Saudi Pharmaceutical Journal 26 (2018) 685–693

Contents lists available at ScienceDirect

Saudi Pharmaceutical Journal

journal homepage: www.sciencedirect.com

Original article

Analysis of antioxidative and antiviral biomarkers β-amyrin, β-sitosterol, lupeol, ursolic acid in *Guiera senegalensis* leaves extract by validated HPTLC methods

Mohammad K. Parvez [a,*], Perwez Alam [a,*], Ahmed H. Arbab [b], Mohammed S. Al-Dosari [a], Tawfeq A. Alhowiriny [a], Saleh I. Alqasoumi [a]

[a] Department of Pharmacognosy, College of Pharmacy, King Saud University, Riyadh 11451, Saudi Arabia
[b] Department of Pharmacognosy, College of Pharmacy, Omdurman Islamic University, Khartoom 14415, Sudan

ARTICLE INFO

Article history:
Received 26 December 2017
Accepted 8 February 2018
Available online 9 February 2018

Keywords:
Guiera senegalensis
HPTLC
β-Amyrin
β-Sitosterol
Lupeol
Ursolic acid
Antioxidant
Antiviral
Anti-HBV

ABSTRACT

Guiera senegalensis J.F. Gmel is a broad-spectrum African folk- medicinal plant, having activities against fowlpox and herpes viruses. Very recently, we have shown the anti-hepatitis B vius (HBV) potential of *G. senegalensis* leaves extract (GSLE). Here, we report the antioxidative and hepatoprotective efficacy of GSLE, including HPTLC quantification of four biomarkers of known antioxidative and antiviral activities. In cultured liver cells (HuH7) GSLE attenuated DCFH-induced oxidative stress and cytotoxicity. This was supported by *in vitro* DPPH radical-scavenging and β-carotene-linoleic acid bleaching assays that showed strong antioxidant activity of GSLE. Further, two simple and sensitive HPTLC methods (I and II) were developed and validated to quantify β-amyrin, β- sitosterol, lupeol, ursolic acid in GSLE. While HPTLC-I (hexane: ethylacetate; 75:25; v/v) enabled quantification of β-amyrin (Rf = 0.39; 20.64 μg/mg) and β-sitosterol (Rf = 0.25; 18.56 μg/mg), HPTLC-II (chloroform: methanol; 97:3; v/v) allowed estimation of lupeol (Rf = 0.47; 6.72 μg/mg) and ursolic acid (Rf = 0.23; 5.81 μg/mg) in GSLE. Taken together, the identified biomarkers strongly supported the antioxidant and anti-HBV potential of GSLE, suggesting its activity via abating the oxidative stress. To our knowledge, this is the first report on HPTLC analysis of these biomarkers in *G. senegalensis* that could be adopted for standardization and quality-control of herbal-formulations.

1. Introduction

Oxidative stress-induced cellular injury is caused by the imbalance between the oxidant and antioxidant molecules or by over-abundance of reactive oxygen species (ROS), produced by endogenous or exogenous sources (Opara et al., 2006). The accumulating excess of ROS can damage lipids, proteins or nucleic acids, and inhibit the normal growth and function of the cells (Ames et al., 1993). Several *in vitro* and *in vitro* studies have suggested the association of oxidative stress and damages with different forms of liver diseases (Ha et al., 2010). The hepatitis B virus (HBV) infection results in acute and chronic liver diseases, such as hepatitis B, cirrhosis and hepatocellular carcinoma (HCC). Evidences have shown that HBV can induce oxidative stress *in vitro* and *in vivo* including chronic hepatitis B patients (Severi et al., 2006; Niu et al., 2009; Bolukbas et al., 2005). Notably, the oncogenic 'X' gene of HBV (HBx) is trans-activated by ROS, and plays a crucial role in the development of HCC. Moreover, the polyunsaturated fatty acids residues of phospholipids of cell membranes and intracellular organelles are highly reactive to ROS that lead to lipid peroxidation (LPO), and produce cytotoxic malondialdehyde and hydroxynonenal (Djordjevic, 2004). It is reported that the total peroxide level, LPO and oxidative DNA damage are significantly higher in hepatitis B patients (Shaban et al., 2014).

Currently, the use of medicinal plants is massively increasing due to fewer or insignificant side effects as well as its low-cost.

* Corresponding authors at: Department of Pharmacognosy, College of Pharmacy, King Saud University, P.O.Box-2457, Riyadh 11451, Saudi Arabia.
E-mail addresses: mohkhalid@ksu.edu.sa (M.K. Parvez), aperwez@ksu.edu.sa (P. Alam), arbabssn@gmail.com (A.H. Arbab), mdosari@ksu.edu.sa (M.S. Al-Dosari), talhowiriny@ksu.edu.sa (T.A. Alhowiriny), sqasoumi@ksu.edu.sa (S.I. Alqasoumi).
Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.jsps.2018.02.022
1319-0164/

The African medicinal plant, *Guiera senegalensis* J.F. Gmel, commonly known as 'Cure all' is a popular folk medicine in the treatment of several types of metabolic and infectious diseases (Suleiman, 2015; Bosisio et al., 1997; Somboro et al., 2011). The dried leaves preparations of *G. senegalensis* is used to treat cough, sexual, gastrointestinal, respiratory and skin diseases, including their use as acaricidal, antimalarial, and antimicrobial, antioxidant, anti-inflammatory and gastroprotective agents (Osman et al., 2014; Bouchet et al., 1998; Sombié et al., 2011; Akuodor et al., 2013). Importantly, the galls of the plant are reported to have *in vitro* antiviral efficacies against fowlpox virus (FPV) (Lamien et al., 2005) and herpes simplex virus (HSV) (Silva et al., 1997). Very recently, we have shown the *in vitro* anti-HBV efficacy of *G. senegalensis* leaves (Arbab et al., 2017).

It is known that the pharmacological activity of a herbal-formulation is attributed to bioactive constituents, and their amount can differ considerably depending on the plant's part used, its geographical origin and the season of harvest. Therefore, development of sensitive methods for quantitative analysis of active biomarker(s) in a claimed plant extract or marketed formulation is fundamental for ensuring its therapeutic quality. Therefore, owing to its low-cost, high-throughput and minimum sample clean-up properties, the high-performance thin-layer chromatography (HPTLC) has become a convenient analytical method. The phytochemical analysis of *G. senegalensis* has identified various bioactive flavonoids, alkaloids, tannins and a naphthyl butenone (Ficarra et al., 1997; Bouchet et al., 1996). However, natural triterpenes (the structurally diverse group of pentacyclic triterpenoids) and phytosterols of known antioxidant and antiviral activities are hitherto, not explored in *G. senegalensis*. The present study was therefore, designed to evaluate the antioxidative and hepatoprotective property of anti-HBV active *G. senegalensis* leaves ethanol-extract (GSLE) and, to quantify biomarkers (β-amyrin, β-sitosterol, lupeol, ursolic acid; Fig. 1) by validated HPTLC methods.

2. Experimental

2.1. Plant material and extract preparation

Fresh and clean leaves of *G. senegalensis* (Family: Combretaceae) were collected from Kordofan region, and authenticated (voucher specimen no. 798) at the Forestry Research Center (FRC), Khartoum, Sudan. A further verification was done by a taxonomist at the herbarium of College of Pharmacy, King Saud University, Saudi Arabia. The leaves were washed and dried at room temperature for a week and powdered (50 g) using mortar-pestle. The extraction was done with 500 mL of 70% ethanol (Merck) for 24 h with intermittent shaking, and repeated twice with fresh solvent. The extracts were pooled, passed through Whatmann filter paper, and dried under reduced pressure using rotary evaporator (R-210, BUCHI).

2.2. Antioxidant activity assays of GSLE

2.2.1. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay

The antioxidant activity was tested by DPPH radical scavenging ability of GSLE in a 96-well microplate as described elsewhere (Bouchet et al., 1998; Wong et al., 2014). Briefly, triplicates of 100 μL of GSLE (0.0, 31.25, 62.5, 125, 250 and 500 μg/mL) was mixed with 50 μL of 0.2 mM DPPH (Sigma, USA) in a 96-well flat-bottom microplate (Becton-Dickinson Labware, USA). Rutin, an antioxidant natural flavonoid was used as positive control. Following a 30 min incubation at 25 °C in dark, the absorbance (λ= 517 nm) was recorded using microplate spectrophotometer (BioRad, USA). The data was analyzed for radical scavenging activity of SGEE, using the following equation:

$$\%Radical\ scavenging\ activity = (1 - As/Ac) \times 100$$ [As : absorbance of sample; As : absorbance of control].

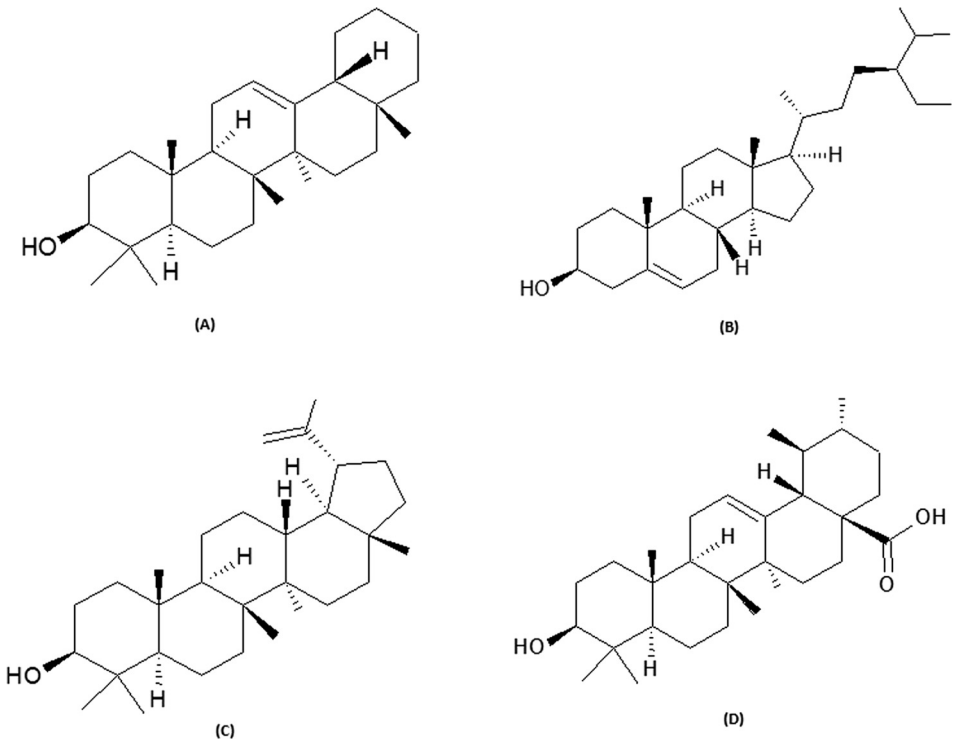

Fig. 1. Chemical structures of antioxidant biomarkers analyzed in the present study. (A) β-amyrin, (B) β-sitosterol, (C) lupeol, and (D) ursolic acid.

2.2.2. β-Carotene-linoleic acid bleaching assay

The antioxidant activity of GSLE was based on peroxidation of β-carotene and linoleic acid in a 96-well microplate as described elsewhere (Wong et al., 2014). Briefly, 0.5 mL β-carotene (500 μg/mL, in chloroform) was mixed with a solution of 12.5 μg of linoleic acid and 100 mg of Tween-40 followed by chloroform evaporation at 43 °C using speed vacuum concentrator (Savant, Thermo Electron Co.). The mixture was immediately diluted to 25 mL with distilled water and shaken vigorously for 2–3 min to form an emulsion. A 150 μL aliquot of the emulsion was added to wells of a 96-well plate containing 50 μL of GSLE or rutin (500 μg/mL), including a negative solvent control The plate was incubated at 50 °C for 2 h, and absorbance (λ = 470 nm) was recorded at 30 min intervals. The antioxidant activity of GSLE or controls was expressed as% inhibition of LPO using the formula:

$$\%Inhibition = ((As_{120} - Ac_{120})/Ac_0 - Ac_{120})) \times 100$$

[As_{120} : Absorbance of sample at 120 min; Ac_{120} : Absorbance of control at 120 min; Ac_0 : Absorbance control at 0 min].

2.3. In vitro hepatoprotective assessment of GSLE

The *in vitro* hepatoprotective activity of GSLE was tested on human hepatoma cell line, HuH7 cells, using MTT cell proliferation assay kits (Tervigen, UAS). HuH7 were grown in RPMI-1640 medium, supplemented with 10% heat-inactivated bovine serum (Gibco, UAS), 1x penicillin-streptomycin, and 1x sodium pyruvate streptomycin (HyClone Laboratories, USA) at 37 °C with 5% CO_2. 2,7-Dichlorofluorescein (DCFH; Sigma, USA) was used to induce *in vitro* chemical cytotoxicity at pre-determined dose (IC_{50}: 100 μg/mL for Huh7). Briefly, cells were seeded (0.5×10^5 cells/well, in triplicate) in a 96-well flat-bottom plate (Becton-Dickinson Labware, USA) and grown over night. Stock of GSLE was prepared in DMSO (100 mg/mL), followed by its dilution with culture media to different doses (250.0, 125.0, 62.5, 31.25, and 0.0 μg/mL). The final concentration of DMSO never exceeded > 0.1%, and therefore, the prepared doses showed non-cytotoxicity, including the highest one. The culture monolayer were replenished with fresh media containing DCFH (100 μg/mL) plus a dose of GSLE, including untreated as well as DCFH only-treated controls. The treated cells were incubated for 48 h at 37 °C followed by MTT assay as per the manufacturer's guidelines. The absorbance (OD; λ = 570 nm) was recorded (BioTek, ELx800), and non-linear regression analysis was performed in Excel software to determine the cell survival.

$$\%Cell\ survival = ((As - Ab)/Ac - Ab)) \times 100$$

[As : Absorbance of sample; Ab : Absorbance of blank; Ac : Absorbance of control].

The treated cells were also visually monitored under an inverted microscope (Optica) for the morphological alternations under the experimental conditions.

2.4. Solvents, standards and stock preparation

The commercial standards: rutin, β-carotene, β-amyrin, β-sitosterol, lupeol, ursolic acid and linoleic acid were procured (Sigma Aldrich, USA). The AR grade organic solvents (hexane, ethyl acetate, chloroform and methanol) and p-anisaldehyde were purchased (BDH, UK). HPLC grade methanol (Merck, Germany) was used for the preparation of standard stock solutions (1000 μg/ml) and their serial dilutions (β-amyrin and β-sitosterol: 10–100 μg/ml; lupeol and ursolic acid: 10–120 μg/mL).

2.5. Apparatus and instruments

For the application of different tracks of standards and GSLE, glass-backed silica gel $60F_{254}$ HPTLC plates (Merck, Germany) were used. CAMAG automatic TLC sampler-4 was used for the application of the standards and GSLE, band-wise to the plates and developed in automatic development chamber (ADC2) (CAMAG, Muttenz, Switzerland). The developed HPTLC Plates were documented by CAMAG TLC Reprostar3 and scanned in CATS 4 (CAMAG).

2.6. Instrumentation and conditions

The HPTLC analyses of β-amyrin and β-sitosterol (Method-I), and lupeol and ursolic acid (Method-II) in GSLE were carried out on 10 × 10 cm precoated HPTLC plates where the band size of each track was 6 mm wide and 7.3 mm apart. All the solvent dilutions (10 μL, each) were applied by micro syringe attached with the applicator on the HPTLC plate to furnish the linearity range of 100–1000 ng/band of β-amyrin and β-sitosterol, and 100–1200 ng/band of lupeol and ursolic acid. The rate of application of all samples on the HPTLC plates was 160 nL/s. The plates were developed in pre-saturated 20 × 10 cm twin-trough glass chamber at room temperature (25 ± 2 °C) and humidity (60 ± 5%). The solvents used were hexane and ethyl acetate (7.5:2.5, v/v) for Method-I, and chloroform and methanol (97:3, v/v) for Method-II. The developed plate was dried, derivatized with p-anisaldehyde, re-dried and quantified at UVmax 540 nm (Method-I) and at 630 nm (Method-II). Since, all marker compounds were soluble in methanol, the extraction of GSLE was carried out in methanol.

2.7. Method validation

Validations of the two developed HPTLC methods (Method-I and -II) were carried out as per the International Conference on Harmonization guidelines for linearity range, precision, recovery as accuracy, robustness, including limit of detection (LOD) and limit of quantification (LOQ) (ICH guideline, 2005). Recovery as accuracy studies involved the addition of a known amount of the analyte to the given sample and to determine how much percentage of analyte was added to the samples. In the present study, for β-amyrin, β-sitosterol, lupeol and ursolic acid known amounts i.e. 50%, 100% and 150% of each standard (200 ng) were added and, the percentage of these added amount were estimated to find out the recovery of the spiked standards. The precision (intra- and inter-day) of the two developed methods were evaluated by performing replicate analysis (n = 6) at low (400 ng/band), medium (600 ng/band), and high (800 ng/band), concentrations of the four biomarkers. The precision was recorded as standard deviation (SD) and% standard deviation of the response (% RSD) of each calibration level. The robustness of the HPTLC methods was performed to analyze their capacity to remain unaffected by small, but deliberate variations in mobile phase composition and volume, used for saturation and saturation-time in developing chamber that indicates reliability of the methods.

The robustness study was performed in replicate analysis (n = 6) for the four biomarkers at 300 ng/band concentration. The results were evaluated in terms of standard SD, %RSD and SEM of peak area. In method-I, the mobile phases were prepared with hexane and ethyl acetate (7.5:2.5, v/v) in different proportions (7.3:2.7 and 7.7:2.3, v/v) for the analysis of β-amyrin and β-sitosterol. In method-II, the different mobile phases (95:5 and 98:2, v/v) were prepared from chloroform: methanol (97:3, v/v) for the analysis

of lupeol and ursolic acid. In addition to the slight variations in the mobile phases for robustness study, the mobile phase volume used for saturation was also varied to 18 mL to 22 mL from 20 mL. In addition, the saturation-time was also varied from 10 min to 30 min from 20 min. The calculation of LOD and LOQ were based on the RSD and slope (S) of the calibration curve, using the formulae: LOD = 3.3 (SD/S); LOQ = 10 (SD/S).

2.8. Statistical analysis

Results were expressed as mean ± SEM. Total variation present in a set of data was estimated by one-way analysis of variance (ANOVA) followed by Dunnet's-test. $P < 0.01$ was considered significant.

3. Results

3.1. Free-radical scavenging and antioxidant activity of GSLE

Two *in vitro* assays were employed to determine the antioxidative potential of GSLE. In the DPPH radical scavenging assay, GSLE (IC_{50}: 82.71 μg/mL) showed dose-dependent activity (Fig. 2A). At concentrations of 31.25, 61.5, 125, 250 and 500 μg/ml, the radical scavenge activity were about 43.74%, 64.25%, 78.72%, 87.5%, and 90.6%, respectively. Interestingly, the radical scavenging activity of the extract at 500 μg/ml was comparable to that of rutin (93.3%). In the β-carotene-linolenic acid assay, GSLE (IC_{50}: 128.7 μg/mL) also inhibited LPO in a dose-dependent manner (Fig. 2B), confirming its strong antioxidative potential.

3.2. In vitro hepatoprotective potential of GSLE

In the HuH7 cell culture mode, DCFH (IC_{50}: 100 μg/mL), induced severe hepatotoxicity as reflected by altered cell morphology compared to untreated control, observed under microscope. Interestingly, the DCFH-treated cells when supplemented with GSLE (125 and 250 μg/mL), showed normal morphology (data not shown). This was in accordance with the MTT assay that demonstrated the protection of DCFH-toxicated HuH7 cells to about 74% and 86% by 125 and 250 μg/mL doses of GSLE, respectively (Fig. 3). In line with our *in vitro* data, our GSLE showed significant hepatoprotective activity via attenuation of DCFH-induced oxidative stress and cytotoxicity.

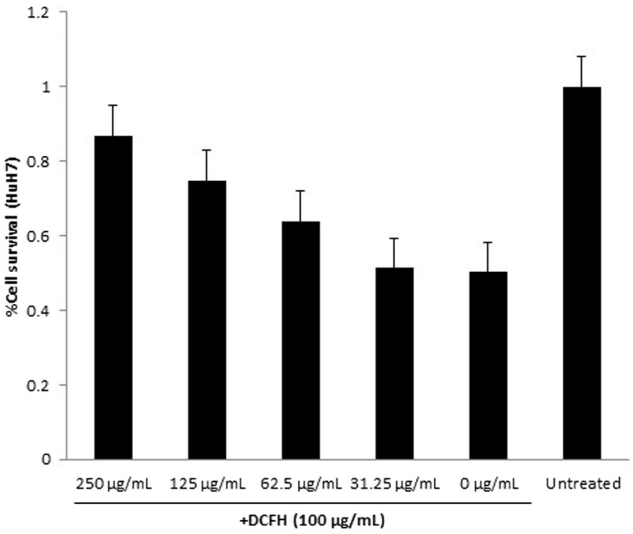

Fig. 3. MTT cell proliferation assay, showing *in vitro* hepatoprotective effect of GSLE on cultured HuH7 cells. Values (Y-axis): means of three determinations.

3.3. Method development

The selection of best mobile phase for the analysis of different biomarkers in Method-I and Method-II were carried out by running several TLC plates with different solvent combinations. The final solvent combination was chosen on the basis of its accurate reproducibility, separation efficiency of maximum phytoconstituents as well as different markers in simultaneous analysis. The mobile phase that fulfilled these criteria for Method-I was hexane and ethyl acetate (7.5:2.5, v/v) (Fig. 4A) whereas for Method-II it was chloroform and methanol (97:3, v/v) (Fig. 4B). The optimized saturation time and volume of mobile phase were observed as 20 min and 20 m, respectively. The developed HPTLC plates were then derivatized by spraying p-anisaldehyde reagent and heated to give compact spots of markers as well as different phytoconstituents present in GSLE.

In Method-I, the densitometric analysis was carried out at 540 nm in the absorbance mode that furnished compact, sharp, symmetrical and high resolution bands of β-amyrin and β-sitosterol at R_f 0.39 ± 0.004 and 0.25 ± 0.005, respectively (Fig. 5A). In Method-II, the analysis was carried out at 630 nm that produced compact, sharp and high resolution band of lupeol and ursolic acid

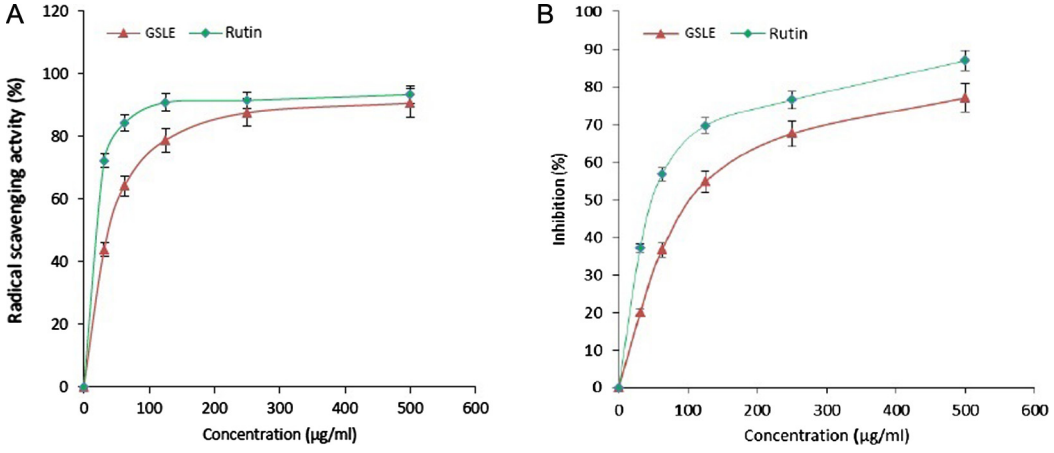

Fig. 2. *In vitro* antioxidant assays of *G. senegalensis* (leaves) ethanol-extract (GSLE). (A) DPPH radical scavenging method showing antioxidative activity, (B) β-carotene-linoleic acid bleaching method showing inhibition of lipid peroxidation by different concentrations (31.25–500 μg/mL) of GSLE compared to the standard antioxidant (rutin).

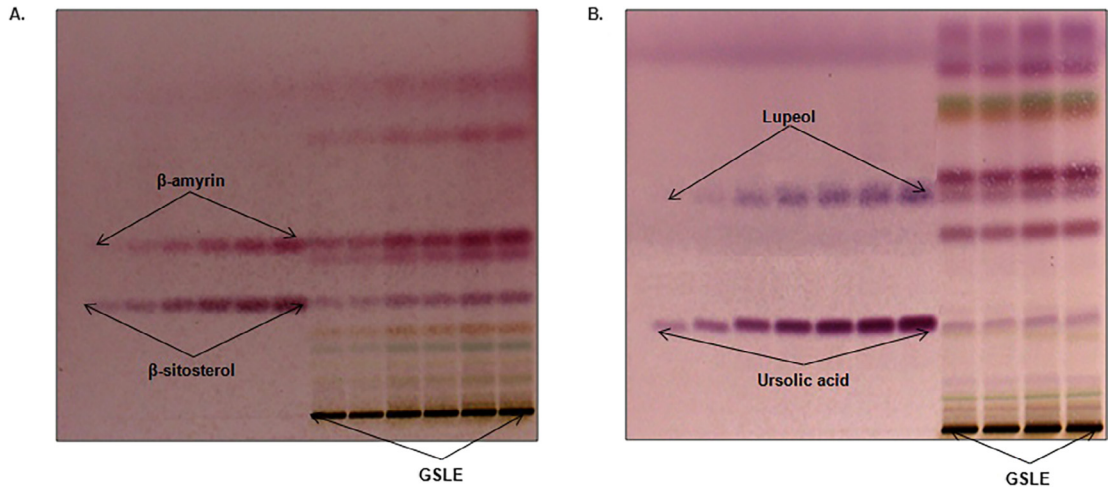

Fig. 4. Pictograms of TLC plates for *G. senegalensis* (leaves) ethanol-extract (GSLE) derivatized with p-anisaldehyde in day light. (A) Method-I (β-amyrin and β-sitosterol): mobile phase- hexane: ethylacetate (7.5:2.5; v/v), (B) Method-II (lupeol and ursolic acid): mobile phase- chloroform: methanol (97:3; v/v).

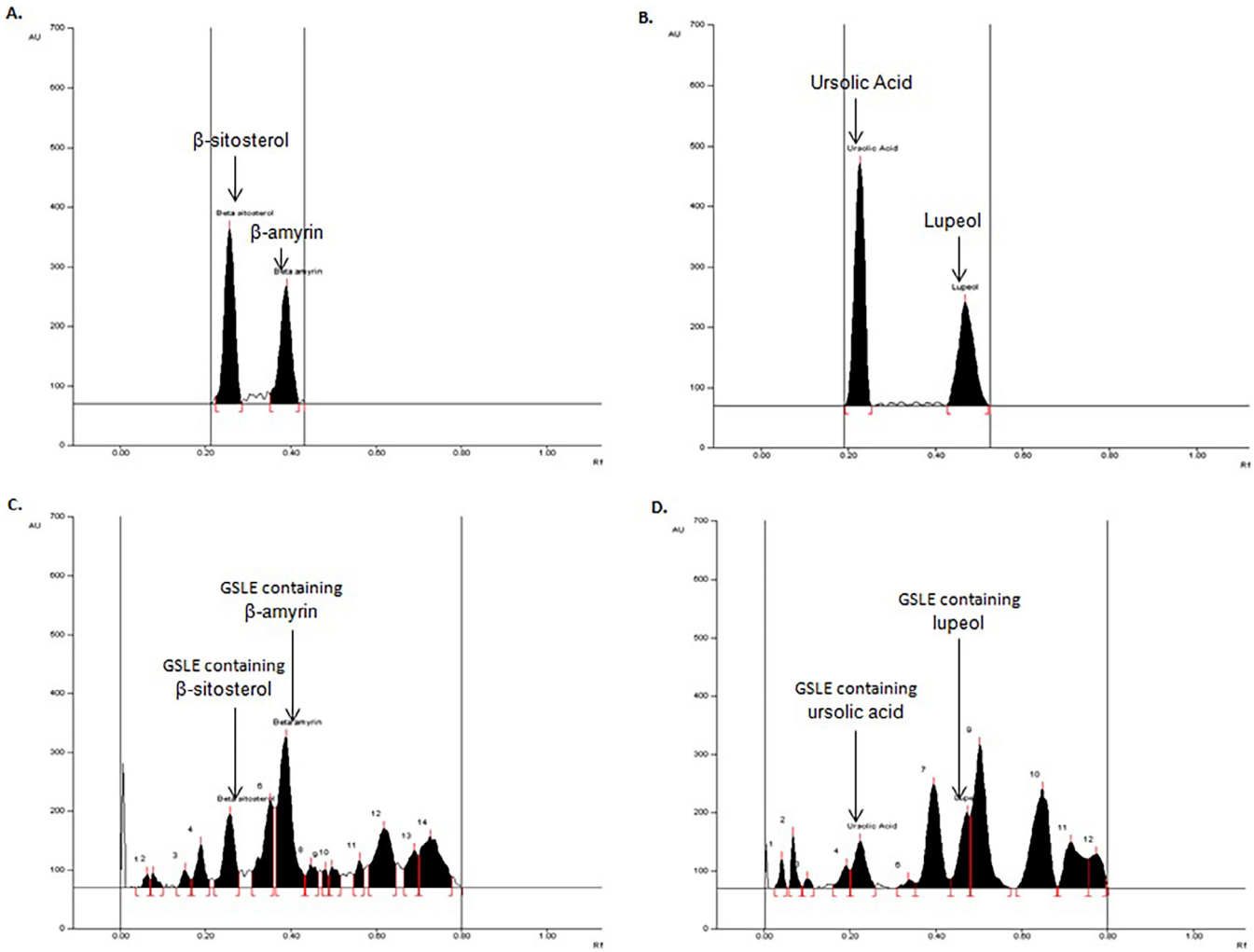

Fig. 5. Chromatograms of antioxidant biomarkers analysis in GSLE. (A) Chromatogram of β-sitosterol (R_f = 0.25) and β-amyrin (R_f = 0.39) scanned at λ_{max} = 540 nm; mobile phase (Method I) - hexane: ethylacetate (7.5: 2.5; v/v). (B) Chromatogram of ursolic acid (R_f = 0.23) and lupeol (R_f = 0.47) scanned at λ_{max} = 630 nm; mobile phase (Method II) – chloroform: methanol (97:3; v/v). (C) Chromatogram of GSLE (β-sitosterol, spot 5, R_f = 0.25; β-amyrin, spot 7, R_f = 0.39) scanned at λ_{max} = 540 nm; mobile phase (Method I) – hexane: ethylacetate (7.5: 2.5; v/v). (D) Chromatogram of GSLE (ursolic acid, spot 5, R_f = 0.23; lupeol, spot 8, R_f = 0.47) scanned at λ_{max} = 630 nm; mobile phase (Method II) - chloroform: methanol (97:3; v/v).

at R_f 0.47 ± 0.004 and 0.23 ± 0.005, respectively (Fig. 5B). Thus, the developed HPTLC methods were found to be quite selective with good baseline resolution.

3.4. Method validation

Linearity of compounds β-amyrin, β-sitosterol, lupeol and ursolic acid were validated by using the linear regression equation (Y) and correlation coefficient (r^2). The six-point calibration curve for β-amyrin and β-sitosterol was found to be linear in the range of 100–1000 ng/band while for lupeol and ursolic acid it was in the range of 100–1200 ng/band. The respective values of Y and r^2 for the biomarkers were β-amyrin (8.727x + 306.63 and 0.9975 ± 0.0002), β-sitosterol (8.843x + 1249.27 and 0.9983 ± 0.0005), lupeol (3.892x + 324.92 and 0.9971 ± 0.0005) and ursolic (9.952x + 146.74 and 0.9987 ± 0.0004). Further analysis revealed a good linearity response for the developed methods (Table 1). The mean value (±SD) of the slope and intercept were found to be 8.727 ± 0.042 and 306.63 ± 11.53 for β-amyrin, 8.843 ± 0.049 and 1249.27 ± 20.955 for β-sitosterol, 3.892 ± 0.061 and 324.92 ± 13.259 for lupeol, and 9.952 ± 0.089 and 146.74 ± 11.432 for ursolic acid, respectively.

The recoveries as accuracy study for the developed methods were recorded for β-amyrin, β-sitosterol, lupeol and ursolic acid (Table 2). The recovery was 99.23–100.14% for β-amyrin, 99.26–100.18% for β-sitosterol, 99.58–100.23% for lupeol and 99.03–100.78% for ursolic acid. The %RSD was 0.338–0.396 for β-amyrin, 0.608–0.703 for β-sitosterol, 0.270–0.331 for lupeol, and 0.204–0.184 for ursolic acid, respectively. The intra-/inter-day precision (n = 6) for β-amyrin and β-sitosterol in Method-I and those for lupeol and ursolic acid in Method-II (Table 3) were recorded. In Method-I, the respective intra-/inter-day %RSD for β-amyrin and β-sitosterol were 0.205–0.235%/0.190–0.232% and 0.355–0.375%/0.337–0.355%, respectively which demonstrated the good precision of this method. Likewise, in Method-II, the respective intra-/inter-day %RSD for lupeol and ursolic acid were 0.238–0.262%/0.229–0.249% and 0.131–0.147%/0.127–0.140% that also demonstrated the good precision of this method.

The data for the robustness studies for β-amyrin and β-sitosterol (Table 4) and for lupeol and ursolic acid (Table 5) were

Table 1
R_f, Linear regression data for the calibration curve of β-amyrin, β-sitosterol, lupeol and ursolic acid (n = 6).

Parameters	β-Amyrin	β-Sitosterol	Lupeol	Ursolic Acid
Linearity range (ng/spot)	100–1000	100–1000	100–1200	100–1200
Regression equation	Y = 8.727x + 306.63	Y = 8.843x + 1249.27	Y = 3.892x + 324.92	Y = 9.952x + 146.74
Correlation (r^2) coefficient	0.9975 ± 0.0002	0.9983 ± 0.0005	0.9971 ± 0.0005	0.9987 ± 0.0004
Slope ± SD	8.727 ± 0.042	8.843 ± 0.049	3.892 ± 0.061	9.952 ± 0.089
Intercept ± SD	306.63 ± 11.53	1249.27 ± 20.955	324.92 ± 13.259	146.74 ± 11.432
Standard error of slope	0.017	0.020	0.025	0.036
Standard error of intercept	4.709	8.553	5.412	4.666
R_f	0.39 ± 0.004	0.25 ± 0.005	0.47 ± 0.004	0.23 ± 0.005
LOD (ng)	16.24	18.56	52.24	29.57
LOQ (ng)	49.21	56.26	158.32	89.61

Table 2
Recovery as accuracy studies of the proposed method of β-amyrin, β-sitosterol, lupeol and ursolic acid (n = 6).

Stand. added to analyte (%)	Theor. conc. of stand. (ng/μL)	Method I						Method II					
		β-Amyrin			β-Sitosterol			Lupeol			Ursolic acid		
		Conc. found (ng/μL) ± SD	% RSD	% Recovery	Conc. found (ng/μL) ± SD	% RSD	% Recovery	Conc. found (ng/μL) ± SD	% RSD	% Recovery	Conc. found (ng/μL) ± SD	% RSD	% Recovery
0	200	199.91 ± 0.69	0.347	99.95	199.94 ± 1.21	0.608	99.97	199.83 ± 0.65	0.326	99.91	201.57 ± 0.41	0.204	100.78
50	300	298.94 ± 1.01	0.338	99.65	300.55 ± 1.96	0.652	100.18	300.69 ± 0.81	0.270	100.23	297.09 ± 0.52	0.176	99.03
100	400	400.56 ± 1.42	0.356	100.14	398.34 ± 2.71	0.682	99.58	398.31 ± 1.16	0.290	99.58	401.25 ± 0.71	0.178	100.31
150	500	496.13 ± 1.96	0.396	99.23	496.32 ± 3.49	0.703	99.26	499.04 ± 1.65	0.331	99.80	500.56 ± 0.92	0.184	100.11

Stand.: Standards; Theor.: Theoretical; Conc.: Concentration.

Table 3
Precision of the proposed HPTLC Method-I & II (n = 6).

Concentration of standard added (ng/spot)	β-Amyrin (Method I)				β-Sitosterol (Method I)			
	Intra-day precision		Inter-day precision		Intra-day precision		Inter-day precision	
	Average Conc. found ± SD	%RSD	Average Conc. found ± SD	%RSD	Average Conc. found ± SD	%RSD	Average Conc. found ± SD	%RSD
400	399.87 ± 0.81	0.205	398.72 ± 0.76	0.190	400.55 ± 1.42	0.355	399.42 ± 1.39	0.348
600	598.71 ± 1.33	0.222	597.56 ± 1.29	0.216	597.02 ± 2.16	0.362	595.89 ± 2.01	0.337
800	797.29 ± 1.87	0.235	793.86 ± 1.84	0.232	796.75 ± 2.99	0.375	793.36 ± 2.82	0.355
Concentration of standard added (ng/spot)	Lupeol (Method II)				Ursolic acid (Method II)			
	Intra-day precision		Inter-day precision		Intra-day precision		Inter-day precision	
	Average Conc. found ± SD	%RSD	Average Conc. found ± SD	%RSD	Average Conc. found ± SD	%RSD	Average Conc. found ± SD	%RSD
400	399.51 ± 0.95	0.238	396.94 ± 0.91	0.229	399.71 ± 0.52	0.131	397.19 ± 0.50	0.127
600	600.69 ± 1.51	0.251	598.12 ± 1.40	0.234	598.79 ± 0.81	0.136	595.77 ± 0.78	0.131
800	798.12 ± 2.09	0.262	795.55 ± 1.98	0.249	794.83 ± 1.17	0.147	791.81 ± 1.11	0.140

Table 4
Robustness of the proposed HPTLC Method I at 300 ng/band (n = 6).

Optimization condition	β-Amyrin		β-Sitosterol	
	SD	%RSD	SD	%RSD
Mobile phase composition; (hexane: ethyl acetate)				
(7.5:2.5)	1.526	0.383	2.662	0.669
(7.3:2.7)	1.589	0.399	2.581	0.650
(7.7:2.3)	1.685	0.425	2.515	0.630
Mobile phase volume (for saturation)				
(18 mL)	1.506	0.378	2.619	0.662
(20 mL)	1.529	0.385	2.513	0.637
(22 mL)	1.585	0.400	2.573	0.649
Duration of saturation				
(10 min)	1.496	0.376	2.589	0.653
(20 min)	1.517	0.383	2.574	0.650
(30 min)	1.581	0.396	2.549	0.641

Table 5
Robustness of the proposed HPTLC Method II at 300 ng/band (n = 6).

Optimization condition	Lupeol		Ursolic acid	
	SD	%RSD	SD	%RSD
Mobile phase composition; (chloroform: methanol)				
(97:3)	0.824	0.276	0.612	0.207
(95:5)	0.832	0.278	0.724	0.246
(98:2)	0.845	0.282	0.916	0.309
Mobile phase volume (for saturation)				
(18 mL)	0.829	0.280	0.912	0.309
(20 mL)	0.851	0.287	0.924	0.314
(22 mL)	0.812	0.273	0.856	0.290
Duration of saturation				
(10 min)	0.831	0.279	0.812	0.277
(20 min)	0.862	0.289	0.844	0.289
(30 min)	0.875	0.294	0.866	0.294

documented. The low value of SD and %RSD obtained after introducing small deliberate changes indicated that the robustness of the two methods. The respective LOD/LOQ for β-amyrin, β-sitosterol, lupeol and ursolic acid were 16.24/49.21, 18.56/56.26, 52.24/158.32 and 29.57/89.61 ng/band (Table 1). This observation further indicated that the two HPTLC methods had good sensitivity for the quantification of the four compounds in GSLE.

3.5. Application of the validated HPTLC methods in the quantitative analysis of β-amyrin, β-sitosterol, lupeol and ursolic acid in GSLE

Further applicability of the developed and validated HPTLC methods were tested for the quantitative analysis of β-amyrin and β-sitosterol (Fig. 5C) and lupeol and ursolic acid (Fig. 5D) in GSLE. With these methods, the contents of β-amyrin, β-sitosterol, lupeol and ursolic acid were estimated to be 20.64 μg/mg, 16.35 μg/mg, 6.73 μg/mg, and 5.81 μg/mg of the dry weight of GSLE. To the best of our knowledge, this is the first report on simple, accurate and rapid HPTLC methods developed and validated for the simultaneous quantification of β-amyrin, β-sitosterol, lupeol and ursolic acid in *G. senegalensis*.

4. Discussion

G. senegalensis J.F. Gmel is a very popular African folk-medicinal plant with antioxidative, gastroprotective, anti-microbial and antiviral potential (Suleiman, 2015; Bosisio et al., 1997; Somboro et al., 2011). DCFH is commonly used to evaluate experimental oxidative stress caused by free-radicals or ROS through the principle of oxidation of DCFH to the fluorescent DCF (Rota et al., 1999). Notably, DCFH is also highly cytotoxic to cultured human cell lines, including hepatoma cells (Al-Yahya et al., 2013; Arbab et al., 2016). In the present study, MTT assay has demonstrated the hepatoprotective activity of GSLE via attenuation of DCFH-induced oxidative stress and cytotoxicity in cultured hepatoma cells, HuH7. Further, our DPPH radical-scavenging and β-carotene-linoleic acid bleaching assays showed dose-dependent antioxidant efficacy of GSLE. Very recently, we have demonstrated a robust anti-HBV activity of *G. senegalensis* (Arbab et al., 2017) in accordance with its previously reported antiviral property against FPV and HSV (Lamien et al., 2005; Silva et al., 1997). The antiviral activities by natural or herbal products are suggested via three mechanisms: firstly, direct inhibition; secondly, enhancing host immunity; and thirdly, abating inflammation and protecting cells from oxidative stress or damages. The indirect antiviral efficacy of *Ampelopsis silica* root extract against HSV (Chen and Yang, 1999) and duck hepatitis B virus (DHBV) (Chen et al., 2000) was thus, attributed to its anti-inflammatory and antioxidative activity (Chen et al., 2005). In line with this, the *in vitro* and *in vitro* antioxidative, hepatoprotective and anti-HBV potential of *Acacia mellifera* leaves extract has also been demonstrated, recently (Arbab et al., 2015).

The phytochemical analysis of *G. senegalensis* has revealed presence of various bioactive flavonoids (eg., catechin, myricitrin, rutin and quercetin), alkaloids (e.g., harman and eleagnine), a naphthyl butenone (guieranone A) and tannins (eg., epicatechin, epigallocatechin gallate and galloylquinic acid (Ficarra et al., 1997; Silva and Gomes, 2003; Bouchet et al., 1998). Of the identified tannins, radical scavenging and antioxidant activities of galloylquinic acid have been studied. However, to our knowledge, the triterpenes of known therapeutic potentials including antioxidant and antiviral activities are not reported in *G. senegalensis*. Natural triterpenes are structurally diverse group of triterpenoids, having linear and, tetra or pentacyclic carbon skeleton. Bioactive triterpenes are isolated from a variety of plants with antimicrobial, anticancer,

hepatoprotective, anti-inflammatory, and antioxidative salutations (Pavlova et al., 2003; Woldemichael et al., 2003; Aiken and Chen, 2005; Fernandez et al., 2011; Sultana and Saify, 2012). Moreover, the pentacyclic triterpenes are shown to have antiviral properties against HSV (Heidary et al., 2014; Joycharat et al., 2008; Tanaka et al., 2004) and HIV (Cichewicz and Kouzi, 2004), the genetically close viruses to HBV. Notably, β-amyrin (oleanane-type pentacyclic triterpenoid) is shown to have antiviral efficacies against influenza A and HSV (Rao et al., 1974). In our analysis, the high quantity of β-amyrin (20.64 μg/mg) in antioxidant GSLE strongly supports its anti-HBV activity, probably by attenuating the cellular oxidative mechanism.

Ursolic acid (ursane-type pentacyclic triterpenoid), also known as urson, prunol, micromerol or malol is one of the most promising therapeutic natural compound. It has shown *in vitro* and *in vitro* hepatoprotective activity against ethanol-toxicity by elevating levels of antioxidant molecules and serum protein while decreasing the total bilirubin and LPO markers (Saraswat et al., 2000; Saravanan et al., 2006). Also, ursolic acid treatment is shown to ameliorate paracetamol- and carbon tetrachloride-induced liver injury by increasing hepatocytes viability and improvement of serum markers (Shukla et al., 1992; Martin-Aragón et al., 2001). Interestingly, ursolic acid is also found to have potent antiviral activity against HSV, human immunodeficiency virus (HIV) and human hepatitis C virus (HCV) (Filho et al., 2010; Hattori et al., 2013; Ma et al., 1999; Quéré et al., 1996). Our identification of ursolic acid (5.81 μg/mg) in the anti-HBV active GSLE thus, endorses its antioxidative potential, and could correlate with its anti-HBV activity via abating cellular oxidative stress.

The triterpenoids, also known as phytosterols have wide spectrum of biological activities including, anti-inflammatory, hypocholesterolemic, and insulin-regulating potential (Kong et al., 2013; Yamamoto et al., 1991; Bouic et al., 1996). Of these, lupeol has *in vivo* and *in vitro* anti-inflammatory, anti-microbial, anti-protozoal, anti-proliferative, anti-invasive, anti-angiogenic and hypocholesterolemic efficacies (Ivorra et al., 1988). Lupeol is reported to decrease the ROS level and restore the antioxidant enzyme activities in mouse liver against chemical-induced oxidative stress (Siddique et al., 2011). Also, lupeol treatment is shown to induce growth inhibition and apoptosis in HCC cell line, SMMC7721 (Prasad et al., 2007). Though lupeol has shown weak antiviral activities in several studies, it has served as a lead drug for the generation of more effective compounds against Influenza A and HSV (Zhang et al., 2009). On the contrary, lupeol isolated from *Strobilanthes cusia* root has demonstrated a robust anti-HSV activity, *in vitro* (Flekhter et al., 2004). Notably, to the best of our knowledge, the antiviral activity of lupeol against hepatitis viruses has not been reported, so far. Nevertheless, lupeol (6.72 μg/mg) identified in the antioxidant GSLE, could have a direct or indirect role in the plant's anti-HBV activity.

β-sitosterol, a phytosterol has been shown to exhibit *in vitro* and *in vitro* anti-HIV activity by immunomodulatory mechanism though stabilization of $CD4^+$ T-lymphocyte counts, and a significant decrease in interleukin-6 level (Tanaka et al., 2004). Very recently, antiviral effect of β-sitosterol isolated from cottonseed oil against tobacco mosaic virus (TMV) is reported (Bouic, 1997). Interestingly, β-sitosterol has been also shown to attenuate *in vitro* chemical-induced hepatotoxicity (Arbab et al., 2016) and cardiotoxicity by enhancing mitochondrial glutathione redox mechanism (Zhao et al., 2015). In line with this, our identification of β-sitosterol (l6.72 μg/mg) as the second most abundant compound in GSLE strongly supports its *in vitro* antioxidative, hepatoprotective and anti-HBV activities, independently or interdependently.

5. Conclusion

The present study has demonstrated antioxidative and hepatoprotective potential of anti-HBV active GSLE. Further, HPTLC analysis of biomarkers (β-amyrin, β-sitosterol, lupeol and ursolic acid) of known antioxidant and antiviral activities, strongly supported the anti-HBV efficacy of GSLE via abating the cellular oxidative stress molecules. Our findings therefore, warrant for the therapeutic potential of *G. senegalensis* against hepatitis B associated chronic liver disease. Nevertheless, bioactivity guided fractionation and isolation of active principles from *G. senegalensis* leaves, and their detailed analysis would be necessary. To our knowledge, this is the first report on HPTLC analysis of triterpenes in *G. senegalensis* that could be further adopted for the standardization and quality-control of marketed herbal-formulations.

Acknowledgments

The authors would like to extend their sincere appreciation to the Deanship of Scientific Research at King Saud University for its funding of this research through the Research Group Project No. RG-1435-053.

Conflict of interest statement

The authors declare no conflict of interest.

References

Aiken, C., Chen, C.H., 2005. Betulinic acid derivatives as HIV-1 antivirals. Trends Mol. Med. 11, 31–36.

Akuodor, G.C., Essien, A.D., David-Oku, E., Chilaka, K.C., Akpan, J.L., Ezeokpo, B., Ezeonwumelu, J.O., 2013. Gastroprotective effect of the aqueous leaf extract of *Guiera senegalensis* in Albino rats. Asian Pac. J. Trop Med. 6, 771–775.

Al-Yahya, Y., Al-Dosari, M.S., Al-Sohaibani, M., AlSaid, M., Mothana, R., Parvez, M.K., Rafatullah, S., 2013. Attenuation of CCl_4-induced oxidative stress and Hepato-nephrotoxicity by Saudi Sidr Honey in Rats. Evid. Based Complementary Altern. Med. 2013, 1–10.

Ames, B.N., Shigenaga, M.K., Hagen, T.M., 1993. Oxidants, antioxidants, and the degenerative diseases of aging. Proc. Proc. Natl. Acad. Sci. U.S.A. 90, 7915–7922.

Arbab, A.H., Parvez, M.K., Al-Dosari, M.S., Al-Rehaily, A.J., 2017. *In vitro* evaluation of novel antiviral activities of 60 medicinal plants extracts against hepatitis B virus. Exp. Ther. Med. 14, 626–634.

Arbab, A.H., Parvez, M.K., Al-Dosari, M.S., Al-Rehaily, A.J., Ibrahim, K.E., Alam, P., AlSaid, M.S., Rafatullah, S., 2016. Therapeutic efficacy of ethanolic extract of *Aerva Javanica* aerial parts in the amelioration of CCl_4-induced hepatotoxicty and oxidative damage in rats. Food Nutr. Res. 60, 30864–30873.

Arbab, A.H., Parvez, M.K., Al-Dosari, M.S., Rafatullah, S., Al-Sohaibani, M., Zorag, E.A., Al-Rehaily, A.J., AlSaid, M.S., 2015. Hepatoprotective and antiviral efficacy of *Acacia mellifera* leaves fractions against hepatitis B virus. BioMed. Res. Int. 2015, 929131.

Bolukbas, C., Bolukbas, F.F., Horoz, M., Aslan, M., Celik, H., Erel, O., 2005. Increased oxidative stress associated with the severity of the liver disease in various forms of hepatitis B virus infection. BMC Infect. Dis. 5, 95–98.

Bosisio, E., Mascetti, D., Verotta, L., Zani, F., Mazza, P., Talbot, P., 1997. *Guiera senegalensis* J.F. Gmelin (Combretaceae): Biological activities and chemical investigation. Phytomed. 3, 339–348.

Bouchet, N., Barrier, L., Fauconneau, B., 1998. Radical scavenging activity and antioxidant properties of tannins from *Guiera senegalensis* (Combretaceae). Phytother. Res. 12, 159–162.

Bouchet, N., LeÂ, vesque, J., Blond, A., Bodo, B., Pousset, J.L., 1996. 1,3-di-O-galloylquinic acid from *Guiera senegalensis*. Phytochemistry 42, 189–190.

Bouic, P.J.D., Etsebeth, R.W., Liebenberg, C.F., 1996. Beta-sitosterol and beta-sitosterol glucoside stimulate human peripheral blood lymphocyte proliferation: implications for their use as an immunomodulatory vitamin combination. Int. J. Immunopharmacol. 18, 693–700.

Bouic, P.J.D., 1997. Immunomodulation in HIV/AIDS: The tygerberg/stellenbosch university experience. AIDS Bull. 6, 18–20.

Chen, K., Yang, Z., 1999. The acting part anti-HSV-1 in infected cells of extract from *Ampelopsis sinica* roots. J. Chin. Drugg. 2, 225–226.

Chen, K., Li, H., Chen, Y., Zhang, C., 2000. Inhibition of extracts of *Ampelopsis sinica* roots on DHB vs. Ag in sera of ducklings. Zhong Yao Cai. 23, 46–47.

Chen, K., Plumb, G.W., Bennett, R.N., Bao, Y., 2005. Antioxidant activities of extracts from five anti-viral medicinal plants. J. Ethnopharmacol. 96, 201–205.
Cichewicz, R.H., Kouzi, S.A., 2004. Chemistry, biological activity, and chemotherapeutic potential of betulinic acid for the prevention and treatment of cancer and HIV infection. Med. Res. Rev. 24, 90–114.
Djordjević, V.B., 2004. Free radicals in cell biology. Int. Rev. Cytol. 237, 57–89.
Fernandez, M.A., de las Heras, B., Garcia, M.D., Saenz, M.T., Villar, A., 2011. New insights into the mechanism of action of the anti-inflammatory triterpene lupeol. J. Pharm. Pharmacol. 53, 1533–1539.
Ficarra, R., Ficarra, P., Tommasini, S., Carulli, M., Melardi, S., Di Bella, M.R., Calabro, M.L., De Pasquale, R., Germano, M.P., Sanogo, R., Casuscelli, F., 1997. Isolation and characterization of *Guiera senegalensis* J.F. Gmel. active principles. Boll Chim Farm. 136, 454–459.
Filho, J.R., de Sousa Falcão, H., Batista, L.M., Filho, J.M., Piuvezam, M.R., 2010. Effects of plant extract on HIV-1 protease. Curr. HIV Res. 8, 531–544.
Flekhter, O.B., Boreko, E.I., Nigmatullina, L.P., Pavlova, N.I., Medvedeva, N.I., Nikolaeva, S.N., Ashavina, O.A., Savinova, O.V., Baltina, L.A., Galin, F.Z., Tolstikov, G.A., 2004. Synthesis and antiviral activity of lupane triterpenoids and their derivatives. Pharm. Chem. J. 38, 355–358.
Hattori, M., Ma, C.M., Wei, Y., Salah El Dine, R., Sato, N., 2013. Survey of anti-HIV and anti-HCV compounds from natural sources. Can. Chem. Tran. 1, 116–140.
Ha, H.L., Shin, H.J., Feitelson, M.A., Yu, D.Y., 2010. Oxidative stress and antioxidants in hepatic pathogenesis. World J. Gastroenterol. 16, 6035–6043.
Heidary, N.M., Laszczyk-Lauer, M.N., Reichling, J., Schnitzler, P., 2014. Pentacyclic triterpenes in birch bark extract inhibit early step of herpes simplex virus type 1 replication. Phytomedicine 21, 1273–1280.
International Conference on Harmonization (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human use, Harmonised Triplicate Guideline on Validation of Analytical Procedures: Text and Methodology Q2 (R1), Comp. Guideline Methodol. incorporated in November 2005 by the ICH Steering Committee, IFPMA, Geneva.
Ivorra, M.D., D'Ocon, M.P., Paya, M., Villar, A., 1988. Antihyperglycemic and insulin releasing effects of beta-sitosterol 3-B-D-glucoside and its aglycone beta-sitosterol. Arch. Int. Pharmacodyn. Therapy 296, 224–231.
Joycharat, N., Greger, H., Hofer, O., Saifah, E., 2008. Flavaglines and triterpenoids from the leaves of *Aglaia forbesii*. Phytochemistry 69, 206–211.
Kong, L., Li, S., Liao, Q., Zhang, Y., Sun, R., Zhu, X., Zhang, Q., Wang, J., Wu, X., Fang, X., et al., 2013. Oleanolic acid and ursolic acid: Novel hepatitis C virus antivirals that inhibit NS5B activity. Antivir. Res. 98, 44–53.
Lamien, C.E., Meda, A., Mans, J., Romito, M., Nacoulma, O.G., Viljoen, G.J., 2005. Inhibition of fowlpox virus by an aqueous acetone extract from galls of *Guiera senegalensis* J. F. Gmel (Combretaceae). J. Ethnopharmacol. 96, 249–253.
Ma, C.M., Nakamura, N., Miyashiro, H., Hattori, M., Shimotohno, K., 1999. Inhibitory effect of constituents from *Cynomorium songaricum* and related triterpene derivatives on HIV-1 protease. Chem. Pharm. Bull. 47, 141–145.
Martin-Aragón, S., de las Heras, B., Sanchez-Reus, M.I., Benedi, J., 2001. Pharmacological modification of endogenous antioxidant enzymes by ursolic acid on tetrachloride-induced liver damage in rats and primary cultures of rat hepatocytes. Exp. Toxicol. Pathol. 53, 199–206.
Niu, D., Zhang, J., Ren, Y., Feng, H., Chen, W.N., 2009. HBx genotype D represses GSTP1 expression and increases the oxidative level and apoptosis in HepG2 cells. Mol. Oncol. 3, 67–76.
Opara, E.C., 2006. Oxidative stress. Dis Mon. 2, 183–198.
Osman, I.M., Mohammed, A.S., Abdalla, A.B., 2014. Acaricidal properties of two extracts from *Guiera senegalensis* J.F. Gmel. (Combrataceae) against *Hyalomma anatolicum* (Acari: Ixodidae). Vet. Parasitol. 199, 201–205.
Pavlova, N.I., Savinova, O.V., Nikolaeva, S.N., Boreko, E.I., Flekhter, O.B., 2003. Antiviral activity of betulin, betulinic and betulonic acids against some enveloped and non-enveloped viruses. Fitoterapia. 74, 489–492.
Prasad, S., Kalra, N., Shukla, Y., 2007. Hepatoprotectic effects of lupeol and mango pulp extract on carcinogen induced alteration in Swiss albino mice. Mol. Nutr. Food Res. 51, 352–359.
Quéré, L., Wenger, T., Schramm, H.J., 1996. Triterpenes as potential dimerization inhibitors of HIV-1 protease. Biochem. Biophys. Res. Commun. 227, 484–488.
Rao, G.S., Sinsheimer, J.E., Cochran, K.W., 1974. Antiviral activity of triterpenoid saponins containing acylated β-amyrinaglycones. J. Pharm. Sci. 63, 471–473.
Rota, C., Chignell, C.F., Mason, R.P., 1999. Evidence for free radical formation during the oxidation of 2'-7'-dichlorofluorescin to the fluorescent dye 2'-7'-dichlorofluorescein by horseradish peroxidase: possible implications for oxidative stress measurements. Free Radic. Biol. Med. 27, 873–881.
Saraswat, B., Visen, P.K.S., Agarwal, D.P., 2000. Ursolic acid isolated from E*ucalyptus tereticornis* protects against ethanol toxicity in isolated rat hepatocytes. Phytother Res. 14, 163–166.
Saravanan, R., Pugalendi, V., Pugalendi, K.V., 2006. Protective effect of ursolic acid on ethanol-mediated experimental liver damage in rats. Life Sci. 78, 713–718.
Severi, T., Ying, C., Vermeesch, J.R., Cassiman, D., Cnops, L., Verslype, C., Fevery, J., Arckens, L., Neyts, J., van Pelt, J.F., 2006. Hepatitis B virus replication causes oxidative stress in HepAD38 liver cells. Mol. Cell Biochem. 290, 79–85.
Shukla, B., Visen, P.K.S., Patnaik, G.K., Tripathi, S.C., Srimal, R.C., Dayal, R., Dobhal, P. C., 1992. Hepatoprotective activity in the rat of ursolic acid isolated from *Eucalyptus hybrid*. Phytother. Res. 6, 74–79.
Siddique, H.R., Saleem, M., 2011. Beneficial health effects of lupeol triterpene: a review of preclinical studies. Life Sci. 88, 285–293.
Silva, O., Barbosa, S., Diniz, A., Valdeira, M.L., Gomes, E., 1997. Plant extracts antiviral activity against Herpes simplex virus type 1 and African swine fever virus. Int. J. Pharmacogn. 35, 12–16.
Silva, O., Gomes, E.T., 2003. Guieranone A, a naphthyl butenone from the leaves of *Guiera senegalensis* with antifungal activity. J. Nat. Prod. 66, 447–449.
Shaban, N.Z., Salem, H.H., Elsadany, M.A., Ali, B.A., Hassona, E.H., Mogahed, F.A.K., 2014. Alterations in lipid peroxidation and antioxidants in patients with different stages of Hepatitis B Virus infection in Egypt. Life Sci. 11, 960–967.
Somboro, A.A., Patel, K., Diallo, D., Sidibe, L., Chalchat, J.C., Figueredo, G., Ducki, S., Troin, Y., Chalard, P., 2011. An ethnobotanical and phytochemical study of the African medicinal plant *Guiera senegalensis J. F. Gmel*. J. Med. Plants Res. 5, 1639–1651.
Sombié, P.A.E.D., Hilou, A., Mounier, C., Coulibaly, A.Y., Kiendrebeogo, M., Millogo, J. F., Nacoulma, O.G., 2011. Antioxidant and anti-inflammatory activities from galls of *Guiera senegalensis* J.F. Gmel. (Combretaceae). Res. J. Med. Plants. 5, 448–461.
Suleiman, M.H., 2015. An ethnobotanical survey of medicinal plants used by communities of Northern Kordofan region, Sudan. J. Ethnopharmacol. 176, 232–242.
Sultana, N., Saify, Z.S., 2012. Naturally occurring and synthetic agents as potential anti-inflammatory and immunomodulants. Antiinflamm Antiallergy Agents Med Chem. 11, 3–19.
Tanaka, T., Ikeda, T., Kaku, M., Zhu, X.H., Okawa, M., Yokomizo, K., Uyeda, M., Nohara, T.A., 2004. new lignan glycoside and phenylethanoid glycosides from *Strobilanthes cusia* BREMEK. Chem. Pharm. Bull. 52, 1242–1245.
Woldemichael, G.M., Singh, M.P., Maiese, W.M., Timmermann, B.N., 2003. Constituents of antibacterial extract of *Caesalpinia paraguariensis* BURK Z. Naturforsch C 58, 70–75.
Wong, H.S., Chen, J.H., Leong, P.K., Leung, H.Y., Chan, W.M., Ko, K.M., 2014a. β-sitosterol protects against carbon tetrachloride hepatotoxicity but not gentamicin nephrotoxicity in rats via the induction of mitochondrial glutathione redox cycling. Molecules 19, 17649–17662.
Wong, H.S., Chen, N., Leong, P.K., Ko, K.M., 2014b. β-Sitosterol enhances cellular glutathione redox cycling by reactive oxygen species generated from mitochondrial respiration: protection against oxidant injury in H9c2 cells and rat hearts. Phytother. Res. 28, 999–1006.
Yamamoto, M., Matsui, T., Sugiyama, K., Yokota, M., Nukagomi, K., Nakazawa, H., 1991. Anti-inflammatory active constituents of *Aloe arborescens* Miller. Agric. Biol. Chem. 55, 1627–1629.
Zhang, L., Zhang, Y., Zhang, L., Yang, X., Lv, Z., 2009. Lupeol, a dietary triterpene, inhibited growth, and induced apoptosis through down-regulation of DR3 in SMMC7721 cells. Cancer Invest. 27, 163–170.
Zhao, L., Feng, C., Hou, C., Hu, L., Wang, Q., Wu, Y., 2015. First discovery of acetone extract from cottonseed oil sludge as a novel antiviral agent against plant viruses. PLoS One 10, e0117496.