Phenolic Profile and In Vitro Assessment of Cytotoxicity and Antibacterial Activity of *Ziziphus spina-christi* Leaf Extracts

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Abstract

**Background:** *Ziziphus spina-christi* is a tropical evergreen tree of Sudanese origin. The plant is commonly used in ethno-medicine for the treatment of many diseases such as malaria, digestive disorders, weakness, hepatic diseases, obesity, urinary problems, diabetes, skin infections, fever, diarrhea and insomnia. However, the pharmaceutical properties and bioactive compounds present in *Z. spina-christi* are still unknown. Therefore, the aim of the study was to significantly improve and deepen our knowledge about the phenolic composition of *Z. spina-christi* leaf extracts as well as their antibacterial and potential cytotoxic effects.

**Methods:** The phytochemical profile of the known compounds in the methanol/water (7:3 v/v) extract of *Z. spina-christi* leaves were tentatively assigned on the basis of reported analytical data from three to four pieces of independent methods including retention time (tR), UV/Vis spectroscopy, high resolution mass spectrometry (HR-MS) and tandem mass spectrometry (MS²). Four bioactive compounds were purified via preparative-HPLC and identified by means of HPLC-ESI-TOF, HPLC-ESI-MS², 1H-NMR, 13C-NMR, literature data and authentic reference standards. Antibacterial activities of aqueous and methanol leaf extracts were carried out with six bacterial strains (*Bacillus subtilis*, *Bacillus aquimaris*, *Clavibacter michiganensis*, *Escherichia coli*, *Erwinia amylovora*, and *Pseudomonas syringae*) using an agar diffusion assay. Cytotoxic effects towards the human epidermal keratinocyte cell line HaCaT and rat intestine epithelial IEC-6 cells was assessed by cytoskeletal and plasma membrane integrity or analyses of mitochondrial and proliferative activities.

**Results:** We tentatively assigned fifty-seven phenolic compounds (fifty-two of them are known) to their regioisomeric level in the methanol/water extract of *Z. spina-christi* leaves. To our knowledge 45 of them were not reported previously in *Z. spina-christi* and five for the first time in nature. Highly glycosylated flavonoids, proanthocyanidins, and chlorogenic acids were identified as the major components. Preparative-HPLC let to the isolation of four known phytochemicals including quercetin 3-O-(6-O-rhamnosyl-glucoside) (rutin) 26, quercetin 3-O-(2-O-rhamnosyl-arabinoside) 27, phloretin 3',5 di-C-glucoside 39 and quercetin 3-O-(2,6-di-O-rhamnosyl-glucoside) 50. Regarding the antimicrobial assays, the methanol extracts of the plant were found to show higher activity than the aqueous extracts indicating hydrophobic substances of *Ziziphus* leaves exerting antibacterial activity. The extracts revealed no cytotoxic effects towards the HaCaT or IEC-6 cells.

**Conclusions:** Several previously known and unknown compounds were indentified in *Z. spina-christi* leaf extracts. The plant possesses strong antimicrobial activity and no toxicity. Our results therefore suggest that *Z. spina-christi* leaf extracts might be potentially suitable for topical applications and support the use of the plant in traditional medicine to treat infectious diseases.

Keywords: *Ziziphus spina-christi*; *Rhamnaceae*; Polyphenols; Antibacterial activity; Cytotoxicity; HPLC-DAD–ESI-MS*

**Background**

*Ziziphus spina-christi* (L.) Desf. (*Rhamnaceae*) is a tropical evergreen tree of Sudanese origin. The plant has very interesting historical and religious aspects. It is repeatedly mentioned in Muslim as well as Christian traditions and was recorded by pilgrims visiting the Holy Land on numerous occasions. The boiled water extracts of the leaves of *Z. spina-christi* are used by Muslims in the cleaning of a dead body before burial suggesting antibacterial properties. In addition, the plant has been used in mummification by the ancient Egyptians [1]. It has been suggested that the plant material referred to in the Bible as the "thorns" or "bramble" (Judges 9; 14-15), "thorns" (Matthew 27:27–29) and the "crown of thorn" (John 19:5) might have been derived from *Z. spina-christi* [2]. The Holy Quran mentions the Lote tree (Cedar) three times (XXXIV: 16; LI: 13-18; LV: 28-32), which was frequently identified as *Z. spina-christi*. Accordingly, this species is highly respected throughout the Middle East, has been widely used as a food and as medicinal as well as a environmental protection plant since antiquity, and is still in use at present [2,3].

*Z. spina-christi* is commonly used in ethno-medicine for the treatment of many illnesses such as digestive disorders, weakness, hepatic disorders, obesity, urinary problems, diabetes, skin infections, fever, diarrhea and insomnia [4,5]. In Sudanese ethno-medicine, the leaves of *Z. spina-christi* are used for the treatment of malaria [6]. In addition, Michel et al. reported an anti-diabetic activity of the leaves of *Z. spina-christi* due to their saponin and polyphenol constituents [7], which was supported in pharmacological studies by Glombitza et al. indicating that extracts of *Z. spina-christi* leaves or its main saponin glycoside, christinin-A, enhanced glucose consumption in diabetic patients.

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The widespread and incorrect use of antibacterial agents has caused emergence of bacterial strains that are resistant to several antibiotics. Bacteria have developed different defense strategies to protect themselves from antimicrobial drugs such as alteration of the drug target, enzymatic inactivation, reduction of intracellular drug concentration by modifications in membrane permeability, or by efflux pumps [12,13]. Accordingly, new antimicrobials with greater effectiveness and better tolerability than existing drugs are urgently required for treatment of bacterial infections. Plant extracts are well-known to come a diversity of phytoconstituents that have a broad spectrum of antimicrobial activities, consequently plant-derived bioactive compounds counted as the main source for lead compound identification and development in pharmaceutical production [14].

Plant polyphenols comprise a large group of secondary metabolites that can extend from simple molecules, such as phenolic acids, to highly polymerized compounds such as tannins. They are constituents with a rich number of derivatives in the plant kingdom, from mosses and ferns to higher angiosperms including food plants. Many of these plant-derived polyphenols are essential components in our diets [15]. They are derivatives of the pentose phosphate, mevalonic acid, shikimate, and phenylpropanoid pathways in plants [16].

Flavonoids have been reported to have numerous biological effects such as antioxidant activity [17], antimicrobial activity [18], anti-inflammatory activities [19], inhibition of platelet aggregation [20], and inhibition of mast cell histamine release [21]. Moreover, antioxidant phenolics have been suggested to possess preventive functions in the progression of heart diseases and different cancers including prostate, breast, lung, colon and rectal cancers [22]. In addition, epidemiological studies have demonstrated that there is an inverse relationship between the intake of flavonoids such as myricetin, apigenin, quercetin, kaempferol and luteolin, which are naturally present in fruits, vegetables and beverages, and the lowering of the risk of suffering from cardiovascular diseases [23].

From different species of Ziziphus, peptide and cyclopeptide alkaloids, flavonoids, sterols, tannins, betulinic acid and triterpenoidal saponin glycosides have been isolated and chemically identified [24,25]. From the methanol extract of the fruits of Z. jujube and Z. spina-christi twelve compounds have been reported as representing quercetin, kaempferol, and phloretin derivatives [15]. In a qualitative as well as quantitative study, flavonoids, saponins, and triterpenic acids were identified and phenylpropanoid pathways in plants [16].

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UV Irradiation

UV irradiation experiments were performed as previously reported [27].

Liquid chromatography–mass spectrometry (LC/ESI/MS*)

The Agilent 1100 LC equipment (Agilent, Karlsruhe, Germany) comprised a binary pump, an auto-sampler with a 100-μl loop, and a diode array detector with a light-pipe flow cell (recording at 254, 280, and 320 nm). This was interfaced with an ion-trap mass spectrometer fitted with an electrospray ionization source (Bruker Daltonics HCT Ultra, Bremen, Germany) operating in full-scan, auto-MS* mode to obtain fragment ion m/z. Tandem mass spectra were acquired in auto-MS* mode (smart fragmentation) using a ramping of the collision energy. Maximum fragmentation amplitude was set to 1 V, starting at 30% and ending at 200%. MS operating conditions (negative mode) had been optimized using procyanidin B1 and 5-O-cafeoylquinic acid with a capillary temperature of 365°C, a dry gas flow rate of 10 l/min, and a nebulizer pressure of 10 psi. The spectra full scan mass were performed within the range from m/z 50 up to 1500 in negative ion mode.

High-resolution LC–MS was performed using the same high performance LC equipped with a microTOF mass spectrometer (Bruker Daltonics, Bremen, Germany) fitted with an electrospray ionization source, and internal calibration was achieved with 10 ml of 0.1M sodium formate solution injected through a six-port valve prior...
to each chromatographic run. Calibration was performed using the enhanced quadratic mode.

High-performance liquid chromatography

The HPLC separation was performed as previously reported [28].

Preparative-HPLC isolation

Preparative-HPLC isolation of compounds 26, 27, 39 and 50 was carried out as in our previous study [29].

Antibacterial activity assay

Three Gram-positive bacterial strains (Bacillus subtilis S168, Bacillus aquimaris MB-2011, and Clavibacter michiganensis GSPB 390 as well as three Gram-negative bacterial strains (Escherichia coli DH5α, Erwinia amylovora 1189, and Pseudomonas syringae pv tomato DC3000) were selected as model organisms to evaluate the antibacterial activity of the methanol and aqueous Z. spina-christi crude plant extracts at a final concentrations of 100 mg/ml. The antimicrobial activity assay was performed using the agar diffusion method according to Nathan et al. with slight modification as follows: Lysogeny broth (LB) agar plates were inoculated with 200 µl of the tester organism (1 x 10^7 cfu/ml) by spreading the bacterial cell suspensions over the agar with the help of sterile glass beads. Holes (5 mm diameter) were punched into the agar with a sterile Pasteur pipette, and 50 µl of the crude extracts was pipetted into the wells [30]. The plates were incubated overnight at 28°C except for E. coli, for which incubation was done at 37°C. Inhibition of microbial growth was determined by measuring the radius of the zone of inhibition. For each bacterial strain, an equal volume of water and methanol was used as negative controls. As positive controls, the following antibiotics were tested effective against the following bacterial organism causing inhibition zones of 10.3 to 23.3 mm: 25 mg/ml kanamycin (grad ≥ 750 IU/mg; Carl Roth, Germany) for B. subtilis and B. aquimaris; 50 mg/ml ampicillin (Carl Roth, Germany) for E. coli and E. amylovora; 25 mg/ml streptomycin (grade 730 IU/mg; Serva, Germany) for P. syringae and C. michiganensis. The experiments were conducted in triplicates, and the data are given as mean values ± standard deviation (SD). Each mean value was calculated from three biological replicas. Data were analyzed with one-way analysis of variance (ANOVA) using Origin lab software.

Cytotoxicity Analysis

Cell culture

HaCaT cells at passages 40 – 55 were used as a model for epidermal keratinocytes [31,32]. They were cultured in Dulbecco’s Modified Eagle Medium (DMEM, DMEM, Lonza Biowhittaker, Switzerland) containing phenol red and supplemented with 10% fetal bovine serum (FBS; Lonza Biowhittaker). The IEC-6 cell line at passage 16 - 40 was used as cellular model of small intestine epithelial cells (European Collection of Cell Cultures, ECACC, UK), and cultured in DMEM containing 0.1 µg/ml insulin (Sigma-Aldrich, USA) and 5% FBS. Cells were grown at 37 °C in a humidified atmosphere at 8% CO₂ for keratinocytes and at 5% CO₂ for IEC-6 cells. Cell culture medium was exchanged every three to four days, confluent cell cultures were used for experimentation.

Cellular integrity analyses by acquisition of propidium iodide (PI) and counter-staining with Draq5™

PI (Carl Roth, Germany) acquisition through ruptured plasma membranes was used as a measure of necrotic cell death, while Draq5™ staining of cellular DNA served to determine total cell numbers [33]. Cells were grown on cover slips for 24 hours and then treated with 50 µl of the plant extract at final concentrations of 100 µg/ml, 10 µg/ml, and 1 µg/ml (weight/volume, final concentrations). Controls were treated with 0.5% DMSO, since all extracts were diluted with DMSO to this final concentration. After incubation of the cells with extract preparations for 24 hours at 37 °C, cells were washed with prewarmed PBS. Staining with 2 µg /µl PI (Carl Roth, Germany) diluted in the respective culture medium was for 30 minutes at 37 °C. Afterwards cells were rinsed with PBS twice before fixation with 4% paraformaldehyde (Applìchem, Germany) in 200 mM Hepes, pH 7.4 for 20 minutes at RT. Then, cells were rinsed in PBS and stained with 5 µM Draq5™ (Biostatus, UK) in PBS for 45 minutes at 37 °C. Finally, cells were washed three times with PBS and then mounted in mowiol (33% glycerol and 14% Mowiol (Carl Roth, Germany) in 200 nM Tris-HCl in ultrapure H₂O at pH 8.5).

Images were taken at a Zeiss LSM 510 Meta confocal laser scanning microscope (Carl Zeiss, Germany) and analyzed using Zeiss LSM Image Browser software version 3.2.0.70.

Staining of the actin cytoskeleton with FITC-Phalloidin and counter-staining with Draq5™

Cells were fixed as described above and stained with 3 µM FITC-Phalloidin (Sigma-Aldrich, Germany) and 5 µM Draq5™ in PBS for 45 minutes at 37 °C [34]. Cells were washed three times before mounting in mowiol.

Determination of cell counts using the Image Analysis Software CellProfiler™

Images were analyzed using Cell Profiler 2.0 [35] as previously described [31,33,34].

MTT Assay

Cells were grown for 24 hours at 37 °C and the respective CO₂ concentration in 24-well plates before treatment with 50 µl plant extracts per 1 ml culture medium to achieve final concentrations of the extracts to reach 100, 10 and 1 µg/ml (wt/vol), respectively. As controls, cells were treated with 0.5% DMSO and mock-treated cells were used, to which no extracts were applied. MTT ([3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Carl Roth, Germany) was applied to which no extracts were applied. MTT assay described [31,33,34].

Results and Discussion

Methanol/water (7:3 v/v) extract of the leaves of Z. spina-christi was separated by reversed-phase chromatography. Identification of compounds was obtained using high resolution-electrospray ionization-mass spectrometry (HR-ESI-MS) for determination of molecular formula and ESI with quadrupole ion trap analyzer MS (ESI-QQT-MS) to obtain tandem MS data for further structure elucidation. Additionally, retention times and UV (320 nm) spectra were used for reversed phase compounds identification (Figure 1). Identification of phenolic compounds, for which commercial standards were available was carried out by the comparison of their retention times, UV–vis spectra and mass spectral data recorded in negative ion mode, while, the identity of other compounds was elucidated using the UV–vis spectrum to assign the phenolic class [36]. For all compounds the high resolution mass data were in good agreement with the theoretical molecular formulas.
Characterization of Proanthocyanidins

(Epi)catechin-(4,8')-(epi)gallocatechin (m/z 593)

One peak (m/z 593) was detected at retention time 11.1 min in the extracted ion chromatogram (EIC) and was tentatively assigned as dimeric B-type proanthocyanidins with (epi)catechin and (epi)gallocatechin monomeric units (epi)catechin-(4, 8')-(epi)gallocatechin 1 (Table 1 and Figures 1S and 2S, Supporting information) as evident from tandem MS data previously reported [38].

(Epi)gallocatechin-(4,8')-(epi)gallocatechin and (epi)gallocatechin-(4,6')-(epi)gallocatechin (m/z 609)

Six isomers (t_R 6.0, 7.7, 9.6, 11.1, 11.9, 14.8 min) were detected at m/z 609 in the methanolic extract of leaves of Z. spina-christi (Figures 3S and 4S, Supporting information). Isomer 2-5 were assigned as (Epi)gallocatechin-(4, 8')-(epi)gallocatechin as previously reported [39]. While, isomers 6-7 were tentatively assigned as (epi)gallocatechin-(4, 6')-(epi)gallocatechin assuming that the latter are less polar and hence

Figure 1: UV Chromatogram (320 nm) (I) base peak chromatogram (II) and expansion (III) for Z. spina-christi leaves extract. Peaks assignment listed in Table 1.
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Characterization of chlorogenic acids

trans-5-O-cafeoylquinic acid 12, trans-5-O-p-coumaroylquinic acid 15 and cis-5-O-p-coumaroylquinic acid 16 were easily detected in the EIC and total ion chromatogram (TIC) Table (1), while 3-O-cafeoylquinic acid 14 at m/z 353, and 4, 5 di-O-cafeoylquinic acid 18 at m/z 515 were observed in low intensities and were not determined in the BPC or total ion chromatogram (TIC). Subsequently, targeted tandem LC-MS experiments at m/z 353 and m/z 515, respectively, have been performed to confirm their presence in the plant extract. The chlorogenic acids were assigned after the comparison with the retention time and fragmentation pattern of authentic standard supported with the hierarchical keys previously reported in the literature [40-43] with retention time 23.2 min, a further small peak (m/z 353) with low intensity (16%) compared to compound 12 (100%) was observed. Targeted tandem-LC-MS experiments at m/z 353 have been performed and it displayed fragmentation patterns identical to the 5-O-cafeoylquinic acid and we suspected that it might be a cis isomer of the 5-O-cafeoylquinic. For confirmation of this isomer, the extract of Z. spina-christi was irradiated with UV light at 245 nm for 60 min. After irradiation, we found that the cis isomer in the chromatogram as peak with considerably increased intensities if compared to trans isomer from the original plant extract (Figures 7S and 8S, Supporting information), which confirmed the presence of the cis-5-O-cafeoylquinic acids 13 [27].

Characterization of caffeic acid 4-O-glucoside (m/z 341)

One peak was readily detected at m/z 341 in the EIC and was tentatively assigned as caffeic acid 4-O-glucoside 17. The neutral loss of a glucosyl moiety (162 u) resulted in a dominant fragment ion at m/z 179.0 corresponding to deprotonated caffeic acid and secondary peak at m/z 161 ([M-H-162-H₂O]⁻) from the neutral loss of a water molecule and an MS² base peak at m/z 135 by the neutral loss of CO₂ (44 u) (Figure 9S, Supporting information). For further evidence the UV spectrum, MS³ fragmentation and the retention time of this compound was compared with the compound (m/z 341) reported in the flowers of Chrysanthemum morifolium Ramat [44] and found that they are identical. Based on the above arguments compound 17 was identified as caffeic acid 4-O-glucoside. We have recently identified 6-O-cafeoyl-α-glucose and 6-O-cafeoyl-β-glucose in the bottle gourd (Lagenaria siceraria Stand) fruits [45]. Here we are unable to assign the anomic structure for the glucose moiety.

Characterization of apigenin 7,4′-di-O-glucoside (m/z 593)

One peak was detected at retention time 21.4 min with m/z 593 in the Z. spina-christi methanol/water extract. It showed fragmentation
Figure 2: Chemical structures of the phytoconstituents identified in Z. spina-christi.
patterns representing apigenin 7, 4'-di-O-glucoside 19. Compound 19 produced an MS\(^\text{1}\) fragment ions at \(m/z\ 431 ([M-H-162]^-)\) by the neutral loss of a glucosyl unit from C-4' (Figure 10S, Supporting information). It produced the MS\(^\text{1}\) base peak at \(m/z\ 269 ([apigenin-H^-]^-)\) by the neutral loss of the second glucosyl unit from C-7 (Table 1). Based on this fragmentation behaviour compound 19 was assigned as apigenin7, 4'-di-O-glucoside.

**Characterization of phloretin derivatives (m/z 597)**

Three C-glycosides derivatives of phloretin (compounds 38-40) were detected in the EIC of Z. spina-christi leaves extract (Table 1). They produced characteristic C-glycosides MS\(^\text{1}\) fragment ions ([M-H-120]^-, [M-H-90]^- and [M-H-30]^-) (Figure 11S, Supporting information). Compound 40 was identified as phloretin 3', 5'-di-C-glycoside. This compound has been previously reported in Ziziphus species [15].

We speculate that compounds 38 and 39 were isomers of compound 40 alternative C-glycosylated hexoses. From the literature and our experiments we have found that the glycosides containing galactoside units are more polar than the glycosides containing glucoside units [46]. Based on the above arguments we have tentatively assigned isomer 38 as phloretin 3', 5'-di-C-galactoside \((t_{\text{R}} 32.6\text{ min})\) and isomer 39 as phloretin 3'-C-glucoside 5'-C-galactoside or phloretin 3'-C-galactoside 5'-C-glucoside \((t_{\text{R}} 36.8\text{ min})\) (Table 1). To our knowledge, compound 38 was not previously reported in the plant kingdom.

**Characterization of diosmetin derivatives (m/z 769)**

Two peaks, 43 and 44, were detected at m/z 769 in the EIC and showed different retention times \((t_{\text{R}} 54.4\text{ and }56.6\text{ min})\). They showed in their MS\(^\text{1}\) fragment ions at m/z 299 (base peak), 255 and 284 corresponding to diosmetin aglycone [47], which suggested that these compounds were diosmetin glycosides (Table 1). Based on the elution order and the data previously reported [48-50] compound 43 was assigned as diosmetin 3'-O-galactoside 7'-O-rutinoside and the later eluting isomer 44 as diosmetin 3'-O-glucoside 7'-O-rutinoside (Table 1). To the best of our knowledge, compounds 43 and 44 were not previously reported in nature. (Further arguments are provided in the Supporting information).

**Characterization isorhamnetin, kaempferol and quercetin derivatives**

Di, tri, tetra and penta glycosylated flavonoids were also detected in Z. spina-christi methanol/water extract. The presence of the aglycone fragment ions at m/z 315, 285 and 301 in their MS\(^\text{1}\) and/or MS\(^\text{2}\) and characteristic fragment ions \((m/z\ 151\) and \(179\) for quercetin, \(m/z\ 151\) for kaempferol and \(m/z\ 300\) for isorhamnetin) [51], in addition to their UV spectra suggested that these compounds were isorhamnetin, kaempferol and quercetin derivatives [52,53].

Compounds 23, 26, 29, 30, 31 and 33 were identified as kaempferol 3-O-(6-O-rhamnosyl-glucoside), quercetin 3-O-(6-O-rhamnosyl-glucoside) (rutin), quercetin 3-O-arabinoside, quercetin 3-O-rhamnoside, quercetin 3-O-glucoside, and kaempferol 3-O-glucoside, respectively, by the comparison of their UV spectra and retention times with commercial standards.

Compounds 20-22, 24, 25, 27, 28, 32, 34, 37 and 45-57 were tentatively identified as isorhamnetin 3-O-(6-O-rhamnosyl-galactoside), isorhamnetin 3-O-(6-O-rhamnosyl-glucoside), kaempferol 3-O-(6-O-rhamnosyl-galactoside), quercetin 3-O-(6-O-rhamnosyl-glucoside), quercetin 3-O-(6-O-rhamnosyl-galactoside) and quercetin 3-O-(2-O-arabinoside), quercetin 3-O-(2-O-rhamnosyl-arabinoside), quercetin 3-O-(2-O-rhamnosyl-glucoside), quercetin 3-O-(6'-O-(3-hydroxyl-3-methylglutaryl)-β-D-galactoside), kaempferol 3-O-[6'-(3-hydroxyl-3-methylglutaryl)-β-D-galactoside], quercetin 3-O-[3, 4'-D-Xylosyl-(-1→2)-α-L-rhamnoside 4'-O-a-L-rhamnoside, kaempferol 3-O-(2, 6-di-O-rhamnosyl-glucoside), quercetin 3-O-(2, 6-di-O-rhamnosyl-glucoside) 7'-O-rhamnoside, quercetin 3-O-(2, 6-di-O-rhamnosyl-glucoside) 7'-O-rhamnoside, kaempferol 3-O-(2, 6-di-O-rhamnosyl-glucoside) 7'-O-rhamnoside, kaempferol 3-O-(2, 6-di-O-rhamnosyl-glucoside) 7'-O-rhamnoside, quercetin 3-O-(2, 6-di-O-rhamnosyl-glucoside) 7'-O-rhamnoside, kaempferol 3-O-(2, 6-di-O-rhamnosyl-glucoside) 7'-O-glucuronide, respectively, as previously reported [15,26,29,48-50] (See also the Supporting information for characterization of these compounds).

Two isomers of flavonoid pentaglycosides (41 and 42) were also detected in the Z. spina-christi methanol/water extract. Both isomers showed a pseudomolecular ion at m/z 1047 ([M-H^-]^-) and the MS\(^\text{1}\) spectra showed a base peak at m/z 901 ([M-H-146^-]^-) due to the neutral loss of rhamnoside residue from position 7 of the flavonoid moiety [54] and a high abundance ions (≥ 60) at 755 ([M-H-146^-]^-) indicating a subsequent neutral loss of another rhamnoside residues from position 7 (Table 1 and Figure 3), this was supported by observing the ion m/z 755 as a base peak in MS\(^\text{2}\) spectra. Their MS\(^\text{2}\) (755 to 300/301) [neutral loss of triglycosidyl (454 u) moieties] were reminiscent to that of quercetin 3-O-(2, 6-di-O-rhamnosyl-glucoside) (50) (Table 1). These data suggested that these isomers were derivatives of compound 50 with an additional two O-rhamnoside substituent at position 7. Accordingly, isomer 42 was tentatively identified as quercetin 3-O-(2, 6-di-O-rhamnosyl-glucoside) 7'-di-O-rhamnoside, and the earlier-eluted isomer 41 as quercetin 3-O-(2, 6-di-O-rhamnosyl-galactoside) 7'-di-O-rhamnoside. To the best of our knowledge compounds 41 and 42 were not reported previously in nature.

**Characterization of the isolated constituents**

In our present work, preparative HPLC was used after the purification on Sephadex G-10 solvent extraction columns to isolate four known compounds from the methanol/water (7:3 v/v) extract of Z. spina-christi leaves. The structures of the isolated compounds were elucidated by HRMS (Table S1 in the Supporting information), tandem MS (Table 1 and Figure 4), UV chromatograms (Figure 1), and authentic standards. (See also Figures 18S and 19S in the Supporting information). Chromatographic resolution and MS data were considered for isolated compounds showing a poor NMR spectral resolution. With these agreements compound 26 (25.0 mg) was identified as quercetin 3-O-(6-O-rhamnosyl-glucoside) (rutin), compound 27 (2.6 mg) as quercetin 3-O-(2-O-rhamnosyl-arabinoside), compound 39 (9.0 mg) as phloretin 3',5'-di-C-glycoside and compound 50 (2.5 mg) as quercetin 3-O-(2,6-di-O-rhamnosyl-glucoside) (Figure 2). Compounds 26, 27 and 39 have been previously isolated from the fruits of Ziziphus species [15]. The UV spectra, MS fragmentation and the retention time of compound 50 were compared with the compound (m/z 755) reported in the leaves of Catharanthus roseus and found that they are identical [55]. The purity of these compounds was determined as in our previous study [29] by total ion chromatograms in negative ion mode and UV chromatograms at 280 and 320 nm. The purity of the compounds were 86% for compound 26, 75% for compound 27, 56% for compound 39 and >98% for compound 50.

**Antibacterial activity**

The antibacterial activities of the plant extracts analysed in this
study were evaluated for their efficacies against Gram-positive and Gram-negative bacteria using the agar diffusion method. As suitable model organisms *B. subtilis* S168, *B. aquimaris* MB-2011, and *C. michiganensis* GSPB 390 were chosen for Gram-positive and *E. coli* DH5α, *E. amylovora* 1189, and *P. syringae pv* tomato DC300 for Gram-negative. The results of the antibacterial activity tests of aqueous and methanol extracts of the leaves of *Z. spina-christi* are given in Table 2 and Figure 5. Interestingly, the plant extracts tested herein showed antibacterial activity only against the Gram-positive bacterium *B. aquimaris* MB2011 and the Gram-negative representative *P. syringae pv* tomato DC300 while the other bacterial strains were not affected at all. These results are in contrast to those of others [56] who found that ethanol and methanol extracts of *Z. spina-christi* leaves inhibited the growth of a variety of Gram-negative bacterial strains Salmonella typhi, *Proteus mirabilis*, Shigella dysenteriae, *E. coli*, Klebsiella pneumoniae, *Brucella melitensis*, Bordetella bronchiseptica and *Pseudomonas aeruginosa*. Previously, Moghadam et al. reported antibacterial activity of ethanolic extracts of the leaves of *Z. spina-christi* against a Gram-positive, Methicillin-resistance *Staphylococcus aureus* strain [9].

**Figure 4:** Tandem MS spectra of the isolated constituents in negative ion mode.

![Figure 4](image)

**Table 2:** Antibacterial activities of *Z. spina-christi* leaf extracts and the standard antibiotics as assessed by the agar diffusion method.

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>Extracts prepared in</th>
<th>Inhibition zone diameters (mm)</th>
<th>Antibiotic</th>
<th>Water</th>
<th>MeOH</th>
<th>Kanamycin</th>
<th>Ampicillin</th>
<th>Streptomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram-positive</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. subtilis (S168)</td>
<td>0.0 ± 0.0</td>
<td>20.0 ± 0.0</td>
<td>Nil</td>
<td>NA</td>
<td>NA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. aquimaris (MB2011)</td>
<td>16.0 ± 1.0</td>
<td>26.0 ± 2.0</td>
<td>19.7 ± 0.6</td>
<td>NA</td>
<td>NA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. michiganensis (GSPB 390)</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>10.3 ± 0.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gram-negative</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli (DH5α)</td>
<td>0.0 ± 0.0</td>
<td>16.3 ± 0.6</td>
<td>Nil</td>
<td>NA</td>
<td>NA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. amylovora (1189)</td>
<td>0.0 ± 0.0</td>
<td>13.3 ± 0.6</td>
<td>Nil</td>
<td>NA</td>
<td>NA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. syringae pv tomato (DC300)</td>
<td>0.0 ± 0.0</td>
<td>18.3 ± 0.5</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>23.3 ± 1.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NA = Not Applicable

**Figure 5:** Inhibition zones as means ± SD generated by extracts prepared from *Z. spina-christi* leaves and the standard antibiotics against bacterial species tested.

![Figure 5](image)
In line with our findings, it has been shown before that many plant metabolites including flavonoids, phenols, tannins, and alkaloids exhibit moderate antimicrobial activity [57]. Our data propose that antibacterial activities might be associated with the phenolic compounds from Z. spina-christi leaves, which were identified in the aqueous and methanol extracts. Our data furthermore indicated that methanol extracts appeared to be more effective in antibacterial activity against Gram-negative bacteria as compared to the aqueous extracts of the leaves from Z. spina-christi suggesting that the bioactive metabolites of Z. spina-christi were rather hydrophilic than hydrophobic. However, B. aquimaris MB-2011 was similarly affected by the aqueous and the methanol extracts suggesting that it is either affected by at least two differently extractable compounds or that it is susceptible to a compound different from that effective against the Gram-negative strain P. syringae. Thus, if traditional use of the leaves of Z. spina-christi involves the preparation of a pre-extract in alcoholic solvents, their effectiveness may be more pronounced than simply brewing the leaves in water [3].

**Cytotoxic activity**

In order to use Z. spina-christi extract as a potential antibiotic and to enable safe use as topical treatment of skin or as systemic drug taken up by oral administration routes, cytotoxicity against the exposed cells and tissues must be analyzed in order to exclude potential negative, off-target effects during treatment. Here, we have used human keratinocyte (HaCaT) and rat intestine epithelial (IEC-6) cell lines in culture to assess potential cytotoxicity exerted by leaf extracts from Z. spina-christi in vitro. For all experiments, the cells were first grown to confluence with the aim to simulate the effect of the extracts on cell monolayers which are considered representative of intact epithelia of the respective tissue, i.e., epidermis of the skin and mucosa of the gastro-intestinal tract lining.

Cytotoxicity was estimated by using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay, which is a routine test evaluating cell viability and proliferative activity by means of cellular enzyme activity. The MTT is converted by mitochondrial NADH-dependent dehydrogenase into an insoluble dye made of dark blue formazan crystals [58]. MTT assays are suited to determine the activity of mitochondrial enzymes reducing the yellow tetrazole MTT to purple formazan in living cells, only [59]. Therefore MTT conversion is proportional to both, cell viability and proliferation rates of treated cell cultures.

The MTT assay has been applied by us and others before. In comparison with other cytotoxicity assays such as the neutral red assay or quantification of LDH latency, the MTT assay was suitable but less sensitive than the former when CdCl₂ effects onto hepatoma cell lines were determined [60]. In another study, the above mentioned three different assays were compared and additionally ATP contents of the cultured cells were determined [61]. While both, the MTT and the LDH assay were somewhat non-suited because of their sensitivity to inhibitory agents contained in the compound mixtures tested, it was also pointed out that the choice of assay depends on the pathway of death that is potentially initiated by the agents applied.

Here, we used the MTT assay to recapitulate the well-known cytotoxicity of DMSO by incubating cultures of HaCaT keratinocytes and IEC6 intestine epithelial cells with different concentrations thereof (Figure 20S, Supporting information). The results demonstrate that indeed DMSO is cytotoxic if applied at concentrations above 1%, because MTT conversion declines in a concentration dependent manner. However, the data also support the notion that concentrations of up to 0.5% DMSO are non-cytotoxic, and can be used as solvent of lipophilic compounds.

We have used this assay before to determine cell viability upon exposure of fibroblasts, keratinocytes, and intestine epithelial cells to other stressors in addition to testing for the safe use of e.g., potential drug delivery tools [33,34,62]. The conversion of MTT by HaCaT keratinocytes (Figure 6A) is not influenced upon exposure to 100 µg or 10 µg/ml Z. spina-christi methanol/water (7:3 v/v) extract. However, upon treatment with 1 µg/ml Z. spina-christi conversion of MTT was decreased in HaCaT cell cultures. On the other hand the MTT conversion by small intestine epithelial IEC-6 cells (Figure 6B) increased with decreasing concentration although this was not statistically significantly different from controls. For the lower concentrations of 10 µg and 1 µg/ml the values of conversion were approximately even. We conclude that cytotoxicity towards IEC-6 cell cultures increased with increasing concentration of Z. spina-christi, and it was safe to apply extracts at a final concentration of 1 µg/ml to intestine epithelial cell monolayers. In contrast, for HaCaT cells the exposure to 1 µg/ml Z. spina-christi extracts, only exerted cytotoxic effects that were detectable by a significant reduction of the MTT-conversion to approx. 75% in comparison to DMSO-treated controls.

In addition, MTT- and LDH-assays are usually backed up by additional tests that involve determination of cellular integrity as we have shown in a study in which the MTT assay was employed to test for cytotoxicity of dust samples applied to keratinocytes and fibroblasts [34]. Results achieved by the MTT assay were comparable with culture
The leaves. Methanol extracts were used in HaCaT or IEC-6 cell cultures treated with extracts of *Z. spina-christi*. This assay is based on the fact that PI intercalates with DNA, thereby visualizing nuclear DNA when PI passes ruptured membrane lipid bilayers. Therefore PI may stain for necrotic cells in which the lipid bilayer integrity of the plasma membrane is compromised [34]. With increasing concentration of *Z. spina-christi* extracts, the number of necrotic or ruptured cells increased for the exposed HaCaT cultures (Figure 7A). For the IEC-6 cell cultures (Figure 7B), the number of ruptured cells decreased upon exposure to leaf extract of *Z. spina-christi*. However, the numbers of ruptured cells in HaCaT or IEC6 cell cultures treated with *Z. spina-christi* leaf extracts remained moderate as they were determined to not even reach one percent upon 24 h of exposure.

As an additional and sensitive measure of cellular integrity, the actin cytoskeleton was analyzed using a Phalloidin Draq5™ staining.

![Figure 7: Plasma membrane integrity and extent of necrotic cell death exerted after 24 hours of exposure of HaCaT keratinocytes (A) or IEC-6 small intestine epithelial cells (B) to leaf extracts from *Z. spina-christi*. The acquisition of PI indicates ruptured plasma membranes due to necrotic cell death. Fluorescence intensities of PI in three arbitrary chosen regions were determined by CellProfiler™ software to quantify the fluorescence of dead cells over that of all cells, which was determined by Draq5™ counter-staining of nuclear DNA. Values are given as permille and expressed as means ± standard deviations.](image)

Consequently, here we performed propidium iodide (PI) staining for both, HaCaT keratinocytes and IEC-6 intestine epithelial cells to determine the extent of potential plasma membrane rupture and the number of necrotic cells per viable cells when the cultures were treated with extracts of *Z. spina-christi*. This assay is based on the fact that PI intercalates with DNA, thereby visualizing nuclear DNA when PI passes ruptured membrane lipid bilayers. Therefore PI may stain for necrotic cells in which the lipid bilayer integrity of the plasma membrane is compromised [34]. With increasing concentration of *Z. spina-christi* extracts, the number of necrotic or ruptured cells increased for the exposed HaCaT cultures (Figure 7A). For the IEC-6 cell cultures (Figure 7B), the number of ruptured cells decreased upon exposure to leaf extract of *Z. spina-christi*. However, the numbers of ruptured cells in HaCaT or IEC6 cell cultures treated with *Z. spina-christi* leaf extracts remained moderate as they were determined to not even reach one percent upon 24 h of exposure.

As an additional and sensitive measure of cellular integrity, the actin cytoskeleton was analyzed using a Phalloidin Draq5™ staining.

![Figure 8: Phallolidin staining of the filamentous actin system of HaCaT keratinocytes and IEC-6 small intestine epithelial cells after exposure to 100 µg/ml leaf extracts of *Z. spina-christi* for 24 h. Single channel fluorescence, phase contrast and merged micrographs taken with a confocal laser scanning microscope of formaldehyde-fixed cells after staining of actin filaments with FITC-phallolidin, and Draq5™ counter-staining of nuclear DNA. Bars represent 20 µm and 50 µm, respectively.](image)

Upon treatment with 100 µg/ml leaf extracts of *Z. spina-christi*, HaCaT cells (Figure 8) showed a slightly altered actin filamentous system, and some cells detached from their neighbors indicating loosening of cell-cells contacts. For the IEC-6 cell cultures (Figure 8), the cortical F-actin system remained intact upon treatment with different concentrations of the extracts prepared from *Z. spina-christi*.

In summary, mild cytotoxic effects can be observed on both, HaCaT and IEC-6 cell cultures upon a 24-h treatment with leaf extracts of *Z. spina-christi*. IEC-6 cells were less sensitive compared to HaCaT cells, suggesting *Z. spina-christi* extracts might be potentially suitable for topical applications.

**Conclusions**

*Z. spina-christi* is a medicinal and edible plant growing wild in Sudan and used for a long time in traditional medicine for the treatment of different diseases caused by infection. In the present work we report that *Z. spina-christi* leaves are a rich source of phenolic compounds including chlorogenic acids, proanthocyanidins, simple and highly glycosylated flavonoids. Fifty-seven components were tentatively detected and characterized on the basis of reported analytical data from three to four pieces of independent methods including retention time, UV/Vis spectroscopy, high resolution mass spectrometry (HR-MS) and tandem mass spectrometry (MS²). To our knowledge 45 of them were not reported previously in *Z. spina-christi* and five for the first time in nature. Besides, four bioactive known compounds were isolated. Next, four known phenolic compounds were isolated and characterized by means of preparative-HPLC and other spectroscopic techniques. We investigated the antimicrobial activities of aqueous and methanol extracts of *Z. spina-christi* leaves. Methanol extracts were more effective in inhibiting bacterial growth than the aqueous extracts, which is most likely attributed to the solubility and hydrophobicity of the antibiotic plant compounds. Remarkably, the methanol extract of *Z. spina-christi* leaves revealed an antibacterial activity against both, a Gram-positive and a Gram-negative tester organism while the aqueous extracts needed with the goal to purify the methanol extracts in order to isolate and identify the specific compound/s that exert/s antimicrobial effects, in particular, against human pathogens. Furthermore, the cytotoxicity of *Z. spina-christi* leaf extracts was tested with a range of different cell biological and biochemical means. The herein established cytotoxicity assessment routine revealed that short-term application of *Z. spina-
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Additional File
High resolution MS data, full assignment arguments, proposed fragmentation pathways and MS spectra of some compounds are provided in the Supporting information.

References


