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Research Article
Metabolite Profiling and Antioxidant Potency of *Couroupita guianensis* Aubl. Using LC-QTOF-MS Based Metabolomics

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Abstract

Background and Objective: *Couroupita guianensis* Aubl. is found throughout India in plains, is native to South India and Malaysia, which is used in traditional medicine to treat colds, stomach aches, skin diseases, malaria and disinfect wounds. Optimization of the best extraction method for the extraction of antioxidant bioactive metabolite and identification of these metabolites by LC-QToF-MS technique has been carried out from this species. Present study was designed to find out the best method for the extraction of antioxidant compounds from *Couroupita guianensis* (*C. guianensis*) and identification of those metabolites. **Materials and Methods:** The extraction was done by six different methods: Decoction extraction, ethanolic maceration extraction, methanolic maceration extraction, cold percolation extraction, microwave assisted extraction and infusion extraction method. Antioxidant activity and total phenol content were determined in all different extracts of various extraction methods of *C. guianensis* leaf, stem and flowers. Antioxidant activity was tested by 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity and ferric reducing antioxidant power. Metabolite profile was carried out by applying non-targeted LC-QToF-MS. **Results:** The results showed that the extracting solvent significantly altered the antioxidant property estimations of *C. guianensis* leaf, stem and flowers. The aqueous extract of leaf obtained by cold percolation method had maximum phenol and showed best DPPH free radical scavenging activity and ferric reducing antioxidant power, therefore, it was selected for the further characterization of active metabolites and total 39 compounds were identified. **Conclusion:** High correlations between phenolic compositions and antioxidant activities of various extracts were observed. Cold percolation extraction method proved to be the best extraction method for the extraction of antioxidant from *C. guianensis*. Total 39 compounds belongs to different groups were detected and identified from the most potent extract of *C. guianensis*.

Key words: *Couroupita guianensis*, metabolomics, metabolite profiling, extraction optimization, characterization, compounds identification, structure elucidation, LC-QToF-MS

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Medicinal plants have been widely used by both ancient and modern man of all cultures for treating different illnesses and for other purposes as well. Plants are a good source of biologically active natural products that are all biodegradable and, more importantly, they are renewable. In recent years, the use of plants based bioactive compounds as a replacement for synthetic drugs has increased. Bioactive plant compounds are preferred over synthetic compounds due to their safety[1-3]. Recently, several extraction techniques have been developed to maximize the extraction of such compounds from different plant sources[4]. During the extraction process some variables need to be evaluated including temperature, time and solvent concentration, therefore, an optimization of the technique is essential in order to reach the maximum potential of extraction.

Antioxidant and other health promoting activities of plants are due to the presence of various biologically active compounds. Antioxidants play an important role in preventing the oxidation of food, which is a deterioration process that involves reactions among lipids, vitamins, proteins and sugars with reactive nitrogen and oxygen species[5-7]. The ROS are constituted by a large amount of reactive molecules derived from molecular oxygen and free radicals formed in organisms by oxygen consumption, water reduction, lipid oxidation, glycosylation and environmental causes such as smoking and exposition to irradiation and air pollutants[8]. Antioxidant activity of phytoconstituents is mainly due to their redox properties, which play an important role in adsorbing and scavenging free-radicals, quenching oxygen and decomposing peroxides[9].

Couroupita guianensis Aubl., commonly known as Cannon ball tree, locally known as 'Kailashpati' and/or 'Shivalingi' is found throughout India in plains, is native to South India and Malaysia[10]. The pharmacological functions of Kailashpati include antibacterial[11-13], antimicrobial[14-17], antibiofilm[18], antioxidant[19-22], ovicidal[23,24], larvicidal[25,26], antiulcer[27,28], anti-arthritic and anti-platelet[29], antioxidant and antimicrobial[30-32], antidiarrhoeal[33], analgestics[34], anti-inflammatory[35], antifertility[36], anticancer[37-39], neuropharmacological[40], anxiolytic[41], antiplasmodial[42], antidepressant[43,44], antinociceptive[45], immunomodulatory[46], anti-quorum sensing[47], antimalarial[48], wound healing[49], antioxidant and anticancer[50], repellency and toxicity[51] and cytotoxicity[52].

The objective of this study was to develop a rapid, reproducible and simple extraction technique, considering significant bioactive metabolites from the *C. guianensis*, also determined as complement to the phytochemical characterization, regardless the effect of extraction technique and antioxidant activity by liquid chromatography coupled to quadrupole-time of flight-mass spectrometry (LC-QToF-MS). However, no studies have so far been reported on effects of different extraction techniques on antioxidant activity of *C. guianensis* with metabolite profiling of potent extract.

MATERIALS AND METHODS

Chemicals and reagents: Petroleum ether, methanol, hydrochloric acid (HCl), Folin-Ciocalteu's reagent, sodium carbonate, ferric chloride ($FeCl_3$), ferrous sulfate ($FeSO_4$), 2, 2-Diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-Tri-(2-pyridyl)-5-triazine (TPTZ), gallic acid and ascorbic acid were obtained from Sigma, Hi-media, Merck and SRL. Water was purified with a Milli-Q system (Millipore, Bedford, USA). All solvents and chemical used were of analytical grade.

Plant collection: The flower, leaf and stem of *Couroupita guianensis* were collected in August, 2015 from Motibaug, Junagadh, Gujarat, India. The plant parts were washed thoroughly with tap water, shade dried and homogenized to fine powder and stored in air tight bottles.

Extraction procedures: Six different extraction processes were employed in this study, i.e., decoction, ethanolic maceration, methanolic maceration, cold percolation, microwave assisted and infusion extraction method. After extraction, the extract was filtered with eight layers of muslin cloth and centrifuged at 5,000 rpm (Remi Centrifuge, India) for 10 min. The supernatant was collected and the solvent was evaporated using a rotary vacuum evaporator (Equitron, India) to dryness. The extract was stored at 4°C in an airtight bottle.

Decoction extraction method: For the decoction, method was followed as previously used by Li *et al.*[53], 5 g of dried powder was extracted with 100 mL of deionized water at 100°C for 30 min in a water bath.

Ethanolic maceration extraction method: For the ethanolic maceration, method was followed as previously used by An[54], 5 g of dried powder was extracted with 100 mL of 50% aqueous ethanol at 25°C for 42 h in static condition.

Methanolic maceration extraction method: For methanolic maceration, method was followed as previously used by Cai *et al.*[55], 5 g of dried powder was extracted with 100 mL of 80% aqueous methanol at 35°C for 24 h in an incubator.

Cold percolation extraction method: For cold percolation extraction, method was followed as previously used by Parekh and Chanda[56], 10 g of dried powder was taken in 150 mL petroleum ether in a conical flask, plugged with cotton wool and then kept on a rotary shaker at 120 rpm for 24 h. After 24 h, it was filtrated through eight layers of muslin cloth and the solvent was evaporated from the powder. This dry powder was then taken in 150 mL of deionized water and was kept on a shaker at 120 rpm for 24 h.

Microwave assisted extraction method: For microwave assisted extraction, method was followed as previously used by Jaitak *et al.*[57], 1 g of dried powder was extracted with 200 mL of deionized water in a conical flask in a microwave (Magicook 20S (Galaxy), India) at different power levels ranging from 20-160 W with extraction time range between 30 sec to 5 min with a temperature range of 10-90°C.

Infusion extraction method: For infusion extraction, method was followed as previously used by Martins *et al.*[58], 2 g of dried powder was extracted with 400 mL of boiling deionized water and were left to stand at room temperature for 5 min.

Quantitative phytochemical analysis by total phenolic content (TPC) estimation: Quantitative phytochemical analysis of the different extracts obtained by different extraction techniques from flower, leaf and stem of *C. guianensis*, was carried out by the estimation of the TPC by modified Folin-Ciocalteu's reagent method[59-61]. The extract (0.5 mL) and 0.1 mL of Folin-Ciocalteu's reagent (0.5 N) were mixed and the mixture was incubated at room temperature for 15 min. Then, 2.5 mL of sodium carbonate (2 M) solution was added and further incubated for 30 min at room temperature and the absorbance was measured at 760 nm using a digital spectrophotometer (Systronic 1823, India), against a blank sample. The calibration curve was made by preparing gallic acid (10-100 μg mL^{-1}) solution in distilled water[62,63]. The TPC is expressed in terms of gallic acid equivalent (mg g^{-1} of extracted compound).

Antioxidant activity-DPPH free radical scavenging assay: The antioxidant activity of the different extracts obtained by different extraction techniques from flower, leaf and stem of *C. guianensis*, was measured by using $DPPH^{\cdot}$ radical scavenging capacity by the modified method of McCune and Johns[64] and Rakholiya *et al.*[65]. The reaction mixture (3.0 mL), consisted of 1.0 mL DPPH in methanol (0.3 mM), 1.0 mL methanol and 1.0 mL (100 μg mL^{-1}) of different extracts diluted by methanol, was incubated for 10 min, in dark, after which the absorbance was measured at 517 nm using a digital spectrophotometer (Systronic 1823, India), against a blank sample. Ascorbic acid (2-16 μg mL^{-1}) was used as positive control[66,67]. Percentage of inhibition was calculated using the following formula[68]:

$$\text{Inhibition (\%)} = 1-\left(\frac{A}{B}\right)\times 100$$

Where:
B = Absorbance of blank (DPPH+methanol),
A = Absorbance of sample (DPPH+methanol+sample)

Ferric reducing antioxidant power: The reducing ability of the different extracts obtained by different extraction techniques from flower, leaf and stem of *C. guianensis*, was determined by ferric reducing antioxidant power (FRAP) assay of Benzie and Strain[69] and Kaneria *et al.*[70]. The FRAP assay is based on the ability of antioxidants to reduce Fe^{3+} to Fe^{2+} in the presence of TPTZ, forming an intense blue Fe^{2+}-TPTZ complex with an absorption maximum at 593 nm. This reaction was pH-dependent (optimum pH 3.6). About 0.1 mL of the different solvent extract was added to 3.0 mL FRAP reagent [10 parts 300 mM sodium acetate buffer at pH 3.6, 1 part 10 mM TPTZ in 40 mM HCl and 1 part 20 mM $FeCl_3$] and the reaction mixture was incubated at 37°C for 10 min. And then, the absorbance was measured at 593 nm using a UV-VIS Spectrophotometer (Shimadzu, Japan), against a blank sample. The calibration curve was made by preparing a $FeSO_4$ (100-1000 μM mL^{-1}) solution in distilled water[71,72]. The antioxidant capacity based on the ability to reduce ferric ions of sample was calculated from the linear calibration curve and expressed as M $FeSO_4$ equivalents per gram of extracted compounds[73].

Metabolite profiling by LC-QToF-MS technique: Metabolite profiling by liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (LC-QToF-MS) technique was done from Food Testing Laboratory, Department of Biotechnology, Junagadh Agricultural University, Junagadh. Metabolite analysis of *C. guianensis* leaf aqueous extract obtained by cold percolation method was carried out using an Agilent 6540 LC-QToF-MS system consisting of an Agilent 1290 LC with a 6540 UHD accurate-mass QToF mass spectrometer. Separation of metabolites was performed using (4.6 mm×100 mm, 3.5 μm) Agilent ZORBAX Eclipse XDB-C18 column at 25°C. The mobile phase consisted of 0.1% of formic acid in water (phase A) and acetonitrile (phase B). Gradient elution was as follows: 5% B for 7 min then increased to 95% B up to 12 min, held for 6 min, followed by decrease to 5% B

and maintained at 5% B for 7 min. Total run time was 30 min. The applied flow rate was 0.7 mL min^{-1} and injection volume was 10.0 µL. MS analysis were carried out using a 6540 Agilent Ultra-High-Definition Accurate-Mass QToF-MS coupled to the LC, equipped with an Agilent Dual Jet Stream electrospray ionization (Dual AJS ESI) interface in negative ionization mode at the following conditions: Drying gas flow (Nitrogen): 8.0 L min^{-1}, nebulizer pressure: 45 psi, gas drying temperature: 325°C, capillary voltage: 4000 V, mass scan range: 100-1700 m/z and fragmentor voltage: 120 V. Integration and data elaboration were performed using Mass Hunter software (Agilent Technologies, Santa Clara, CA, USA)[74]. Agilent Technologies has provided the METLIN Personal Compound Database with accurate mass MS/MS Library (PCDL). The METLIN PCDL includes all compounds and additionally accurate mass Q-TOF MS/MS library reference spectra.

Statistical analysis: All the experiments were performed in triplicate and results are presented as Mean±SEM (Standard Error of Mean).

RESULTS AND DISCUSSION

Metabolomic approaches allow a comprehensive profiling of the cell metabolome or "Library of metabolites" that provides chemical signatures of cell dynamics and metabolic activity. Various analytical approaches are used to identify metabolites. Metabolic profiling is often referred to as targeted or nontargeted. In the targeted approach, specific metabolites of known identity are profiled. Nontargeted profiling involves the use of NMR or MS for simultaneous measurement of as many metabolites as possible in a biological specimen. The major approaches in metabolomics studies include MS-based techniques. Modern MS platforms such as those that incorporate time of flight mass analyzers offer very high mass resolution and mass accuracy. Coupling such MS instrumentation with high-resolution chromatographic technologies has made it possible to resolve literally thousands of individual small molecules. The high mass accuracy of these methods facilitates peak identification through databases such as METLIN, HMDB and KEGG.

Polyphenols have many favorable effects on human health like inhibiting the oxidation of low-density proteins, thereby decreasing the risk of heart diseases[75,76]. They have anti-inflammatory and anti-carcinogenic properties. Flavonoids and many other phenolic compounds of plant origin have also been reported as scavenger of reactive oxygen species (ROS) and are viewed as promising therapeutic drugs for free radical pathogens[77]. Thus, measurements of polyphenols and antioxidant activity in herbs have become important tools to understand the reactive values of plant species[78] from a health point of view. The total phenol content of different extracts of all extraction methods of flower, leaf and stem of *C. guianensis* are shown in Table 1. Highest amount of total phenol content was in cold percolation method in leaf, while lowest amount of total phenol content was in microwave assisted method in leaf. Different extracts of flower and stem had almost similar total phenol content (Table 1).

Several methods have been used to measure free radical scavenging capacities of plant. The DPPH radical scavenging activity has been widely used as a model system to investigate the scavenging activity of natural compounds[79-82]. Among the various methods to evaluate the radical scavenging activity of natural compounds, DPPH method received more attention due to its fast, reliable results, relatively simple, stable and the DPPH was available commercially in high purity[83,84]. The DPPH is a commercial oxidizing radical, which can be reduced/scavenged by antioxidants through the donation of a proton forming the reduced DPPH. The color changes from purple to yellow after reduction, which can be quantified by its decrease of absorbance at wavelength 517 nm[85,86]. Radical scavenging activity increases with increasing percentage of the free radical inhibition. The DPPH free radical scavenging activity of different extracts at 100 µg mL^{-1} of all extraction methods of flower, leaf and stem of *C. guianensis* are shown in Table 1. All the different extracts showed varied level of percentage inhibition at a fix concentration of 100 µg mL^{-1}. Ascorbic acid was used as a positive control and it shows 43.9% inhibition at 10 µg mL^{-1} concentration. The highest DPPH free radical scavenging activity was showed by aqueous extract obtained by cold percolation method in leaf followed by maceration ethanol method in flower (Table 1).

The antioxidant activity was also determined on the basis of the ability of antioxidant in the plant extracts to reduce ferric (III) iron to ferrous (II) iron in FRAP reagent[87]. Generally, FRAP assay is used due to its reproducibility. The ferric reducing antioxidant power (FRAP) of different extracts of all extraction methods of flower, leaf and stem of *C. guianensis* are shown in Table 1. All the different extracts showed varied level of antioxidant potential. Amongst all the extracts, maximum FRAP was in aqueous extract obtained by cold percolation method in leaf followed by aqueous extracts obtained by infusion and decoction methods in flower (Table 1). Meanwhile, FRAP assay was used to determine the antioxidant ability by utilizing the electron-donating capacity of the antioxidant to reduce Fe^{3+} to Fe^{2+} [88].

Table 1: Total phenol content and antioxidant potency of different extracts of *C. guianensis*

Parts used	Extracts	TPC (mg g^{-1})*	DPPH (% inhibition at 100 µg mL^{-1} concentration)*	FRAP (M g^{-1})*
Flower	DcAq	83.91±0.205	47.54±0.068	9.18±0.084
	McEt	82.08±0.236	74.78±0.279	8.56±0.188
	McMe	82.09±0.229	50.11±0.124	8.37±0.128
	CpAq	81.72±0.236	48.60±0.124	7.75±0.061
	MaAq	71.53±0.150	22.58±0.062	6.97±0.041
	InAq	87.45±0.150	69.46±0.186	9.59±0.173
Leaf	DcAq	86.63±0.164	59.46±0.062	8.42±0.088
	McEt	85.03±0.118	63.55±0.062	8.03±0.046
	McMe	77.07±0.066	54.62±0.186	6.95±0.049
	CpAq	91.87±0.113**	85.43±0.155**	11.85±0.155**
	MaAq	62.92±0.118	6.29±0.031	5.65±0.110
	InAq	81.72±0.515	69.03±0.124	8.66±0.184
Stem	DcAq	75.46±0.397	30.54±0.186	6.36±0.047
	McEt	77.82±0.150	48.87±0.155	6.68±0.098
	McMe	67.96±0.215	52.26±0.186	5.56±0.150
	CpAq	56.01±0.284	9.03±0.124	4.69±0.215
	MaAq	43.69±0.164	7.28±0.078	3.36±0.134
	InAq	52.01±0.291	12.47±0.186	4.85±0.044

*Values are expressed in Mean±Standard error of the mean (n = 3), DcAq: Decoction aqueous extract , McEt: Maceration ethanol extract , McMe: Maceration methanol extract , CpAq: Cold percolation aqueous extract , MaAq: Microwave assisted aqueous extract , InAq: Infusion aqueous extract , TPC: Total phenol content , FRAP: Ferric reducing antioxidant power , **Indicating potent activity

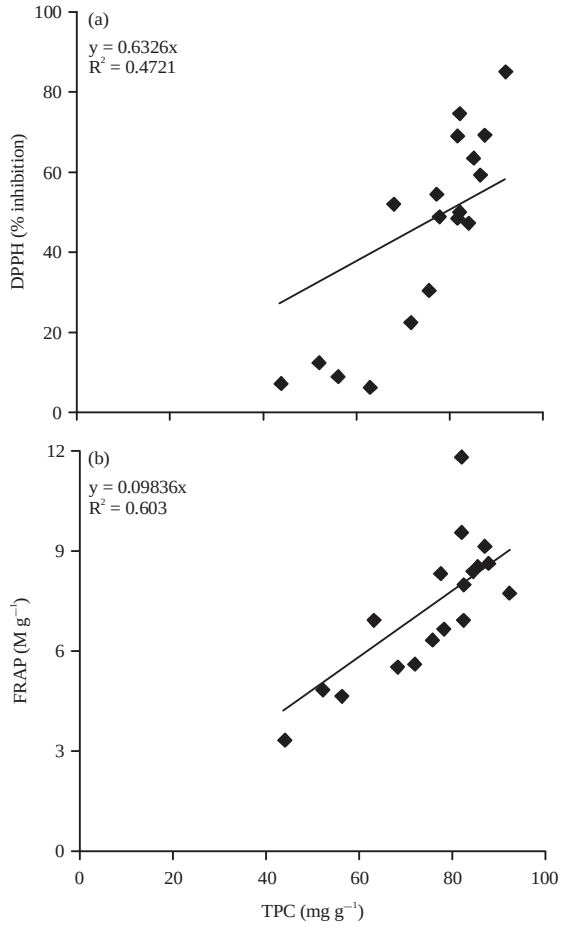


Fig. 1(a-b): Correlation between TPC and antioxidant activity, (a) DPPH and (b) FRAP of different extracts from various extraction methods of different parts of *C. guianensis*

In the present study, it was observed that the greatest antioxidant activity, i.e., DPPH and FRAP had a direct correlation with quantities of total phenols (Fig. 1). Phenolic compounds are the main class of natural antioxidants and there is a close relationship between the phenolic content and antioxidant activity of plant extracts[89-92]. Several studies have shown that higher antioxidant activity associated with medicinal plants is attributed to their total phenolic compounds[93-95].

The Leaf Cold Percolation Aqueous Extract (LeCpAq) showed significant activity, therefore, it was selected for the further characterization of active metabolites. Metabolite profile of *C. guianensis* LeCpAq assessed by applying non-targeted LC-QToF-MS using ESI in negative ionization mode is shown in Table 2. Name of the detected and identified compounds, IUPAC name of the compounds, molecular formula, PubChem compound identification number, retention time (t_R), experimental mass (m/z), height of the peak and area covered by the individual peak of about the identified compounds is given in Table 2. Molecular structures of the detected and identified metabolites of *C. guianensis* LeCpAq by applying non-targeted LC-QToF-MS in negative ionization mode is shown in Fig. 2a-d. The total ion current (TIC) base peak chromatogram (BPC) of *C. guianensis* leaf aqueous extract obtained in extracted ion chromatogram in negative ionization mode is shown in Fig. 3. Total 39 metabolites were detected in LC-QToF-MS and identified as shown in Table 2 by using Mass Hunter software of Agilent Technologies. From the detected and identified compounds, 14 were belongs to lipid group, 5 flavonoids, 4 glycosides, 3 alkaloids, 2 triterpenes, 2 tripeptides, 2 vitamins, remaining

Table 2: Metabolite profiling of *C. guianensis* Le-CpAq by applying non-targeted LC-QToF-MS using ESI in negative mode

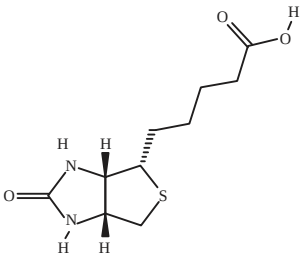
Name of compound (Cpd# No.)	IUPAC Name	Molecular formula	PubChem CID*	t_R (min.)	m/z value (Mass)§	Peak height	Peak area
Fluconazole glucuronide (386)	(2S,3S,4S,5R,6S)-6-[2-(2,4-difluorophenyl)-1,3-bis(1,2,4-triazol-1-yl) propan-2-yl]oxy-3,4,5-trihydroxyoxane-2-carboxylic acid	$C_{19}H_{20}F_2N_6O_7$	71316779	1.671	482.1357	34083	205833
E-64c (545)	(2S,3S)-3-[[(2S)-4-methyl-1-(3-methylbutylamino)-1-oxopentan-2-yl]carbamoyl] oxirane-2-carboxylic acid	$C_{15}H_{26}N_2O_5$	123664	3.975	314.1848	12553	173762
Lonchocarpin (530)	(E)-1-(5-hydroxy-2,2-dimethylchromen-6-yl)-3-phenylprop-2-en-1-one	$C_{20}H_{18}O_3$	6283743	4.398	306.1271	18140	398579
Gossypol (898)	7-(8-formyl-1,6,7-trihydroxy-3-methyl-5-propan-2-yl naphthalen-2-yl)- 2,3,8-trihydroxy-6-methyl-4-propan-2-yl naphthalene-1-carbaldehyde	$C_{30}H_{30}O_8$	3503	10.45	518.1929	9325	129067
Trans-3,4-Dihydro-3,4-dihydroxy-7,12-dimethylbenz [a]anthracene (180)	(3S,4S)-7,12-dimethyl-3,4-dihydrotetraphene-3,4-diol	$C_{20}H_{18}O_2$	-	10.698	290.131	71152	510008
Trp Thr Asn (538)	-	$C_{19}H_{25}N_5O_6$	-	10.856	419.1824	25105	141708
Ipriflavone (14)	3-phenyl-7-propan-2-yl oxychromen-4-one	$C_{18}H_{16}O_3$	3747	11.037	280.11	1238560	11495027
Gnididin (472)	-	$C_{37}H_{44}O_{10}$	5476509	11.063	648.2928	28357	183411
Lys Leu Glu	-	$C_{17}H_{32}N_4O_6$	-	11.106	388.2319	134589	98654
Icariside II (44)	5,7-dihydroxy-2-(4-methoxyphenyl)-8-(3-methylbut-2-enyl)-3-[(2S,3S,4S,5S,6R)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxychromen-4-one	$C_{27}H_{30}O_{10}$	44587252	11.121	514.1834	292920	2606059
Cucumerin B (521)	5,7-dihydroxy-2-(4-hydroxyphenyl)-8-[1-(4-hydroxyphenyl)ethyl]-6-[(2S,4R,5S)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]chromen-4-one	$C_{29}H_{28}O_{11}$	44257648	11.188	552.1634	19866	183357
Matteuorienate B (69)	3-hydroxy-3-methyl-5-oxo-5-[[(2R,3S,4S,5R,6S)-3,4,5-trihydroxy-6-[[(2S)-5-hydroxy-6,8-dimethyl-4-oxo-2-phenyl-2,3-dihydrochromen-7 -yl]oxy]oxan-2-yl]methoxy]pentanoic acid	$C_{29}H_{34}O_{13}$	190959	11.212	590.2008	225274	2350832
Haemocorin (345)	2-[(2S,3R,4R,5S,6R)-3,4-dihydroxy-6-(hydroxymethyl)-5-[(2S,3R,4S,5S,6R)- 3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl] oxyoxan-2-yl]oxy-6-hydroxy-5-methoxy-9-phenylphenalen-1-one	$C_{32}H_{34}O_{14}$	165899	11.237	642.1956	49104	292993
Thalicarpine (1090)	(6aS)-9-[2-[[(1S)-6,7-dimethoxy-2-methyl-3,4-dihydro-1H-isoquinolin- 1-yl]methyl]-4,5-dimethoxyphenoxy]-1,2,10-trimethoxy-6-methyl-5,6,6a,7-tetrahydro-4H-dibenzo[de,g]quinoline	$C_{41}H_{48}N_2O_8$	21470	11.295	696.341	14838	67419
PG(O-18:0/22:6 (4Z, 7Z, 10Z, 13Z, 16Z, 19Z)) (465)	[(2R)-1-[[(2S)-2,3-dihydroxypropoxy]-hydroxyphosphoryl]oxy -3-octadecoxypropan-2-yl] (4Z,7Z,10Z,13Z,16Z,19Z)-docosa-4,7,10,13,16,19-hexaenoate	$C_{46}H_{81}O_9P$	52927330	11.312	808.5635	41325	186797
PI(P-20:0/17:2(9Z,12Z)) (1838)	[(2R)-1-[hydroxy-[(5R)-2,3,4,5,6-pentahydroxycyclohexyl] oxyphosphoryl]oxy-3-[(Z)-icos-1-enoxy]propan-2-yl] (9Z,12Z)-heptadeca-9,12-dienoate	$C_{46}H_{85}O_{12}P$	52928574	11.312	860.5789	6860	27172
(22S)-1α±,22,25-trihydroxy-26,27-dimethyl-23,23,24,24-tetradehydro- 24a,24b,24c-trihomovitamin D3 (1291)	(1R,3S,5Z)-5-[(2E)-2-[(1R,3aS,7aR)-1-[(2S,3S)-9-ethyl-3,9-dihydroxyundec-4- yn-2-yl]-7a-methyl-2,3,3a,5,6,7-hexahydro-1H-inden-4- ylidene]ethylidene]-4-methylidenecyclohexane-1,3-diol	$C_{32}H_{50}O_4$	-	11.42	498.3724	13661	57564
N-oleoyl alanine (184)	(2S)-2-[[(Z)-octadec-9-enoyl]amino]propanoic acid	$C_{21}H_{39}NO_3$	44423663	11.42	353.2926	99947	458843
Squamoxinone (1709)	(5R)-5-[(7S,13R)-7,13-dihydroxy-13-[(2R,5R)-5-[(1R)-1- hydroxytridecyl]oxolan-2-yl]tridecyl]-3-(2-oxopropyl)oxolan-2-one	$C_{37}H_{68}O_7$	10531987	11.478	624.4955	7149	30747
Ophirasterol (1016)	(3S,8S,9S,10R,13R,14S,17R)-10,13-dimethyl-17-[(E,2R,5R)-6-methylidene- 5-propan-2-yloct-3-en-2-yl]-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol	$C_{31}H_{50}O$	21589230	11.486	438.3862	13509	79107

Table 2: Continue

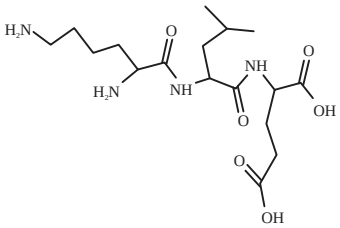
Name of compound (Cpd# No.)	IUPAC Name	Molecular formula	PubChem CID*	t_R (min.)	m/z value (Mass)$	Peak height	Peak area
Bruceantin (26)	-	$C_{28}H_{36}O_{11}$	5281304	11.503	548.228	676532	4256814
PI(21:0/20:0) (593)	[(2R)-3-[hydroxy-[(5R)-2,3,4,5,6-pentahydroxycyclohexyl] oxy phosphoryl] oxy-2-icosanoyloxypropyl] henicosanoate	$C_{50}H_{97}O_{13}P$	52928114	11.668	936.6685	25863	137554
TG(12:0/12:0/15:1(9Z)) [iso3] (1003)	[(2R)-2,3-di(dodecanoyloxy) propyl](Z)-pentadec-9-enoate	$C_{42}H_{78}O_6$	56936434	11.677	678.5823	13022	73254
Licorice glycoside E (948)	[(4S,5S)-5-[(2S,4S,5S)-4,5-dihydroxy-6-(hydroxymethyl)-2-[4-[(2S)-7-hydroxy- 4-oxo-2,3-dihydrochromen-2-yl]phenoxy]oxan-3-yl] oxy-3,4-dihydroxyoxolan- 3-yl]methyl 1H-indole-3-carboxylate	$C_{35}H_{35}NO_{14}$	42607811	11.735	693.2043	12289	82844
PG(O-18:0/12:0) (676)	[(2R)-1-[[(2S)-2,3-dihydroxypropoxy]-hydroxyphosphoryl]oxy-3-octadecoxypropan-2-yl] dodecanoate	$C_{36}H_{73}O_9P$	52927266	11.892	680.4989	32704	122228
Carpaine (461)	13,26-dimethyl-2,15-dioxa-12,25-diazatricyclo[22.2.2.2^{11,1}] triacontane-3,16-dione	$C_{28}H_{50}N_2O_4$	442630	11.9	478.3771	32031	173641
5-Hydroxy-10-prenyl-7,8-dihydro-7,8-trans-dimethyl-4 -phenyl-2H, 6H-benzo [1,2-b:5,4-b'] dipyran -2,6-dione (713)	8-(3,4-Dihydroxyphenyl)-5-hydroxy-7-methoxy-2,2-dimethyl-10-(3-methyl-2-butenyl)-2H,6H-benzo[1,2-b:5,4-b']dipyran-6-one	$C_{25}H_{24}O_5$	-	11.917	404.1629	17010	100021
β-Amyrin Acetate (700)	(4,4,6a,6b,8a,11,11,14b-octamethyl-1,2,3,4a,5,6,7,8,9,10,12,12a, 14,14a-tetradecahydropicen-3-yl) acetate	$C_{32}H_{52}O_2$	345510	12.116	468.3967	19486	100624
(+)-trans-Carveol glucoside (2)	(1R,5S)-5-Isopropenyl-2-methyl-2-cyclohexen-1-yl β-D-glucopyranoside	$C_{16}H_{26}O_6$	-	12.224	314.1736	4967676	39667567
4',6'-Dihydroxy-2'-methoxyacetophenone 6'-glucoside (168)	1-(4-hydroxy-2-methoxy-6-{[3,4,5-trihydroxy-6-(hydroxymethyl) oxan-2-yl]oxy}phenyl)ethan-1-one	$C_{15}H_{20}O_9$	-	12.24	344.1101	92955	520597
N-Phospho-D-lombricine (99)	(2R)-2-amino-3-[2-[[amino-(phosphonoamino) methylidene] amino] ethoxy-hydroxyphosphoryl] oxypropanoic acid	$C_6H_{16}N_4O_9P_2$	439827	12.663	350.0384	171711	1085642
3beta-Fluoro-5alpha-androstan-17beta-ol (48)	(3S,5S,8R,9S,10S,13S,14S,17S)-3-fluoro-10,13-dimethyl-2,3,4,5,6,7,8,9,11, 12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-ol	$C_{19}H_{31}FO$	247285	13.484	294.2359	115313	723596
6beta,17beta-Dihydroxyandrost-4-en-3-one diacetate (1032)	[(6R,8R,9S,10R,13S,14S,17S)-6-acetyloxy-10,13-dimethyl-3-oxo-1,2,6,7,8,9, 11, 12,14,15,16,17-dodecahydrocyclopenta[a] phenanthren-17-yl] acetate	$C_{23}H_{32}O_5$	224006	14.023	388.2235	8246	61187
Sparteine (342)	(1S,2S,9S,10S)-7,15-diazatetracyclo [7.7.1.0^{2}, .0^{1}, ^{1}]heptadecane	$C_{15}H_{26}N_2$	7014	14.122	234.2084	35820	249588
Plakortic acid (1)	2-[4-ethyl-6-[(E)-2-ethylhex-3-enyl]-6-methyldioxan-3-yl]acetic acid	$C_{17}H_{30}O_4$	10402441	14.128	298.2135	4077834	112325405
20-oxo-heneicosanoic acid (910)	Methyl 20-oxohenicosanoate	$C_{21}H_{40}O_3$	536908	15.001	340.298	7196	149885
TG (22:3(10Z,13Z,16Z)/ 22:6 (4Z,7Z,10Z,13Z,16Z,19Z)/ 22:6(4Z,7Z,10Z,13Z,16Z,19Z)) [iso3] (128)	[(2R)-2,3-bis[[(4Z,7Z,10Z,13Z,16Z,19Z)-docosa-4,7,10,13,16,19 - hexaenoyl]oxy]propyl](10Z,13Z,16Z)-docosa-10,13,16-trienoate	$C_{69}H_{104}O_6$	9546560	18.176	1028.783	43558	799577
D-Biotin	5-[(3aS,4S,6aR)-2-oxo-1,3,3a,4,6,6a-hexahydrothieno [3,4-d]imidazol-4-yl]pentanoic acid	$C_{10}H_{16}N_2O_3S$	171548	21.76	244.088	9455	135638
Cyclomorusin (1142)	11,19-dihydroxy-7,7-dimethyl-15-(2-methylprop-1-en-1-yl)-2,8,16-trioxapentacyclo [12.8.0.0^{3},^{12}.0, .0^{1}, ^{22}]docosa-1(14), 3(12),4(9),5,10,17(22),18,20-octaen-13-one	$C_{25}H_{22}O_6$	5481969	21.865	418.1418	7034	59429

#Cpd: Compound numbers as matched with the library of the Agilent Mass Hunter Software of the model QTOF/LCMS 6540. *CID (Compound identifier): A compound identifier (CID) is the permanent identifier for a unique chemical structure. These are found in the PubChem Compound database. Each stereoisomer of a compound has its own CID. It is also possible for different tautomeric forms of the same compound to have different CID's. $m/z value (mass-to-charge ratio): m/z represents mass divided by charge number and the horizontal axis in a mass spectrum is expressed in units of m/z. Since z is almost always 1 with MS, the m/z value is often considered to be the Mass (experimental)

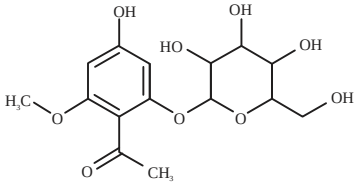
(a)



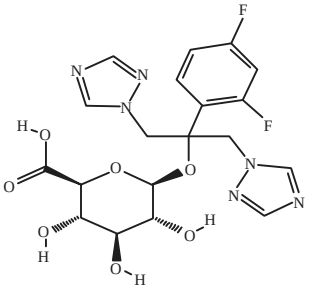
D-Biotin



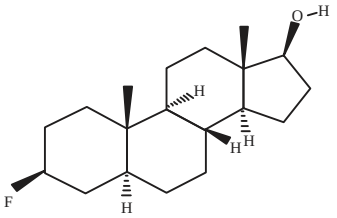
Lys Ley Glu



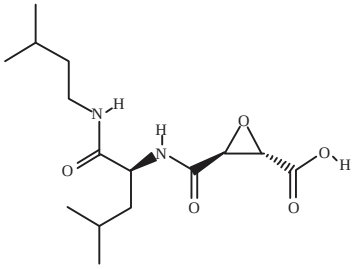
4',6'-Dihydroxy-2'-methoxyacetophenone-6'-glucoside



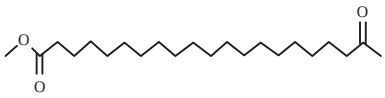
Fluconazole glucuronide



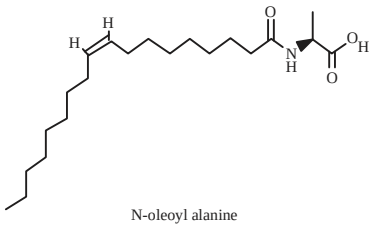
3beta-Fluoro-5alpha-androstan-17beta-ol



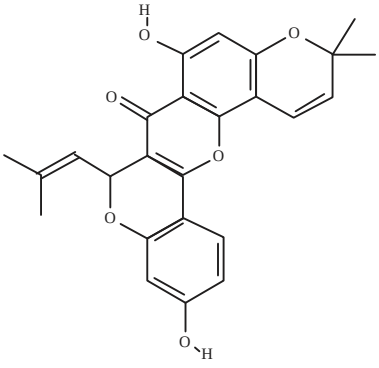
E-64C



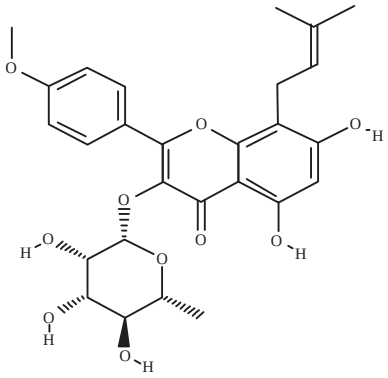
20-Oxo-heneicosanoic acid



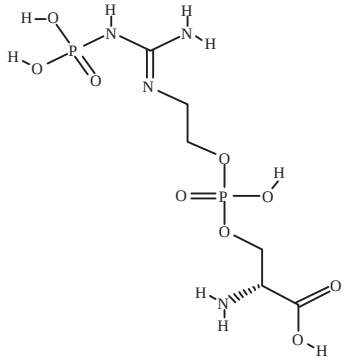
N-oleoyl alanine



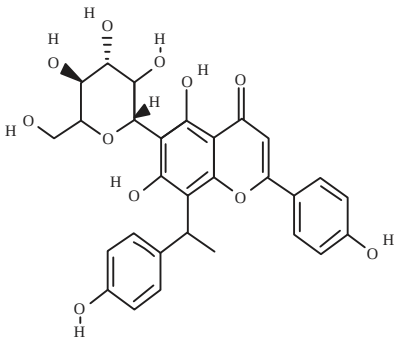
Cyclomorusin



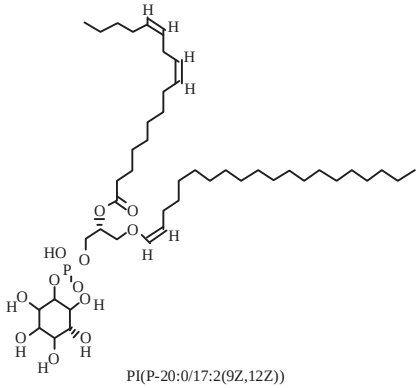
Icariside II



N-Phospho-D-lombricine



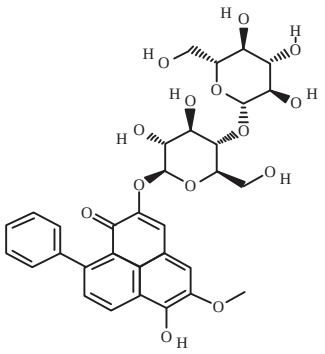
Cucumerin B



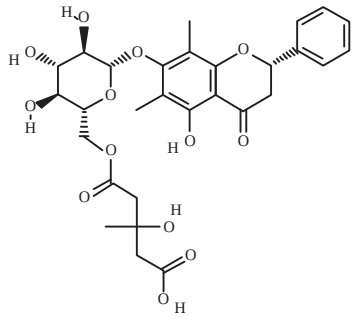
PI(P-20:0/17:2(9Z,12Z))

Fig. 2(a-d): Continue

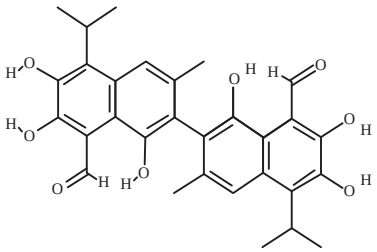
(b)



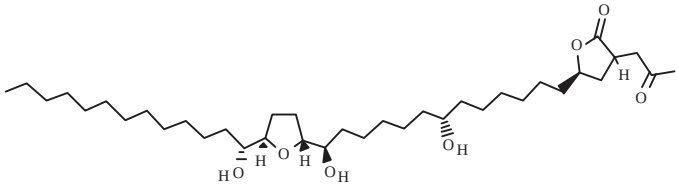
Haemocorin



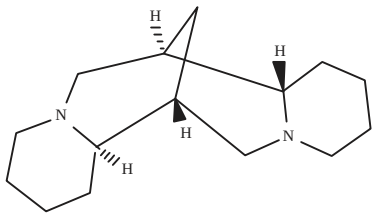
Matteuorienate B



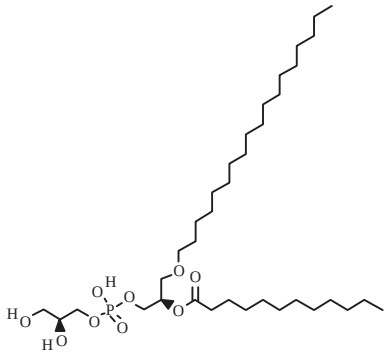
Gossypol



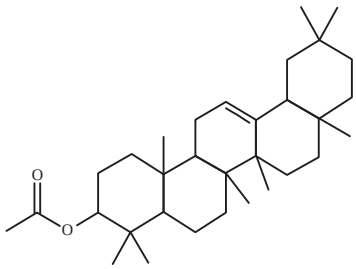
Squamoxinone



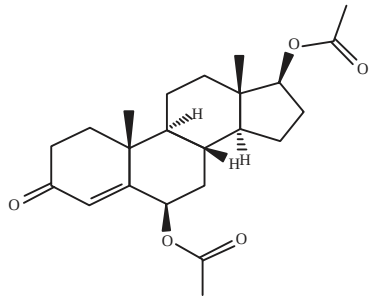
Sparteine



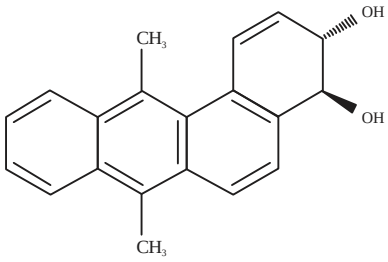
PG(O-18:0/12:0)



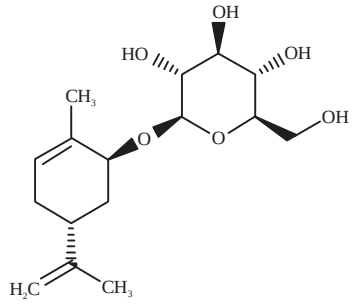
β-Amyrin Acetate



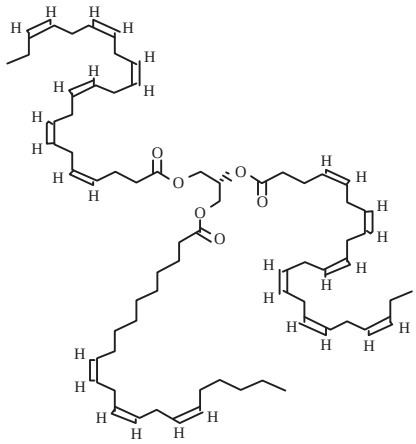
6beta,17beta-Dihydroxy- androst-4-en-3-one diacetate



Trans-3,4-Dihydro-3,4-dihydroxy-7,12 -dimethylbenz[a]anthracene



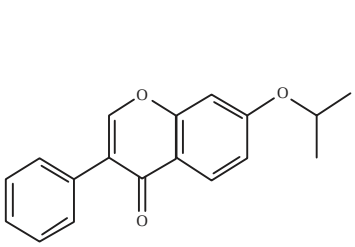
(+)-trans-carveol glucoside



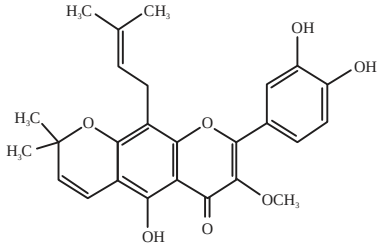
TG(22:3(10Z,13Z,16Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z)/ 22:6 (4Z,7Z,10Z,13Z,16Z,19Z))[iso3]

Fig. 2(a-d): Continue

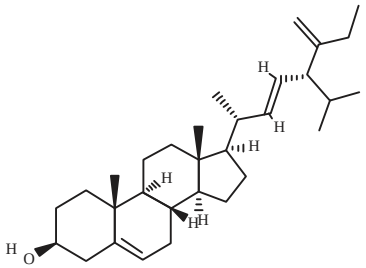
(c)



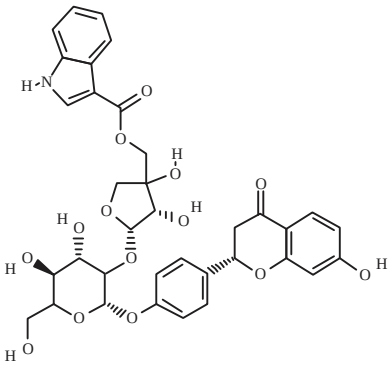
Iprif lavone



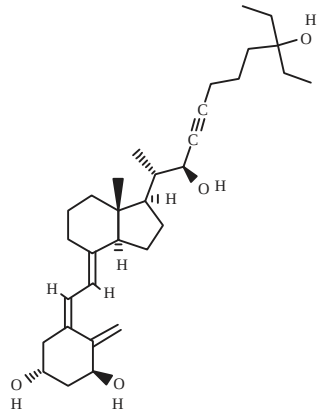
5-Hydroxy-10-prenyl-7,8-dihydro-7,8-trans-dimethyl-4-phenyl-2H, 6H-benzo [1,2-b:5,4-b'] dipyran -2,6-dione



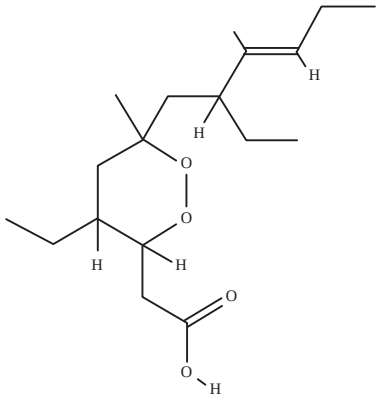
Ophirasterol



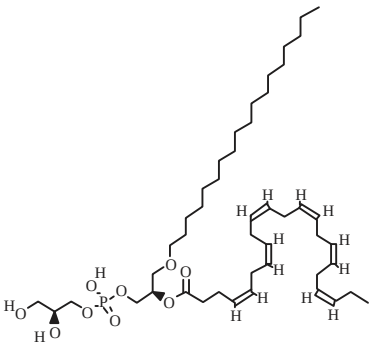
Licorice glycoside E



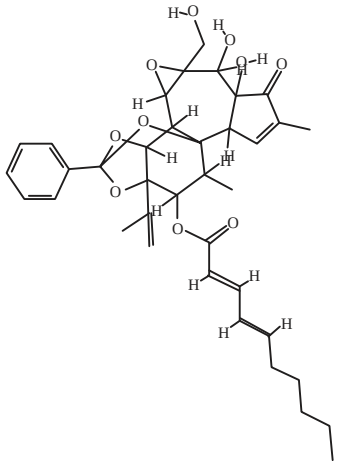
(22S)-1a±,22,25-trihydroxy-26,27-dimethyl-23,23,24,24-tetradehydro- 24a,24b,24c-trihomovitamin D3



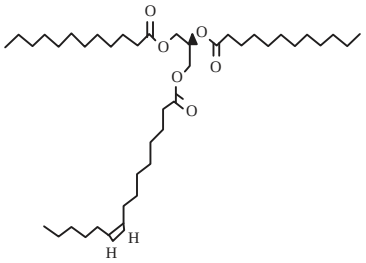
Plakortic acid



PG(O-18:0/22:6 (4Z,7Z,10Z,13Z, 16Z,19Z))



Gnididin



TG(12:0/12:0/15:1(9Z))[iso3]

Fig. 2(a-d): Continue

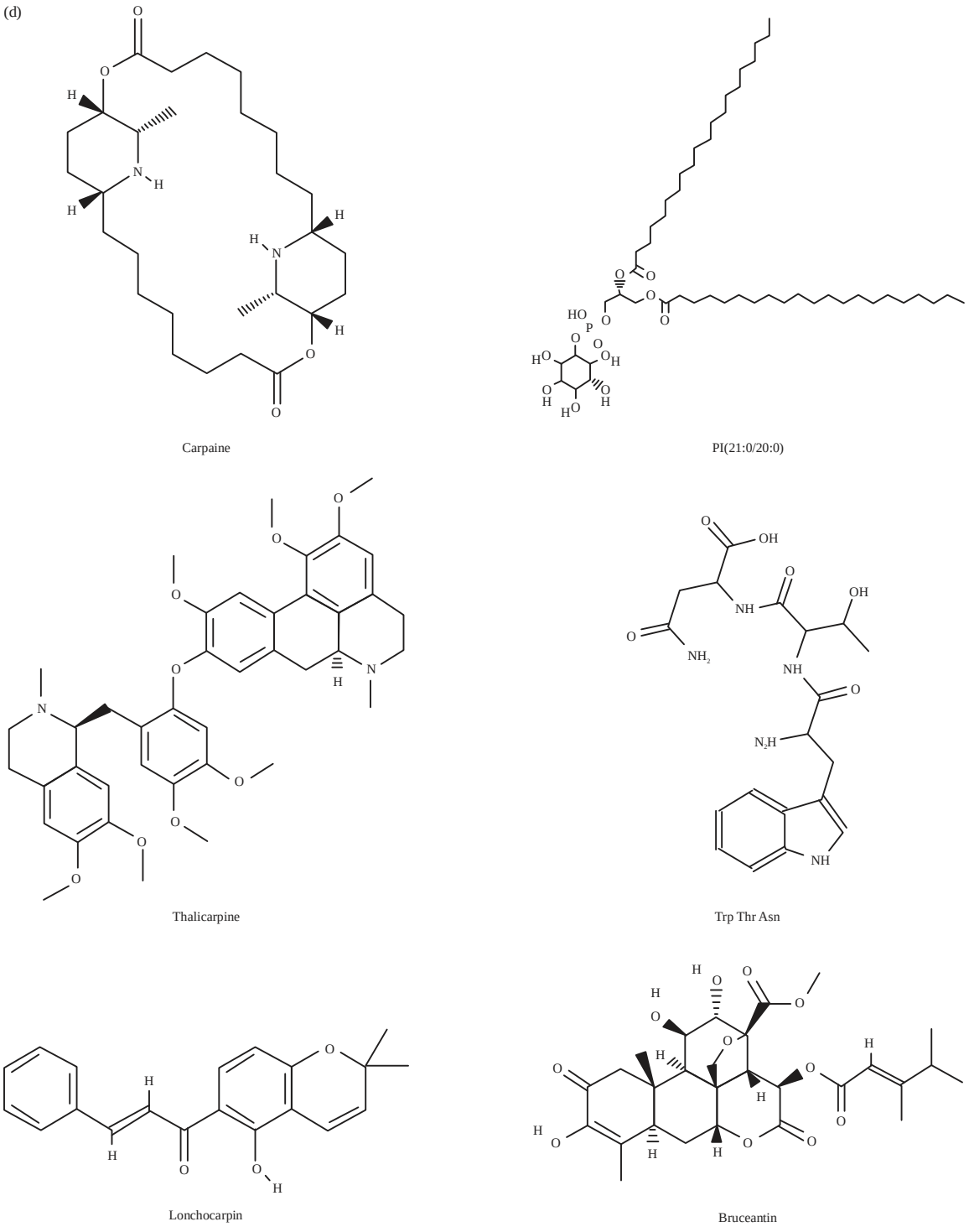


Fig. 2(a-d): Molecular structures of detected and identified metabolites from *C. guianensis* LeCpAq by employing LC-QToF-MS in negative mode

were polycyclic and aromatic compounds. Moreover, one derivative of fluconazole antifungal drug fluconazole glucuronide was detected as well as, one diterpenoid potent anticancer compound bruceantin was also detected.

Metabolomics is the profiling of the total set of metabolites or low molecular weight biochemical intermediates, resulting from the physiological, developmental or pathological state of a cell, tissue, organ or

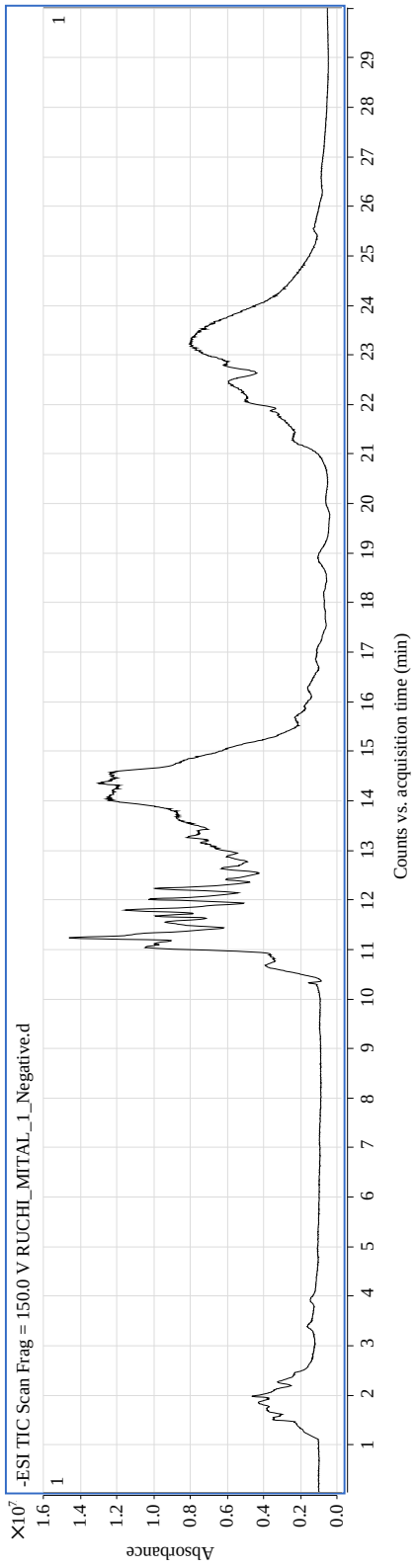


Fig. 3: Representative TIC base peak chromatogram (BPC) of *C. guianensis* LeCpAq obtained in extracted ion chromatogram in negative ionization mode

organism. Compound identification is a key element in untargeted metabolomics experiments. The level of confidence in the identification is directly dependent on the quality of the database used to assign compound identity. Metabolomics databases used for the accurate identification of the detected compounds were: HMDB, BiGG, PubChem Compound, SYSTOMONAS, LIPID MAPS (LMSD), MetaCyc (MetaCyc is a database of nonredundant, experimentally elucidated metabolic pathways). Encyclopedia of Metabolic Pathways; The Molecular Ancestry Network (MANET: MANET database traces evolution of protein architecture onto biomolecular networks), Metabolite and Tandem MS database (METLIN: The METLIN Metabolite Database is a repository of metabolite information as well as tandem mass spectrometry data)[96-98].

Begum *et al.*[99] reported three triterpenes from the n-hexane and carbon tetrachloride soluble fractions of a methanolic extract of the stem bark of the *C. guianensis* using NMR spectra. Identified compounds were (1) β-amyrin, (2) Betulin-3β-caffeate and (3) Lupeol-3β-caffeate, as structures are shown in Fig. 4a and b. Al-Dhabi *et al.*[18] reported one compound, Indirubin from the chloroform extract of the fruit of *C. guianensis* using HPLC-DAD technique, as structure is shown in Fig. 4c. Prabhu and Ravi[37] reported two compounds, Stigma sterol and Quercetin from methanol extract of the fresh flowers of *C. guianensis* using HPTLC, IR, NMR and MS techniques, as structures are shown in Fig. 4d and e. Sukumar and Shakira[100] reported one compound, Quercetin-3-O-rutinoside (rutin) from 85% ethanol fraction of the fresh pinkish white flowers of *C. guianensis* using NMR, as structure is shown in Fig. 4f. Tayade and Adivarekar[101] reported two pigments, namely blue pigment-Indigotin and pink pigment-Indirubin from fruits of *C. guianensis* using UV, FTIR and NMR techniques, as structures are shown in Fig. 4g and h. The 3D structures of some detected and identified compounds generated using freely available online structure generator-Molinspiration Galaxy 3D Structure Generator v2016.01 beta (http://www. molinspiration.com/cgi-bin/galaxy) from *C. guianensis* LeCpAq is shown in Fig. 5a and b. According to the results, it can be stated that cold percolation extraction method is an efficient method for the determination antioxidant activity and this method should be considered for extracting higher quality and quantity of antioxidants. Further docking studies on interaction and elucidation of mechanism of antioxidant actions is underway.

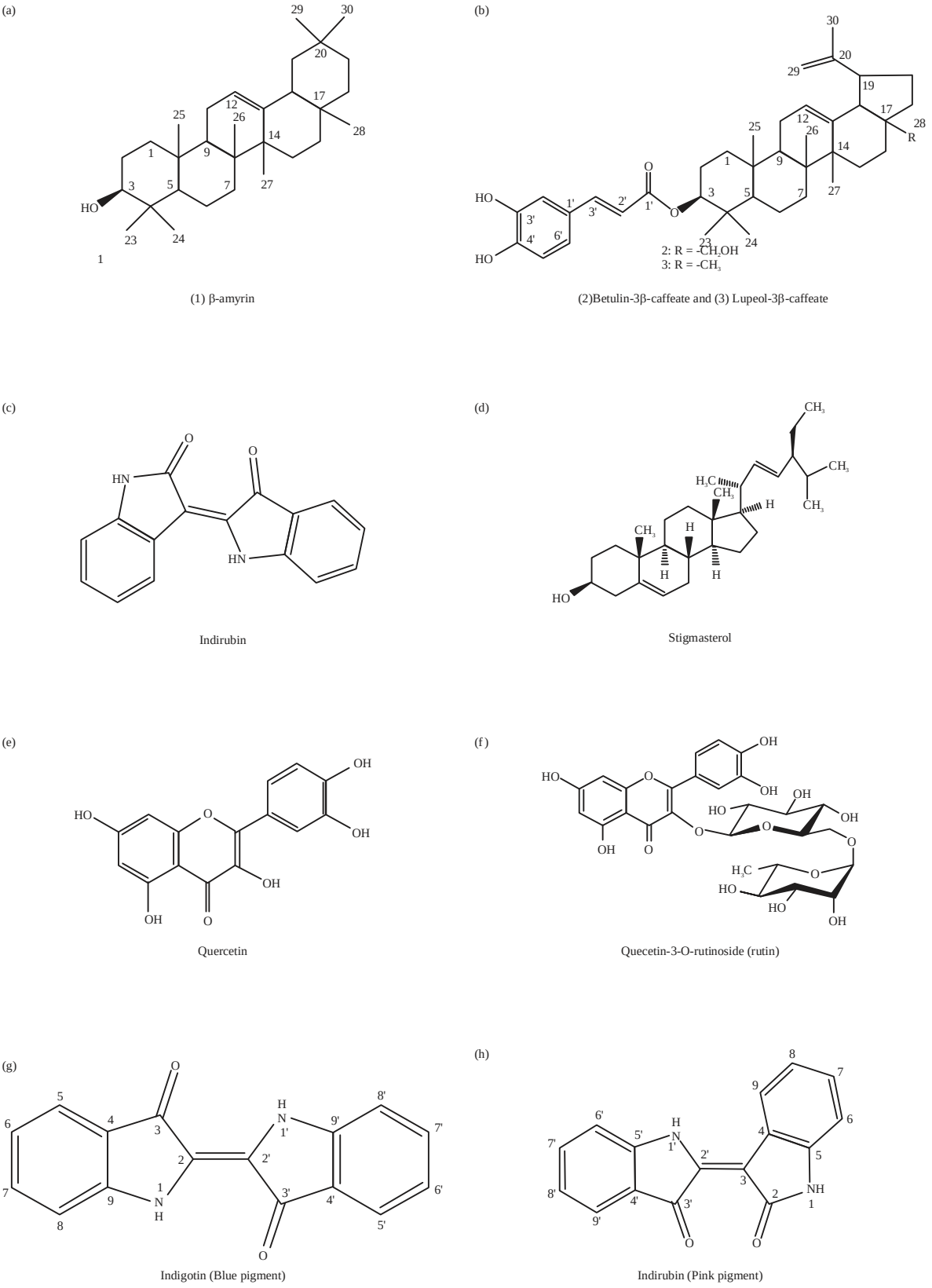
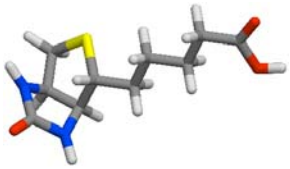
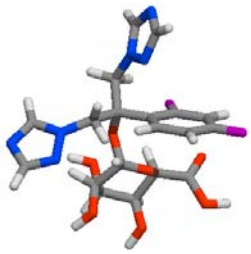


Fig. 4: Reported compounds from different parts of *C. guianensis*

(a)

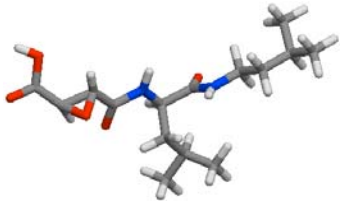


D-Biotin

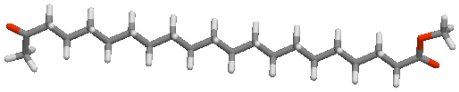


Fluconazole glucuronide

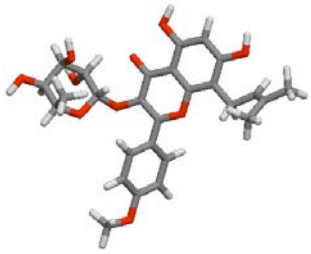
3beta-Fluoro-5alpha-androstan-17beta-



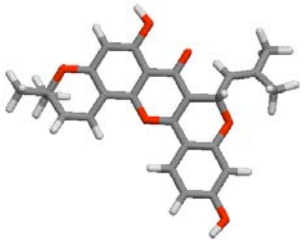
E-64c



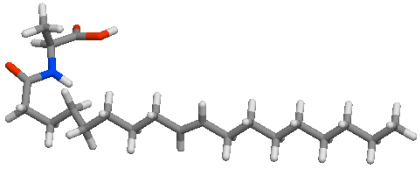
20-Oxo-heneicosanoic acid



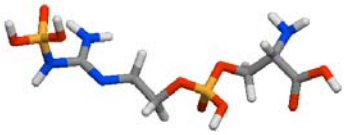
Icariside II



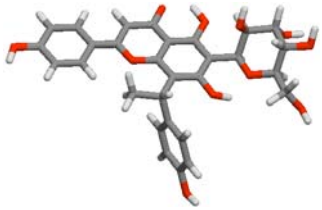
Cyclomorusin



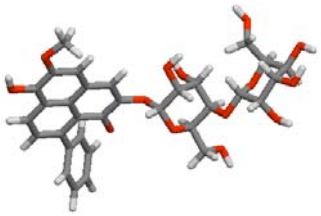
N-oleoyl alanine



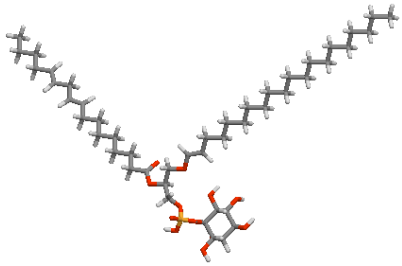
N-Phospho-D-lombricine



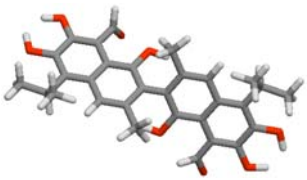
Cucumerin B



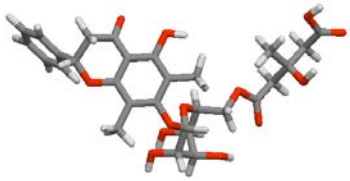
Haemocorin



PI(P-20:0/17:2(9Z,12Z))



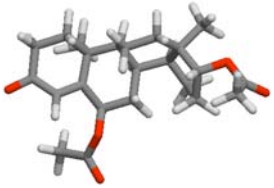
Gossypol



Matteuorienate B

β-Amyrin Acetate

Sparteine



6beta,17beta-Dihydroxyandrost-4-en-3-one diacetate

Fig. 5(a-b): Continue

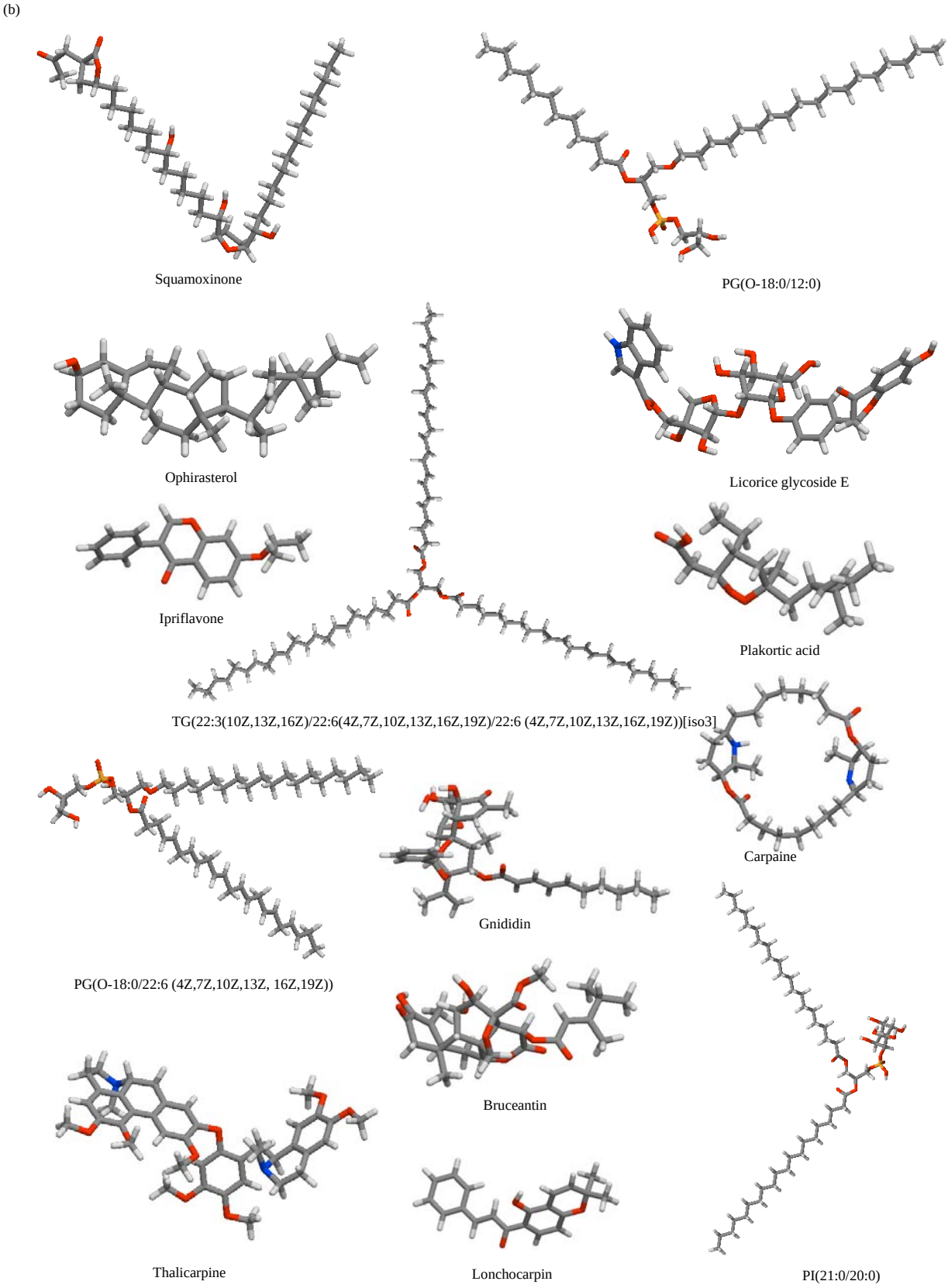


Fig. 5(a-b): 3D structure of detected and identified compounds from *C. guianensis* LeCpAq

CONCLUSION

The results of the present study showed that the cold percolation extraction method was best than the other extraction methods used in the present investigation, maybe by concentrating active principles and by removing interferences to substances of the plant *C. guianensis* leaves. This could be due to the presence of an enormous amount of the bioactive compounds, which are responsible for the immense antioxidant property, a number of thirty nine were here identified and reported for the first time. The study also revealed the possible antioxidant mechanism of the extracts may be due to hydroxyl groups existing in almost each of the detected compounds that can scavenge the free radicals.

SIGNIFICANCE STATEMENTS

The measurement and interpretation of the endogenous metabolite profile from a biological sample have provided many opportunities to investigate the changes induced by external stimuli or enhance knowledge of inherent biological variation. This article focused on the metabolic profiling of the plant *C. guianensis* by using LC-QToF-MS technique. Total 39 metabolites were detected and identified belongs to various groups. This study will help the researcher to uncover the positive relation of metabolites and antioxidant activity of *C. guianensis*.

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