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ORIGINAL PAPER

2-Benzhydrylsulfinyl-N-hydroxyacetamide-Na extracted from fig as a novel cytotoxic and apoptosis inducer in SKOV-3 and AMJ-13 cell lines via P53 and caspase-8 pathway

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Abstract

In this study, the fruits of both mature and air-dried figs were exposed to selective sequential extracting processes using soxhlet. Different polarity and non-polarity solvents were used to increase the yield of the isolated extracts. Methanol, ethyl acetate, chloroform, and *n*-hexane were used to precipitate and isolate the effective compound *2-benzhydrylsulfinyl-n-hydroxyacetamide-Na* from 11 extracts and 7 compounds extracted from the organic layer. Various spectral techniques were applied, including UV-spectroscopy, FT-IR, GC-Mass, ^{1}H-NMR, and ^{13}C-NMR for the detection of the precipitate. The aim of current work is to deal with the synthesis of a novel compound *2-benzhydrylsulfinyl-n-hydroxyacetamide-Na* from fig fruit and study its effect as anticancer and anti-proliferative agent against SKOV-3, and AMJ-13 cells. The effect of the active compound on breast cancer cells, ovarian cancer cell proliferation was measured by the MTT assay, while its ability to induce apoptosis was detected using DAPI, acridine orange/ethidium bromide (AO/EtBr) staining, flow cytometry assay and finally mitochondrial membrane potential (MMP). Real-Time PCR was used to detect changes in the expression of mRNA for Bax and Bcl-2, P53, caspase-8, and caspase-9. Treated cancer cells with *2-benzhydrylsulfinyl-n-hydroxyacetamide-Na* significantly increased ROS synthesis, with subsequent reduction of the MMP through mechanisms that included Bax upregulation P^{53}, Caspase-8, and Caspase-9 and Bcl-2 downregulation. The outcomes of the current study show that *2-benzhydrylsulfinyl-n-hydroxyacetamide-Na* extracted from fig fruit suppressed cancers cells' proliferation, resulting in apoptosis as a novel pathway that involves mitochondrial mechanism via activated P53 and caspase-8 signaling. In addition, the extract exerted no toxic effects, neither on serum levels of liver functional enzymes nor on the normal histological architecture of the lungs and spleen. We demonstrated how the *2-benzhydrylsulfinyl-n-hydroxyacetamide-Na* affects cancer cells along with a study the possible mechanisms involved in this effect which is mitochondrial damage and P^{53} pathway. We conclude that *2-benzhydrylsulfinyl-n-hydroxyacetamide-Na* holds a promising potential as an anticancer therapeutic agent.

Keywords *2-Benzhydrylsulfinyl-n-hydroxyacetamide-Na* · Anticancer activity · P53 · Caspase-8

Introduction

Cancer is ranked second among the global causes of death in humans, being a serious threat to health and livelihood [1]. The most common cancers surrounded by women are breast cancer and is another main cause of doom after lung cancer [2]. Breast cancer is triggered by frequent exposure of breast cells to prevalent ovarian hormones [3]. These hazard agents point across endogenous estrogen as possible players in the origination, development and promotion of breast cancer [4]. Amongst the gynecological malignancies, ovarian cancer is the principal cause of death in advanced countries. In spite of the global impact of this disease, the lifetime risk of evolving ovarian cancer in even in the postmenopausal population that

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is at extreme risk. Therefore, ovarian cancer is not a common or rare disease but the overall treatment rate is less than 40% in all stages [5]. Presently, cancer treatment consists of surgery, radiotherapy, immunotherapy and chemotherapy. Regardless of the progression in cancer treatment, breast cancer leftovers a tragic disease [6]. In recent years, plant derivatives have acquired increased attention as a result of their diverse applications, most importantly their ability to abolish ROSs and thereby to ensure anti-oxidative stress protection for crucial cellular molecules such as proteins, DNA, and lipids [7]. These plant derivatives might also exert their activities through interference with various intracellular signaling events, including proliferation, apoptosis, and responding to oxidative stress [8, 9]. Animal experiments as well as epidemiological investigations showed that more than half of the anticancer agents were derived from natural, particularly plant, sources, while specifically indicating a link between flavonoids intake and decreased cancer risk [10]. Above 50% of anticancer drugs have been produced from natural sources, particularly from plants, and the anticancer activity of some plants may be owing to their antioxidant properties. Furthermore, numerous plant antioxidants are cast-off as anticancer drugs and cancer cells can induce cancer cells apoptosis [11]. *Ficus carica* L. is a short-treed plant of African origin that is classified under the family Moraceae, with fruits, known as figs, which are globally used for nutritional and medicinal purposes. Elevated antioxidant potentials have been described to be served by the flavonoids, phenols, and anthocyanin's derived from both the dry and fresh fruits of this plant [12]. Several in vivo, as well as in vitro, studies demonstrated that these products can be used as a treatment in numerous disorders such as gastrointestinal, respiratory, inflammatory, and cardiovascular disorders along with cancers [13]. In addition to these, *Ficus carica* L. also has noticeable pharmacological properties such as antioxidant, anticancer, cytotoxic, anti-inflammatory, and lipid deficiency [11]. Preceding studies have exposed that of fig fruit extracts revealed modest cytotoxic efficiency against MCF7, HepG2, U2OS cells, cervical cancer cell line and HeLa [14]. Moreover, further studies have established that fig leaves, fruit, and latex wholly comprise anticancer components such as bergapten, psoralen, benzaldehyde, amylose, and selenium [15–17]. Several opener molecules in breast cancer are closely participatory in proliferation or apoptosis of breast cancer cells, comprising p53, Bax, Bcl-2, Bcl-2 which can inspire the viability of cancer cells by influencing the cell cycle, reforming spoiled DNA, or persuading apoptosis [18]. One peculiar and essential mechanisms of programmed cell death is embodied by the proceedings of apoptosis, which ensures normal development and homeostasis of tissues through the eradication of abnormal cells. Also during the actions of anticancer agents, triggering of apoptosis is considered as an indispensable event [19]. Two pathways of apoptosis can be recognized, one is extrinsic and the other is intrinsic and mediated by the mitochondria, with the latter being the target of most of the anticancer agents through the essential actions of the Bcl-2 proteins family in the activation of the caspase-mediated apoptosis [20]. Among the members of this family, some proteins are pro-apoptotic, such as Bax, whereas others are anti-apoptotic, as in the case of Bcl-2 which, upon its activation, hinders the triggering of Bax and thereby permits malignant cells to escape apoptosis [21, 22]. Bcl-2 are enzymes that belong to the protease group and perform two functions, one is the degradation of proteins forming the skeletons of the cell and the nucleus, and the other is the activation of endonuclease enzymes in the cytoplasm which accomplish the degradation of the nuclear material [23]. Furthermore, P53 is another pathway described to play a key role in chemo resistance of human breast cancer cell line (AMJ-13) and human ovary cancer cell line (SKOV-3), supporting their roles as good models for investigating the responsiveness to *Ficus carica* extracts and to ultimately determine whether active materials from fig fruit are sufficiently effective to be used in cancer treatment [24]. Usually, all the previous studies are depended on a crude extract from leave, fruit, and root of fig. Additionally, the extraction of the active compound and their cytotoxicity against SKO-3, AMJ-13 cells and normal HBL cells was not found. Therefore, the main objective of this study is for the first time to find out if this compound *2-benzhydrylsulfinyl-N-hydroxyacetamide-Na* has the ability to inhibit cancer cells and thus use it as an anticancer therapeutic agent. This is achieved only through a series of laboratory experiments for this compound. Thus, the present study has aimed to: (i) selective sequential extracting processes from fresh and dried figs to precipitate and isolate the effective *compound 2-benzhydrylsulfinyl-N-hydroxyacetamide-Na* from 11 extracts and 7 compounds extracted from the aquatic and organic layer found on whole figs fruits. (ii) study the chemical characterization of 2 *-benzhydrylsulfinyl-N-hydroxyacetamide-Na* active compound by various spectral technique. (iii) anti-proliferative of 2-*benzhydrylsulfinyl-N-hydroxyacetamide-Na* on human breast cell line (AMJ-13, human ovary cancer cell line (SKOV-3) and HBL cell, and its ability to pro-apoptotic effects on same human cell line, Mitochondrial membrane potential (MMP) Function, RT-PCR was used to detect changes in the expression of Bax and Bcl-2, P53, caspase-8 proteins. and finally the toxicity of *2-benzhydrylsulfinyl-N-hydroxyacetamide-Na* in vivo.

Materials and methods

Unless elsewhere referred to, all solutions and materials used in the current study are of analytical grade. Also, the experiments were conducted using deionized water (resistivity of 18.2 Ω-cm at 25 °C).

Collection of fig fruit samples

The fresh and dried figs were obtained from the endemic plant collected during July 2018 in Al Kifl, a town located on the Euphrates River to the south-east of Iraq on the road connecting the cities of Al-Hilla and Al-Najaf. (Is a town in southeastern Iraq on the Euphrates River, between Najaf and Al Hillah) on July 2018. A plant sample was classified by an herbalist from the Center of Agricultural Research and Applied Natural Sciences, University of Basra, and dedicated to the annex number 0031150.

Extraction of crude extracts

After collection and proper washing of ripe fig fruits with water to remove dust and impurities, 4 kg were weighed out, the fruits were cut up into small pieces (up to 1 g per piece). Both fresh and dried fruits were selectively and sequentially extracted with the use of increasingly polarized solvents as described below. This process includes the extraction and isolation of fatty components from black ripe fig fruits using suitable volumes of *n*-hexane in the bottom round flask of a Soxhlet apparatus. The process was performed at 75–80 °C temperature for continuous 16 h using 3-cm thimble tubes to prevent the blockage of the Soxhlet tubes. Reflux with the same solvent was performed until the red color of *n*-hexane has disappeared in the condenser Soxhlet. The volume of the *n*-hexane extract was further reduced to the lowest possible volume by a rotary evaporator. The solid plant material which was extracted from the organic components was moved into a Petri plate to dry out at room temperature, while the leachate was filtered using a 0.45 µm filter paper (Millipore-USA). After purification, the filtered material was placed in a separating funnel and a 50:50% of ethanol:water solution was added to the pure leachate. After shaking for 20 min, the solution of the mixture in the separation funnel was separated into two layers; the aquatic layer (aq1) and the organic layer (OR1). The pure liquid filter *2-benzhydrylsulfinyl-N-hydroxyacetamide* was then measured by GC-mass, ^{1}H-NMR, ^{13}C-NMR, and FT-IR. A number of fatty acids and other organic components were detected and removed from the extracts of both layers [25].

Extraction of alkaloids and nitrogen substances

A quantity of the fat-free, dried plant was placed in a circular flask containing an appropriate volume of 5% hydrochloric acid and 70% anhydrous solution. The mixture was replaced each 4 h and then filtered in a Buckner funnel. Rotary evaporation was applied next to reduce the volume of the filtration extract to a quarter of the original volume. Using a separating funnel, a mixture of ethyl acetate and chloroform (30:30) with some drops of ammonium hydroxide was added to extract the aquatic solution layer. Two layers were again produced, one aqueous (aq2) and one organic (OR2). A number of organic compounds with different molecular weights were obtained from the organic and aquatic classes in the fig fruit extract found in each of the layers by mass spectrometry, NMR and infrared spectroscopy. Table 1 shows all compounds that extract from two layers and measured by GC-mass Technique [26]. The liquid compound *2-benzhydrylsulfinyl-N-hydroxyacetamide* was isolated and diagnosis from the aquatic layer.

Characterization of plant extract

Eighteen compounds derived from the fruit extract were found and characterized by UV–Vis (Jena Model 1100, UV–Visible spectrophotometer, Germany), FT-IR, and GC-Mass (Tolerance = 5.0 PPM, DBE: Min = − 1.5, max = 50.0, isotope cluster parameters: separation = 1.0, abundance 1%). ^{1}H-NMR was used with Magnus type AVIIIHD4005 mm PABBO BB/zg30, TD65536, SWH8012.820 Hz, 1HP1 10.00 usecPLW1 16.00000000 W, CHANNEL f1 SFO1 400.2324716 MHz. ^{13}C-NMR. Measurement using Magnus type AVIIIHD 400 5 mm PABBO BB/zg30, TD 65536, SWH 8012.820 Hz, CHANNEL SFO1 100.6525060 MHz-NUC1 13CP1 10.00 usecP13 2000.00 was also performed. For the purposes of the current work, only *2-benzhydrylsulfinyl-N-hydroxyacetamide* was used to evaluate anticancer activity.

Percentage yield of solid compound *2-benzhydrylsulfinyl-N-hydroxyacetamide-Na* (adrafinil form) extracts

After the liquid compound *2-(benzhydrylsulfinyl)-N-hydroxyacetamide* ($C_{15}H_{15}NO_3S$) has been converted into a solid sodium compound, *2-(benzhydrylsulfinyl)-N-hydroxyacetamide-Na* ($C_{15}H_{15}NO_3NaS$). The percentage yield of the approximately 1 kg from the air-dried powdered fig, mean percentage yields of 16.0%. The practical all components analysis are in agreement with the theoretical formula of sodium adduct of ($C_{15}H_{15}NO_3S$) [27]. The spectroscopic of sodium adduct of ($C_{15}H_{15}NO_3S$) was recorded to prove the expected structure which has the molecular formula $C_{15}H_{15}NO_3NaS$, as that is shown in Table 1. A number of solvents were used for the purpose of precipitation and isolation of the biologically active compound $C_{15}H_{15}NO_3NaS$. Methanol (SD = 0.24) 12.5%, chloroform 0.5% (SD 0.23), ethyl acetate 2% (SD 0.22) and *n*-hexane 1% (0.43) Table 2. Methanol extract gave the highest percentage yield at 10 °C. Most of the components extracted were of a polar nature.

The sodium adducts of $C_{15}H_{15}NO_3NaS$ were extracted by the Methanol [28]. Physical properties of the compound

Table 1 The compounds discovered by GC-mass technique in the aquatic (aq) and Organic (OR) extract for the black mature

No. of aq	RT (min)	Name of the compounds	Molecular formula	MW g/mol	Peak area%
$aq_{1.}$	4.890	1-Tetradecene	$C_{14}H_{28}$	196.378	1.14
$aq_{1.}$	5.818	2(1H)-Pyridinethione,3-hydroxy-	C_5H_5NOS	127.161	1.15
$aq_{1.}$	5.115	(2-benzhydrylsulfinyl)-*N*-hydroxyacetamide)-Na	$C_{15}H_{15}NO_3S$	289.35	1.02
$aq_{1.}$	4.97	2-(benzhydrylsulfinyl) acetamide	$C_{15}H_{14}NO_2S$	272.18	2.33
$aq_{1.}$	4.195	Benzyl chloride	C_7H_7Cl	126.58	1.82
$aq_{1.}$	5.58	Benzyl methyl ketone	$C_9H_{10}O$	134.18	1.14
$aq_{1.}$	13.597	2,6,10,14,18,22-tetracosahexaene,2,6,10,15,19,23-hexamethyl-,(all-E)-	$C_{30}H_{50}$	410.718	1.44
$OR_{1.}$	12.124	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270.4507	2.17
$OR_{1.}$	13.32	9-Octadecenoic acid, methyl ester,(E)-	$C_{19}H_{36}O_2$	296.4879	1.13
$OR_{1.}$	13.34	9,12,15-Octadecatrienoic acid, methyl ester, (z, z, z)-	$C_{19}H_{32}O_2$	292.4562	2.32
$OR_{1.}$	5.032	2-benzhydrylsulfinyl)-*N*-sec-butylacetamide	$C_{19}H_{23}NO_2S$	329.35	2.07
$OR_{1.}$	5.78	4H-Pyran-4-on,2,3-dihydrro-3,5-dihydroxy-6-methyl-	$C_6H_8O_4$	144.1253	1.97
$OR_{1.}$	6.704	2-Furancarboxaldehyde, 5-(hydroxymethyl)-	$C_6H_6O_3$	126.111	1.17
$aq_{2.}$	1.561	Benzyl chloride	C_7H_7Cl	126.58	0.19
$aq_{2.}$	4.890	2-Tetradecene,(E)-	$C_{14}H_{28}$	196.378	1.22
$aq_{2.}$	16.49	Phenol,2,4-bis(1,1-dimethylethyl)-	$C_{14}H_{22}O$	206.329	1.84
$3aq_{2.}$	17.190	Urs-12-en-24-oic acid, 3-oxo-, methyl ester,(+)-	$C_{31}H_{48}O_3$	468.722	2.59
$OR_{2.}$	18.400	Lup-20(29)-en-3-ol, acetate, (3.beta.)-	$C_{32}H_{52}O_2$	468.754	3.56

Table 2 Amount and yield of *2-benzhydrylsulfinyl-N-hydroxyacetamide-Na* extracted from fig fruit

Extract	Amount (g)	Yield (w/w) %
Methanol	0.125	12.5
Chloroform	0.005	0.5
Ethyl acetate	0.02	2
Hexane	0.01	1

$C_{15}H_{15}NO_3NaS$ is the pale orange compound as a precipitate powder, stable in air and non-hygroscopic compound.

UV spectral diagnosis of extracted compounds

Using UV spectral for detection and evaluation at different wavelengths indicated the presence of 18 active compounds in two organic layers and two aquatic layers extracted from fig fruits, of which 11 aqueous degradation compounds and 7 compounds in the organic layer. Attention was paid to studying and diagnosing absorption spectra of the compound (*2-benzhydrylsulfinyl-N-hydroxyacetamide*)*-Na*. By means of the standard solution of the extracted compound that is converted into a sodium form (*2-benzhydrylsulfinyl)-N-hydroxyacetamide-Na,* the directly was the measurement for the compound (1.0 ml) of diverse concentrations (2.0, 4.0, 6.0, 8.0. and 10.0) µg/ml volume 1.0 ml. The absorbance of the (*2-benzhydrylsulfinyl*)*-N-hydroxyacetamide-Na* was measured at 280 nm with double beam UV–Vis spectrophotometer against blank (deionized water). The quantity of $C_{15}H_{15}SO_3NaS$ was calculated from the linear regression equation got from the quercetin standardization. The $C_{15}H_{15}SO_3NaS$ was determined using a linear regression equation achieved from the standard plot of $C_{15}H_{15}SO_3NaS$. This compound was calculated as mean SD ($n = 3$) and expressed as mg/g $C_{15}H_{15}SO_3NaS$ equivalent to dry extract [29].

MTT assay

Cells (1×10^5 cells mL^{-1}) were seeded in RPMI + 10% FCS within 96-well micro-titer plates. Overnight incubation was performed to reach efficient attachment of cells, which were next treated with an inhibitory concentration (IC_{50}) of *2-benzhydrylsulfinyl-N-hydroxyacetamide-Na* followed by further incubation for 72 h. After three washing steps with PBS, staining with 2 µg mL^{-1} MTT and incubation for 3 h at 37 °C [30]. Following addition of DMSO to the cells, a final step of absorbance reading was performed with a microplate reader at 492 nm, while growth inhibition rate was extracted as in the following equation [31]:

$$\text{Inhibitionrate}\% = (A - B/A) \times 100$$

where A and B represent optical densities for the control and treated samples, respectively.

Crystal violet staining of cancer cells

After 24 h of treatment of cancer cells with the IC_{50} dose of the extracted active compound, replacement of the culture medium with 100 µL crystal violet was performed for 10 min. Careful washing of cells with tap water was then applied for three times, followed by rinsing with DW and air-drying. Morphological properties of the cells were finally examined with the phase contrast inverted microscope [32].

AO/EtBr staining

Based on our previously described protocol, cells were treated with *2-benzhydrylsulfinyl-N-hydroxyacetamide-Na* (IC_{50}) within 96-well plates, incubates for 24 h. After incubation time, the cells were washed for three times with PBS, stained with 100 µL of AO/EtBr for 5 min, and finally visualized with a fluorescent microscope [33].

DAPI staining for the nucleus

For this test, cells were subjected to 12-well plating, 24 h incubation with *2-benzhydrylsulfinyl-N-hydroxyacetamide-Na* (IC_{50}), PBS washing, and 30 min fixation with 1% glutaraldehyde. After removal of fixative with PBS, 30 min DAPI staining was performed, followed by another PBS washing, and final fluorescence microscopic observation of the morphology of the nucleus microscopy BX51 UV fluorescent microscope (Olympus, Tokyo, Japan) [34].

Flow cytometry

Apoptosis detection using flow cytometry was done according to manufactured protocol. SKOV-3, AMJ-13, and HBL cells treated by *2-benzhydrylsulfinyl-N-hydroxyacetamide-Na* were analyzed by determining the ratio of cells with nucleus concentration and fragment. Cells were seeded for 24 h then treated with *2-benzhydrylsulfinyl-N-hydroxyacetamide-Na* at IC_{50} concentration. Cells were suspended in the FACS buffer. Then, all treated and non-treated cancer cells were stained with special marker annexin V-FITC (Invitrogen, Carlsbad, CA) and measured to investigate the apoptotic cells using flow cytometer assay.

Mitochondrial membrane potential assay

Simultaneous determination of the critical apoptotic events in the cells treated with *2-benzhydrylsulfinyl-N-hydroxyacetamide-Na,* dye (JC-1) for probing the membrane potential of the mitochondria was employed. In brief, 24 h seeding of cells in 96-well plates was followed by treatment with the IC_{50} dose of the extracted active compound and staining with MMP dyes [35].

Real-time PCR

Treated cells were also tested with respect their expression levels of BAX, Bcl-2, P^{53}, Caspase-8, and Caspase-9 mRNA using QPCR. The primer sets which are used in our experiments were calculated based totally on the standard sequences from the NCBI database. The sequences of primers used within the quantitative RT-PCR assay involved: 1, Bax (forward: 5′-ATG GAG GGGTCC GGG GAG-3′ (reverse: 5′-ATC CAG CCC AACAGC CGC-3′). 2, Bcl-2 (forward: 5′-AAG CCG GCG ACGACT TCT) (reverse: 3′-GGT GCC GGT TCA GGTACT CA). 3, P53 (forward:5′-CCGTCGCAAGCAATGGATG-3′) (reverse:5′-GAAGAT GACAGGGGCCAGGAG-3′). 4, Caspase-8 (forward: 5′-GACCACGACCATTGA AGAGCTTC-3′) (reverse: 5′-CAGCCTCATCCGCG ATATATC-3′); and 5, Caspase-9 (forward: 5′-C TCTTGAGCTGTGGCTGGTC-3′) (reverse: 5′-GCT GATCTATGACCGATACT-3′). Based on the suppliers' protocol, total RNA was purified from the cells (RNeasy Mini kit, Qiagen. Cat. No. 74104, UK) and treated with DNase, while the product was treated with Superscript II reverse transcriptase for the synthesis of cDNA (Invitrogen. Cat. No. 18064-071, USA). For quantitative reverse transcription polymerase chain reaction (q RTPCR), each reaction contained 1 µL cDNA, 7.5 µL SYBR green, 0.3 µL ROX, and 0.3 µL related primers; the final quantity was topped up to 15 µL via adding 5.6 µL of distilled water. Fast SYBR Green master mix was applied (Applied Biosystems. Cat. No. 4385612, UK) with 7900HT fast system (Applied Biosystems). Following normalization of the levels of expression of genes to TATA-binding protein (TBP), their mean relative values were calculated based on adopted methods (Livak and Schmittgen) [36]. Triplicate measurements were used in all experiments.

Toxicity of *2-benzhydrylsulfinyl-N-hydroxyacetamide-Na* in mice

Balb/c mice (20–30 g, 6–8 weeks) were arbitrarily divided into five groups ($n = 5$), and kept in polypropylene cages with wood dust under standard experimental conditions (24–26 °C, 55–65% RH, 12 h light/dark cycle), while both water and food were provided *adlibitum*. The experiments were approved by the Animal Ethical Committee, Biotechnology Division, Applied Science Department, University of Technology, Baghdad, Iraq. In Group 1 (control), intraperitoneal (i.p.) injection of PBS (250 µL) was applied, whereas Groups 2, 3, 4, and 5 received i.p. injections of *2-benzhydrylsulfinyl-N-hydroxyacetamide-Na* (1 mg kg^{-1}) for 1, 2, 3, and 4 weeks, respectively. Following end of the injection periods, mice were anesthetized with sodium pentobarbital and cardiac puncture was used to collect blood which was subjected to standard serum extraction procedures. Based

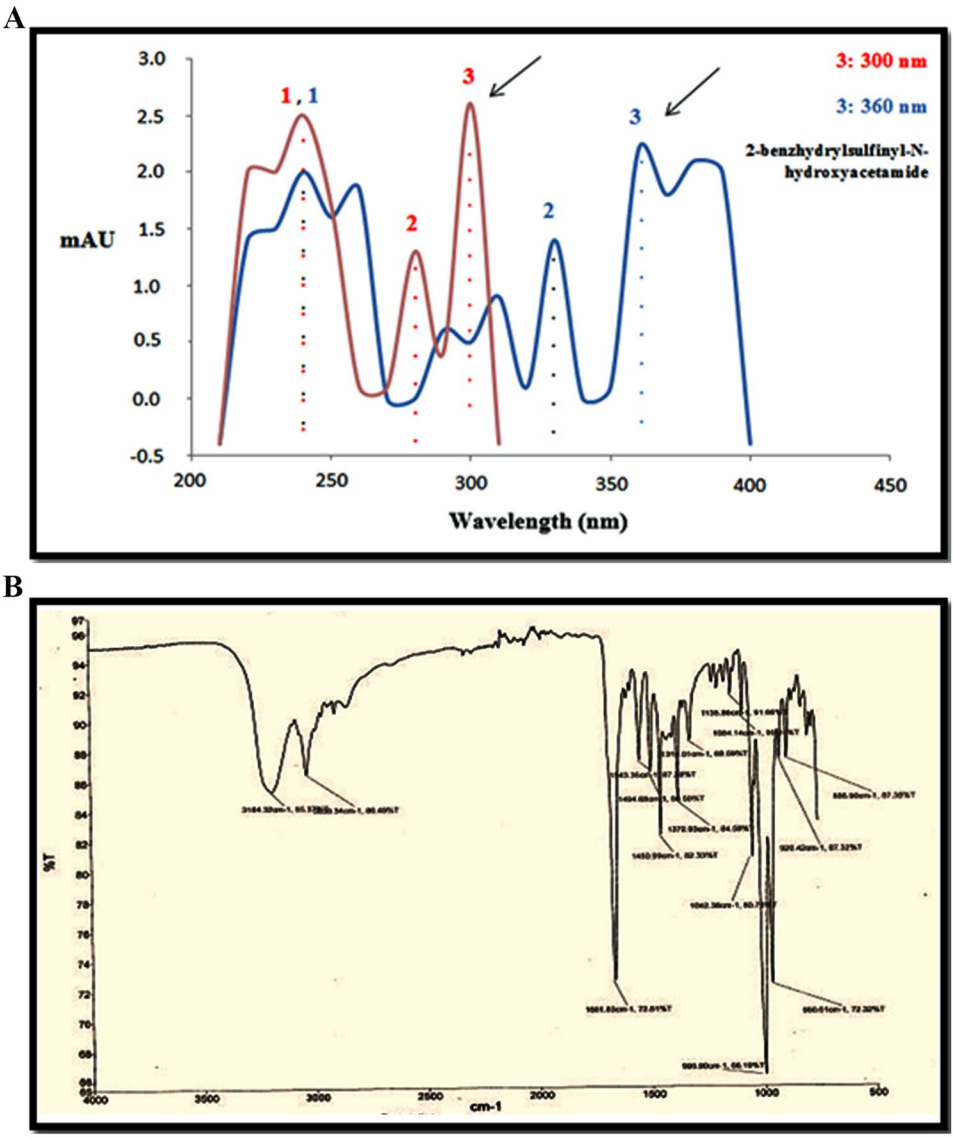

Fig. 1 UV–visible spectrum and FT-IR for *2-benzhydrylsulfinyl-N-hydroxyacetamide-Na*

on suppliers' protocols, serum levels of enzymes related to the liver function (GOT, GPT, and alkaline phosphatase), whereas excision, fixation, and haematoxylin–eosin staining steps were applied and the resulting sections were microscopically examined for possible treatment-associated tissue alterations [37].

Statistical analysis

Graph Pad Prism version 6 (Graph Pad, USA) was employed to analyze the data which were represent as mean ± SEM. Unpaired *t* test was selected to detect differences with statistical significance at a limit of $p < 0.05$ [38].

Results and discussion

Characterization of *2-benzhydrylsulfinyl-N-hydroxy acetamide-Na*

In the UV/Vis technique, the concentration of *2-benzhydrylsulfinyl-N-hydroxyacetamide-Na* crude extract was determined through the use of a linear regression equation ($y = 0.010x + 0.040$, $R^2 = 0.9998$) resulting of the standard plot for the compound. The highest concentricity of the crude extract of the compound following the use of different solvents was recorded with methanol, followed by ethyl acetate, hexane and chloroform (94 ± 2.3, 88.56 ± 3.53,

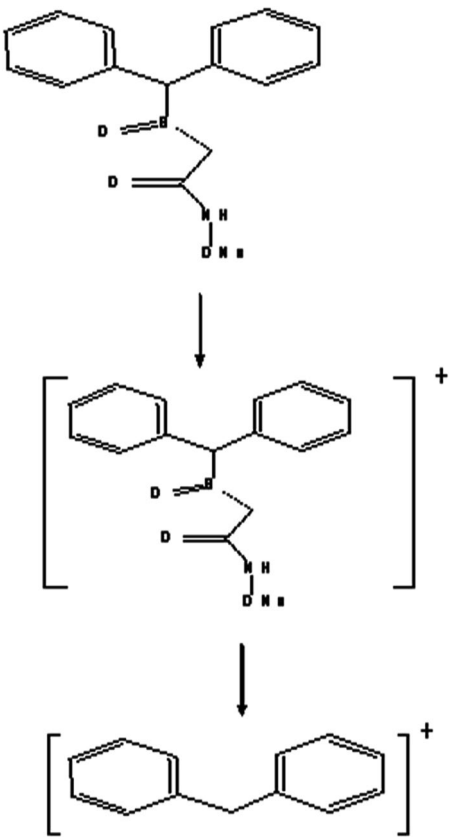

Fig. 2 The chemical structure ($C_{15}H_{15}NO_3NaS$) of *2-benzhydrylsulfinyl-N-hydroxyacetamide-Na*

82.53 ± 1.65, 0.150 ± 0.02, respectively), with significant differences observed at $p \leq 0.001$. As indicated by the results, the concentration of the crude extract obtained by the chloroform solvent was very low. As shown in Fig. 1a, three main regions can be observed in the UV–visible spectrum absorbance. First, a group of spectral beams located at approximately 210–240 nm. These packages represent two benzene rings in the compound and result from π–π* transition (packages 1.1). Second, a strong broad beam located between 280 and 330 nm given by the sulfur, oxygen and nitrogen groups, resulting from transfers of the type n–π* (packages 2: 2). Third, a very strong beam located within 300–360 nm, which represents the maximum absorption values and results from transitions of the type δ–δ* (beams 3:3). These beams represent the ultraviolet region spectra extending to the beginnings of the visible region, which include all absorption spectra of the compound. Transitions as a whole confirms the physical diagnostic character of the compound.

The infrared spectrum assay

The infrared spectrum of ($C_{15}H_{15}NO_3NaS$) is shown in Fig. 1b, with positions and intensities of peaks which correspond to various groups of these components. The IR spectrum of ($C_{15}H_{15}NO_3NaS$) shows a broad band centered at 3148 cm^{-1} due to an N–H extending. A weak band at 3033 cm^{-1} is attributed to an aromatic C-H extending while several weak bands at the range of 886–710 cm^{-1} are impute to the bending of C–H. A weak band at 2091 cm^{-1} can be assigned to aliphatic C-H extending. Also, the ($C_{15}H_{15}NO_3NaS$) IR spectrum displays two strong bands at 1661 cm^{-1} and 1450 cm^{-1} which can be impute to asymmetrical and symmetrical bands of stretching (C=O). The symmetrical extending vibration of the aromatic C=C is at 1490 and 1372 cm^{-1}, respectively. Finally, the adrafinil spectrum expressions three strong bands at 1042, 995, 960 cm^{-1} which can be referred to C-N, S=O, and N–O stretching bands, respectively (Fig. 2).

GC-mass analysis

As seen in Fig. 3, the gas chromatography/mass spectrum (GC/MS) of sodium adduct of ($C_{15}H_{15}NO_3NaS$) was performed in the electron ionization mode monitored at m/z 312 and 167 ions, and revealed monoisotopic mass (Fig. 3a), mass spectrum (Fig. 3b) and single mass analysis (Fig. 3c) for the *2-benzhydrylsulfinyl-N-hydroxyacetamide-Na* structures. The two essential peaks are the important characteristic fragments of sodium adduct of ($C_{15}H_{15}NO_3NaS$) which confirm the suggested structure of the extracted component. The *2-benzhydrylsulfinyl–N-hydroxyacetamide-Na* compound was analyzed using GC-Mass to find the molecular ion for each fragment, which were shown to be equal to formula weight minus one. All diagrams confirm the weights of all the molecules in the compound, indicating sufficient isolation and identification of the extracted compounds. The results of the GC–MS measurement of the bioactive components of the different fig extracts are shown in Table 3. In general, previous studies revealed that all the extracted compounds can be more precisely detected through the application of the GC–MS technique (MSDCHEM\1\METHODS\MUAFAQ.M) to determine M/Z negative ions with 30 m length × 250 mm diameter, and 0.25 mm thickness of the film. GC–MS spectroscopic determination involved a system for ionization of electrons that employed electron with elevated energy level (70 eV). Helium gas with 99.995% purity was employed as a carrier gas (flow rate = 1 mL min^{-1}). The

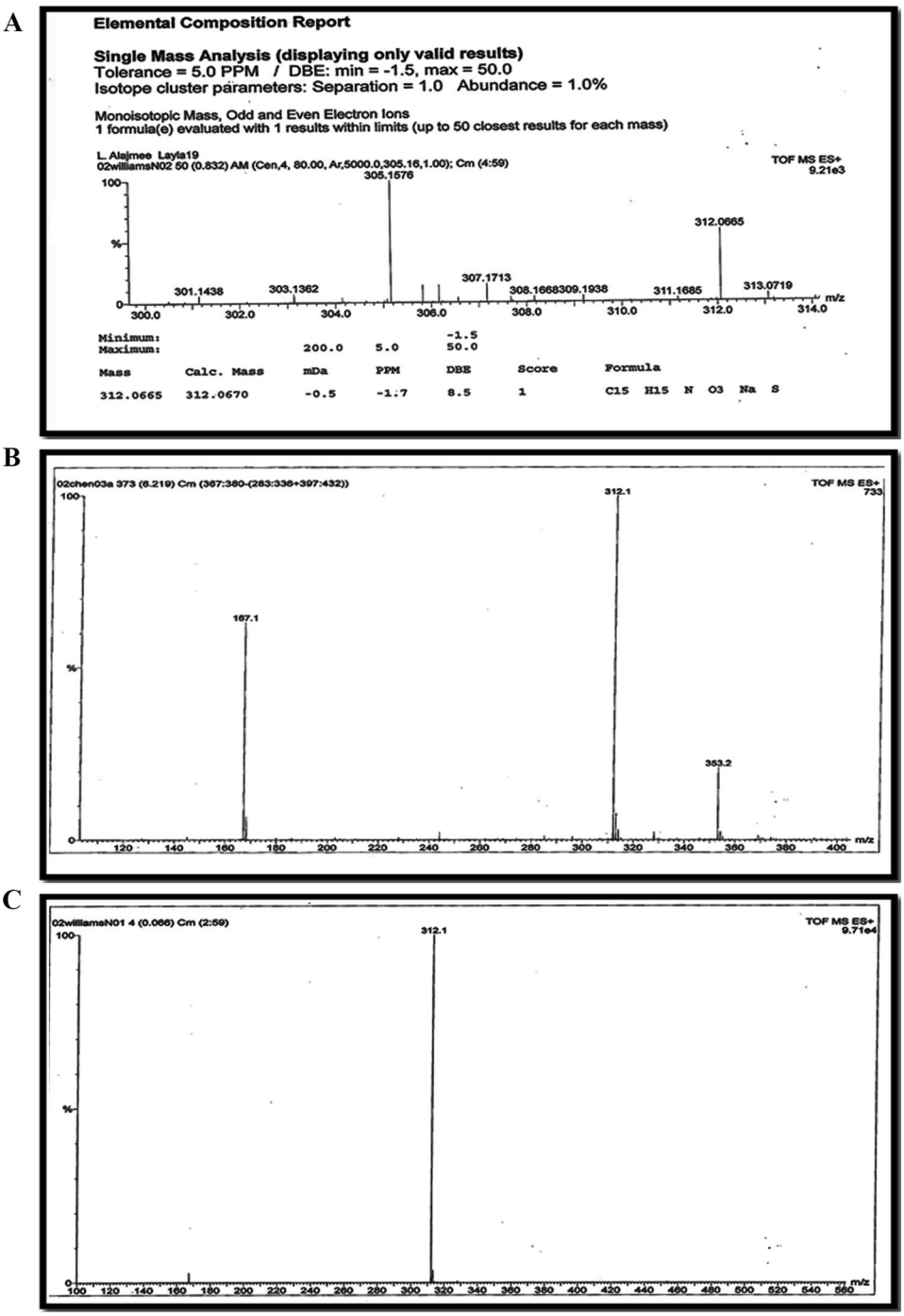

Fig. 3 Mass spectrum of purified *2-benzhydrylsulfinyl-N-hydroxyacetamide-Na*. **a** Monoisotopic mass for the *2-benzhydrylsulfinyl-N-hydroxyacetamide-Na* structures. **b** Mass spectrum for the *2-benzhydrylsulfinyl-N-hydroxyacetamide-Na* structures. **c** Single mass analysis for the *2-benzhydrylsulfinyl-N-hydroxyacetamide-Na* structures

system was initially set at 100–250 °C and a rate of elevating temperature that is equal to 3 °C per minute, along with a 10 min retention time. In addition, an increased temperature up to 400 °C was reached at an elevation rate of 10 °C per minute. The splits method was employed to vaccinate 1 µl of the extract which was diluted with special solvents. One microliter of the intended 1% of the extracts diluted with specific solvents was vaccinated by the splits method. The comparative amount of the chemical components found in each of them extracted figs was presented as a percentage, depending on the peak area resulted in the chromatogram. Figure 2 shows the pattern of the purified 2-*benzhydrylsulfinyl-N-hydroxyacetamide-Na* with the fragments of the basic compound after being placed in the mass spectrometry device. We show the mass of the mother compound and the fragments derived from it, represented by the active compounds S=O, NH and $O^{-}Na^{+}$.

Table 3 Specific identification of fig fruit extracts in the GC-mass spectrum and separation data analysis parameters

Column	HP-5MS, 5% Phenyl methyl Sillox (1629.5), 30 m × 0.250 µm I.D. × 0.25 µm, SS., Inlet He
EMV mode	Gain factor (1.00)
Resulting EM voltage	1306
Low mass	301.1438
High mass	312
Threshold	149
Minimum quality for all fig extracts	95–99%
Flow rate	1 ml/min
Run time	24 min
Hold up time	1.5388 min
Solvent delay	2.59 min
Average velocity	36.796 cm/sec
Temperature	Initial 100 °C to maximum 400 °C
Pressure	8.5 Psi

^{1}H-NMR spectrum analysis

MeOD was employed as a solvent agent during the recording the ^{1}H-NMR spectrum of the sodium adduct of ($C_{15}H_{15}NO_3NaS$) while all the protons were detected in their regular regions (Fig. 4a). At the NH group, the proton is responsible for the signal at δ 7.80 ppm exerted by the sodium salt of ($C_{15}H_{15}NO_3NaS$). At δ 7.36, 7.42 and 7.38 ppm, the proton signals of the aromatic rings of this compound were represented by doublet signals (attributed ti H1′, H5 and H5′; H2, H2′, H4 and H4′; and (H3 and H3′, respectively). Two signals that appeared at δ 5.45 and 3.45 ppm can be considered as belonging to aliphatic protons of CH and CH2 groups, respectively.

^{13}C-NMR spectrum analysis

^{13}C-NMR spectrum for the sodium salt of ($C_{15}H_{15}NO_3NaS$) provided further evidence about the characterization of the extracted components, showing low field signals of carbonyl carbon atoms (C1) at 162.20 ppm, being consistent with previously published works. The high chemical shift of the carbonyl carbon signal can be associated to the presence of the oxygen atom which are highly electronegative. ^{13}C-NMR spectrum shows high field signals at 52.59 and 70.74 ppm which can be related to the aliphatic carbon atoms C2 and C3, respectively. Generally, the other signals between 135.50 and 128.24 ppm can be related to the aromatic carbon atoms C4–C9 and C4′–C9′ as, shown in Fig. 4b.

2-Benzhydrylsulfinyl-N-hydroxyacetamide-Na inhibits cancer cells proliferation

In present study, we tested the cytotoxic impacts of *2-benzhydrylsulfinyl-N-hydroxyacetamide-Na* at the different indicated concentrations against cancer cells, using the MTT assay. Treatment of cells with this compound for 72 h caused a significant decrease in the proliferative activity of SKOV-3 and AMJ-13 cells. Figure 5 demonstrates this effect with the different concentrations of the compound (100, 50, 25, 12.5, 6.25 µg mL^{-1}) in comparison with the untreated cells. The highest cytotoxic activity was recorded when the compound was used at IC_{50} values. These results propose selectivity towards cancer cells when the compound exerts its proliferation inhibition impacts. The physical properties of the pale orange compound *2-benzhydrylsulfinyl-N-hydroxyacetamide-Na* are peculiar while its biological actions are remarkable. Khodarahmi et al. showed that approximate IC_{50} values of 10–20 µg mL^{-1} of the fruits, leaves, and latex of *Ficus carica* show cytotoxicity against HeLa cell line [39]. In another study, that plant extracts had the ability to significantly decrease cell viability, an effect that depended on both dose and time [40]. Rubnov et al. showed that fig's latex has anti-proliferative activity in several cancer cell lines [41]. In vivo studies performed via Ullman et al. exposed that fig's fresh latex has antitumor activity in xenografted and spontaneous tumors in mice [42].

2-Benzhydrylsulfinyl-N-hydroxyacetamide-Na induce apoptosis in SKO-3, and AMJ-13 cells

Apoptosis is primarily characterized by biochemical pathways that depend on energy and morphological properties that are unique. Apoptosis is a critically important cellular homeostatic regulation mechanism for controlling the population size of various cell types. Cells undergoing early stages of apoptosis characteristically exert chromatin condensation pyknosis as well as cell shrinkage, with the former being the most crucial event and the latter being a consequence of condensed organelles and dense cytoplasm. In the late stages of apoptosis are associated with massive formation of buds or blebs from the plasma membrane which are tightly loaded with cell organelles [43]. The current study involved the examination of the ability of *2-benzhydrylsulfinyl-N-hydroxyacetamide-Na* to induce apoptosis through the use of fluorescence microscopy. Following treatment with *2-benzhydrylsulfinyl-N-hydroxyacetamide* for 24 h, alterations in the morphology of the treated and untreated SKOV-3 and AMJ-13 cells. While the control cells retained intact structure (Fig. 6),

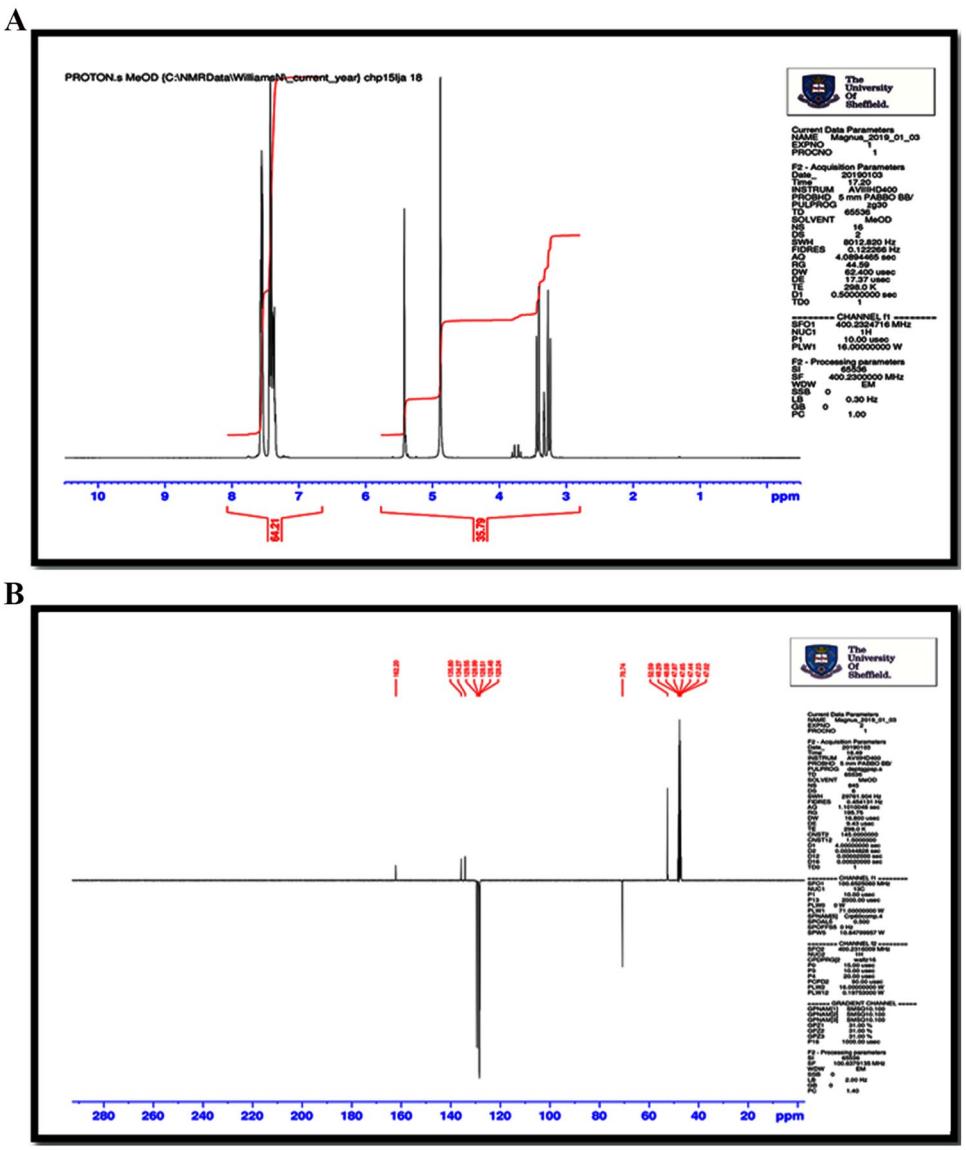

Fig. 4 ^{1}H-NMR and ^{13}C-NMR of *2-benzhydrylsulfinyl-N-hydroxyacetamide-Na*. **a** Spectrum of ^{1}H-NMR for *2-benzhydrylsulfinyl-N-hydroxyacetamide-Na*. **b** Spectrum of ^{13}C-NMR for *2-benzhydrylsulfinyl-N-hydroxyacetamide-Na*

cells treated with the *2-benzhydrylsulfinyl-N-hydroxyacetamide-Na* showed obvious indications of apoptosis, as manifested by shrinkage of the cytoplasm and formation of membranous blebs. Cells suffering early apoptosis were identified through the incorporation of the AO stain into the fragmented DNA, leading to the emission of fluorescence with bright green color. Following 24 h of the treatment, moderate apoptosis was observed in the form of condensed chromatin and membrane blebbing. Apoptosis-related morphological characteristics were time-dependent. Furthermore, the same treatment caused late apoptosis events that were identified through certain changes that include the binding between ethidium bromide and denatured DNA leading to emission of reddish-orange color. Both cancer cell lines were exposed to the IC_{50} concentration of *2-benzhydrylsulfinyl-N-hydroxyacetamide-Na* for 24 h, with the use of staining with DAPI for observing the morphology of the nucleus. The nuclear morphology of the cells was observed using DAPI staining. The results revealed condensation of the chromatin in the treated cells as opposed to normal morphology of the nucleus in the control ones. To confirm our results, the percentage of apoptotic cells was determined by staining the cancer cells with the annexin V-FITC using the cytometry flow. A sizable increase of apoptosis due to

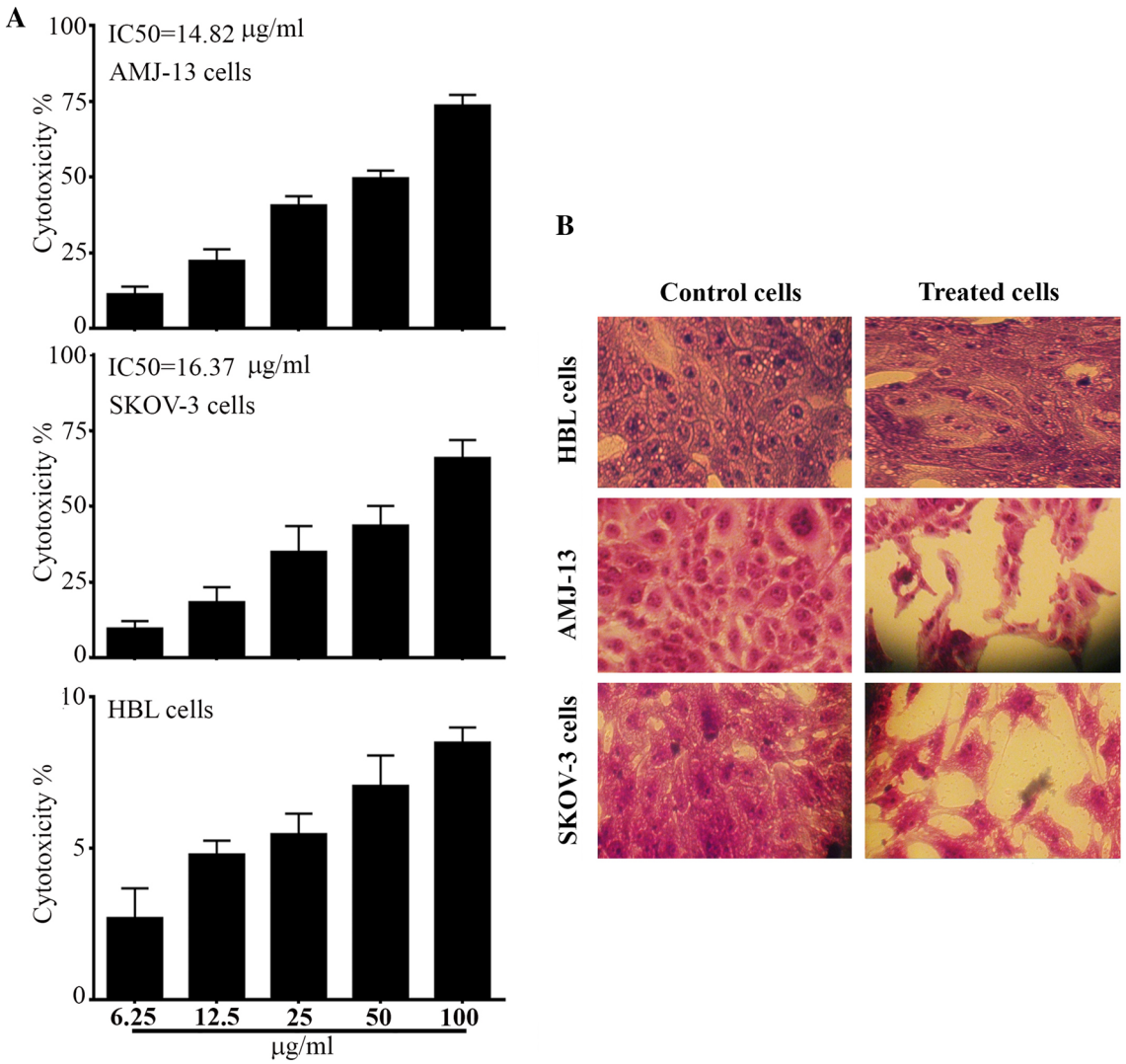

Fig. 5 Anti-proliferative activity of *2-benzhydrylsulfinyl-N-hydroxyacetamide-Na* against AMJ-13, SKOV-3, and HBL cells. **a** The values are expressed as Mean ± SEM. **b** Morphological changes in AMJ-13, SKOV-3, and HBL cells treated with *2-benzhydrylsulfinyl-N-hydroxyacetamide-Na*

2-benzhydrylsulfinyl-N-hydroxyacetamide-Na in SKOV-3, AMJ-13, and HBL cells is obviously presented in Fig. 7. Results of this study showed that the proportion of apoptotic cells increased significantly compared with the control group.

Effects of *2-benzhydrylsulfinyl-N-hydroxyacetamide-Na* on mitochondrial function

The mitochondria take a critical part in the induction of the apoptotic events through different stimuli of cellular death. Alterations in this organ are represented by the loss of its membrane potential (Δψm) and the secretion of cytochrome c to the cytoplasm, resulting in the stimulation of caspase-3 through a caspase-9 pathway. Thus, we tested the impacts of *2-benzhydrylsulfinyl-N-hydroxyacetamide-Na* on the Δψm in SKOV-3 and AMJ-13 cells. The cells were exposed *to 2-benzhydrylsulfinyl-N-hydroxyacetamide-Na* for 24 h, followed by staining with a sensitive dye (JC-1) for probing the membrane potential of the mitochondria, and examination with the fluorescent microscope. JC-1 molecule is known for its selective entry into the mitochondria, based on differential membrane potential, spontaneously forming J-aggregates that emit strong red fluorescence. Treatment of cells caused higher percentage of cells emitting red fluorescence as related to that in the control

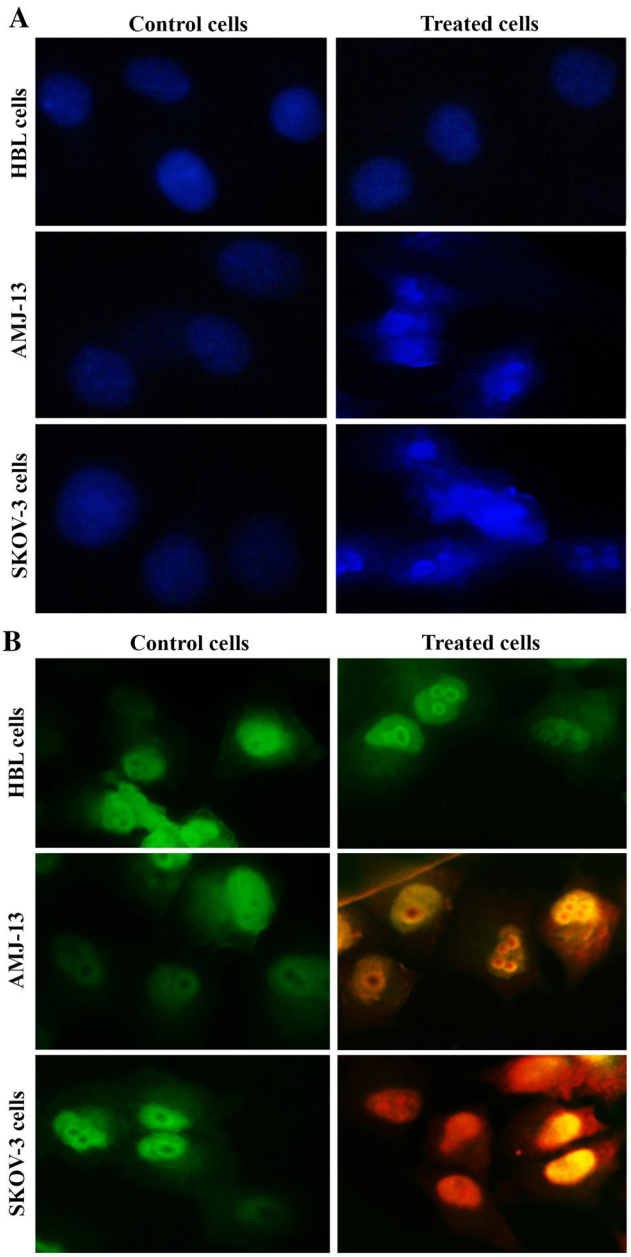

Fig. 6 Apoptosis marker in AMJ-13, SKOV-3, and HBL cells treated with *2-benzhydrylsulfinyl-N-hydroxyacetamide-Na*. **a**, **b** Fluorescent micrographs of DAPI and AO/EtBr-double-stained cells. Scale bar 10 µm

group as in Fig. 8. Loss of the membrane potential leads to cytochrome c release from its location in the mitochondrial inter membrane space into the cytosol. Our findings demonstrate the involvement of mitochondrial dysfunction in the apoptosis of cancer cells induced by treatment with *2-benzhydrylsulfinyl-N-hydroxyacetamide-Na.*

Effect of *2-benzhydrylsulfinyl-N-hydroxyacetamide-Na* in gene expression

Proteins from the Bcl-2 family exert tight regulation on the events initiated by the mitochondria, as in the case of pro- and anti-apoptotic proteins which are controlled by 25 genes to regulate the apoptosis process. Under regulation by the pro-apoptotic protein of Bax family, cytochrome c is dimerized and translocated to the outer membrane of the mitochondria before being released to the cytoplasm. However, this translocation is suppressed by the anti-apoptotic proteins of the Bcl-2 family. The present study involved the measurement of the levels of both types of protein families with the employment of western blot, Immunofluorescent, and QPCR technologies. As shown by the results, treatment of cancer cells with *2-benzhydrylsulfinyl-N-hydroxyacetamide-Na* caused significantly lower Bcl-2 levels as compared to the control, while the same treatment resulted in increased Bax gene expression as well as cells treated with *2-benzhydrylsulfinyl-N-hydroxyacetamide-Na* indicate increase in P53, and caspase-8 expression levels (Fig. 9). Hence, upregulated Bcl-2 and downregulated Bax levels as a result of treatment with the extracted compound could result in MMP loss and, thereby, facilitated release of cytochrome c as well as stimulation of the caspase cascade. Such alterations in the gene expression levels of these two proteins further supported the involvement of a mitochondrial-dependent intrinsic pathway in the induced apoptosis.

Toxicity of *2-benzhydrylsulfinyl-N-hydroxyacetamide-Na* in vivo

In this study, body weight and the liver, kidney, lung, and spleen histopathology were also measured in mice following the i.p. injection of

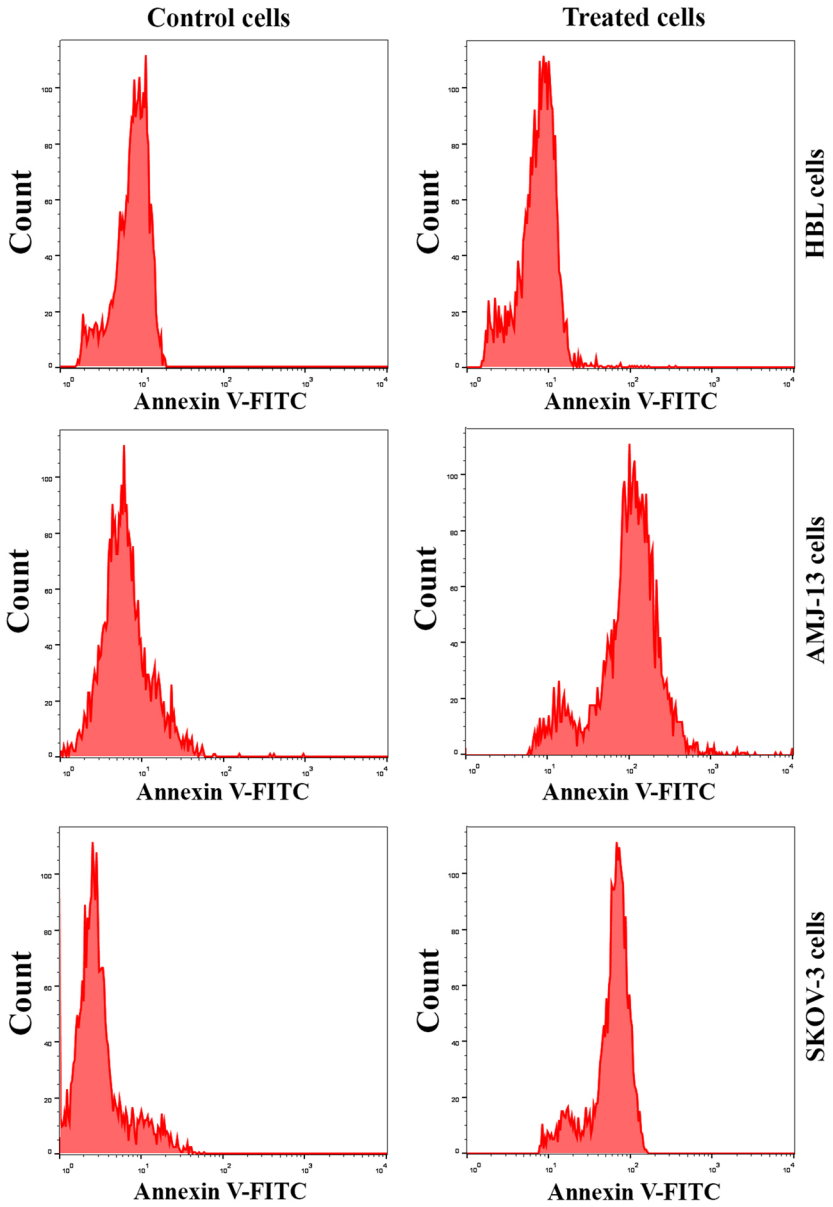

Fig. 7 Flow cytometry results represented proportion of apoptosis cells after treated with *2-benzhydrylsulfinyl-N-hydroxy-acetamide-Na*

2-benzhydrylsulfinyl-N-hydroxyacetamide-Na. The results revealed the absence of significant changes in the animal's body organs as illustrated in Fig. 10. The results revealed the absence of significant alterations in the lungs, spleen, and hepatic cords, with mild vacuolar and hepatic lobules in the treated groups. As well as no significant changes in liver enzymes GOT, GPT, and ALP levels.

Conclusions

In the existing study, we have evaluated for the foremost time the anti-proliferative and pro-apoptotic effects of 2-*benzhydrylsulfinyl-N-hydroxyacetamide-Na* extract separated from *Ficus carica* against SKOV-3 and AMJ-13 cell lines via P53 and caspase-8 pathways. Various raw

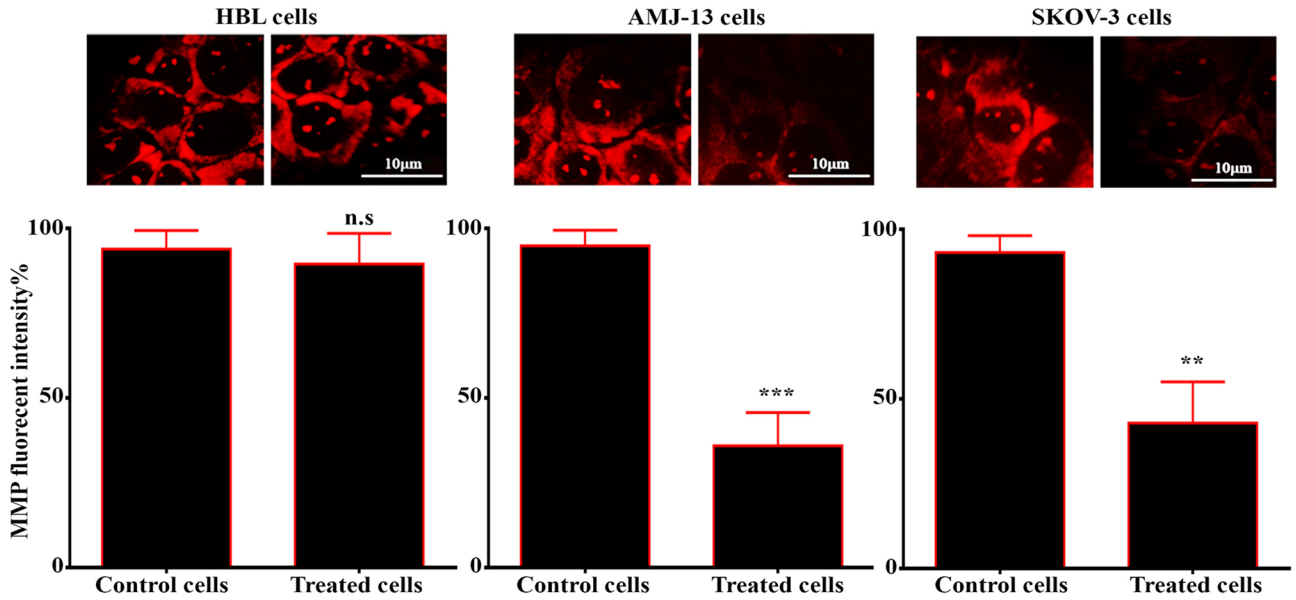

Fig. 8 Effect of *2-benzhydrylsulfinyl-N-hydroxyacetamide-Na* on mitochondrial membrane activity in Control and treated cells. Scale bar 10 µm. The values represents the Mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$

extracts of dried fig fruits were prepared through selective sequential extraction with different polar solvents (methanol, ethyl acetate, chloroform, and *n*-hexane, based on the existence of altered active ingredients in *Ficus* spp. with diverse solubility properties. The results indicated that the number of organic compounds detected in fig extracts using GC-Mass, NMR and other techniques is eighteen aromatic and non-aromatic compounds with medium and large molecular weights that have potential biological effects. However, the identified bioactive compound (*2-benzhydrylsulfinyl-N-hydroxyacetamide-Na*) was one of the most important organic compounds in fig extracts which is less abundant in other fruits and plants. This compound is characterized by unique physicochemical properties since it contains both sulfur and nitrogen elements, being biologically more active than synthetic chemical compounds manufactured due to their synergistic effects. Moreover, the results of the present study proposed the potential biological effects of the *2-benzhydrylsulfinyl-n-hydroxyacetamide-Na* spectrum due to the active groups such as S=O, NH and $O^{-}Na^{+}$. The study assumed the mechanical action of this compound through the association of the electronic couplings of the groups with the walls of the cell causing damage, destruction, and elimination. In addition, the *2-benzhydrylsulfinyl-n-hydroxyacetamide-Na,* shows three strong bands that can be attributed to C–N, S=O, and N–O stretching bands. Chemical analysis of plants point out the existence of many organic chemicals including alkaline flavonoids, tannins, steroids, glycosides, saponins, and other medical organic compounds. The results demonstrated that the *benzhydrylsulfinyl-n-hydroxyacetamide-Na* had anticancer activity against AMJ-13, SKOV-3 cells and provided a confirmation to a role of intrinsic pathways in the induction of apoptosis in these cells. Additionally, more extensive, well-controlled clinical experiments are needed to fully evaluate the potential of 2-*benzhydrylsulfinyl-n-hydroxyacetamide-Na* in terms of the optimal dose, route of administration, cancer targets and potential interactions with other drugs.

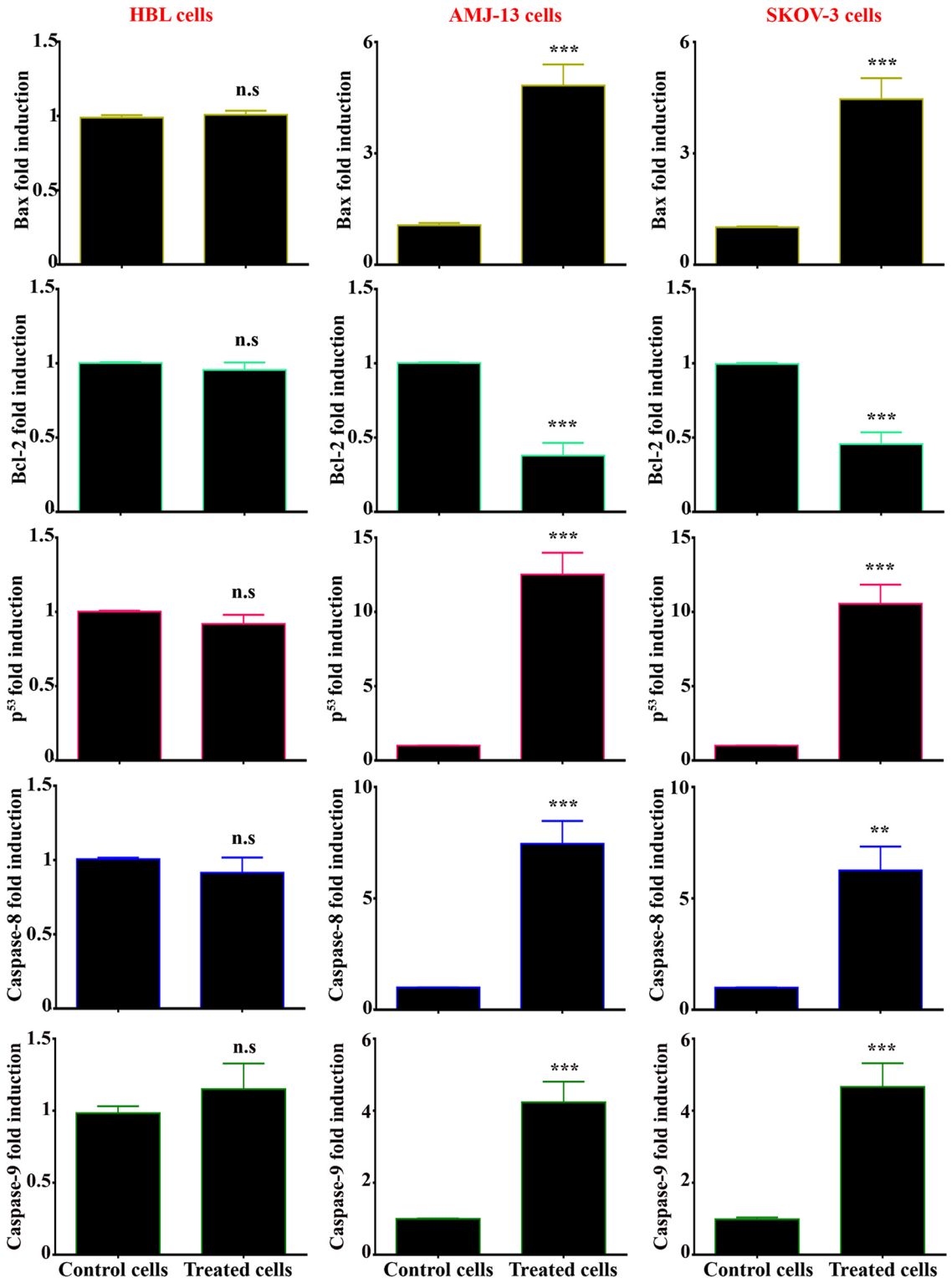

Fig. 9 Effect of *2-benzhydrylsulfinyl-N-hydroxyacetamide-Na* on Bax, Bcl-2, P53, Caspase-8, and Caspase-9 genes expression. Real-time PCR results are expressed as Mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$

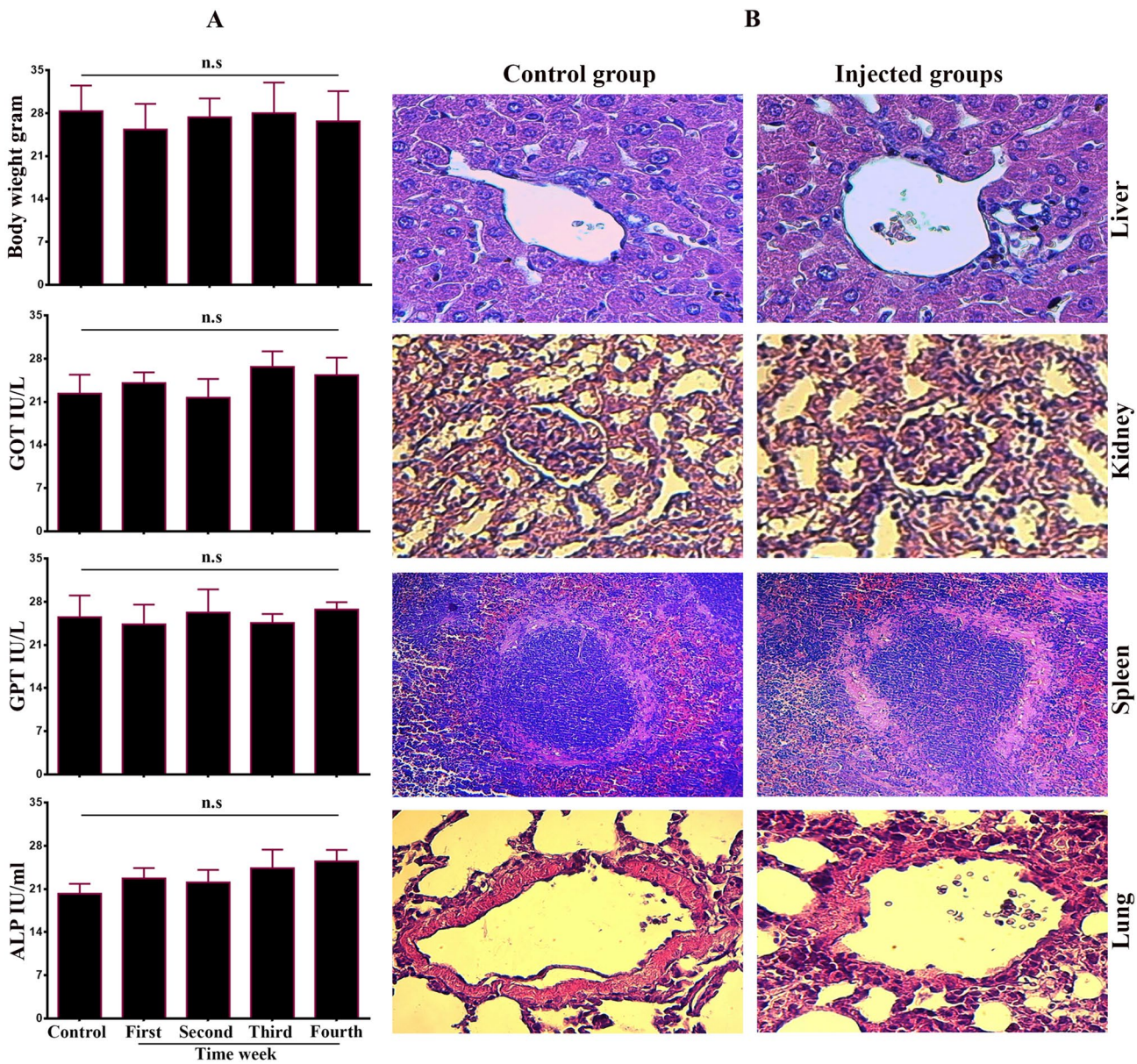

Fig. 10 In vivo effect of *2-benzhydrylsulfinyl-N-hydroxyacetamide-Na* in body weight and serum enzymes level (**a**). Effect of *2-benzhydrylsulfinyl-N-hydroxyacetamide-Na* in organs of injected animals (**b**)

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Compliance with ethical standards

Conflict of interest We authors declare that we do not have any conflict of interest.

Compliance with ethics requirements All Institutional and National Guidelines for the care and use of animals were followed and approved by Animal Care and Ethics Committee at Biotechnology Division, Applied Sciences Department, University of Technology, Baghdad, Iraq according to Ref. No. AS 569/12/01/2019.

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