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Full Length Research Paper

Edraianthus pumilio (Schult.) A. DC.: Phytochemical and biological profile

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***Edraianthus pumilio* is stenoendemic plant native to Dalmatia, Croatia. This paper deals with its phytochemical and biological profile. Phytochemical profile of volatile oil was performed by the gas chromatography–mass spectrometry (GC/MS), while total phenolic content of its aqueous extract was performed by Folin-Ciocalteu method. The phytochemical analysis showed that the main volatile oil compounds were nonanal (21.2%) and myristicin (16.4%). This oil could be characterized as nonanal-myristicin type. Total phenolic content of aqueous extract was 30.6 ± 1.1 mg GAE/g extract. Results of testing antioxidant potential of *E. pumilio* volatile oil and aqueous extract showed low antioxidant potentials as tested by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) methods. Results also showed low acetylcholinesterase inhibition potential of volatile oil and low to moderate potential of aqueous extract, as tested by Ellman method.**

Key words: *Edraianthus pumilio* (Schult.) A. DC., volatile oil, aqueous extract, gas chromatography–mass spectrometry (GC/MS), total phenolic content, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP), Ellman.

INTRODUCTION

In terms of biological diversity Croatia is one of the richest European countries. Croatian flora, with about 5000 species and subspecies is characterized by a markedly high level of diversity per unit of surface (Nikolic et al., 2015). Genus Edraianthus in flora Europe includes 9 species, 6 of which belong to the flora Croatica (Tutin et. al., 1980: Nikolic, 2018). *Edraianthus pumilio* (Schult.) A. DC. is stenoendemic species of flowering plant in the family Campanulaceae, native to Dalmatia in Southern Croatia. It is a strictly protected and almost endangered species. It is widespread on Mount Biokovo, near Makarska (Nikolic, 2018). The plant is highly heliophilic, thermophilic and xerophilic. It grows on limestone-rock cracks. This is a low-growing perennial plant that grows in dense pads. Linear and silver leaves grow in tufts. The underside of leaves is naked, while upper side of leaves

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is hairy. The flowers are violet-blue bells, while fruits are capsules. The plant belongs to alliance *Seslerion juncifoliae* Horvat 1930 (Nikolic et al., 2015).

Previous Edraianthus studies mainly focused on systematization of plants. The genus was the subject of cytogenetic investigations (Siljak-Yakovlev et al., 2010), molecular phylogenetic and phylogeographic studies (Park et al., 2006; Lakušić et al., 2016) as well as finding new species and determining their taxonomic status (Boštjan et al., 2009).

According to molecular phylogeny analysis the *E. pumilio* and *E. dinaricus* are two very closely related species. Both species are closely restricted to the mountains of Middle Dalmatia. These two species are quite different from other *Edraianthus* species in terms of morphological features such as leaf, inflorescence and habits (Stefanović et al., 2008).

Chemical taxonomy at this genus is poorly researched. The chemical structures of phenolics and terpenoids are often specific and restricted to taxonomically related organisms and hence useful in classification (Singh, 2016). Secondary metabolites and beneficial biological effects of *E. pumilio* have not been investigated so far. These compounds have shown to have antioxidant, antimicrobial, antiinflammatory, anticarcinogenic, antimutagenic and antiallergic properties (Roy et al., 2017; Murugesan and Deviponnuswamy, 2014; Bharti et al., 2012: Namita and Mukesh, 2012; Allesiani et al., 2010).

Antioxidants are vital substances, which possess the ability to protect the body from damages caused by free radical-induced oxidative stress (Birben et al., 2012). A large amount of evidence has demonstrated that oxidative stress is intimately involved in age-related neurodegenerative diseases. There have been a number of studies which examined the positive benefits of antioxidants to reduce or to block neuronal death occurring in the pathophysiology of these disorders (Loizzo, 2009; Ramassamy et al., 2006).

Acetylcholinesterase (AChE) is the enzyme involved in the hydrolysis of acetylcholine neurotransmitter which plays important role in memory and cognition. Low level of AChE in brain is usually connected with neurodegenerative disease, that is, Alzheimer's disease (AD). The most promising approaches in treating AD are usually based on AChE inhibitors (Wszelaki et al., 2010; Menichini et al., 2009).

Therefore, the aim of this work was to determine the phytochemical composition of volatile oil, total phenolic content of aqueous extract as well as antioxidant and acetylcholinesterase inhibition potential of volatile oil and aqueous extract isolated from *E. pumilio*. There are no known records of this plant being used for medicinal purposes but this or another research may contribute otherwise. Also, the analysis of volatile components can contribute to a better determination of the species *E. pumilio*. To the best of our knowledge, this is the first work that shows the phytochemical and biological profile of this species.

MATERIALS AND METHODS

Plant material

Plant material (whole plants, without roots) was collected from its natural habitat, *via* St. Ilija, Biokovo Mountain, Croatia (Central Dalmatia, Makarska) during flowering in July, 2017, at 1500 m a.s.l.; *Gauss-Krüger* coordinates: X=5663111, Y=4804120. The botanical identity of the plant material was confirmed by a botanist PhD Mirko Ruscic, associate professor, Department of Biology, Faculty of Science, University of Split. Voucher specimens were deposited in herbarium at Department of Biology, Faculty of Science, University of Split, Croatia, under number EP_7/17.

Volatile oil isolation

Air-dried aerial parts of plants were hydro-distilled using Clavenger apparatus for 3 h. Obtained essential oil was dried over anhydrous Na_2SO_4 and stored in sealed vials, under - 20 ^oC before use (Nikbin et al., 2014).

Preparation of aqueous extract

Air-dried aerial parts of plants and water were placed in an *Erlenmeyer* flask and refluxed in an ultrasound bath for 2 h. The mixture was then filtered through a filter paper and evaporated under vacuum at 40°C and stored at -20°C in fridge before use.

Gas chromatography-mass spectrometry analysis

The analysis of the volatile oil was carried out using Shimadzu gas chromatography–mass spectrometry (GC/MS), QP2010 system equipped with an AOC 20i autosampler, using fused silica capillary column Inert Cap (5% diphenyl, 95% dimethylpolysiloxane, 30 m × 0.25 mm i.d., film thickness 0.25 µm). The operating conditions were as follows: injection volume: 1.0 µl of volatile oil solution (1:500 *v/v* in pentane); injection mode: splitless; injection temperature: 260 ^oC; carrier gas: helium, 1.11 ml/min; the oven temperature program: 50°C (5 min), 50 - 260°C (3°C/min); MS conditions: ion source temperature: 200°C, ionization voltage: 70 eV, mass range: m/z 40 - 400 u. GCMSolution 2.5 (Shimadzu) was used to handle data. Identification of volatile oil components was based on (a) retention indices on non-polar column relative to a homologous series of *n*-alkanes (C_8 - C_{40}), (b) on the comparison of their mass spectra and retention indices with the NIST and Wiley spectra library and with those reported in the literature (Adams, 2007; Linstrom and Mallard, 2014). GC/MS analysis was performed in triplicate and results were averaged.

Total phenolic content

Total phenolic content was measured using Folin-Cocalteu spectrophotometric method (Singleton and Rossi, 1965; Katalinic et al., 2013) and gallic acid as a standard. 15 ml water and Folin-Ciocalteu reagent (1.25 ml, diluted 1:2) were added to the sample solution (0.25 ml, 1 mg/ml). The resulting solution was mixed. After 3 min, in solution were added Na_2CO_3 (3.75 ml, 20%) and water to volume of 25 ml. The resulting mixture was then left for 2 h. The absorbance of the resulting blue color was measured at 765 nm. The concentration of the total phenolic content was calculated by using an equation obtained from gallic acid calibration curve and

expressed as mg gallic acid equivalent per gram of extract (mg GAE/g extract). The determination of total phenolic content was carried out in triplicate and the results were expressed as mean ± standard deviations.

Antioxidant capacity

Antioxidant capacity of volatile oil and aqueous extract from *E. pumilio* were tested using two different methods: 2,2-diphenyl-1-picrylhydrazyl (DPPH) method and ferric reducing antioxidant power (FRAP) method.

Capacity of DPPH radical scavenging by volatile oil and aqueous extract was measured according to the method of Brand-Williams et al. (1995) (Katalinic et al., 2010). DPPH method is based on the reaction between free DPPH radicals and antioxidants. As a result, a stable non-radical form of the DPPH is obtained, with simultaneous change of the violet color to pale yellow. The decrease in absorbance was measured at 517 nm. DPPH radical solution was prepared by dissolving the stock solution (4 mg of DPPH in 100 ml of ethanol). To optimize the conditions used to run the DPPH assay in microplates, 10 µl of sample (1 mg/ml) was placed in a well and 290 µl of DPPH solution was added. The mixture was shaken vigorously and left to stand at room temperature in the dark. The decrease in the absorbance was measured after 1 h, with ethanol as blank. The DPPH radical scavenging activity of the sample was calculated according to the formula:

$$\% \text{ inhibition} = [(A_0 - A_{sample})/A_0] \times 100$$

where A_0 was absorbance of the DPPH ethanol solution measured at the beginning and A_{sample} was absorbance of the sample measured after 60 min. The results were expressed as percentage inhibition of DPPH. Butylated hydroxyanysole (BHA) was used as positive control.

The reducing power of volatile oil and aqueous extract were also performed using FRAP method (ferric reducing antioxidant power) (Skroza et al., 2015; Benzie and Strain, 1996). FRAP method is based on the reduction of ferric-tripyridyltriazine (Fe^{3+}-TPTZ) complex to ferrous (Fe^{2+}) complex with an intense blue color and maximum absorption at 593 nm. The method was performed in 96-well microplates, with slight modifications. The FRAP solution was freshly prepared by mixing 0.3 M acetate buffer (pH = 3.6) and 10 mM TPTZ in 40 mM HCl and 20 mM $FeCl_3$ in a ratio of 10:1:1 (by volume). The assay was carried out by placing 10 µl of the sample (1 mg/ml) and 300 µl of FRAP reagent in a well. The absorbance was measured after 4 min. The reducing power of sample was calculated by comparing with the reaction signal given by solution of Fe^{2+} ions in known concentration and expressed as µmol/l Fe^{2+}. BHA was used as positive control. The determination of antioxidant capacity, performed by both methods was carried out in triplicate and the results were expressed as mean ± standard deviations. Tested stock solution concentration was 1 mg/ml.

Acetylcholinesterase inhibition potential

Acetylcholinesterase (AChE) inhibition potential of volatile oil and aqueous extract were carried out by a slightly modified Ellman assay (Politeo et al., 2018; Ellman, 1961). A typical run consisted of 180 µl of phosphate buffer (0.1 M, pH 8), 10 µl of DTNB (at a final concentration of 0.3 mM prepared in 0.1 M phosphate buffer pH 7 with 0.12 M sodium bicarbonate added for stability), 10 µl of sample solution (dissolved in 80% EtOH), and 10 µl of AChE solution (with final concentration 0.03 U/ml). Reactants were mixed in a 96-well plate wells and reaction was initiated by adding 10 µl of acetylthiocholine iodide (ATChI to reach a final concentration of 0.5 mM). As a negative control, 80% EtOH was used instead of sample solution. Non-enzymatic hydrolysis was also monitored by measurement of two blank runs for each run. All spectrophotometric measurements were performed at 405 nm and at room temperature for 6 min. The results are expressed as percentage inhibition of enzyme activity. Eserine was used as positive control. The determination of acetylcholinesterase inhibition potential was carried out in triplicate and the results were expressed as mean ± standard deviations. Tested stock solution concentration of *E. pumilio* samples was 1 mg/mL, while stock solution concentration of eserine was 0.1 mg/ml.

RESULTS AND DISCUSSION

Phytochemical composition of *E. pumilio* volatile oil

E. pumilio volatile oil yield was 0.31% (w/w). The analysis revealed forty two compounds separated into five classes: nonterpene compounds, phenyl propanes, terpene compounds, norisoprenoids, and other compounds (Table 1). Nonterpene compounds (54.6%) were predominated compound class in *E. pumilio* volatile oil. Among them nonterpene aldehydes (41.8%) were the most common class of compounds with nonanal (21.2%) as a major one. (*E,E*)-2,4-Decadienal (3.9%), tridecanal (3.4%), decanal (2.5%), octanal (2.5%), (*E,Z*)-2,4-decadienal (2.1%) and others nonterpene aldehydes were identified in lower quantity. Nonterpene hydrocarbons (7.4%), ketones (4.0%), alcohols (1.1%) and esters (0.3%) were also identified in lower quantity. Among these compounds, the most common compound was 4-methyldecane (2.9%), while other compounds were identified in quantity lower than 2%. Second one quantitatively important compound class, phenyl propanes (17.3%), was presented with two compounds: myristicin (16.4%) as predominant compound and anisole (0.9%). Terpene compounds (15.1%) were mainly presented with monoterpene compounds. Among them the main ones were hexahydrofarnesyl acetone (5.1%), prenol (2.2%) and ß-myrcene (2.0%). Other monoterpene compounds were identified in quantity lower than 2%. Sesquiterpene compounds were present only with one compound, ß-caryophyllene (0.4%). Norisoprenoids (0.4%) were presented with two compounds: ß-ionone (0.1%) and ß-ionone epoxide (0.3%). 4-Vinyl phenol (1.6%), benzothiazole (0.7%) and indole (0.5%) were presented as other compounds (2.8%).

Total phenolic content of *E. pumilio* aqueous extract

The total phenolic content of *E. pumilio* aqueous extract (the extraction yields was 14.8%, w/w) was determined using Folin-Ciocalteu reagent and external calibration with gallic acid, according to the method previously described by Singleton and Rossi (Katalinic et al., 2013; Singleton and Rossi, 1965). The total phenolic content was 30.6 ± 1.1 mg GAE/g extract (Table 2).

Table 1. *E. pumilio*: Volatile oil constituents.

S/N	Compounds	%	RI[a]	Mode of identification
	Nonterpene compounds	**54.6**		
	Nonterpene aldehydes	***41.8***		
1	Benzaldehyde	1.4	961	RI, MS
2	Octanal	2.5	1001	RI, MS
3	Phenyl acetaldehyde	1.0	1044	RI, MS
4	Nonanal	21.2	1104	RI, MS
5	(*E*)-2-Nonenal*	1.1	1161	RI, MS
6	Decanal	2.5	1204	RI, MS
7	(*E*)-2-Decenal*	0.8	1263	RI, MS
8	(*E,Z*)-2,4-Decadienal*	2.1	1299	RI, MS
9	Undecanal	1.0	1305	RI, MS
10	(*E,E*)-2,4-Decadienal*	3.9	1314	RI, MS
11	(*E*)-2-Undecenal*	0.2	1360	RI, MS
12	Tridecanal	3.4	1504	RI, MS
13	Pentadecenal	0.7	b	-, MS
	Nonterpene hydrocarbons	***7.4***		
14	3,5,5-Trimethyl-2-hexene	0.8	986	RI, MS
15	4-Methyldecane	2.9	1060	RI, MS
16	2- Methyldecane	0.4	1072	RI, MS
17	1-Dodecene	1.1	1193	RI, MS
18	1-Tetradecene	1.8	1390	RI, MS
19	Pentadecane	0.4	1500	RI, MS
	Nonterpene ketones	***4.0***		
20	(*E,E*)-3,5-Octadien-2-one*	0.8	1093	RI, MS
21	(*E*)-2-Methyl-2-nonen-4-one*	1.7	1216	RI, MS
22	(*Z*)-3-Nonen-2-one*	0.6	1332	RI, MS
23	6,10-Dimethyl-undecan-2-one	0.9	1403	RI, MS
	Nonterpene alcohols	***1.1***		
24	3,5-Octadien-2-ol	1.1	1037	RI, MS
	Nonterpene esters	***0.3***		
25	*cis*-3- Hexenyl tiglate	0.3	1319	RI, MS
	Phenyl propanes	**17.3**		
26	Anisole	0.9	1235	RI, MS
27	Myristicin	16.4	1513	RI, MS
	Terpene compounds	**15.1**		
	Monoterpene ketones	***5.5***		
28	Neryl acetone	0.4	1443	RI, MS
29	Hexahydrofarnesyl acetone	5.1	b	-, MS
	Monoterpene hydrocarbons	***5.1***		
30	*α*-Thujene	1.3	925	RI, MS
31	*β*-Myrcene	2.0	991	RI, MS
32	*p*-Cymene	0.7	1022	RI, MS
33	Limonene	1.1	1028	RI, MS

Table 1. Cont.

	Monoterpene alcohols	***4.1***		
34	Prenol	2.2	778	RI, MS
35	Linalool	1.6	1101	RI, MS
36	*cis*-Sabinol	0.3	1142	RI, MS
	Sesquiterpenes	***0.4***		
37	ß-Caryophyllene	0.4	1410	RI, MS
	Norisoprenoids	**0.4**		
38	*β*-Ionone	0.1	1475	RI, MS
39	(*E*)-5,6-Epoxy-ß-ionone*	0.3	1479	RI, MS
	Other compounds	**2.8**		
40	*p*-Vinylphenol	1.6	1222	RI, MS
41	Benzothiazole	0.7	1224	RI, MS
42	Indole	0.5	1292	RI, MS
	Total	**90.2**		

* Correct isomers were not identified; RI = Kovats index determined on a Inert Cap column using the homologous series of *n*-hydrocarbons C_8-C_{40}; MS = mass spectra; b = The RI was outside of the RI interval of series of *n*-alkanes.

Table 2. Total phenolic content, antioxidant and anticholinesterase inhibition capacity of volatile oil and water extract from *E. pumilio*

Variable	Total phenolic content mg GAE/g extract	Antioxidant capacity		AChE inhibition %
		DPPH inhibition %	FRAP µmol/L Fe^{2+}	
Ep-volatile oil[a]	-	ni	15.3 ± 0.7	26.6 ± 2.1
Ep-aqueous extract[a]	30.6 ± 1.1	10.7 ± 0.7	118.7 ± 7.2	46.9 ± 4.7
BHA[a]	-	91.9 ± 2.9	5586.3 ± 72.6	-
eserine[b]	-	-	-	95.9 ± 1.9

Ep = *Edraianthus pumilio*; [a]stock solution concentration was 1 mg/ml (0.048 mg/mL for DPPH, 0.029 mg/ml for FRAP and 0.045 mg/ml for AChE in reaction system); [b]stock solution concentration was 0.1 mg/mL (4.5 µg/ml for AChE in reaction system); ni = inhibition not identified.

Antioxidant capacity of volatile oil and aqueous extract from *E. pumilio*

Antioxidant potential of volatile oil and aqueous extract from *E. pumilio* (Ep) are presented in Table 2 and Figure 1. Results obtained were compared with those of well-known antioxidant BHA. Result showed no antioxidant potential of *Ep*-volatile oil and low antioxidant potential of Ep-aqueous extract (10.7±0.7%) tested by DPPH method as well as low antioxidant capacity of Ep-volatile oil (15.3±0.7 ek. µmol/L Fe^{2+}) and Ep-aqueous extract (118.7±7.2 ek. µmol/L Fe^{2+}) tested by FRAP method for tested stock solution concentration of 1 mg/ml (0.048 mg/ml for DPPH and 0.029 mg/ml for FRAP in reaction system). In comparison, well-known antioxidant compound BHA inhibited DPPH with 91.9±2.9%, while antioxidant capacity tested by FRAP method was eq.. 5586.3 ± 72.6 µmol/L Fe^{2+} for the same tested concentration. Low antioxidant capacity of Ep-volatile oil and slightly better, but still low, antioxidant capacity of Ep-aqueous extract could be connected to chemical composition of these extracts. Namely, tested Ep-volatile oil does not contain components responsible for antioxidant potential such as phenolic or monoterpenoid compounds (eugenol, carvacrol, thymol, menthol) (Brewer, 2011; Bakkali et al., 2008), while low antioxidant capacity of Ep-aqueous extract is in correlation with low total phenolic content of tested extract (30.6±1.1 mg GAE/g extract). Therefore, Ep-aqueous extract probably contains low quantity of phenolic components with good antioxidant capacity such as phenolic acids (gallic, protochatechuic, caffeic and rosmarinic acids), phenolic

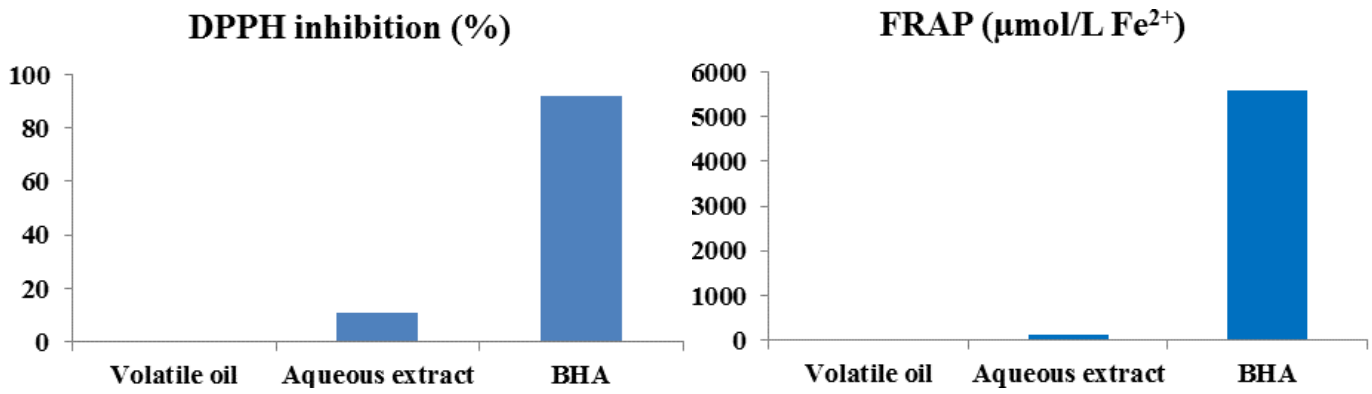

Figure 1. Antioxidant capacity of volatile oil and aqueous extract from *E. pumilio* (tested concentrations were 1 mg/ml).

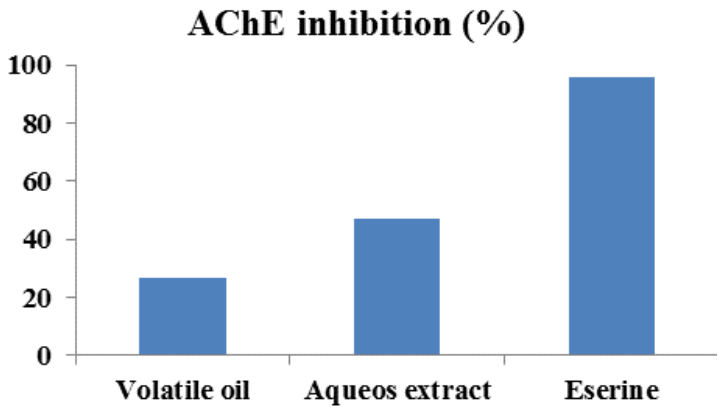

Figure 2. Acetylcholinesterase inhibition of volatile oil and aqueous extract from *E. pumilio* (tested concentrations were 1 mg/ml and 0.1 mg/ml for eserine).

diterpenes (carnosol and carnosic acid) and flavonoids (quercetin and catechin) (Brewer, 2011) but these compounds cannot significantly contribute to antioxidant capacity of Ep-aqueous extract.

Acetylcholinesterase inhibition potential of volatile oil and aqueous extract from *E. pumilio*

Acetylcholinesterase inhibition potential of Ep-volatile oil and Ep-aqueous extract is presented in Table 2 and Figure 2. Results obtained were compared with those of well-known AChE inhibition agent, eserine. Results showed low AChE inhibition potential of Ep-volatile oil (26.6±2.1%) and low to moderate AChE inhibition potential for Ep-aqueous extract (46.9±4.7%) in tested concentration of 1 mg/ml (0.045 mg/ml in reaction system). In comparison, well-known AChE inhibitor eserine showed 95.9±1.9% AChE inhibition in tested concentration of 0.1 mg/ml (4.5 µg/ml in reaction system).

Low AChE inhibition potential of Ep-volatile oil and low to moderate AChE inhibition potential for Ep-aqueous extract could also be connected to chemical composition of tested extracts. Namely, tested volatile oil probably does not contain compounds responsible for AChE inhibition potential, such as *α*-pinene, *δ*-3-carene, 1,8-cineole, *α*- and *β*-asarone (Burcul et al., 2018), while Ep-aqueous extract probably contains low quantity of compounds responsible for AChE inhibition, such as compounds with catechol moiety in their structure (Ji and Zhang, 2006), polymers of resveratrol (Jang et al., 2007), stilbene oligomers (Sung et al., 2002) or others (Szwajgier, 2014; Suganthy et al., 2009; Mukherjee, 2007).

Conclusions

The phytochemical analysis of *E. pumilio* volatile oil revealed forty two compounds separated into five

classes: nonterpene compounds, phenyl propanes, terpene compounds, norisoprenoids, and other compounds. Among identified compounds, the main *E. pumilio* volatile oil compounds were nonanal (21.2%) and myristicin (16.4%) and this oil could be characterized as nonanal-myristicin type. The total phenolic content of *E. pumilio* aqueous extract was 30.6 ± 1.1 mg GAE/g extract.

Results of testing antioxidant potential of *E. pumilio* volatile oil and aqueous extract showed no antioxidant potential of volatile oil and low antioxidant potential of aqueous extract tested by DPPH method as well as low antioxidant capacities of volatile oil and aqueous extract tested by FRAP method, in comparison with BHA. Results of acetylcholinesterase inhibition potential test showed low potential of volatile oil and low to moderate potential of aqueous extract in comparison with eserine. The volatile oils rich in nonanal as well as nonanal isolated from volatile oil may have other useful biological properties such as antidiarrhoeal (Zavala-Sanchez et al., 2002). Myristicin (a phenylpropanoid) was reported to have cytotoxic effects (Lee et al., 2005), anti-cholinergic, antibacterial, hepatoprotective effects and anti-inflammatory properties (Lee and Park, 2011) and also can inhibit tumorigenosis in mice (Zheng et al., 1992).

Future studies of phytochemical compound should focus on other possible biological effects such as anti-inflammatory, antidiarrheal, and anticancer.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Protective effects of *Pleurotus ostreatus* in ameliorating carbon tetrachloride (ccl_4) induced liver injury in Wistar rats

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Since oxidative stress is implicated in the pathogenesis of liver diseases, this study was carried out to evaluate the hepatoprotective, hematoprotective and hypolipidemic effects of Pleurotus ostreatus (Oyster mushroom) in Carbon Tetrachloride (CCl_4) induced liver injury in wistar rats. Thirty rats were used for this study. The rats were divided into five groups of six rats per cage. Group I that served as the normal control received distilled water only. Groups II to V served as test groups. Group II received CCl_4 at a dose of 1 ml/kg body weight on the 14^{th} day and 28^{th} day only. Groups III, IV and V received CCl_4 at a dose of 1 ml /kg body weight on the 14^{th} day and 28^{th} day, then, silymarin (100 mg/kg), 25% w/w and 50% w/w of powdered Oyster mushroom respectively. The results revealed that CCl_4 caused a significant ($p<0.05$) increase in lipid peroxidation judging from the significant ($p<0.05$) elevated level of malondialdehyde MDA in the hepatic tissues whereas the level or activities of reduced glutathione (GSH), catalase (CAT), suproxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione S-transferase (GST) in the liver tissues were significantly ($p<0.05$) reduced. Liver peroxidation was followed by disruption of proper functioning of the hepatocytes as indicated by the significant increase of liver biomarker enzymes (AST, ALT, and ALP) and decreased serum levels of albumin and total protein. Dyslipidemia and hematotoxicity were also manifested via a significant ($p<0.05$) increase in the serum levels of triglyceride TG, total cholesterol TC, very low-density lipoprotein cholesterol VLDL-C, low density lipoprotein cholesterol LDL-C and white blood cells count. These were accompanied by significant ($p<0.05$) reduction in the serum level of high density lipoprotein cholesterol HDL-C and RBC count and its differentials. Supplementation of powdered Oyster mushroom daily for a period of 28 days to rats led to reversal of these signs of toxicities. The ability of the powdered Oyster mushroom to mitigate against CCl_4-induced hepatotoxicity is probably due to its antioxidant and enzyme modulatory effects.

Key words: Hepatoprotective, hematoprotective, hypolipidemic, *Pleurotus ostreatus*, carbon tetrachloride, peroxidation.

INTRODUCTION

Toxic effects of chemicals on internal organs and cellular components such as liver, kidneys, brain, hematopoiesis, lipids, Proteins and nucleic acids have been a matter of public health concern (Rizwan et al., 2014). Carbon

tetrachloride is used as an organic solvent in many industries. It is also used as a hepatotoxic agent for the purpose of studying pathogenesis of liver injury (Boll et al., 2001a). Its mechanism of toxicity is in connection with its ability to interact with membrane lipids thus causing peroxidation (Boll et al., 2001b). This process, however, involves series of biotransformation of CCl_4 by hepatic microsomal P_{450} to produce the trichloromethyl radical (CCl_3^-) which, in the presence of oxygen, is further converted to a peroxy radical (CCl_3OO^-) involved in peroxidation of internal organs and cellular components (Sharifudin et al., 2013).

The end product of peroxidation that is malondialdehyde (MDA) or 4-hydroxynonenal (HNE), which is highly reactive aldehydes forms adducts with protein and DNA (Kadiiska et al., 2005). When excess production of this Reactive Oxygen Species (ROS) overwhelms the endogenous antioxidant defense system a condition called oxidative stress occurs (Renugadevi. and Prabu, 2010). This has been implicated in the pathogenesis of many diseases. The major antioxidant defenses systems are composed of antioxidant enzymes that include superoxide dismutate SOD, catalase CAT, glutathione peroxidase GPxand non-antioxidant enzymes (GSH) (Klivenyi et al., 2000). SOD defends against oxidative stress by catalyzing the dismutation of superoxide radicals (O_2^-) into molecular oxygen (O_2) and hydrogen peroxide (H_2O_2) (Mates, 2000). H_2O_2 is neutralized by the combined action of CAT and GPx in all vertebrates (Mourelle et al., 1988).

Reports have shown that carbon tetrachloride causes increased lipid synthesis and a concomitant decrease in the transport of lipids out of the hepatocyte (Okolo et al., 2017; Jayakumar et al., 2008). This imbalance between the synthesis and degradation of lipids may be the direct cause of steatosis or fatty liver associated with CCl4-induced hepatotoxicity (Kiezcka and Kappus, 1980). Silymarin and colchicine are natural products having antioxidant effects that were found to protect the liver from CCl_4-induced damage (Letteron et al., 1990) by inhibiting cytochrome P450 enzymes. Another natural substance is the hepatic stimulator substance (HSS), also an antioxidant; it protects the liver from failure induced by $CC1_4$ due to its ability to reduce peroxidation (Mao-Hua et al., 1993). Despite the acclaimed therapeutic uses of these natural products; there relatively scarcity limits their use for the treatment of liver and liver-related diseases. This implies there is a compelling need for a continued search for an efficient and readily available hepatoprotective agent from natural source that could either be used directly or serve as lead compounds for the treatment of liver disease.

Pleurotus ostreatus (Oyster mushroom) is a wood-rotting fungus produced on ligno-cellulose substrates that grow in the tropical and subtropical part of the world. It belongs to the class of basidiomycetes and *Agaricaceae* family. A hypolipidemic agent called Lovastatin has been isolated as the active chemical principle of *P. ostreatus* (Liu et al., 1997). Also, its high dietary fiber content, protein, microelements coupled with the presence of plant sterols make it a valuable therapeutic agent (Wasser and Weis, 1999). Vitamin B_1 and B_2 had been reported as constituents of the fungus. Oyster mushrooms contain polyphenols which are scavengers of free radicals (Antonia et al., 2002). Other therapeutic uses of *P. ostreatus* include inhibition of platelets aggregation and reduction of blood cholesterol (Borchers et al., 1999).

The presence of valuable therapeutic agents in the Oyster mushroom has captured our interest to investigate this fungus as a prospective candidate for the remedy of liver necrosis, hematotoxicity and dyslipedemia.

MATERIALS AND METHODS

Collection and Identification of oyster mushrooms

Oyster mushrooms were collected from decaying dead trees in Edo State, Nigeria. The plant materials were identified and authenticated by a mycologist in the Department of Botany, University of Benin, Edo State. The fresh mushroom samples were crushed to powder by using electronic blender. The powdered oyster mushrooms were then stored in an air-tight container and kept in the refrigerator at 4°C until use.

Animals

Thirty (30) adult Wistar rats of both sexes weighing 170-240 g were purchased from National Veterinary Research Institute (NVRI), Vom, Plateau State. Food and water were provided *ad libitum*. Animals were exposed to controlled environmental temperature (28 ± 2°C), relative humidity (50 ± 5%) and 12-h light or darkness. After obtaining ethical approval (ABUCAUC/2018/028), this study was conducted in accordance with the principle governing the handling procedures of experimental animals as laid down by the Ahmadu Bello University Committee on Animal Use and Care. All the animal care and treatment procedures were respected following the guidelines established by the University's committee on animal use and care. Efforts were made to minimize the number of animals used and their sufferings by strictly following the ethical guidelines for investigations of experimental pain in conscious animals as described.

Acute toxicity study

Acute oral toxicity (AOT) of oyster mushrooms was investigated using Wister rats in a method described by Lorke (1983).The animals were fasted for 12 h (overnight) prior to the experiment.

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The animals were divided into five groups of six animals each and were administered with single dose of oyster mushroom powder orally at doses of 1, 2, 4, 6 and 8 g/kg body respectively. The animals were observed for mortality up to 48 h (acute) and for another 14 days for sub chronic toxicity. Calculated LD_{50} was done after oral administration of variable doses.

Preparation of oyster mushroom

The grower mash fed to the animals consisted of 25 and 50% w/w of oyster mushroom.

Grouping of the experimental animals

The CCl_4 was prepared as described previously (Okolo et al., 2017): rats were divided into five groups, each group consisting of six animals thus: Group I: (Control) received 0.5 ml of distilled water daily for a period of 28 days; Group II: (CCl_4 –induced) animals received 0.5 ml of distilled water daily for 28 days and CCl_4 at a dose of 1 ml /kg body weight on the 14^{th} day and 28^{th} day; Group III: animals (Standard control) received aqueous form of silymarin at a dose of 100 mg/kg body weight daily for 28 days and CCl_4 at a dose of 1 ml /kg body weight on the 14^{th} day and 28^{th} day; Group IV: animals received grower mash supplemented with 25% w/w of powdered Oyster mushroom daily for 28 days and $CCl_{4\ at}$ at a dose of 1 ml /kg body weight on the 14^{th} day and 28^{th} day; Group V: animals received grower mash supplemented with 50% w/w of powdered Oyster mushroom daily for 28 days and CCl_4 at a dose of 1 ml /kg body weight on the 14^{th} day and 28^{th} day. After 24 h of the last treatment, all the animals were fasted overnight and were anaesthetized with chloroform. They were then sacrificed by cervical decapitation and the blood was collected into heparinized and non-heparinized tubes. Serum was collected from the non-heparinised tubes by centrifugation at 4000 rpm for 15 min and was stored at 20°C till analysis. The liver tissue was collected and perfused with normal saline to remove blood and used for the preparation of tissue homogenate.

Assay for liver function biomarkers

The activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were measured spectrophotometrically by the methods described by Reitman and Frankel (1957), and Belfield and Goldberg (1971) respectively. Total protein was determined by the method of Henry (1964).

Lipid profiles assay

The serum levels of triglyceride and total cholesterol were determined according to the principle described by Tietz (1990) and Siedel (1983), respectively. The HDL-cholesterol levels were determined by the method of Assmann (1983) and LDL-cholesterol levels were calculated using Friedwald's formula (1972).

Antioxidant assay

Lipid peroxidation (LPO)

This was estimated by measuring MDA in the liver homogenate using the method described by Ohkawa et al (1979). The principle involves measuring the absorbance of pink colour complex formed from the reaction of MDA with thiobarbituric acid in acidic medium at 534 nm. Absorbance was expressed in nmol/g protein.

Determination of enzymatic antioxidants in liver homogenate

Catalase activity (CAT)

The activity of CAT was measured as described by Aebi (1984). The principle involves the reaction of CAT with excess H_2O_2. Exactly after a minute, the remaining H_2O_2 reacts with 3,5-dichloro-2-hydroxybenzene sulfonic acid (DHBS)and 4-aminophenazone (AAP) to form a chromophore. The colour intensity was inversely proportional to the absorbance measured at 240 nm. The enzymatic activity of CAT was expressed in units/mg cellular protein.

Superoxide dismutase activity (SOD)

The activity of SOD was assayed according to the method of Nishikimi et al. (1979).The principle of this method involves the ability of the enzyme to inhibit the phenazine methosulphate-mediated reduction of nitro blue tetrazolium dye (NBT). The absorbance was read at 505 nm using a spectrophotometer and the enzyme activity of SOD was expressed in units/mg cellular protein. All CCl_4 administrations were made intraperitoneally.

Glutathione peroxidase activity (GPx)

The activity of GPx was determined spectrophotometrically (Paglia and Valentine, 1967). GPx catalyzes the oxidation of glutathione. Where glutathione reductase and NADPH are present, the oxidized glutathione is immediately converted to the reduced form with a simultaneous oxidation of NADPH to $NADP^+$. GSH-Px activity was measured at 340 nm by the decrease of NADPH absorbance using extinction coefficient of 6.22 mM expressed in unit/mg-protein.

Assay of reduced glutathione (GSH) level

The level of reduced glutathione was assayed using the colorimetric method described by Beutler et al. (1963).

Determination of glutathione transferase (GST) level

The activity of GST was assayed by monitoring the reaction between 1-chloro-2,4-dinitrobenzene (CDNB) and reduced glutathione. The reaction was accompanied by an increase in absorbance which was measured at 340 nm. The rate of increase is directly proportional to the GST activity in the sample (Habig et al., 1974).

Hematological assay

White blood cells (WBCs), Red blood cells (RBCs) counts, hemoglobin concentration (Hb), hematocrit percent (HCt %), mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) were determined using a Neubauer counting chamber Japan, (Dacie and Lewis, 1991).

Statistical analysis

The data were expressed as mean± SD and the difference among treatment groups was analyzed using one-way analysis of variance

Table 1. Effect of P*leurotus ostreatus* on liver enzymes and serum total protein in CCl4- induced hepatotoxity in Wistar rats.

Parameter	Group I	Group II	Group III	Group IV	Group V
ALT U/L	42.82±3.6	78.55±6.4[a]	50.22±5.3[ab]	73.7±6.3[a]	45.52±4.2[b]
AST U/L	86.34±5.8	120.63±7.8[a]	92.65±7.4[ab]	163.41±8.1[a]	88.49±6.7[b]
ALP U/L	98.76±8.7	175.36±10.2[a]	114.64±7.6[ab]	127.2±.6[ab]	103.9±7.7[b]
ALB g/dl	5.4 ±1.2	3.6±0.6[a]	4.8±1[b]	3.2±0.72[a]	5.1±0.22[b]
TP mg/dl	7.42 ±1.3	4.76±2.2[a]	6.83±1.5[ab]	6.73±1.3[a]	6.4±1.7[ab]

Data are expressed as Mean ±SD for the six animals in each group. [a]p<0.05 Compared with control (Group I). [b]p<0.05. Compared with CCl_4 –induced group control (Group II).

Table 2. Effect of P*leurotus ostreatus* on serum lipid profiles in CCl4- induced hepatotoxity in Wistar rats.

Parameter (mg/dl)	Group I	Group II	Group III	Group IV	Group V
TG	82.46±3.2	148.32±11.7[a]	104.72±8.92[ab]	138.56±10.42[a]	85.61±6.19[b]
TC	96.53±7.46	207.97±13.7[a]	I63.46±11.90[ab]	205.1±10.48[a]	98.48±6.51[b]
HDL-C	26.74±3.77	24.84±2.95[a]	29.26±4.28[ab]	26.18±2.65[b]	29.52 ±1.86[ab]
LDL-C	53.30±2.57	147.26±15.7[a]	118.66±7.21[ab]	133.62±10.36[a]	53.84±5.3[b]

Data are expressed as Mean ±SD for the six animals in each group. [a]p<0.05 Compared with control (Group I). [b]p<0.05. Compared with CCl_4 – induced group control (Group II).

Table 3. Effect of P*leurotus ostreatus* on Enzymatic and Non-enzymatic Antioxidants in Liver Homogenate of CCl4- induced Hepatotoxity in Wistar Rats.

Parameter	Group I	Group II	Group III	Group IV	Group V
MDA(nmol/g prt)	6.62±1.77	22.61±2.55[a]	10.62±3.95[ab]	12.83±2.13[ab]	8.61±1.71[a]
CAT (U/mg prt)	30.42±4.71	19.58±1.96[a]	24.87±1.84[ab]	22.4±3.32[ab]	28.47±2.82[b]
SOD(U/mg prt)	27.42±4.23	15.84±2.65[a]	20.30±3.36[ab]	18.64±4.17[ab]	25.39±2.77[b]
GPx(U/mg prt)	35.3±2.71	20.87±1.72[a]	29.77±3.11[ab]	21.18±1.55 [a]	29.75±2.86[ab]
GSH(nmol/g prt)	24.85±1.66	13.76±2.33[a]	19.88±2.37[ab]	16.94±2.88[ab]	32.85±1.96[ab]
GST(nmol/g prt.)	340.21±15.46	242.95±13.86[a]	298.70±12.85[ab]	269.37±14.82[a]	339.71±16.1[b]

Data are expressed as Mean ±SD for the six animals in each group. [a]p<0.05 Compared with control (Group I). [b]p<0.05. Compared with CCl_4 – induced group control (Group II).

(one-way ANOVA, Version 9, SAS institute Inc. USA) followed by Duncan test. P<0.05 was considered as statistically significant.

RESULTS

The results obtained from this study revealed that CCl_4 causes a significant (p<0.05) increase in lipid peroxidation; judging from the significant (p<0.05) elevated level of malondialdehyde MDA in the hepatic tissues whereas the levels or activities of reduced glutathione, catalase, superoxide dismutase, glutathione peroxidase and glutathione S-transferase in the liver tissues were significantly reduced (p<0.05). This was also followed by a significant increase in liver biomarker enzymes such as AST, ALT, and ALP with a corresponding decrease in the serum levels of albumin and total protein. Dyslipidemia and hepatotoxicity were also manifested via a significant (p<0.05) increase in the serum levels of TG, TC, VLDL-C, LDL-C and WBC count. These were accompanied by a significant (p<0.05) reduction in the serum levels of HDL-C and RBC count and its differentials. Supplementation of powdered oyster mushroom daily for a period of 28 days to the sample animals studied led to reversal of these signs of toxicities. There was no death recorded following the acute toxicity studies, suggesting that *Pleurotus ostreatus* has no associated toxicity (Tables 1 to 4; Figure 1).

DISCUSSION

The liver is susceptible to toxic substances because of its key role in neutralizing and excreting xenobiotics. In fact,

Table 4. Effect of P*leurotus ostreatus* on blood haematological parameters in CCl4- induced hepatotoxity in Wistar rats.

Parameter	Group I	Group II	Group III	Group IV	Group V
RBC(X1012/L)	7.4±0.42	5.3±1.32[a]	6.5±0.62[a]	6.0±1.43[a]	6.8±0.71[b]
WBC(X106/L)	5.8±0.83	7.6±0.11[a]	6.2±0.72[b]	7.3±0.84[ab]	5.9±0.81[b]
Hb(gdl-1)	8.4±0.51	5.2±0.86[a]	7.7±1.61[b]	5.9±0.88[a]	7.5±1.61[ab]
Hct (%)	41±1.8	32.5±2.61[a]	35.6±1.8[a]	38.2±1.20[b]	43.6±0.2[b]
MCV (fL)	55.41±0.22	51.59±1.21	54.77±1.3[b]	63.67±0.81[ab]	64.1±0.7[ab]
MCH(10-12G)	11.35±0.61	8.25±1.21[a]	11.85±0.33[b]	8.83±1.43[a]	11.0±0.78[b]
MCHC(g/dl)	20.5±1.48	16±0.43[a]	21.63±0.84[b]	15.44±1.35[a]	17.20±1.65[ab]

Data are expressed as Mean ±SD for the six animals in each group. [a]$p<0.05$ Compared with control (Group I). [b]$p<0.05$. Compared with CCl_4 –induced group control (Group II).

Figure 1. *Pleurotus ostreatus.*
Source: Adopted from Nigerian Mushroom farm.

it has been reported that over 700 drugs are involved in the pathogenesis of liver injury (Friedman et al., 2003).The mechanism of CCl_4- induced hepatic injury involves rapid biotransformation of CCl_4 to trichloromethyl radical (CCl_3), and trichloromethyl peroxyl which are very reactive radicals that causes lipid peroxidation and decreased activities of antioxidant defense system in the liver tissues (Muriel, 1997). The alarming increase of drug-induced liver injury indicated that there is need for continuous search for hepato-protective agents from medicinal plants.

In this present study, intraperitoneal injection of CCl_4 resulted in a significant ($p<0.05$) increase of malondialdehyde MDA level in the liver accompanied by a significant ($p<0.05$) decrease in the activities or level of GSH, CAT, SOD, GPx and GST in the hepatic tissues. This observation could be attributed to free radical generation from the peroxidation of polyunsaturated fatty acids measured as MDA and lipid hydroperoxides LPO. These findings conform with previous studies as reported by Poli (1993). The increase in MDA level in this study confirmed the pro-oxidant and hepatotoxic effect of CCl_4, manifested likely due to the failure of antioxidant defense mechanisms in the hepatocytes to prevent the formation of excessive free radicals (Al-Dosari, 2010).

The observed decreased in the activities of the enzymatic and the non-enzymatic antioxidant system in the hepatic tissue of the sample animals injected with CCl_4 may perhaps be an indication of overwhelming of the system by the free radicals generated. This does not however mean reduction in the amount of the enzymes but an indication of saturation as a result of increased

utilization of non-enzymatic and enzymatic antioxidant system. Supplementation of powdered Oyster mushroom daily for a period of 28 days to the sample animals investigated in this study led to increased level and activities of these non-enzymatic and enzymatic antioxidant systems respectively. This strongly suggests the antioxidant potential of Oyster mushroom.

Consequent to the liver membrane damage by CCl_3OO- following the intraperitoneal injection of CCl_4, activities of liver biomarker enzymes such as AST, ALT and ALP, assayed in this study commonly refer to as liver function indices or markers increased tremendously in the plasma. The observed increase in the activities of these liver enzymes was likely as a result of leakage of these enzymes from liver tissue into the plasma. These leakages could be due to LPO of cell membranes which had been reported to be the cause of loss of membrane fluidity, changes in membrane potential and an increase in membrane permeability. Interestingly, from this study, Supplementation of powdered Oyster mushroom daily for a period of 28 days reversed these hyper activities of these enzymes to nearly normal activities as compared to normal rats (group 1). This above observation strongly suggests the hepato-protective effect of Oyster mushroom and may be related to its ability to mitigate against LPO which in turn stabilizes the integrity of the hepatic tissue membranes thus preventing the leakage of these liver enzymes. Albumin and other proteins are synthesized in the liver and an impairment of the liver affects its ability to synthesize these biomolecules. Low serum levels of these proteins are considered a sign of hepatotoxicity.

In this study, intraperitoneal injection of CCl_4 resulted in a significant ($p<0.05$) decrease in the serum levels of albumin and total protein as compared to the normal rats (group I). This may suggest the inhibition of these proteins synthesis by CCl_4-generated peroxy radical (CCl3OO-). Our findings is inconsistent with previous studies as reported by Wessam (2013).The results from this study also show that supplementation of grower mash with 50% powdered Oyster mushroom daily for 28 days caused an increase ($p<0.05$) in serum level of albumin and total protein as compared to the CCl_4 injected rats (group II).This may indicate the ability of the Oyster mushroom to mitigate against oxidative liver damage thus restoring liver synthetic function. Liver plays a key role in the metabolism and transport of lipids. The results of this findings reveal a significant ($p<0.05$) increase in the serum levels of triglyceride TG, total cholesterol TC, very low density lipoprotein cholesterol VLDL-C and low density lipoprotein cholesterol LDL-C accompanied by a significant ($p<0.05$) reduction in the serum level of high density lipoprotein cholesterol HDL-C upon intraperitoneal injection of CCl_4 as compared to normal rats (control I). These results are in tandem with the findings of El-Habibi et al. (2009). Hyperlipidemic effect of CCl_4 as evident in this study may be related to its positive effect on acetate transport to the liver, esterification of fatty acids and disruption of membrane phospholipid. Others may include inhibition of synthesis of the bile acids and fatty acid β oxidation (Elshater et al., 2013).

Supplementation of grower mash with 50% powdered Oyster mushroom daily for 28 days reversed these elevated lipid profiles with concomitant increase in serum level of high density lipoprotein cholesterol HDL-C. Although an Oyster mushroom hypolipidemic effect is poorly understood, it may likely be related to inhibition of oxidative stress and hydroxymethylglutary-CoA (enzyme that catalyses the rate of limiting step of cholesterol biosynthesis) (Maduka et al., 2014). Intraperitoneal injection of CCl_4 to rats in this study also revealed alteration in hematological parameters of the rats.

The results showed that there was significant increase ($P < 0.05$) in WBC count with a corresponding decrease in RBC count and its differentials as compared to normal control. Our findings are consistent with the findings of Saba et al. (2010). The decreased RBCs count and its indices might be attributed to the oxidative stress imposed by CCl_4 injection (Sule et al., 2012). Destruction of hematopoiesis with consequent reduction in the rate of formation of RBC and its indices may be another reason for this reduction (Essawy et al., 2010).Whereas the increase in the WBCs count might be due to the defensive mechanism of immune system (Oluyemi et al., 2007).

Supplementation of powdered Oyster mushroom daily for 28 days to sample rats investigated led to a reversal of the altered hematological parameters to near normal level, suggestive of hepato-protective potentials. In this study, supplementation of powdered Oyster mushroom at the dose of 50% of the feed given to the rats appears to be more effective as compared to group iii which received silymarin.

Conclusion

In conclusion, daily supplementation of powdered Oyster mushroom subsided greatly dyslipidemia, hepatotoxicity and hematotoxicity associated with injection of CCl_4. Although its mechanism of action is unknown, it is believed to be associated with its ability to mitigate against LPO which in turn stabilizes the integrity of the hepatocyte membrane. Others may include enhancing the activities of enzymatic and non-enzymatic antioxidants in the liver. Research is in progress to isolate the bioactive compound responsible for these observed positive effects.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Identification of annotated metabolites in the extract of *Centella asiatica*

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Plants contribute to 75% of molecular medicines (MM) either directly or indirectly. *Centella asiatica* (CA) is being extensively used in experimental and clinical studies. However, its scientific approval is not forthcoming. It is well known that every plant contains useful as well as some harmful compounds. Subjecting whole plant extracts containing harmful compounds to modern pharmacological methods will only demonstrate that they are not safe for use as medicines. To ascertain both the useful and harmful compounds in CA extract, all the compounds of the extract must be identified. In the present study, a methanol extract was prepared from the whole plant. The compounds were identified using liquid chromatography-mass spectrometry with database confirmation. 3,201 compounds were identified using the METLIN database. Database searches yielded 1,187 biological compounds of which 154 were for human/human cell lines. These 154 compounds were classified based on their already reported effects. Two contemporary medicines found in this extract were quantified. Here, we report both beneficial and harmful compounds in the methanol extract of CA. We propose that the harmful compounds can be removed to yield safe medicines from CA.

Key words: *Centella asiatica*, mass spectrometry, secondary metabolites, traditional medicine, medicinal plant, molecular medicine.

INTRODUCTION

Traditional medicine (TM) is the result of accumulated knowledge and practices based on past experiences. As different TMs evolved 5000 years ago, they mainly depended on natural products. However, the TM system appears to have become stagnant over the past millennium with regard to new theories and practices. TM lacks a suitable means to review its principles and practices. Cochrane reviews of available studies reveal that clinical trials in TM have poor controls and lack statistical power or comparisons (Nahin and Straus,

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2001; Narahari et al., 2010; Telles et al., 2014).

Conversely, traditional herbal medicines are claimed to be effective and safe. This assertion has existed throughout history and is a common claim by millions who have different medical options. About one-fifth of the US population uses some form of TM (Barnes et al., 2004), more than 40% of adults with neuropsychiatric symptoms in the US used complementary or alternative medicine in the year 2007 (Purohit et al., 2012), and 35.3% of persons diagnosed with cancer in the US took aid of complementary health approaches (Clarke, 2018).

Of all cancer patients in China, 93.4% were using complementary medicines during the period of 2009-2010 (Teng et al., 2010). About three-fourths of molecular medicines originate from plants. Molecules of plant origin are used in many therapeutic applications, including: cancer (paclitaxel from *Taxus brevifolia*) (Rao, 1993; Weaver, 2014), malaria (artemisinin from *Artemisia annua*) (Klayman et al., 1984; Levesque and Seeberger, 2012), Alzheimer's disease (galantamine from *Galanthus nivalis*) (Heinric and Lee, 2004; Libro et al., 2016), hypertension (reserpine from *Rauvolfia serpentina*) (Sheldon and Kotte, 1957; Lobay, 2015), and pain (codeine and morphine from *Papaver somniferum*) (Tookey et al., 1976; Maurya et al., 2014). It is the presence of these types of molecules that provide the medicinal value found in plants.

Importantly, plants appear to have molecules that may be helpful in certain clinical applications for which contemporary medicine has no solution, such as neurodegenerative disorders. Though it evolved before the concepts and tools of modern pharmacology, TM remains empirical, and the molecules devised by it can be used therapeutically without significant manipulation. Rather than blaming TM, these molecules should be engineered for efficacy and to avoid unwanted interactions. There were early attempts to identify molecules from the extracts of many medicinal plants in different contexts. Some studies identified plant extract molecules in the serum because only the absorbed molecules matter as medicines (Sun et al., 2012).

Few studies have reported the compounds that are present in specific fractions (Ding et al., 2014; Sun et al., 2016). Numerous interaction studies on herbal formulations with conventional drugs are available (Chen et al., 2012). Knowledge about herbal drugs has grown in recent years, but the recommendation of their use is not forthcoming. They are acceptable only if there is no contemporary treatment available and no herb-drug interaction is known (Mörike and Gleiter, 2014). The number, concentrations, and identities of all compounds consumed as herbal medicines are not known.

These preparations along with many beneficial compounds may contain compounds such as the phytotoxins, excess heavy metals, apoptotic inducers, and psychoactive and drug interacting chemicals. Available evidence has shown that several herbal products that have been put to extensive use in traditional medications for generations may possess carcinogenic, hepatotoxic, cardiotoxic and other harmful activities (Gromek et al., 2015). Thus, such harmful compounds should be removed to make traditional herbal medicines safer and more acceptable. It is imperative that we identify all of the compounds present in an extract. Only then can we know which useful, harmful or conditionally useful compounds are present in traditional medicines. The fingerprinting of extracts is helpful for molecular medicine to better understand the active components the plants contain and for TM to avoid unwanted reactions (Miyata, 2007; Yuan et al., 2016).

Centella asiatica, a member of Apiaceae family, is native to the tropical countries of India, China, Malaysia, Sri Lanka, Indonesia, South Africa, and Madagascar (Orhan, 2012; Sabaragamuwa et al., 2018). This perennial, herbaceous creeper has small fan-shaped green leaves, white flowers and bears small oval fruits. The medicinal qualities of this plant have been utilized in Ayurvedic and Chinese traditional medicines for centuries (Meulenbeld and Wujastyk, 2001; Yuan et al., 2016). The whole plant of CA can be used for medicinal purposes.

CA is reported to possess an array of therapeutic properties such as wound healing (Somboonwong et al., 2012; Jenwitheesuk et al., 2018), anti-inflammatory (Park et al., 2017), anti-cancer (Rai et al., 2014), anti-ulcer (Cheng and Koo, 2000; Zheng et al., 2016), anti-diabetic (Chauhan et al., 2010; Emran et al., 2016), anti-convulsant (Visweswari et al., 2010; Manasa et al., 2016), immunostimulant (Wang et al., 2003; Sushen et al., 2017), neuroprotective (Kumar et al., 2009; Sabaragamuwa et al., 2018), hepatoprotective (Antony et al., 2006; Sivakumar et al., 2018), cardioprotective (Gnanapragasam et al., 2004; Kumar et al., 2015), anti-bacterial (Dash et al., 2011; Soyingbe et al., 2018), anti-viral (Yoosook et al., 2000; Sushen et al., 2017), anti-fungal (Naz and Ahmad, 2009; Senthilkumar, 2018), insecticidal (Senthilkumar et al., 2009), and anti-oxidant (Pittella et al., 2009; Gulumian et al., 2018). Triterpene saponosides are the major class of active compounds in CA and are generally accredited for its therapeutic physiological effects (Gohil et al., 2010).

CA is a popular medicinal herb that is widely utilized for its therapeutic properties in a number of traditional medicinal systems. However, individual compounds responsible for different therapeutic physiological effects or compounds that might have adverse effects are not known. Since it is a widely consumed plant, there is an urgent need for in-depth analysis of compounds present in this plant rather than only focusing on the gross effects of the herb. Often prescribed in many traditional medicine systems, the whole plant extract of CA was used to identify all the compounds present in this plant during the present study.

Methanolic extraction was employed to get high percentage yield of extracts from the plant (Dhawan and Gupta, 2017) and extract maximum bioactive compounds such as alkaloids, steroids, flavinoids, saponins, and tannins from CA.

Here, we report the identification of a large number of compounds in the methanol extract of CA (Gotu Kola) using mass spectrometry and database confirmation, an effective way of performing large scale untargeted plant screening. We understand that this knowledge is critically needed to realize the medicinal value of a plant by identifying the presence of (1) the active therapeutic molecules (efficiency) and (2) the harmful molecules that should be removed from the preparation.

MATERIALS AND METHODS

The extract

CA was obtained from the Mother India Nursery, Najafgarh, New Delhi and identified by Dr. Vandana Mishra, Department of Environmental Studies, University of Delhi. The plant was submitted to the herbarium in the same department with the herbarium voucher DUH 14337. The plants were collected from the same place within a period of 15 days to avoid the metabolite variability due to seasons, geography and environmental causes. Surface disinfection was performed by brushing the fresh plants with a soft brush under running tap water before processing. The whole plant was cut into small pieces and dried in the laboratory. The methanol extract of the dried plant material was prepared by incubating it with 100% methanol for 24 h at room temperature with mechanical stirring. The extract was centrifuged at 10,000 g for 10 min at room temperature. The supernatant was collected. The methanol from the supernatant was removed in a centrifugal evaporator. The dried sample was weighed and stored at -20°C in an airtight vial

Identification of compounds

The dried methanolic extract was re-suspended in 0.1% formic acid, centrifuged and injected into a Shimadzu Prominence SIL HTC system equipped with a C18 column, 1.8 µm, 2.1×100 mm (ACQUITY UPLC HSS T3 Column). A gradient elution using water and acetonitrile containing 0.1% formic acid was used at a flow rate of 0.3 ml/min for 20 min. The injection volume was 5 µl. An AB SCIEX TripleTOF® 5600 LC-MS/MS system with a DuoSpray™ Source and electrospray ionization (ESI) probe was used for data acquisition. TOF MS scan mode acquisition with simultaneous information dependent acquisition MS/MS was performed. Each sample was analyzed in positive polarity. Data were acquired over a mass range of 100 to 1100 m/z with IDA MS/MS performed with a collision energy of 35 eV and a spread of ±20 eV. From the MS/MS data collected, the compounds corresponding to 100 to 1100 m/z were searched. Only the monoisotopic molecule ions were considered. The generated peak list with a peak above the quality of 30 was searched in METLIN database using its m/z ratio. All the possible compounds for a given monoisotopic mass were screened for known biological activity using Pubmed and Google. The data of shortlisted compounds was processed using PeakView® Software, AB Sciex for features identification and ID confirmation. The structure elucidation of the compound was confirmed by matching experimental fragments of the m/z to theoretical fragments of the structure. The molfile file of the possible structure was uploaded in PeakView software which then generated the fragmentation pattern for that structure. The fragments generated from molfile were matched with the experimental fragments. The percentage of matching was generated from the software. Only the compounds having 100% match were considered in the present analysis.

Quantification of compounds

In order to substantiate that the compounds identified by software are actually present in the extract, two compounds, methotrexate and cytarabine were selected at random for quantification. The stock solutions of methotrexate, cytarabine, and homatropine (internal standard) were separately prepared at a concentration of 1 mg/ml in acetonitrile. The stock solutions were appropriately diluted with 75% acetonitrile containing 0.1% formic acid to reach the required lower working concentrations.

The calibration curves of methotrexate and cytarabine were plotted with concentrations ranging from 3.9 to 62.5 ng/ml. A working internal standard solution was used at a concentration of 250 ng/ml in 75% acetonitrile. A ZIC HILIC 4.6×50 mm column was used for analytical separation with an isocratic mobile phase consisting of 10 mM ammonium formate and acetonitrile containing 0.1% formic acid at a ratio of 25:75 with a flow rate of 0.5 ml/min. The autosampler tray and the column were kept at 22°C. 20 µl of the sample was injected into the UPLC with a run time of 5 min.

Chromquest software version 4.1 was used to control all UPLC parameters. The tandem mass spectrometric detection of analyte and internal standard (IS) was performed on a 4000QTrap (AB SCIEX) equipped with a Turbo Ion Spray (ESI) source operating in positive ion mode. Data acquisition and integration were performed by Analyst 1.5.2 software (AB SCIEX). The source dependent and compound dependent parameters were optimized in positive ion mode using the inbuilt algorithm of Analyst 1.5.2 software to yield the maximum intensity for precise detection.

RESULTS

Identification of compounds

The non-redundant number of compounds containing both MS and MS2 spectra was found to be 3,201 in positive polarity. About half of the compounds (46.8%) had less than 1 ppm mass error when compared to the METLIN database. More than three-fourths of the compounds (77.4%) were within 5 ppm mass error. The rest of the compounds had a mass error between 6 and 10 ppm. The methanolic extract of CA was separated on UPLC coupled to mass spectrometer. The duplicate runs are shown in Figure 1.

Molecular identification of m/z 244.09 is as shown in Figure 2. Figure 2, Panel A shows chromatogram of CA methanolic extract while Panel B shows the compound eluted at 1.43 min and had m/z value of 244.09. The identification of the compound was done based on the fragment matching of the theoretical and experimental MS/MS pattern. The m/z of the peak was uploaded into METLIN software and a list of all possible compounds was generated from METLIN.

The molfile of each compound was then used to generate the theoretical fragments and was compared with the experimental MS/MS spectrum for identification

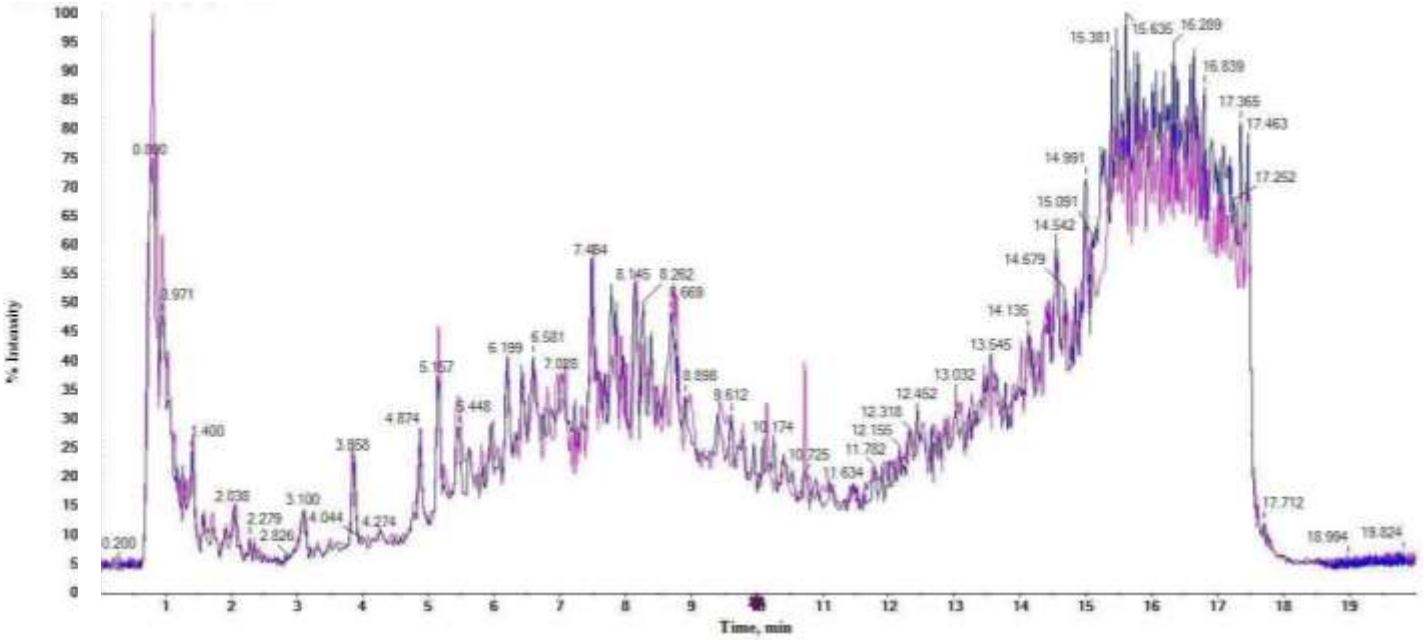

Figure 1. Chromatogram of the *Centella asaitica* methanolic extract. Two different runs are indicated in red and blue.

of the compound. The percentage matching was obtained for each compound and a list of all the fragments assigned by matching theoretical fragments of the structure to the experimental spectrum was displayed. The molfile of cytarabine showed 100% matching with the experimental fragments of 244.09 (Figure 2, Panel C).

Thus, the m/z 244.09 was identified as Cytarabine (Figure 2, Panel D). Highlighted in blue are the peaks that have been matched. If there is no matching, then the peak will be highlighted in red. All the m/z matched to molfile are listed in panel E of Figure 2. The m/z 244.09 fragmented to 227.10, 169.04, 112.05, 95.02 and 84.04 m/z. The determined compound has 100% matching of the experimental and theoretical peaks (Figure 2, Panel E). Major fragments in the spectrum (m/z 227.10, 169.04, and 112.05) were investigated, and each m/z was also seen in MS mode with the same elution profiles at a retention time of 1.43 min.

Categorization of compounds

Among these 1,187 compounds identified in the extract, 154 compounds have been studied in humans or human cell lines and have 100% matches with the theoretical and observed MS/MS spectra (fragments). These compounds had varying effects on living systems. They had properties that were inflammatory, anti-oxidative, anti-bacterial, anti-nociceptive, anti-viral, anti-fungal, anti-apoptotic, anti-parasitic, anti-insecticidal, neuroprotective, anti-tumor, anti-diabetic, etc. They also had volatile compound, including insect attractants and repellants, some of which were quorum sensing compounds for plants (Supplementary Information, Tables S1, S2, S3, S4, S5, and S6). These 154 compounds were categorized based on their effect on normal physiology. The present report is based on the analysis of these compounds. Table 1 lists 37 compounds that are listed as drugs in the Drugbank database (DrugBank Version 4.3) (Wishart et al., 2006). 17 toxins were detected in the extract (Table 2). In addition to these compounds, 11 environmental pollutants were identified (Table 3).

Quantification of compounds

The quantification of methotrexate and cytarabine was performed in multiple reaction monitoring (MRM) mode based on molecular adduct ion and its fragment ion with the transitions of 244.3 → 112.1 for cytarabine (Figure 3, Panel A) and m/z 455.2 → 308.3 for methotrexate (Figure 3, Panel B). The transition of m/z 276.1 → 142.2 was used for the internal standard (homatropine). All of the calibration curves showed good linearity ($r > 0.999$) within the test ranges. The precision was evaluated by intra and inter day tests, which revealed relative standard deviation (RSD) values of less than 3.88%. The recoveries for the quantified compounds were between 96.3 and 103.7%, with RSD values below 2.89%. Methotrexate and cytarabine were detected at concentrations of 60 and 400

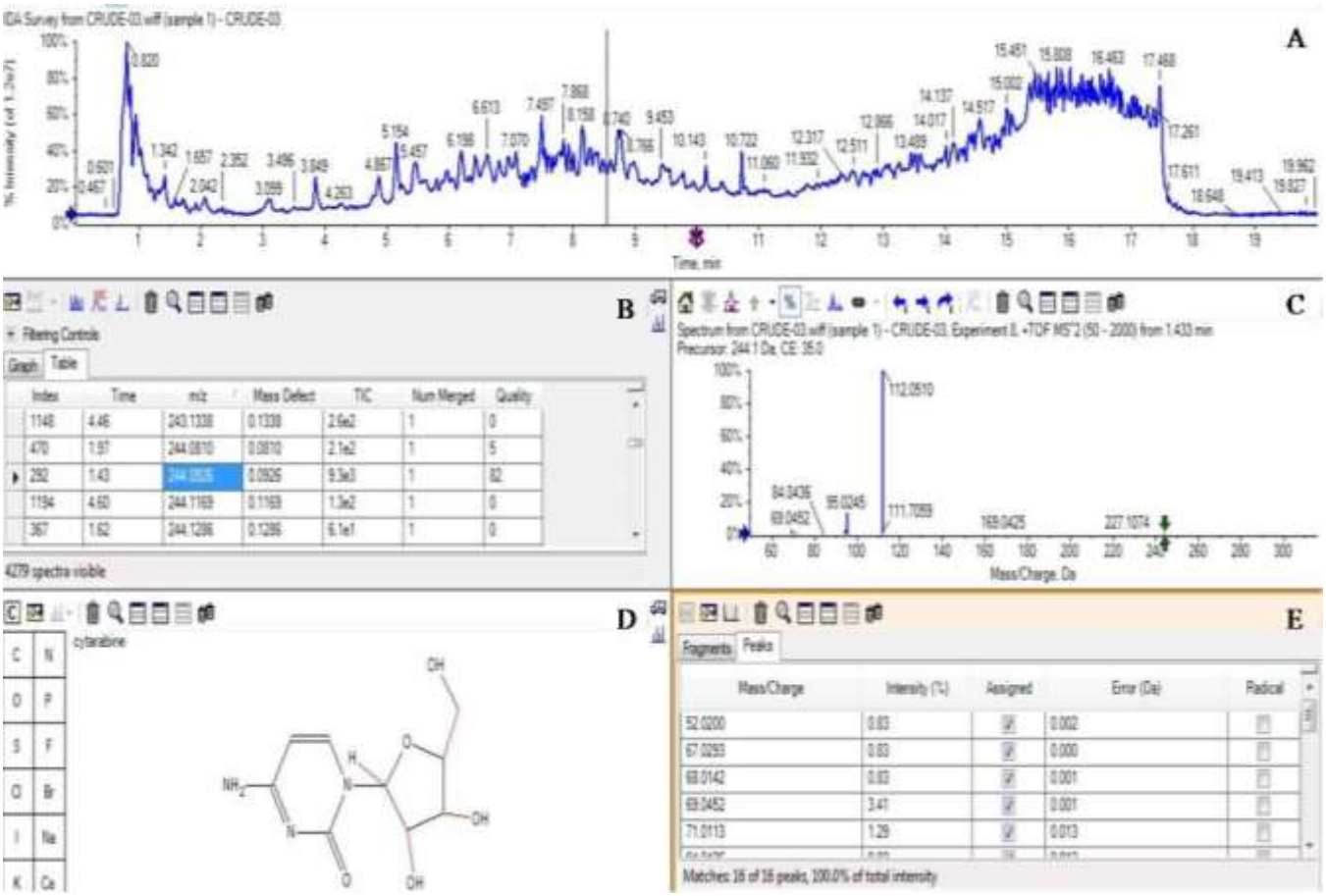

Figure 2. Structural Elucidation of m/z 244.09 in PeakView™ Software. Panel (A) shows the elution pattern, panel (B) shows the m/z and retention time, panel (C) indicates the fragmentation pattern, panel (D) shows the identity, and panel (E) shows peak matching of both experimental and theoretical fragments for the compound shown, cytarabine.

ng/mg of methanol extract, respectively.

DISCUSSION

The contemporary medicines

Thirty seven contemporary drugs were identified in the methanolic extract of CA (Table 1). The pharmacological activities of these drugs are already established. Upon reviewing the literature, it was found that CA is reported to exhibit same therapeutic effects as these 30 contemporary drugs. CA manifests anti-diabetic (Rahman et al., 2011; Emran et al., 2015), anti-ulcer (Sairam et al., 2001; Abdulla et al., 2010), anti-fungal (Lalitha et al., 2013), anti-cholinergic (Arora et al., 2018), anti-cancer (Hamid et al., 2016; Ren et al., 2016), anti-bacterial (Arumugam et al., 2011; Idris and Nadzir, 2017), anxiolytic (Wijeweea et al., 2006; Wanasuntronwong et al., 2012), anti-convulsant (Manasa and Sachin, 2016; Sudha et al., 2002), anti-inflammatory (Somchit et al., 2004; Chippada et al., 2011), anti-diarrheal (George et al., 2009), anti-psychotic (Chimbalkar et al., 2015; Chen et al., 2003), anti-nociceptive (Somchit et al., 2004; George et al., 2008), analgesic (Saha et al., 2013; Qureshi et al., 2015) and sedative (Hossain et al., 2005; Rocha et al., 2011) properties. Though it has been proved that CA possesses the aforesaid properties in various studies, the specific compounds or the mechanism of action responsible for all these properties are not known. There is a concurrence of the pharmacological effects of the drugs identified in extract and reported effects of CA. This indicates that the drugs identified in the study may be responsible for the remedial effects illustrated by CA extract.

The toxins

Seventeen toxins (Table 2) have been identified in the

Table 1. Contemporary medicines identified in the *Centella asiatica* extract.

Compound name	m/z	Functions	Drug Bank Reference
Acarbose	646.2553	Anti-diabetic	DB00284 (APRD00656)
Cinitapride	403.2013	Gastroprokinetic agent and anti-ulcer agent	DB08810
Clotrimazole	344.108	Anti-fungal	DB00257 (APRD00244)
Conivaptan	499.2176	Vasopressin receptor antagonist	DB00872 (APRD01302)
Cyclopentate	292.1911	Anti-cholinergic	DB00979 (APRD00893)
Cytarabine	244.0928	Anti-leukemia	DB00987 (APRD00499)
Demeclocycline	464.0986	Anti-bacterial	DB00618 (APRD00272)
Desflurane	168.001	Anesthetic	DB01189 (APRD00907)
Ecabet	380.1657	For reflux esophagitis and peptic ulcer disease.	DB05265
Eplerenone	415.2120	Aldosterone receptor antagonist	DB00700 (APRD00707)
Epothilone D	492.2778	Colorectal cancer	DB01873 (EXPT01350)
Eprosartan	424.1457	Diabetic Nephropathy and Hypertension	DB00876 (APRD00950)
Estrone acetate	312.1725	17-beta-isomer of estradiol	DB00783 (Estradiol)
Eszopiclone	389.1123	Nonbenzodiazepine hypnotic	DB00402 (APRD0043)
Ethynodiol diacetate	384.23	A synthetic progestational hormone	DB00823 (APRD00760)
Fludiazepam	302.0622	Anxiolytic, anti-convulsant, sedative and skeletal muscle relaxant	DB01567
Fludrocortisone	380.1999	Synthetic adrenocortical steroid	DB00687 (APRD00756, DB02478)
Flunitrazepam	314.0936	Sedative, anti-convulsant	DB01544
Isothipendyl	285.13	Anti-histamine, anti-cholinergic	DB08802
Loperamide	476.2231	Anti-diarrheal	DB00836 (APRD00275)
Lumichrome	243.0194	Activates the LasR bacterial quorum-sensing receptor.	DB04345 (EXPT02065)
Meclizine	390.1863	Anti-histamine	DB00737 (APRD00354)
Methotrexate	455.1786	Chemotherapy drug	DB00563 (APRD00353)
Mitoxantrone	445.2082	Anti-neoplastic agent	DB01204 (APRD00371)
Netilmicin	476.3060	Anti-biotic	DB00955 (APRD00232)
Nitisinone	329.0511	Inhibitor of HPPD	DB00348 (APRD01141)
Norethindrone acetate	340.2038	Progestational hormone	DB00717 (APRD00679)
Penbutolol	291.2198	Beta-blocker	DB01359
Perphenazine	404.1558	Anti-psychotic drug	DB00850 (APRD00429)
Phenacetin	180.1018	Anti-pain, fever-reducing	DB03783 (DB08243, EXPT03306)
Prazepam	324.1029	Anxiety disorders	DB01588
Prochlorperazine	374.1449	Anti-psychotic	DB00433 (APRD00624)
Remoxipride	371.0976	Anti-psychotic agent	DB00409 (APRD00316)
Ridogrel	367.1283	Systemic thrombo-embolism	DB01207 (APRD00271)
Rimonabant	463.0854	Anorectic anti-obesity	DB06155

Table 1. Cont.

Vigabatrin	130.0863	Anti-epileptic drug	DB01080 (APRD00282)
Zidovudin	268.1045	Anti-retroviral drug	DB00495 (APRD00449)

Table 2. Toxins from *Centella asiatica* extract.

Compound name	m/z	Origin	Functions	References
(-)-Batrachotoxin	538.3043	Fungi	Potent cardiotoxic and neurotoxic steroidal alkaloid	Tokuyama et al. (1969)
1-Desulfoyessotoxin	1062.522	Marine	Cytotoxic	Korsnes et al. (2006); Korsnes et al. (2013)
Anatoxin-a(s)	252.0987	Fungi	Neurotoxic cyanobacterial toxin	Hyde et al. (1991); Mejean et al. (2014)
Daphnin	341.0872	Plant	Binds to HAS. Plant toxin	Zhu et al. (2012)
Dihydrosterigmatocystin	326.079	Fungi	Aflatoxin biosynthesis	Yabe et al. (1998)
Gitoxin	781.4386	Plant	Cardenolides	Haustein et al. (1975)
Huratoxin	584.3349	Plant	From *Pimelea simplex*	Mcclure et al. (1984)
Illudin M	249.1482	Fungi	Fungal cytotoxin illudin M	Schobert et al. (2008)
Ipomeamaronol	267.1592	Plant	Furanoterpenoid toxins from sweet potato	Shen et al. (1997)
Miserotoxin	267.0954	Plant	Plant toxin	Patocka et al. (2000)
Nivalenol	312.1209	Fungi	Mycotoxin	Li et al. (2014)
Onchidal	277.1761	Marine	Neurotoxins with anti-cholinesterase activity	Pita et al. (2003)
Oscillatoxin A	578.3091	Marine	Toxins from blue-green algae	Fujiki et al. (1983)
Patulin	154.0266	Fungi	Cytotoxic and Cytopathic	Schaeffer et al. (1975)
Radicinin	237.0862	Fungi	Produced by *Alternaria radicina* on carrots	Solfrizzo et al. (2004)
Sambutoxin	453.2879	Fungi	Mycotoxin by *Fusarium* spp.	Kim et al. (1994)
Zinniol	267.1592	Fungi	Phytotoxins from *Alternaria solani.*	Moreno et al. (2005)

CA extract. Five of these toxins were of plant origin, nine toxins were from fungi and three toxins were from marine origin. The plant origin toxins could be inherent but fungi and marine origin toxins seem to be acquired. Plant origin toxins ipomeamaronol, huratoxin, daphnin, gitoxin, and miserotoxin have been reported in diverse plants in the literature. It is known that a large number of endophytic fungi and bacteria reside in CA (Rakotoniriana et al., 2008). 45 different taxa of fungi and 31 endophytic bacteria were isolated from healthy leaves of CA. Endophytes alter the composition of secondary metabolites in their plant hosts (Gao et al., 2015; Zhang et al., 2013). Climatic stress, wounding, metals, bacterial infections, fungal infections, and environmental chemicals also control and contribute to plant metabolomics (Ramakrishna and Ravishankar, 2011; Ncube et al., 2013; Nasim and Dhir, 2010). Plant secondary metabolites have microbicidal, insecticidal or herbicidal functions. It is surprising that the toxins oscillatoxin A (blue-green algae), 1-desulfoyessotoxin (dinoflagellate) and onchidal (onchidioideans) are present in CA considering that these three species are of marine origin. These three species are also known to thrive in freshwater environments. The early land-dwelling plants were effective in the uptake of DNA (broken by the harsh conditions), which might have helped in the acquisition of many self-defense functions (Cove, 2015). Notwith-standing the apical meristem, plants do acquire foreign entities through various mechanisms. These toxin molecules

Table 3. Environmental pollutants seen in *Centella asiatica*.

Compound	m/z	Function	References
Benfuresate	256.0769	Pesticide	Albert-García et al. (2008)
Chlorphoxim	332.0151	Insecticide	Bown et al. (1984)
Coumachlor	343.1239	Rodenticide	Dam et al. (1953)
Cycloprothrin	481.0848	Insecticide	Jiang et al. (2008)
Dodemorph (93% matching)	282.2806	Pesticide	Leenheers et al. (1992)
HC Blue no. 2	286.1398	Hair colorant	Kari et al. (1989)
Isopropalin	310.2307	Pesticide	West et al. (1988)
Propargite	350.1552	Pesticides	Zhang et al. (2014)
Thionazin (95.6% matching)	249.1482	Pesticide	Skrbić et al. (2007)
Tolfenpyrad	383.1401	Insecticide	Hikiji et al. (2013)
Xylylcarb	180.1018	Pesticide	Hayatsu et al. (2001)

provide a glimpse of evolutionary interactions between plants, marine, and microbial genomes.

Environmental pollutants

Eleven environmental pollutants were identified in the present study of CA extract (Table 3). The soil of medicinal plants is also a major cause for concern since the toxicity of traditional medicinal plants is associated with environmental pollutants. The unacceptable level of heavy metals in traditional medicine is well known. Plants take up nutrients, fertilizers and other chemicals from their environment. Environmental pollutants such as synthetic pesticides, rodenticide, etc., can be taken up. Some of the pollutants detected are organo-phosphorous and c-organochlorine compounds. Long-term exposure of experimental animals to organo-phosphorous compounds resulted in glucose intolerance with hyperinsulinemia, a hallmark of insulin resistance (Nagaraju et al., 2015). Some of the pollutants are acetylcholine esterase inhibitors and carcinogens. A previous study reported 198 types of pesticides in 120 types of traditional Chinese medicine (Wang et al., 2014). Furthermore, the types of pesticides were not the same in different parts of the plant. Typically, any acute effect (toxicity of pesticides, the benefit of medicines, etc.) has been largely measured at the acute median lethal dose or concentration. In addition to direct mortality induced by pesticides, their sub-lethal effects on physiology and behavior must be considered for a complete analysis of their impact. To appreciate the potential effects of a pest or pollutant, ecologically relevant experimental approaches that take into account the sub-lethal exposure over the long-term should be developed. The results of one such study indicated that insecticide mixtures continue to affect natural systems over many weeks, despite no traces of the mixture being detectable in the environment. Direct and indirect consequences across many levels could be observed (Hasenbein et al., 2016). The data of the present study revealed that care should be exercised while disposing biological and industrial waste.

Quantification of compounds

Cytarabine and methotrexate are prescribed for cancer treatment. As the beginning of therapy, a low dose of cytarabine produced maximal anti-leukemic effects a evidenced by all response endpoints. This suggests saturation in the dose-response relationship at this dose level. High-dose cytarabine results in excessive toxic effects without the therapeutic benefit (NTR 230). When used for induction, methotrexate in doses of 3.3 mg/m^2 in combination with 60 mg/m^2 of prednisone, given daily, produced remission produced remission in 50% of treated patients, usually within a period of 4 to 6 weeks. These concentrations are at least ten times greater than the concentrations of cytarabine and methotrexate estimated in the CA extract. The two drugs were detected at concentrations of 0.4 µg and 0.06 µg/mg of methanol extract. A normal prescribed dose of up to 1 to 10 g/kg/day of CA extract is recommended for enhanced cognition over a 52 week period (Manyam et al., 2004; Kumari et al., 2016).

Over this span, 2.0 and 0.3 mg of these drugs will be consumed, respectively. Methotrexate is at the top of the list of high-risk drugs in a hospital setting causing prolonged hospitalization (Saedder et al., 2014). Methotrexate and cytarabine can exert their effects at low doses (Hocaoglu et al., 2008). Another set of compounds that could be actively harmful are hormones. There are plant and insect related hormones in CA. Four compounds, megestrol acetate, norethindrone acetate, conivaptan and ∆4-tibolone, are hormonal drugs used in humans. There are also compounds that stimulate hormones. In TM, CA extract is taken over a long period. Effectively, one is consuming over twenty cancer drugs at

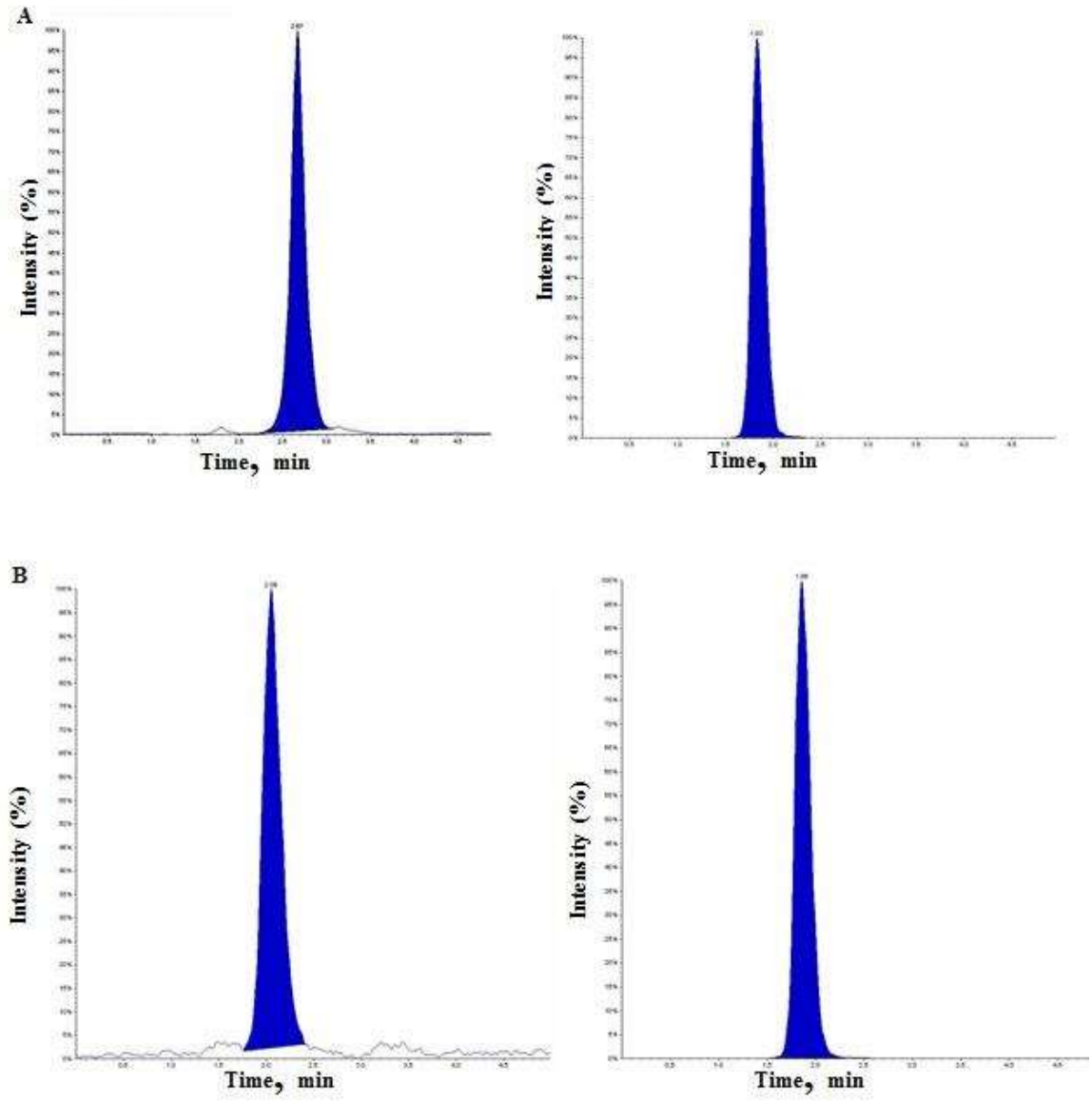

Figure 3. Quantification of cytarabine and methotrexate using LC-ESI-MS/MS. (A) representative chromatogram of cytarabine m/z 244.3 → 112.1 at 2.67 min and internal standard homatropine m/z 276.1 → 142.2 at 1.83 min, respectively, in the plant extract. (B) Representative chromatogram of methotrexate m/z 455.2→ 308.3 at 2.06 min and the internal standard homatropine at m/z 276.1 → 142 at 1.83 min in the plant extract.

sub-lethal doses over an extended period. If the intention is neuroprotection or anti-oxidation, the consumption of cytotoxic compounds is unnecessary.

CA has been actively used as a medicinal herb in Ayurveda for various ailments from ancient times. For many years, CA extracts have been considered acutely and chronically non-toxic, even at doses as high as 5000 mg/kg (Chivapat et al., 2011; Chauhan and Singh, 2012; Deshpande et al., 2015). A clinical case study has reported the development of hepatotoxicity in three women after consuming CA tablets for 30, 20 and 60 days in order to lose weight (Jorge and Jorge, 2005). In the same study, it was found that not only did the discontinuation of the tablets help cure the symptoms such as jaundice, hepatitis, hepatomegaly, choluria, etc., but the commencement of the tablets again reinstated the same symptoms. Another study reported a 15-year girl to develop hepatotoxicity after ingesting lymecycline and a herbal medicine with active ingredient CA (20 mg/day) for 6 weeks to treating acne (Dantuluri et al., 2011).

The study concluded that the deranged liver function and coagulation profile were in fact caused by herbal medicine containing CA. Methotrexate and Cytarabine are well-known hepatotoxicity inducers (Sotoudehmanesh et al., 2010; Thatishetty et al., 2013). Consequently, chemotherapic drugs methotrexate and cytarabine might play a role in hepatotoxicity caused by CA ingestion in different cases. Ingestion of crude plant extract can cause many such interactions.

Library of secondary metabolites

Since the pharmacological activities of 154 compounds have been reported in human or human cell lines, we have the name, chemical formulae, therapeutic activity in humans as well as the MS/MS data for these 154 plant products (many given under different categories). This list should be combined with compounds from aqueous and other extracts of CA in the future. This compilation should be continued to include all medicinal plants. A detailed database of secondary metabolites from all medicinal plants will be of great pharmaceutical use. To complete the knowledge, (1) all the compounds are to be studied *in vitro*/*ex vivo*/*in vivo* and (2) the peaks with MS and MS/MS data (including compounds with less than 100% matching) should be identified *de novo* as well as their effects should be studied. Natural products are the most proven source of novel drug candidates. An integrated approach involving virtual screening, automated high content assays and high impact technologies for fractionation as well as assay development is urgently needed in drug discovery research (Cremin and Zeng, 2002; Koehn, 2008). The availability of purified natural products and experimental high-throughput screening can synergize to yield new therapies.

Conclusions

TM should make sure of the identity of the compounds present in the extract. This will identify the harmful compounds. The presence of the active molecules indicates the efficacy of the extract. TM should undergo pharmacological tests to gain acceptance. Equally important issues are quality assurance and Good Manufacturing Practices (GMP). TM can assure the safety of plant compounds after enzyme inhibitors, toxins, environmental pollutants, and cytotoxic substances are removed. The approach used in this study is affordable, efficient, and safe.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Table S1. Anti-cancer compounds from CA extract:

S/N	Compound name	M/z	Functions	References
1.	Alloantolactone	233.1897	Induces Apoptosis and Cell Cycle Arrest in Lung Squamous Cancer SK-MES-1 Cells	Zhao et al. (2015)
2.	Alismol	221.1894	Cytotoxic, cytostatic and HIV-1 PR inhibitory activities	Ellithey et al. (2015)
3.	Alpha-phellandrene	137.1320	anticancer and antioxidant activities of Schinusmolle L. and SchinusterebinthifoliusRaddi berries essential oils	Bendaoud et al. (2010)
4.	Altersolanol	337.0925	in vitro cytotoxic activity against 34 human cancer cell lines with mean IC50 (IC70) values of 0.005 µg ml(-1) (0.024 µg ml(-1), kinase inhibitor, induces cell death by apoptosis through the cleavage by Caspase-3	Mishra et al. (2015)
5.	Alpha-santalol	221.1894	Chemopreventive mechanism for a traditional medicine: induces autophagy and cell death in proliferating keratinocytes	Dickinson et al. (2014)
6.	Amurensin	534.1737	Amurensin G inhibits angiogenesis and tumor growth of tamoxifen-resistant breast cancer via Pin1 inhibition	Kim et al. (2012)
7.	Asiatic Acid	489.3569	Anti-tumor and anti-angiogenic activity evaluations of asiatic acid	Jing et al. (2015)
8.	Beta-cyclocostunolide	233.1536	anticancer activity study	Robinson et al. (2008)
9.	beta-Thujaplicin	164.0837	β-Thujaplicinmodulates estrogen receptor signaling and inhibits proliferation of human breast cancer cells	Ko et al. (2015)
10.	Broussoflavonol F	422.1729	Broussoflavonol B restricts growth of ER-negative breast cancer stem-like cells	Guo et al. (2013)
11.	Carbestrol	275.0761	Anti-cancer drug in trial	Kogler et al. (1972)
12.	Ceanothic acid	487.3433	Potent cancer chemopreventive agents	Nakagawa etal. (2009)
13.	Chicoric Acid	475.0873	Antioxidant and antiproliferativeactivities	Elansaryetal. (2015)
14.	Costunolide	233.1897	Potential Anti-Cancer Activitiy	Lin et al. (2015)
15.	Cryptocaryone	283.0966	Induces apoptosis in human androgen independent prostate cancer cells	Chen et al. (2010)
16.	Cytrabine	244.0932	A pyrimidine nucleoside analog that is used mainly in the treatment of leukemia, especially acute non-lymphoblastic leukemia	Larrueet al. (2015)
17.	Damsin	249.1482	Multiple anticancer effects	Villagomez et al. (2013)
18.	Delphinidin	303.0383	Inhibits vascular endothelial growth factor receptor-2 phosphorylation, sensitizes prostate cancer cells to TRAIL-induced apoptosis	Ko et al., 2015
19.	Dextrin	505.1763	Anti-tumor effect of pH-responsive dextrin nanogels delivering doxorubicin on colorectal cancer	Manchun et al. (2015)
20.	Elesclomol	400.1028	Cellular mechanisms of the cytotoxicity of the anticancer drug elesclomol and its complex with Cu(II)	Hasinoff et al. (2015)
21.	Ginsenoyne K	277.2161	Cytotoxic phenylpropanoids from carrot	Yang et al. (2008)
22.	Isoalantolactone	233.1536	Induces reactive oxygen species mediated apoptosis in pancreatic carcinoma PANC-1 Cells	Khan et al. (2012)
23.	Jatrophone	312.1725	Plant anticancer agents. 28. New antileukemicjatrophone derivatives from Jatrophagossypiifolia: structural and stereochemical assignment through nuclear magnetic resonance spectroscopy	Pessoa et al. (1999)
24.	Methotrexate	455.1596	Chemotherapy drug	Chu et al. (2015)

Table S1. Contd.

25.	Mitoxantrone	445.2065	Anthracenedioneantineoplastic agent.	Hussain et al. (2015)
26.	Parthenolide	249.1481	Inducing apoptosis in acute myelogenous leukemia cells,	Kim et al. (2015)
27.	Perillyl alcohol	153.1269	Perillyl alcohol for cancer including lung cancer, breast cancer, colon cancer, prostate cancer, and brain cancer	Chen et al. (2015)
28.	Sorafenib	465.1030	Sorafenib inhibit colonies formation into human hepatocarcinoma cells	Bondì et al. (2015)
29.	Usambarensine	432.2314	Potential anticancer and antiparasitic	Bonjean et al. (1996)

Table S2. Antioxidant compounds from CA extract.

S/N	Compound name	M/z	Functions	References
1.	1,4-Di-O-caffeoylquinic acid	515.1194	Evaluation of antioxidant, antidiabetic and anticholinesterase activities of small anthussonchifolius landraces and correlation with their phytochemical profile	Russo et al. (2015)
2.	5,7-dihydroxy-4-methylcoumarin	193.0343	Antioxidants	Kancheva et al. (2010)
3.	Cernuoside	449.1088	Antioxidant properties of cernuoside by the DFT method	Güçlütürk et al. (2012)
4.	Chicoric Acid	475.0873	*In vitro* antioxidant and antiproliferative activities of six international basil cultivars	Elansary et al. (2015)
5.	Chlorogenic Acid	355.1029	Anantioxidant, may also slow the release of glucose into the bloodstream after a meal	Zhen et al. (2015)
6.	Cyclandelate	277.2161	Scavenger and antioxidant properties of prenylflavones isolated from Artocarpusheterophyllus	Ko et al. (2015)
7.	Epicatechinpentaacetate	501.1361	Cyclooxygenase inhibitory and antioxidant compounds	Seeram et al. (2003)
8.	Isorhoifolin	577.1619	Antidiabetic, antihyperlipidemic and antioxidant effects of the flavonoid rich fraction of Pileamicrophylla (L.) in high fat diet/streptozotocin-induced diabetes in mice.	Bansal et al. (2012)
9.	N-Acetylleucine	172.0986	First observation of N-acetyl leucine and N-acetyl isoleucine in diabetic patient hair and quantitative analysis by UPLC-ESI-MS/MS	Min et al. (2015)
10.	Naringin	579.1767	Naringin ameliorates cognitive deficits via oxidative stress, proinflammatory factors and the PPARγ signaling pathway in a type 2 diabetic rat model.	Qi et al. (2015)
11.	Norbixin	379.1895	Bixin and Norbixin Have Opposite Effects on Glycemia, Lipidemia, and Oxidative Stress in Streptozotocin-Induced Diabetic Rats	Antonio et al. (2005)
12.	Mangiferin	423.0921	Antimicrobial and antioxidant activities, inhibitory effects on type II 5α-reductase in vitro, and gastroprotectiveand antidiabetic effects in rodents	Sahoo et al. (2016)
13.	Phloridzin	435.1282	Phloridzin reduces blood glucose levels and improves lipids metabolism in streptozotocin-induced diabetic rats	Paganga et al. (1999)
14.	Quercetin-3-O-glucuronide	479.0825	An antioxidant effect in human plasma. In vitro studies indicate that miquelianin is able to reach the central nervous system from the small intestine.	Ishisaka et al. (2014)

Table S3. Cytotoxic compounds from CA extract.

S/N	Compound name	M/z	Functions	References
1.	Altersolanol A	336.0845	Altersolanol A: a selective cytotoxic anthraquinone from a Phomopsis sp.	Mishra et al. (2015)
2.	Biflorin	355.1030	Cytotoxic effects on tumor cells showing antimicrobial, antitumor and antimutagenic activities.	Wisintainer et al. (2014)
3.	Beta-thujaplicin	165.0904	*In vitro* cytotoxic and antileishmanial activities	Capello et al. (2015)
4.	Dorsmanin I	422.1729	Cytotoxicity of two naturally occurring flavonoids (dorsmanin F and poinsettifolin B) towards multi-factorial drug-resistant cancer cells	Kuete et al. (2015)
5.	Egonol	327.2015	Cytotoxic activity egonol-derived hybrid molecules against Plasmodium falciparum and multidrug-resistant human leukemia cells	Reiter et al. (2014)
6.	Elesclomol	400.1028	Cellular mechanisms of the cytotoxicity of elesclomol and its complex with Cu(II)	Hasinoff et al. (2011)
7.	gamma-Thujaplicin	164.0837	Cytotoxicity of the hinokitiol-related compounds, gamma-thujaplicin and beta-dolabrin	Morita et al. (2004)
8.	Ginsenoyne C	277.1787	Cytotoxicity in vitro	Yang et al. (2008)
9.	Huratoxin	584.3349	Some cytotoxic effects of mixtures of simplexin and huratoxin obtained from the desert rice flower, Pimelea simplex	McClure et al. (1984)
10.	Melampodin A	420.142	Melampodiumleucanthum, a source of cytotoxic sesquiterpenes with antimitotic activities.	Robles et al. (2015)
11.	Scytophycin C	806.5409	Potent antifungal and cytotoxic activities.	Parker et al. (2008)
12	Ustiloxin A	674.2082	Cytotoxic and is an inhibitor of microtubule assembly *in vitro*. Because of its resemblance to phomopsin A	Li et al. (1995)

Table S4. Apoptotic compounds from CA extracts.

S/N	Compound name	M/z	Functions	References
1	Alloantolactone	233.1897	Alantolactone induces apoptosis and cell cycle arrest on lung squamous cancer SK-MES-1 Cells	Zhao et al. (2015)
2	Altersolanol	337.0925	*In vitro* cytotoxic activity against 34 human cancer cell lines with mean IC50 (IC70) values of 0.005 µg ml(-1) (0.024 µg ml(-1), kinase inhibitor, induces cell death by apoptosis through the cleavage by Caspase-3	Mishra et al. (2015)
3	Asiatic Acid	489.3569	Anti-tumor and anti-angiogenic activity evaluations of asiatic Acid	Jing et al. (2015)
4	Cryptocaryone	283.0966	Induces apoptosis in human androgen independent prostate cancer cells by death receptor clustering in lipid raft and nonraft compartments.	Chen et al. (2010)
5	Delphinidin	303.0383	Inhibits vascular endothelial growth factor receptor-2 phosphorylation, Delphinidin sensitizes prostate cancer cells to TRAIL-induced apoptosis	Ko et al. (2015)

Table S4. Contd.

6	Isoalantolactone)	233.1536	Isoalantolactone Induces Reactive Oxygen Species Mediated Apoptosis in Pancreatic Carcinoma PANC-1 Cells	Khan et al. (2012)
7	Parthenolide	249.1481	Inducing apoptosis in acute myelogenous leukemia (AML) cells,	Kim et al. (2015)
8	Pheophorbide A	593.1481	Induces apoptosis in human hepatocellular carcinoma cells	Chan et al. (2006)
9	Scutellarein	288.1438	Induce apoptosis of ovarian and breast tumor cells *in vitro*	Shi et al. (2015)
10	Spathulenol	221.1894	dose-dependent death (apoptosis) Of lymphocytes . Due to inhibition of the MDR protein 1 (also *PGP pump*) *in vitro* has the potential spathulenol, a chemotherapy at a cancer support	Mishra et al. (2015)
11	Zidovudin	268.1045	Zidovudine (INN) or azidothymidine (AZT) (also called ZDV) is a nucleoside analog reverse-transcriptase inhibitor (NRTI), a type ofantiretroviral drug used for the treatment of HIV/AIDS infection.	Kinpara et al. (2013)

Table S5. Anti-Inflammatory compounds from CA.

S/N	Compound name	M/z	Functions	References
1	Alloantolactone	233.1897	Induces apoptosis and cell cycle arrest on lung squamous cancer SK-MES-1 Cells	Zhao et al. (2015)
2	Madecassic acid	505.3523	Anti-inflammatory effects via the suppression of NF-kappaB pathway in LPS-induced RAW 264.7 macrophage cells	Won et al. (2009)
3	Madecassoside	975.5157	Attenuates inflammatory response on collagen-induced arthritis in DBA/1 mice	Li et al. (2009)
4	Myrcene	137.1326	The anti-inflammatory, anti-catabolic and pro-anabolic effects of myrcene	Rufino et al. (2015)
6	Parthenolide	249.1481	Anti-Inflammatory and Cytostatic activities	Talhouk et al. (2015)
7	Phytosphingosine	318.3003	Ameliorate skin inflammation by inhibiting NF-κB and JAK/STAT signaling in keratinocytes and mice	Kim et al. (2014)
8	Quillaic acid	483.1322	Anti-inflammatory activity	Rodríguez-Díaz et al. (2014)
9	Rhododendrin	329.1016	Analgesic/anti-inflammatory	Kim et al. (2011)
10	Ridogrel	367.1283	Anti-inflammatory profile inflammatory bowel disease.	Carty et al. (2000)
11	Sericoside	667.2290	Anti-inflammatory triterpenoid	Rode et al. (2003)

Table S6. Agonist /Antagonist of receptors from CA extract.

S/N	Compound name	M/z	Functions	References
1	Idazoxzn	205.0974	A2-adrenoceptor antagonist	Smith et al. (1992)
2	Isovelleral	233.1897	A noxious fungal sesquiterpene, excites sensory neurons through activation of TPRA1	Escaleraet al. (2008)
3	L-902,688	420.2230	Agonists on prostaglandin E2	Kay et al. (2013)
4	N6-methyl deoxy adenosine	266.1383	A competitive antagonist specific to the P2Y1 receptors in the P2Y receptor family	Lee et al. (2001)
5	Thujone	153.1267	Modulator of the gamma-aminobutyric acid (GABA) type A receptor	Höldet al. (2000)

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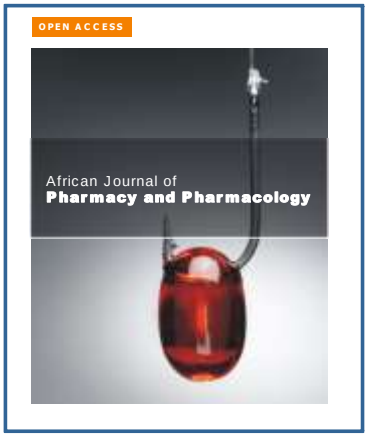

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