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LC-ESI/MS and GC-MS Methanol Extract Analysis, Phytochemical and Antimicrobial Activity Studies of *Centella asiatica*

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Authors' contributions

This work was carried out in collaboration among all authors. Author DAO designed the study, did experiments, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors BFJ and LDB designed experiments, wrote the protocol, managed the analyses of data, literature surveys and wrote the manuscript. Author PKN did the bioassay experiments and analyses, managed the literature searches and wrote the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To determine chemical constituents of the Leaf extracts of *Centella asiatica* using the LC-MS and GC-MS and their antimicrobial activities.
Study Design: Structural determination of compounds from the leaf extracts was done using GC-MS and LC-MS analysis. The antimicrobial properties of the extracts were done using disc diffusion method.
Place and Duration of Study: Pure and Applied Chemistry Department, Masinde Muliro University of Science and Technology, Kenya: Between 2016-2019.
Methodology: Plant materials of *C. asiatica* were sequentially extracted separately based on the

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polarity *viz*., hexane, ethyl acetate and methanol. Determination of chemical constituents was done using LC-MS and GC-MS analysis and phytochemical screening. The extracts were assayed against *Staphylococcus aureus*, *Klebsiella pneumonia*, *Escherichia coli* and *Candida albicans*. Standard antimicrobials *viz*. ampicillin and Nystatin were used as the control. Disc diffusion method was used and zones of inhibition, after respective incubation periods, were used to quantify antibacterial and antifungal activity.

Results: Phytochemical screening of the hexane, ethyl acetate and methanolic extracts showed that terpenoids, flavonoids, saponins, alkaloids, steroids, amino acids and proteins, reducing sugars and carbohydrates were present. LC-MS and GC-MS analyses of the methanolic extracts identified 22 and 33 compounds, respectively, by use of the national institute of standards and technology (NIST) library. The extracts showed appreciable activity against common microbes tested.

Conclusion: This study forms the basis for the biological characterization and significance of the compounds identified in the leaf and stem extracts of *C. asiatica*. These compounds are known to possess antibacterial and antifungal activities that could be established as potential candidates for future drug development. However, these extracts, need to be subjected to further chromatographic procedures to isolate the identified compounds and their bioactivities determined.

Keywords: Antibacterial activity; antifungal activity; Centella asiatica.

1. INTRODUCTION

Centella asiatica (L.) Urban {synonym *Hydrocotyle asiatica* Linn} is a perennial herbaceous creeping plant that has found great significance in the traditional and current medicine in the Middle East, Southern Africa, Eastern Europe and Central Asia. The European Pharmacopoeia, the German Homeopathic Pharmacopoeia (GHP) and the Pharmacopoeia of the People's Republic of China all recognize this plant as a drug [1]. It belongs to the family Apiaceae (previously known as Umbelliferae) and to the genus *Centella* which comprises approximately 53 species [2,3]. *C. asiatica* has been used traditionally in Africa for the treatment of wounds, leprosy, throat infections, bronchitis, stomachic, steam massage formulations, etc [4-7]. In other parts of the world it has been used as a blood purifier, remedy for high blood pressure, for memory enhancement, revitalizing the nerves and brain cells (Ayurveda), treatment of emotional disorders (e.g. depression) and in wound healing [8-10]. This plant is known to possess a number of biological activities including neuroprotective activity, anti-inflammatory, antiulcer, hepatoprotective, anticonvulsant sedative immunostimulant, cardioprotective, antioxidant, antimicrobial amongst others [4,11-18]. A number of phytochemicals have been isolated from the various extracts of the plant. These include terpeneoids, phenolic compounds, polyacetylenes, alkaloids, carbohydrates, vitamins, mineral and amino acid [19-21]. The main group of compounds in *C. asiatica* is the triterpene saponins, examples being madecassoside, asiaticosides A to G, centelloside and brahmoside. Examples of flavonoids isolated include Quercetin and kaempferol. Phytosterols, amino acids and a bitter principle vallerin are also known components of the plant [2]. These isolated compounds have shown similar activities to those observed in the crude extracts albeit with varying levels of activity [22-25]. It has been suggested in some studies that asiaticoside content in extracts is responsible for dissolving the waxy coat of leprosy bacteria, thus exposing it to destruction by the immune system of the host [26,27]. This group of compounds is also regarded as phytoanticipins due to their antimicrobial activities and protective role against pathogenic infections [28]. In this work, chemical composition of the East African species was analyzed using LC-MS and GC-MS (Methanol extract) and phytochemical screening of the Hexane, ethylacetate and methanol extracts done using standard methods. Further, antimicrobial activity tests were done against the microbs, *Escherichia coli*, *Klebsiela pneumonia*, *Staphylococcus aureus* and *Candida albicans*.

2. MATERIALS AND METHODS

2.1 Collection and Plant Preparation

Fresh plant stem and leaf materials of *C. asiatica* were collected from Maseno University Arboretum, Vihiga County, Kenya. Identification was done at the herbarium, botany unit of the Department of Biological sciences of Masinde Muliro University of Science and Technology (MMUST) and stored (Voucher specimen

number MMUST/CA/001/2018). The plant materials were spread separately for uniform drying at room temperature and the dried plant material pulverized into fine powder using an electric grinder and stored in labeled airtight plastic bags, until when required.

2.2 Extractions

About 300 g of dried powdered plant material was soaked sequentially in Hexane, Ethyl acetate and methanol at room temperature for 24 hour, three times in each case. This was followed by filtration and solvent removal using a rotary evaporator under reduced pressure, to obtain the various extracts.

2.3 Analysis for Constituents

2.3.1 Phytochemical screening of *C. asiatica*

Qualitative phytochemical screening was done using standard methods:- Alkaloid contents by Dragendorf, Mayer and Wagner, Terpenoids and steroids by Salkowski and Liebermann-Burchard reaction, Flavonoids by Shinoda method, Proteins by Biuret and Million's test, Saponnins Foam test, Anthraquinones by Borntrageŕs, reducing sugars by Fehling's A and B and Benedict's and amino acids by Ninhydrin test [2, 29-32].

2.3.2 Gas chromatography - mass spectrometry (GC-MS) procedure

GC-MS analysis of the methanol extract *of C. asiatica* were performed using a perkin-Elmer GC clauses 500 system and Gas chromatograph interfaced to a mass spectrometer (GC-MS) equipped with an Elite-1, fused silica capillary column (30 m x 0.25 mm IDX 1 μ DF, composed of 100 % Dimethylpolysiloxane). For GC/MS detection, an electron ionization system with ionizing energy of 70 eV was used. Helium gas (99.999 %) was used as the carrier gas at constant flow rate 1 ml/min and an injection volume of 2 μl was employed (split ratio of 10:1), injector temperature 250 $^{\circ}$C; ion–source temperature 280 $^{\circ}$C. The oven temperature was programmed from 110 $^{\circ}$C (isothermal for 2 min) with an increase of 100 $^{\circ}$C/min to 200 $^{\circ}$C, then to 280 $^{\circ}$C, ending with a 9 min isothermal at 280 $^{\circ}$C. Mass spectra were taken at 70 eV; a scan interval of 0.5 seconds and fragments from 45 - 450 Da. Total GC running time was 40 min. The relative % amount of each component was calculated by comparing its average peak area to the total areas. Interpretation of mass spectrum of GC-MS was done using the database of National Institute Standard and Technology (NIST). This is similar to the method used by Neelamegam *et al*. [33] with some modification.

2.3.3 Liquid chromatography - mass spectrometry (LC-MS) procedure

Chromatographic and on-line mass spectral analyses of methanol extract of *C asiatica* were performed on an Acuity UPLC I-class system (Waters corp., Milford, MA). The UPLC system was interfaced by electro spray ionization to Synapt G2-Si QTOF-MS (WATERS) operated in full scan MS^E in positive mode. The Mass Lynx version 4.1 SCN 712 (Waters) Software was used for data acquisition and for qualitative and quantitative analysis. Ultra Performance Liquid Chromatographic Conditions: Column: (250 mm × 4.6 mm, 5 μm; ACE-18 column Advance Chromatography Technologies, Aberdeen, Scotland. Eluents: Formic acid in water A (0.01 % formic acid); Formic acid in Methanol B (0.01 % formic acid). Flow Rate: 0.2 μl/min. Injection: 0.2 μl. Calibration mass range: 50-1500-Da. Triple Quadruple Mass Spectrometric Conditions:- Ion source: ESI, positive. Source temperature: 100 $^{\circ}$C. Disolvation temperature: 350 $^{\circ}$C. Nebulizer pressure: 45 psi (N2). Nitrogen disolvation flow rate: 9 L min^{-1} (N2). Sampling cone voltage: 40 V. Fragmentor voltage: 130 V. Capillary voltage: 0.5k V. Scan range: *m/z* 100 - 700 (cycle time: 1S). Before quantitation both fragmentor voltage (from 70 to 140 V, with steps of 10 V) and collision energy (from 25 to 45 eV, with steps of 5 eV) were optimized by parameter ramping in the T-wave collision cell using ultrahigh purity argon (≥ 99.999 %) as the collision gas. Optimal setting for collision energy was 35 eV, and for fragmentor voltage, 130 V. Quantification was achieved in MRM mode. This is similar to the method by Shen et al. [34] with some modifications.

2.3.3.1 Analysis of the extracts for LC-MS

1 mg of each of the supplied standards were separately weighed and dissolved in 1 ml acidified methanol (+ 0.01 % formic acid) to make a stock solution (1 mg /ml) from which an experimental sample whose final concentration was 100 ng/μL was prepared using the same solvent. The samples of the methanol extract were analyzed on LC-QTOF-MS with the following condition. A mobile phase of water (A) and MeOH (B), each with 0.01 % formic acid was

employed. The following gradient was used 0-1.50 min, 95% A 5 % B; 1.50−2.50 min, 60% A 40 % B; 2.50−4.50 min, 60% A 40 % B; 4.50−6.00 min, 20% A 80 % B; 6.00-9.00 min, 20% A 80 % B; 9.00-12.00 min, 100 % B, 12.00-15.00 min, 100 % B; 15.00-16.50 min 100 % B, 16.50-20.00 min, 100% B. The flow rate was held constant at 0.2 □L / min. The injection volume was 0.2 *µ*L. Interpretation of spectra using the database of National Institute Standard and Technology (NIST).

2.4 Susceptibility Testing

2.4.1 Preparation of crude extracts for susceptibility testing

Samples for antibacterial and antifungal assays were prepared by dissolving 3 mg of each extract in 10 ml of DMSO to get a 300 *µ*g/ml concentration. This stock solution was used for further dilutions to 200, 150 and 100 *µ*g/ml with DMSO. Solutions of standard drugs (Ampicillin and Nystatin) in DMSO were prepared for positive control and DMSO as negative control. Susceptibility tests were performed using the disc diffusion method for the concentrations given [35,36].

2.4.2 Susceptibility testing for bacterial strains

Standard strains of *E. coli* (ATCC25922), *K. pneumoniae* (ATCC49620) and *S. aureus* (ATCC43320) were used for quality control. The strains were obtained from the Microbiology Laboratory of Masinde Muliro University of Science and Technology. All the test strains were maintained on nutrient agar slants (Oxoid, UK) at 4 ℃ and sub-cultured into Nutrient Broth for 24 hours at 37 ℃ prior to testing.

2.4.3 Susceptibility testing for fungal strains

The *in-vitro* tests of anti-fungal agents were similar in design to those of antibacterial agents. Standard fungal strain of *C. albicans* (ATCC90028) obtained from Microbiology Laboratory of Masinde Muliro University was maintained on Potato Dextrose Agar slant (Oxoid, UK) at 4 ℃ and sub-cultured into to Potato Dextrose Broth for 24 - 48 hours at 28 ℃ prior to testing.

3. RESULTS AND DISCUSSION

3.1 Phytochemical Screening of *C. asiatica*

Analysis of the extracts of *C. asiatica* revealed the presence of various classes of compounds (Table 1, 2 and 3). Constituents of this plant tended to be polar as most of them were picked by methanol. Hexane extracts tested positive for terpenoids and steroids. The ethyylacetate extract was found to possess alkaloids, flavonoids and steroids. Interestingly no mid polar terpenoids were found both in the plant leaves and stem. The methanol extract was found to be rich in all the compounds tested except anthraquinines. Saponins and Tannins were exclusive to this extract which could support the plant's reported medicinal properties. Tannins are known to possess antiviral activity, antibacterial and antiparasitic effects [2, 37]. Methanol extract also contained flavanoids, alkaloid and terpenoids.

Table 1. Qualitative analysis for secondary metabolites in *C. asiatica* hexane extracts

Phytochemicals	Test	Leaves	Stems
Alkaloids	Dragendroffs, Mayers, Wagner's	-	-
Proteins	Biuret test & Million's test	-	-
Flavonoids	Shinoda	-	-
Terpenoids & Triterpenoids	Salkowski , Liebermann-Burchard reaction	X	X
Tannins	5% Ferric chloride solution,10% Lead acetate solution & 10% Potassium dichromate solution	-	-
Steroids	Salkowski reaction & Liebermann-Burchard reaction	X	X

KEY: Compounds present (X); Compounds absent (-)

Table 2. Qualitative analysis for secondary metabolites in *C. asiatica* ethylacetate extracts

Phytochemicals	Test	Leaves	Stems
Alkaloids	Dragendroffs, Mayers, Wagner's	X	X
Proteins	Biuret test & Million's test	X	X
Flavonoids	Shinoda	X	X
Terpenoids& Triterpenoids	Salkowski ,Liebermann-Burchard reaction	-	-
Steroids	Salkowski reaction & Liebermann-Burchard reaction	X	X

KEY: Compounds present (X); Compounds absent (-)

Table 3. Qualitative analysis for secondary metabolites in *C. asiatica* methanol extracts

Phytochemicals	Test	Leaves	Stems
Alkaloids	Dragendroffs, Mayers, Wagner's	X	X
Proteins	Biuret test & Million's test	X	X
Flavonoids	Shinoda	X	X
Terpenoids & Triterpenoids	Salkowski ,Liebermann-Burchard reaction	X	X
Tannins	5% Ferric chloride solution, 10% Lead acetate solution &10% Potassium dichromate solution	X	X
Steroids	Salkowski reaction & Liebermann-Burchard reaction	X	X
Saponnins	Foam test	X	X
Anthraquinones	Borntrager's	-	-
Anthraquinone glycosides	Borntrager's	-	-
Reducing sugars	Fehling's A and B, Benedict's	X	X
Carbohydrates	Molisch's	X	X
Amino acids	Ninhydrin	X	X

KEY: Compounds present (X); Compounds absent (-)

3.2 GC-MS and LC-MS Analysis of *C. asiatica*

A total of 22 compounds (Table 4; Fig.1) were identified in LC-MS analysis of *C. asiatica* with the structural characterization being based on accurate mass and fragmentation patterns registered for the various chemical constituents. The classes of compound identified in this plant included Alkaloids (2), Alkylresorcinols (1), Anthocyanins (3), Aromatic amide (1), Flavonoid glycosides (4), Coumarin (1), Polyaromatics (1), Lignans (1), Phenolic acids (2), Phytosterols (1), Prenol lipids (1), Saturated fatty acid (1), Stilbenes (1), Triterpene Glycosides (1), Xanthones (1) and 10 other compounds that were not identified. Some of these classes of compounds have been mentioned in previous studies [36,37]. Structure determination of coumarin (Coumestrol), xanthone and anthrocyanins is unique to the current research. Coumestrol is a phytoestrogen known in the treatment of lupus and its existence in this plant gives valuable information to the therapeutic potential of *C. asiatica* in treatment of this condition [19,38-41]. The alkaloid structures Dioncopeltine A and Dipyridamole are also unique to the current work. Dipyridamole is an antiplatelet drug and helps in keeping blood flowing by stopping platelets from clumping together and keeping heart blood vessels open. Some of the most abundant compounds in this work were the glycoside Resveratol 3-O-glucoside (TR 7.222), an unidentified compound (RT 6.672), 5-Heptadecylresorcinol (RT 4.607) and 18Z-pentadecosenoic acid (RT 14.24). In the GC-MS analysis of the methanolic extract of *C. asiatica*, 32 chemical constituents (Table 5; Fig. 2) were identified by comparing their chromatograms and mass spectral data with those of reference standard compounds from NIST library. Groups of compounds identified included Alkaloids (2), Alkane hydrocarbons (3), Aromatic amines (1), Aromatic hydrocarbons (2), Carboxylic acid esters (1), Diterpenes (2), Fatty acids (11 – both saturated and unsaturated), sesquiterpenes (2), Ketones (2), Phytosterols (3) and Vitamin E (2). The most abundant compound was 9,12,15-octadecatrienoic acid,(z,z,z) (RT 24.8725) followed by Hexadecanoic acid (RT

23.176). Some of these compounds have been reported [42-45]. Identification of the alkaloid (+)-Norreticuline is unique to the current work.

3.3 Susceptibility Testing

The susceptibility of the test was based on microbial inhibition zones with strength values compared to the criteria for microbial susceptibility [46] (Table 7). Comparisons with t-test were made between the activity of the extracts and that of the control drugs (Table 6). The negative control DMSO recorded a zero inhibition for all the microbes tested with standard drugs.

3.3.1 Susceptibility tests for the leaf extracts of *C. asiatica*

Methanolic extract of *C. asiatica* leaves were active against the tested bacteria for the concentrations used. Mean inhibition zone against *S. aureus* range was 17 - 35 mm (Table 8), *K. pneumoniae* 13 - 26 mm (Table 9) while *E. coli* 15 - 23 mm (Table 10). A two tailed t-test for paired samples to compare the activities of different extracts and the standard drug ampicillin on microbes was computed. The results showed that methanol extract activities were not significantly different among the bacteria compared to that of the standard control drug ampicillin ($t = 0.5507$, $df = 2$, $p = 0.6371$ and $t_{crit} = 4.303$). The methanolic extracts of *C. asiatica* leaves had mean zone of inhibition in the range of 14 - 25 mm (Table 11) against *C. albicans* compared to that of the standard drug Nystatin 17 mm.

Ethyl acetate extracts of *C. asiatica* leaves recorded sensitivity with mean inhibition range between 12 - 26 mm for S. *aureus*, 15 - 33 mm for *K. pneumoniae* and 9 - 25 mm for *E. coli*. The ethyl acetate extracts activities were not significantly different among bacteria in comparison to Ampicillin ($t = 0.4132$, $df = 2$, $p = 0.7196$ and $t_{crit} = 4.303$). The ethyl acetate extracts of *C. asiatica* leaves had mean zone inhibition range between 13 - 25 mm against *C. albicans* in comparison to 17 mm for the standard drug Nystatin.

Hexane was active against *S. aureus* with a mean zone inhibition range between 15 - 32 mm for S. *aureus*, 17 - 33 mm for *K. pneumoniae* and 16 - 32 mm for *E. coli*. These activities were not significantly different among bacteria compared to the standard drug ampicillin ($t = 0.8968$, $df = 2$, $p = 0.4645$ and $t_{crit} = 4.303$). The hexane extracts of *C. asiatica* leaves had mean zone inhibition between 12 - 23 mm against *C. albicans* in comparison to 17 mm for Nystatin.

3.3.2 Susceptibility tests for stem of extracts of *C. asiatica*

Methanolic extracts of *C. asiatica* stems were active against bacteria with mean zone inhibition range between 16 - 32 mm against *S. aureus* (Table 8), *K. pneumoniae* 12 - 22 mm (Table 9) while *E. coli* was 15 - 21.5 mm (Table 10). These activities were not significantly different among bacteria with methanolic extract compared to that of standard control drug ampicillin ($t = 0.33$, $df = 2$, $p = 0.7726$ and $t_{crit} = 4.303$). The methanolic extracts of *C. asiatica* stems had mean zone inhibition range between 13 - 26.5 mm (Table 11) against *C. albicans* in comparison to 17 mm for Nystatin.

Ethyl acetate extracts of *C. asiatica* stems recorded sensitivity against *S. aureus* with mean inhibition range between 12.5 - 23.5 mm, *K. pneumoniae* 17 - 32 mm and *E. coli* 8 - 23 mm. These activities were not significantly different among bacteria as compared to that of control drugs ampicillin ($t = 0.233$, $df = 2$, $p = 0.8376$ and $t_{crit} = 4.303$). The ethyl acetate extracts of *C. asiatica* stems had a mean zone inhibition range between 12 - 30 mm against *C. albicans* in comparison to 17 mm for the standard drug Nystatin.

Hexane was active against *S. aureus* with mean zone of inhibition between 16 - 26 mm, *K. pneumoniae* 18 - 31.5 and *E. coli* 14.5 - 28 mm. These activities were not significantly different among bacteria in comparison to ampicillin ($t = 0.687$, $df = 2$, $p = 0.5631$ and $t_{crit} = 4.303$). The hexane extracts of *C. asiatica* stems had a mean zone inhibition between 15 - 25 mm against *C. albicans* as compared to 17 mm for Nystatin.

All the extracts of *C. asiatica* were able to inhibit *S. aureus* a bacteria that is common in human skin infection causing boils and food poisoning thus affecting the digestive system [47]. *S. aureus* is among microbes that have exhibited resistance to synthetic drugs such as the Methicillin Resistant *Staphylococcus aureus* (MRSA) which is a major nosocomial pathogen causing serious morbidity and mortality in immunosuppressed patients [48].

Table 4. LC-MS results of *C. asiatica* methanol leaf extract

	Compound	TIC $t_{R(min)}$	M + $H_{(M/Z)}$	CID Product ions $_{(M/Z)}$	Molecular Formula
	Alkaloid				
1	Dioncopeltine A	1.148	380	274,122	$C_{23}H_{25}NO_4$
2	Dipyridamole	4.266	504	265,186,142	$C_{24}H_{40}N_8O_4$
	Alkylresorcinols				
3	5-Heptadecylresorcinol	4.607	349	163	$C_{23}H_{40}O_2$
	Anthocyanins				
4	Delphinidin 3,5-O-diglucoside	5.153	628	409,307	
5	Delphinidin 3-O-glucosyl-glucoside	5.226	628	413,307	$C_{27}H_{31}O_{17}$
6	Pelargonidin-3-O-(6”-malonyl-glucoside)	12.8	520	375,353,243	$C_{24}H_{23}O_{13}$+
	Aromatic Amides				
7	N-(,4-Bis{1,2-(hydraxinylidenemethyl)hydrzinylidene ethyl}phenyl)decanamid	6.879	444	349,163	ND
	Flavonoid Glycosides				
8	Isorhamnetin 3-O-rutinoside	6.992	463	287,145	$C_{28}H_{32}O_{16}$
9	Kaempferol 7-O-glucoside	5.082	448	402,307	$C_{21}H_{20}O_{11}$
10	Dihydroquercetin 3-O-rhamnoside	7.417	451	333,145	$C_{21}H_{22}O_{11}$
11	Isorhamnetin 3-O-glucuronide	14.49	493	441,163	$C_{22}H_{20}0_{13}$
	Coumarin				
12	Coumestrol	4.689	267	163	$C_{15}H_8O_5$
	Polyaromatic				
13	6,13-Dihexyl-2,3,9,10-termethylpentacene-1,4,8,11-tetrone	4.988	563	411,307,209	$C_{38}H_{42}O_4$
	Lignans				
14	Todolactol A	4.432	377	163,145	$C_{20}H_{24}O_7$
	Phenolic Acid				
15	Dihydrocaffeic acid	1.003	183	139	$C_9H_{10}O_4$
16	Cinnamoyl glucose	4.028	311	292,166	$C_{15}H_{18}O_7$
	Phytosterols				
17	Stigmasterol	14.08	413	413,	$C_{29}H_{48}O$
	Lipids				
18	Delta-carotene-1,2-epoxide	11.27	553	453,317,203	$C_{40}H_{56}O$
	Saturated Fatty Acid				
19	18Z-pentadecosenoic acid	14.24	381	341	$C_{25}H_{48}0_2$
	Stilbenes				
20	Resveratol 3-O-glucoside	7.22	391	309,189,171	$C_{20}H_{22}O_8$
	Triterpene Glycoside				
21	Ziziphin	7.963	981	453,291,154	$C_{51}H_{80}O_{18}$
	Xanthones				
22	8-Desoxygartatin	1.24	381	248,203,182	$C23H_{24}O_5$
	Not Determined				
23	Unknown	4.906	460	307,186	ND
24	Unknown	6.339	499	337,163	ND
25	Unknown	6.475	499	349,289,163	ND
26	Unknown	6.672	502	303,186,131	ND
27	Unknown	7.561	981	635,331,287	ND
28	Unknown	8.057	965	301	ND

	Compound	TIC $t_{R(min)}$	M + $H_{(M/Z)}$	CID Product ions $_{(M/Z)}$	Molecular Formula
29	Unknown	8.718	1031	527,451	ND
30	Unknown	11.45	1023	699,529,317	ND
31	Unknown	11.67	861	699	ND
32	Unknown	13	677	496,163	ND
34	Unknown	13.19	496	377	ND
35	Unknown	14.97	607	ND	ND

Table 5. GC-MS results of *C. asiatica* methanol leaf extract

S. no	Name of compound	RT	Molecular formula	MW
	Alkaloid			
1	(+)-Norreticuline	21.3391	$C_{18}H_{21}NO_4$	315.37
2	6-Azaestra-1,3,5(10),6,8-pentaen-17-one,-3-methoxy-	32.4892	$C_{18}H_{19}NO_2$	281.36
	Alkane Hydrocarbon			
3	Tetracosane	20.2392	$C_{24}H_{50}$	338.66
4	Pentadecane,3-methyl-	20.5844	$C_{16}H_{34}$	226.45
5	Heptadecane ,9-octyl-	21.0583	$C_{25}H_{52}$	352.68
	Aromatic Amine			
6	1-Naphthalenamine,N-methyl-	24.1354	$C_{11}H_{11}N$	157.22
	Aromatic Hydrocarbon			
7	Benzene,1-(1-Buten-3-yl)-2-vinyl-	23.6089		158.24
8	Phenol,2,6-bis(1,1-dimethylethyl)-	18.3205	$C_{16}H_{26}O$	234.38
	Carboxylic Acid Esters			
9	Oxalic acid, bis(6-ethyloct-3-yl) ester	18.6422	$C_{22}H_{42}O_4$	370.57
	Diterpene			
10	Phytol	24.6209	$C_{20}H_{40}O$	120.17
11	Phytol, acetate	21.9533	$C_{22}H_{42}O_2$	
	Fatty Acid Ester			
12	Methyl linoleate	24.4513	$C_{19}H_{34}O_2$	294.48
13	Octadecanoic acid,2,3-dihydroxypropyl ester	29.5467	$C_{21}H_{42}O_4$	358.56
14	9,12,15-octadecatrienoic acid, methyl ester,(z,z,z)-	22.9771	$C_{19}H_{32}O_2$	292.46
15	7,10,13-Hexadecatrienoic acid, methyl ester	24.5156	$C_{17}H_{28}O_2$	264.40
16	Methyl8,11,14,17-eicosatetraenoate	22.5851	$C_{21}H_{34}O_2$	318.49
17	Methyl octadecanoate	24.7321	$C_{22}H_{44}O_2$	298.51
18	Hexadecanoic acid, ethyl ester	23.486	$C_{18}H_{36}O_2$	284.48
19	Tetradecanoic acid	21.1343	$C_{14}H_{28}O_2$	228.38
20	Hexadecanoic acid	23.176	$C_{16}H_{32}O_2$	256.43
21	Octadecanoic acid	25.0421	$C_{18}H_{36}O_2$	284
22	9,12,15-octadecatrienoic acid,(z,z,z)-	24.8725	$C_{18}H_{30}O_2$	278
	Ketone			
23	2-Tridecanone	18.1508	$C_{13}H_{26}O$	198.35
24	4-Hexen-2- one,3,3-diethyl-4,5-dimethyl-	21.0115	$C_{12}H_{22}O$	182.30
	Phytosterols			
25	Stigmasterol	35.736	$C_{29}H_{48}O$	412.70
26	Beta-sitosterol	36.6428	$C_{29}H_{50}O$	414.72
27	Taraxasterol	38.029	$C_{30}H_{50}O$	426.73
	Prenol Lipids			
28	Gamma-Elemene	17.3844	$C_{15}H_{24}$	204.36
	Sesquiterpene			

S. no	Name of compound	RT	Molecular formula	MW
29	1H-Cycloprop(E) azulene,1a,2,3,4,4a,5,6,7b-octahydro-1,1,4,7-tetramethyl-	17.9402	$C_{15}H_{24}$	204.16
30	Naphthalene,1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-,(1s-cis	18.5252	$C_{15}H_{24}$	204.35
	Vitamin E			
31	Alpha-Tocopherol	33.7646	$C_{29}H_{50}O_2$	430
32	Gamma-Tocopherol	33.8172	$C_{28}H_{48}O_2$	416.68

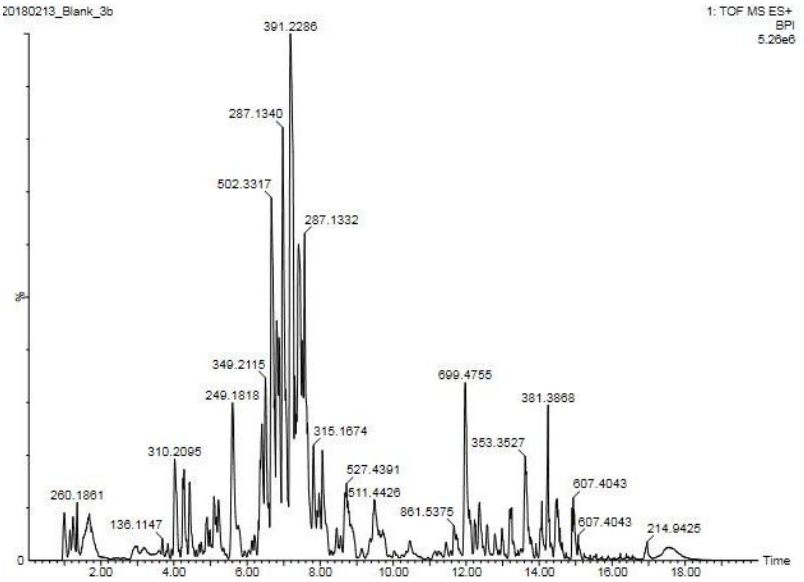

Fig. 1. Total Ion Content (TIC) Chromatograms of UPLC-QTOF-MS Methanol Extracts of *C. asiatica*

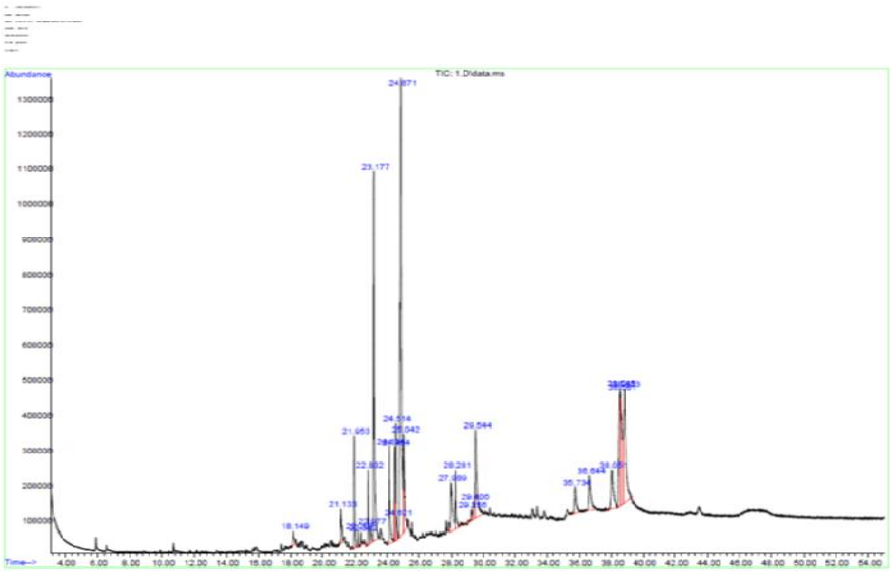

Fig. 2. Total Ion Content Chromatogram (TIC) for GC-MS Methanolic Extracts of *C. asiatica*

Table 6. Inhibition zones with standard drugs

Microbes	Standard Drugs/Zones of Inhibition (mm)		Negative Control
	Ampicillin	Nystatin	DMSO
S. aureus	30		0
K. pneumonia	20		0
E. coli	6		0
C. albicans		17	0

Table 7. Criteria for microbial susceptibility

Diameter (mm)	Activity
9-12	Non-significant
13-15	Low
16-18	Good
Above 18	Significant

Table 8. Inhibition zones for *C. asiatica* extracts against *S. aureus*

Plant material	Solvent	50 µg/ ml	150 µg/ ml	300 µg/ ml	600 µg/ ml
C. asiatica leaves	Hexane	15±0	22±0.24	27±0.25	32±0.29
	Ethyl acetate	12±0.24	21±0.25	24±0.24	26±0.25
	Methanol	17±0	22±0.25	30±0.24	35±0.24
C. asiatica stems	Hexane	16±0.24	22±0	25±0.29	26±0.24
	Ethyl acetate	12.5±0.25	20±0.24	23±0.29	23.5±0.24
	Methanol	16±0	23±0.29	30±0.24	32±0.24

Table 9. Inhibition zones for *C. asiatica* against *K. pneumonia*

Plant material	Solvent	50 µg/ ml	150 µg/ ml	300 µg/ ml	600 µg/ ml
C. asiatica leaves	Hexane	17± 0	23± 0.24	33±0.41	33± 0.24
	Ethyl acetate	15± 0	22± 0.62	30± 0.47	33± 0.41
	Methanol	13± 0.41	18± 0.24	22± 0.41	26± 0.82
C. asiatica stems	Hexane	18± 0.24	22± 0	30± 0.62	31.5± 0.24
	Ethyl acetate	17±0.71	20± 0.41	28± 0.71	32± 0.47
	Methanol	12± 0.47	17± 0	20± 0.47	22± 0.41

Table 10. Inhibition zones for *C. asiatica* extracts against *E Coli*

Plant material	Solvent	50 µg/ ml	150 µg/ ml	300 µg/ ml	600 µg/ ml
C. asiatica leaves	Hexane	16± 0	20.5± 0.79	30± 0.24	32± 0.29
	Ethyl acetate	9± 0.82	16± 0.78	22±0.24	25±0.24
	Methanol	15± 0.24	16± 0.29	20±0.29	23± 0.24
C. asiatica stems	Hexane	14.5± 0.29	22± 0.1	28± 0.82	28.5± 0.76
	Ethyl acetate	8± 0	15± 0.05	19± 0.82	23± 0.29
	Methanol	15± 0.24	17± 0.25	19.5± 0.76	21.5± 0.29

Table 11. Inhibition zones for *C. asiatica* extracts against *Candida albicans*

Plant part	Crude extract	50 µg/ ml	150 µg/ ml	300 µg/ ml	600 µg/ ml
C. asiatica leaves	Hexane	12± 0.41	17± 0.41	20± 0	23± 0.29
	Ethyl acetate	13± 0.41	20± 0.41	24± 0.24	25± 0
	Methanol	14±0	20±0.82	23± 0.41	25± 0
C. asiatica stems	Hexane	15±0.71	17±0	22± 0	25± 0.41
	Ethyl acetate	12± 0.71	22±0	27±0.47	30± 0
	Methanol	13± 0.71	19±0.41	22± 0.41	26.5±0.24

4. CONCLUSION

The leaf and stem extracts of *C. asiatica* contains Alkaloids, proteins, flavonoids, terpenoids, tannins, steroids, saponins, reducing sugars , carbohydrates, amino acids, steroids. Main compounds observed include Terpenoids and flavonoids, which are known to contain varied medicinal properties. From GC-MS and LC-MS analysis 22 and 32 compounds, respectively, were established with structure of some of these reported for the first time. The antimicrobial assays revealed that the extracts of *C. asiatica* were active against *S. aureus*, *K. pneumonia*, *E. coli* and *C. albicans*. Further studies will be undertaken aimed at isolating compounds and their individual antibacterial and antifungal activity studied. Synergy studies also need to be undertaken for future drug formulations with related plants to *C. asiatica*.

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DISCLAIMER

The products used for this research are commonly and predominantly used products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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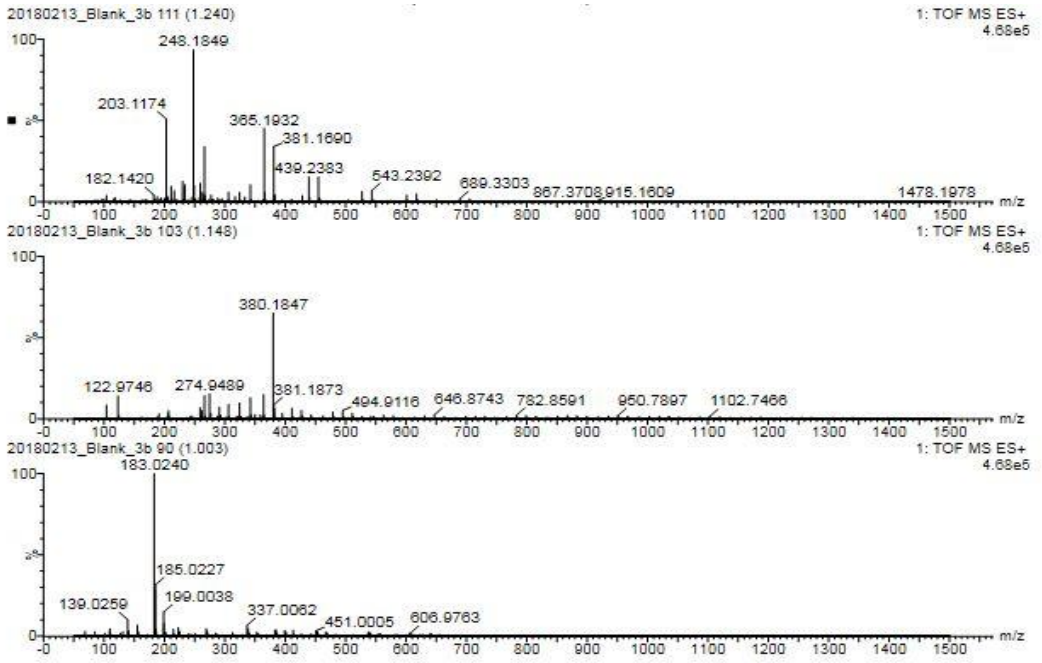
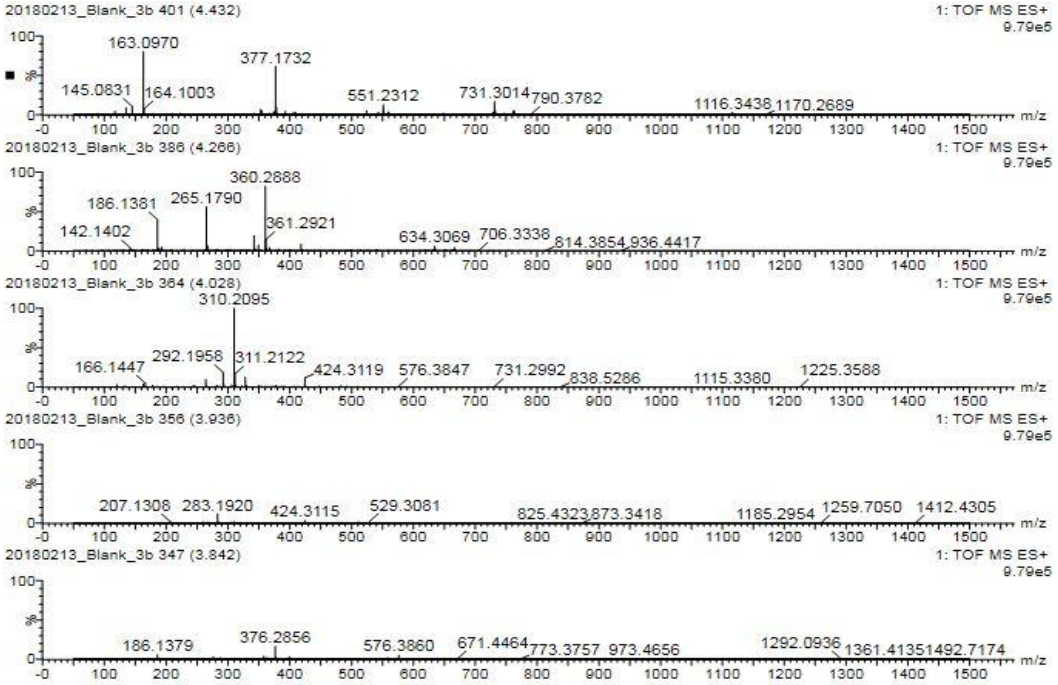
APPENDICES

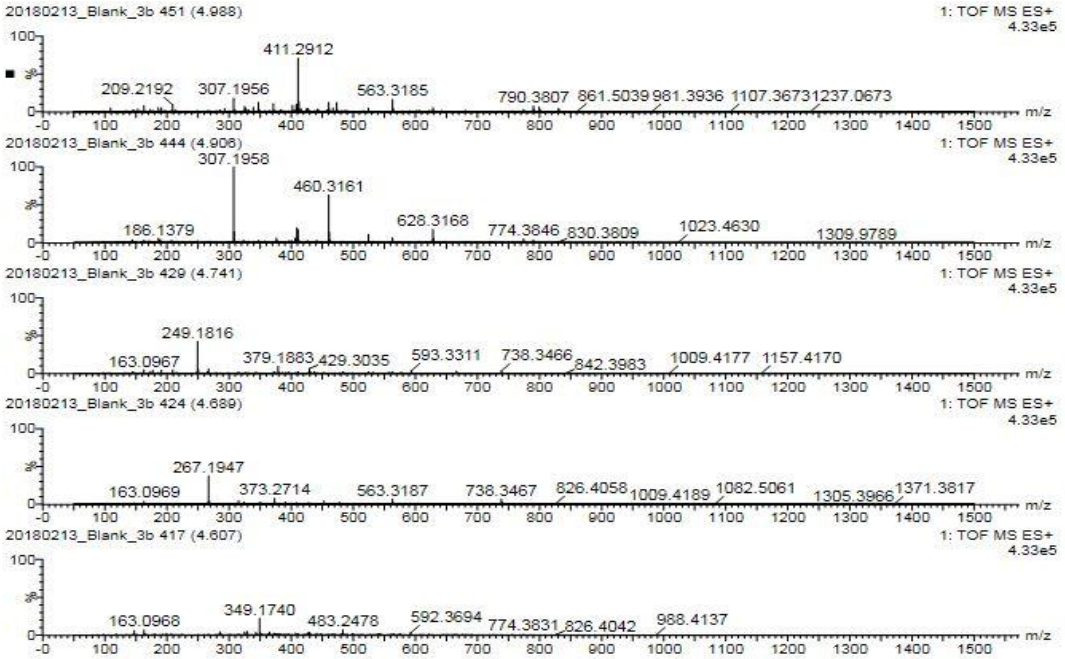
Appendix 1. ESI-MS Positive Ion Spectra for *C. asiatica* R_t = 1.003 -1.240



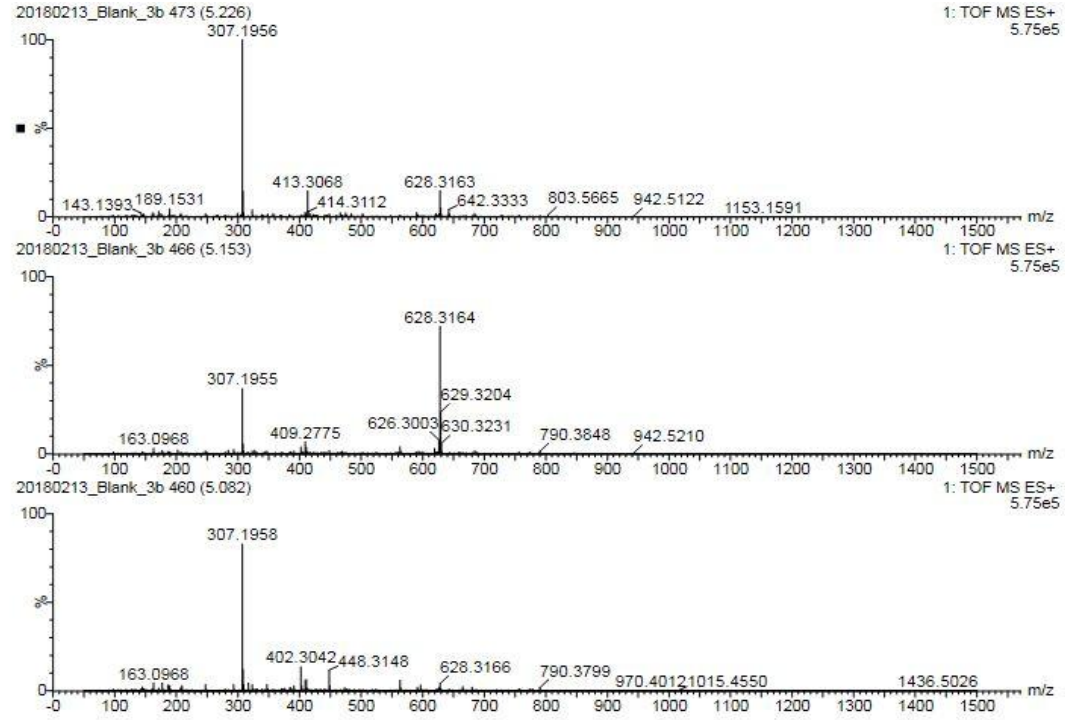
Appendix 2. ESI-MS Positive Ion Spectra for *C. asiatica* R_t = 3.842 - 4.432



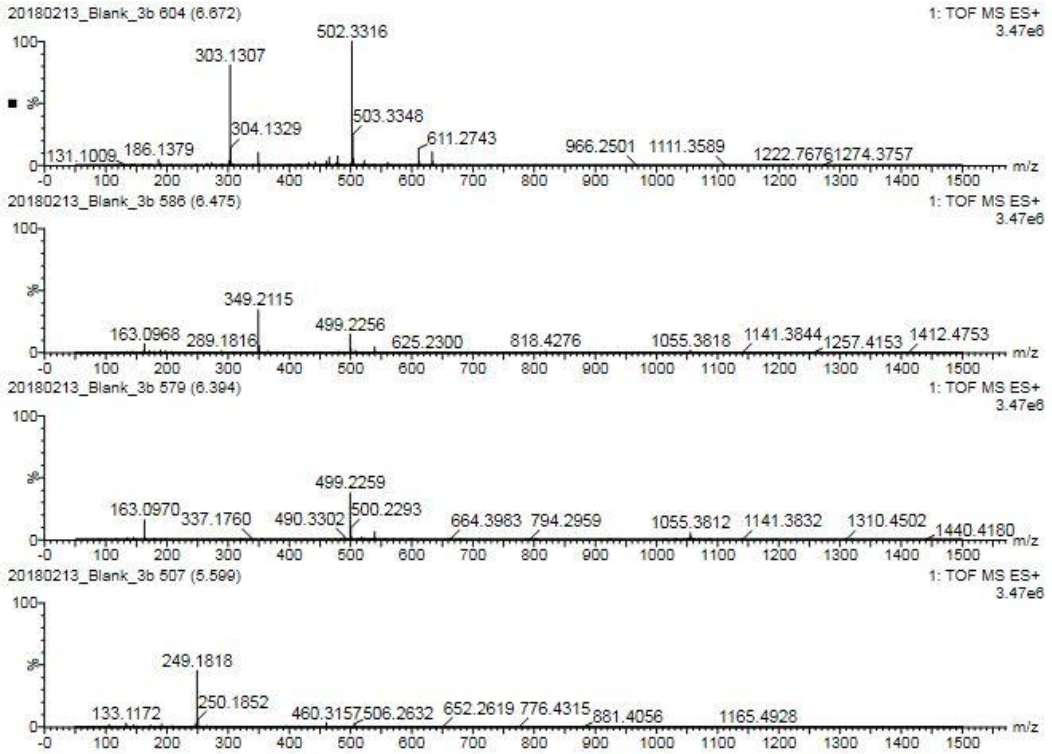
Appendix 3. ESI-MS Positive Ion Spectra for *C. asiatica* R_t = 4.607 - 4.988



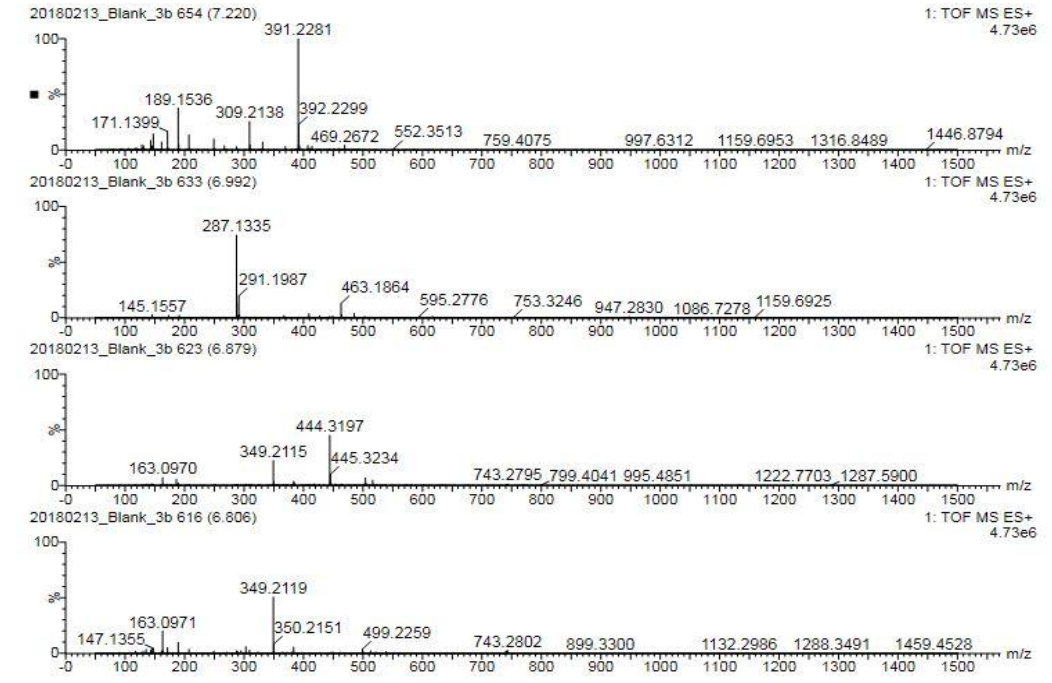
Appendix 4. ESI-MS Positive Ion Spectra for *C. asiatica* R_t = 5.602 -5.226



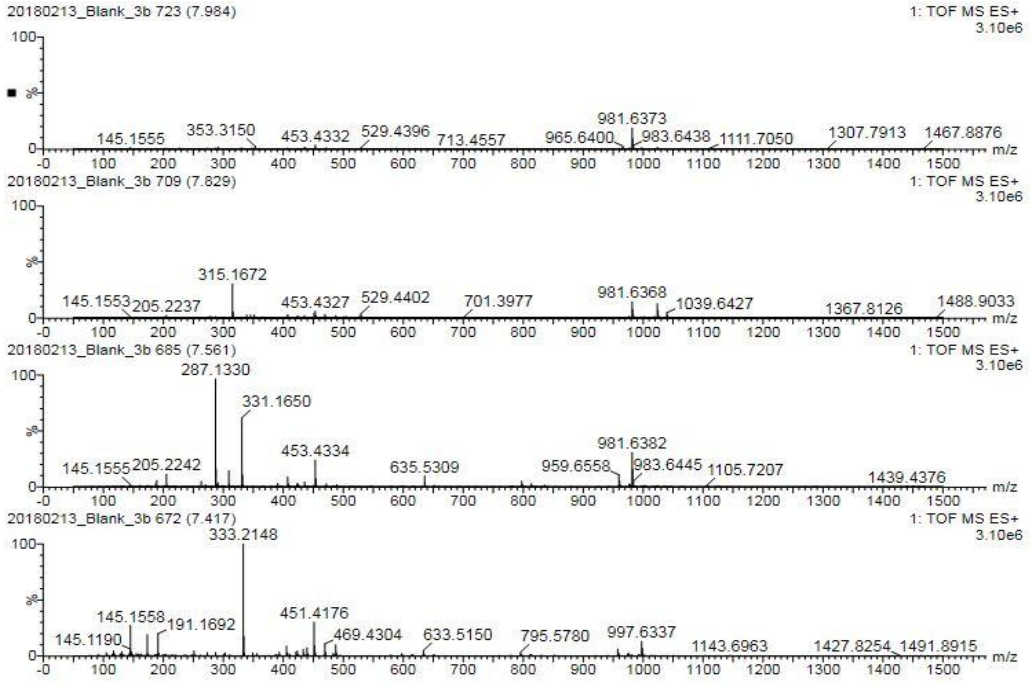
Appendix 5. ESI-MS Positive Ion Spectra for *C. asiatica* R_t = 5.599 - 6.672



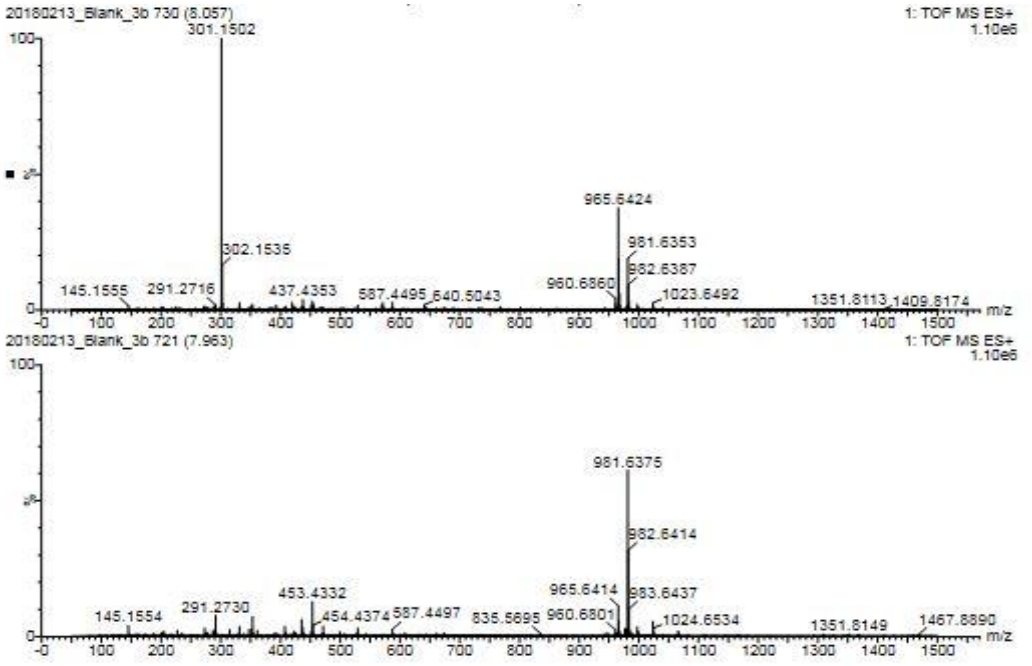
Appendix 6. ESI-MS Positive Ion Spectra for *C. asiatica* R_t = 6.806 -7.220



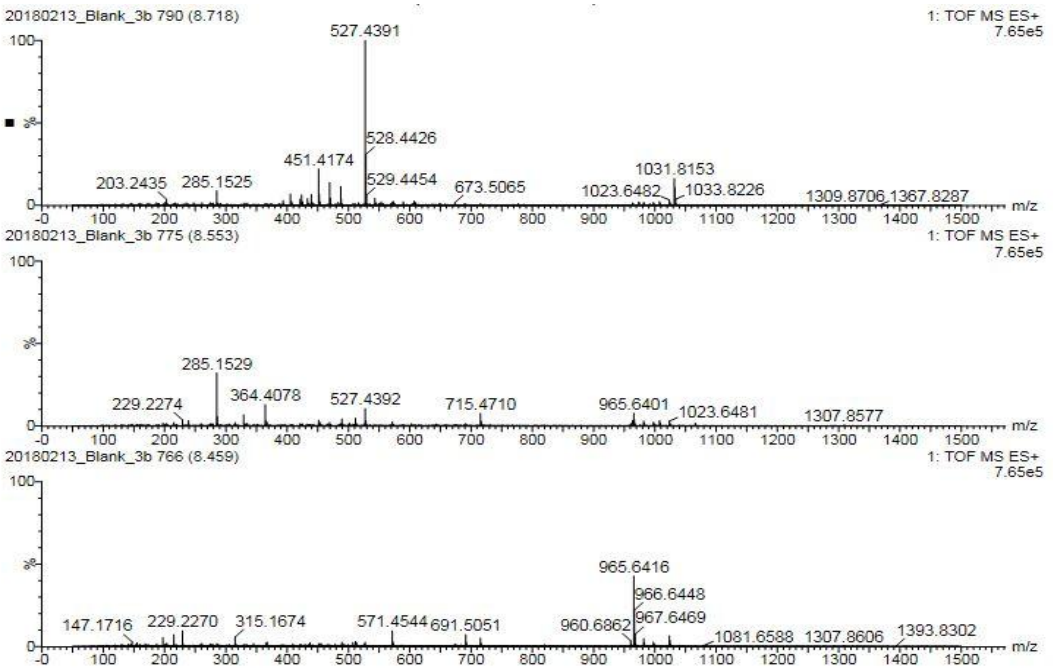
Appendix 7. ESI-MS Positive Ion Spectra for *C. asiatica* R_t = 7.417 -7.984



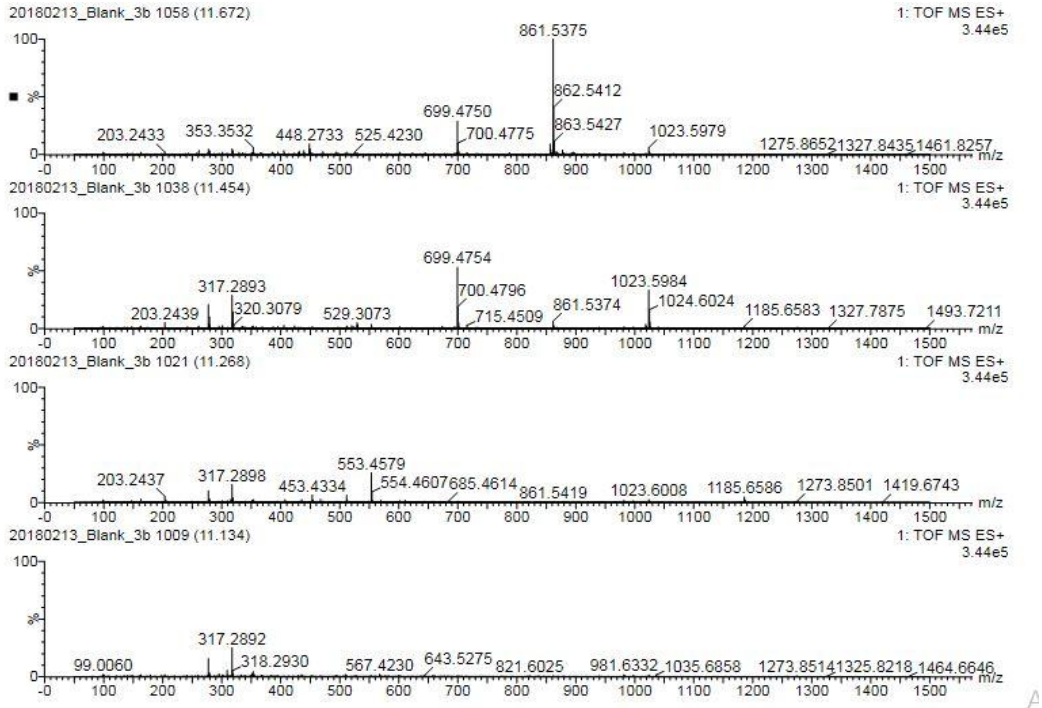
Appendix 8. ESI-MS Positive Ion Spectra for *C. asiatica* R_t = 7.963 - 8.057



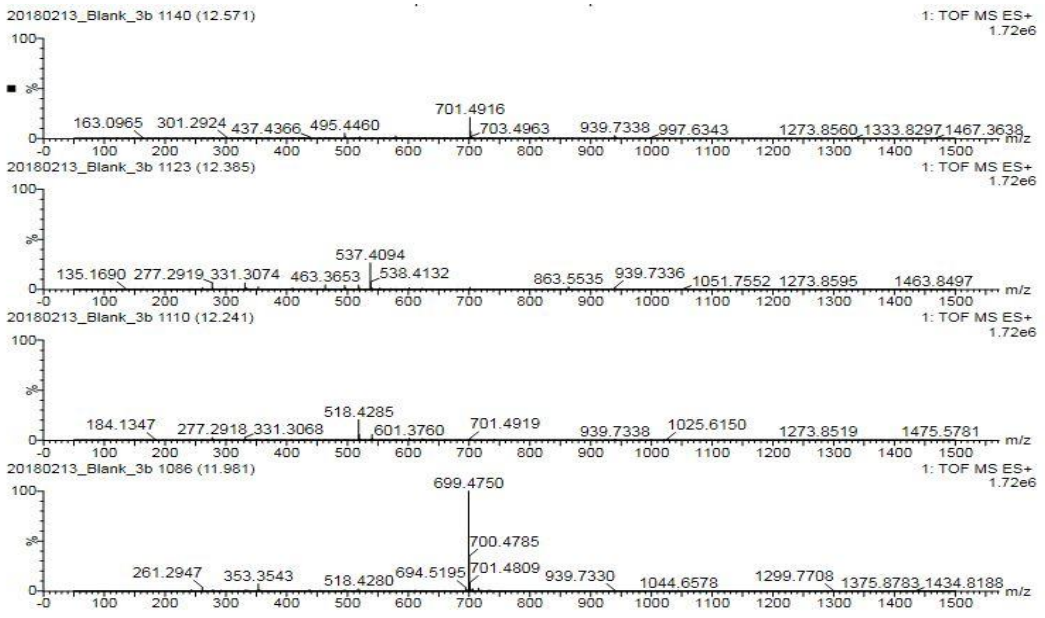
Appendix 9. ESI-MS Positive Ion Spectra for *C. asiatica* R_t = 8.4599 - 8.718



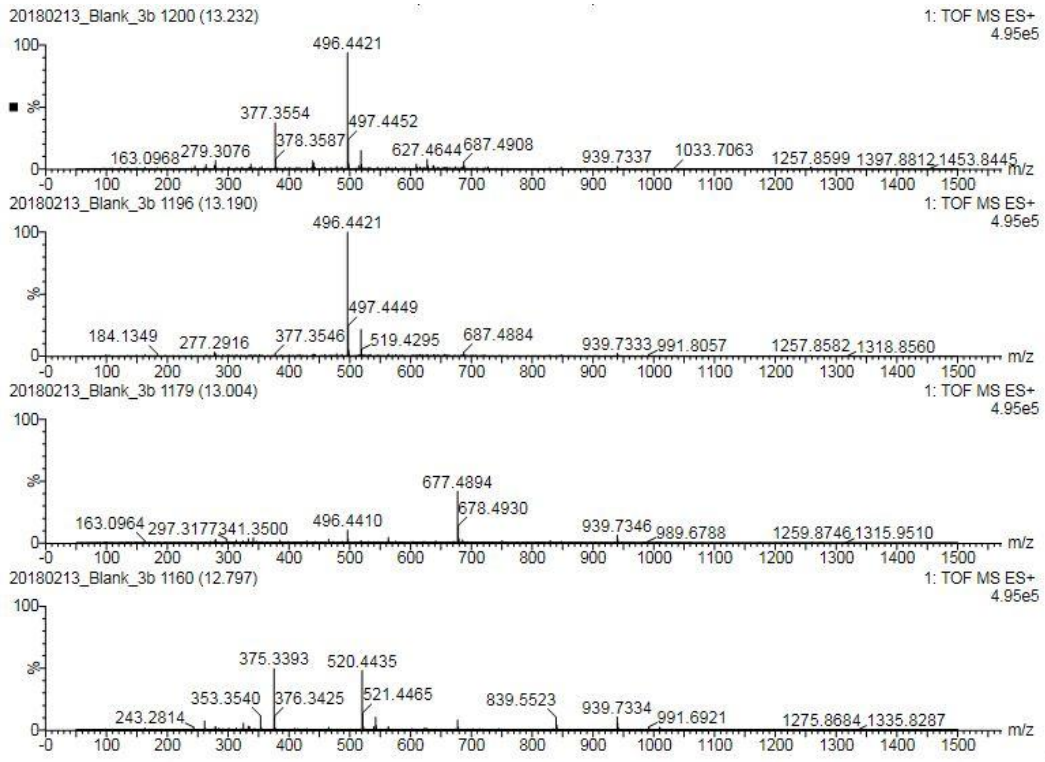
Appendix 10. ESI-MS Positive Ion Spectra for *C. asiatica* R_t = 11.134 -11.672



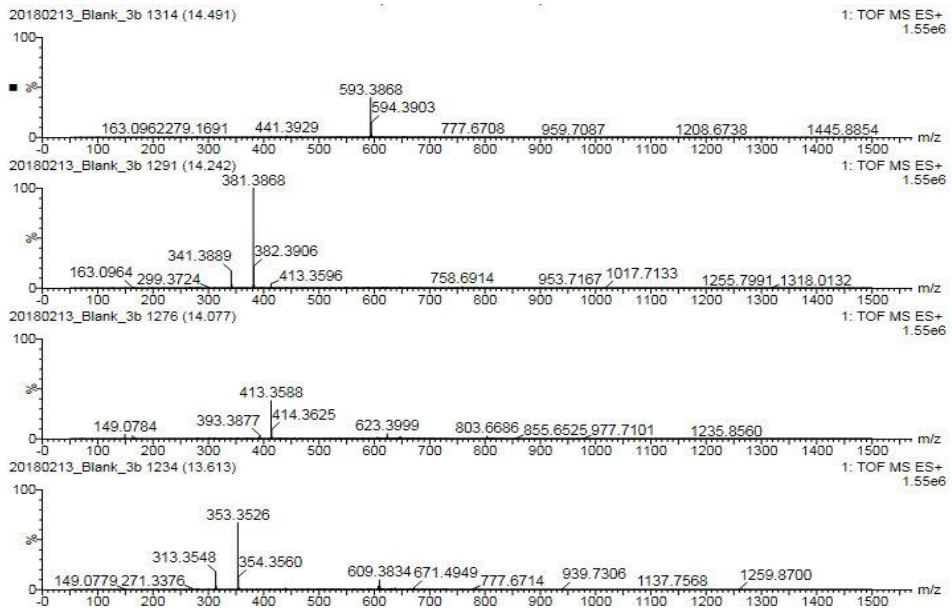
Appendix 11. ESI-MS Positive Ion Spectra for *C. asiatica* R_t = 11.981 - 12.571



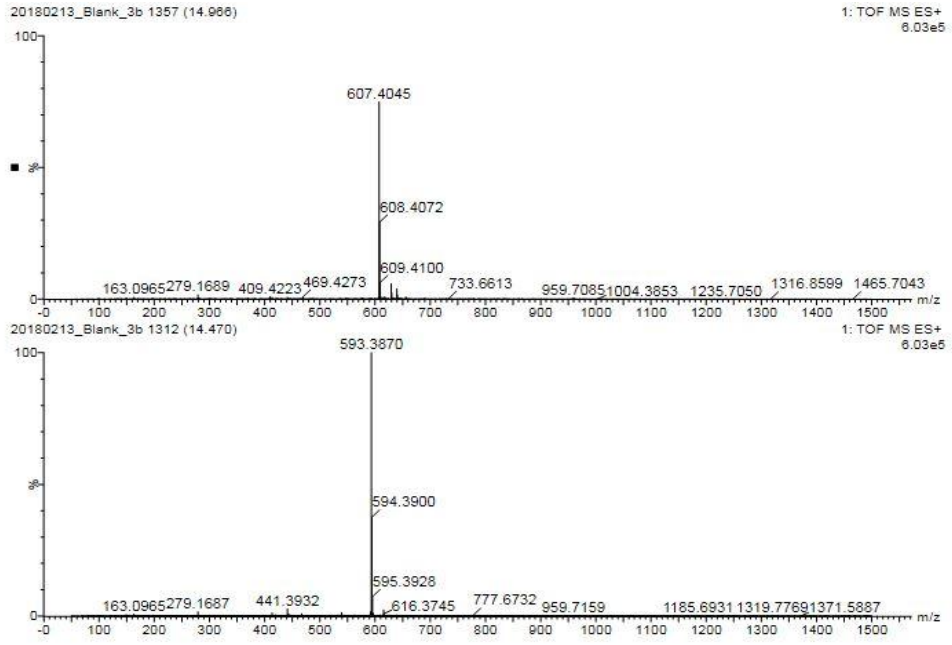
Appendix 12. ESI-MS Positive Ion Spectra for *C. asiatica* R_t = 12.797 - 13.232



Appendix 13. ESI-MS Positive Ion Spectra for *C. asiatica* R_t = 13.613 - 14.491



Appendix 14. ESI-MS Positive Ion Spectra for *C. asiatica* R_t = 14.470 - 14.966



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