Tropical Journal of Pharmaceutical Research January 2020; 19 (1): 129-136
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v19i1.20

Original Research Article

Anticancer property of hexane extract of *Suaeda fruticose* plant leaves against different cancer cell lines

Kamel A Saleh[1]*, Tahani H Albinhassan[1], Adel M Al-Ghazzawi[2], Abdulrahman Mohaya[3], Ali A Shati[1], Huda J Ayoub[4], Qasem M Abdallah[5]

[1]Department of Biology, [2]Department of Chemistry, Science College, King Khalid University, PO Box 9004, Abha, [3]Asser Toxicology Center, King Abduallah Street, PO Box 1988, Abha 61441, Saudi Arabia, [4]Department of Pharmacognosy, Pharmacy College, Alzaytoonah University, [5]Department of Pharmacology, Pharmacy College, University of Petra , Amman, Jordan

For correspondence:* ***Email:* *kasaleh@kku.edu.sa*

Sent for review: 3 April 2019 *Revised accepted: 19 December 2019*

Abstract

Purpose: *To evaluate the bioactivity of hexane extract of S. fruticosa leaves against the cancer cell lines HepG2, MCF-7, and HCT-116, and to determine the chemical composition-function relationship.*
Methods: *Using the liquid-liquid extraction method, the nonpolarL constituent compounds were isolated from the leaves. The cytotoxicity of the hexane extract was evaluated using an SRB assay. Mechanism of action was verified by observing the appearance of apoptotic bodies using fluorescence microscopy, while anti-proliferative activity was assayed via flow cytometry.*
Results: *The results revealed that secondary metabolites in the hexane extract demonstrated the highest cytotoxicity, and thus anticancer activity, against HCT-116 cells, with an IC_{50} of 17.15 ± 0.78 mg/mL. The presence of apoptotic bodies indicate an ability to induce apoptosis. Flow cytometry results suggest that the secondary metabolites stalled the cell cycle at the G0/G1 phase.*
Conclusion: *The results indicate that S. fruticosa hexane extract may be considered a potential new source of the anti-cancer compound, momilactone B.*

Keywords: *Anticancer, Apoptosis, Colon Cancer, Liver cancer, Breast cancer, Liquid chromatography–mass spectrometry, Suaeda fruticose, Momilactone B*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Cancer is an invasive disease, with global impacts in both developed and developing countries; 23.6 million new cases are expected by 2030 [1]. The cancer cell engages in complicated pathways to ensure its survival inside the body and resistance to different therapies [2]. Understanding these pathways can lead to innovations in the activation of appropriate mechanisms to control cancer cell proliferation and induce programmed cell death [3]. Apoptosis is cited as a favourable pathway and informs many strategies for the development of novel cancer therapies [4]. However, their success is currently hampered by unpleasant side effects.

Natural products have recently received increased attention among those searching for new anticancer therapeutics [5,6]. Plant-derived therapies have had significant success against different cancer types both *in vivo* and *in vitro* through the induction of apoptosis [7,8]. Plants produce bioactive compounds known as secondary metabolites that enable them to survive in and adapt to different habitats. Halophyte plants are able to manage stress conditions using antioxidant system mechanisms [9]. Medicinal halophytes are rich in bioactive secondary metabolites such as antioxidants, polyphenols, and flavonoids; these compounds have shown antimicrobial, antiviral, anticancer, and anti-inflammatory activities while remaining nontoxic to normal cells [10-11].

Suaeda fruticosa, a member of the *Chenopodiaceae* family, is a halophytic medicinal plant and is highly salt-tolerant. Seeds and leaves of the plant have been classified as safe for human consumption or forage, and are used as a phytoremediation tool [12,13]. *S. fruticosa* is known for being rich in a bioactive compound; a recently isolated polysaccharide from the plant demonstrated antioxidant, anti-filamentary, anti-nociceptive, hypoglycaemic and anti-hyperlipidaemic properties in *in vitro* and *ex vivo* assays [14,15]. The shoots and leaves of the plant are rich in phenols, flavonoids, tannins, alkaloids, saponins, proanthocyanins, and carotenes, indicating an impressive pharmacological spectrum as compared to other halophytes in the same family, such as *Salsola kali* [16].

Juice and decoction from the *S. fruticosa* Leaf have been used to treat fever, flu, skin disease, rheumatism, and helminthiasis livestock diseases [17,18]. Different extracts of the shoots have also been tested against different cancer cell lines, with the most active extract being dichloromethane against colon carcinoma cell lines DLD-1 and HT-29, with IC_{50} values of 10 ± 1 and 12 ± 14 µg/mL, respectively. Conversely, root methanolic extract showed less toxicity against human lung carcinoma (LU-1) and hormone-dependent prostate carcinoma (LnCaP), with an $IC_{50} \leq 50$ µg/mL [16,21]. Despite this plant's richness in bioactive compounds, no studies have been conducted that explored the bioactivity of the plant's leaves against a range of cancer cell lines. The current study aims to investigate the ability of the non-polar crude extracts from *S. fruticosa* leaves to activate different mechanisms to prevent cancer cell proliferation or induce cancer cell apoptosis; it will also conduct LC\MS-MS profiling of each of the different extracts.

EXPERIMENTAL

Cell lines, chemicals, and biochemicals

Ethanol, methanol, and SRB stain were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were obtained from Gibco/Life Technologies (Carlsbad, CA, USA) unless otherwise indicated. Cell culture vessels were obtained from Nunc A/S (Roskilde, Denmark). Human colon (HCT 116), liver (HepG-2) and breast (MCF-7) cancer cell lines were acquired from Vacsera (Giza, Egypt). Cells were maintained in RPMI 1640 cell culture medium supplemented with 1 mM sodium pyruvate, 2 mM L. glutamine, 100 units/mL penicillin-streptomycin, and 10 % fetal bovine serum. Incubation was in a humidified environment at 5 % CO_2 and 37 ºC.

Plant collection and crude extract preparation

Fresh leaves from *S. fruticosa* plant were collected from Al-Birk beach located in Geographic coordinates ($18^{o}12'44.8"N$ $41^{o}32'09.7"E$) in the Asser region of Saudi Arabia on 21 July 2017 Plant authentication was carried out by Zouhair Barnoumy, and a voucher specimen was kept in King Khalid University herbarium (voucher no. kku-2017-2526). For crude extract preparation, 400 g of fresh leaves were washed with distilled water and ground with a grinder, then immersed in 300 mL of hexane and left with stirring at room temperature (18 - 24 ^{o}C) for seven days. The extract was filtered using filter paper and concentrated to dryness under reduced pressure using a rotary evaporator (Ika, Germany) at 40 ºC.

Hexane crude extract was 2.38 g. Following evaporation, 0.01 g of crude extract was diluted in 1 mL of dimethyl sulfoxide (DMSO) to provide a stock solution for bioactivity assay. The crude extract was stored at 4 ºC until used.

Evaluation of cytotoxicity activity of *S. fruticosa* crude extracts

The cytotoxicity and anticancer properties of prepared *S. fruticosa* leaves hexane crude extracts were tested against human breast (MCF-7), colon (HCT 116), and liver (HepG-2) cancer cell lines using a SulfoRhodamine B (SRB) assay as described by Skehan *et al* [19]. Briefly, the different cancer cell lines were exposed to a range of concentrations (0.01 to 100 µg/mL) of hexane crude extract and incubated in a humidified incubator aerated with 5 % CO_2 at 37 °C for 72 h. Doxorubicin was used as a positive control. Treated cells were fixed

with TCA (10 %) for 1 h at 4°C. Cells were washed with water several times to remove the TCA, and then a 0.4 % SRB solution was used to stain cells in the dark for 10 min. Stained cells were then washed with 1 % glacial acetic acid. Finally, Tris–HCl was used to dissolve the SRB-stained cells. After drying overnight, the colour intensity of the remaining cells was measured at a wavelength of 540 nm with an ELISA plate reader.

Determination of apoptosis activity

For apoptotic body detection, treated cells were washed twice with PBS and then collected using 0.25 % trypsin–EDTA. Cells were then stained using ethidium bromide (EtBr) and Acridine Orange (AO) at a 1:1 concentration and transferred to slides. Stained apoptotic bodies were detected and photographed with a Nikon Fluorescent microscope (Japan).

Assessment of cell cycle distribution using DNA flow-cytometry

Adherent cancer cells were exposed to IC_{50} equivalent concentrations of extract solutions for 48 h. Cells were then suspended using 0.25 % trypsin-EDTA, washed with ice-cold PBS, and re-suspended in 0.5 mL of PBS. Cells were then fixed in 70 %, ice-cold ethanol at 4 °C for 1 h before being stored at -20 °C until analysis. Upon analysis, fixed cells were washed with ice-cold PBS and re-suspended in 1 mL of PBS containing 50 µg/mL RNase A and 10 µg/mL propidium iodide (PI). After a 20 min incubation at 37 ℃, cells were assayed for DNA content by a FACSVantage^{TM} Flow Cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA). For each sample, 10,000 events were acquired. Cell cycle distribution was calculated using CELLQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA) [20].

LC-ESI-QTOF-MS/MS analysis

Analysis performed on SCIEX X500R QTOF system includes UPLC-MS/MS (Woodlands Central Indus. Estate. SINGAPORE). The separation was performed using Phenomenex Kinetex 2.6 µm Phenyl-Hexyl 100 A (50 × 4.6 mm). The mobile phase consists of, phase A (10 mM ammonium formate in water) and phase B (0.05 % formic acid in methanol). A variable gradient flow rate was used, which is described in (table 1). A Positive Non-targeted mode was used for the analytes (Table 1).

Table 1: Gradient flow rate for LC-MS/MS

Time (min)	Flow rate (mL/min)	A conc.	B conc.
0.00	0.7	90.0	10.0
7.00	0.7	2.00	98.0
8.50	0.7	2.00	98.0
8.60	0.7	90.0	10.0
9.50		**Stop**	

Statistical analysis

To present statistically accurate results, all experiments were performed in triplicates. The results were expressed as mean ± SEM. Statistical analyses were performed by SBSS V. 16. Statistical differences among treated and untreated cells were determined by two-way ANOVA to compare cell lines response and cell viability values and one-way ANOVA for IC_{50}. $P < 0.05$ was considered significant (Table 2, Table 3).

Table 2: Dependent variable for the different cancer cell lines (HepG-2, MCF-7, HCT-116) treated with *S. fructose* hexane leaf extract

Source	Type III sum of squares	df	Mean square	F	Sig.
Corrected model	125090.598	35	3574.017	664.919	.000
Intercept	455732.077	1	455732.077	8.479E4	.000
Cell type	15469.132	5	3093.826	575.583	.000
Control	95500.267	5	19100.053	3.553E3	.000
Cell type*Control	14121.198	25	564.848	105.086	.000
Error	387.009	72	5.375		
Total	581209.683	108			
Correct total	125477.606	107			

a. R. squared = .997 (Adjusted R squared = .995)

Table 3: The IC_{50} for different cancer cell lines (HepG-2, MCF-7, HCT-116) treated with *S. fructose* hexane leaf extract., based on Duncan test

Var	N	Subset for Alpha = 0.05		
		a	b	c
1	3	0.37		
3	3	0.37		
5	3	0.60		
2	3		46	
4	3		46	
6	3			56
Sig.		0.831	0.749	1.000

a: b: c: IC_{50} calculations were performed using Sigma Plot version 12.0.

RESULTS

Cytotoxicity

Prepared crude extract was tested against the cancer cell lines MCF-7, HCT-116, and HepG2. Results revealed that hexane significant effects (Table 2, Figure 1). Hexane extract IC_{50} value was the highest at 17.15 ± 0.78 µg/mL against the HCT-116 cell line. These results were confirmed by cell viability curves (Figures 1a, 1b and, 1c) respectively.

Table 2: IC_{50} (µg\mL) of hexane extracts of *S. fruticosa* against three solid tumour cell lines

Extract/cell line	MCF-7	HCT-116	HepG2
Hexane (µg/mL)	28.1 ± 0.93	17.15 ± 0.78	33.2 ± 1.11
Doxorubicin (µg/mL)	0.6 ± 0.022	0.45 ± 0.0516	0.42 ± 0.103

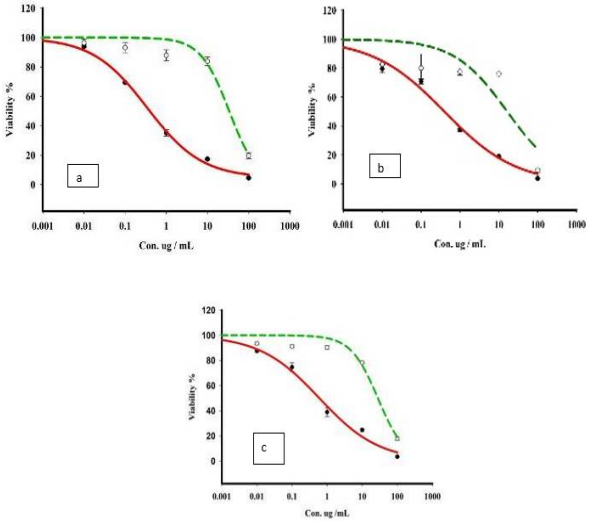

Figure 1: Concentration-response curve of hexane Leaf extract of *S. fruticosa* against solid tumor cell lines: (a) MCF-7, (b) HCT-116, and (c) HepG2. Cells were exposed to the extract for 72 h. Cell viability was determined using an SRB-U assay. Data are expressed as mean ± SD (n = 3)

Fluorescence microscopy analysis of cell viability and apoptosis

Results obtained from fluorescence microscopy showed that hexane extract was able to induce apoptosis. As shown in Figure 2, general morphological characteristics of apoptotic processes such as chromatin condensation, membrane blebbing, and apoptotic bodies were observed. The presence of a necrotic cell death pathway was also detected (Figure 2).

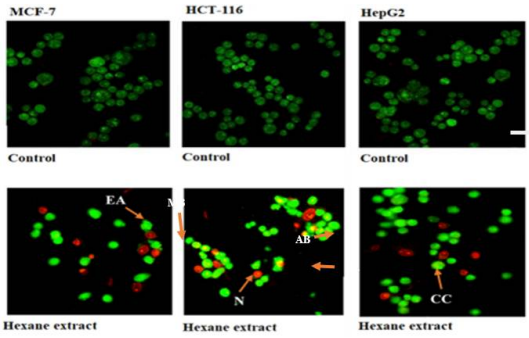

Figure 2: Morphological features of HCT-116, MCF-7 and HepG2 cells induced by the IC_{50} concentration of Hexane extract of S. fruticosa, stained with AO/EB. The images were taken using fluorescence microscopy at 20×. C: control, no treated cells; T: treated cells. MB: membrane blebbing; CC: chromatin condensation; EA: early apoptosis; LA: late apoptosis; AB: an apoptotic body; N: necrosis. Scale bar: 2µm

Cell cycle

To identify which phase in the cell cycle was affected by *S. fruticosa* hexane extract we used flow cytometry for tracking DNA through the cell cycle. Results revealed that the hexane extract exerted similar activity on the different cancer cell types. The hexane extract was able to arrest the cell cycle at the G0-G1 phase (Table 3 and Figure 3).

Table 3: Effect of hexane extract of S. fruticosa on the cell cycle distribution of three tumour cell lines; HCT116, MCF-7 and HepG2 for 24 h and compared with control cells

Cell type	Extract	Cell cycle phase/DNA content		
		G0-G1	***S***	***G2-M***
HCT-116	Control	44.8 ± 0.85	51.79 ± 0.59	3.41 ± 0.29
	Hexane extract	80.79 ± 0.83	19.17 ± 0.83	0.03 ± 0.063
MCF-7	Control	45.4 ± 0.97	46.2 ± 0.81	8.34 ± 0.93
	Hexane extract	70.84 ± 0.65	24.37 ± 0.06	4.78 ± 0.71
HepG2	Control	50.48 ± 0.62	43.3 ± 0.71	6.21 ± 0.36
	Hexane	71.59 ± 0.84	22.03 ± 0.83	6.37 ± 0.776

LC\MS-MS

The non-polar secondary metabolites of the plant were isolated by solid extraction with hexane then qualitatively analysed using LC-ESI-TOF-MS/MS. The result of the analysis revealed the presence of monoterpenes, diterpenes and phenolic compounds beside some fatty alcohols, fatty acids, steroidal and miscellaneous compounds. Table 4, Table 5 and Table 6 show

Table 4: LC-MS/MS results for monoterpene compounds detected in *S. fruticose* hexane extract

No	rt	Compound Name	Compound formula	(M+H) found for ms	(M+H) found for ms/ms	(M+H) Calculated	Mass error (ppm)	Uncertainty in m/z	Conc. Of compound in extract (ppm)	Conc. Of compoun d in dry plant (ppm)	ref
1	5.41	Dihydrojasmone	$C_{11}H_{18}O$	167.1432	79.0542, 59.0487, 31.01182	167.1441	-5.3846	±0.0009	540	3.21	26
2	5.50	Jasmolone	$C_{11}H_{16}O_2$	181.1222	163.0389, 149.0236, 84.9599	181.1234	-6.6253	±0.0012	4120	24.51	26
3	6.32	Terpinene-4-ol	$C_{10}H_{18}O$	155.1427	114.0646, 98.9835, 80.9722, 43.0179	155.1441	-9.0238	±0.0014	30	0.18	27

Table 5: LC-MS/MS results for diterpene compounds in *S. fruticose* hexane extract

No	RT	Compound Name	Compound formula	(M+H) found for ms	(M+H) found for ms/ms	(M+H) Calculated	Mass error (ppm)	Uncertainty in m/z	Conc. Of compound in extract (ppm)	Conc. Of compound in dry plant (ppm)	Ref
1	6.39	Pimaric acid	$C_{20}H_{30}O_2$	303.2321	147.1176, 105.0709, 91.0547	303.2330	-2.9680	±0.0009	710	4.22	
2	6.92	Steviol	$C_{20}H_{30}O_3$	319.2269	273.1846, 135.0805, 105.0697, 91.0542	319.2279	-3.1326	±0.0010	4610	27.23	28
3	7.65	Momilactone B	$C_{20}H_{26}O_4$	331.1903	167.0342, 149.0229,	331.1915	-3.6233	±0.0012	3210	19.10	29

Table 6: LC-MS/MS data for phenolics in *S. fruticose* hexane extract

No	RT	Compound Name	Compound Formula	(M+H) found for ms	(M+H) found for ms/ms	(M+H) Calculated	Mass error (ppm)	Uncertainty in m/z	Conc. Of compound in extract (ppm)	Conc. Of compound in dry plant (ppm)	Ref
1	4.76	Quercinol	$C_{11}H_{12}O_3$	193.0856	147.1180, 105.0707, 91.0543	193.0870	-7.2506	±0.0014	40	0.24	
2	5.35	Zingerone	$C_{11}H_{14}O_3$	195.1016	163.0366, 107.0856, 45.0331, 43.0174	195.1027	-5.6381	±0.0011	180	1.07	30
3	4.10	Zingerol	$C_{11}H_{16}O_3$	197.1172	179.1069, 133.1012, 105.0700, 91.0542	197.1183	-5.5804	±0.0011	2950	17.56	
4	6.53	Neoflavan	$C_{15}H_{12}O_2$	225.091	105.0336, 77.0385	225.0921	-4.8869	±0.0011	290	1.73	
5	5.03	Abscisic acid	$C_{15}H_{20}O_4$	265.1437	247.1347, 191.0703, 187.1123	265.1445	-3.0172	±0.0008	120	0.71	31

the name, molecular formula, molecular mass found and calculated, main mass fragments, retention times and percentage error of monoterpenes, diterpenes and phenolic compounds while, other compounds were neglected because they do not contribute to bioactivity against cancer cell lines.

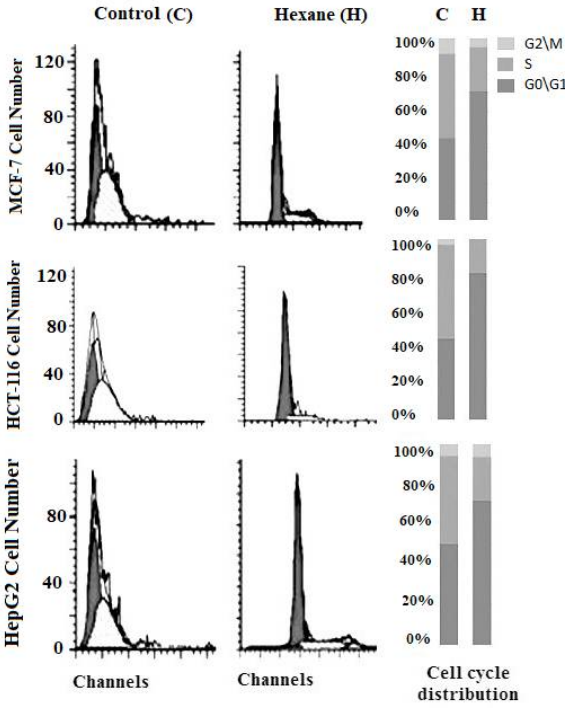

Figure 3: Effect of hexane fraction of S. fruticosa aqueous ethanol crude extract on the cell cycle distribution of MCF-7, HCT-116, and HepG2 cancer cell lines. Cells were exposed to hexane extract for 48 h (B) and compared with cell control (A). Cell cycle distribution was determined using DNA cytometry analysis, and different cell phases plotted. (C) Percent of total events (n = 3)

DISCUSSION

All natural product working with crude extract are suffering from the complexity of the determination of the structure-function relationship of the extracts, generally due to the numerous number of the particles in the extract. Moreover, not all particles have a function or even help to presents the estimated function. Quite the contrary, some ingredients may be inhibiting the work of the active ingredients. On the other hand, the positive view reveals the presence of the cofactors or coenzymes of the secondary metabolites, which may can induce one or more pathways like apoptosis to prevent cancer cell proliferation. Current study took upon itself the clearance of this complicity not only to determine the activity of the hexane crude extract but also to analyse the chemical composition of the extract in order to reduce or eliminate the probabilities of the inactive ingredients by do a correlation between the impact and the composition [7,8].

S. fruticosa is an edible, medicinal, halophytic plant rich in bioactive compounds that used in folk medicine for the treatment of many different diseases [16,21].

In this study, we evaluated the anticancer activity of hexane leaf extract of *S. fruticosa* against three different cancer cell lines (HCT-116, MCF-7, and HepG2) in a concentration-dependent manner. Recent studies reported that hexane, dichloromethane, water, and methanol extracts of different parts of the *S. fruticosa* plant can inhibit the growth of certain cancer cell lines *in vitro* [16,21]. Among these, the dichloromethane and methanol extracts had the most significant effects on colon cancer cell line DLD-1 (IC_{50} 10 ± 1 and 15 ± 1 µg/mL, respectively). The current study used hexane extract which is more nonpolar than methanol and dichloromethane but yielded almost similar results (17.15 ± 0.78 µg/mL, Table 1).

The effects of the extract on MCF-7 and HepG2 cancer cell lines were less impressive. Interestingly, the extracts have been tested against normal human skin fibroblast cell lines and no significant toxicity was found [16]. This harmony between the previous results and what has been obtained in this study indicates that plant *S. fruticosa* even different extracts have a good potential to have anti colon cancer prodrugs. Furthermore, the ability of plant chemical composition to induce apoptosis in colon cancer cells (HCT116) (Figure 1) as demonstrated by the morphological cell changes more than other cell line (liver and breast) types, may open a promising specific pathway for colon cancer different types lead to the development of new anticancer therapeutics. These conclusions encouraged us to go through the cell cycle in hope to determine the phase that hexane extract shows its impact. Current study indicated that hexane extract was able to arrest cell cycles at the G0-G1 phase in all the three cancer cell lines (Table 2).

Many existing anticancer drugs also work by arresting the cell cycle, either at the G0-G1 or G2-M transitions [24]. Halting the cell at G0-G1 may present an avenue for the induction of apoptosis by activating cell cycle exit at this checkpoint via cyclins D or E or other restriction signals [24].

On the other hand, our LC\MS-MS results confirmed the presence of momilactone B compound (Table 5), which had been reported to have anticancer activity (potential chemotherapy) against and promising inhibition in pre-clinical models, not only that but also can inhibit cell cycle in G1 which explain the prevention of cells to enter S phase. [25]. Furthermore, recent studies revealed the ability of momilactone to inhibit the growth of Breast cancer cell line through STAT5b and a caspase-3 dependent pathway [25].

CONCLUSION

The findings of this study reveal that *S. fruticosa* plant is a new natural source of a potential chemotherapeutic agent, namely, the momilactone B compound. The compound is a non-toxic natural primary source for manufacturing several drugs such as anticancer, antiviral, antifungal, antioxidative, and anticoagulant.

DECLARATIONS

Acknowledgement

The authors extend their appreciation to the Deanship of Scientific Research at King Khalid University for funding this work through General Research Project (grant no. RGP 1/9/38).

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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