



## Phytochemical analysis of *Canna indica* L. roots and rhizomes extract

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### ABSTRACT

*Canna indica* L. (Cannaceae) roots and rhizomes were reported to possess various biological properties like antimicrobial, anthelmintic potential and HIV-1 reverse transcriptase inhibition. In our previous studies, they showed antidiabetic activity on normal rats and rats co-addicted with caffeine and nicotine. In the pursuit of the phytochemical/s responsible for these biological activities, present study was aimed at phytochemical evaluation of hydroalcoholic extract (HAE) of *C. indica* L. roots and rhizomes; including preliminary screening, thin layer chromatography, H<sup>1</sup>-NMR and HR-LC/MS-MS analysis. After preliminary detection of flavonoids, tannins and sterols, HAE was tested for presence of  $\beta$ -sitosterol using TLC. H<sup>1</sup>-NMR spectrum of HAE revealed the presence of around 761 deshielded protons corresponding to different polar compounds. HR-LC/MS-MS analysis carried out at both positive and negative ion mode, indicated the presence of more than 90 compounds including short fragment of peptide. As per METLIN database, predicted major phytochemicals were 3'-hydroxytrimethoprim, 3,7-epoxycaryophyllan-6-one, swietenine, typhasterol, hexacosanedioic acid and 3 $\beta$ , 6 $\alpha$ ,7 $\alpha$ -trihydroxy-5 $\beta$ -cholan-24-oic acid few of which, are biologically active.

### 1. Introduction

Crude plant extracts are the complex mixture of different biologically active secondary metabolites. Their rapid and accurate identification and quantification is thereby very crucial in phytochemical analysis. Recently developed advanced instrumental techniques like chromatographic separation under high pressure (HPLC) hyphenated with mass fragmentation (MS) of separated compounds and their Nuclear Magnetic Resonance (NMR) spectrum made this phytochemical investigation possible.

*Canna indica* L. (Cannaceae) (Fig. 1) is an ornamental, perennial herb; native of tropical regions of America but also found in other tropical countries of world [3]; widely used as a folklore medicine with beneficial effects in, hepatitis, infection, rheumatism [5]. Roots and rhizomes of *C. indica* L. are thick, cylindrical and creamy white or pinkish in colour. Roots are about 2–5 mm in diameter with numerous root hairs. Rhizomes may be sympodial, stoloniferous or tuberous. Secondary lateral roots are also present [1]. Its roots and rhizomes were shown to exhibit variety of pharmacological activities. Woradulayapinij et al. 2005 [24] demonstrated inhibitory activity on HIV-1 reverse transcriptase; Nirmal et al. 2007 [19] proved anthelmintic potential of *C. indica* roots and rhizomes while Gaur et al. 2014 [8] reported *C. indica* L. roots to have antimicrobial activity against bacteria. A

decoction of the root with fermented rice is used in the treatment of gonorrhoea and amenorrhoea [13]. In our previous research articles [15,16] we reported antidiabetic activity of hydroalcoholic extract in normal rats as well as rats co-addicted with caffeine and nicotine. Since few decades, it has been used in constructed wetland for removal of organic pollutants, nitrogen, phosphorous and heavy metals [18,6].

Considering the wide uses of *C. indica* L. roots and rhizomes, the present research work is an attempt to explore phyto-compounds present in *Canna indica* L. roots and rhizomes probably responsible for different pharmacological activities exhibited and thereby therapeutic uses attributed to them.

### 2. Materials and methods

#### 2.1. Collection of plant material and extraction

The plant *C. indica* L. was identified and collected from the valley of Pawana River, Pimpri-Chinchwad region of Pune, India and then authenticated from Western Regional Centre- Botanical Survey of India, Pune (Voucher Specimen No. SK01). Plant material was washed under tap water and allowed to dry in shade. Dried material was then pulverised to powder. About 50 gm of powder was then extracted with 300 ml of mixture of ethanol and water (1:1) using Soxhlet apparatus.

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Fig. 1. *Canna indica* L. plant.

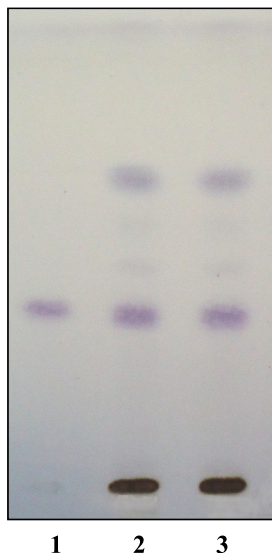


Fig. 2. TLC profile of hydro-alcoholic extract after derivatization. 1:  $\beta$ -sitosterol standard; 2–3: Test samples.

After 6 h, the resulting hydro-alcoholic extract (HAE) was filtered and concentrated using rota evaporator. It was stored in refrigerator till further use.

## 2.2. Phytochemical analysis of HAE of *Canna indica* L. roots and rhizomes

### 2.2.1. Preliminary screening

HAE was tested for the presence of different secondary metabolites (alkaloids, terpenoids, tannins, flavonoids, sterols). Test specific for each class of secondary metabolites was based on change in colour or formation of precipitate on addition of specific reagent.

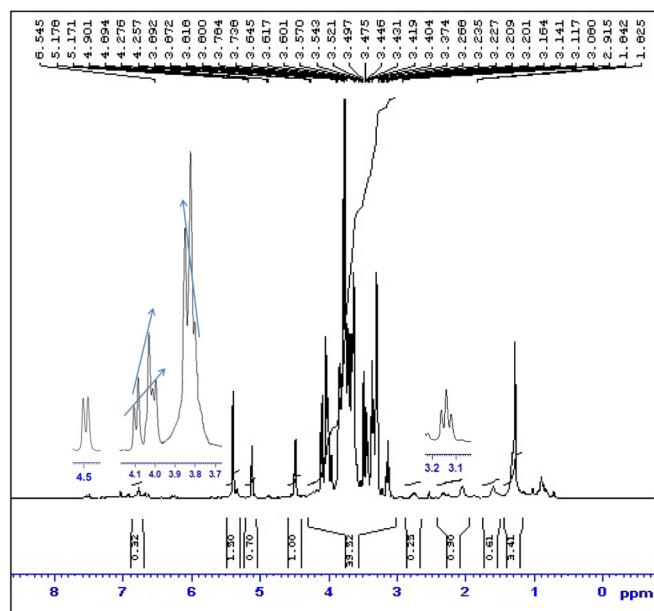


Fig. 3.  $^1\text{H-NMR}$  spectrum of HAE.

### 2.2.2. Thin layer chromatographic analysis

HAE was applied on a precoated silica gel 60 F254 TLC plate (E. Merck) of uniform thickness of 0.2 mm. Plate was developed in the mobile phase Toluene: Ethyl acetate (93:07) in a twin trough chamber to a distance of 8 cm and then envisioned by spraying anisaldehyde-sulphuric acid reagent and heating at 105 °C for 5–10 min.

### 2.2.3. $^1\text{H-NMR}$ spectrum analysis

Facility for  $^1\text{H-NMR}$  analysis was outsourced from Sophisticated Analytical Instrument Facility (SAIF) - North-Eastern Hill University (NEHU), Shillong, India. Test sample of HAE was dissolved in deuterated methanol ( $\text{CD}_3\text{OD}$  with characteristic peak at  $\delta$  3.34) and  $^1\text{H-NMR}$  spectrum was obtained via Bruker Avance II, 400 MHz instrument.

### 2.2.4. HR-LC/MS-MS analysis

To analyse HAE by HR-LC/MS-MS technique, facility was sub-contracted to Sophisticated Analytical Instrument Facility (SAIF) - Indian Institute of Technology, Bombay (IIT Bombay), India. Here, firstly chromatographic separation was achieved using extra densely bonded and double end capped active silanols as stationary phase packed in Agilent Eclipse XDB-C-18  $2.1 \times 150$  mm,  $5 \mu\text{m}$  column and acetonitrile-water (with 0.1% formic acid) as mobile phase at flow rate of 0.2 ml/min. Then, LC-ESI-MS analysis of HAE was performed in dual (positive and negative) ion mode using Agilent Jet Stream technology with a hexabore capillary sampling array and dual-stage ion funnel for increased ion sampling and transmission where fragmentation achieved on collision induced dissociation (CID) by varying the collision cell voltage. Firstly chromatogram was obtained and studied for retention volume and column efficiency

## 3. Results and discussion

On solvent evaporation using Rota evaporator and refrigeration, a highly viscous, sticky, dark brown HAE was obtained which was then used for further analysis

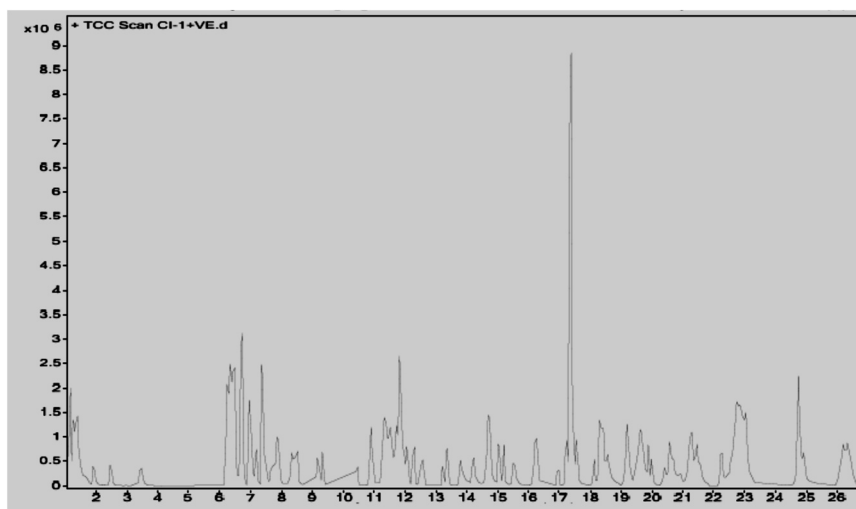


Fig. 4. HPLC chromatogram of HAE with acetonitrile as mobile phase flowing at rate of 0.2 ml/min.

### 3.1. Preliminary screening

On simple chemical tests, based on change in colour or precipitation, HAE showed presence of mainly polar compounds like polyphenolics (flavonoids, tannins), triterpenoids, steroids and some sugars. There was no alkaloid or terpene present in the extract.

### 3.2. Thin layer chromatographic analysis

TLC analysis of HAE showed presence of various coloured bands at different  $R_f$  values; 0.36, violet ( $\beta$ -sitosterol); 0.48 and 0.56, light violet; 0.68, violet (Fig. 2).

Considering the specificity of visualising anisaldehyde-sulphuric acid reagent and number of spots observed, it can be concluded that HAE may contain several triterpenoids and/or related compounds.

### 3.3. $^1\text{H-NMR}$ spectrum analysis

$^1\text{H-NMR}$  spectrum (Fig. 3) of HAE dissolved in  $\text{CD}_3\text{OD}$  was generated on Bruker Avance II, 400 MHz at SAIF-NEHU, Shillong. It showed several peaks downfield/low field to those of standard TMS at  $\delta$  0, revealing the presence of around 761 deshielded protons (included 4 hydrogens in deuterated methanol) may be attributed to different compounds present in HAE (Supplementary data file).

Singlet at  $\delta$  0.8 was indicative of hydrogens of free and terminal methyl groups. Occurrence of several peaks like singlets at  $\delta$  1.28 and 1.60 (3H each) for its tertiary methyl groups; doublet at  $\delta$  2.061 ( $J \approx 6.4$  Hz) for a methyl group at its C-21 and multiplet at  $\delta$  2.35 for 3H reflected the presence of compound with steroids nucleus. Prominent doublets at  $\delta$  4.49 ( $J \approx 8$  Hz) claimed the presence of chiral compound. Peculiar triplets at  $\delta$  5.39 ( $J \approx 1.5$  Hz) could be assigned to olefinic protons. As per the 'structural-reporter-group' concept introduced by Vliegthart et al. 1983 [23], signals in region of  $\delta$  3.0–3.9 ppm (except that at  $\delta$  3.34 which is assigned to solvent methanol-d [11] indicated the occurrence of carbohydrates, may present in the form of glycosides). Singlet at  $\delta$  3.96 reflected occurrence of compound with methoxy group.  $^1\text{H-NMR}$  spectrum exposed presence of

compounds with terminal ethyl group, designated by quartet at  $\delta$  4.07 and triplet at  $\delta$  3.77 which consisted of peaks slanted in upward towards the signal of protons responsible for its splitting. Proton NMR spectrum of HAE also revealed the presence of flavan-3-ol as function of condensed tannins, its H-12 was observed as doublet at  $\delta$  6.78 with  $J \approx 7.2$  Hz, downfield to distorted doublets at  $\delta$  6.69 and 6.62 for H-15 and H-16; with  $J \approx 8$  and 12 Hz, respectively. Doublet at  $\delta$  6.9 ( $J \approx 8$  Hz) redirected the *ortho*-hydrogens in B-ring of flavonoids. The singlet at  $\delta$  7.04 ppm could be readily assigned to the H-3 proton of flavone [25].

Comparing with literature available,  $^1\text{H-NMR}$  spectrum of HAE could also be considered to reveal the presence of few amino acids with characteristic hydrogen positioning. Peaks as *s* at  $\delta$  1.6, *d* at  $\delta$  2.7, *d* at  $\delta$  3.2 and *s* at  $\delta$  7.04 could be assigned to  $\beta$  CH- of Arg,  $\epsilon$  CH<sub>2</sub>- of Lys,  $\delta$  CH<sub>2</sub>- of Arg and hydrogens of aromatic rings of Tyr and/or Trp, respectively; reflecting the existence of peptide fragment [4].

### 3.4. HR-LC/MS-MS analysis

On high resolution liquid chromatographic separation, extract showed several peaks (Fig. 4) indicative of different compounds. Addition of formic acid to mobile phase of acetonitrile – water eluents improved peak resolution. Now, because of similarity in polarity and chemical properties, various compounds may exhibit same retention time, accordingly each resolved peak may correspond to more than 1 compound. Another proof of allocation of 2 or more compounds to single peak was detection of flavonoid and/or tannin in preliminary phytochemical analysis, but surprisingly, not a single has been predicted in HR-LC-MS/MS analysis, as per METLIN database. Hence, the probability of this aspect is greater than 0.9. So, as per Hirschfeld T. 1980 given equation [Eq. 1],

$$N_c = \sqrt{2R \ln 1/\alpha} \quad (1)$$

where,  $N_c$  is the probable number of compounds present under each peak,  $R$  is the number of resolved peaks in the entire LC run and  $\alpha$  is probability of each peak attributed to more than 1 compound; the probable number of compounds present under each peak was found to

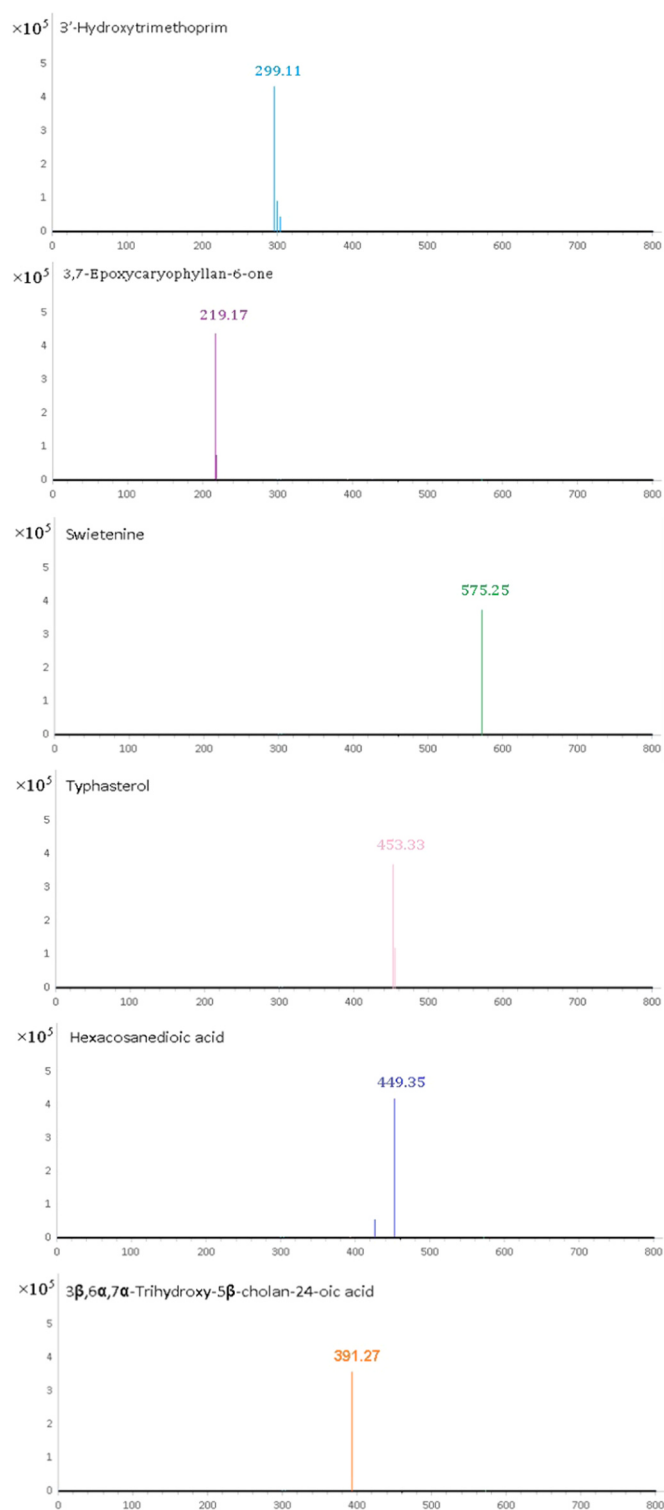


Fig. 5. MS/MS spectra of major compounds depicted by METLIN database.

be  $2.86 \approx 3$ ; as number of resolved peaks in LC separation was 39 and probability was 0.9. Therefore, probable number of compounds present in extract was found to be 117 ( $39 \times 3$ ).

These LC separated compounds later on, in ionizing chamber, got split-up into fragments of different masses. Now, prediction of phyto-compounds attributable to mass fragments obtained was based on fragmentation at particular mass ion and its matching with already available database, METLIN (Fig. 5). On the basis of compounds predicted, it can be noted that hydrophobic interactions between analytes and a stationary phase used in chromatographic separation increase with decrease in number of double bonds.

Here in this article, only compounds (Fig. 6) corresponding to prominent LC peaks with sufficiently larger area under curve have been mentioned in Table 1 (Entire list of compounds predicted compounds were provided in Supporting data file).

As per METLIN database, major phytochemicals likely to be present in HAE were found to be 3'-Hydroxytrimethoprim, 3,7-Epoxyecaryophyllan-6-one, Swietenine, Typhasterol, Hexacosanedioic acid and 3β, 6α,7α-Trihydroxy-5β-cholan-24-oic acid.

As such, Swietenine was previously isolated as tetranortriterpenoid from *Swietenia macrophylla* (Meliaceae) seeds [22] which were shown to possess anti-inflammatory [9] and hypoglycaemic effects [17] in rodents. Initially, typhasterol was identified as one of plant growth regulator [21] and one of the intermediate in biosynthesis of brassinolide in shoots of *Arabidopsis thaliana* [7]. Typhasterol was also found to be present in black tea (*Camellia sinensis*, Theaceae) which exhibited an antihyperglycemic effect in rats [10] by insulin-enhancing activity [2] and reduced most of the diabetes-associated abnormalities [12] including hyperglycemia-induced renal oxidative stress and inflammation in streptozotocin-induced diabetic rats [14]. Presence of these pharmacologically active phytochemicals in HAE, supported the previously reported anti-diabetic potential exhibited by *C. indica* L. roots and rhizomes [15,16].

In addition to some of these secondary metabolites, mass spectrum showed few peaks which were predicted as peptide fragments by METLIN database. As it has already been made clear that each LC peak may represent more than 1 compound, noticing the presence of different peaks at different  $m/z$  ratio, it can be taken revealed that these peptide fragments are the part of large protein fraction. As per Roepstorff and Fohlman [20] proposed peptide fragment nomenclature, these fragments were observed as b-ions, supported by presence of a-ions with the difference of 28  $m/z$  (Fig. 7, Table 2), which represents the mass of C=O fragment.

On this basis, the sequence of amino acids of fragment in large protein fraction can be predicted as in Fig. 8. In this pattern, fragments with different molecular ion peaks were placed in increasing order of  $m/z$  ratio. Going right to left, two successive observed fragments were separated by red line which corresponds to peak at  $m/z$  slighter by 28 than peak at  $m/z$  assigned for fragment placed right to red line.

Considering the length of fragment with specified sequence of amino acids, it can be concluded that, fragment was extracted out in hydro-alcoholic extract at very early stage of concerned protein synthesis (translation).

#### 4. Conclusion

The present investigation of HAE of *C. indica* L. roots and rhizomes has revealed the presence of several constituents most of which are described for the first time in the species. TLC profiling,  $^1\text{H-NMR}$  analysis of HAE supported the reported claim of β-sitosterol and; steroidal compounds and flavonoids presence, respectively while HR-LC/MS-MS analysis predicted several compounds, surprisingly peptide fragments as well, in HAE. Most of the phytochemicals found or predicted (like swietenine and typhasterol) have been reported to possess significance

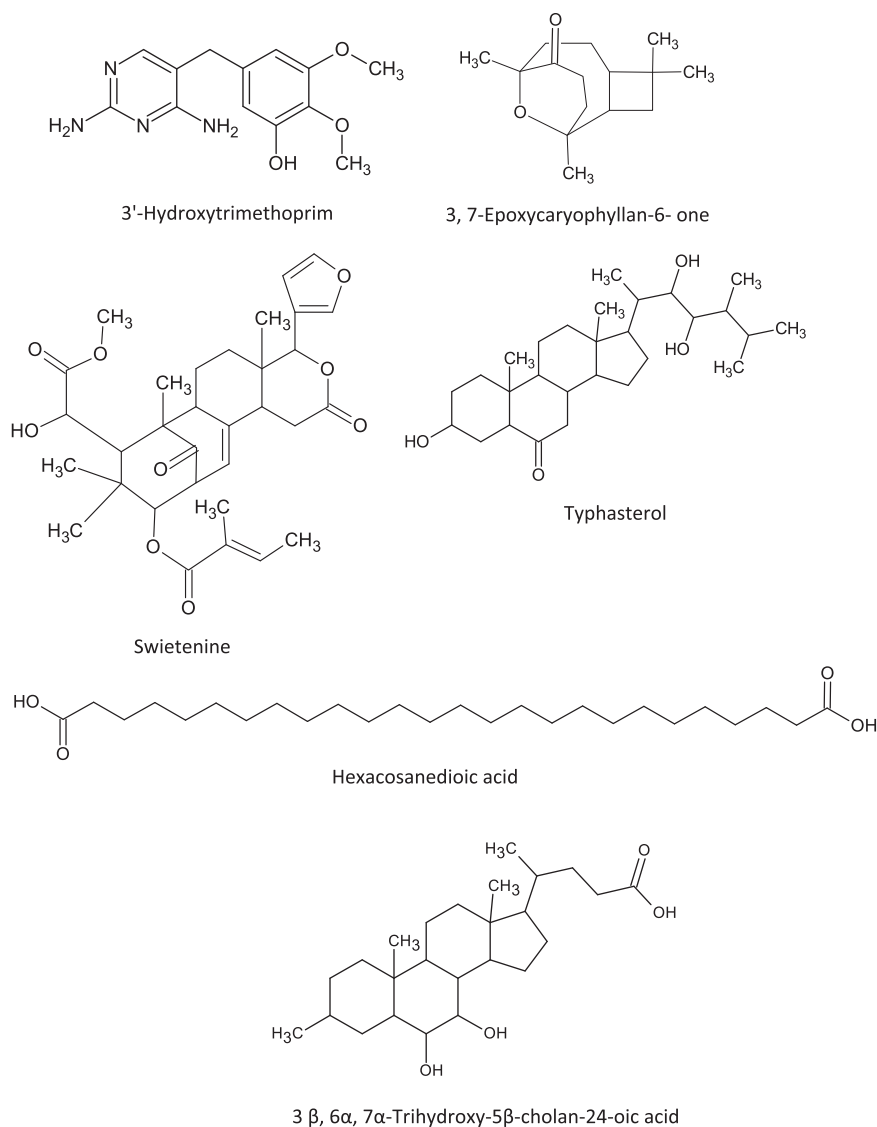


Fig. 6. Structures of major phytochemicals predicted using METLIN on HR-LC/MS analysis.

Table 1

Compounds predicted on the basis of mass fragments.

<i>tR</i> (min)	Mass fragments ( <i>m/z</i> )	Compound predicted
8.427	299.11	3'-Hydroxytrimethoprim
10.917	219.17	3,7-Epoxycaryophyllan-6-one
11.761	575.25	Swietenine
17.387	453.33	Typhasterol
22.844	449.35	Hexacosanedioic acid
24.739	413.26, 391.27	3β, 6α,7α-Trihydroxy-5β-cholan-24-oic acid

Table 2

Predicted mass fragments.

Fragment	Molecular ion peak ( <i>m/z</i> )	Peak corresponding to difference of CO mass ( <i>m/z</i> )
Pro Ile	228	200
Arg Gly Cys	334	306
Glu Lys Leu	372	344
Lys Thr Tyr	410	382
Arg Arg Gln	458	430
Trp Arg Asp	476	448

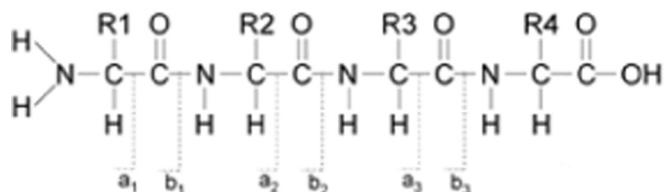


Fig. 7. Mass fragmentation pattern of peptide as per Roepstoff et al. 1984 [20].

in biological activities and/or in basic plant physiology. Hence, these compounds can be considered responsible pharmacological undertakings reported for *C. indica* L. roots and rhizomes. Therefore, present phytochemical exploration on *C. indica* L. roots and rhizomes may found supportive in chemotaxonomy of *C. indica* and thereby Cannaceae; and it which will also support analytical approaches for the characterisation of herbal preparations from *C. indica* in the future.

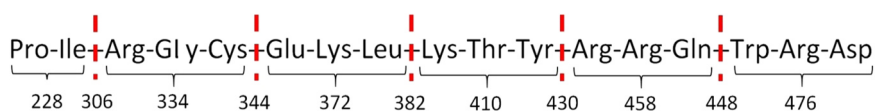


Fig. 8. Amino acid sequence in peptide fragment.

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## Appendix A. Transparency document

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbrep.2018.09.002.

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