

Rapid Detection of Eight Volatile Alkaloids from *Caryota mitis* Lour. by LC-MS/MS and Antimicrobial Effects of their Extracts

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Abstract

Caryota mitis Lour. palm belongs to Family: Arecaceae. Pyridine and piperidine alkaloids are the most bioactive secondary metabolites reported in this Family. The dried leaves of *Caryota mitis* Lour. were subjected to defatting using n-hexane. The alkaloids were extracted from the defatted powder (marc) by acid base method. Eight pyridine/piperidine alkaloids were detected by LC-MS and reported for the first time in the genus *Caryota*. The alkaloid fraction as well as other plant extract fractions showed antibacterial and antifungal activities (MIC) against selected strains.

Keywords: *Caryota mitis*; LC-MS/MS; Alkaloids; Antimicrobial; Mass profiling

Introduction

Caryota mitis Lour. palm belongs to Family Arecaceae that contains about 230 genera and 3000 species [1]. The plants of this Family are distributed in tropical and subtropical areas [1]. In folk medicine, the leaves and fruits of *C. mitis* Lour. are used for treatment of constipation, hemorrhoids, loss of virility, rheumatoid arthritis, vomiting and stomachache [2,3]. The chemical screening Tests on the

leaves of *C. mitis* Lour. showed the presence of different chemical classes; steroids, triterpenoids, flavonoids, saponins and alkaloids [4]. Both piperidine and pyridine alkaloids such as; arecoline, guvacoline, nicotine and ethyl nicotinate were reported in the Family Arecaceae [5]. These alkaloids possess wide range of biological activities include antidepressant [6], anticonvulsant [7], hypoglycemic [8], salivary stimulant [9] and anthelmintic to eradicate worms [10]. In this study; eight of piperidine and pyridine alkaloids (Table 1) are first detected in the genus *Caryota mitis* Lour. by LC-MS/MS. The fraction of alkaloids showed antibacterial activity (MIC) as shown in Table 2.

Peaks no.	t _R	MS (m/z)	[M+H] ⁺	Formula	DBE	Diff (ppm)	Score	Compound
			Major MS peaks					
1	3.406	162.1155	163.1231, 134.0789, 120.0814, 106.0662, 93.0642, 79.0556	C ₁₀ H ₁₄ N ₂	5	1.24	99.56	Nicotine
2	15.117	157.1097	158.1168, 143.0702, 99.1027	C ₈ H ₁₅ NO ₂	2	3.78	97.16	Methyl N-methylpiperidine-3-carboxylate
3	15.585	185.1409	186.1484, 171.0732, 157.0836, 143.0725, 99.1020	C ₁₀ H ₁₉ NO ₂	2	3.44	78.4	Propyl N-methyl piperidine-3-carboxylate
4	15.662	171.1259	172.1326, 157.0828, 143.0715, 99.1018	C ₉ H ₁₇ NO ₂	2	0.22	96.35	Ethyl N-methyl piperidine-3-carboxylate
5	15.749	141.0785	142.0858, 127.0745, 83.0502	C ₇ H ₁₁ NO ₂	3	3.59	78.7	Guvacoline
6	15.850	169.1086	170.1163, 141.1009, 97.0679, 82.0654	C ₉ H ₁₅ NO ₂	3	10.14	63.42	Ethyl N-methyl-1,2,5,6-tetrahydro-pyridine-3-carboxylate

7	17.376	155.094	156.1016, 141.0917, 97.0664, 82.0663	C ₈ H ₁₃ NO ₂	3	3.94	76.16	Arecoline
8	21.353	151.0611	152.0681, 123.0486, 79.0542	C ₈ H ₉ NO ₂	5	14.79	57.7	Ethyl nicotinate

Table 1: Retention times and mass spectral data of the chromatographic peaks depicted in Figure 1 obtained from (+)-ESI LC-MS spectral analysis of the constituents of the alkaloid fraction extract.

Inhibition Zone Diameter in mm and MIC (µg/ml)			Group
<i>C. albicans</i>	<i>E. coli</i>	<i>S. aureus</i>	
	10 mm (5)	8 mm (2.5)	Total extract (20 mg/ml)
			n-Hexane fr. (20 mg/ml)
			Chloroform fr. (20 mg/ml)
	11 mm (5)	20 mm (2.5)	Ethyl acetate fr.(20 mg/ml)
12 mm (5)	14 mm (5)	17 mm (2.5)	n-Butanol fr.(20 mg/ml)
12 mm	7 mm (5)	7 mm (2.5)	Aqueous fr.(20 mg/ml)
16 mm (2.5)	18 mm (5)	19 mm (2.5)	Alkaloids fr.(20 mg/ml)
Controls			
	26 mm (0.12)		Gentamicin (5 µg/ml)
		28 mm (0.24)	Ampicillin (10 µg/ml)
(0.24)			Clotrimazole (5 µg/ml)
			Negative control DMSO:H ₂ O

Table 2: Inhibitory activity (MIC) of the leave extract fractions of *C. mitis* Lour. at (20 µg/ml).

Experimental Methods

Materials and chemicals

The leaves of *C. mitis* Lour. were collected in June 2014 from El-Orman Botanical Garden, Giza, Egypt. The extraction of the alkaloids from the dried powdered leaves of *C. mitis* Lour. was performed in the Natural Product Chemistry Lab, Faculty of Pharmacy, Assiut University, Egypt. LC-MS and LC-MS/MS analyses of the alkaloid fraction were achieved in the Department of Drug Discovery and Development, Harrison School of Pharmacy, Auburn University, USA. All organic solvents were of HPLC grade and were purchased from Thermo Fisher (Hanover Park, IL, USA).

Extraction of alkaloids

Extraction of alkaloid rich fraction from *C. mitis* Lour. leaves were performed by acid base extraction method [11]. Ten grams of the dried powdered leaves were defatted by maceration in n-hexane followed by filtration. The marc was then soaked in 70% ethanol at 25°C for 2 hrs. The methanol extract was filtered using Whatman No.1 filter paper and concentrated by Rota vapor under reduced pressure at 40°C. The dried alkaloid fraction (100 mg) was mixed with 300 ml tartaric acid (2% W/V) then filtered. The aqueous filtrate was rendered alkaline at PH 10 by adding 20% NH₄OH. The alkaline aqueous solution was transferred into 1 L separating funnel containing equal amount of dichloromethane. The dichloromethane layer was collected after shaking, concentrated and confirmed for presence of alkaloids by Dragendorff reagent (repeated three time). This alkaloid rich fraction was subjected to LC-MS and LC-MS/MS analyses.

LC-MS and LC-MS/MS analysis

A ZORBAX Eclipse XDB-C18 column (Agilent Technologies, New Castle, DE, USA) (5 µm, 4.6 × 150 mm) was used for separation of the alkaloids. A gradient mobile phase consisting of (A) 0.1% formic acid in water and (B) methanol was used for chromatographic separation of alkaloids at 25°C with flow rate 0.6 mL/min. Identification of compounds was achieved by using an Agilent 6520 Q-TOF mass spectrometer equipped with a 1220 RRLC system (Agilent Technologies, Little Falls, DE, USA) and electrospray ion (ESI) source was used for detection of plant alkaloids. ESI-MS analysis was performed with a capillary voltage of 3200 and 3400 V, respectively [12]. MS/MS experiments were carried out under similar LC-MS conditions described above with collision energies of 20 eV.

Antibacterial and antifungal assays

Preparation of extracts for antimicrobial study: Fourty mg of each fraction (total extract, n-hexane, chloroform, n-butanol, aqueous and alkaloid fraction) were separately reconstituted in the least amount of mixture of DMSO (Dimethylsulfoxide) and Water (1:1% v/v) and the volume was completed to 2 ml with DMSO: H₂O (20 mg/ml). DMSO: H₂O mixture served as a negative control.

Antibacterial assay: The inoculum size of each test strain was standardized according to the national committee for the clinical laboratory standard (NCCLS). Strains were grown in Mueller-Hinton broth or Mueller-Hinton agar medium at 37°C. A single isolated colony of each bacteria was picked from the agar plate culture and suspended in 0.9% (w/v) sterile aqueous saline solution to prepare bacterial suspensions of 0.5 McFarland standard and diluted to give a final concentration of 10⁸ CFU/ml. Antibacterial screening of *C. mitis* Lour. leaf extracts were investigated using agar well diffusion method [13,14]. Bacterial suspensions of either *Staphylococcus aureus* or *E.*

coli strains were mixed with Sabouraud agar (20 ml) in sterile petri dishes (9 cm in diameter) and the agar plates were allowed to solidify. After solidification, wells were made in the agar plates using a pore-maker of size 10 mm and filled with 100 μ L of different extracts (20 mg/ml). Ampicillin (10 μ g/ml) and Gentamicin (5 μ g/ml) were used as positive controls, while DMSO:H₂O mixture (1:1) served as a negative control. Plates were incubated at 37°C for 24 h and the diameters of inhibition zone were measured using a digital caliber. Inhibition zone diameter was an indicator for the antibacterial activity of the studied solutions.

Antifungal activity: The antifungal activity of the tested extracts was evaluated by the agar dilution method [15]. Using Emmon's Sabouraud Dextrose Agar (ESDA) as a growth medium. Stock solutions of the test extracts were prepared at initial concentration of 20 mg/ml in DMSO-Water mixture (1:1), and the reference standard antifungal drug (clotrimazole) was prepared in the same mixture at concentrations (0.1-100 mg/ml) and incorporated into the growth medium and plates were poured. ESDA incorporating only DMF was used as a negative control. Plates were inoculated with 0.05 ml of the fungal suspension (approximately 5-10 conidia or hyphal element/ml 0.9% normal saline) and incubated at 30°C until macroscopically visible growth appeared in the control (48-96 hrs post inoculation).

Determination of the minimal inhibitory concentration (MIC): MIC of *C. mitis* Lour. leaf extracts were determined against *Staphylococcus aureus* and *E. coli* strains using microtiter broth dilution method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2012). Sterile 96-well rounded bottom plates were used. Samples were vortexed before the experiment to ensure homogenous distribution of the particles. Mueller Hinton broth (100 μ L) was dispensed into all wells of the microtiter plate. Each tested extract was added to the first well and 10 serial two-fold dilutions were made (20-0.039 mg/ml). Bacterial suspensions were added into all wells except for control wells. Plates were incubated in 37°C for 24 h and evaluated for the minimum inhibitory concentration by visual examination of the culture turbidity.

Results and Discussion

Identification of alkaloids

Eight different peaks appeared in the Extractive Ion Chromatogram (EIC) of *C. mitis* Lour. (Figure 1), the peaks were prescribed according to their retention times and were identified by LC-MS and LC-MS/MS analyses. Based on accurate mass measurement, molecular formula, double bond equivalent (DBE), MS/MS fragmentation (Table 1) and comparison with previously available MS data, alkaloids of *C. mitis* Lour. were identified.

Compound 1 appeared at ($t_R=3.406$ min) and displayed a protonated molecule at m/z 163.1231 [M+H]⁺ with molecular formula C₁₀H₁₄N₂, DBE=5 supporting one cyclic ring and a pyridine nucleus. MS/MS fragmentation (Figure 2) showed peaks at m/z 134.0987 [M+H-29.0244]⁺ empathized loss of N-CH₃ group, m/z 120.0814 [M+H-NC₂H₅]⁺, m/z 106.0662 [M+H-C₃H₇N]⁺ and m/z 79.0556 for pyridinium cation [C₅H₄N]⁺ which confirmed the MS fragmentation pattern of nicotine [16] previously isolated from *Nicotiana tabacum* L. [17] and this is the first report in the genus *Caryota*.

Compound 2 eluted at ($t_R=15.117$ min) and displayed a protonated molecule at m/z 158.1168 [M+H]⁺ with molecular formula C₈H₁₅NO₂, DBE=2 supporting one cyclic ring and a carbonyl group. MS/MS

fragmentation (Figure 2) showed peaks at m/z 143.0702 [M+H-CH₃]⁺, m/z 99.1027 [M+H-59.0141]⁺ corresponding to loss of methyl carboxylate group and this confirmed the MS fragmentation pattern of methyl N-methyl piperidine-3-carboxylate previously detected in *Areca catechu* L. [5] and this is the first report in the genus *Caryota*.

Compound 3 with ($t_R=15.585$ min) appeared as a protonated molecule at m/z 186.1484 [M+H]⁺ with molecular formula C₁₀H₁₉NO₂, DBE=2 expressed by presence of one cyclic ring and a carbonyl group. MS/MS fragmentation (Figure 2) showed peaks at m/z 171.0732 [M+H-CH₃]⁺, m/z 157.0836 [M+H-C₂H₅]⁺, m/z 143.0725 [M+H-C₃H₇]⁺, m/z 99.1020 [M+H-C₄H₇O₂]⁺ explaining loss of propyl carboxylate group. This MS fragmentation pattern helped us to identify the compound as propyl N-methyl piperidine-3-carboxylate suggesting it to be a new compound that is firstly isolated from the genus *Caryota*.

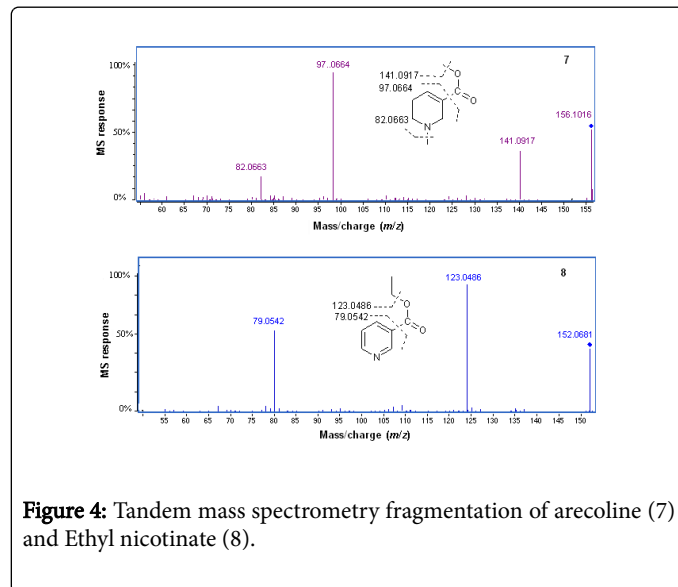
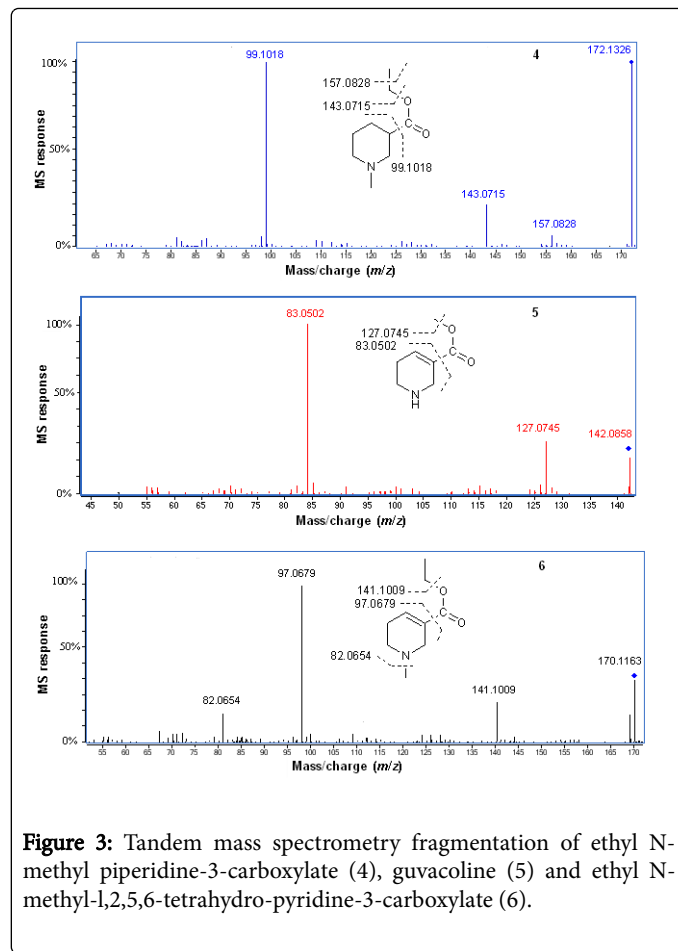
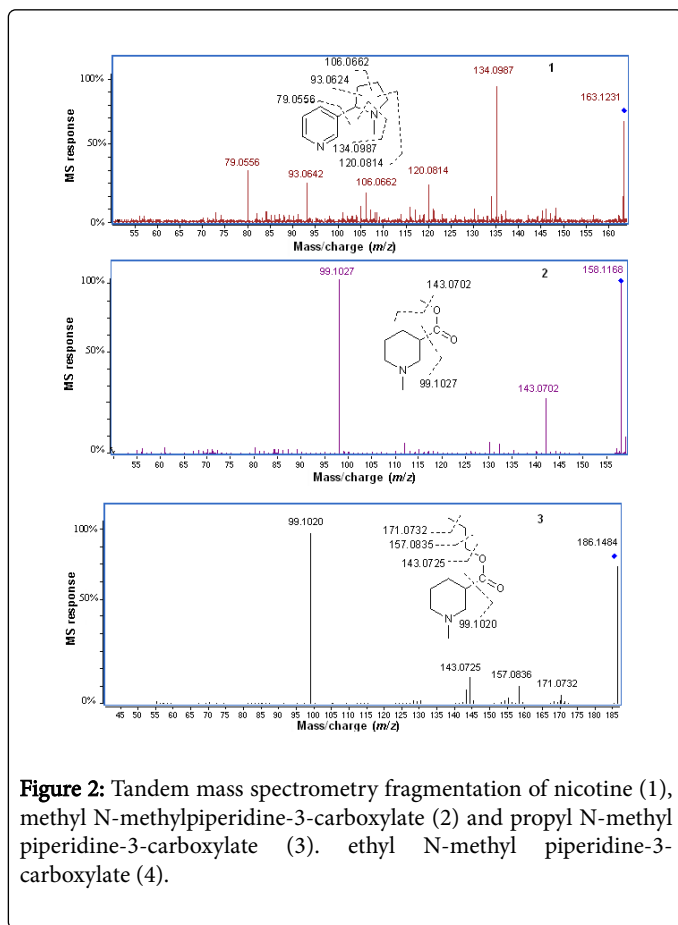
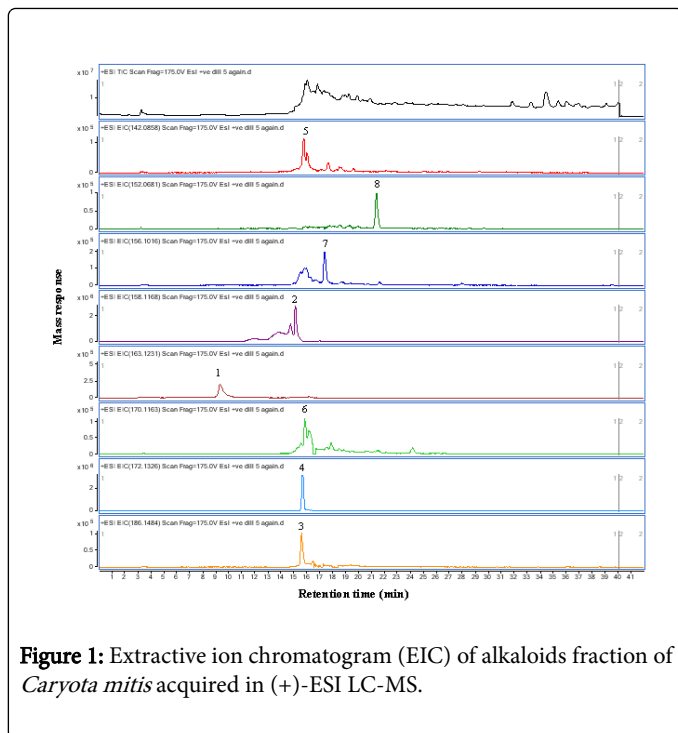
Compound 4 appeared at ($t_R=15.662$ min) exhibited a protonated molecule at m/z 172.1326 [M+H]⁺ with molecular formula C₉H₁₇NO₂, DBE=2 confirmed the presence of one cyclic ring and a carbonyl group. MS/MS fragmentation (Figure 3) showed peaks at m/z 157.0828 [M+H-15.0498]⁺ with loss of methyl group, m/z 143.0715 [M+H-C₂H₅]⁺, m/z 99.1018 [M+H-C₃H₅O₂]⁺ corresponding to loss of ethyl carboxylate group and matched with MS fragmentation pattern of ethyl N-methyl piperidine-3-carboxylate previously detected in *Areca catechu* L. [5] and this is the first report in the genus *Caryota*.

Compound 5: ($t_R=15.749$ min) resulted in a protonated molecule at m/z 142.0858 [M+H]⁺ with molecular formula C₇H₁₁NO₂, DBE=3, which mean that one monounsaturated cyclic ring and a carbonyl group are present. MS/MS fragmentation (Figure 3) exhibited peaks at m/z 127.0745 [M+H-CH₃]⁺, m/z 83.0502 [M+H-59.0356]⁺ with loss of methyl carboxylate group which is similar to MS fragmentation of guvacoline previously identified in *Areca catechu* L. [5] and this is the first report in the genus *Caryota*.

Compound 6: eluted at ($t_R=15.850$ min) showed a protonated molecule at m/z 170.1163 [M+H]⁺ with molecular formula C₉H₁₅NO₂, DBE=3 supporting the presence of one monounsaturated cyclic ring and a carbonyl group. MS/MS fragmentation (Figure 3) showed peaks at m/z 141.1009 [M+H-29.0154]⁺ with loss of ethyl group, m/z 97.0679 [M+H-73.0484]⁺ with loss of ethyl carboxylate group, m/z 82.0654 [M+H-C₄H₈O₂]⁺ that confirm MS fragmentation of Ethyl N-methyl-1,2,5,6-tetrahydro-pyridine-3-carboxylate previously identified in *Areca catechu* L. [5] and this is the first report in the genus *Caryota*.

Compound 7: ($t_R=17.376$ min) displayed a protonated molecule at m/z 156.1016 [M+H]⁺ with molecular formula C₈H₁₃NO₂, DBE=3 supporting the presence of one monounsaturated cyclic ring and a carbonyl group. MS/MS fragmentation (Figure 4) showed peaks at m/z 141.0917 [M+H-CH₃]⁺, m/z 97.0664 [M+H-59.0352]⁺ with loss of methyl carboxylate group, m/z 82.0663 [M+H-C₃H₆O₂]⁺ which is similar to MS fragmentation of arecoline previously detected in *Areca catechu* L. [5] and this is the first report in the genus *Caryota*.

Compound 8: ($t_R=21.353$ min) displayed a protonated molecule at m/z 152.0681 [M+H]⁺ with molecular formula C₈H₉NO₂, DBE=5 confirming one cyclic ring and a pyridine nucleus. MS/MS fragmentation (Figure 4) showed peaks at m/z 123.0486 [M+H-29.0195]⁺ with loss of ethyl group, m/z 79.0542 [M+H-73.0139]⁺ with loss of ethyl carboxylate group, which was in accordance with the MS fragmentation pattern of ethyl nicotine previously identified in *Areca catechu* L. [5] and this is the first report in the genus *Caryota*.



Antibacterial and antifungal activities

The leaf different extracts including the alkaloid fraction showed promising antibacterial and antifungal activities as shown in Table 2. Gentamicin (5 µg/ml) and ampicillin (10 µg/ml) were used as reference antibiotics. All the tested bacterial and fungal strains showed different

susceptibility to each extract. The n-hexane and chloroform fractions demonstrated weak activity against the tested strains with zone of inhibition range from 3-5 mm. The total and aqueous fractions exhibited moderate bactericidal activity against both *S. aureus* and *E. coli* relative to the positive controls and caused inhibition zone of 7-10 mm diameter (MIC; 2.5 and 5 mg/ml). The ethyl acetate fraction displayed the strongest bactericidal activity against *S. aureus*, relative to the positive control (Ampicillin) and caused inhibition of 20 mm diameter (MIC; 2.5 mg/ml), and also showed moderate activity against *E. coli* relative to control (gentamycin) and caused zone of inhibition of 11 mm diameter. Both n-butanol and alkaloids fractions demonstrated strong bactericidal activity against both *S. aureus* and *E. coli* relative to positive controls and exhibited inhibition zones of 17-19 and 14-18 mm diameters respectively, (MIC; 2.5 and 5 mg/ml, respectively).

The results of the antifungal study against *C. albicans* strains revealed that the alkaloids fraction has powerful antifungal activity with zone of inhibition of (16 mm MIC; 2.5 mg/ml) more than the n-butanol and aqueous fractions with zone of inhibition (12 mm MIC; 5 mg/ml). The other extracts exhibited no antifungal activity.

Conclusion

Caryota mitis Lour. is a member of Family Arecaceae that biosynthesize alkaloids among their secondary metabolites. Using LC-MS and LC-MS/MS analyses, eight alkaloids were identified and firstly reported in *C. mitis* according to their accurate mass measurement, molecular formula, double bond equivalent (DBE), MS/MS fragmentation (Table 1) and comparison with previously available MS data. Alkaloids fractions demonstrated strong antibacterial and antifungal activities against tested bacteria.

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References

- Nelson G (1996) The shrubs and woody vines of Florida: a reference and field guide. 1st edn. Sarasota, Florida.
- Mollik AH, Hassan AI, Paul TK, Sintaha M, Khaleque HN, et al. (2010) A survey of medicinal plant usage by folk medicinal practitioners in two villages by the Rupsha River in Bagerhat district, Bangladesh. *Am Eurasian J Sustain Agric*, pp: 349-357.
- Sharief MU (2007) Plants folk medicine of Negrito tribes of Bay Islands. *Indian J Tradit Knowl* 6: 468-476.
- Ajani OO, Owoye TF, Olasehinde GI, Audu OY, Owolabi FE, et al. (2016) Preliminary studies on the seed oil of *Caryota mitis*: Proximate composition, phytochemical screening and evaluation of antimicrobial activity. *Am J Food Technol* 11: 253-263.
- Holdsworth DK, Jones RA, Self R (1998) Volatile alkaloids from *Areca catechu*. *Phytochemistry* 48: 581-582.
- Dar A, Khatoon S (2000) Behavioral and biochemical studies of dichloromethane fraction from the *Areca catechu* nut. *Pharmacol Biochem Behav* 65: 1-6.
- Lodge D, Johnston GAR, Curtis DR, Brand SJ (1977) Effects of the *Areca* nut constituents arecaidine and guvacine on the action of GABA in the cat central nervous system. *Brain Res* 136: 513-522.
- Tung TH, Chiu YH, Chen LS, Wu HM, Boucher BJ, et al. (2004) A population-based study of the association between areca nut chewing and Type 2 diabetes mellitus in men (Keelung Community-based Integrated Screening Program No. 2. *Diabetologia* 47: 1776-1781.
- Harvey W, Scutt A, Meghji S, Canniff JP (1986) Stimulation of human buccal mucosa fibroblasts in vitro by betel-nut alkaloids. *Arch Oral Biol* 31: 45-49.
- Dewick PM (2002) Medicinal natural products: A biosynthetic approach. 2nd Edn. Nottingham, UK.
- Houghton PJ, Raman A (1998) Laboratory handbook for the fractionation of natural extracts. 1st edn, London, UK.
- Zaher AM, Moharram AM, Davis R, Panizzi P, Makboul MA, et al. (2015) Characterisation of the metabolites of an antibacterial endophyte *Botryodiplodia theobromae* Pat. of *Dracaena draco* L. by LC-MS/MS. *Nat Prod Res* 29: 2275-2281.
- Kora AJ, Manjusha R, Arunachalam J (2009) Superior bactericidal activity of SDS capped silver nanoparticles: Synthesis and characterization. *Mater Sci Eng B* 29: 2104-2109.
- Perez C, Pauli M, Bazerque P (1990) An antibiotic assay by the agar well diffusion method. *Acta Biol Med Exp* 15: 113-115.
- Okunji CO, Okeke CN, Gugnani HC, Iwu MM (1990) An Antifungal Spirostanol Saponin from Fruit Pulp of *Dracaena mannii*. *Int J Crude Drug Res* 28: 193-199.
- Pellegrini M, Marchei E, Rossi S, Vagnarelli F, Durgbanshi A, et al. (2007) Liquid chromatography/electrospray ionization tandem mass spectrometry assay for determination of nicotine and metabolites, caffeine and arecoline in breast milk. *Rapid Commun Mass Spectrom* 21: 2693-2703.
- Waller GR, Nowacki EK (1978) Alkaloid biology and metabolism in plants. 1st edn. Oklahoma.