Isolation and characterization of anti-tumor compounds from ethyl acetate extract of *Rumex japonicus* houtt roots and their cytotoxic effects

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Abstract

To isolate and characterize anti-tumor compounds through nuclear magnetic resonance (NMR) spectroscopy of ethyl acetate extract of *R. japonicus* Houtt root and their cytotoxic effects were evaluated in terms of measuring inhibition of growth of three cancer cell lines: human hepatoma cells (HepG2), cerebral glioma cells (U251) and ovarian carcinoma cells (SK-OV-3/DDP). The roots of *R. japonicus* Houtt were extracted with ethanol, and partitioned with petroleum ether, dichloromethane and ethyl acetate, respectively. The ethyl acetate extract was separated chromatographically using macroporous resin, positive and negative phase silica gel and high performance liquid chromatography. The structures of isolated compounds were elucidated by nuclear magnetic resonance (NMR) spectral data. Ten compounds were isolated form the ethyl acetate extract of *R. japonicus* roots. Their structures were determined as emodin (1), chrysophanol (2), aloe emodin (3), physcion (4), quercetin (5), resveratrol (6), β-sitosterol (7), 3β-acetoxy-28-hydroxyurs-12-ene (8), sucrose (9) and dibutyl phthalate (10). Among of them, compounds 1, 3, 4, 6 and 8 exhibited highly significant ($p<0.05$) cytotoxicities against HepG2 to a certain extent. Compounds 3-6 exhibited moderate cytotoxicities against U251 with IC_{50} values of 19.8, 23.5, 23.8 and 17.8 μM, respectively. Compounds 1, 2 and 7 also exhibited cytotoxicities against U251 to a certain extent with IC_{50} values of 48.3, 50.1 and 85.3 μM, respectively. Compounds 1-6 exhibited moderate cytotoxicities against (SK-OV-3/DDP) with IC_{50} values of 62.8, 64.2, 31.3, 61.0, 47.4 and 42.0 μM, respectively.

Keywords: *Rumex japonicus* Houtt; ethyl acetate extract; chemical constituents; anti-tumor activity; NMR; characterization.

Practical Application: Ethyl acetate extract of R. japonicus Houtt root Isoltaion, NMR charcatrization and cytotoxic effects were evaluated on three cancer cell lines (HepG2, U251, SK-OV-3/DDP).

1 Introduction

Since prehistoric times, human beings have been using plants as folkloric sources of phytoconstituents of medicinal and pharmaceutical significance. In modern medicine age, single-compound based purified drugs have been emerged with promising potential as anti-tumor agents (Jenks & Smigel, 1995). Plant-derived chemical compounds and their semi-synthetic and synthetic analogues are in use by pharmaceutical and oncology researchers. Since, 1961, United Stated Food and Drug Administration (FDA) has approved plant-derived anti-cancer and anti-tumor chemicals to prepare anti-cancer drugs (Lee, 1999). As perennial herb, *Rumex japonicus* Houtt belongs to the Polygonaceae family and is known in Traditional Chinese Medicine (TCM) as *Yang-Ti*. It is widely distributed in Eastern China, Central China, Southern China, Korea and Japan (Xie & Yang, 2014). *Rumex japonicus* Houtt root has medicinal significance and in folklore and alternate medicines, it is employed for various medicinal benefits like as a antitumor, anti-inflammatory, purgative and detox agent and as an alternate medicine for skin treatment, scabies, regulation of blood circulation and wastes clearance out of body (Lee et al., 2006; Xie & Yang, 2014). Moreover, *R. japonicus* Houtt comprises of rich quantities of several phytoconstituents of pharmaceutical significance, such as oxanthrones, anthraquinones, nepodin, rutin and phenolic compounds in its roots (Zhou et al., 2005).

Cancer is one of the main chronic diseases that affect the health of people worldwide including China. Globally, cancer is categorized as the second leading death cause next to cardiovascular diseases. Lung cancer is one of the primary cancer types as cause of death in developing countries and nearly 85% cases of lung cancer are reported because of smoking as the main factor. As far as the mortality rate of females and males is concerned, colon cancer is the second most widely known death cause. (Thakurta et al., 2015). Colon cancer affects almost one million people every year worldwide. On the other hand, each year about 10,000 cases of malignant gliomas are reported in United States only (Alwarsamy & Ravichandran, 2011).

The treatment and prevention of cancer has always been a difficult problem for researchers all over the world (Lichota & Gwozdzinski, 2018). If not effectively controlled, metastatic cancer cell will cause tissue failure and necrosis, thus threatening life (Steenkamp & Gouws, 2006). Adjunct therapies including cisplatin, interferon alpha epirubicin and doxorubicin are commonly used to overcome abnormal growth of cancer

cells, however, these chemotherapy agents are ineffective against resistance mechanism of cancer cells owing to up-regulation of multi-drug resistant proteins (MDR) and lower levels of apoptotic proteins. Therefore, more effective antitumor and tumor suppressions chemotherapy agents are needed to effectively overcome apoptotic induction for successfully treating cancer (Machana et al., 2012).

At present, there are many studies available on inhibitory and therapeutic effects of TCM on cancer cells. Therefore, pure plant extracts are of great significance for cancer treatment as natural alternative of synthetic and semi-synthetic chemotherapy agents. Until now, there have been few systematic studies on in-vitro anti-tumor effects of *R. japonicus* Houtt root extracts (He et al., 2016; Xie & Yang, 2014; Yang et al., 2019).

Therefore, the objective of this study was to isolate and characterize anti-tumor compounds through nuclear magnetic resonance (NMR) spectroscopy of ethyl acetate extract of *R. japonicus* Houtt root and their cytotoxic effects were evaluated in terms of measuring inhibition of growth of three cancer cell lines (HepG2, U251, SK-OV-3/DDP).

2 Materials and methods

2.1 Materials

Roots of *Rumex japonicus* Houtt were collected in nearby mountain area of Jilin City, Jilin province, China. After collection, the roots of *Rumex japonicus* Houtt were subjected to drying in conjunction with active ventilation at normal room temperature of 25 °C. Then, polyethylene bags were utilized to pack the dried *R. japonicus* roots followed by -80 °C prior to further use.

2.2 Extraction and isolation of *Rumex japonicus* compounds

Dried roots were amounted to 10 kg after drying. Dried *R. japonicus* Houtt roots were subjected to ethanol (95%) extraction separately extracted and total extraction time was 24 h. After extraction time completion, the root extracts were filtered using Whatman' filter paper No. 2 (Whatman Int. Ltd., Maidstone, England), and concentrated in vacuum at 40 °C. Concentrates (546.8 g) were then suspended with distilled water and petroleum ether (4 L, 3 times) (78.5 g) was employed for partition in successive manner: dichloromethane (4 L, 3 times) (138.4 g), and ethyl acetate (4 L, 3 times) (185.6 g); Flow chart of *Rumex japonicus* Houtt roots extraction is depicted in in Figure 1. Ethyl acetate extract (150 g) from the roots of *R. japonicus* Houtt was eluted with macroporous resin. Eluents were water, 20% ethanol, 40% ethanol and 95% ethanol, respectively. The collected components were concentrated to obtain water fraction (13.7 g), 20% ethanol fraction (55.6 g) (YT1), 40% ethanol fraction (48.0 g) (YT2) and 95% ethanol fraction (25.7 g) (YT3).

Column chromatography aided with Merck silica gel (mesh size ranges 60, 200-300, 10 i. d. × 40 cm) was employed as means for YT1 fraction (55.6 g) elution with a ethyl acetate/methanol (100:0-0:100) to give 4 fractions (YT1-1-YT1-4). Same Merck silica gel was utilized for column chromatographic separation of YT1-1 fraction (7.8 g) by eluting with a petroleum ether/ethyl acetate (100:1-1:1) to give 7 fractions (YT1-1-1-YT1-1-7). Compound 9 (3.4 mg) was obtained from YT1-1-2 petroleum ether/ethyl acetate (100:1-1:1) fraction (1.8 g). YT1-1-5 fraction (85.6 mg) was processed to get YT1-1-5-2 fraction (25 mg). Compound 3 (12.5 mg) was obtained from YT1-1-5-2 fraction using medium-pressure preparative liquid chromatograph eluting with methanol/water (1:1-3:1).

Moreover, 10 fractions (YT1-3-1-YT1-3-10) of YT1-3 fraction (19.8 g) were obtained through silica gel-aided column chromatography as per TLC results. Among them, the YT1-3-1 fraction was needle crystal, and after recrystallization repeatedly to give compound 1 (9.5 mg). Similarly, YT1-3-5-6 fraction (220.5 mg) was obtained from YT1-3-5 fraction (3.5 g) separation through silica gel-based column chromatography. Then, compound 4 (6.9 mg) recovery was carried out using medium-pressure preparative liquid chromatograph by elution through methanol/water (4:1-8:1). YT1-3-8 fraction (2.2 g) was used to give YT1-3-8-5 fraction (95 mg). Then, compound 2 (15.3 mg) was obtained through MPPLC elution with methanol/water (4:1-8:1).

YT1-4 fraction (8.6 g) was used to give 6 fractions (YT1-4-1-YT1-4-6) petroleum ether/ethyl acetate (50:1-1:1) followed by recovery of compound 6 from YT1-4-5 (178.5 mg) group by MPPLC with methanol/water (1:1-3:1) obtained compound 6 (8.2 mg). YT2 fraction (48 g) yielded 4 fractions (YT2-1-YT2-4), YT2-2 fraction (18.4 g) yielded 8 fractions (YT2-2-1-YT2-2-8), YT2-2-3 fraction (3.6 g) yielded YT2-2-3-1 fraction (280.5 mg), YT2-3 fraction (15 g) yielded 8 fractions (YT2-3-1-YT2-3-10), YT2-3-2 fraction (654.5 mg) yielded YT2-3-2-3 fraction (25.8 mg), YT2-3-5 fraction (1.2 g) yielded YT2-3-5-2 fraction (34.5 mg). Compounds 7 and 10 were obtained from these fractions through gradiet elution. The obtain fraction was eluted with a MeOH-H2O (4:1-8:1) to give YT2-2-3-1-3 fraction (85.6 mg) based on TLC results. The fraction continue eluted with a MeOH-H2O (2:1) to obtain compound 5 (6.4 mg). Similarly, YT3 fractions (YT3-1-YT3-3) and YT3-2 fractions (YT3-2-1-YT3-2-5) according to TLC results were obtained. The YT3-2-3 fraction (982.5 mg) eluting with a petroleum ether / ethyl acetate (50:1-1:1) to give YT3-2-3-1 fraction (53.6 mg), and the continue eluted with a MeOH-H2O (1:1-4:1) to give compound 8 (2.6 mg).

2.3 NMR measurement for structural analysis

Different NMR experimentation modalities were carried out, such as ^1H NMR, APT, J-resolved, ^1H-^1H COSY, TOCSY, ^1H–^{13}C HMBC, ^1H–^{13}C HSQC, and NOESY at 600 MHz using a Micromass mass spectrometer (Synapt, Waters, USA). TMS was utilized as a standard (Maltese et al., 2009).

2.4 Antitumor activity of cancer cells (HepG2, U251, SK-OV-3/DDP)

Antitumor activity of HepG2, U251, SK-OV-3/DDP cancer cells were determined by reported methods of Qin et al. (2017, 2019) and Emerson et al. (2007). Tumor cell lines of human beings, known as HepG2, U251 and SK-OV-3/DDP, along with normal liver cell line HL-7702 were purchased from the Shanghai Cell Bank of Chinese Academy of Sciences. Cells cultures were prepared in Dulbecco's modified Eagle's medium (DMEM) followed by 10% supplementation of fetal bovine serum (FBS)

Rumex Japonicus Houtt roots（10 kg）

95% ethanol，room temperature

Extract（546.8 g）

PE extract 3 times

Water layer

CH$_2$Cl$_2$ extract 3 times   PE extract（78.5 g）

Water layer   CH$_2$Cl$_2$ extract（138.4 g）

EtOAc extract 3 times

Water layer   EtOAc extract（185.6 g）
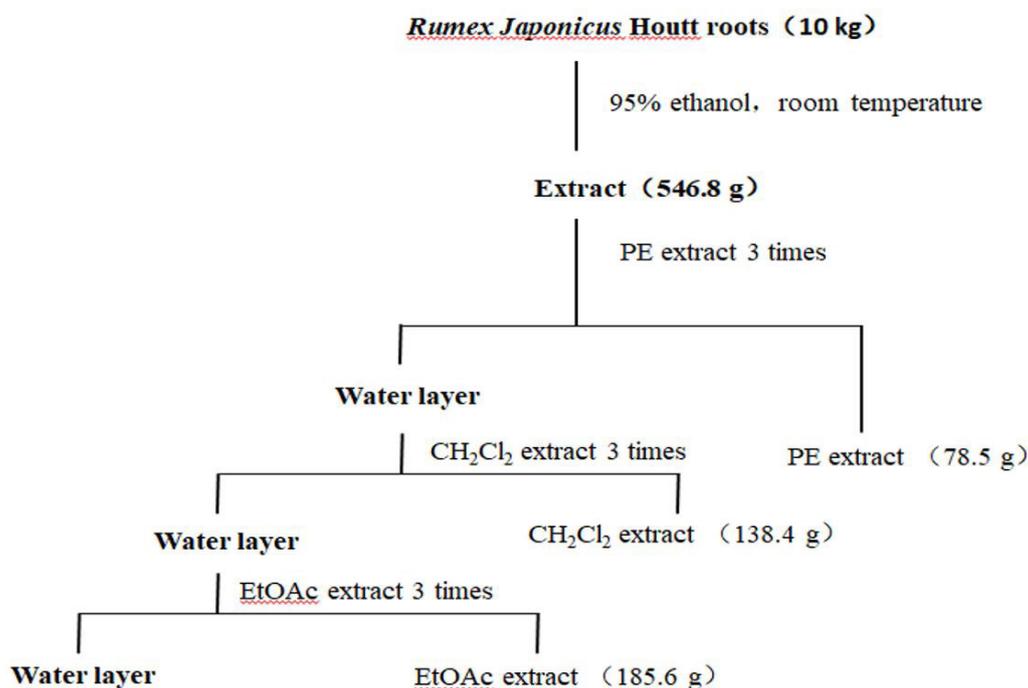
Figure 1. Flow chart of _Rumex japonicus_ Houtt roots extraction.

along with 100 U/mL penicillin and streptomycin. Cell cultures were subjected to atmospheric humidification at 37 °C with combination of 95% air and 5% CO_2. Evaluating extracts and compounds (2.0 mM) were prepared through DMSO stock solution method prior to dilution to make working concentrations of 0.5% DMSO-containing PBS buffer. Whereas, cisplatin was employed as positive control and 0.9% sodium chloride was utilized for dissolution.

2.5 Statistical analysis

Duplicate experiments were carried out for all measurements and values were recorded in triplicate manner. Means ± standard deviations were calculated for all measurements. SPSS software (SPSS Inc., Chicago, IL, USA) version 18.0 was the software package utilized for data analysis consisting of one-way analysis of variance (ANOVA). Duncan's multiple-range test was utilized to evaluate significance at $p < 0.05$.

3 Results and discussion

3.1 Yields and structure of isolated compounds

Yields of compounds isolated from roots of _Rumex japonicus_ Houtt (mg/g on dry weight basis of fractions) were determined. Table 1 shows that chrysophanol was the highest compound (~20%) present in EtOH fraction. Aloe emodin, emodin, physcion, quercetin, resveratrol, dibutyl phthalate were found relatively in significant quantities ranging from 20 to 40% in EtOH fractions. The yields of isolated chemical compounds fractions from _R. japonicus_ Houtt root, such as aloe emodin, chrysophanol, emodin, physcion, quercetin, resveratrol, β-sitosterol, 3β-acetoxy-

Table 1. Yields of compounds isolated from roots of _Rumex japonicus_ Houtt (mg/g dry wt of fractions).

Compounds	Yield (mg/g dry wt. of fractions)
Aloe emodin	9.5
Chrysophanol	15.3
Emodin	12.5
Physcion	6.9
Quercetin	6.4
Resveratrol	8.2
β-sitosterol	2.8
3β-Acetoxy-28-hydroxyurs-12-ene	2.6
Sucrose	3.4
Dibutyl phthalate	9.8

28-hydroxyurs-12-ene, sucrose and dibutyl phthalate were 9.5, 15.3, 12.5, 6.9, 6.4, 8.2, 2.8, 2.6, 3.4, and 9.8 mg/g, respectively on dry weight (DW) basis (Table 1). Similar findings have been reported by Guo et al. (2008) whereby a liverwort plant (_Scapania verrucosa_) was screened for antitumor compounds. The ether-extract based yield of dried _S. verrucosa_ was 0.6% and about 59 compounds were isolated from _S. verrucosa_. Flow chart for isolation of ethyl acetate extract from _Rumex japonicus_ Houtt roots is shown in Figure 2.

The structures of compounds showed in Figure 3 elucidated by ^{1}H-NMR and ^{13}C-NMR analyses are given below as follows:

Compound 1: Aloe emodin, yellow needle crystal, ^{1}H-NMR (500 MHz, DMSO): δ_H (ppm) 7.86 (1H, t, _J_ = 7.9 Hz, H-6), 7.77 (1H, d, _J_ = 7.5 Hz, H-7), 7.74 (1H, s, H-4), 7.44 (1H, d, _J_ = 9.5 Hz, H-5), 7.35 (1H, s, H-2), 12.00 (1H, s, OH), 4.69 (2H, d, _J_ = 6.0 Hz, H-1'). ^{13}C-NMR (125 MHz, DMSO): δC (ppm) 192.1 (C-9), 181.9
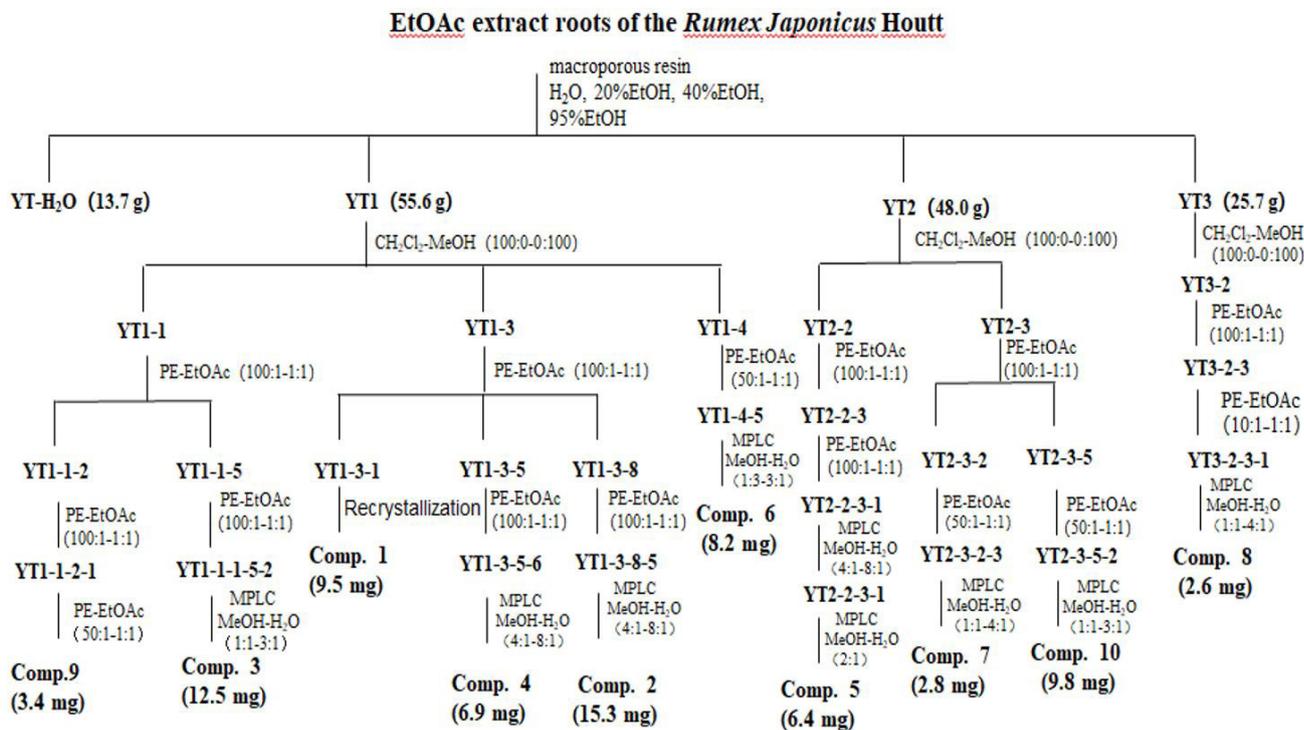
Figure 2. Flow chart for isolation of ethyl acetate extract from *Rumex japonicus* Houtt roots.

(C-10), 162.1 (C-8),161.8 (C-1),154.2 (C-3),133.8 (C-5a),133.6 (C-4a),124.9 (C-5),122.9 (C-4),121.1 (C-6), 119.8 (C-2), 117.6 (C-7), 116.4 (C-1a), 114.9 (C-8a), 62.5 (C-1').

Compound 2: Chrysophanol, yellow needle crystal, ^{1}H-NMR (500 MHz,DMSO): δ_H (ppm) 11.95 (1H,s,1-OH),7.81(1H, d,*J* = 7.0 Hz,H-5),7.72 (1H,d,*J* = 7.5 Hz,H-6),7.57 (1H,d,*J* = 1.5 Hz,H-4),7.39 (1H,d,*J* = 8.3 Hz,H-7), 7.23 (1H,d,*J* = 1.5 Hz,H-2), 2.45 (3H,s,-CH$_3$). ^{13}C-NMR (125 MHz,DMSO):δC (ppm) 192.1(C-9),181.9 (C-10),162.0 (C-1),161.8 (C-8),149.6 (C-3),137.8 (C-6),134.1 (C-5a),133.8 (C-4a),124.9 (C-2),124.6 (C-7),121.0 (C-4),119.8 (C-5),116.3 (C-1a),112.9 (C-8a), 21.2 (3-CH$_3$).

Compound 3: Emodin, aurantium needle crystal, ^{1}H-NMR (500 MHz,DMSO): δ_H (ppm) 12.04 (1H,s,1-OH),11.96 (1H,s,8-OH), 7.42 (1H,d,*J* = 2.2 Hz,H-4),7.11 (1H,d,*J* = 2.1 Hz,H-5),7.07 (1H,d,*J* = 2.2 Hz,H-2),6.56 (1H,d,*J* = 2.1 Hz,H-7),2.39 (3H,s,3-CH$_3$). ^{13}C-NMR (125 MHz,DMSO):δC (ppm) 190.1 (C-9),181.8 (C-10),166.0 (C-6),164.9 (C-8),161.9 (C-1),148.7 (C-3),135.5 (C-5a),133.2 (C-4a),124.6 (C-2),120.9 (C-4),113.8 (C-1a) 109.4 (C-5),109.2 (C-8a),108.4 (C-7),21.2 (3-CH$_3$).

Compound 4: Physcion, aurantium needle crystal, ^{1}H-NMR (500 MHz,DMSO): δ_H (ppm) 12.13 (1H,s,8-OH), 7.55 (1H,d,*J* = 2.3 Hz,H-4), 7.22 (1H,d,*J* = 2.3 Hz,H-2), 7.21 (1H,d,*J* = 2.5 Hz,H-5), 6.90 (1H,d,*J* = 2.5 Hz,H-7), 3.94 (3H,s,6-OCH$_3$), 2.44 (3H,s,3-CH$_3$). ^{13}C-NMR (125 MHz,DMSO): δC (ppm) 190.5 (C-9),181.8 (C-10),166.7 (C-8),164.9 (C-1),161.9 (C-6),149.1 (C-3),135.4 (C-5a),133.4 (C-4a),124.7 (C-2),121.1 (C-4),114.0 (C-1a),108.1 (C-5),108.1 (C-8a),107.1 (C-7),56.9 (6-OCH$_3$),22.0 (3-CH$_3$).

Compound 5: Quercetin, yellow needle crystal, ^{1}H-NMR (500MHz,DMSO):δ_H (ppm) 12.46 (5-OH),7.66 (1H,d,*J* =2.1 Hz,H-2'),7.53 (1H,dd,*J* = 8.5 Hz,*J* = 2.1 Hz,H-6'),6.89 (1H,d,*J* = 8.5 Hz,H-5'),6.41 (1H,d,*J* = 2.0 Hz,H-8), 6.19 (1H,d,*J* = 2.0 Hz,H-6). ^{13}C-NMR (125 MHz,DMSO): δC (ppm) 176.3 (C-4), 164.3 (C-7),161.2 (C-5),156.7 (C-9),148.2 (C-4'),145.5 (C-3'),122.5 (C-1'),120.5 (C-6'),116.1 (C-5'),115.5 (C-2'),103.5 (C-10),98.7 (C-6),93.9 (C-8),147.3 (C-2),136.2 (C-3).

Compound 6: Resveratrol, white needle crystal, ^{1}H-NMR (500MHz,CD$_3$OD): δ_H (ppm) 7.04 (1H,d,*J* = 16.3 Hz,H-7), 6.90 (1H,d,*J* = 16.3Hz,H-8), δ_H (ppm) 7.43 (2H,d,*J* = 8.4Hz,H-2,6), 6.84 (2H,d,*J* = 8.4 Hz,H-3,5), 6.53 (2H,d,*J* = 2.2 Hz,H-10,14), 6.24 (1H,t,*J* = 2.2 Hz,H-12). ^{13}C-NMR (125 MHz,CD$_3$OD):δC (ppm) 158.3 (C-11,13),156.9 (C-1),139.9 (C-4),129.0 (C-9),127.4 (C-3,5),115.1 (C-2,6),104.4 (C-10,14),101.3 (C-12), δC (ppm) 128.0 (C-7),125.6(C-8).

Compound 7: β-sitosterol, white needle crystal, ^{1}H-NMR (300 MHz,CDCl$_3$): δ_H (ppm) 5.34 (1H,d,*J* = 5.0 Hz,H-6), 3.49 (m,H-3), 0.67 (3H,s,H-18), 1.01 (3H,s,H-19), 0.84 (3H,d,*J* = 6.0 Hz,H-21), 0.92 (3H,d,*J* = 7.6 Hz,H-26), 0.91 (3H,d,*J* = 7.6 Hz,H-27), 0.82 (3H,t,*J* = 6.8 Hz,H-29). ^{13}C-NMR (75 MHz,CDCl$_3$):δC (ppm) 71.9 (C-3),140.9 (C-5),121.8 (C-6), 140.9 (C-5),36.6 (C-10),42.4 (C-13),19.9 (C-26),19.5 (C-19),19.1 (C-27),18.9 (C-21),12.1 (C-29),12.0 (C-18).

Compound 8: 3β-Acetoxy-28-hydroxyurs-12-ene, white powder, ^{1}H-NMR (500 MHz,CDCl3): δH (ppm) 5.26 (1H,t,J = 3.8Hz,H-12),4.09 (1H, d, J = 10.3 Hz,H-28a),4.32 (1H, d, J = 10.8 Hz, H-28b),2.05 (3H,s,H-2'), 1.08 (3H,s,H-27),0.96 (3H,s,H-26),0.93 (3H,s,H-25),0.92 (3H,s,H-29),0.87 (3H,s,H-30),0.85 (3H,s,H-23), 0.79 (3H,s,H-24). ^{13}C-NMR(125 MHz,CDCl3): δC

(ppm) 126.0 (C-12),138.1 (C-13),171.1 (C-1′),28.3 (C-23),23.8 (C-27),21.4 (C-30),21.3 (C-2′),17.3 (C-26),17.2 (C-29),16.9 (C-24),15.7 (C-25), 68.4 (C-28).

Compound 9: Sucrose, light yellow granular crystal, ^{1}H-NMR (300 MHz,CD$_3$OD): δ$_H$ (ppm) 4.07 (2H,d,J = 8.0 Hz,H-1), 4.06 (2H,d,J = 8.1Hz,H-6′), 3.6 (2H,d,J = 9.5 Hz,6-H), 5.15 (1H,d,J
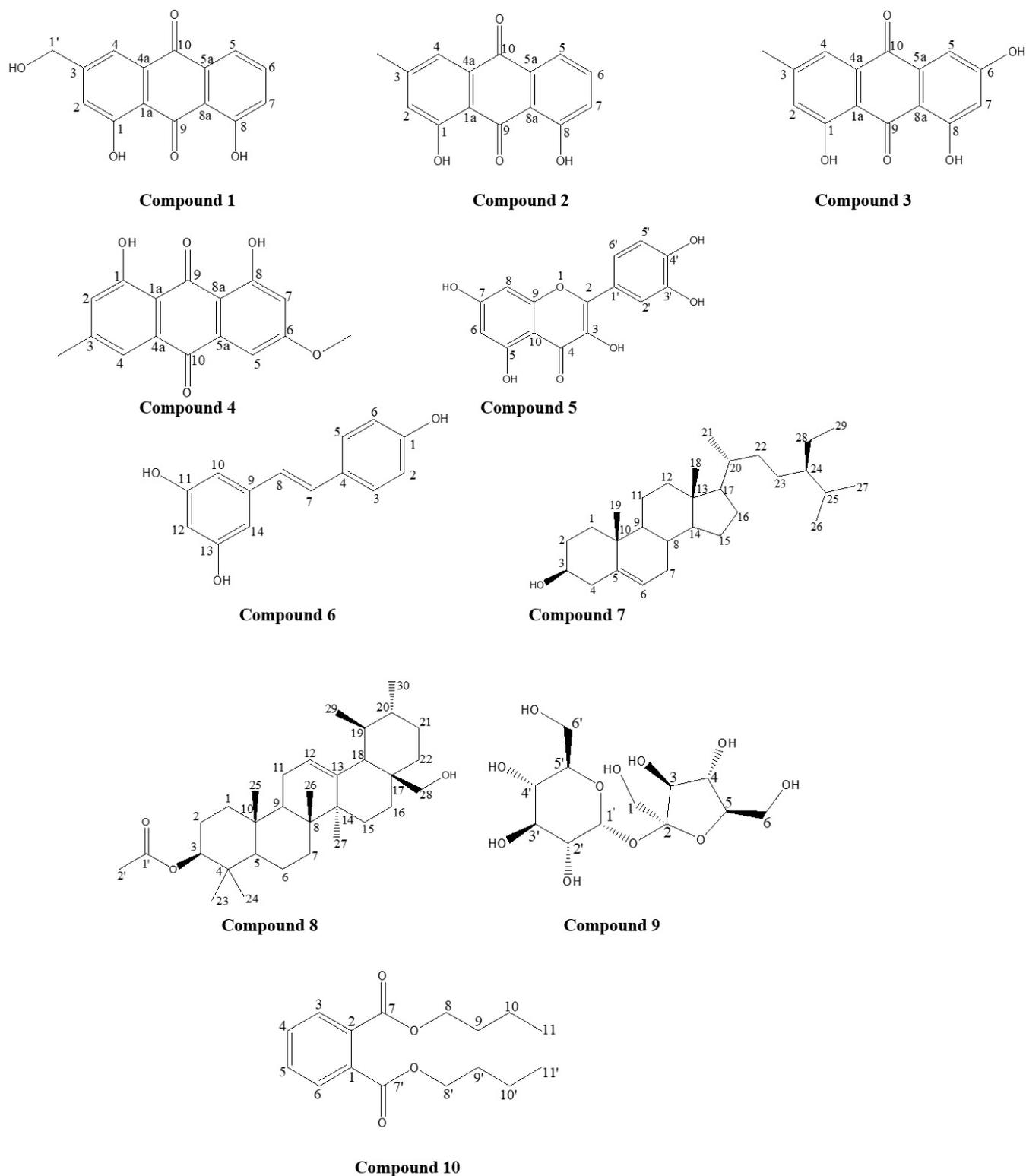
Figure 3. Chemical structures of compounds 1-10. (1) aloe emodin; (2) chrysophanol; (3) emodin; (4), physcion; (5) quercetin; (6) resveratrol; (7) β-sitosterol; (8) 3β-Acetoxy-28-hydroxyurs-12-ene; (9) sucrose; (10) dibutyl phthalate.

= 3.4 Hz,H-1′), 3.89 (1H,d,*J* = 3.6 Hz,H-4), 3.74 (1H,m,H-3′), 3.72 (1H,s,H-4′), 3.52 (1H,s,H-2′), 3.36 (1H,m,H-5′). ^{13}C-NMR (75 MHz,CD$_3$OD):δ$_C$ (ppm) 99.8 (C-1′),71.6 (C-2′),70.4 (C-5′),69.8 (C-3′),67.9 (C-4′),63.1 (C-6′),64.4 (C-1),101.8 (C-2),81.7 (C-5),76.5 (C-4),76.1 (C-3),62.8 (C-6).

Compound 10: Dibutyl phthalate, light yellow oil, ^{1}H-NMR (300 MHz,CD$_3$OD): δ$_H$ (ppm) 7.73 (2H,m,H-3,H-6), 7.64 (2H,m,H-4,H-5), 1.00 (6H,t,*J* = 7.4 Hz,H-11,H-11′), 4.31 (4H,t,*J* = 6.6 Hz,H-8,H-8′), 1.74 (4H,m,H-9 H-9′), 1.47 (4H,m,H-10,H-10′). ^{13}C-NMR (75 MHz,CD$_3$OD):δ$_C$ (ppm) 169.3 (C-7,C-7′); δ$_C$ (ppm) 1.00 (C-11,C-11′), 66.7 (C-8,C-8′), 31.7 (C-9,C-9′), 20.3 (C-10,C-10′), 133.6 (C-1,C-2), 132.4 (C-4,C-5), 129.9 (C-3,C-6).

3.2 Antitumor and cytotoxic effects of ethyl acetate extract of *Rumex japonicus* Houtt root

Extract apoptosis effects in HepG2 cells

For confirmation of anticancer potential of ethyl acetate extract of *R. japonicus* Houtt root, apoptosis induction in HepG2 cancer cells was evaluated in terms of inhibitory effect and results are presented as IC$_{50}$ values in Table 2. According to Food and Drug Administration (FDA) of Unites States, IC$_{50}$ value is indicative as measure of half maximal (50%) inhibitory concentration of potent chemical substance/compound exerting antitumor effect. The IC$_{50}$ values showing inhibitory effect on HepG2 cancer cells for all extract types, such as EtOAc, aqueous, 20%, 40% and 95% EtOH extracts were 86.7 µM, 111.3 µM, 85.6 µM, 108.21 µM, 414.23 µM as shown in in Table 2. EtOAc and 20% EtOH extracts exhibited comparable inhibitory effects, whereas gradual rises in IC$_{50}$ values were observed when ethanolic extracts concentration was varied from 40 to 95%. This implied that extract yield increased with corresponding rises in ethanol as extraction solvent and hence cytotoxicity of cancer cells increased from 85.6 µM to 414.23 µM when ethancolic extracts concentration was varied from 20 to 95%. These results are in coherence with findings of Machana et al. (2012) who reported anticancer effects of Finet & Gagnep (*Polyalthia evecta*) against human HepG2 cell line. Authors reported that 50% ethanol-water crude leaf extract of *P. evecta* led to moderate inhibitory effect (46.4%) in human HepG2 cell line. Inhibitory effect caused by antitumor activity of crude extract led to apoptosis which is the essential and reported end point of all anticancer therapies in terms of pharmaco-dynamics (Cummings et al., 2004). Moreover, the programmed cell death of cancer cells caused by antitumor activity of natural plant byproducts metabolites like *R. japonicus* Houtt root provides a balancing effect between

uncontrolled cell proliferation and cell death (Bruin & Medema, 2008; Mondal et al., 2011).

Extract apoptosis effects in U251 cells

Similar to HepG2 cell line results, the anticancer activity of human cerebral glioma cells (U251) was determined in terms of cytotoxicity. In vitro cytotoxicity evaluation was carried out by MTT assay and results are tabulated in terms of IC$_{50}$ values in Table 2. Petroleum ether/EtOAc (100:1-1:1) fraction showed IC$_{50}$ value of 85.6 µM. The aqueous extract of *R. japonicus* Houtt root exhibited higher inhibitory effect (IC$_{50}$ value: 165.5 µM) which implied that aqueous extracts were more potent to inhibit proliferation of U251 cancer cell lines. Among ethanolic extracts, 40% EtOH extracts showed the most significant inhibitory effect (IC$_{50}$ value: 224.3 µM) as compared to those observed in case of 20% (IC$_{50}$ value: 89.1 µM) and 95% EtOH extracts (IC$_{50}$ value: 80.1 µM). Extracts extracted using >40% ethanol exhibited corresponding decreasing tendency in inhibitory effect whereas 40% EtOH extracts were the most potent to exhibit cytotoxic effects to minimize cancerous cells proliferation. These results are in line with the findings of Alwarsamy & Ravichandran (2011) who reported anticancer activity of red seaweed (*Kappaphycus alvarezii*) and the active compounds Fucoidan demonstrated promising results in terms of its oncological significance. The methanolic extract exhibited significantly potent antitumor activity and cytotoxicity in glial cell carcinoma (U251) with 55 µg/mL as the most effective IC$_{50}$ value.

3.3 Extract antiproliferative effects in SK-OV-3/DDP cell line

For investigating antitumor properties of *R. japonicus* Houtt root extract, cytotoxicity to SKOV-3 cell line was assessed through MTT assay. It was found that cells treated with *R. japonicus* Houtt root extracts exhibited striking reduction in cell viability. The IC$_{50}$ values of EtOAc, water, 20% EtOH, 40% EtOH and 95% EtOH were 65.4 µM, 100.8 µM, 101.4 µM, 288.1 µM, and 311.36 µM, respectively. 95% EtOH showed the highest cytotoxic effect in SKOV-3 cell line followed by 40% EtOH and 20% EtOH extracts.

Concentration-dependent reductions in cell viability was found for EtOH extracts. Similar results have been reported by Paramee et al. (2018) for anti-proliferative effects of *Kaempferia parviflora* extract on SKOV3 ovarian cancer cells. SKOV3 has been reported as high-grade cell line for ovarian cancer and is resistant to many cytotoxic agents. Moreover, majority of the synthetically developed anti-tumor compounds have serious side effects (Cristea et al., 2010), therefore, natural anti-tumor chemical compounds of plant-origin like *R. japonicus* Houtt root extract might be good candidature for natural cancer therapies

Table 2. IC$_{50}$ value of the inhibitory effect of extract from roots of *Rumex japonicus* Houtt on three kinds of cancer cells (µM).

Sample	HepG2	U251	SKOV-3
Petroleum ether/EtOAc (100:1-1:1)	86.7	85.6	65.4
H$_2$O	111.3	165.5	100.8
20% EtOH	85.6	89.1	101.4
40% EtOH	108.2	224.3	288.1
95% EtOH	414.22	80.0	311.36

exhibiting potent cytotoxic effects to suppress growth of the most commonly diagnosed gynecologic cancer like ovarian cancer as adenocarcinoma.

3.4 Antitumor activity of R. japonicus Houtt root compounds 1-10

After evaluation of inhibitory effects of *R. japonicus* Houtt root extract, individual compounds (1-10) were also tested for their antitumor potential on the inhibition of three kinds of cancer cells and results are expressed in terms of IC_{50} value for each compound shown in Table 3. The lowest antitumor activity for HepG2 cells was shown by compound 5: quercetin (52.1), compound 4: physcion (62.3 μM), compound 6: resveratrol (68.2 μM) and compound 8: 3β-acetoxy-28-hydroxyurs-12-ene (86.4 μM). Four compounds: compound 2 (chrysophanol), 7 (β-sitosterol), 9 (sucrose), and 10 (dibutyl phthalate) exhibited highly significant and potent antitumor activity (>100 μM) for HepG2 cell lines whereas, compounds 3 (aloe emodin), 1 (emodin), and 8 (3β-acetoxy-28-hydroxyurs-12-ene) demonstrated antitumor activities of 93.4 μM, 90.8 μM and 86.4 μM, respectively. The possible mechanism for apoptotic effects of anticancer compounds might be ascribed to their modification ability of mitochondrial transmembrane potential and transcriptional activity by means of genetic or nuclear factors and owing to probable inhibition or activation of anti- or pro-apoptotic proteins (Méndez-Cuesta et al., 2019).

Similarly, cerebral glioma cells (U251) have also exhibited cytotoxicity conferred by ten isolated compounds from *R. japonicus* Houtt root extract. Highest anti-tumor activity of >100 μM was measured for compound 7 (β-sitosterol), compound 8 (3β-acetoxy-28-hydroxyurs-12-ene), compound 9 (sucrose) and compound 10 (dibutyl phthalate). While, compound 6 (resveratrol) was found with least inhibitory effect (17.8 μM) followed compound 3: aloe emodin (19.8 μM), compound 4: physcion (23.5 μM) and compound 5: quercetin (23.8 μM). Conversely, compound 1 (emodin) and compound 2 (chrysophanol) showed moderate cytotoxic effects of 48.3 μM and 50.1 μM in U251 cancer cell line, respectively.

Individual compounds from 1 to 10 were also assessed for their cytotoxic and tumor suppression effects on ovarian cancer SK-OV-3/DDP cell line and the antitumor potential on inhibition is expressed in terms of IC_{50} value for each compound shown in Table 3. Similar to cytotoxicity of U251 cell line, compound 7 to 10, such as β-sitosterol, 3β-acetoxy-28-hydroxyurs-12-ene, sucrose and dibutyl phthalate exhibited highly significant tumor suppression activity (>100 μM) in SK-OV-3/DDP cell line which implied that extracted and isolated *R. japonicus* Houtt root compounds were effective to inhibit tumor growth of highly resistant cancer cell lines like SK-OV-3/DDP. However, the compound 3 (aloe emodin) showed the least IC_{50} value (31.3 μM), while IC50 values of compounds 4 (physcion), 5 (quercetin) and 6 (resveratrol) were 61.1 μM, 47.4 μM and 42.1 μM, respectively which implied that these compounds exhibited moderate cytotoxic activity. The IC_{50} values of compound 1 (emodin) and compound 2 (chrysophanol) were 62.8 μM and 64.2 μM, respectively. Similar to antitumor compounds of *R. japonicus* Houtt root extract reported in this study, the published literature

Table 3. IC50 value of compound 1-10 on the inhibition of three kinds of cancer cells (μM).

Compound	HepG2	U251	SKOV-3
1	90.8 ± 4.8	48.3 ± 1.7	62.8 ± 3.4
2	>100	50.1 ± 2.2	64.2 ± 2.8
3	93.3 ± 3.2	19.8 ± 1.6	31.3 ± 1.2
4	62.3 ± 2.9	23.5 ± 0.9	61.0 ± 2.4
5	52.1 ± 2.4	23.8 ± 1.1	47.4 ± 2.1
6	68.2 ± 3.2	17.8 ± 0.8	42.0 ± 2.4
7	>100	85.3 ± 3.6	>100
8	86.4 ± 3.8	>100	>100
9	>100	>100	>100
10	>100	>100	>100

also reports several anticancer compounds of plant origin like cucurbitacins which are primarily a type of tetracyclic triterpenoids and are found in plants of *cucurbitaceae* family. The main notable plants as source of cucurbitacins include *Trichosanthes cucumerina* L. and *T. kirilowii* Maximowicz (Thai medicinal plants), plant by-products (leaves and fruits) of the Tunisian plant *Ecballium elaterium, Hemsleya amabilis* roots (ancient Chinese medicinal plant), *Cucumis melo* L., Balsam pear *(Momordica balsamina* L.), winter squash *(Cucurbita andreana),* and bitter cucumber *(Citrullus colocynthis)* (Florento et al., 2012; Lichota & Gwozdzinski, 2018; Zyad et al., 2018).

4 Conclusion

In this study, water, 20% EtOH, 40% EtOH and 95% EtOH were obtained by eluting the ethyl acetate fraction of *Rumex japonicus* Houtt roots. The results showed that ethyl acetate extract and 20% EtOH layer had a strong inhibitory effect on HepG2, U251 and SKOV-3 cells, while 95% EtOH layer had a strong inhibitory effect on U251 cells. The compounds of 20% EtOH, 40% EtOH and 95% EtOH were isolated and purified. Ten compounds, emodin (1), chrysophanol (2), aloe emodin (3), physcion (4), quercetin (5), reveratrol (6), β - sitosterol (7), 3 β - acetoxy-28-hydroxyurs-12-ene (8), sucrose (9) and dibutyl phalate (10), were identified through NMR spectral data. In vitro cytotoxic assay of 10 compounds showed that compounds 1, 3, 4, 5, 6 and 8 can inhibit the growth of HepG2 cells. Among them, compound 5 can strongly inhibit the growth, and the IC_{50} value is 52.1 μM. Compounds 3, 4, 5 and 6 have a strong inhibitory effect on glioma (U251) cells, IC_{50} values are 19.8, 23.5, 23.8 and 17.8 μM respectively, while compounds 1, 2 and 7 have a moderate inhibitory effect on glioma (U251) cells growth with IC_{50} values of 48.3, 50.1 and 85.3 μM respectively. Compounds 1, 2, 3, 4, 5 and 6 have a certain inhibitory effect on ovarian cancer (SKOV-3) cells, with IC_{50} values of 62.8, 64.2, 31.3, 61.0, 47.4 and 42.0 μM, respectively. Moreover, majority of the synthetically developed anti-tumor compounds have serious side effects, therefore, natural anti-tumor chemical compounds of plant-origin like *R. japonicus* Houtt root extract might be good candidature for natural cancer therapies exhibiting potent cytotoxic effects to suppress growth of the most commonly diagnosed adenocarcinomas.

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