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Phenolic compounds from *Syzygium jambos* (Myrtaceae) exhibit distinct antioxidant and hepatoprotective activities *in vivo*

Mansour Sobeh[a,*], Ahmed Esmat[b], Ganna Petruk[c], Mohamed A.O. Abdelfattah[d], Malak Dmirieh[a], Daria Maria Monti[c], Ashraf B. Abdel-Naim[e], Michael Wink[a,*]

[a] Institute of Pharmacy and Molecular Biotechnology, Heidelberg University, Heidelberg, Germany
[b] Department of Pharmacology and Toxicology, Faculty of Pharmacy, Ain Shams University, Cairo 11566, Egypt
[c] Department of Chemical Sciences, University of Naples Federico II, Complesso Universitario Monte Sant'Angelo, via Cinthia 4, 80126 Naples, Italy
[d] Department of Chemistry, American University of the Middle East, Eqaila 54200, Kuwait
[e] Department of Pharmacology and Toxicology, Faculty of Pharmacy, King Abdulaziz University, Jeddah, Saudi Arabia

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ABSTRACT

HPLC-PDA-MS/MS analysis of *Syzygium jambos* leaf extract allowed the identification of 17 secondary metabolites, including flavonol glycosides, flavonol di-glycosides, and flavones as well as ellagitannins and phenolic acids. The extract showed promising antioxidant activities in different experimental models. In *Caenorhabditis elegans*, the extract mediated a dose dependent survival rate and decreased the intracellular ROS level and HSP-16.2 expression. In a rat model against acute CCl_4 intoxication, the extract reduced the levels of all tested liver markers ALT, AST, TB, TC, TG, and MDA and increased GSH and SOD; effects were similar to those of silymarin. In hepatocytes, pre-treatment with the extract inhibited ROS production, against the deleterious effects of sodium arsenite, and increased GSH levels, without alteration in the phosphorylation levels of p38 and of its direct target, MAPKAPK-2. These results demonstrate the high efficacy of *S. jambos* extract in free radical-scavenging, and inhibition of reactive oxygen species.

1. Introduction

Liver plays a central role in filtering and clearing blood received from digestive tract prior to pass it to other body tissues and organs. Moreover, it is involved in detoxifying the body from xenobiotics, toxins, and in mediating drug transformations and metabolism. However, despite its physiological role, the liver is highly susceptible to damage from different toxins, viruses and reactive oxygen and nitrogen species (ROS and RNS, respectively) (De Abajo, Montero, Madurga, & Garcia Rodriguez, 2004; Hoek & Pastorino, 2002). Such damage is often associated with metabolic and synthetic dysfunctions which can lead to fatal complications (Orhan, Orhan, Ergun, & Ergun, 2007).

Reactive oxygen and nitrogen species are produced as an inevitable outcome of cell metabolism and as a result of environmental stress as well. Even if ROS and RNS show some beneficial effects, such as cytotoxicity against bacteria and other pathogens, a high concentration of these reactive free radical species can cause oxidative damage of cellular macromolecules, such as lipids, proteins, and DNA, thus, in turn, leading to DNA mutations and disrupting cellular functionality that enhances the pathogenesis of various disorders affecting liver and other organs and systems (Valko et al., 2007).

Keeping the levels of ROS under control is achieved through some enzymatic cellular mechanisms, such as superoxide dismutase (SOD) which catalyses the transformation of super oxide radicals into either molecular oxygen, or the less reactive hydrogen peroxide which can be then destroyed by catalase (CAT) (Fridovich, 1993). The endogenous glutathione (GSH) is crucial for cells as it is able to scavenge many free radicals, peroxides, lipid peroxides, electrophiles, physiological metabolites, and xenobiotics forming soluble mercapturates (Rees & Sinha, 1960).

In case of enhanced ROS levels, the ingestion of dietary antioxidants. i.e. carotenoids, vitamin C, anthocyanins, vitamin E and several other plant secondary metabolites, such as polyphenolics, can support the organism in the defence pathway (Sies & Stahl, 1995). In this context, hepatic diseases, often associated with increased intracellular ROS levels are of special concern, as they can evolve into fatal complications. Some plants and their secondary metabolites can provide safe, efficacious, antioxidant multi-mechanistic agents that may be useful as hepatoprotective drugs (Abbas & Wink, 2014; Youssef et al., 2016).

* Corresponding authors.
E-mail addresses: sobeh@uni-heidelberg.de (M. Sobeh), wink@uni-heidelberg.de (M. Wink).

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The genus *Syzygium* comprises 1200 species occurring in Africa and the Pacific region. *Syzygium* is one of 131 genera belonging to the complex, well-studied myrtle family, Myrtaceae (with 5500 species). This genus, along with the other strongly aromatic genera (i.e. *Myrtus, Eugenia, Melaleuca, and Eucalyptus*), are rich in essential oils, flavonoids, flavonols, anthocyanins, ellagitannins, and phenolic acids (Haron, Moore, & Harborne, 1992; Kuo, Yang, & Lin, 2004; Nawwar et al., 2016; Sobeh et al., 2016).

The phytochemistry and pharmacology of several members of this genus (including *S. cumini*, *S. aqueum, S. samarangense*, *S. aromaticum*, and *S. jambolanum*) have been extensively investigated. In traditional medicine, some of them have been used to treat several disorders, such as hemorrhage, dysentery and gastrointestinal disorders, as well as diabetes and inflammation as these extracts possess antifungal, antimicrobial, antihypertensive, analgesic and antiviral (against herpes virus) properties (Kuiate, Mouokeu, Wabo, & Tane, 2007; Kuo et al., 2004; Raga et al., 2011; Sharma, Kishore, Hussein, & Lall, 2013).

The rose apple, *Syzygium jambos* (syn. *Eugenia jambos* L.), is a large shrub or small to medium in size tree, known to grow originally in Southeast Asia, but now widely distributed in the tropics. The plant has been traditionally used for its antipyretic and anti-inflammatory properties and to treat hemorrhages, syphilis, leprosy, wounds, ulcers, and lung diseases. In Indo-China, all plant parts are employed to treat digestive tract and tooth disorders. The leaves decoction is used as antirheumatic, diuretic, and to relieve sore eye conditions (Kuiate et al., 2007; Nawwar et al., 2016).

Generally, leaf extracts of *S. jambos* and isolated flavonoid glycosides have shown substantial anti-inflammatory activity (Slowing, Carretero, & Villar, 1994). A study from Venezuela has reported promising anti-inflammatory and analgesic activities for the leaf extract when compared to the positive controls diclofenac and morphine, respectively (Avila-Peña, Peña, Quintero, & Suárez-Roca, 2007). In another study, leaf extracts from plants grown in Pretoria, South Africa, have been subjected to bioassay guided fractionation resulting in the isolation of ursolic acid and the anacardic acid analogue, squalene (Sharma et al., 2013). Kuiate et al. (2007) have reported antidermatophytic activity of the ethyl acetate extract of the stem bark and its isolated compounds against three dermatophyte species.

The phytochemical investigation of the plant parts revealed several secondary metabolites. For instance, a recent study from plants cultivated in Egypt has reported three unknown compounds from the aqueous ethanol extract of the whole plant along with 8 known compounds including myricetin 3-*O*-xylosyl-(1 → 2) rhamnoside, quercetin 3-*O*-xylosyl-(1 → 2) rhamnoside and ellagic acid (Nawwar et al., 2016). In Taiwan, two hydrolyzable tannins, 1-*O*-galloylcastalagin and casuarinin, were isolated from the leaf extract; they turned out to exhibit potent anticancer activity *in vitro* (Yang, Lee, & Yen, 2000).

From plants coming from Sri Lanka, the phytochemical profiling of the methylene chloride extract of the leaves revealed three dihydrochalcones, myrigalone G, myrigalone B, and phloretin 4 -*O*-methyl with appreciable radical scavenging properties (Jayasinghe, Ratnayake, Medawala, & Fujimoto, 2007). The phytochemical investigation of the fruits from Chinese plants revealed seven new phloroglucinol derivatives (jambone A, B to G) along with four known triterpenoids and two known flavones, some of which showed promising cytotoxic activities against melanoma cells (Li et al., 2015).

In the current study, the polyphenols of *S. jambos* were investigated using HPLC-PDA-MS/MS. The potential antioxidant activity of a methanol extract was evaluated both *in vitro* using DPPH and FRAP assays, on hepatocytes, and *in vivo* using the well-established nematode *Caenorhabditis elegans* model. Moreover, the potential hepatoprotective activity of a methanol extract was demonstrated in stressed hepatic cells and in an acute CCl_4-induced hepatic injury rat model.

2. Materials and methods

2.1. Chemicals

Carbon tetrachloride (CCl_4) was purchased from Sigma Aldrich, St Louis, MO, USA. All colorimetric kits for biochemical parameters were obtained from Biodiagnostic Co, Cairo, Egypt. All other chemicals and kits were of the highest analytical grade.

2.2. Plant material and extraction

During the spring season (April-May 2015), fresh mature plant leaves were collected from trees grown in a private garden in El Obour city (Egypt). A voucher specimen is kept under accession number P8617 at IPMB, Heidelberg, Germany. Air dried leaves (50 g) were ground and exhaustively extracted with 100% methanol at room temperature during an overall extraction period of 3 d. The combined extracts were evaporated under vacuum at 40 °C until dryness. The residues were further dissolved in methanol, centrifuged, and then only the methanol soluble extract was evaporated under vacuum at 40 °C until dryness. After freezing at −70 °C, the extract was lyophilized yielding fine dried powder (8 g).

2.3. High performance liquid chromatography (HPLC-PDA-MS/MS)

HPLC-PDA-MS/MS system was Thermofinnigan (Thermo electron Corporation, USA) coupled with an LCQ–Duo ion trap mass spectrometer with an ESI source (ThermoQuest). The separation was achieved by using a C18 reversed-phase column (Zorbax Eclipse XDB-C18, Rapid resolution, 4.6 × 150 mm, 3.5 μm, Agilent, USA). A gradient of water and acetonitrile (ACN) (0.1% formic acid each) was applied from 5% to 30% ACN in 60 min in flow rate of 1 mL/min with a 1:1 split before the ESI source. The sample was injected automatically using autosampler surveyor ThermoQuest. The instrument was controlled by Xcalibur software (Xcalibur™ 2.0.7, Thermo Scientific). The MS conditions were set as described before (Sobeh et al., 2017). The ions were detected in the negative mode, a full scan mode and mass range of 50–2000 *m/z*. In brief, to quantify the concentration of phenolic compounds, gallic acid was used as a standard and expressed as its equivalents, whereas the diglucoside flavonoid rutin was used for flavonoids.

2.4. Biological activity experiments

2.4.1. Antioxidant activities in vitro

Determination of total phenolic contents was investigated using the Folin-Ciocalteu method (Ghareeb et al., 2017). The antioxidant activities were investigated by DPPH radical scavenging activity and FRAP assay as previously described (Ghareeb et al., 2017).

2.4.2. Biocompatibility of SJE extracts on HepG2 cells

Human hepatic carcinoma cells (HepG2) were from ATCC and were cultured in Dulbecco's Modified Eagle's Medium (EuroClone), supplemented with 10% foetal bovine serum (HyClone), 2 mM L-glutamine and antibiotics (EuroClone) in a 5% CO_2 humidified atmosphere at 37 °C. Every 48–72 h the culture medium was removed and cells were rinsed with PBS (EuroClone), detached with trypsin-EDTA (EuroClone) and diluted in fresh complete growth medium.

The influence of SJE on proliferation of HepG2 cells was assessed by MTT assay. In this set of experiments, cells were seeded in 96-well plates at a density of 2 x 10^3/well, and 24 h after seeding, increasing concentrations of the methanolic extract (from 25 to 200 μg/mL) were added to the cells for 24 and 48 h. Cell viability was assessed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, as described in Guglielmi et al. (2009). Cell survival was expressed as the percentage of viable cells in the presence of the extract compared to controls. Two groups of cells were used as control, i.e. cells

untreated with the extract and cells supplemented with identical volumes of methanol. Each sample was tested in three independent analyses, each carried out in triplicates.

2.4.3. Oxidative stress

To study the antioxidant effect of SJE, cells were plated at a density of 5 x 10^4 cells/cm^2. After 24 h, cells were incubated for 2 h in the presence or absence of 50 μg/mL of the extract and then exposed to 300 μM sodium arsenite (SA) for 2 h.

2.4.4. Intracellular reactive oxygen species measurement

The 2′,7′-dichlorofluorescein diacetate (H_2-DCFDA, Sigma-Aldrich) method was used to measure the intracellular levels of ROS by using the protocol described by Del Giudice et al. (2017). Briefly, at the end of incubation, cells were incubated with the DCFDA probe (20 μM). Fluorescence intensity was measured by a Perkin-Elmer LS50 spectrofluorimeter (525 nm emission wavelength, 488 nm excitation wavelength, 300 nm/min scanning speed, 5 slit width for both excitation and emission). ROS production was expressed as percentage of DCF fluorescence intensity of the sample under test, with respect to the untreated sample. Each value was assessed by three independent experiments, each with three determinations.

2.4.5. Intracellular GSH levels measurement

DTNB assay was performed as described by Petruk et al. (2016) and it was used to analyze total GSH levels. Briefly, at the end of the experiment, cells were detached by trypsin, centrifuged at 1000*g* for 10 min and resuspended in lysis buffer (0.1 M Tris HCl, pH 7.4 containing 0.3 M NaCl, 0.5% NP-40 and protease inhibitors (Roche). Upon 30 min incubation on ice, lysates were centrifuged at 14,000*g* for 30 min at 4 °C. Protein concentration was determined by the Bradford assay and 50 μg of proteins were incubated with 3 mM EDTA, 144 μM 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) in 30 mM Tris HCl pH 8.2, centrifuged at 14,000*g* for 5 min at 4 °C and the absorbance of the supernatant was measured at 412 nm by using a multiplate reader (Biorad). Total GSH content in the lysate was expressed as the percentage of TNB, as its production is directly related to the rate of this recycling reaction, which, in turn, is directly related to the concentration of GSH in the sample. Values are the mean of three independent experiments, each with triplicate determinations.

2.4.6. Western blot analyses

To investigate the activation of MAPK cascade, cells were plated at a density of 5 x 10^4 cells/cm^2 in complete medium for 24 h and then treated as described above. After treatment, cells were lysed and then analyzed by Western blotting by using protocol described by Galano, Arciello, Piccoli, Monti, and Amoresano (2014). Phosphorylation levels of p38 and MAPKAPK-2 were detected by using specific antibodies purchased from Cell Signal Technology (Danvers, MA, USA). To normalize protein intensity levels, specific antibodies against internal standards were used, i.e. anti-GAPDH (Thermo Fisher, Rockford, IL, USA). The chemiluminescence detection system (SuperSignal® West Pico) was from Thermo Fisher.

2.4.7. Antioxidant activity in vivo

2.4.7.1. Caenorhabditis elegans strains and maintenance. Nematodes were maintained under the following conditions: 20 °C, on nematode growth medium (NGM), fed with living *E. coli* OP50. Age synchronized cultures were obtained by treating gravid adults with sodium hypochlorite. The eggs were kept in M9 buffer for hatching and the larvae were transferred to S-media seeded with living *E. coli* OP50 ($O.D._{600nm}$ = 1.0). The *C. elegans* strains such as Wild type (N2), TJ375 [*hsp-16.2: GFP (gpls1)*] and TJ356 were obtained from the *Caenorhabditis* Genetic Center (CGC). The *in vivo* assays, including survival rate and ROS concentration, were done according to our previous description (Sobeh et al., 2018).

2.5. Hepatoprotective activity in vivo

2.5.1. Animals

The study was conducted with male Sprague-Dawley rats, weighing 200–250 g, obtained from the animal facility, King Abdulaziz University, Geddah (KSA). Rats were kept under air-conditioned environment at 22 ± 2 °C, with a 12 h light–dark cycle. They were supplied with rodent chow and water *ad libitum*. Animal care and experiments were conducted in accordance with the protocols approved by the Unit of Biomedical Ethics Research Committee, Faculty of Medicine, King Abdulaziz University, following the Institutional Animal Care and Use Committee guidelines.

2.5.2. Experimental design

Rats were divided into 4 groups of 6 rats each. The first group served as a control and took water orally followed by intraperitoneal (IP) injection of corn oil after 4 h. The second group was injected once with 1 mL/kg of CCl_4-corn oil 50% mixture. Group 3 was pretreated with the known hepatoprotective lignan silymarin (200 mg/kg orally) as a positive control. Group 4 was pretreated with *S. jambos* extract (200 mg/kg orally). Four hours after the pretreatment, groups 3 and 4 have received an IP injection of CCl_4-corn oil 50% mixture.

After 24 h, blood samples were collected by cardiac puncture and allowed to clot. The sera were obtained by centrifugation for 10 min at 3000 rpm, and then kept at −80 °C till analysis. The sera were used to measure hepatotoxicity parameters: alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TB), total cholesterol (TC), and triglycerides (TG). Rats were then sacrificed and liver tissues were dissected. Representative tissue from each lobe was cut and fixed in 10% formalin/saline and then embedded in paraffin for histopathological examination. The remaining liver tissues were re-weighed, washed, and homogenized in ice-cold PBS to yield 10% w/v homogenates and then stored at −80 °C till analyses.

2.5.3. Biochemical analyses

Activities of serum ALT and AST, serum levels of TB, TC, and TG were determined colourimetrically using Mindray BS-120 clinical chemistry auto-analyzer (Shenzhen Mindray Bio-medical Electronics Co. Ldt., Shenzhen, China). Levels of glutathione (GSH), lipid peroxidation marker malondialdehyde (MDA) and the activity of the antioxidant enzyme superoxide dismutase (SOD) were determined using the commercially available kits (Biodiagnostics, Cairo, Egypt).

2.5.4. Histopathological examination

Storage of tissue samples was done in 10% buffered neutral formalin for 24 h and tap water was used for washing. For dehydration, serial dilutions of methyl, ethyl and absolute ethyl alcohols were used. Specimens were embedded in xylene for clarification, immersed in paraffin, and kept for 24 h at 56 °C inside hot air oven. Slide microtome was used to prepare paraffin bee wax tissue sections at 4 μm thickness that were placed on glass slides, had the paraffin cleared, and stained either with eosin and hematoxylin for histopathology. Glass slides were examined through the light electric microscope (Olympus BX-50 Olympus Corporation, Tokyo, Japan) (Banchroft, Stevens, & Turner, 1996).

2.6. Statistical analysis

Statistical analyses for biological data were carried out three times unless otherwise mentioned in the procedure. Data are presented as mean and S.D. One way analysis of variance (ANOVA) was used to carry out the comparisons that were followed by post hoc analysis using Tukey's test. A level of $p < .05$ was taken as cut off to accept statistical significance. GraphPad Prism software, version 5.00 (GraphPad Software, Inc. La Jolla, CA, USA) and (SigmaPlot® 11.0) were used to perform all the required statistical analyses.

Table 1
Chemical composition of a methanol extract of *S. jambos* leaves using LC-MS/MS.

Peak No	Proposed compounds	t_R (min.)	$[M-H]^-$ (*m/z*)	MS/MS fragments	μg gallic equivalent	References
1	Malic acid	1.61	133	–	13.56	Sobeh et al. (2017)
2	Citric acid	1.64	191	111, 173	14.55	Mena et al. (2012)
3	Hexahydroxydiphenoyl-hexoside	1.96	481	257, 301	62.31	Mena et al. (2012)
4	Castalagin/vescalagin isomer	2.85	933	301, 569, 613, 871	31.60	Nonaka, Aiko, Aritake, and Nishioka (1992)
5	Galloyl-HHDP-DHHDP-hexoside*	3.32	951	301, 569, 613, 915	8.47	
6	bis-HHDP-hexoside*	3.43	783	229, 257, 301, 481	59.91	Bresciani et al. (2015)
7	Castalagin/Vescalagin isomer	3.86	933	301, 569, 631, 915	44.38	Nonaka et al. (1992)
8	Galloyl-HHDP-DHHDP-hexoside*	4.86	951	301, 783	11.36	
9	bis-HHDP-hexoside*	5.39	783	229, 257, 301, 481	23.57	Bresciani et al. (2015)
10	Galloyl-bis-HHDP-hexoside (casuarinin)*	8.73	935	301, 481, 571, 633	42.75	Yang et al. (2000)
11	Ellagic acid pentoside	18.52	433	257, 301	55.40	Mena et al. (2012)
12	Ellagic acid rhamnoside	19.64	447	257, 301	105.04	Mena et al. (2012)
13	Ellagic acid	20.58	301	229, 257, 301	35.29	Nawwar et al. (2016)
14	Myricetin rhamnoside[1]	20.89	463	151, 179, 317	5.38	Celli, Pereira-Netto, and Beta (2011)
15	Myricetin 3-*O*-xylosyl-(1 → 2) rhamnoside[§,1]	21.96	595	179, 317, 463	68.07	Nawwar et al. (2016)
16	Rosmarinic acid rhamnoside	23.99	505	359	3.21	
17	Quercetin 3-*O*-xylosyl-(1 → 2) rhamnoside[§,1]	27.74	579	179, 300, 301, 447	121.60	Nawwar et al. (2016)

* HHDP = hexahydroxydiphenoyl.
§ Previously isolated from the plant.
[1] μg rutin equivalent.

3. Results and discussion

3.1. Identification of constituents by HPLC-PDA-MS/MS

The polyphenol content of a methanol extract of *S. jambos* leaves has been of interest from both phytochemical and biological points of view. HPLC-PDA-ESI-MS/MS analyses (full scan and product ion scan mode) have provided comprehensive structural information that successfully led to the identification and characterization of 17 secondary metabolites representing different classes of compounds including phenolic acids, flavonol glycosides, flavonol di-glycosides, flavones, and ellagitannins.

Compounds detected in the current work were identified by means of their MS data, together with the structural information of both the daughter MS ions and the spectral data from the PDA detector. These data were compared to those of formerly identified compounds from plants of the same family or described in the reported online data. Table 1 shows the proposed chemical composition of a methanol extract of *S. jambos* based on LC-MS analysis; the corresponding total ion chromatogram is documented in Fig. 1.

A molecular ion peak of $[M-H]^-$ (*m/z*) 595 with daughter ions at 463 [M − 132], 317 [M − 132 − 146] was identified as myricetin 3-*O*-xylosyl-(1 → 2) rhamnoside as previously described (Nawwar et al., 2016). Another peak exhibiting $[M-H]^-$ (*m/z*) 579 with MS^2 fragments of 447 [M-132], 301 [M − 132 − 146] was assigned to quercetin 3-*O*-xylosyl-(1 → 2) rhamnoside (Nawwar et al., 2016).

Two compounds showed molecular ion peaks at $[M-H]^-$ (*m/z*) 433, 447 and daughter ion at *m/z*301 and two characteristic fragment ions at 257 and 229; they were assigned to ellagic acid pentoside and ellagic acid rhamnoside, respectively (Mena et al., 2012) along with ellagic acid aglycone at $[M-H]^-$ (*m/z*) 301 (Nawwar et al., 2016). Compound 10 was identified as casuarinin with $[M-H]^-$ (*m/z*) 935 as previously reported (Yang et al., 2000). Malic and citric acids were identified also along with several ellagitannins (Table 1).

3.2. Biological activities

3.2.1. In vitro antioxidant activity

Polyphenols are known to exhibit a wide range of biological activities. In addition to scavenging free radicals and ROS, they have been shown to cure or slow the progress of various diseases and health conditions (Abbas & Wink, 2014). Flavonoids represent an important subclass of polyphenols and are widely distributed among many plant families at varying levels. Several studies have highlighted the potent health benefits of flavonoids, substantially, the antioxidant, anticancer, anti-inflammatory effects, and the hepatoprotective activities as well (Cheng, Yi, Wang, Huang, & He, 2017; Lesjak et al., 2018).

Our leaf extract (SJE) contained a high total phenolic content of 466 mg gallic acid equivalents (GAE)/g extract. The extract showed a strong antioxidant activity, as evaluated *in vitro* by using DPPH and FRAP assays. In particular, DPPH indicated an EC_{50} of 5.7 ± 0.45 μg/mL and FRAP exhibited an EC_{50} of 19.77 ± 0.79 mM $FeSO_4$ equivalent/mg sample when compared to the reference compounds ascorbic acid (EC_{50} of 2.92 ± 0.29) and quercetin (24.04 ± 1.23 mM $FeSO_4$ equivalent/mg sample), respectively. Such high free radical-scavenging properties of the crude extracts have been reported only for few other plants as well (Abbas & Wink, 2014; Nawwar et al., 2016).

3.2.2. Antioxidant activity in vivo in C. elegans

3.2.2.1. Survival assay and intracellular ROS content under juglone induced oxidative stress. The extract was able to decrease the intracellular ROS level of wild-type nematodes in a dose dependent manner; ROS levels were reduced in the SJE groups by 59.22% (at 200 μg/mL extract) when compared to control group. Data are shown in Fig. 2(a).

In the current study, the natural pro-oxidant juglone (5-hydroxy-1,4-naphthoquinone) (80 μM) was used to induce fatal oxidative stress in *C. elegans*. Worms which were pre-treated with antioxidant compounds or extracts are often able to survive juglone toxicity. In our experiments, worms, pre-treated with SJE showed a dose-dependent survival activity when compared to the control group that was treated with juglone only (survival rate as low as 18%). Data are shown in Fig. 2(b) indicating that the polyphenols are absorbed and exhibit antioxidant activity inside the nematodes.

3.2.2.2. Quantification of Phsp-16.2::GFP expression and subcellular DAF-16::GFP localization. To get an insight in the corresponding antioxidant mechanisms, we have used a transgenic worm strain in which the stress-responding heat shock protein HSP-16.2 is fused with GFP. Worms were incubated with 20 μM juglone for 24 h to induce oxidative stress followed by measuring the expression of P*hsp*-16.2::GFP by fluorescence microscopy. As shown in Fig. 2(c), SJE was able to significantly decrease the HSP-16.2 expression in a dose dependent manner. These findings indicate that the studied extract is not only scavenging reactive radicals but also it modulates the expression of stress-response genes.

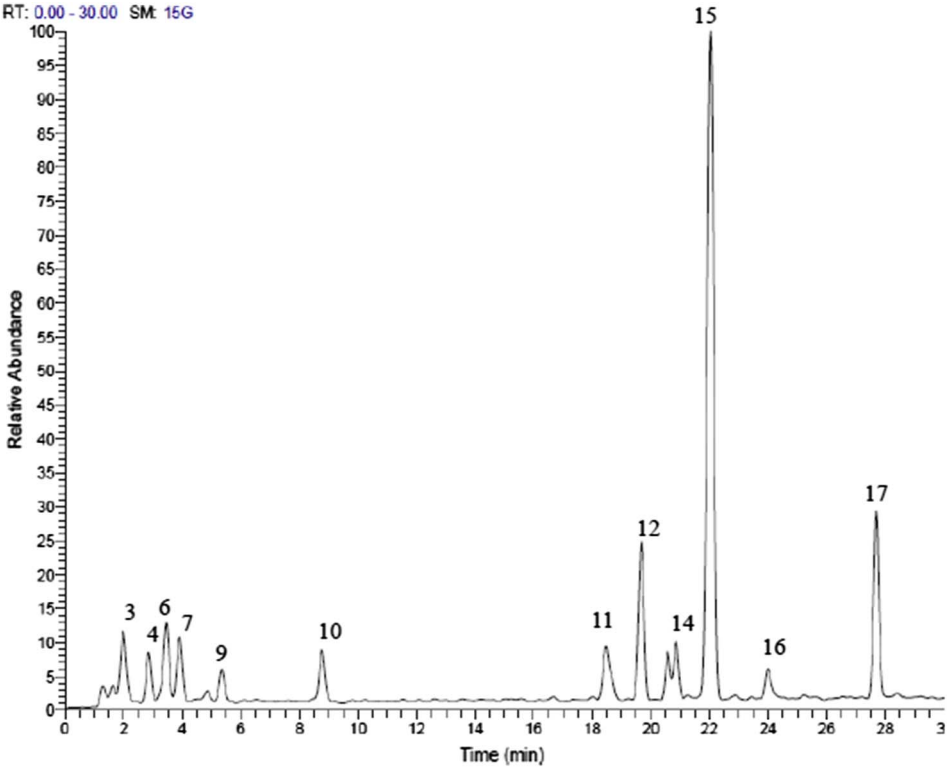

Fig. 1. Total ion chromatogram of the methanol extract from *S. jambos* leaves (LC-MS base peak in the negative ionization mode ESI).

The DAF-16/insulin pathway is often associated with antioxidant response in *C. elegans*. A critical element is the transcription factor DAF-16/FOXO which regulates the expression of heat shock and other stress-related genes. When inactive, DAF-16 is located in the cytoplasm; only when translocated in the nucleus, DAF-16 can regulate stress genes. The extract induced a nuclear translocation of DAF-16::GFP at low concentrations (Fig. 2d), indicating that the *in vivo* antioxidant effect could involve DAF-16/FOXO signaling pathway.

It is worth to mention that this appreciable antioxidant potential of *S. jambos* extract is comparable to that of some polyphenols such as aspalathin in Rooibos tea extracts, EGCG in green tea, and anthocyanins in purple wheat (Abbas & Wink, 2014; Chen, Rezaizadehnajafi, & Wink, 2013; Chen, Sudji, et al., 2013).

3.2.3. Hepatotoxicity markers

Carbon tetrachloride is one of the chemicals associated with severe hepatic toxicity, as it is known that a single exposure is enough to cause serious necrosis and steatosis of liver tissues. It thus represents an optimum choice to induce liver injury in the models required to screen for the hepatoprotective potential of new drugs (Al-Sayed, Abdel-Daim, Kilany, Karonen, & Sinkkonen, 2015). The pathogenesis of the damage is multivariate, involving propagation of free radicals such as trichloromethyl radical ($CCl_3^{\cdot}$) and trichloromethyl peroxy radical ($CCl_3O_2^{\cdot}$), leading to lipid peroxidation, cell membrane destruction and DNA damage, followed by triggering an inflammatory response by the body (Fahmy, Al-Sayed, Abdel-Daim, Karonen, & Singab, 2016).

In this study, a one day CCl_4-induced liver injury model was implemented, as CCl_4 deleterious toxic effects have a peak 24 h following injection, and then slowly normalize. Drugs able to reverse CCl_4 damage during its peak period represent promising candidates for treating liver diseases and intoxication (Al-Sayed & Abdel-Daim, 2014).

Table 2 shows the effects of SJE on the hepatotoxicity markers. Following the CCl_4 administration, there was a marked leakage of the liver enzymes into the blood, manifested as significant elevation of ALT and AST serum activities. In addition, acute CCl_4 intoxication caused a significant increase in TB, TC and TG serum levels compared to the control group. These effects could be attributed to hepatocellular damage (Al-Sayed & Abdel-Daim, 2014). Pretreatment with the known liver-protecting silymarin reduced the levels of all tested markers comparable to normal values that were shown to be statistically different from the CCl_4-challenged group while being not from the control group. Pretreatment of animals with SJE significantly reduced the levels of the hepatic markers relative to the CCl_4-challenged group; effects are similar to those of silymarin (Table 2). Similar activities were reported for other plant crude extracts (Abbas & Wink, 2014; Youssef et al., 2016).

3.2.3.1. Oxidative stress markers. The pathogenesis and progression of liver diseases is often associated with oxidative stress, which is also pivotally involved in promoting drug-induced hepatotoxicity (Al-Sayed, El-Lakkany, Seif el-Din, Sabra, & Hammam, 2014). Toxicity of CCl_4 is, to a wide extent, a matter of free radical mediated damage where the formed reactive species covalently bind cell macromolecules leading to formation of protein, nucleic acid, and/or lipid adducts (Azab, Abdel-Daim, & Eldahshan, 2013). This in turn results in disturbed cellular functionality and protein synthesis that finally ends up in apoptosis and/or necrosis (Williams & Burk, 1990).

The determination of intracellular GSH and lipid peroxidation levels are among the most common techniques used to detect liver injuries owing to oxidative stress (Lykkesfeldt, 2007; Matés, Pérez-Gómez, & De Castro, 1999). As shown in Fig. 3, CCl_4 intoxication induced a significant depletion in GSH level and caused about 3-fold increase in MDA level compared to the control group. Alterations in GSH content (Fig. 3a) and MDA level (Fig. 3b) were inhibited by pretreatment with silymarin or SJE.

While GSH represents the non-enzymatic side of the host antioxidant defense mechanism, superoxide dismutase (SOD) and others constitute the major enzymatic part. Accordingly, SOD activity was significantly decreased by the CCl_4 challenge and restored to almost normal levels by silymarin and *S. jambos* extract (200 mg/kg) pretreatments, (Fig. 3c). These findings further confirm a substantial free radical scavenging and antioxidant potential for *S. jambos*.

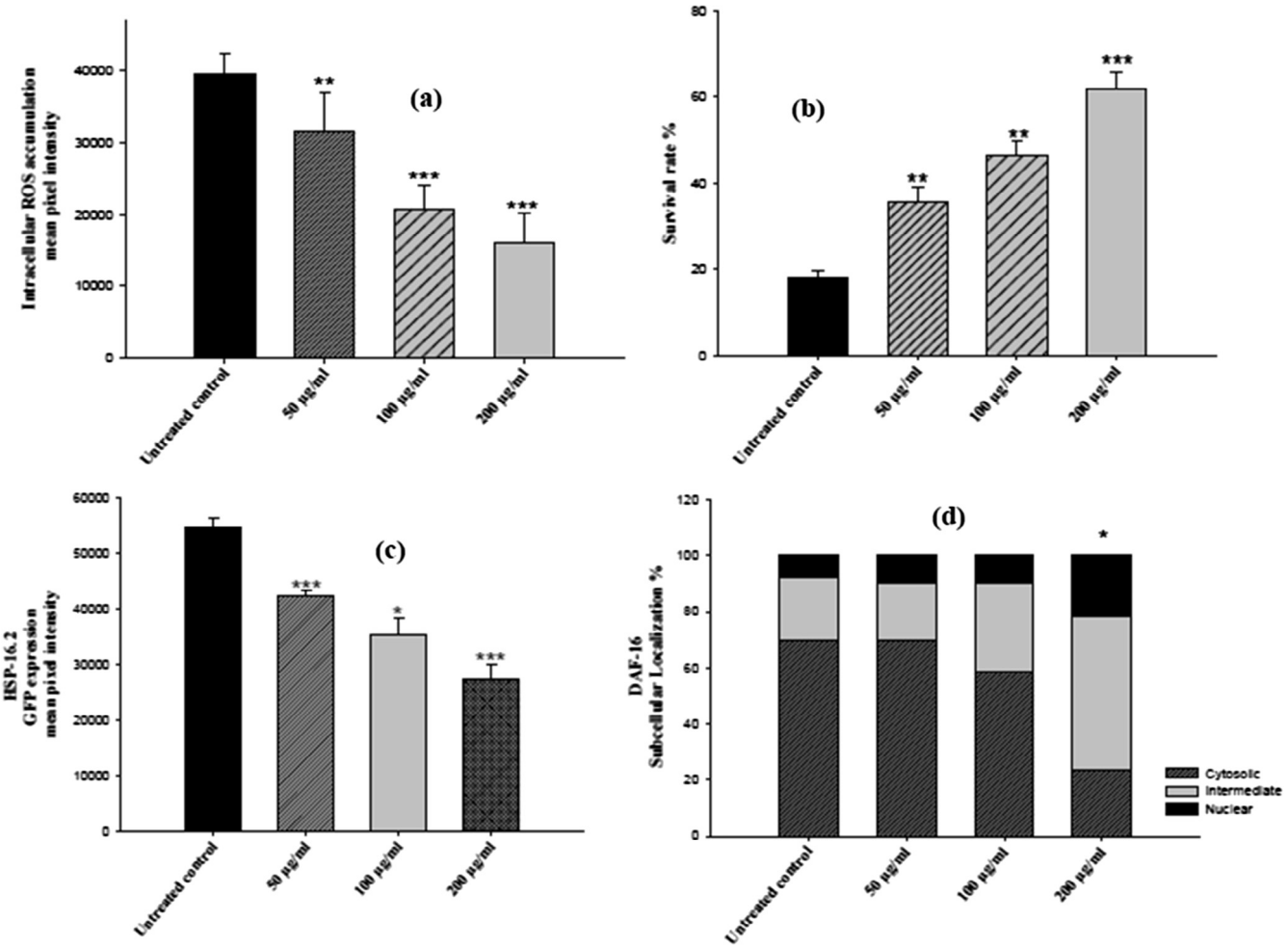

Fig. 2. (a) Intracellular ROS content in N2 wild-type nematodes treated with the fluorescent ROS indicator dye H2DCF-DA. Data are presented as the percentage of fluorescent pixel related to control. ROS content were significantly decreased after pre-treatment of the nematodes with SJE in a concentration dependent manner. (b) SJE effect on stress resistance under juglone treatment (80 µM). Survival rates of the worms (N2, wild-type) were significantly increased after pre-treatment with SJE in a dose dependent manner. (c) P*hsp*-16.2::GFP expression in mutant TJ375 worms. Data are presented as the intensity of fluorescent pixels. P*hsp-16.2*::GFP was significantly decreased after pre-treatment of the nematodes withSJE in a dose dependent manner. (d) Translocation of DAF-16::GFP in mutant TJ356 worms. Data are presented as the percentage of worms exhibiting a DAF-16 subcellular localization pattern, namely, cytosolic, intermediate, and nuclear translocation of DAF-16::GFP in mutant TJ356 worms. Data are presented as percentage of survival (mean ± SEM, n = 3). $^{***}p < .001$, $^{**}p < .01$, $^{*}p < .05$, related to control was analyzed by one-way ANOVA. Survival of untreated nematodes was set 100%.

3.2.3.2. Histopathological examination. The antioxidant properties of SJE were further confirmed by histopathological examination of representative liver tissues, as shown in Fig. 4D. Sections taken from a liver of control rats showed normal hepatic architecture, hepatocytes structure and central veins (Fig. 4A), while CCl_4-intoxicated rat liver was characterized by dilated central vein and centrilobular necrosis (Fig. 4B). Pretreatments with silymarin and/or SJE were able to preserve hepatic architecture with only scattered cytoplasmic vacuolization (Fig. 4C and D). Similar protective activities were determined in plants containing flavonoids, tannins, and ellagitannins (Al-Sayed et al., 2014; Azab et al., 2013; Fahmy et al., 2016).

3.2.4. Antioxidant activity in a hepatic cell model

Human hepatic cells (HepG2) were incubated in the presence of increasing concentrations of the extract (from 25 to 200 µg/mL) for 24 and 48 h. At the end of the incubation, cell survival was analyzed (Fig. 5A) and no significant toxicity was observed up to 50 µg/mL up to 48 h. Thus, this concentration value was selected to analyze the

Table 2
Effect of pretreatment of methanol extract of *S. jambos* on hepatotoxicity markers in the rat model of acute CCl_4 intoxication (number of rats = 6).

Group	ALT (U/L)	AST	TB (mg/dL)	TC	TG
Control	15.4[b] ± 4.07	13.4[b] ± 0.9	0.24[b] ± 0.06	56.7[b] ± 2.9	73.6[b] ± 3.06
CCl_4	137.5[a] ± 6.77	95.2[a] ± 4.49	1.27[a] ± 0.12	135.0[a] ± 7.05	174.2[a] ± 6.45
CCl_4 + silymarin (200 mg/kg)	27.6[b] ± 2.12	24.4[b] ± 1.77	0.28[b] ± 0.03	65.2[b] ± 3.29	80.3[b] ± 3.38
CCl_4 + SJE(200 mg/kg)	29.6[a,b] ± 2.26	31.4[a,b] ± 0.86	0.37[b] ± 0.092	70.1[b] ± 3.15	84.8[b] ± 2.03

Statistical analysis was carried out by one-way ANOVA followed by Tukey post hoc test.
[a] Statistically significant from the corresponding control at $p < .05$
[b] Statistically significant from CCl_4-treated group at $p < .05$

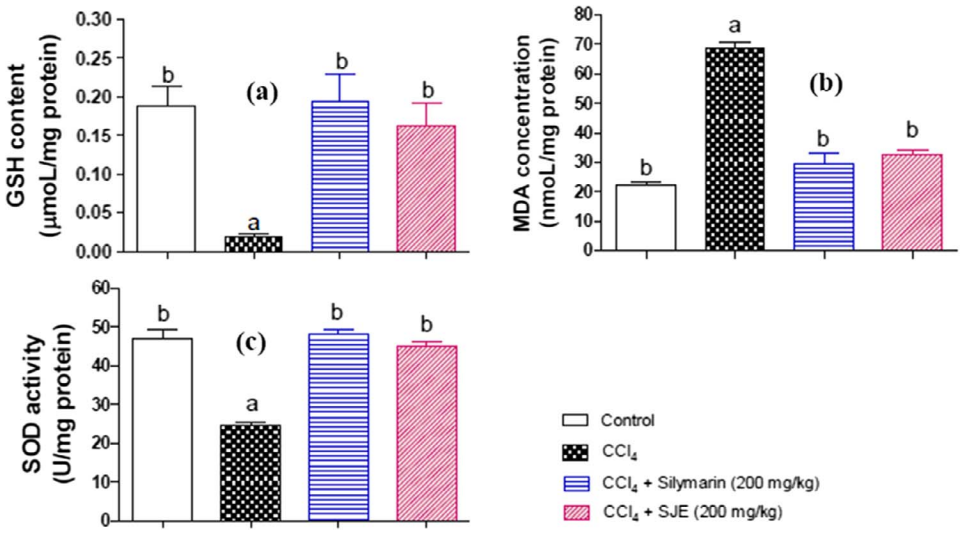

Fig. 3. Effect of pretreatment with SJE on oxidative stress markers in rats with acute CCl_4 intoxication on (a) GSH content; (b) MDA level; (c) SOD activity, (n = 6). Statistical analysis was carried out by one-way ANOVA followed by Tukey post hoc test. [a]Statistically significant from the corresponding control at $p < .05$. [b]Statistically significant from CCl_4-treated group at $p < .05$.

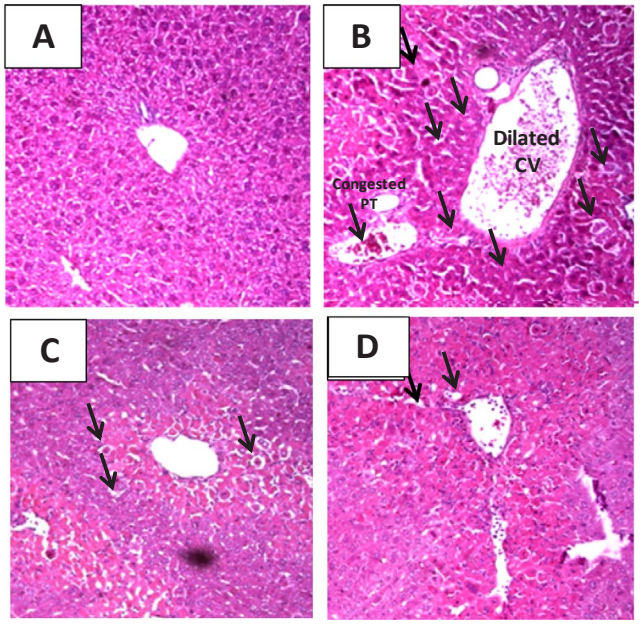

Fig. 4. Representative photomicrographs of liver sections stained by hematoxylin and eosin (×100); (A) Section taken from a liver of control rat showing normal hepatic architecture, hepatocyte structure and central vein; (B) Section taken from a liver of CCl_4 intoxicated rat showing dilated central vein with central hepatocellular necrosis (arrows) and congested portal triad; (C) Section taken from a rat liver pretreated with silymarin with preserved hepatic architecture and only scattered cytoplasmic vacuolization (arrows); (D) Section taken from a rat liver pretreated with SJE showing scattered cytoplasmic vacuolization (arrows).

antioxidant activity in cells in which oxidative stress was induced by sodium arsenite (SA). Trivalent inorganic arsenic (iAs^{3+}) is a toxic and carcinogenic environmental contaminant that humans are inadvertently exposed to every day through drinking water, food and air. It exerts its toxic effect through ROS generation that, in turn, causes loss of GSH homeostasis and oxidations of different molecules (such as formation or lipid peroxides, DNA strand breaks, and chromosome breakages), and leads to cell death.

Cells were incubated with SJE for 120 min prior to induce oxidative stress by 300 µM SA for 120 min. At the end of incubation, intracellular ROS levels were determined (Fig. 5B). No alteration in ROS levels was observed when cells were incubated in the presence of SJE, whereas, as expected, a 70% increase in ROS levels was observed in the presence of oxidative stress. Interestingly, pre-incubation of hepatic cells with SJE prior to exposure to SA resulted in an inhibition of ROS production. The protective effect was confirmed by the analysis of intracellular GSH levels, whose homeostasis plays a crucial role in counteracting iAs^{3+}-induced oxidative stress. As shown in Fig. 5C, a significant decrease (about 40%) was observed after SA-treatment, but no oxidation in GSH was observed when cells were protected by SJE extract. The antioxidant activity of SJE was finally confirmed by Western blotting analyses, in which the phosphorylation levels of p38 and of its direct target, MAPKAPK-2, were analyzed. These two proteins belong to a MAPK family, which are evolutionarily highly conserved enzymes that manage the response to growth stimulatory signals, such as insulin or EGF, as well as adverse signals, such as cytotoxic and genotoxic substances or radiations (Klotz et al., 1999). During oxidative stress, the phosphorylation level of these two proteins increases. Fig. 5D clearly shows that SA induces high phosphorylation levels of these two markers, whereas, in the presence of SJE, the phosphorylation levels of p38 and MAPKAPK-2 are similar to those observed in untreated cells. Therefore, SJE is able to prevent SA activated signaling cascade.

4. Conclusions

In the current study, the chemical profiling of *S. jambos* leaf extract by HPLC-PDA-ESI-MS/MS revealed 17 compounds including flavonol glycosides, flavonol di-glycosides and flavones as well as ellagitannins and phenolic acids. The antioxidative and hepatoprotective activities of SJE were investigated *in vitro*, on human hepatic cells and *in vivo*. As expected, from the phytochemical data SJE showed promising antioxidant activities in two reliable and commonly used assays (DPPH and FRAP), as well as high total phenolic contents. *S. jambos* leaves extract exerted substantial antioxidant activity *in vivo* in *C. elegans* organism. Moreover, potent hepatoprotective activities in human hepatic cells in which oxidative stress was induced using sodium arsenite, and in an acute CCl_4-induced hepatic injury rat model were observed. This protective effect may be ascribed to antioxidant molecules present in the leaf extract.

In conclusion, our results suggest that *S. jambos* leaf extract may provide an interesting candidate in pharmaceutical formulations for the treatment of diseases resulting from elevated reactive oxygen species. However, further experiments are still needed to investigate the pharmacodynamics and pharmacokinetics of the extract.

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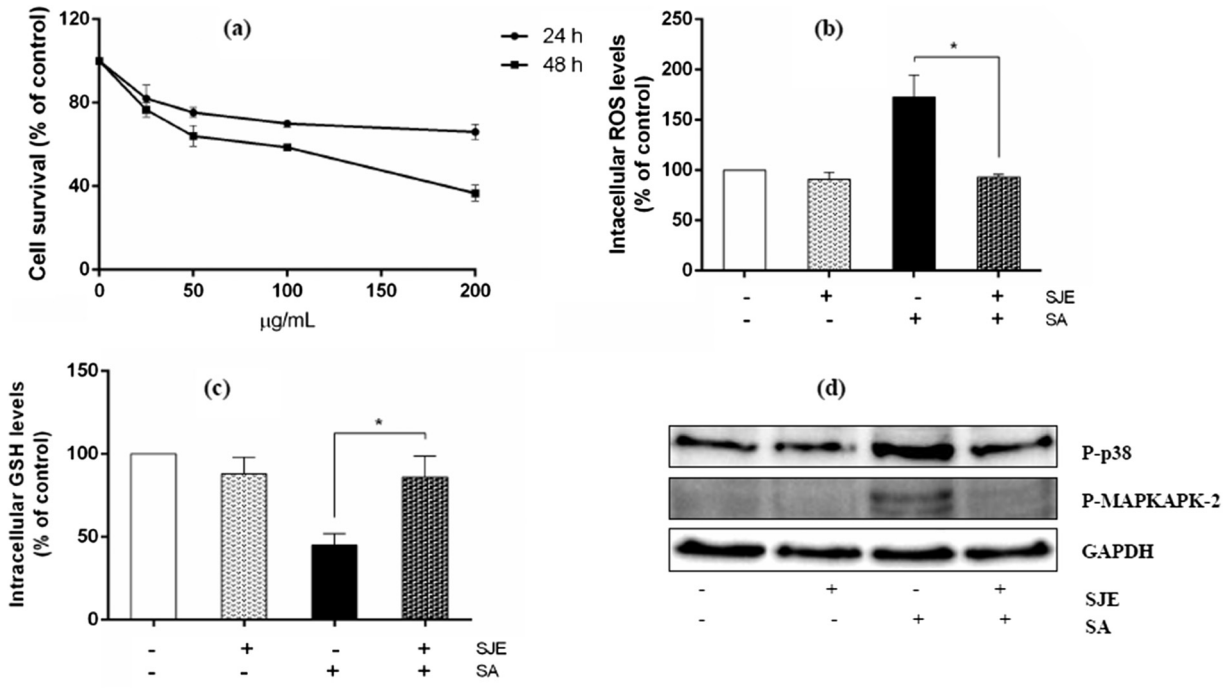

Fig. 5. Effects of SJE on human hepatic cells. (a) MTT analysis: cells were treated with increasing concentrations of extract (25–200 µg/mL) for 24–48 h. Cell viability was assessed by the MTT assay and values are given as means ± SEM (n ≥ 3). (b) and (c), DCFDA and DTNB assays: cells were pre-incubated in the presence of 50 µg/mL of the extract for 120 min and exposed to 300 µM SA for 120 min. White bars refer to control cells, black bars to stressed cells, dotted bars to cells incubated with SJE, and black dotted bars to cells pretreated with SJE and then stressed by SA. Asterisks (*) indicate values that are significantly different from SA treated cells ($p < .05$) as determined by Student's *t*-test. (d) Western blotting analysis: cells were pre-incubated as described and western blot was performed using specific antibodies against phospho-p38 and phospho-MAPKAPK-2. GAPDH was used as loading control.

Conflict of interest

The authors declare no conflict of interest.

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