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Structural Characterization of Polyphenolics in *Livistona chinensis* Using HPLC-PDA-MS

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ABSTRACT

Objectives: This study focused on the identification and characterization of secondary metabolites tentatively from aqueous ethanolic leaf extract of *Livistona chinensis* by HPLC-PDA-ESI-MS/MS and evaluation of its cytotoxic activity. **Methods:** The aqueous ethanolic extract was analyzed by high performance liquid chromatography (HPLC) coupled to photodiode array detection of mass spectroscopy (PDA-MS/MS), to detect the secondary metabolites in *L. chinensis* leaves extract. It was also estimated for its cytotoxicity against human prostate carcinoma (PC3) and hepatocellular liver carcinoma (HepG2) cancer cell lines using SRB (3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay. **Results:** Forty-two secondary metabolites were tentatively identified; the most major compounds were *C*-glycoside derivatives of apigenin, luteolin and tricin together with phenolic acids. The 70% alcoholic extract of *L. chinensis* leaves exhibit more antitumor activity against PC3 than against HepG2. **Conclusion:** *L. chinensis* is a privileged source of *C*-flavonoids that revealed the efficiency of HPLC-MS metabolomics in natural products drug discovery.

Keywords: *Antitumor; HPLC; Livistona chinensis; Mass spectroscopy; Phenolics*

INTRODUCTION

Arecaceae known as Palmae, is one of the well recognized and established plant families, it encompasses 181 genera with approximately 2600 species. The majority of palms are characterized by their enormous evergreen fronds. Palms show a variety of physical traits and inhabit almost each type of habitat within their area, from rainforests to deserts[1]. Among its genera is *Livistona* which is vastly cultivated in tropical environments as a landscaping tree; it is a genus of 36 species native to Africa, southeastern and southern Asia and Australasia[2]. There are about four *Livistona* species in Egypt namely *L. rotundifolia*, *L. australis*, *L. decipiens*, and *L. chinensis*, and three species in South China[3]. Traditionally they have been utilized as analgesics and for leukemia, esophageal cancer and nasopharyngeal carcinoma[4]. Reviewing the current literature, it was found that plants belonging to genus *Livistona* are rich in flavonoids, triterpenes, alkaloids, phenolic acids, amino acids and fatty acids[5-10]. In this study, the aqueous ethanolic extract of *L. chinensis* leaves were qualitatively and quantitatively characterized for the first time by HPLC-PDA-MS/MS, and the total extract was assessed for its anticancer activity.

MATERIALS AND METHODS

Plant material

The leaves of *L. chinensis* (Jacq) R.Br. (Fam. Arecaceae) were collected from El–Orman Botanical Garden, Giza, Egypt and identified by Dr. M. Elgebaly Former Researcher of Taxonomy in National Research Centre and Consultant at Central Administration of Plantation and Environment according to the New Royal Horticultural Society dictionary of gardening[11]. A voucher specimen (No. M-140) was deposited at the herbarium of the National Research Centre (Egypt).

Extraction and HPLC-PDA-MS/MS

Air–dried powdered leaves (2 kgm) of *L. chinensis* were extracted with aqueous ethanol, the filtrates were collected and evaporated to dryness under vacuum to yield a dark brown amorphous powder. The aqueous ethanolic extract of *L. chinensis* was analyzed by HPLC-PDA-MS/MS using a Thermo Finnigan LC system (Thermo Electron Corporation, Austin, TX, USA)[12]. A Zorbax Eclipse XDB-C18, Rapid resolution, 4.6 × 150 mm, 3.5 µm column was used (Agilent, Santa Clara, CA, USA). A gradient consists of water and acetonitrile (ACN), each having 0.1% formic acid, was applied and acetonitrile was increased from 5 to 30% within 60 min in 1 mL/min flow rate and a 1:1 split before the ESI source. The sample was injected using autosampler. LCQ-Duo ion trap having a Thermo Quest ESI source was used for MS analysis. Xcalibur software (Xcalibur™ 2.0.7, Thermo Scientific, Waltham, MA, USA) was used to control the system. MS operating parameters in the negative mode were used[13].

Measurement of potential cytotoxicity by SRB assay

Cells were plated in 96-multiwell plate (10^4 cells/ well) for 24 h before treatment with the tested samples to allow the attachment of cells to the wall of the plate. Different concentrations of the tested samples (0, 1, 2.5, 5 and 10) were added to the cell monolayer, triplicate wells were prepared for each individual dose. Monolayer cells were incubated with the tested samples for 48 h at 37 °C and in atmosphere of 5% CO_2. After 48 h cells were fixed, washed and stained with Sulfo-Rhodamine-B stain. Excess stain was washed with acetic acid and attached stain was recovered with Tris EDTA buffer. Finally, the colour intensity was measured in an ELISA reader. The relation between surviving fraction and drug concentration is plotted to get the survival curve of each tumor cell line after the specified extract[14].

Determination of total phenolic and total flavonoid contents

Total phenolic content was estimated by the Folin–Ciocalteu method[15]. The reaction mixture composed of 250 µL of diluted sample, 1.25 ml of 1:10 diluted Folin–Ciocalteu reagent and 100 µL of gallic acid was added. After 2 min, 1000 µl of saturated sodium carbonate (7.5%) was added. After 2 h of incubation at room temperature, the absorbance at 760 nm was measured using spectrophotometer. Gallic acid was used for the standard calibration curve. The results were expressed as gallic acid equivalent (GAE)/ g dry weight of plants, and calculated as mean value ± SD (n = 3). Colorimetric assay of total flavonoid was measured[16]. A 100 µl aliquot of diluted sample and standard solution of quercetin were added to 1 mL volumetric flask containing 250 µL of distilled H_2O, seventy five µL of 5% Na NO_2 was added to the flask, after 5 min, seventy five µl of 10% Al Cl_3 was added to the mixture followed by 500 µL of 1M NaOH after another 5 min. Absorbance of the mixture was measured at 410 nm. Total flavonoid content of the extract was expressed as mg quercetin equivalents (QE) / g dry weight.

RESULTS AND DISCUSSION

Phytochemical Profiling of *L. chinensis* Leaf Extract

The aqueous ethanolic extract of the leaves of *L. chinensis* was analyzed by the combination of HPLC-PDA-ESI-MS/MS. The chromatogram showed a mixture of 42 polyphenolic compounds. LC-MS base peaks in the negative ionization mode ESI (−) are demonstrated in **Figure 1** and the identified peaks are illustrated in **Table 1**.

Six hydroxycinnamic acid derivatives have been identified. Three peaks showed a molecular ion peaks $[M-H]^-$ at *m/z* 353 that were assigned to caffeoylquinic acid isomers along with caffeic acid at *m/z* 179. The three caffeoylquinic acid isomers come out at Rt 7.06, 10.05 and 11.94 minutes, the MS/MS spectra of the two isomers trans-5-caffeoylquinic acid and trans-3-caffeoylquinic acids showed a base peak product ion of *m/z* 191, while trans-4-caffeoylquinic acid had the base peak ion *m/z* 179[17]. In addition to a pseudo molecular ion peak at *m/z* 335 $[M-H]^-$, which is characteristic for caffeoylshikimic acid at Rt 19.5 minute, MS/MS spectra of this compound contained the main ions at m/z 179, 161 and 135, characteristic of caffeoyl moiety[16]. Generally the deprotonated phenolic acids $[M-H]^-$ characterized by the loss of a CO_2 (44 u) from the carboxylic acid group and produce a typical fragmentation pattern due to collision stimulated dissociation, giving an anion of $[M-H-CO_2]^-$. Another peak corresponding to sinapoyl-*O*-β-hexoside appeared at retention time 18.79 minute showed in the negative ion mode the signal of the deprotonated substance at *m/z* 385, and a fragment at *m/z* 223 (sinapic acid) due to loss of 162 amu (the hexose moiety)[19,20]

Table 1. Chemical constituents of the aqueous ethanolic extract of *Livistona chinensis* R.Br. Leaves

Peak No.	Rt	λmax	[M-H]	MS/MS	Proposed structure
1	5.97	259, 292	153	109	Protocatechuic acid
2	7.06	Not detected	353	179, 191	5-caffeoyl quinic acid (Neochlorogenic acid)
3	9.80	248	137	93	p-hydroxy benzoic acid
4	10.05	318	353	179, 191	3-caffeoyl quinic acid (chlorogenic acid)
5	11.94	320	353	173, 179, 191	4-caffeoyl quinic acid (crypto chlorogenic acid)
6	13.35	260, 291	167	108, 123, 152	Vanillic acid
7	14.65	297, 321	179	135	Caffeic acid
8	15.55	276	197	182, 153, 137	Syringic acid
9	17.97	255, 321	181	93, 137, 166	Homovanillic acid
10	18.79	331	385	223	Sinapic acid hexoside
11	19.5	244, 326	335	135, 179, 291	caffeoyl shikimic acid
12	21.19	269, 322	527	447	Orientin sulphate
13	21.41	270, 334	593	353, 383, 473, 503, 575	Apigenin-6,8-di-*C*-glycoside
14	22.21	269, 322	527	447	Isoorientin sulphate
15	24.70	270, 336	511	431	Vitexin sulphate
16	25.80	270, 336	511	431	Isovitexin sulphate
17	26.38	255,267,346	447	327, 357, 429	Orientin
18	27.64	269, 333	541	341, 443, 461	Diosmetin-6-*C*-hexoside sulphate
19	27.99	271, 334	563	353, 383, 443, 473, 503, 545	Apigenin-6-*C*-β-D-hexoside-8-*C*-α-L-Pentoside (Schaftoside)
20	29.12	267, 336	431	311, 341	Apigenin-8-hexoside (Vitexin)
21	30.14	269, 336	431	311, 341	Apigenin-6-hexoside (Isovitexin)
22	32.19	270, 339	461	341, 371	Diosmetin-8-hexoside
23	33.24	268, 336	461	341, 371	Diosmetin-6-hexoside
24	35.74	268, 325	447	151,179, 285	Lueolin-7-*O*- hexoside
25	35.74	268, 325	447	151,179, 285	Lueolin-7-*O*- glucoside
26	36.78	267, 334	571	329, 491	Tricin-7-hexoside sulphate
27	39.06	267, 346	637	329	Tricin-*O*-rutinoside
28	42.39	Not detected	473	311, 413, 431	Acetyl vitexin
29	45.93	Not detected	687	525,329	Tricin 4′-*O*-(*erythro*-*β*-guaiacylglyceryl ether) glucoside (salcolin A)
30	46.14	Not detected	687	525,329	Tricin 4′-*O*-(*threo* -*β*-guaiacylglyceryl) ether) glucoside.
31	48.23	266, 343	285	151	Luteolin
32	48.76	270, 332	611	593,431,113, 341	Syringoylvitexin
33	49.64	268, 304, 325	551	311, 341, 431, 533	Hydroxybenzoyl vitexin
34	50.02	269, 299, 327	581	311, 341, 431,	Vaniloyl vitexin
35	54.22	270, 325	637	311, 341, 431, 601, 619	Sinapoyl vitexin
36	57.93	268, 350	329	299, 315	Tricin
37	58.29	268, 346	299	284, 271	7-methoxy luteolin
38	60.01	Not detected	495	329	Tricin-4′-*O*- (*erythro* -*β*-4-hydroxyphenylglyceryl ether) (Calquiquelignan D)
39	61.00	Not detected	525	329, 507	Tricin-4′-*O*-(*erythro*-*β*-guaiacylglyceryl) ether (Salcolin A)
40	62.68	Not detected	495	329	Tricin-4′-*O*-(*threo*-*β*-4-hydroxyphenylglycery) ether (Calquiquelignan E)
41	63.77	Not detected	525	329, 507	Tricin-4′-*O*-(*threo*-*β*-guaiacylglyceryl) ether (Salcolin B)
42	71.98	273,307,325	671	525,507,329	Tricin-4'-*O*-(coumarylerythro-β-guiacylglycerol ether) glucoside

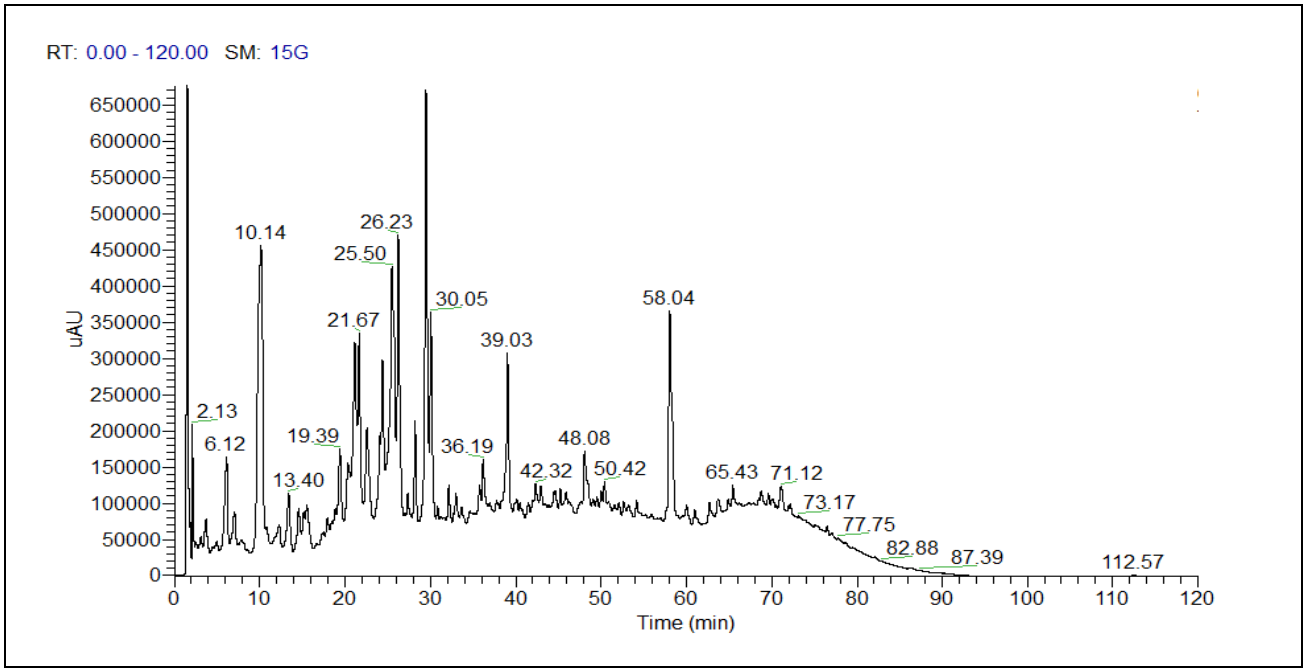

Figure 1. Total ion chromatogram of the aqueous ethanolic extract *L.chinensis* leaves [LC-MS-ESI (-)].

The ESI-MS signals at *m/z* 153, 137, 167, 197 and 181 were identified as, protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, syringic acid and homovanillic acid, respectively. The fragmentation of vanillic acid, protocatechuic acid and *p*-hydroxybenzoic acid produced the ions at *m/z* 123.0, 109.0 and 93.0, respectively at different retention times, due to loss of CO_2 from their respective precursor ions, which is a characteristic feature of hydroxybenzoic acid derivatives like other phenolic acids. On the other hand the fragmentation of syringic acid homovanilic acid produce molecular ion peaks at m/z 182 and 166, respectively due to the loss of methyl group, together with molecular ion peaks at m/z 153 and 137, respectively due to the loss of CO_2[21].

A series of peaks related to *C*-glycosides were detected, the metabolite of these compounds showed fragment ions [M-H-90] and [M-H-120], which are characteristic for *C*-hexosides[22]. In detail, a peak at Rt 21.41 minute that showed $[M-H]^-$ at *m/z* 593 was identified as apigenin-6,8-di-*C*-hexoside exhibited fragment ions at *m/z* 575 (M-H-18), 503 (M-H-90), 473 (M-H-120), 383 (aglycone+113), and 353 (Aglycone+83) that is characterized for di-*C*-glycosyl flavonoids[23]. Two Peaks related to the two isomers apigenin-8-*C*-hexoside (vitexin) and apigenin-6-*C*-hexoside (isovitexin), were detected at 29.12 and 30.14 minutes representing molecular ion peak at *m/z* 431, showed a neutral loss of 120, 90, and 18 amu which is indicative for *C*-hexoside; the MS/MS spectrum of the both isomers presented fragment ion *m/z* 311 $[M-H-120]^-$ as base peak with slight intensity differences in the fragment ions *m/z* 341 $[M-H-90]^-$, as the higher intensity of [M-H-90] suggested that the attachment of sugar occurs at the 8-position compared with that for 6-glycosides[24]. As well as the three *C*-glycosides peaks appeared related to luteolin and diosmetine aglycones at Rt 26.83 minute for orientin and 32.19 and 33.24 minutes for diosmetin-8-hexoside and diosmetin-6-hexoside.

A peak with *m/z* 563 $[M-H]^-$ was tentatively identified as apigenin-*C*-hexosyl-*C*-pentoside (schaftoside), that showed neutral losses loss of 120 and 90 amu due to *C*-hexoside in addition to the loss of 90 and 60 amu, which are characteristic for a *C*-pentoside[22].

Several peaks at *m/z* 527, 511 and 541 were detected at different retention times as illustrated in table 1; they were tentatively identified as orientin sulphate and isoorientin sulphate for *m/z* 527 $[M-H]^-$, vitexin sulphate and isovitexin sulphate for *m/z* 511 $[M-H]^-$, and at *m/z* 541 $[M-H]^-$ for diosmetin-6-*C*-hexoside sulphate, the fragments of these compounds showed fragment ions $[M-H-80]^-$ at *m/z* 447,431 and 461, due to the loss of sulphate group.

Acetyl, syringoyl, hydroxybenzoyl, vanilloyl and sinapoyl derivatives of apigenin-*C*-8-hexoside (vitexin) were detected at *m/z* 473, 611, 551, 581 and 637 they all give a pseudo molecular ion peak at *m/z* 431 which is related to apigenin-*C*-8-hexoside.

A series of peaks showed molecular ion peaks $[M-H]^-$ at *m/z* 447, 299 and a daughter ion at *m/z* 285; they were tentatively identified as luteolin-7-*O*-hexoside, 7-methoxy luteolin and luteolin, respectively. Luteolin-7-*O*-hexoside and 7-methoxy luteolin gave two fragments at *m/z* 285 and 284, respectively attributed to the elimination of a hexose [M-H-162], which is characteristic for *O*-hexoside and loss of methyl group [M-H-15] confirming the presence of methoxyl group, respectively[25].

Tricin and its derivatives showed characteristic peaks at different retention times, the *m/z* of 329 (negative mode) has been usually used to identify tricin[26]. Signals at *m/z* 315 and 299 are suggestive of cleavage of one and two molecular methyl from tricin as displayed for peak 36 which gave a molecular ion peak at *m/z* 329 that's attributed to tricin itself[27]. Two guaiacylglyceryl tricin isomers of molecular ion peak $[M-H]^-$ at *m/z* 525 that were defined as salcolin A and salcolin B[28]. in addition to their glycosides that displayed molecular ion peak $[M-H]^-$ at *m/z* 687, and tentatively identified as tricin 4′-*O*-(*erythro*-β-guaiacylglyceryl ether) glucoside and its *threo* isomer[29]. Another two isomers of tricin derivatives are detected at Rt 60.01 and 62.68 minutes of molecular ion peak $[M-H]^-$ at *m/z* 495 that was identified as hydroxyphenylglyceryl tricin derivatives; calquiquelignan D and calquiquelignan E[30]. Two characteristic fragments for tricin-7-hexoside sulphate appeared at *m/z* 491 $[M-H-80]^-$, due to the loss of sulphate group and at *m/z* 329 [M-H-80-162]-. Tricin-dihexoside was eluted at Rt 39.06 and displayed a molecular ion peak $[M-H]^-$ at *m/z* 637 as giving a fragment ion peaks at *m/z* 491 $[M-H-rhamnose]^-$ and *m/z* 329 $[M-H-\ rhamnose-hexose]^-$[28]. Also another signal of tricin derivatives appeared at Rt 71.98 and showed a molecular ion peak $[M-H]^-$ at *m/z* 671, this mass signal was tentatively identified as tricin-4′-*O*-(coumarylerythro-β-guiacylglycerol ether) glucoside, that give a molecular ion peak at *m/z* 525 due to loss of coumaric acid[31].

Evaluation of Antitumor activity

It was verified that an increase in the number of the OH ring substituents in the compounds investigated leads to higher antiproliferative and cytotoxic activities, triphenols were found to be more effective than diphenols. The length of the carbon chain between the aromatic ring and the terminal carboxylic group affects the anticancer activity differently according to each phenolic acid and each particular cell line.[32]. The anticancer effect of the aqueous ethanolic extract of *L. chinensis* leaves against PC3 and HEPG2 cancer cell lines was evaluated and expressed by median inhibitory growth concentration (IC_{50}) using doxorubicin as a standard (IC_{50} = 2.93 µg/mL for PC3 and IC_{50} = 4.85 µg /mL for HEPG2). The extract of *L. chinensis* showed IC_{50} = 40 µg/mL against PC3 as shown in **Figure 2**, on the other hand the extract showed lower activity towards HEPG2 with IC_{50} > 50 µg/mL as demonstrated in **Figure 3**. In conclusion the extract has more prominent anti-prostate cancer activity than its anti-liver cancer activity.

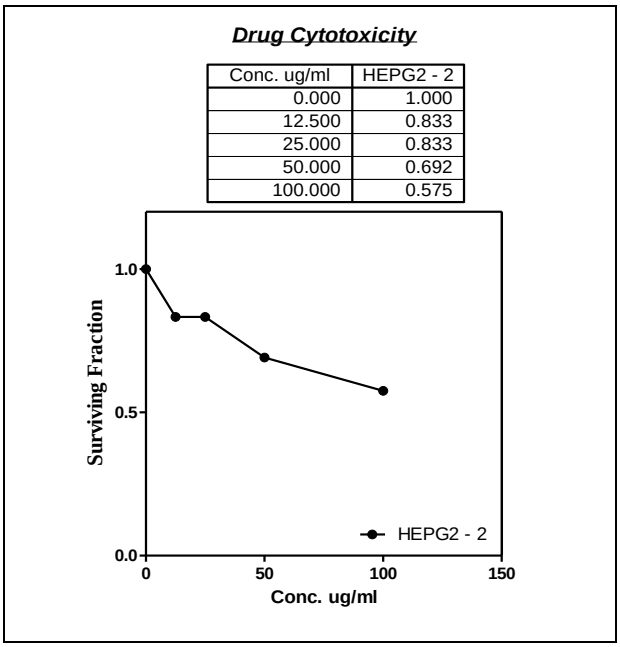

Figure 2. Antitumor activity of aqueous ethanolic extract of *L. chinensis* against surviving fraction of HEPG2 cancer cells.

Total phenolic and total flavonoid

The total phenolic content (TPC) was determined using Folin-Ciocalteu reagent (FCR) according to the method described by Singleton and Rossi, 1965, which depends on the reduction of (FCR) by phenols to a mixture of blue oxides that have a maximal absorption at 760 nm[15]. The content of total phenolic was estimated by reference to the standard calibration curve of gallic acid. The results were expressed as gallic acid equivalent (GAE)/ g dry weight of plants, and calculated as mean value ± SD (n=3). The total flavonoid content (TF) was determined spectrophotometerically by aluminum chloride method, using quercetin as a reference compound[16]. The total flavonoid was determined by reference to the calibration curve of standard quercetin. All determinations were carried out in three replications. The total phenolic as well as total flavonoid contents were chemically estimated in the alcoholic extract of the leaves of *L. chinensis*. The content of total phenolic in the extract was measured using FCR assay and is

expressed as gallic acid equivalent (GAE), while the total flavonoid was estimated using aluminum chloride method (expressed as quercetin equivalent, QE). Total flavonoid and total phenolic contents of *L. chinensis* leaves extract were found to be 139.51 ± 5.90 µg/mL and 112.07 ± 2.24 µg/mL, respectively.

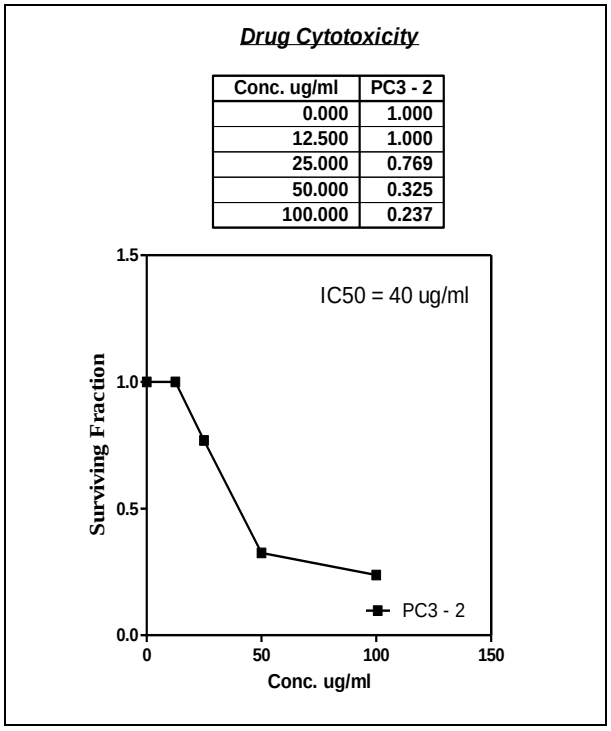

Figure 3. Antitumor activity of aqueous ethanolic extract of *L. chinensis* against surviving fraction of PC3 cancer cells.

CONCLUSION

From LC-MS structural analysis of *L. chinensis* leaf extract 42 secondary metabolites were tentatively identified, we concluded that the investigated plant is rich in phenolic compounds; *C*-glycoside derivatives of apigenin, luteolin and tricin together with phenolic acids. The contents of total flavonoid and phenolic were found to be 139.51 ± 5.90 µg/mL and 112.07 ± 2.24 µg/mL, respectively. 70% alcoholic extract of *L.chinensis* leaves has more prominent anti-prostate cancer activity than its anti-liver cancer activity. *L. chinensis* is a privileged source of *C*-flavonoids that revealed the efficiency of HPLC-MS metabolomics in natural products drug discovery.

Conflict of Interest

The authors declare that they do not have any conflict of interest.

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