ID : 53791
9	Quadranoside IV	5.90	650.85	651.41	-	$C_{36}H_{58}O_{10}$	325.08, 183.99	Pubchem (CID 10372074)
10	Madecassosside	5.91	975.13	-	973.50	$C_{48}H_{78}O_{20}$	191.06	METLIN, ID : 94663
11	Quercetin	6.33	302.24	-	301.04	$C_{15}H_{10}O_7$	151.00, 121.03, 107.01	Massbank : BML82026
12	Asiaticoside	6.48	959.13	959.52	957.59	$C_{48}H_{78}O_{19}$	453.34, 407.33	Massbank : BS003708
13	Asiaticoside B	7.20	975.13	-	974.50	$C_{48}H_{78}O_{20}$	577.14, 361.19	METLIN, ID : 86571
14	Kaempferol	7.39	286.23	-	285.04	$C_{15}H_{10}O_6$	229.28, 150.04, 143.05	Massbank : BML81511
15	Campesterol	7.39	400.69	-	399.03	$C_{28}H_{48}O$	229.28. 183.04, 159.04	METLIN, ID : 167

Table 1 *(Continue)*

No.	Tentative assignment	R_T (min)	Molecular Weight	$[M+H]^+$ (m/z)	$[M-H]^-$ (m/z)	Molecular formula	MS/MS fragment ions	References
16	Patuletin	7.77	332.26	-	331.25	$C_{16}H_{12}O_8$	332.16, 277.18, 205.05	METLIN, ID : 51298
17	Madecassic acid	9.36	504.71	-	503.34	$C_{30}H_{48}O_6$	503.34	Massbank : BS003837
18	Asiatic acid	10.52	488.71	-	487.34	$C_{30}H_{48}O_5$	487.34	Massbank : BML80786
19	Naringin	13.69	580.54	-	579.28	$C_{27}H_{32}O_{14}$	225.01, 164.99	Massbank : TY000029

Table 2

Calibration parameters, linear ranges, limit of detection (LOD) and limit of quantification (LOQ) values for HPLC method for quantitative analysis of marker triterpenoid compounds in leaf ethanolic extract of Centella asiatica.

Reference standards	R_T (min)	Regreassion line equation	Linear range (mg/mL)	R^2	LOD (µg/mL)	LOQ (µg/mL)
Asiaticoside	*16.01*	*y=0.874x+10.715*	0.0625 - 1.0	0.997	2.8842	8.7400
Madecassic acid	20.46	y=0.4045x+12.95	0.189 - 1.27	0.999	1.3348	4.0450
Asiatic acid	22.72	y=2.7694x+73.41	0.0625-1.0	0.997	1.1915	3.6100

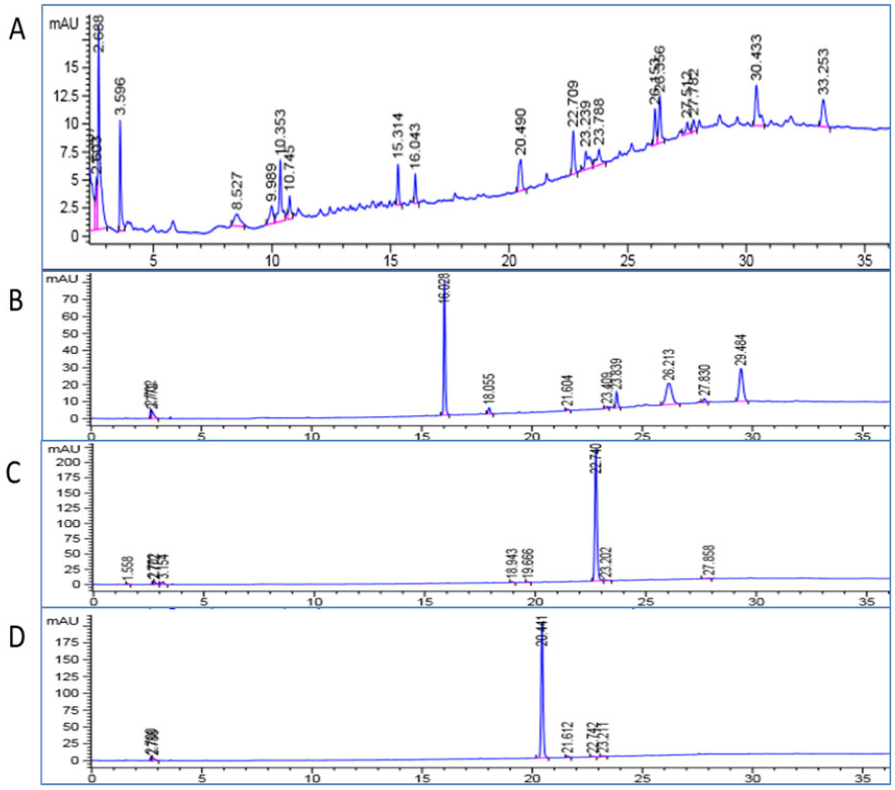

Figure 3. HPLC chromatogram of (A) leaf ethanolic extract of *Centella asiatic*a (500 µg/L) (B) asiaticoside (C) madecassic acid (D) asiatic acid, analyzed at 256 nm.

Table 3

Concentration of three marker triterpenoids in the leaf ethanolic extract of Centella asiatica (n=3)

Sample No	Asiaticoside (mg/g)	RSD (%)	Asiatic acid (mg/g)	RSD (%)	Madecassic acid (mg/g)	RSD (%)
1	0.61±0.01	1.60	0.39±0.002	0.51	0.46±0.01	2.17
2	0.63±0.003	0.47	0.40±0.002	0.5	0.49±0.002	0.41
3	0.59±0.001	0.17	0.39±0.01	2.50	0.47±0.001	0.21

Generally, asiaticoside and madecassoside are the most abundant triterpenoids found in *C. asiatica*, and the concentrations of these compounds may vary depending on the origin, environment, and time of harvest, as well as the processing methods used. In the present study, the range of concentrations for asiatic acid, asiaticoside and madecassic acid were between 0.39-0.40, 0.59-0.63, and 0.46-0.49 mg/g of extract, respectively. The amount of asiaticoside in was slightly higher compared to the content reported for the plant from India with 0.31 mg/g (Gupta, et al., 2014), and from Germany with 0.18-0.52 mg/g (Günther & Wagner, 1996). However, another recent study reported higher contents of the triterpenes in *C. asiatica* from Malaysia, with 7.9 and 11.5 mg/g of asiaticoside and 9.7 and 16.5 mg/g of madecassoside (Azerad, 2016). Due to unavailability of the reference standard, madecassoside was not quantified in the present study. However, the contents of the triterpenoid glycosides are usually reflective of the triterpenoid aglycones (Günther & Wagner, 1996). In the present study, the content of madecassic acid was found to be slightly higher than asiatic acid. Thus, in the same way, the content of madecassosisde in the present extract was also expected to be higher than that of asiaticoside.

Acute Toxicity Effect of *Centella asiatica* Extract on Zebrafish Model

The acute toxicity effect of the leaf ethanolic *C. asiatica* extract (RECA) on adult wild-type zebrafish was further assessed. Exposing the zebrafishes to the extract at test concentrations of 156.5 and 312.5 mg/L for up to 96 hours did not produce any mortality in the zebrafishes. However, at higher test concentrations, the mortality of the zebrafish model was affected. Half of the zebrafishes died at a test concentration of 1250 mg/L, while at the highest test concentration of 2500 mg/L, 100% death was recorded. From the dose-response curve shown in Figure 4, the LD_{50} for the extract was determined to be 1250 mg/L.

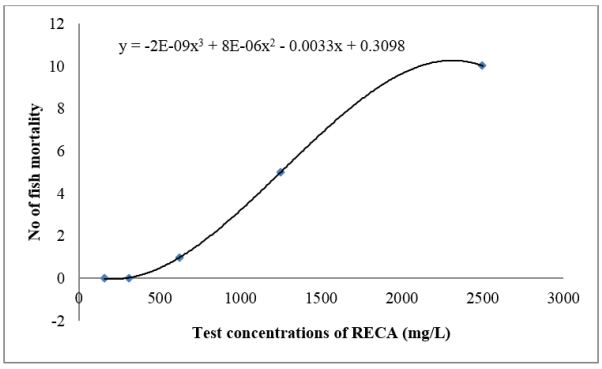

Figure 4. Dose-response curve for acute toxicity effect of leaf ethanolic extract of *Centella asiatic*a (RECA)

A previous experiment on rodents showed that up to 1000 mg/kg BW of *C.asiatica* water extract showed no mortality on the animals (Chivapat et al., 2011). An LD_{50} value of 1000 mg/kg on the rodents was reported for the triterpenoid saponin-rich fraction of the extract. Deshpande et al. (2015) also reported no toxicity on rodents when they were exposed for up to 14 days to a standardized water extract of *C. asiatica* at a test concentration of 2000 mg/kg BW. In another acute and sub-acute toxicity study of the acetone leaf extract of *C. asiatica*, it was found that up to 1000 mg/kg BW of oral administration did not cause any significant effect on behaviour, sensory nervous system, breathing and sensory nervous system response in mice (Chauhan & Singh, 2012).

The acute toxicuty results in the present study showed that as a test organism, zebrafish is more robust and sensitive compared to rodents. Since it is a smaller animal compared to rodents, a zebrafish's ability to detect toxicity of a substance is better because the range of toxicity can be better observed. Another advantage is that the experiment is easier to perform because the fishes need to be only immersed in the test solution, whereas in rodents, the test materials have to be administered orally or via injection. Nevertheless, a major drawback in using zebrafish as model for toxicity studies compared to rodents is that some pathological protocol may be quite difficult to perform due to the small size of the zebrafish's organs.

The acute toxicity effect of four marker triterpenoids of the extract, i.e asiatic acid, madecassic acid, asiaticoside and madecassoside, were also assessed. A ninety-six hours exposure to each of the compounds at test concentrations of 10 to 500 mg/kg did not cause any mortality in the adult, wild-type zebrafishes.

CONCLUSION

Toxicity evaluation is an important aspect of pharmacological research and the quality control of plant-based health products. Due its special characteristics, the zebrafish have become an emerging vertebrate model organism in toxicological and pharmacological studies because it offers a viable, quick and inexpensive alternative to test hypothesis and generate strategies for complementary research using rodent models and human subjects (Altemus et al., 2014; Greenleaf et al., 2014). In the present study, undertaken to determine the lethal concentration prior to its use in further experimental pharmacological studies, the standardized leaf ethanolic extract of *C. asiatica* affected the adult zebrafish survivability at concentrations above 1000 mg/kg BW for exposure up to 96 hours. This was in contrast to previous reports on the medicinal herb's non-toxic effect on rodents at similar range of test concentrations. However, the toxicity was not due to asiatic acid, madecassic acid, asiaticoside and madecassoside, the marker constituents which were qualitatively and quantitatively shown to be present in the ethanolic extract, based on the zero mortality

shown up to 500 mg.kg BW. It is possible that other constituents may be contributing to the toxicity of the extract.

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