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ORIGINAL ARTICLE

Chemical Composition and Biological Activities of the Aqueous Fraction of *Parkinsonea aculeata* L. Growing in Saudi Arabia

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C and *O*-flavone glycosides;
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UPLC-ESI-MS/MS

Abstract Polyphenolic constituents and chromatographic fingerprint of the aqueous fraction obtained from the ethanolic extract of the aerial parts of *Parkinsonea aculeata* L. growing in Saudi Arabia were investigated for the first time using UPLC-ESI/MS/MS in negative mode. Forty compounds were tentatively identified including sixteen *C*-flavone glycosides, twenty-two *O*-flavone glycosides, and two polymethoxylated flavonoids. Compounds identification was based on the MS/MS fragmentation and literature comparison. The aqueous fraction fingerprint is rich in *C*- and *O*-flavone glycosides, like apigenin-8-*C*-*β*-D-glucopyranoside (vitexin), vitexin 2″-*O*-rhamnoside, luteolin-8-*C*-glucoside (orientin), luteolin-8-*C*-*β*-D-glucopyranoside-7-*O*-rhamnoside and luteolin-7-*O*-rutinoside. These compounds were identified for the first time in the aqueous fraction of Saudi *P. aculeata* L. plant. Additionally, the antioxidant and anticancer activities were investigated. The aqueous fraction showed a strong DPPH scavenging activity with IC_{50} 48.3 ± 1.5 μg/mL compared to ascorbic acid 14.2 ± 0.5 μg/mL. However, this fraction showed a very weak cytotoxic activity against HepG-2 (Hepatocellular carcinoma) and MCF-7 (Breast carcinoma) with IC_{50} 222 ± 1.8 and 304 ± 9.2 μg/ml respectively compared to cisplatin IC_{50} 3.67 ± 8.1 and 5.71 ± 3.8 μg/ml respectively.
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1. Introduction

Parkinsonia aculeata L. family Fabaceae is a spinous shrub or a small tree with green or brown bark; bipinnate leaves; the flowers are yellow fragrant racemes. The fruit is pod containing several seeds (Divya et al., 2011). The chemical literature survey of *P. aculeata* showed the presence of flavonoidal aglycones, *C*-glycosides, flavanones (Besson et al., 1980; Divya et al., 2011; Nabil et al., 1991; Shaiq Ali et al., 2005; Sharma

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and Vig, 2014). Sterols, glycerols (Nabil et al., 1991; Rani et al., 1999), alkaloids, terpenoids, saponins, tannin and macro cyclic-monoterpene-*O*-glycosides (Al-Youssef and Hassan, 2015; Bhatia et al., 1966; Kamal and Mathur, 2007; Kamba and Hassan, 2010; Marzouk et al., 2013), carotenoids (Kamal and Mathur, 2007). Extracts of *P. aculeata* L. showed antibacterial (Bhatia et al., 1966; Kamba and Hassan, 2010; Marzouk et al., 2013), antioxidant and hepatoprotective activities (Hassan et al., 2008; Sharma and Vig, 2014). Few studies were performed on the aerial parts *P. aculeata* L. growing in Saudi Arabia. Al-Youssef and Hassan (2015), reported that, the essential oil of the aerial parts of *P. aculeata* L. growing in Saudi Arabia has antimicrobial activity. LC-MS-MS analysis of *P. aculeata* L. growing in Saudi Arabia has not been previously studied. Hence, the aims of the current study are, the characterization of the polyphenolic constituents of *P. aculeata* L. aqueous fraction using UPLC-ESI/MS/MS and, the assessment of the antioxidant and anticancer activities. Forty phenolic compounds were identified, for the first time in this plant. In addition, the DPPH scavenging and anticancer activities against HepG-2 (Hepatocellular carcinoma) and MCF-7 (Breast carcinoma) cell lines were investigated.

2. Materials and methods

2.1. Plant material

P. aculeata L. aerial parts were collected from Diriyah region, Riyadh city, Saudi Arabia in 2013. The plant was kindly identified by Prof Dr. Jakob Thomas; College of science, KSU, a voucher specimen was prepared and deposited at the herbarium in Department of Pharmacognosy, College of Pharmacy, King Saud University. *P. aculeata* aerial parts were air dried and grinding into coarse particles until use.

2.2. Preparation of extract

Ethanol 80% was used for the extraction of the air-dried powdered aerial parts of *P. aculeata* (800 g). The resulting ethanol extract (111 g) was dissolved in methanol water mixture (1:9, 300 ml) then partitioned with petroleum ether, dichloromethane and ethyl acetate to give 10.0 g, 4.0 g, 7.0 g and 53.0 g of petroleum ether, dichloromethane, ethyl acetate and aqueous fraction respectively.

2.3. LC/MS instrument and separation technique

The sample was analyzed by UPLC-ESI-MS/MS (Ultra performance liquid chromatography-electrospray ionization Mass/Mass) negative ion acquisition mode on a XEVO TQD triple quadrupole instrument. Waters Corporation, Milford, MA01757 U.S.A, mass spectrometer; column: ACQUITY UPLC - BEH C18 1.7 μm - 2.1 × 50 mm column. Solvent system: consists of HPLC analytical grade solvents (A) water containing 0.1% formic acid and (B) methanol containing 0.1% formic acid. The gradient was programmed as follows: 0 min, 10% B; 5 min, 30% B; 15 min, 70% B; 22 min, 90% B; 25 min, 90% B; 26 min, 100% B, 29 min, 100% B; 32 min, 10% B and finally, the initial conditions were held for 3 min as a re-equilibration step. The flow rate was 0.2 ml/ min, the sample at a concentration of 100 μg/ml was prepared in HPLC methanol then degassed and filtered using a membrane disc filter (0.2 μm) then subjected to LC-ESI-MS analysis, the sample injection volume was 10 μl.

The parameters for analysis were carried out using negative ion mode as follows: source temperature 150 °C, cone voltage 30 eV, capillary voltage 3 kV, desolvation temperature 440 °C, cone gas flow 50 l/h, and desolvation gas flow 900 l/h. Mass spectra were detected in the ESI negative ion mode between *m/z* 100–1000. The peaks and spectra were processed using the Maslynx 4.1 software and tentatively identified by comparing its retention time (R_t) and mass spectrum with reported data.

2.4. Biological activities

The biological activities were carried out at Regional Center for Mycology and Biotechnology (RCMB) at Al-Azhar University, Cairo, Egypt.

2.4.1. Antioxidant activity (Yen and Duh, 1994)

According to Yen and Duh (1994), the antioxidant activity of the aqueous fraction of the ethanolic extract of aerial parts of *P. aculeata* was determined at different concentration, 10, 20, 40, 80, 160 and 320 μg/ml methanol. The assay was performed by the DPPH (0.1 mM) solution 2, 2-diphenyl-1-picrylhydrazyl) free radical scavenging assay in triplicates and average values were considered. The percentage of DPPH radical scavenging was calculated according to the formula: % DPPH radical scavenging = (AC-AS)/AC × 100, Where **AC** is the absorbance of the control and **AS** is the absorbance of the sample in DPPH solution. SC_{50} (μg/ml) of both of fraction and the standard (ascorbic acid) were calculated and the results were shown in Figs. 5 and 6.

2.4.2. Antitumor activity (Al-Enazi et al., 2018; Kameyama et al., 2005)

The cell lines HepG-2 (Hepatocellular carcinoma) and MCF-7 (Breast carcinoma) were used for determination of antitumor activity of *P. aculeata* by the MTT assay and according to the method and statistics reported by Al-Enazi et al. (2018), Kameyama et al. (2005).

3. Results and discussion

The qualitative analysis using UPLC-ESI-MS/MS of the polyphenolic compounds of the aqueous fraction of the aerial parts of *p. aculeate* L., antioxidant and antitumor activities were evaluated.

3.1. Identification of P. aculeata aqueous fraction polyphenols by UPLC–ESI-MS/MS

Forty compounds were identified (Figs. 1 and 2) and listed with their spectral data, retention times, and MS/MS fragmentation in Table 1. Some of the identified compounds such as orientin, isoorientin, vitexin, isovitexin and vicenin-3 were coincident with those compounds already reported in *P. aculeata* (Divya et al., 2011). In case of *O*-flavone glycosides, the common losses of 132, 146 and 162 Dal indicated the loss of

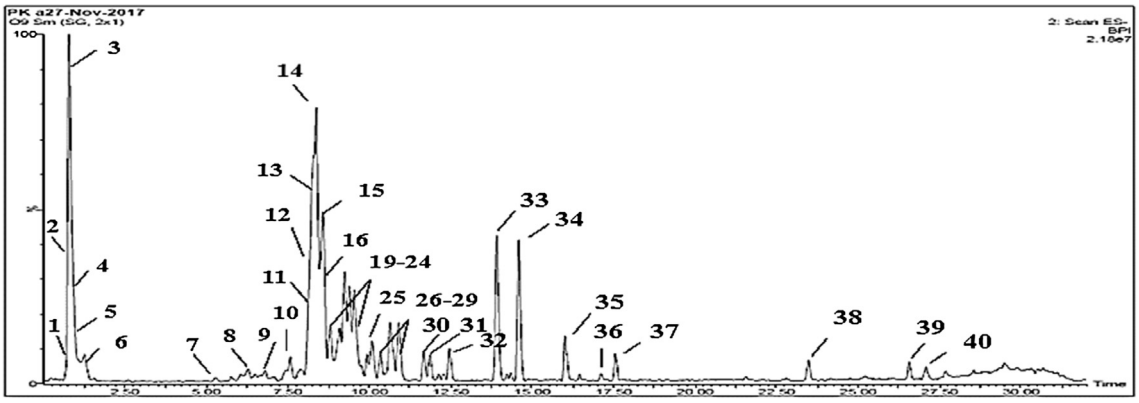


Fig. 1 Negative mode UPLC-ESI-MS/MS chromatogram of aqueous fraction of the ethanolic extract of *P. aculeata* L.

pentose (arabinose or xylose), rhamnose and hexose (glucose or galactose) respectively. Additionally, the loss of 308 or 294 Dal indicated the disaccharide structure rutinose/neohesperidose or pentosyl-hexoside respectively, other losses of 338 Dal represent the loss of either glucuronosyl-glucoside or feruloyl-glucoside (Benayad et al., 2014; Geng et al., 2016; Wojakowska et al., 2013). Furthermore, *O,C*-glycosides of flavones give characteristic fragment ions at [(M−H)-120], [(M−H)-90] and [(M−H-120)-162] or [(M−H-90)-146]. However, *C*-flavone glycosides (Figs. 3, 4) showed the key fragmentation ions at *m/z* [(M−H)-150 (^{0,2}X)], [(M−H)-120 (^{0,3}X)] [(M−H)-90 (^{0,4}X)], [(M−H)-60 (^{0,5}X)] with or without [(M−H)-18]. These peaks are characteristic for 6 and 8-*C*-hexoside respectively. *C*-pentoside characteristic fragments are [(M−H) - 90 (^{0,3}X)], [(M−H)-60 (^{0,4}X)], (Fig. 3). Moreover, the fragments showing [aglycone + 113] and [aglycone + 83] are characteristic for di-*C*-glycosides. While the fragment showing [aglycone + 41] is characteristic for mono-*C*-glycoside (Abu-Reidah et al., 2013; Brito et al., 2014; Figueirinha et al., 2008; Geng et al., 2016; Zhang et al., 2011).

3.1.1. Identification of flavone, flavonol-C-glycosides

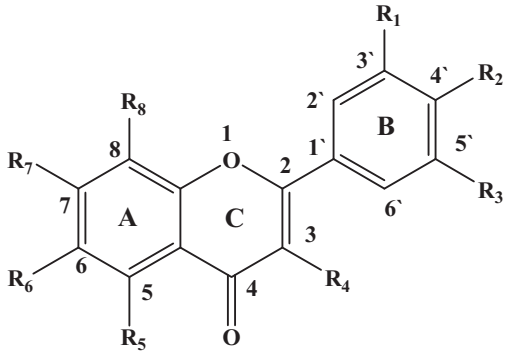
Sixteen *C*-flavonoidal glycoside (1–3, 5, 7, 8, 10, and 12, 14–16, 18–20 and 32–33) of apigenin, luteolin, kaempferol, chrysoeriol and diosmetin were identified in the aqueous fraction of *P. aculeata* L. To our knowledge apigenin, kaempferol, luteolin, chrysoeriol and diosmetin aglycones were reported in *P. aculeata* L. (Divya et al., 2011; Nabil et al., 1991).

- ***Characterization of Luteolin/kaempferol -C- glycosides:***

Peak 1 showed pseudo molecular ion peak at *m/z* 447 [M−H]. MS^2 showed fragment ions at *m/z* 357.4 [M−H-90], 326.9 (100%) [M−H-120] and 297.0 [M−H-150] corresponding to fragments $^{0,4}X_O$, $^{0,3}X_O$ and $^{0,2}X_O$ respectively which revealed the presence of hexose nature of *C*-glycoside flavone. Moreover, from the observed base peak fragment at *m/z* 326.9 (aglycone + 41) and short retention time at 0.81 it could be concluded that compound **1** was kaempferol -8-*C*- *β*-D-glucopyranoside (Brito et al., 2014; Geng et al., 2016). Similarly compounds **8**, **32 and 33** were assigned as luteolin-8-*C*-*β*-D-glucopyranoside (orientin), while compound **12** was identified as luteolin-6-*C*-*β*-D-glucopyranoside (isoorientin). To the best of our knowledge orientin and isoorientin were reported in *P. aculeata* L. (Divya et al., 2011; Sharma and Vig, 2014). Compound **14** (R_t 8.54 min), The ESI-MS^1 at *m/z* 593 [M−H] and MS^2 ion at *m/z* 473.4 [M−H-120] indicated the presence of mono- *C*- glucoside. Another fragment at *m/z* 326.7 [M−H -120–146 (rha)] showed loss of $^{0,3}X_O$ fragment and one rhamnose moity. From these results and based on the previous reports (Brito et al., 2014; Geng et al., 2016). Compound **14** was suggested to be luteolin-8-*C*-*β*-D-glucopyranoside-7-*O*-rhamnoside.

- ***Characterization of apigenin -C-glycosides:***

Compounds **7, 18** (R_t 5.72 and 9.22 mins) exhibited the same [M−H] ion at *m/z* 431, and the MS^2 spectra showed typical fragment ions of mono-*C*-glycosides as they exhibited fragment ions at *m/z* 310.7 and 311.0 (100%) corresponding to [M−H-120] respectively. In addition the MS^2 spectra showed no loss of water molecule which is characteristic for *C*-6- glycoside, so these compounds were identified as apigenin-8- *C*-*β*-D-glucopyranoside (vitexin) (Llorent-Martínez et al., 2015), to our knowledge vitexin, was previously isolated from *P. aculeata* L. (Divya et al., 2011; Sharma and Vig, 2014). Compound **19** at R_t 9.37 min and molecular ion peak at **577** [M−H], its MS^2 data exhibited a fragment ion at *m/z* 341.0 [(M−H)–90–146]$^-$ corresponding to the loss of rhamnose and 90 amu ($^{0,3}X_O$). Also the fragment ion at *m/z* 322.9 which displayed the loss of [90 + 146 + 18] which is characteristic for 6-*C*-glucoside -*O*- rhamnoside (Sakalem et al., 2012). From the previous studies and through comparison with the present study, compound **19** was identified as apigenin-6-*C*-hexoside 2″-rhamnoside (isovitexin-2′-rhamnoside) (Abu-Reidah et al., 2013). Similarly compound **15** was identified as vitexin-2″-rhamnoside from [M−H] at *m/z* 577 and MS^2 fragment ions at *m/z* 356.7 [M−H -120], 311.0 [M−H -120–146] (Ag + 41), 292.9 (100%) (Ferreres et al., 2007; Karar MGE, 2015; Slimestad, 2003). These compounds were previously isolated from *P. aculeata* (Divya et al., 2011). Based on previous literature (Benayad et al., 2014; Brito et al., 2014; Cao et al., 2014; Qiao et al., 2011), another apigenin derivatives at R_t of 0.92 and 9.13 mints were tentatively identified as apigenin 8-*C*-(-6″-*O*-feruloyl-)glucoside and apigenin-7-*O*-acetylglucoside-6-*C*-hexoside-8-*C*-pentoside for compounds **5** and **16** respectively. This is the first report about the presence of these compounds in *P. aculeata* L. Compound **10** with retention time of 7.58 min was tentatively



No.	R1	R2	R3	R4	R5	R6	R7	R8
1	H	OH	H	OH	OH	H	OH	*C*-glu
2	OH	O-CH3	H	H	OH	H	OH	*C*-glu
3	OH	O-CH3	H	H	OH	*C*-pent	OH	*C*-glu
5	H	OH	H	H	OH	H	OH	*C*-feruloyl-glucose
6	H	O-CH3	H	H	OH	H	*O*-glu-pent	H
7	H	OH	H	H	OH	H	OH	*C*-glu
8	OH	OH	H	H	OH	H	OH	*C*-glu
9	H	O-CH3	H	H	OH	H	*O*-glu-pent	H
10	H	OH	H	H	OH	*C*-glu	OH	*C*-glu
11	O-CH3	OH	H	H	OH	H	*O*-glu-pent	H
12	OH	OH	H	H	OH	*C*-glu	OH	H
13	H	OH	H	*O*-glu	OH	H	OH	H
14	OH	OH	H	H	OH	H	*O*-rha	*C*-glu
15	H	OH	H	H	OH	H	OH	*C*-glu-2``-rha
16	H	OH	H	H	OH	*C*-glu	*O*-acetylglu	*C*-pent
17	H	OH	H	*O*-dicoumaroul-glu	OH	H	OHH	
18	H	OH	H	H	OH	H	OH	*C*-glu
19	H	OH	H	H	OH	*C*-glu-2``-rha	OH	H
20	OCH3	OH	H	H	OH	H	OH	*C*-glu
22	OH	OH	OH	*O*-glu-glu A	OH	H	OH	H
23	OH	OH	OH	*O*-glu A	OH	H	OH	H
24	H	OH	H	*O*-rut	OH	H	*O*-rha	H
25	H	OH	H	*O*-rut	OH	H	H	H
26	H	OH	H	*O*-glu	OH	H	H	H
27	OCH3	OH	H	H	OH	H	*O*-neohesp	H
28	H	OCH3	H	H	OH	H	*O*-glu-glu	H
29	OCH3	OH	H	H	OH	H	*O*-glu	H
30	H	OH	H	*O*-rut	OH	H	H	H
31	H	OH	H	*O*-rha	H	H	OH	H
32	OH	OH	H	H	OH	H	OH	*C*-glu
33	OH	OH	H	H	OH	H	OH	*C*-glu
34	OCH3	OH	OCH3	H	OH	H	*O*-pent-pent	H
35	OCH3	OH	OCH3	H	OH	H	*O*-pent-pent	H
36	OCH3	*O*-pent	H	H	OH	H	*O*-rut.	H
37	OCH3	*O*-pent	H	H	OH	H	*O*-rut.	H

Fig. 2 Chemical structures of some identified compounds in the aqueous fraction of *P. aculeata* L. glu, glucose; neohesp.; neohesperidose; pent, pentose; rha, rhamnose; rut, rutinose.

Table 1 *O* and *C*- Flavonoidal glycosides identified in the aqueous fraction of the ethanolic extract of the aerial parts of *P. aculeata* L. by using UPLC–DAD/ESI-MS in negative ionization mode.

No.	Tentative assignment	Molecular Formula	R_t (min)	[M−H]	MS/MS	Reference
1	Kaempferol-8-*C*-*β*-D-glucopyranoside	$C_{21}H_{20}O_{11}$	0.81	447	357.4 [M−H-90], 326.9 (100%) [M−H-120], 297.0 [M−H-150], 299.1	Brito et al. (2014)
2	Diosmetin -8-*C*-*β*-D-glucopyranoside	$C_{22}H_{22}O_{11}$	0.81	461	340.9 [M−H-120], 312.5, 241.1 (100%) 215.7, 136.4	Brito et al. (2014), Geng et al. (2016)
3	Diosmetin 8-*C*-glucoside-6-*C*-pentoside	$C_{27}H_{29}O_{15}$ $C_{21}H_{20}O_{11}$	0.87	593	473.0 [M−H-120], 412.9 [M−H-120-60], 338.0, 325.5, 298.2, 297.0 (100%)	Benayad et al. (2014)
4	Diosmetin-*O*-acetyl glucopyranoside - malonyl glucopyranoside	$C_{33}H_{36}O_{20}$	0.87	751	298.7 (100%) [(M−H) -204–248]	Kajdžanoska et al. (2010)
5	Apigenin 8-*C*-(-6″-*O*-feruloyl)-glucopyranoside	$C_{31}H_{28}O_{13}$	0.92	607	311.4 [Ag + 41] (100%) [M−296 (−176 to 120)	Qiao et al. (2011)
6	Methyl apigenin-7-arabinosyl (1‴-6″)-*β*-D-glucopyranoside	$C_{27}H_{30}O_{14}$	1.43	577	293.3 (100%), [M−H-284]	Al-Shammari et al. (2015)
7	Apigenin-8-*C*-glucoside (vitexin)	$C_{21}H_{20}O_{10}$	5.72	431	310.7 (100%) [M−H-120], 282.8	Llorent-Martínez et al. (2015)
8	Luteolin-8-*C*-*β*-D-glucopyranoside (orientin)	$C_{21}H_{20}O_{11}$	6.67	447	356.9 [M−H-90], 326.6 (100%) [M−H-120], 296.9 [M−H-150]	Karar MGE (2015)
9	Methyl apigenin-7-*O*–arabinosyl (1‴-6″)-*β*-D-glucopyranoside	$C_{27}H_{30}O_{14}$	7.13	577	293.3 (100%) [M−H-284 (aglycone)]	Al-Shammari et al. (2015)
10	Apigenin 6,8-di-*C*-glucoside (vicenin 2)	$C_{27}H_{30}O_{15}$	7.58	593	502.7 [M−H-90], 412.6 [M−H-90-90], 395 [M−H-180-18], 382.8 (Ag + 113) (100%) [M−H-90-120], 353.1 [M−H-120-120], 284	Brito et al. (2014)
11	Diosmetin -7 -*O*-arabinosyl (1‴-6″) -*β*-D-glucopyranoside	$C_{27}H_{30}O_{15}$	8.18	593	299.1 [M−H-294]	Al-Shammari et al. (2015)
12	Luteolin-6-*C*-*β*-D-glucopyranoside (isoorientin)	$C_{21}H_{20}O_{11}$	8.27	447	357.2 [M−H-90], 326.9 (100%) [M−H-120], 339 [M−H-90-18], 297.2 [M−H-150]	Divya et al. (2011), Karar MGE (2015)
13	kaempferol -7-*O*-glucorunide	$C_{21}H_{18}O_{12}$	8.48	461	285.0 (100%) [M−H−gluA]	Karar MGE (2015), Lin and Harnly (2010)
14	luteolin-8-*C*-*β*-D-glucopyranoside-7-*O*-rhamnoside	$C_{27}H_{30}O_{15}$	8.54	593	473.4 [M−H-120], 326.9 [M−H-120-146 (rha)], 297.9 (100%)	Wojakowska et al. (2013)
15	Vitexin 2″-*O*-rhamnoside	$C_{27}H_{30}O_{14}$	8.89	577	356.7 [M−H-120], 311.0 [M−H-120-146], 292.9 (100%)	Ferreres et al. (2007), Slimestad (2003)
16	Apigenin-7-*O*-acetylglucoside-6-C-hexoside-8-*C*-pentoside	$C_{34}H_{40}O_{20}$	9.13	767	503.1 [M−H-264 (60 + 204], 485.0 [M−H-264-18], 442.0 [503–60], 413.1 [503.0–90], 383.0 (100%) [503.0–120], 383.0 [ag + 113]	Benayad et al. (2014), Brito et al. (2014), Cao et al. (2014)
17	kaempferol -3-*O*- (3″, 6″-dicoumaroyl glucoside)	$C_{39}H_{32}O_{15}$	9.13	739	285.0 [M−H-454 (162–146-146)], 283.8 (100%), 254.3, 227.1,	Slimestad (2003)
18	Apigenin-8-*C*-*β*-D-glucopyranoside (vitexin)	$C_{21}H_{20}O_{10}$	9.22	431	310.9 [M−H-120], 283.0 (100%), 173.3, 148.9	Divya et al. (2011), Karar MGE (2015)
19	Isovitexin 2″-*O*-rhamnoside	$C_{27}H_{30}O_{14}$	9.37	577	341.0 [M−H-90-146]. 322.9 [M−H-90-146-18], 292.9 (100%)	Karar MGE (2015), Slimestad (2003)
20	Chrysoeriol 8-*C*-glucoside (Scoparin)	$C_{22}H_{22}O_{11}$	9.37	461	341 [M−H-120], 311.0 [M−H-150], 297.8 (100%)	Brito et al. (2014)
21	Pinocembrin -7-*O*-feruloyl glucoside	$C_{31}H_{30}O_{12}$	9.44	593	255.2 (100%) [M−H-338 (162 + 176)]	Marczak et al. (2016), Simirgiotis et al. (2015)
22	Myricetin-*3*- *O*-*β*-D-glucoside-Glucuronide	$C_{27}H_{28}O_{19}$	9.68	655	317.1 [M−H-338 (162 + 176)], 61.7 (100%)	Marczak et al. (2016)

(*continued on next page*)

Table 1 (*continued*)

No.	Tentative assignment	Molecular Formula	R_t (min)	[M−H]	MS/MS	Reference
23	Myricetin -3-*O*-glucuronide	$C_{21}H_{18}O_{14}$	9.78	493	317.0 (100%) [M−H-H-176]	Abu-Reidah et al. (2015), Barbosa et al. (2006)
24	kaempferol-3- *O*-rutinoside -7-O-rhamnoside	$C_{33}H_{40}O_{19}$	10.44	739	307.4 (100%) [M−H-432, 285.0	Koike et al. (2015)
25	Luteolin -7-*O*-rutinoside	$C_{27}H_{30}O_{15}$	10.50	593	285.3 (100%) [M−H-rut], 283.6	Llorent-Martínez et al. (2015), Oniszczuk et al. (2016)
26	kaempferol -7-*O*-*β*-D-glucopyranoside	$C_{21}H_{20}O_{11}$	10.58	447	284.9 (100%) [M−H-162], 150.7	Downey and Rochfort (2008), Kajdžanoska et al. (2010), Omezzine and Haouala (2017)
27	Chrysoeriol 7-*O*-neohesperidoside	$C_{28}H_{32}O_{15}$	10.74	607	299.0 [M−H-308], 283.8 [M−H-308-CH_3]	Brito et al. (2014)
28	Acacetin 7-*O*-diglucoside	$C_{28}H_{32}O_{15}$	10.79	607	283.0 (100%) [M−H-324]	Lin and Harnly, 2010
29	Chrysoeriol-*O*-*β*-D-glucopyranoside	$C_{22}H_{22}O_{11}$	10.83	461	299.1 [M−H-glu], 297.0 (100%), 284.0	Plazonić et al. (2009)
30	Luteolin-7-*O*-rutinoside	$C_{27}H_{30}O_{15}$	11.55	593	285.0 (100%) [M−H-rut]	Brito et al. (2014), Llorent-Martínez et al. (2015)
31	Kaempferol-3-*O*-rhamnoside	$C_{21}H_{19}O_{10}$	11.68	431	285.0 (100%), 254.5, 252.8, 169.0	Slimestad (2003)
32	Luteolin-8-*C*-glucoside (orientin)	$C_{21}H_{20}O_{11}$	12.50	447	327.2 [M−H-120], 284.8 (100%)	Llorent-Martínez et al. (2015), Negri et al. (2012)
33	Luteolin -8-C-glucoside (orientin)	$C_{21}H_{20}O_{11}$	13.78	447	327.1 [M−H-120], 149.1, 131.0, 125.5, 112.7	Llorent-Martínez et al. (2015), Negri et al. (2012)
34	Tricin -7-*O*-dipentoside	$C_{27}H_{30}O_{15}$	14.04	593	329 [M−H-264], 163.1, 118.1 (100%), 89.1	Goławska et al. (2012)
35	Tricin -7-*O*-dipentoside	$C_{27}H_{30}O_{15}$	14.43	593	329.2 (100%), 263.0, 247.0, 165.0, 163.0, 145.0, 118.6, 101.0, 85.0, 59.0	Goławska et al. (2012)
36	Chrysieriol 4′-*O*-pentoside-7′ *O*-rutinoside	$C_{33}H_{40}O_{19}$	16.10	739	461.0, 308. 3 (100%) [M−H-431], 299.2	Brito et al. (2014), Plazonić et al. (2009)
37	Tetrahydroxy trimethoxy dihydroflavone	$C_{18}H_{18}O_9$	17.60	377	303.0 [100%, M−H-74 (2OCH_3 + CH_3)]	Zhang et al. (2011)
38	Trimethoxy tetrahydroxy dihydroflavone	$C_{18}H_{18}O_9$	23.54	377	270.7 [100%, M−H-106 (M−3OCH_3 –OH)]	Zhang et al. (2011)
39	Naringenin -7-*O*-acetyl-glucopyranoside	$C_{23}H_{24}O_{11}$	26.58	475	203.3 (100%) [M−H-272]	Brito et al. (2014)
40	Luteolin 7, 4′ dimethyl ether-*O*- *β*-D-glucopyranoside	$C_{23}H_{24}O_{11}$	27.10	475	313.0 (100%) [M−H-162]	Nabil et al. (1991), Wang et al. (2008), Zhang et al. (2011)

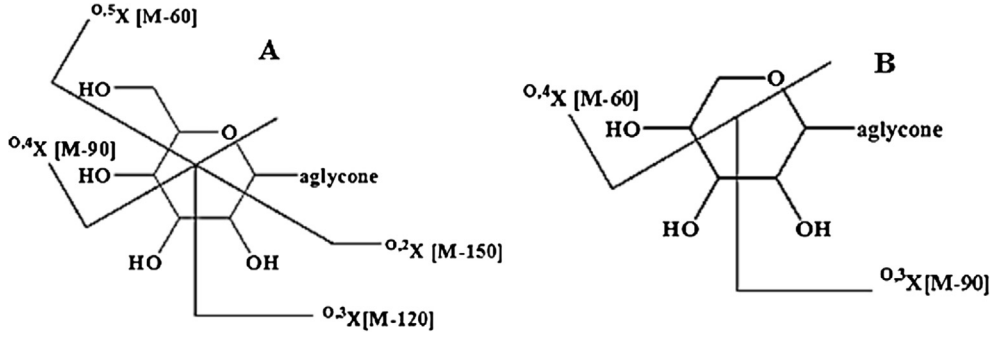


Fig. 3 Fragmentation pattern of *C*-hexose **(A)** and *C*- pentose **(B)**.

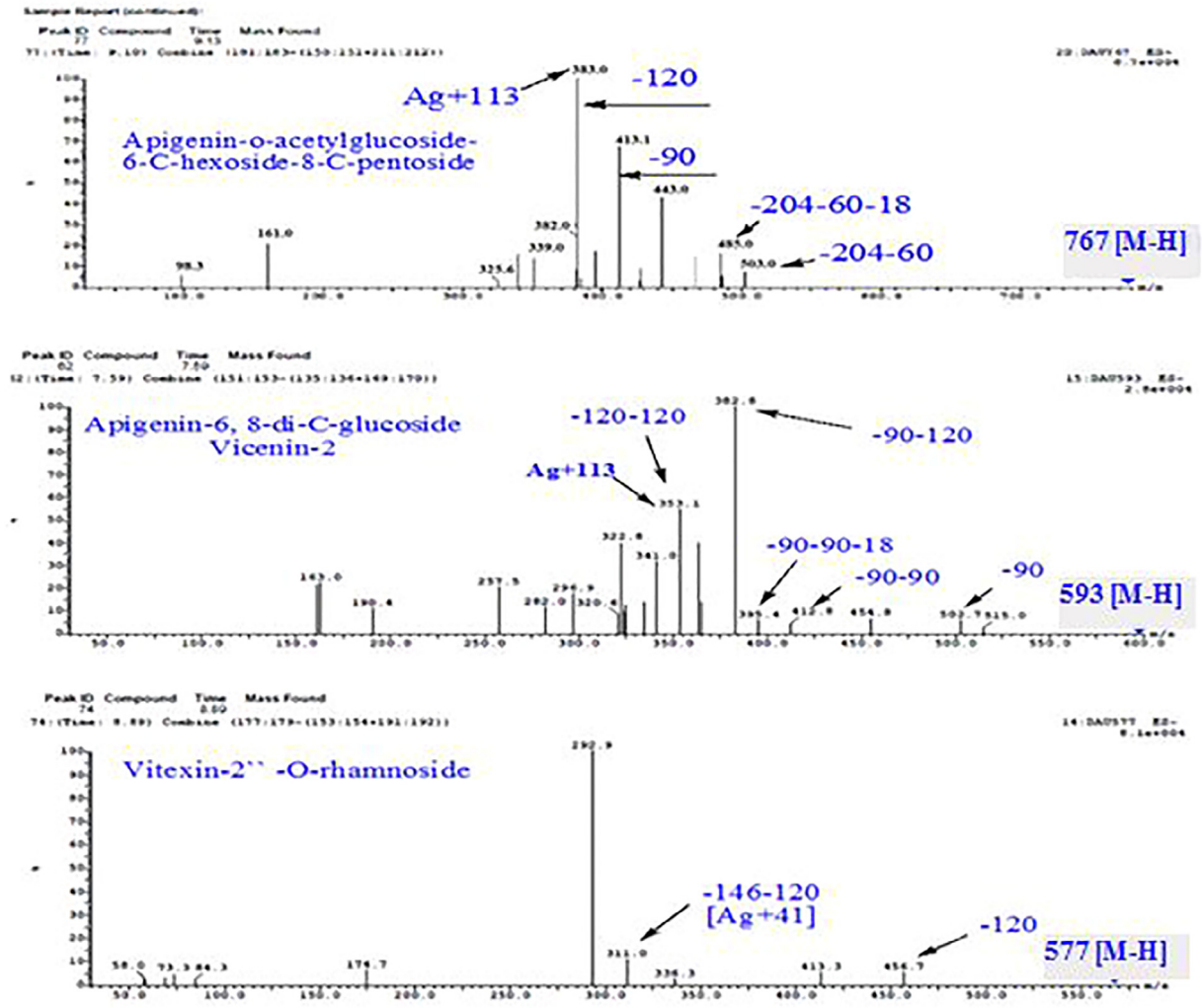


Fig. 4 Negative mode ESI-MS/MS spectra of some flavone -*C*-glycoside.

identified as apigenin 6,8-di-*C*-glucosides (vicenin 2) (Brito et al., 2014).

- ***Characterization of chrysoeriol and diosmetin -C-glycosides:***

Peak at R_t of 0.87 min showed two components with the molecular ion peaks at *m/z* 461 and 593 [M−H] respectively. The MS^2 spectrum of the first component produced fragment ion at *m/z* 340.9 [M−H -120]. This fragment is equal to (aglycone + 41) which is characteristic for mono-*C*- glycoside of aglycone with *m/z* at 300 amu (diosmetin). These results suggest that, this component (compound **2**) was diosmetin -8-*C*-*β*-D-glucopyranoside (Brito et al., 2014; Gattuso et al., 2007), while the second component (compound **3**) MS^2 displayed fragment ion at *m/z* 473.0 [M−H-120], indicating the presence of 8-*C*- glucoside. The characteristic fragment ion at *m/z* 412.9 [M−H-120-60] gave the loss of 60 amu for *C*-pentose, this fragment represents aglycone plus 113 and indicates the presence of di-*C*-glycoside with diosmetin aglycone (Benayad et al., 2014; Geng et al., 2016). According to the previous studies, compound **3** was suggested to be diosmetin -8-*C*-hexoside -6-*C* pentoside (Brito et al., 2014; Cao et al., 2014) and compound **20** was identified as chrysoeriol 8-*C*-glucoside (scoparin) (Brito et al., 2014).

3.1.2. Identification of flavone, flavonol-O-glycosides

The LC-MS analyses of aqueous fraction obtained from the ethanolic extract of the aerial parts of *P. aculeata* L. revealed the presence of twenty-two flavonoid-*O*- glycosides.

- ***Characterization of luteolin/kaempferol -O-glycosides:***

Compounds **17** and **24** (R_t 9.13 and 10.44 min) with the same [M−H]- at *m/z* 739 were tentatively identified as kaempferol -3-*O*- (3″, 6″-dicoumaroyl glucoside) and kaempferol-3-*O*-rutinoside-7-*O*-rhamnoside respectively. Based on the ESI-mass data in Table 1 and the one step neutral loss of (146 + 146 + 162 amu) for two coumaroyl and one glucose (compound **17**) and two losses of 308 amu and 146 amu (compound **24**) leaving fragment ion at *m/z* 285.0 kaempferol aglycone (Lin and Harnly, 2010). Compound **13** (R_t 8.48 min) with [M−H]- at *m/z* 461 was tentatively identified as kampferol 3-*O*-glucuronide from ESI- mass data in (Table 1) and the neutral losses of 176 amu for glucouronyl moiety (Lin and Harnly, 2010). By the same manner compounds **25, 30** and **26** (R_t 10.50, 11.55 and 10.58 mins) were identified as luteolin -7-*O*-rutinoside (**25** and **30**) (Llorent-Martínez et al., 2015; Omezzine and Haouala, 2017) and kaempferol-3-*O*-*β*-D-

glucopyranoside (**26**) (Llorent-Martínez et al., 2015; Omezzine and Haouala, 2017). Kaempferol-3-*O*-rhamnoside (compound **31**) was tentatively identified from The MS profile of peak at retention time of 11.55 min with [M−H] at *m/z* 431 and MS^2 base peak fragment ion at *m/z* 285.0 which gave the loss of 146 Da (rhamnose moiety) (Downey and Rochfort, 2008; Kajdžanoska et al., 2010; Plazonić et al., 2009). In addition compound **40** was identified as luteolin 7, 4′ dimethyl ether-*O*- *β*-D-glucopyranoside. It is worth mentioning that luteolin 7, 4′ dimethyl ether-*6*-*C*-glucopyranoside was previously isolated from *P. aculeata* L which confirmed the presence of luteolin 7, 4′ dimethyl ether as aglycone in compound **40** (Nabil et al., 1991).

- ***Characterization of chrysoeriol and diosmetin -O-glycosides:***

Compound 4 with a $[M-H]^-$ ion at *m/z* 751 was tentatively identified as diosmetin-7-*O*-acetyl hexoside -malonyl hexoside (Table 1). The base peak fragment ion at *m/z* 298.7 [M−H-204-248] showed the loss of (acetylglucose and malonyl glucoside) (Brito et al., 2014; Kajdžanoska et al., 2010). Diosmetin -7-arabinosyl (1‴- 6″)-*β*-D-glucopyranoside was proposed for compound **11,** R_t 8.18 min, (*m/z* 593 [M−H]). In the MS/MS spectra, the loss of pentosyl-hexoside (294 Da) gave base peak fragment ions at *m/z* 299.1 (diosmetin) (Al-Shammari et al., 2015). Compound **27** with R_t 10.74 min, molecular ions at *m/z* 607 and fragments at *m/z* 299.0 and 283.8, suggesting that it might be chrysoeriol 7-*O*-neohesperidoside (Brito et al., 2014; Plazonić et al., 2009). Compound **29** shows a molecular ion at *m/z* 461 $[M-H]^-$ (Table 1). The MS data showed fragment signal at *m/z*: 299.1 [(M−H-162) which indicate glucose moiety, this result shows that compound **29** could be chrysoeriol-7-*O*- glucopyranoside (Plazonić et al., 2009). Compound **36** with R_t 16.10 min, molecular ion peak at *m/z* 739 [M−H] and MS/MS fragments at *m/z* 461.0 [M−H-132-146], showed the loss of rhamnose and pentose moieties, while the base peak fragment ion at *m/z* 308. 3 [M−H-431] show the presence of rutinose as sugar moiety and leaving chrysoeriol-pentoside, the MS^2 showed another fragment at *m/z* 299.2 corresponding to chrysoeriol aglycone. From these results compound **36** was tentatively identified as Chrysieriol 4′-*O*-pentoside-7′-*O*-rutinoside (Brito et al., 2014; Plazonić et al., 2009).

- ***Characterization of methyl apigenin -O-glycosides***

Peaks at 1.43 and 7.13 min (compounds **6** and **9**) (Table 1), gave the $[M-H]^-$ ion at *m/z* 577. The main fragmentation step of this compound in MS/MS spectrum yielded one type of ion with 100% abundant [M−H − aglycone (284)]$^-$ ions at *m/z* 293.3 which showed the loss of 284 amu which corresponding to methyl apigenin as an aglycone (Marczak et al., 2010). The fragment at *m/z* 293.3 [162 + 132-H] displayed the presence of hexose and pentose moieties interlinkage with each other as reported by Al-Shammari et al. (2015), so compounds **6** and **9** were tentatively characterized as Methyl apigenin-7-*O*-arabinosyl (1‴-6″)-*β*-D-glucopyranoside. Compound **28** with molecular ion peak at *m/z* 607 was characterised as acacetin-7-*O*-di glucoside based on the MS^2 base peak fragment ion at *m/z* 283.0 which showed the loss of two hexose moieties (Lin and Harnly, 2010) To the best of our knowledge, this is the first characterization of these compounds in *P. aculeata*. L.

- ***Characterization of myricetin –O-glycosides*:**

Compounds **22** (R_t 9.68 min) described as myricetin-*3*-*O*-*β*-D- glucuronyl-glucoside (Marczak et al., 2016). Depending on the data obtained by MS^1 molecular ion peak at *m/z* 655 and MS^2 fragment ion at *m/z* 317.1 [M−H-162-176] indicating the loss of glucose and glucoronyl moieties. Compound **23** (R_t 9.78 min) was suggested to be myricetin-3-*O*-glucuronide, As its ESI-MS spectrum displayed MS^1 at *m/z* 493 [M−H] in addition, the MS^2 data showed base peak fragment at *m/z* 317 [M−H -176] corresponding to the loss of glucoronyl moiety (Abu-Reidah et al., 2015). To the best of our knowledge, this is the first characterization of compounds **22** and **23** in *P. aculeata* L.

- ***Characterization of pinocembrin –O-glycosides:***

Compound **21** R_t 9.44 min exhibited a molecular ion peak [M−H] at 593, we observed characteristic losses of 338 Da for feruloyl glucoside (Marczak et al., 2016). The aglycone fragment at 255.2 [aglycone – H] is characteristic for dihydroxyflavone (pinocembrin). From the previous results compound **21** was tentatively identified as pinocembrin -7-*O*- feruloyl glucoside (Simirgiotis et al., 2015).

- ***Characterization of tricin –O-glycosides:***

Tricin-7-*O*-dipentoside (compound **34** and **35**) is proposed to peak (R_t 14.04 and 14.43 min, *m/z* 593, [M−H]). In the MS/MS spectra, the loss of dipentose (264 Da) moieties gave a fragment ion at *m/z* 329.0 which corresponds to tricin in structure (Goławska et al., 2012).

- ***Characterization of naringenin -O-glycoside:***

Compound **39** R_t 26.58 min exhibited a molecular ion peak [M−H] at 475, we observed characteristic losses of 272 Da for an aglycone (naringenin) leaving a base peak fragment at *m/z* 203.3 which is a characteristic fragment for acetyl hexoside [acetyl glucoside -H]. From this result compound **39** could be naringenin-7-*O*- acetyl glucopyranoside (Simirgiotis et al., 2015). This is the first report in *P. aculeata* L.

- ***Characterization of polymethoxylated compounds:***

Compound **37** R_t 17.60 min showed the precursor ion at *m/z* 377 [M−H]. The compound had been already identified as tetrahydroxy trimethoxy dihydroflavone (may be dihydroquercetin derivative) from the MS^2 base peak fragment at *m/z* 303.0 [100%, M−H -74 ($2OCH_3$ + CH_3)] (Zhang et al., 2011) Similarly compound **38** with R_t 23.54 min with the same molecular ion peak was tentatively identified as an isomer of trimethoxy tetrahydroxy dihydroflavone from the MS^2 base peak fragment at 270.7 [100%, M−H -106 ($M-3OCH_3$ – OH)] (may be naringenin derivative) (Zhang et al., 2011).

3.2. Biological activity results

3.2.1. Antioxidant activity

The DPPH scavenging potential of the aqueous *P. aculeata* L. aqueous fraction showed significant antioxidant activity with IC_{50} = 48.3 ± 1.5 μg/mL compared to ascorbic acid 14.2 ± 0.5 μg/mL. These results support the previous literature about

Table 2 IC_{50} of Pk a fraction against Hep-G2 and MCF-7 carcinoma cell lines.

Tested fraction		
Cell line	IC_{50} (μg/ml)	
	Pk a	Cisplatin
HepG-2 (Hepatocellular carcinoma)	222 ± 1.8	3.67 ± 8.1
MCF-7 (Breast carcinoma)	304 ± 9.2	5.71 ± 3.8

These are the mean of three determinations.

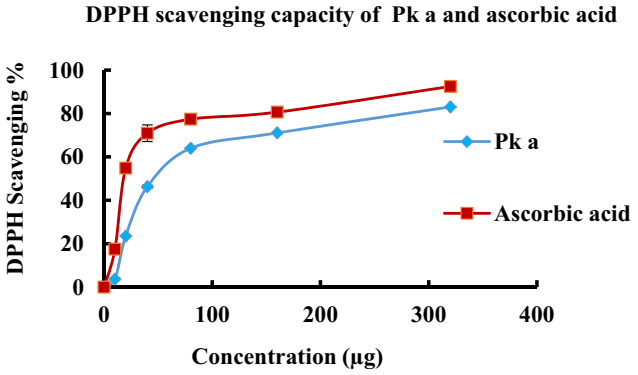


Fig. 5 DPPH scavenging capacity of *P. aculeata* L. aqueous fraction (PK a) and ascorbic acid.

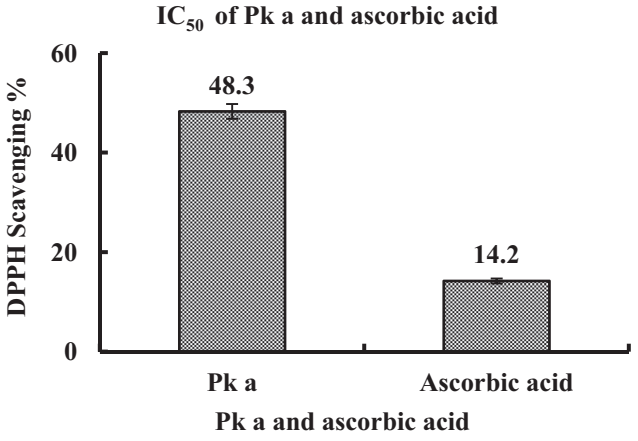


Fig. 6 IC_{50} of *P. aculeata* L. aqueous fraction (PK a) and ascorbic acid.

the antioxidant potential of *P. aculeata* L. and confirmed its ethnomedical use in the treatment of jaundice (Mruthunjaya and Hukkeri, 2008).

3.2.2. Antitumor activity

The antitumor activity of *P. aculeata* L. aqueous fraction was investigated against **HepG-2 (Hepatocellular carcinoma)** and **MCF-7 (Breast carcinoma)** cell lines using **Cisplatin** as a positive standard. NCI screening showed that, the crude extract and the pure compounds are cytotoxic with IC_{50} less than

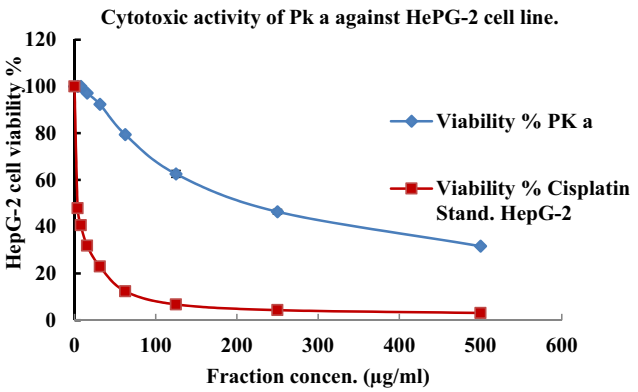


Fig. 7 Cytotoxic activity of *P. aculeata* L. aqueous fraction (PK a) against Hep-G2 cell line.

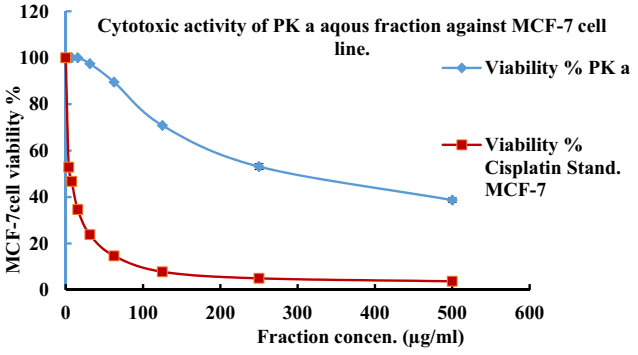


Fig. 8 Cytotoxic activity of *P. aculeata* L. aqueous fraction (PK a) against MCF-7 cell line.

20 μg/ml and less than 4 μg/ml respectively (Boik, 2001). Unfortunately the aqueous fraction of *P. aculeata* L. exhibited a very weak cytotoxic activity against **HepG-2** and **MCF-7** cell lines with IC_{50} of **222 ± 1.8 and 304 ± 9.2** μg/ml respectively, when compared to cisplatin **3.67 ± 8.1** and **5.71 ± 3.8** μg/mL (Figs. 7, 8 and Table 2).

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