

High resolution UPLC-MS/MS profiling of polyphenolics in the methanol extract of *Syzygium samarangense* leaves and its hepatoprotective activity in rats with CCl₄-induced hepatic damage



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ABSTRACT

Oxidative stress plays a crucial role in the development of several liver diseases. Many natural polyphenols can attenuate oxidative stress and liver injury. In this study, a phytochemical profiling of a methanol extract from leaves of *Syzygium samarangense* revealed 92 compounds belonging to flavonoids, phenolic acids, condensed tannins, and ellagitannins. The *S. samarangense* extract exhibited a noticeable antioxidant activity with an EC₅₀ of 5.80 µg/mL measured by DPPH scavenging capacity assay, 2632 Trolox equivalents, 10 mM Fe²⁺ equivalents/mg of samples by TEAC and FRAP assays, respectively. The total phenolic content was 419 mg gallic acid equivalent GAE/g extract. In a cell-based model (HaCaT cells), the extract completely inhibited ROS production induced by UVA, and prevented GSH-depletion and p38 phosphorylation. In addition, the extract exhibited a substantial antioxidant and hepatoprotective activities in CCl₄-treated rats, with an increase in GSH (reduced glutathione) and SOD (superoxide dismutase) activities by 84.75 and 26.27%, respectively, and a decrease of 19.08, 63.05, 52.21, 37.00, 13.26, and 15.15% in MDA, ALT, AST, TB (total bilirubin), TC (total cholesterol), and TG (total glycerides), respectively. These results were confirmed by histopathological analyses. We believe that *Syzygium samarangense* is a good candidate for further evaluation as an antioxidant and liver protecting drug.

1. Introduction

The liver constitutes the largest and most critical detoxifying organ within the human body. In spite of the necessity of oxygen to maintain life, sometimes it can become toxic through the generation of reactive oxygen species (ROS), which play an important role in the liver pathology because of lipid peroxidation and DNA damage (Panovska et al., 2007).

Infections, autoimmune disorders, multiple hazardous substances such as acetaminophen, isoniazid, carbon tetrachloride, non-steroidal anti-inflammatory drugs, and alcohol can cause liver inflammation, serious damage and liver cirrhosis. Although the liver is known to possess a great regeneration power after damage, the aforementioned factors can eventually lead to serious hepatic ailments (Pandit et al., 2012).

In modern medicine, evidence-based synthetic liver-protective

Abbreviations: ABTS⁺, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid); ALT, alanine transaminase; ANOVA, one way analysis of variance; AST, aspartate transaminase; CCl₄, carbon tetrachloride; DCFDA, 2',7'-dichlorodihydrofluorescein diacetate; DPPH, 2,2-diphenyl-1-picrylhydrazyl radical; DTNB, 5,5'-dithiobis-2-nitrobenzoic acid; ESI, electrospray ionization; FRAP, Ferric reducing antioxidant power; GAE, gallic acid equivalent; GSH, reduced glutathione; HaCaT cells, normal human keratinocytes; HR-UPLC-MS/MS, high resolution ultra-high performance liquid chromatography mass-mass spectrometry; MDA, malondialdehyde; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ROS, reactive oxygen species; SOD, superoxide dismutase; TB, total bilirubin; TC, total cholesterol; TEAC, Trolox equivalent antioxidant capacity; TG, total glycerides; UVA, near ultra violet radiation (315–400 nm)

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agents are nearly absent. However, several medicinal plants with phenolic secondary metabolites are known for their hepatoprotective properties. A lignan mixture from *Silybum marianum*, termed silymarin, is well-known and clinically applied (van Wyk and Wink, 2015, 2017).

The Myrtaceae comprises about 131 genera and 5500 species that are characterized by their abundant antioxidants, mainly flavonoids, flavonols, anthocyanins, ellagitannins as well as phenolic acids (Nair et al., 1999). Among its genera, *Syzygium* is a large genus that includes about 1100 species, many of which had been taxonomically confused with the genus *Eugenia* (Wrigley and Fagg, 2013).

Syzygium samarangense (Blume), Merr. & Perry (synonym *Eugenia javanica* L.) is commonly known as wax apple and characterized by its bell-shaped edible fruit which shows different colors. Extracts from this plant possess antibacterial, anti-inflammatory, analgesic, and spasmolytic properties. Additionally, immune-stimulant, antipyretic, and diuretic activities were reported from *S. samarangense*, as well as anti-hyperglycemic activity in diabetes mellitus type II (Shen et al., 2013). These pharmacological properties are probably attributed to the existence of various secondary metabolites, such as flavonoids, chalcones exemplified by 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone and its isomer 5-O-methyl-4'-desmethoxy matteucinol. Gallic and ellagic acids, in addition to many tannins and ellagitannins as vescalagin are present in *S. samarangense* (Nair et al., 1999).

Although *S. samarangense* has been investigated for a plethora of biological activities, its antioxidant activity and its potential hepatoprotective activity, related to the ability to counteract liver deterioration caused by xenobiotics, has not been reported in literature. Thus, in this study, the chemical profiling of a leaf extract was carried out using HR-UPLC-MS/MS. In addition, we investigated the antioxidant potential of the methanol leaf extract both *in vitro*, using DPPH, TEAC, and FRAP assays and in a cell-based model, using normal human keratinocytes (HaCaT cells) in which oxidative stress was induced by UVA irradiation. Finally, the antioxidant and hepatoprotective activities were analyzed *in vivo* using rats with CCL₄-induced hepatic toxicity.

2. Materials and methods

2.1. Plant material

Leaves of *S. samarangense* were collected from fully mature trees that were cultivated in a private botanical garden during the spring season in the period between April to May 2014. The plant was authenticated morphologically by Mrs. Therese Labib, Consultant of Plant Taxonomy at the Ministry of Agriculture and El-Orman Botanical Garden, Giza, Egypt (Sobeh et al., 2016). A voucher specimen of the plant material is being kept at Pharmacognosy Department, Faculty of Pharmacy, Ain Shams University with voucher number PHG-P- SS-182.

2.2. Preparation of the plant extract

Air dried leaves (100 g) of *S. samarangense* were ground into a coarse powder and exhaustively percolated in methanol (3 × 0.5 L). After filtering the extract, it was consequently evaporated under reduced pressure at 40 °C until dryness. Upon lyophilization, 15 g dried extract was obtained.

2.3. Chemicals and kits

Diphenylpicrylhydrazyl (DPPH) and carbon tetrachloride (CCl₄) were purchased from Sigma® (Sigma-Aldrich, St. Louis, USA). Quercetin and gallic acid were purchased from Gibco® (Invitrogen; Karlsruhe, Germany). All kits for the assessment of the biochemical parameters as alanine transaminase (ALT), aspartate transaminase (AST), total bilirubin (TB), total cholesterol (TC), total glycerides (TG), superoxide dismutase (SOD) activity, concentration of reduced glutathione (GSH), and lipid peroxidation marker malondialdehyde (MDA) were obtained

from Biodiagnostics (Cairo, Egypt). LC-MS analysis was performed using UPLC solvents grade. All other chemicals and kits were of the highest grade commercially available.

2.4. Chemical profiling

The phytochemical profiling of the plant polyphenolics was done using LC consisting of Agilent 1200 series. The LC column was Gemini® 3 μm C18 110 Å, 100 mm × 1 mm i. d (Phenomenex, USA). The column was protected with a guard column (RP-C18, 5 μm 100 Å, 5 mm × 300 μm i. d). Two solvents (A) water, and (B) 90% MeOH (2% acetic acid each) were used as a mobile phase at a flow rate of 50 μL/min 10 μL sample was injected via autosampler. Gradient from 5% B at 0 min to 50% B in 60 min and then increased to 90% in 10 min and kept for 5 min was adopted. A Fourier transform ion cyclotron resonance mass analyzer coupled with electrospray ionization (ESI) system was used (Thermo Fisher Scientific, Bremen, Germany). The instrument was set to the following conditions: capillary voltage of 36 V, a temperature of 275 °C. Voltage of 5 kV, and desolvation temperature of 275 °C were used for the API source. As a nebulizing gas, nitrogen with a flow rate of 15 L/min was used. The ions were collected in a high resolution up to 100,000 and full mass scan mass range of 150–2000 *m/z*. Xcalibur® software (Thermo Fisher Scientific, USA) was used for recording and integrating the chromatograms.

2.5. *In vitro* antioxidant evaluation

The Folin-Ciocalteu method was applied to determine the total phenolic content as previously described by Zhang et al. (2006), DPPH' (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity of the extract was assessed using the standard technique described by Blois (1958), trolox equivalent antioxidant capacity (TEAC) persulfate decolorizing kinetic assay was carried out as previously reported by Re et al. (1999), ferric reducing antioxidant power (FRAP) assay was used to measure reduction ability of the extract to convert the ferric complex (2, 4, 6-tripyridyl-s-triazine - Fe³⁺-TPTZ) to its ferrous form (Fe²⁺-TPTZ) at low pH following the protocol described by Benzie and Strain (1996) and they were adapted to 96-well plate as previously described (Ghareeb et al., 2017).

2.6. Cell culture and MTT assay

Human epidermal keratinocytes (HaCaT), provided by Innoprot (Biscay, Spain), were cultured in Dulbecco's Modified Eagle's Medium (EuroClone), supplemented with 10% fetal bovine serum (HyClone), 2 mM L-glutamine and antibiotics (EuroClone) in a 5% CO₂ humidified atmosphere at 37 °C. Every 48 h, HaCaT cells were sub-cultured in a ratio of 1:3. Briefly, the culture medium was removed and cells were rinsed with PBS (EuroClone), detached with trypsin-EDTA (EuroClone) and diluted in fresh complete growth medium.

For dose and time dependent cytotoxicity experiments, cells were seeded in 96-well plates at a density of 2 × 10³/well. 24 h after seeding, increasing concentrations of the methanol extract (from 25 to 200 μg/mL) were added to the cells. After 24 and 48 h incubation, cell viability was assessed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, as described in Monti et al. (2015). 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 buffer. Each sample was tested in three independent analyses, each carried out in triplicates.

2.7. Oxidative stress

UVA light (100 J/cm²) was used as source of oxidative stress. To investigate oxidative stress, cells were plated at a density of

2×10^4 cells/cm². 24 h after seeding, cells were incubated for 2 h in the presence or absence of 50 µg/mL of the extract and then exposed to UVA light (365 nm) for 10 min.

2.7.1. DCFDA assay

ROS production was measured as described by Del Giudice et al. (2016). Briefly, immediately after UVA irradiation, cells were incubated with 2',7'-dichlorodihydrofluorescein diacetate (H₂-DCFDA, Sigma-Aldrich). 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.

2.7.2. DTNB assay

DTNB assay which was performed according to Petruk et al. (2016) was used to analyze glutathione levels. Briefly, immediately after UV-A irradiation, 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 Diagnostics Ltd, Mannheim, Germany)). 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 14,000g for 5 min at 4 °C and the absorbance of the supernatant was measured at 412 nm by using a multiplate reader (Biorad). GSH levels were expressed as the percentage of TNB as the rate of TNB production is directly related to the rate of this recycling reaction, which is in turn directly related to the concentration of GSH in the sample. Values are the mean of three independent experiments, each with triplicate determinations.

2.8. Western blot analyses

To investigate signaling stress pathways, cells were plated at a density of 2×10^4 cells/cm² in complete medium for 24 h and then treated as described above. Untreated and treated cells were incubated for 90 min at 37 °C and cell lysates analyzed by Western blotting performed as reported in Galano et al. (2014). Phosphorylation levels of p38 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.

2.9. In vivo antioxidant and hepatoprotective assessment

2.9.1. Animals and animal treatment

Male Sprague-Dawley rats, weighing 200–250 g, obtained from the animal facility of King Abdulaziz University (Jeddah, KSA) were used in the current study. In an air-conditioned atmosphere at 22 ± 2 °C, rats were housed under a 12 h light–dark cycle. The rats had access to food (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.

2.9.2. Experimental design

Animals were randomly assigned into 4 groups (n = 6). The control group obtained water orally followed by intraperitoneal injection of corn oil after 4 h and was considered as the first group. A dose of 1 mL/kg from CCl₄-corn oil 50% mixture was intraperitoneally injected into

rats of the second group. Groups 3 and 4 were pretreated orally with 200 mg/kg of the known hepatoprotective silymarin and *S. samarangense* extract, respectively. Four hours after the pretreatment, groups 3 and 4 received an IP injection of the CCl₄-corn oil mixture.

2.9.3. Preparation of tissue and serum samples

Blood samples were collected from the rats after 24 h from CCl₄ injection; the sera were prepared and as previously described (Breikaa et al., 2013). Rats were sacrificed and liver tissues were dissected and prepared for the histopathological examination. Liver markers ALT, AST, TB, TC, and TG were determined colourimetrically using Mindray BS-120 clinical chemistry auto-analyzer (Shenzhen Mindray Bio-medical Electronics Co. Ltd., Shenzhen, China). Oxidative stress markers GSH, MDA and SOD were quantified using available commercial kits (Biodiagnostics, Cairo, Egypt).

2.9.4. Histopathological examination

Tissue samples were kept for 24 h in 10% neutral buffered formalin and water; then they were dehydrated with methyl, ethyl and absolute ethyl alcohol, and finally clarified using xylene. Tissues were then embedded in paraffin at 56 °C in hot air oven for 24 h. A slide microtome was used to prepare the sections at 4 µm thickness and they were then collected on glass slides, deparaffinized, and stained with hematoxylin and eosin stain for histopathological examination. Light microscopy (Olympus BX-50 Olympus Corporation, Tokyo, Japan) was used to investigate the glass slides (Bancroft et al., 1996).

2.10. Statistical analysis

Biological experiments were run in triplicates and the data are presented as mean \pm S.D. The statistical significance was carried out using one way analysis of variance (ANOVA) followed by Tukey's test for post hoc analysis. A value of $p < 0.05$ was considered as statistically significant. Graphs were plotted using GraphPad Prism software, version 5.00 (GraphPad Software, Inc. La Jolla, CA, USA).

3. Results

3.1. Phytochemical composition

Chemical profiling of the total methanol extract of *S. samarangense* leaves using LC-ESI-MS/MS revealed 92 compounds belonging to several classes, mainly flavonoids, phenolic acids as well as tannins and ellagitannin (Table 1). The main compounds were (epi)-catechin-(epi)-gallocatechin (20 and 23), (epi)-gallocatechin gallate (35), (epi)-catechin-afzelechin (50), myricetin pentoside (71), myricetin rhamnoside (72), guaijaverin (81), and isorhamnetin rhamnoside (84). The total ion chromatogram [LC-MS base peak in the negative ionization mode ESI (–)] is represented in Fig. 1.

A series of phenolic acids represented by gallic acid (previously isolated from the plant), *p*-coumaroylquinic acid, and galloylquinic acid were identified; their retention times and fragmentation patterns are reported in Table 1.

Proanthocyanidins were detected in the studied extract via several ions, for instance, (epi)-gallocatechin was detected with [M-H][–] at *m/z* 305, (epi)-catechin gallate with [M-H][–] at *m/z* 441, (epi)-gallocatechin gallate [M-H][–] at *m/z* 457 and fragments *m/z* 169, 305, and 331, that match previously described data (Tala et al., 2013). Furthermore, proanthocyanidin dimers, trimers, tetramers and their galloylated esters in different isomeric forms were identified: Three peaks, as an example, showed [M-H][–] at *m/z* 577 and fragment ions at *m/z* 289, 407 and 599; they were identified as (epi)-catechin-(epi)-catechin as previously reported (Sobeh et al., 2017a).

Additionally, several ions were detected at [M-H][–] *m/z* at 561, 593, 609, 761, 745, and 913 which were identified as (epi)-catechin-afzelechin, (epi)-catechin-(epi)-gallocatechin, (epi)-gallocatechin-(epi)-

Table 1
Chemical composition of the methanol extract of *S. samarangense* leaves.

No.	t _R (min.)	[M-H] ⁺ (m/z)	MS/MS fragment	Tentatively identified compound	References
1	1.78	191	127	Quinic acid	(Sobeh et al., 2017a)
2	1.95	325	133	Malic acid derivative	
3	2.11	337	191	<i>p</i> -Coumaroylquinic acid	(Pereira et al., 2007)
4	2.27	337	191	<i>p</i> -Coumaroylquinic acid	(Pereira et al., 2007)
5	2.51	343	125, 169, 191	Galloylquinic acid	
6	3.10	609	305, 441, 417, 591	(epi)-Galocatechin-(epi)-galocatechin	(Tala et al., 2013)
7	3.65	331	125, 169	Galloyl-hexose	
8	3.94	169	125	Gallic acid ^b	(Sobeh et al., 2017a)
9	4.13	933	301, 425, 631, 915	Castalagin/Vescalagin	
10	5.38	435	169, 271, 313	Malferin B	
11	5.95	633	301, 463, 613.	Gallotannin/ellagitannin	
12	6.15	609	305, 423, 441	(epi)-Galocatechin-(epi)-galocatechin	(Tala et al., 2013)
13	6.39	1583	765, 935, 1395	Eugeniflorin D2	(Lee et al., 2000)
14	6.69	343	125, 169, 191	Galloylquinic acid	
15	7.80	305	125, 179, 287	(epi)-Galocatechin	(Sobeh et al., 2017b)
16	8.25	593	305, 423, 441	(epi)-Catechin-(epi)-galocatechin	(Sobeh et al., 2017b)
17	9.90	1417	451, 595, 765, 785	Unknown tannin	
18	10.21	761	305, 423, 441, 609	(epi)-Galocatechin-(epi)-galocatechin gallate	
19	11.39	1585	633, 765, 907, 1541	Ellagitannin	
20	12.54	593	289, 407, 425, 557	(epi)-Catechin-(epi)-galocatechin	(Sobeh et al., 2017b)
21	13.50	1567	451, 765, 935, 1548	Oenothetin B	(Lee et al., 2000)
22	17.32	609	305, 423, 441, 591	(epi)-Galocatechin-(epi)-galocatechin	
23	17.40	593	305, 423, 441, 575	(epi)-Catechin-(epi)-galocatechin	(Sobeh et al., 2017b)
24	17.97	761	305, 423, 591	(epi)-Galocatechin-(epi)-galocatechin gallate	(Tala et al., 2013)
25	19.78	305	125, 179, 287.	(epi)-Galocatechin	
26	19.97	647	305, 493, 577	Unknown tannin	
27	21.38	599	301, 447	Quercetin galloyl-rhmanoside	
28	22.25	745	305, 407, 423, 593	(epi)-Galocatechin-(epi)-catechin gallate	(Tala et al., 2013)
29	25.55	745	305, 423, 575, 619	(epi)-Galocatechin-(epi)-catechin gallate	(Tala et al., 2013)
30	26.87	593	289, 407, 575	(epi)-Catechin-(epi)-galocatechin	(Sobeh et al., 2017b)
31	26.94	577	271, 289, 407, 558	(epi)-Catechin-(epi)-catechin	(Sobeh et al., 2017b)
32	27.78	609	305, 423, 441, 591	(epi)-Galocatechin-(epi)-galocatechin	
33	27.79	913	285, 423, 591, 761	(epi)-Galocatechin gallate-(epi)-galocatechin gallate	(Tala et al., 2013)
34	28.16	447	285	Kaempferol hexoside	
35	29.53	457	169, 305, 331	(epi)-Galocatechin gallate	
36	31.17	729	305, 423, 577	Procyanidin dimer monogallate	(Tala et al., 2013)
37	31.66	935	301, 633	Casuarinin	
38	31.69	745	289, 423, 593	(epi)-Galocatechin-(epi)-catechin gallate	(Tala et al., 2013)
39	31.81	761	285, 423, 607, 635	(epi)-Galocatechin-(epi)-galocatechin gallate	(Tala et al., 2013)
40	32.00	1217	423, 761, 913	(epi)-Galocatechin-(epi)-galocatechin-(epi)-galocatechin-(epi)-galocatechin	(Tala et al., 2013)
41	32.22	577	289, 407, 599	(epi)-Catechin-(epi)-catechin	(Sobeh et al., 2017a)
42	33.15	625	317, 479	Myricetin rhamnosyl-hexoside	
43	33.95	1017	407, 729, 865	(epi)-Catechin-(epi)-catechin-(epi)-catechin gallate	(Tala et al., 2013)
44	34.70	1049	423, 591, 761, 879	(epi)-Galocatechin-(epi)-galocatechin-(epi)-galocatechin gallate	(Tala et al., 2013)
45	34.95	1353	407, 754, 1013, 1201	(epi)-Galocatechin-(epi)-galocatechin-(epi)-galocatechin-(epi)-catechin gallate	
46	35.05	745	289, 423, 559, 725	(epi)-Galocatechin-(epi)-catechin gallate	(Tala et al., 2013)
47	35.15	1217	423, 761, 912, 1199	(epi)-Galocatechin-(epi)-galocatechin-(epi)-galocatechin-(epi)-galocatechin	(Tala et al., 2013)
48	35.64	441	151, 271, 289	(epi)-Catechin gallate	(Tala et al., 2013)
49	35.84	1201	407, 745, 863, 1049	(epi)-Catechin-(epi)-galocatechin-(epi)-galocatechin-(epi)-galocatechin	(Tala et al., 2013)
50	36.26	561	271, 273, 289	(epi)-Catechin-afzelechin	(Sobeh et al., 2017b)
51	38.45	1169	407, 559, 729, 881	(epi)-Catechin-(epi)-catechin-(epi)-catechin-(epi)-galocatechin	(Tala et al., 2013)
52	39.55	937	301, 465, 767	Tellimagrandin II	
53	40.51	881	407, 559, 577, 729	(epi)-Catechin gallate-(epi)-catechin gallate	
54	40.63	729	289, 407, 559, 577	Procyanidin dimer monogallate	(Sobeh et al., 2017a)
55	41.25	441	169, 289	(epi)-Catechin gallate	(Tala et al., 2013)
56	41.64	1809	575, 1639, 1657	Unknown tannin	
57	42.17	1017	287, 407, 729, 847	(epi)-Catechin-(epi)-catechin-(epi)-catechin gallate	(Tala et al., 2013)
58	43.36	913	285, 423, 743, 761	(epi)-Galocatechin gallate-(epi)-galocatechin gallate	(Tala et al., 2013)
59	43.90	785	423, 591, 617, 659	(epi)-Galocatechin-(epi)-galocatechin- <i>O</i> -glucuronide	(Tala et al., 2013)
60	44.65	1169	407, 729, 1017	(epi)-Catechin-(epi)-catechin-(epi)-catechin-(epi)-galocatechin	
61	45.33	1353	423, 1013, 1199	(epi)-Galocatechin-(epi)-galocatechin-(epi)-galocatechin-(epi)-catechin gallate	
62	45.91	1169	407, 729, 881, 1017	(epi)-Catechin-(epi)-catechin-(epi)-catechin-(epi)-galocatechin	(Tala et al., 2013)
63	46.49	609	287,305, 439, 457	(epi)-Galocatechin-digallate	
64	47.05	745	305, 457, 575, 593	(epi)-Galocatechin-(epi)-catechin gallate	(Tala et al., 2013)
65	47.64	577	287, 423, 541, 559	(epi)-Catechin-(epi)-catechin	(Tala et al., 2013)
66	48.90	599	285, 447	Myriganon H galloyl-hexoside	
67	49.25	479	179, 316	Myricetin hexoside	(Celli et al., 2011)
68	50.38	449	317	Myricetin pentoside	(Celli et al., 2011)
69	51.13	1169	407, 729, 881, 1043	(epi)-Catechin-(epi)-catechin-(epi)-catechin-(epi)-galocatechin	
70	51.68	595	179, 301, 463	Quercetin pentosyl-hexoside	(Ieri et al., 2015)
71	51.93	449	317	Myricetin pentoside	(Celli et al., 2011)
72	53.88	463	317	Myricetin rhamnoside	(Celli et al., 2011)
73	54.28	615	301, 463	Quercetin galloyl-hexoside	

(continued on next page)

Table 1 (continued)

No.	t _R (min.)	[M-H] ⁻ (m/z)	MS/MS fragment	Tentatively identified compound	References
74	54.70	503	165, 209	Lambertianoside.	
75	56.95	729	289, 407, 559	Procyanidin dimer monogallate	(Tala et al., 2013)
76	57.23	881	407, 559, 729	(epi)-Catechin gallate-(epi)-catechin gallate	(Tala et al., 2013)
77	57.84	609	179, 301	Quercetin rutinoside	
78	58.82	503	165, 209	Lambertianoside isomer	
79	59.68	431	269	Cryptostrobinhexoside	
80	60.75	343	329	Methyltricin	
81	62.94	433	301	Guaijaverin ^a	
82	64.32	417	151, 284	Myrigalone H pentoside	
83	64.91	615	317, 463	Myricetin galloyl-rhmanoside	(Wang et al., 2016)
84	66.21	461	315	Isorhamnetin rhamnoside	(Liu et al., 2016)
85	66.93	331	316	2-C-Methylmyricetin (Mearnsitrin) ^a	(Nair et al., 1999)
86	68.18	417	151, 285	Myrigalone H pentoside	
87	68.78	431	285	Myrigalone H rhamnoside	
88	69.19	585	179, 301, 433	Quercetin galloyl-pentoside	
89	70.46	329	315	Tricin	
90	71.16	285	151, 285	Myrigalon H ^a	
91	72.53	343	329	Methyltricin	
92	73.22	269	269	Cryptostrobin ^a	(Mamdouh et al., 2014)

^a Previously isolated from the plant.

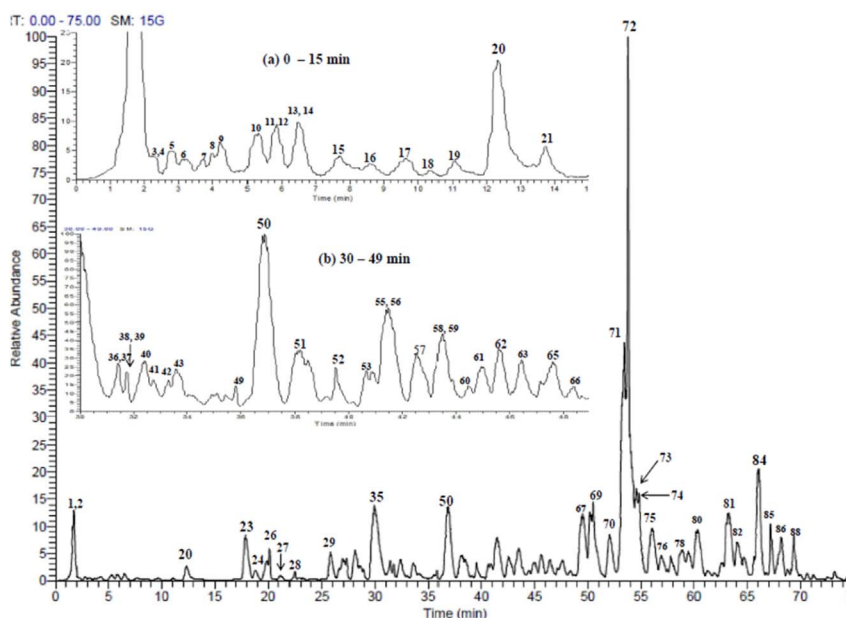


Fig. 1. LC-MS base peak in the negative ionization mode ESI of the methanol extract of *S. samarangense*. (A) A representative chromatogram of the area between 0 and 15 min; (b) A representative chromatogram of the area between 30 and 49 min.

Table 2

Antioxidant activities of the methanol extract from *S. samarangense* leaves using DPPH, TEAC and FRAP assays.

Assay	Extract	EGCG _{Control}
DPPH (EC ₅₀ µg/mL)	5.80	3.50
TEAC (Trolox equivalents/mg of sample)	2632	5293
FRAP (Fe ²⁺ equivalents/mg of sample)	10	25

EGCG_{Control}: *epi*-gallocatechin gallate used as a positive control.

gallocatechin, (epi)-gallocatechin-(epi)-gallocatechin gallate, (epi)-gallocatechin-(epi)-catechin gallate, and (epi)-gallocatechin gallate-(epi)-gallocatechin gallate, respectively as previously described (Tala et al., 2013); their retention times and fragmentation pattern are shown in Table 1.

Flavonoids were also detected. A series of ions showed [M-H]⁻ at *m/z* 449, 463, 479, and 615 with a main fragment ion at *m/z* 317; they were identified as myricetin pentoside, myricetin rhamnoside (previously isolated of the plant), myricetin hexoside, and myricetin

galloyl-rhmanoside as previously reported (Celli et al., 2011; Ieri et al., 2015; Wang et al., 2016).

Another series of compounds showed [M-H]⁻ at *m/z* 585, 595, 609, and 615 and a daughter ion at *m/z* 301; they were assigned to quercetin galloyl-pentoside, quercetin pentosyl-hexoside, quercetin rutinoside, and quercetin galloyl-hexoside, respectively as previously shown in the literature. However, a peak showing [M-H]⁻ at *m/z* 433 and a daughter ion *m/z* 301 was assigned to guaijaverin as previously described from the plant. Similarly, mearnsitrin, myrigalone H, cryptostrobin were isolated from the plant (Nair et al., 1999; Mamdouh et al., 2014; Liu et al., 2016). Data are presented in Table 1.

3.2. Biological activities

3.2.1. Total phenolic content and in vitro antioxidant activity

The total phenolic content of the extract was 419 mg GAE/g extract, as assessed by the Folin-Ciocalteu method. *In vitro* antioxidant activity of the extract was evaluated using three widely used assays: DPPH, TEAC, and FRAP. *S. samarangense* extract exhibited a noticeable

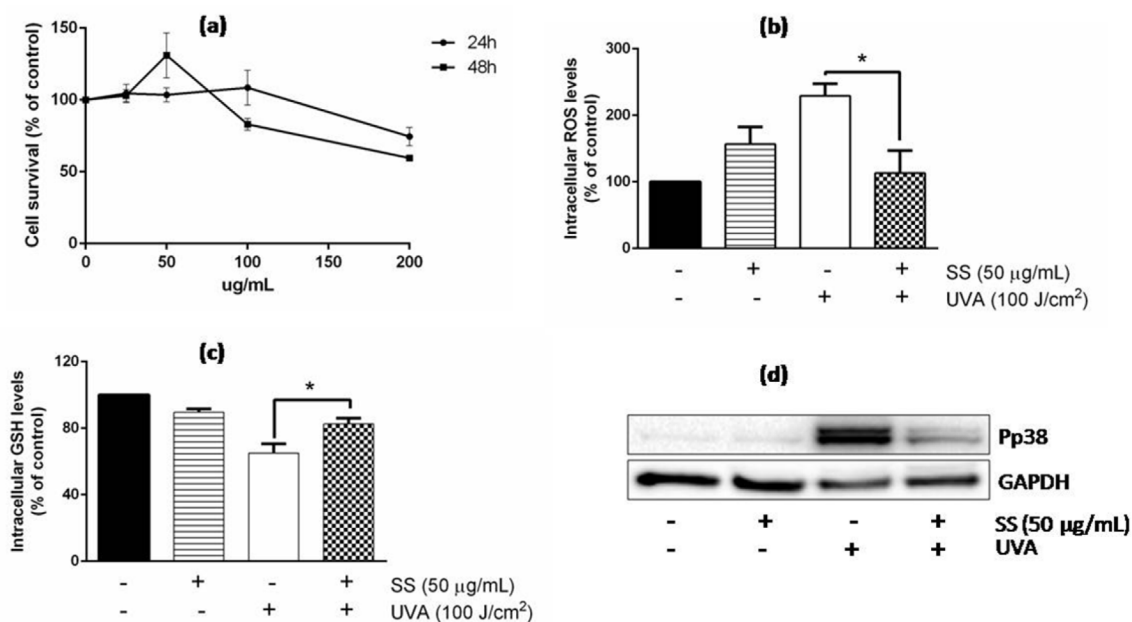


Fig. 2. Effects of *S. samarangense* extract on normal human keratinocytes (HaCaT cells). (a) MTT analysis: cells were treated with increasing concentrations of extract (25–200 µg/mL) for 24–48 h. Cell viability was assessed by the MTT assay and values are given as means ± SD (n ≥ 3). (b) DCFDA and (c) DTNB assays: cells were pre-incubated in the presence of 50 µg/mL of the extract for 2 h and exposed to UVA (100 J/cm²). Black and white bars refer to control cells, untreated (black) or treated with UVA (white), whereas cell incubated with the extract are indicated by horizontal line bars or dotted bars. Asterisks (*) indicate values that are significantly different from UVA treated cells (p < 0.05) as determined by Student's t-test. (d) Western blotting analysis: cells were pre-incubated as described above and western blot was performed after 90 min incubation using anti-phospho-p38 antibody. GAPDH was used as loading control.

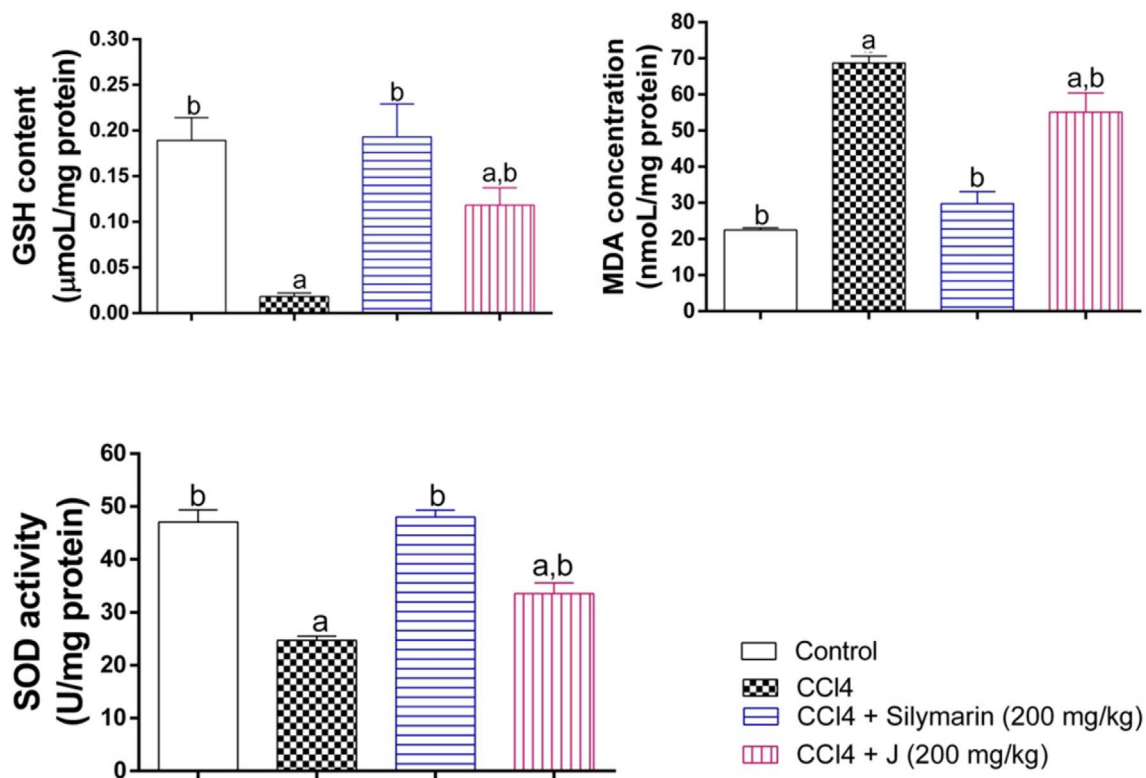


Fig. 3. Effect of pretreatment with *Syzygium samarangense* (SS) leaf extract on GSH content, SOD activity and MDA concentration in the rat model of acute CCl₄ intoxication. Values are means ± SD, n = 6. Statistical analysis was carried out by one-way ANOVA followed by Tukey post-hoc test. a: Statistically significant from the corresponding control at p < 0.05. b: Statistically significant from CCl₄-treated group at p < 0.05.

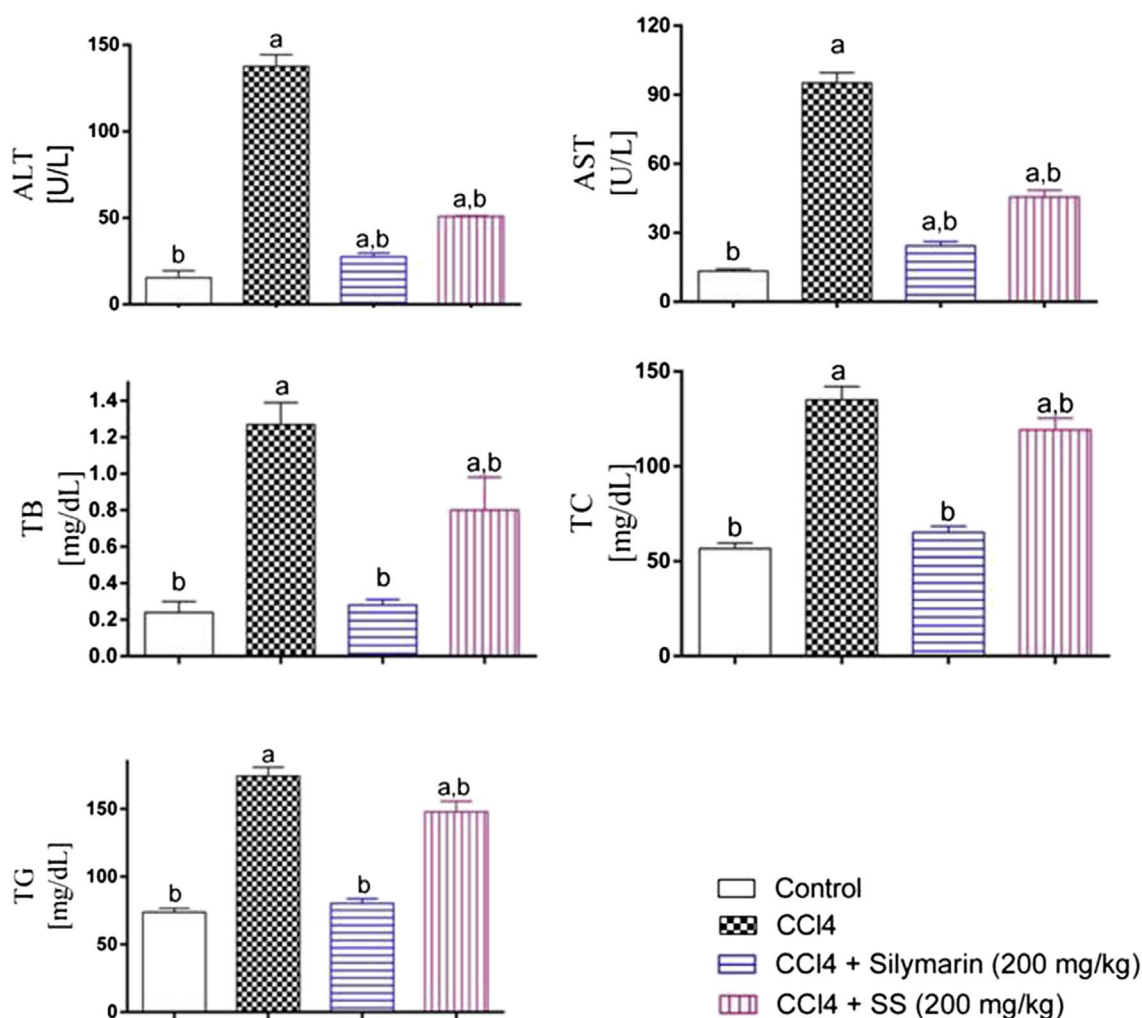


Fig. 4. Effect of pretreatment of leaf extract of *S. samarangense* (SS) on hepatotoxicity markers in the rat model of acute CCl₄ intoxication.

Values are means \pm SD, 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 $p < 0.05$.

b: Statistically significant from CCl₄-treated group at $p < 0.05$.

antioxidant activity (Table 2) comparable to that of EGCG (*epi-gallo-catechin gallate*).

3.2.2. *S. samarangense* extract mitigates UVA-induced ROS production

We first determined the biocompatibility of the extract on HaCaT cells by the MTT assay. Cells were incubated in the presence of increasing concentration of the extract (25–200 $\mu\text{g}/\text{mL}$) for 24 and 48 h. As shown in Fig. 2A, no significant toxicity was observed up to 100 $\mu\text{g}/\text{mL}$ after 24 h incubation, whereas an increasing toxicity was found above 100 $\mu\text{g}/\text{mL}$ after 48 h. This may probably due to the high concentration of antioxidant molecules. The concentration of 50 μg extract/mL was selected for antioxidant experiments on HaCaT cells.

To analyze the antioxidant activity of *S. samarangense* extract against UVA-induced oxidative stress, ROS levels were measured in cells pre-treated with the extract, exposed to UVA and immediately incubated with H₂-DCFDA. This is a cell-permeable dye, which is converted in its fluorescent form in the presence of ROS. As shown in Fig. 2B, a small increase in ROS production was observed upon exposure to the extract (horizontal lines bars), whereas UVA (white bars) induced an increase of 50% in ROS levels compared to control cells (black bars). Noteworthy, UVA-induced ROS production was completely inhibited when cells were pre-treated with the extract (50% decrease with respect to UVA-stressed cells, dotted bars).

3.2.3. *S. samarangense* extract prevents GSH-depletion and p38 phosphorylation

We then evaluated intracellular glutathione (GSH) levels of human keratinocytes treated with *S. samarangense* extract, as intracellular GSH is the most important antioxidant defence molecule. As shown in Fig. 2C, pre-treatment of cells with the extract had no significant effect on the intracellular GSH levels. Oxidative stress induced by UVA resulted in about 30% GSH depletion, but no decrease in GSH oxidation levels was observed upon pre-incubation of keratinocytes with the extract prior to UVA-induced stress. The protective effect of *S. samarangense* extract was further confirmed by the analysis of p38 phosphorylation levels by Western blotting. As shown in Fig. 2D, when cells were stressed with UVA, we observed a 30% decrease in phospho-p38 level in cells pre-incubated with the extract with respect to stressed cells. This result is in agreement with the activation pathway induced by UVA radiations (Petruk et al., 2016).

3.2.4. *In vivo* antioxidant and hepatoprotective assessment

The antioxidant activity was evaluated *in vivo* using rats with CCl₄-induced hepatic toxicity. We determined various antioxidant parameters, such as GSH and SOD together with MDA as oxidative stress markers (Fig. 3). Single intraperitoneal injection of a 1 mL/kg of CCl₄-corn oil mixture caused a dramatic decline in GSH content as well as

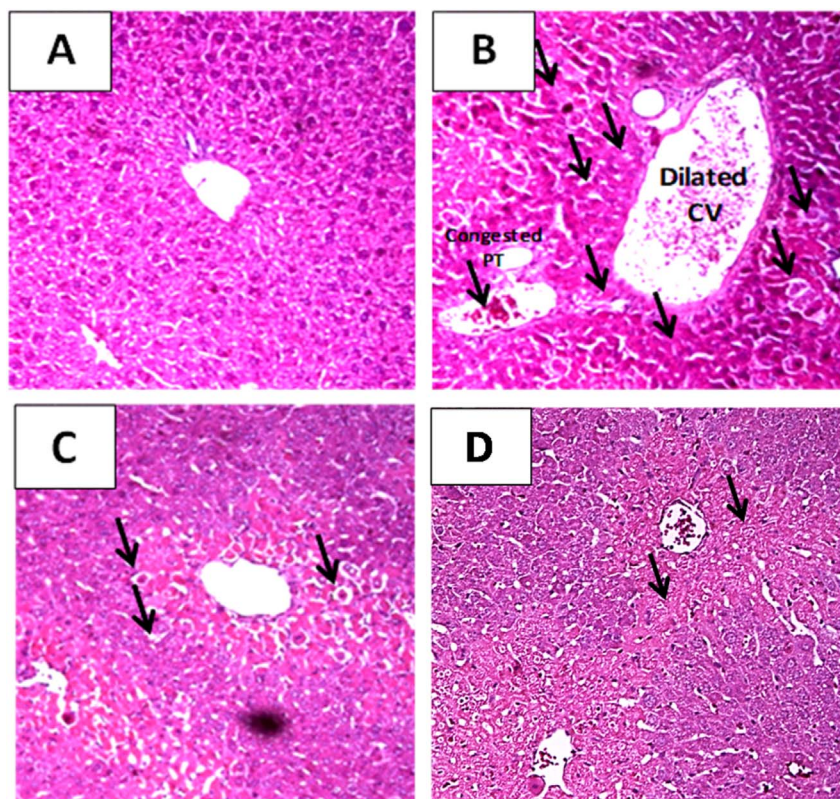


Fig. 5. Representative photomicrographs of liver sections stained by hematoxylin & eosin ($\times 100$): (A) Section taken from a liver of control rat showing normal hepatic architecture, hepatocyte structure and central vein, (B) Section taken from a liver of CCl_4 intoxicated rat showing dilated central 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 *S. samarangense* leaf extract showing alleviated hepatic injury with mild congestion of the central vein and few cytoplasmic vacuolization.

Group	Central vein dilation	BV congestion	Cytoplasmic vacuolization (indicated by arrows)
(A) Control	–	–	–
(B) CCl_4 -intoxication	++++	++++	++++
(C) Silymarin + CCl_4	+	+	++
(D) Extract + CCl_4	+	++	++

SOD activity (90.47, 47.56%, respectively) with a concomitant increase in MDA levels of 305.33%, when compared to untreated controls. After an oral administration of 200 mg/kg b. w. of the leaf extract, a significant antioxidant activity was observed, manifested by significant increases in the levels of GSH and SOD with a moderate amelioration in MDA concentration, similar to that observed with silymarin, the known hepatoprotective drug. Furthermore, the leaf extract caused 84.75 and 26.27% rise in GSH and SOD activity, respectively and a 19.08% decline in MDA concentration relative to the CCl_4 -treated group.

As shown in Fig. 4, CCl_4 caused a prominent elevation in all the hepatotoxic markers comprising ALT, AST, TB, TC, and TG estimated by 792.86, 610.45, 429.17, 138.10, and 136.68%, respectively with respect to the values of the untreated control group. The leaf extract showed a pronounced hepatoprotective activity as evidenced by the amelioration of the hepatic damage manifested by a significant decline in ALT, AST, TB, TC, and TG activities evaluated by 63.05, 52.21, 37.00, 13.26, and 15.15%, respectively relative to CCl_4 -treated group.

The hepatoprotective activity was further evidenced by the histopathological examination of liver sections obtained from treated animals (Fig. 5). As expected, the liver of the control group showed normal hepatic architecture, with distinct hepatic cells, sinusoidal spaces as well as central vein (Fig. 5A). On the other hand, the liver of CCl_4 -treated rats showed a dilated central vein with central hepatocellular necrosis (marked by arrows in Fig. 5B) and congested portal triad. For the silymarin treated group, liver sections showed preserved hepatic architecture with only few scattered cytoplasmic vacuolization as indicated by the arrows in Fig. 5C. However, a certain degree of congestion in the central vein and portal triad with the appearance of hepatocellular necrosis was observed in the group pretreated with the leaf extract, but to lesser extent with respect to CCl_4 intoxicated group

(Fig. 5D). In addition, a mild congestion in the central vein and portal triad with the appearance of few hepatocellular vacuolization were observed in the group pretreated with the leaf extract, showing alleviated hepatic injury compared to CCl_4 intoxicated group (Fig. 5D).

4. Discussion

Here, we analyzed the antioxidant activity of *S. samarangense* *in vitro*, using three different assays, on a cell-based model and *in vivo*, using a different oxidative stress inducer for each experimental system. UVA radiations have been selected to induce oxidative stress on normal keratinocytes as they are known to increase reactive oxygen species (ROS) production, thus causing oxidative stress (Petruk et al., 2016). Carbon tetrachloride (CCl_4) causes severe hepatic toxicity because it is converted by cytochrome P450s in the liver into highly active halogenated free radicals. The latter results in a serious lipid peroxidation and hepatic tissue destruction and this might be attributed to the formation of covalent bonds with membrane lipids. Thus, the ability of drugs to counteract the liver damage induced by CCl_4 has widely been implemented as a marker to study potential hepatoprotective activity.

In the present paper, independently from the source of oxidative stress, a strong protection of *S. samarangense* leaves extract was observed. The antioxidant and hepatoprotective activity of *Syzygium* species in general and of *S. samarangense* in particular, depends upon its richness in many flavonoids, phenolic acids and tannins. Similar hepatoprotective activities were demonstrated by some *Syzygium* species such as *S. alternifolium*, *S. aromaticum*, *S. cumini*, and *S. jambolana* (Hasan et al., 2009).

The extract is rich in flavonoids such as myricetin, tricetin, quercetin, kaempferol, and isorhamnetin and their glycosides; they can counteract

the hepatotoxic effect caused by CCl_4 via multiple mechanisms. These antioxidants show prominent free radical, reactive oxygen and nitrogen species scavenging activity. Besides, they promptly inhibit the enzymes that are responsible for the production of superoxide anion, comprising xanthine oxidase and protein kinase C (Soobrattee et al., 2005). In addition, they concomitantly prohibit cyclooxygenase, lipoxygenase, NADH oxidase, glutathione S-transferase, and mitochondrial succinoxidase that are responsible for ROS production (Soobrattee et al., 2005). Moreover, they act as transition metals chelators, such as copper and iron, through their adjacent –OH groups, thus inhibiting free radical chain reactions (Soobrattee et al., 2005; Youssef et al., 2017). Interestingly, myricetin exhibits the highest scavenging activity among all flavonoids, followed by quercetin and rhamnetin (Tapas et al., 2008).

Also, many other molecules, such as casuarinin and tannins, and more in detail (epi)-catechin gallate, (epi)-gallocatechin gallate and (epi)-catechin-(epi)-catechin exert powerful radical scavenging properties due to their high number of phenolic hydroxyl groups (Fahmy et al., 2016; Landete, 2011; Amarowicz, 2007). All these antioxidant molecules are present in *S. samarangense* leaf extract, thus let us to conclude that *S. samarangense* is a good candidate for a further evaluation as an antioxidant and liver protecting drug.

Transparency document

Transparency document related to this article can be found online at <http://dx.doi.org/10.1016/j.fct.2018.01.031>.

Conflicts of interest

The authors declare no conflict of interest.

References

- Amarowicz, R., 2007. Tannins: the new natural antioxidants? *EJLST* 109, 549–551.
- Bancroft, J., Stevens, A., Turner, D., 1996. *Theory and Practice of Histological Technique* (Edinburgh London, Melbourne and New York).
- Benzie, I.F., Strain, J., 1996. The ferric reducing ability of plasma (FRAP) as a measure of “antioxidant power”: the FRAP assay. *Anal. Biochem.* 239, 70–76.
- Blois, M.S., 1958. Antioxidant determinations by the use of a stable free radical. *Nature* 188, 1199–1200.
- Breikaa, R.M., Algandaby, M.M., El-Demerdash, E., Abdel-Naim, A.B., 2013. Biochanin A protects against acute carbon tetrachloride-induced hepatotoxicity in rats. *Biosci. Biotechnol. Biochem.* 77, 909–916.
- Celli, G.B., Pereira-Netto, A.B., Beta, T., 2011. Comparative analysis of total phenolic content, antioxidant activity, and flavonoids profile of fruits from two varieties of Brazilian cherry (*Eugenia uniflora* L.) throughout the fruit developmental stages. *Food Res. Int.* 44, 2442–2451.
- Del Giudice, R., Petruk, G., Raiola, A., Barone, A., Monti, D.M., Rigano, M.M., 2016. Carotenoids in fresh and processed tomato (*Solanum lycopersicum*) fruits protect cells from oxidative stress injury. *J. Sci. Food Agric.* 97, 1616–1623.
- Fahmy, N.M., Al-Sayed, E., Abdel-Daim, M.M., Karonen, M., Singab, A.N., 2016. Protective effect of *Terminalia muelleri* against carbon tetrachloride-induced hepato and nephro-toxicity in mice and characterization of its bioactive constituents. *Pharmaceut. Biol.* 54, 303–313.
- Galano, E., Arciello, A., Piccoli, R., Monti, D.M., Amoresano, A., 2014. A proteomic approach to investigate the effects of cadmium and lead on human primary renal cells. *Metallomics* 6, 587–597.
- Ghareeb, M.A., Mohamed, T., Saad, A.M., Refahy, L.A.-G., Sobeh, M., Wink, M., 2017. HPLC-DAD-ESI-MS/MS analysis of fruits from *Firmiana simplex* (L.) and evaluation of their antioxidant and antigenotoxic properties. *J. Pharm. Pharmacol.* <http://dx.doi.org/10.1111/jphp.12843>.
- Hasan, S.R., Hossain, M.M., Akter, R., Jamila, M., Mazumder, M.E.H., Rahman, S., 2009. DPPH free radical scavenging activity of some Bangladeshi medicinal plants. *J. Med. Plants Res.* 3, 875–879.
- Ieri, F., Innocenti, M., Possieri, L., Gallori, S., Mulinacci, N., 2015. Phenolic composition of “bud extracts” of *Ribes nigrum* L., *Rosa canina* L. and *Tilia tomentosa* M. *J. Pharm. Biomed. Anal.* 115, 1–9.
- Landete, J., 2011. Ellagitannins, ellagic acid and their derived metabolites: a review about source, metabolism, functions and health. *Food Res. Int.* 44, 1150–1160.
- Lee, M.-H., Chiou, J.-F., Yen, K.-Y., Yang, L.-L., 2000. EBV DNA polymerase inhibition of tannins from *Eugenia uniflora*. *Canc. Lett.* 154, 131–136.
- Liu, L., Zhang, L.-Y., Wang, S.-L., Niu, X.-Y., 2016. Analysis of anthocyanins and flavonols in petals of 10 *Rhododendron* species from the sygera mountains in southeast tibet. *Plant Physiol. Biochem.* 104, 250–256.
- Mamdouh, N.S., Sugimoto, S., Matsunami, K., Otsuka, H., Kamel, M.S., 2014. Taxiphyllin 6'-O-Gallate, actinidioionoside 6'-O-gallate and myricitrin 2"-O-Sulfate from the leaves of *Syzygium samarangense* and their biological activities. *Chem. Pharm. Bull.* 62, 1013–1018.
- Monti, D.M., Guarnieri, D., Napolitano, G., Piccoli, R., Netti, P., Fusco, S., Arciello, A., 2015. Biocompatibility, uptake and endocytosis pathways of polystyrene nanoparticles in primary human renal epithelial cells. *J. Biotechnol.* 193, 3–10.
- Nair, A.G.R., Krishnan, S., Ravikrishna, C., Madhusudanan, K.P., 1999. New and rare flavonol glycosides from leaves of *Syzygium samarangense*. *Fitoterapia* 70, 148–151.
- Pandit, A., Sachdeva, T., Bafna, P., 2012. Drug-induced hepatotoxicity: a review. *J. Appl. Pharm. Sci.* 2, 233–243.
- Panovska, T., Kulevanova, S., Gjorgoski, I., Bogdanova, M., Petrushevska, G., 2007. Hepatoprotective effect of the ethyl acetate extract of *Teucrium polium* L. against carbon tetrachloride-induced hepatic injury in rats. *Acta Pharm.* 57, 241–248.
- Pereira, J.A., Oliveira, I., Sousa, A., Valentão, P., Andrade, P.B., Ferreira, I.C., Ferreres, F., Bento, A., Seabra, R., Esteveiro, L., 2007. Walnut (*Juglans regia* L.) leaves: phenolic compounds, antibacterial activity and antioxidant potential of different cultivars. *Food Chem. Toxicol.* 2287–2295.
- Petruk, G., Raiola, A., Del Giudice, R., Barone, A., Frusciant, L., Rigano, M.M., Monti, D.M., 2016. An ascorbic acid-enriched tomato genotype to fight UVA-induced oxidative stress in normal human keratinocytes. *J. Photochem. Photobiol., B* 163, 284–289.
- Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C., 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic. Biol. Med.* 26, 1231–1237.
- Shen, S.-C., Chang, W.-C., Chang, C.-L., 2013. An extract from wax apple (*Syzygium samarangense* (Blume) Merrill and Perry) effects glycogenesis and glycolysis pathways in tumor necrosis factor- α -treated FL83B mouse hepatocytes. *Nutrients* 5, 455–467.
- Sobeh, M., Braun, M.S., Krstin, S., Youssef, F.S., Ashour, M.L., Wink, M., 2016. Chemical profiling of the essential oils of *Syzygium aqueum*, *Syzygium samarangense* and *Eugenia uniflora* and their discrimination using chemometric analysis. *Chem. Biodiv.* 13, 1537–1550.
- Sobeh, M., Mahmoud, M.F., Abdelfattah, M., El-Beshbishy, H.A., El-Shazly, A.M., Wink, M., 2017a. Hepatoprotective and hypoglycemic effects of a tannin rich extract from *Ximenia americana* var. *caffra* root. *Phytomedicine* 33, 36–42.
- Sobeh, M., Mahmoud, M., Hasan, R., Cheng, H., El-Shazly, A., Wink, M., 2017b. *Senna singuana*: antioxidant, hepatoprotective, antiapoptotic properties and phytochemical profiling of a methanol bark extract. *Molecules* 22, 1502.
- Soobrattee, M.A., Neergheen, V.S., Luximon-Ramma, A., Aruoma, O.I., Bahorun, T., 2005. Phenolics as potential antioxidant therapeutic agents: mechanism and actions. *Mut. Res.* 579, 200–213.
- Tala, V.R.S., Candida da Silva, V., Rodrigues, C.M., Nkengfack, A.E., Campaner dos Santos, L., Vilegas, W., 2013. Characterization of proanthocyanidins from *Parkia biglobosa* (Jacq.) G. Don. (Fabaceae) by flow injection analysis-electrospray ionization ion trap tandem mass spectrometry and liquid chromatography/electrospray ionization mass spectrometry. *Molecules* 18, 2803–2820.
- Tapas, A.R., Sakarkar, D., Kakde, R., 2008. Flavonoids as nutraceuticals: a review. *Tropical J. Pharma. Res.* 7, 1089–1099.
- van Wyk, B.-E., Wink, M., 2015. *Phytomedicines, Herbal Drugs, and Poisons*. University of Chicago Press, USA.
- van Wyk, B.-E., Wink, M., 2017. *Medicinal Plants of the World*, second ed. .
- Wang, L., Li, W., Ma, L., Chen, J., Lü, H., Jian, T., 2016. Salt stress changes chemical composition in *Limonium bicolor* (Bag.) Kuntze, a medicinal halophytic plant. *Ind. Crop. Prod.* 84, 248–253.
- Wrigley, J.W., Fagg, M., 2013. *Australian Native Plants* (New Holland) .
- Youssef, F.S., Labib, R.M., Eldahshan, O.A., 2017. Synergistic hepatoprotective and antioxidant effect of artichoke, fig, mulberry herbal mixture on HepG2 cells and their metabolic profiling using NMR coupled with chemometrics. *Chem. Biodiv.* <http://dx.doi.org/10.1002/cbdv.201700206>.
- Zhang, Q., Zhang, J., Shen, J., Silva, A., Dennis, D.A., Barrow, C.J., 2006. A simple 96-well microplate method for estimation of total polyphenolic content in seaweeds. *J. Appl. Phycol.* 18, 445–450.