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Hepatoprotective, cytotoxic, antimicrobial and antioxidant activities of *Dioon spinulosum* leaves Dyer Ex Eichler and its isolated secondary metabolites

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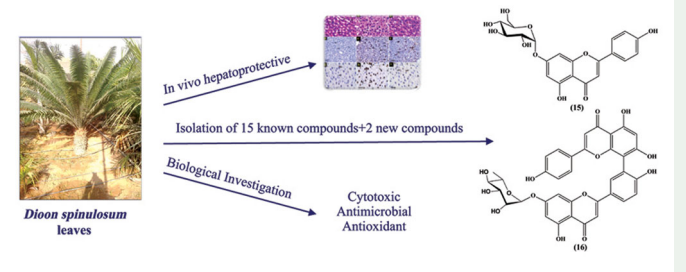
ABSTRACT

Given the lack of adequate research on *Dioon spinulosum* (*D. spinulosum*) Dyer Ex Eichler, this study was conducted focusing on different biological activities and phytochemical investigation of *D. spinulosum* for the first time. *D. spinulosum* showed strong protective activity against DNA damage and potent activity against VERO cell line. It also presented antimicrobial and hepatoprotective activity. Phytochemical investigation of the leaves resulted in isolation of two new flavonoids, apigenin 7-*O*-α-D-glucopyranoside (15) and amentoflavone 4′-*O*-α-L-rhamnopyranoside (16), in addition to fifteen known compounds: phytone (1), trans-phytol (2), β-sitosterol (3), stigmasterol (4), oliveriflavone (5), 7,4′,7″,4‴-tetramethylamentoflavone (6), 7,4′,7″-trimethylamentoflavone (7), scaidopitysin (8), bilobetin (9), isoginkgetin (10), aromadendrin (11), sotusflavone (12), engeletin (14) and eriocitrin (17) for the first time together with amentoflavone (13). Compounds (11) and (13) displayed very strong cytotoxic activity and showed the highest protective activity against DNA damage.



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1. Introduction

Gymnosperms are still a dark area in scientific research. Cycadales order as living representative of gymnosperms is classified into 11 genera in three families; Cycadaceae,

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Zamiaceae and Stangeriaceae, and distributed all over the world (Walters and Osborne 2004). It is regrettable that cycads are approaching the extinction line (Agrawal 2017). Zamiaceae was chosen to be the target of this study. Zamiaceae is considered a rich family of flavonoids, biflavonoids, their glycosides and methoxylated biflavonoids (Negm et al. 2016). *Dioon spinulosum* (*D. spinulosum*) is the largest Cycad in the Americas and the second largest in the world. *D. spinulosum* gains its Latin name from the spiny margins of its leaflets (Whitelock 2002). The lack of biological and phytochemical research motivates us to undertake the present study.

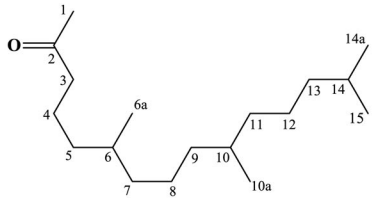
Liver diseases are one of the most challenging health care problems worldwide (Gaafar et al. 2019). Baking and frying food at high temperatures cause acrylamide formation at high levels. Acrylamide is a dangerous chemical substance having genotoxic and carcinogenic effects. The mechanism involved in acrylamide induced liver damage is attributed to the significant reduction in the glutathione concentration in liver cells inducing oxidative stress. This results in oxidative damage to various cell components, which affects liver cell function and leads to liver cell damage (Ansar et al. 2016). The aim of this study was to investigate the hepatoprotective effect of *D. spinulosum* total extract using acrylamide induced liver cell damage in albino rats by assessment of the morphological changes. We also investigated the cytotoxic, antimicrobial and antioxidant activities.

2. Result and discussion

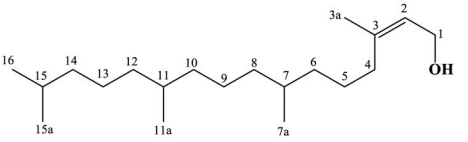
2.1. Phytochemical investigation

Seventeen compounds were isolated from petroleum ether, methylene chloride, ethyl acetate and *n*-butanol fractions of the MeOH extract of the leaves of *Dioon spinulosum* Dyer Ex Eichler family Zamiaceae using extensive column chromatography. Their structures were identified by spectroscopic analysis (1D, 2D NMR and HRMS) and comparison with the literature data. Among them from ethyl acetate fraction compounds 15 and 16 were new compounds (Figure 1).

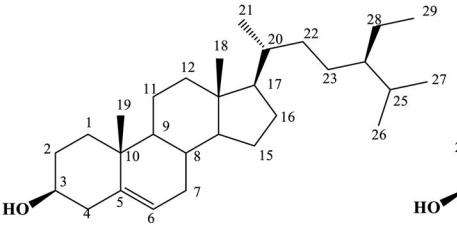
Compound 15 is a yellowish white amorphous powder, HR-ESI-MS of 15 showed a pseudo molecular ion at m/z 433.335 for $[M+H]^+$ with a molecular formula $C_{21}H_{20}O_{10}$. UV spectrum showed λ_{max} at 267 and 329 nm which suggested a flavonoid structure. ^{1}H NMR spectrum indicated *meta*-coupled doublets at δ 6.43 and 6.82 ($J = 2.5$ Hz) assigned to H-6 and H-8 protons in ring A. The two protons resonating at δ 7.95 (d, $J = 9.0$ Hz) can be assigned as ring B protons at 2′, 6′ positions *ortho*-coupled to the signal at δ 6.92 assigned to H-3′ and H-5′ protons. A singlet signal at δ 6.86 corresponding to H-3 proton. ^{1}H NMR spectrum is characteristic for a 5,7,4′-trisubstituted flavone. The presence of sugar protons resonating at δ 3.16–3.74 (m) and one anomeric proton at δ 5.40 (d, $J = 4.0$ Hz) suggested the presence of α-D-glucose. ^{13}C NMR spectrum showed 21 carbons, 15 were assigned to apigenin and 6 signals at δ 99.5, 73.1, 77.1, 69.5, 76.4 and 60.5 confirmed the presence of D-glucose (Agrawal 1989). The site of the attachment of the D-glucose moiety was confirmed by HMBC correlations between the D-glucose anomeric proton at δ 5.40 (d, $J = 4.0$ Hz) to C-7 at δ 164.2, to C-6 at δ 99.6 and also to C-8 at δ 94.8, respectively (Supporting Information Figure S3). Acid hydrolysis of compound 15 yielded apigenin



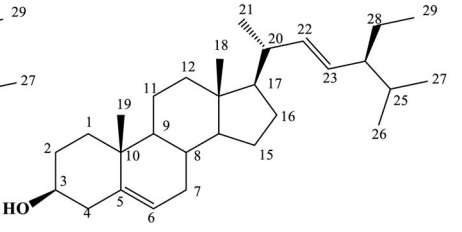
Compound 1



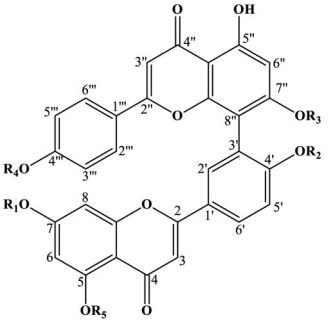
Compound 2



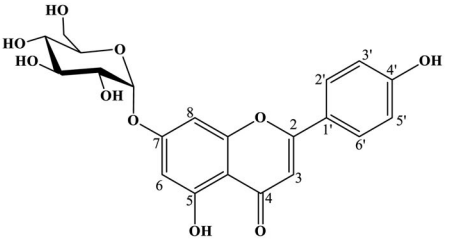
Compound 3



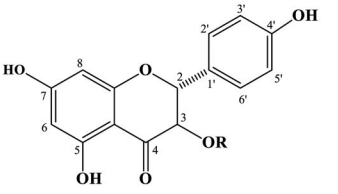
Compound 4



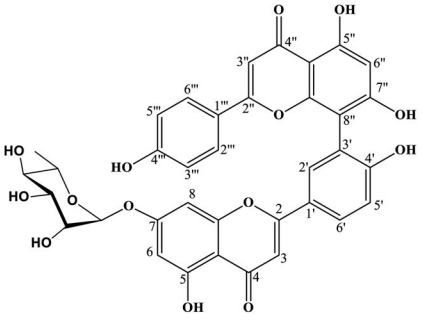
Compound 5 $R_1=R_2=R_3=R_4=R_5=CH_3$
Compound 6 $R_1=R_2=R_3=R_4=CH_3$, $R_5=H$
Compound 7 $R_1=R_2=R_3=CH_3$, $R_4=R_5=H$
Compound 8 $R_1=R_2=R_4=CH_3$, $R_3=R_5=H$
Compound 9 $R_2=CH_3$, $R_1=R_3=R_4=R_5=H$
Compound 10 $R_2=R_4=CH_3$, $R_1=R_3=R_5=H$
Compound 12 $R_1=CH_3$, $R_2=R_3=R_4=R_5=H$
Compound 13 $R_1=R_2=R_3=R_4=R_5=H$



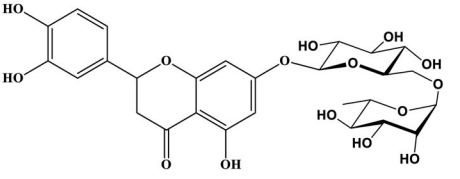
Compound 15



Compound 11 R=H
Compound 14 R=α-L-rhamnose



Compound 16



Compound 17

Figure 1. Chemical structures of isolated compounds.

the aglycone part which was confirmed by direct comparison with the authentic sample by TLC. Paper chromatography of the sugar part after acid hydrolysis showed that D-glucose is the sugar moiety. Compound 15 was identified as apigenin 7-*O*-α-D-

glucopyranoside. This new compound is isolated for the first time from *Dioon* species leaves.

Compound 16 is a yellowish white amorphous powder, HR-ESI-MS of 16 showed a pseudo molecular ion at *m/z* 684.578 for $[M+H]^+$ with a molecular formula $C_{36}H_{28}O_{14}$. UV spectrum showed λ_{max} at 282 and 333 nm which suggested a flavonoid structure. The ^{1}H NMR spectrum showed an AA′BB′ systems for ring E protons as indicated by δ 7.56 (2H, d, 9.5 Hz) for H-2‴, 6‴ and δ 6.62 (2H, d, 9.5 Hz) for H-3‴, 5‴. The ^{1}H NMR spectrum showed signals for *meta*-coupled protons at δ 6.18 (d, $J = 1.5$ Hz) and 6.41 (d, $J = 1.5$ Hz) for H-6 and H-8 in ring A protons, respectively, the only signal at δ 6.38 was assigned to H-6″. Additionally an ABX system was also shown by the signals at δ 7.95 (d, $J = 2.5$ Hz) for H-2′, δ 7.14 (d, $J = 9.5$ Hz) for H-5′ and δ 7.97 (dd, $J = 9.5$, 2.5 Hz) for H-6′ in ring B protons. These data indicated that C-8″ and C-3′ are involved in the biflavonoid linkage. The presence of one anomeric proton at δ 4.60 singlet and doublet methyl protons at δ 1.20 (d, $J = 6.0$ Hz) confirmed the L-rhamnose sugar moiety. ^{13}C NMR spectrum showed 36 carbons and confirmed the conjugation linkage between C-8″ at δ 105.5 and C-3′ at δ 123.2 due to the downfield shift of C-8″ by 10 ppm and of C-3″ by 6.00 ppm, respectively (Harborne et al. 1975; Markham et al. 1978; Markham et al. 1987; Andersen and Markham 2006). The sugar was identified as L-rhamnose and the anomeric carbon was at δ 100.1. HMBC correlations confirmed the involvement of C-3′ and C-8″ in the interflavonoid linkage via the correlation between H-5′ at δ 7.14 to C-3′ at δ 123.2 and H-2′ at δ 7.95 to C-8″ at δ 105.5. The attachment site of L-rhamnose was confirmed through HMBC correlation between the anomeric proton at δ 4.60 to C-7 at δ 166.1 (Supporting Information Figure S7). Acid hydrolysis of compound 16 yielded amentoflavone as the aglycone part which was confirmed by direct comparison with the isolated amentoflavone (13) by TLC. Paper chromatography of the sugar part after acid hydrolysis showed that it is α-L-rhamnopyranoside. Compound 16 was identified as amentoflavone 4′-*O*-α-L-rhamnopyranoside. This is the first report of this compound from plants.

Other isolated known compounds were identified as 6,10,14-trimethylpentadecan-2-one, also known as hexahydrofarnesylacetone or phytone (1) (Duke 2004), trans 3a,7a,11a,15a-tetramethyl-2-hexadecen-1-ol (trans-phytol) (2) (Wong and Brown 2002), β-sitosterol (3) (Sen et al. 2012), stigmasta-5,22-dien-3β-ol (stigmasterol) (4) (Habib et al. 2007), 5,7,4′,7″,4‴-pentamethylamentoflavone, oliveriflavone (5) (Wu et al. 1986), 7,4′,7″,4‴-tetramethylamentoflavone (6) (Markham et al. 1987; Moawad and El Amir 2016), 7,4′,7″-trimethylamentoflavone (7) (Markham et al. 1987), 7,4′,4‴-trimethylamentoflavone (scaidopitysin) (8) (Markham et al. 1987; Moawad and El Amir 2016), bilobetin (9) (Markham et al. 1987; Moawad and El Amir 2016), isoginkgetin (10) (Markham et al. 1987; Moawad et al. 2010; Moawad and El Amir 2016), aromadendrin (dihydrokaempferol) (11) (Xiang et al. 2011; Zhang et al. 2016), sotusflavone (12) (Lopez-saez et al. 1994), amentoflavone (13) (Markham et al. 1987; Negm et al. 2016), dihydrokamepherol 3-*O*-α-L-rhamnopyranoside or engeletin (14) (Huang et al. 2011; Ali et al. 2014; Gulluce et al. 2015) and eriocitrin or eriodictyol glycoside (17) (Miyake et al. 1997). Compounds (1, 2, 4, 5, 7, 11, 14 and 17) were isolated for the first time from *Dioon* species leaves. Compounds (3, 6, 8, 9, 10 and 12) were isolated for the first time from *D. spinulosum*. (Supporting Information).

2.2. Biological investigation

2.2.1. Histopathological effects of acrylamide and D. spinulosum methanol extract on liver cells (Aschner et al. 2005; Chen et al. 2009; Molina et al. 2013)

Examination of hematoxylin and eosin H & E stained sections of liver of control group (Group I) as well as *D. spinulosum* extract-treated rats (Group II) revealed normal histological architecture of the hepatocytes. The cells were arranged in anastomosing cords and were separated by the blood sinusoids. They were polyhedral shaped cells and had large rounded vesicular nuclei with prominent nucleoli (Supporting Information Figure S10(a)).

Examination of liver sections of acrylamide-treated group (Group III) showed distortion of the normal hepatocyte architecture. Many hepatocytes showed severe histological changes in the form of markedly vacuolated cytoplasm and deeply stained shrunken nuclei (pyknotic nuclei) as presented in (Supporting Information Figure S10(b)). Conversely, liver sections of rats in Group IV (*D. spinulosum* and acrylamide-treated group) showed apparently more or less normal hepatocyte architecture nearly that comparable to the control group (Supporting Information Figure S10(c)).

2.2.2. Immunohistochemical results

P53 immunostaining: p53-immunostained liver sections of the control (Group I) as well as *D. spinulosum* extract-treated rats (Group II) revealed negative immunoreaction for p53 in the liver cells (Supporting Information Figure S10(d)). In acrylamide-treated group (Group III), strong positive cytoplasmic and/or nuclear p53 immunoreactions were observed in many liver cells (Supporting Information Figure S10(e)), while in *D. spinulosum* extract and acrylamide-treated group (Group IV), moderate positive immunoreaction for p53 was detected only in some liver cells (Supporting Information Figure S10(f)).

PCNA immnunostaining: PCNA-immunostained liver sections of the control (Group I) and *D. spinulosum* extract-treated rats (Group II) revealed weak positive nuclear immunoreaction for PCNA in a few liver cells (Supporting Information Figure S10(g)). In acrylamide-treated group (Group III), strong positive nuclear PCNA immunoreaction was observed in many liver cells (Supporting Information Figure S10(h)), while in *D. spinulosum* extract and acrylamide-treated group (Group IV), moderate positive nuclear immunoreaction for PCNA was detected only in some liver cells (Supporting Information Figure S10(i)).

2.2.3. Morphometric and statistical results

The mean p53% (the percentage number of p53 positive liver cells) in the acrylamide-treated group (Group III) showed a significant increase (38.51 ± 1.15) compared to the control group (1.11 ± 1.22), while *D. spinulosum* extract and acrylamide-treated group (Group IV) showed a significant increase (9.62 ± 1.15) compared to control group and a highly significant decrease compared to Group III.

The mean PCNA labeling index showed a significant increase (68.44 ± 4.78) in the acrylamide-treated group (Group III) compared to the control group (9.55 ± 1.83). Moreover, *D. spinulosum* and acrylamide-treated group (Group IV) showed a

nonsignificant increase (11.11 ± 1.48) compared to the control group. (Supporting Information Table S3)

D. spinulosum was introduced to examine its probable role in alleviating the liver cell damage induced by acrylamide. Results from this study clearly demonstrated that *D. spinulosum* offered an evident improvement in the hepatocyte structural changes. In Conclusion: Concomitant treatment with *D. spinulosum* ameliorated the liver cell damage induced by acrylamide.

2.2.4. Cytotoxic activity of D. spinulosum methanol extract

Cytotoxic effect was carried out using MTT assay (Skehan et al. 1990). Breast cancer (MCF7), Liver cancer (HepG2), kidney cancer African green monkey (VERO), Colorectal carcinoma (HCT-116) and Normal cell (WI-48) lines are used. The results revealed that *D. spinulosum* methanol extract showed very strong activity against MCF7, HCT-116, VERO and HepG2 cell line with IC_{50} of 9.55 ± 0.8, 7.53 ± 0.9, 8.35 ± 0.7 and11.27 ± 1.0 μg/mL, respectively. The cytotoxic activity of *D. spinulosum* methanol extract (IC_{50} = 8.35 ± 0.7) was comparable to that of the positive control (IC_{50} = 9.01 ± 0.9) against VERO cell line. *D. spinulosum* methanol extract showed moderate cytotoxic activity against Normal cell line (WI-38) with IC_{50} of 26.73 ± 1.7 μg/mL which showed safer effect comparable to that of positive control (IC_{50} of 9.68 ± 0.3). Different fractions of *D. spinulosum* were tested against Kidney (VERO) and Colorectal carcinoma (HCT-116) cell lines. All the tested extract and different fractions of *D. spinulosum* showed different inhibition percentages against Colorectal carcinoma (HCT-116) and Kidney carcinoma (VERO) cell line. Inhibition percentage against Kidney carcinoma (VERO) was higher than that in Colorectal carcinoma cell line (HCT-116). *n*-Butanol fraction showed very strong cytotoxic activity with IC_{50} of 8.55 ± 0.9, 6.67 ± 0.7 μg/mL that exceeds 5-Fluorouracil (IC_{50} of 9.01 ± 0.9 and 7.19 ± 0.8) against both VERO and HCT-116 cell lines, respectively, according to Ayyad et al. (2012) classification. Ethyl acetate fraction presented strong cytotoxic activity with IC_{50} of 12.61 ± 1.4 and 11.86 ± 1.1 μg/mL against both VERO and HCT-116 cell lines, respectively. Methylene chloride fraction presented moderate cytotoxic activity with IC_{50} of 35.59 ± 1.9 and 44.10 ± 2.3 μg/mL against both VERO and HCT-116 cell lines, respectively (Supporting Information Table S4 and Figure S11). These results are in accordance to that reported by (Rejon et al. 2009), concerning the cytotoxic activity of petioles of *D. spinulosum* on Hep-2 (laryngeal carcinoma). Our results are in agreement with that reported by (Moawad et al. 2010; Negm et al. 2016) on other Cycadales plants.

2.2.5. Antioxidant activity

Antioxidant activity of *D. spinulosum* extract and different fractions are carried out using 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) ABTS method (Lissi et al. 1999). *D. spinulosum n*-butanol fraction and total methanol extract presented the highest antioxidant activity (87.8%, 84.2% inhibition) followed by ethyl acetate fraction (81.6% inhibition), that comparable to ascorbic acid which used as a positive control (89.3% inhibition; Supporting Information Figure S12).

The DNA protective activity of *D. spinulosum* extract and different fractions was carried out using (Aeschbach et al. 1994) method. The results are presented in

(Supporting Information Figure S13). The absorbance of *D. spinulosum* total methanol extract and *n*-butanol fraction was 0.058 and 0.073 for sample concentration of 0.1 mg/mL, respectively. It showed the highest protective activity against DNA damage which exceeded the positive control (absorbance = 0.075).

2.2.6. Antimicrobial activity

D. spinulosum n-butanol fraction showed high activity against Gram-negative bacteria, e.g. *Escherichia coli* and *Pseudomonas aeruginosa*, than Gram-positive bacteria, e.g. *Staphylococcus aureus* and *Bacillus subtilis*. *n*-Butanol fraction presented high antifungal activity against *Aspergillus flavus* more than *Candida albicans*. Ethyl acetate fraction possessed also high effect against *Candida albicans*, followed by methylene chloride fraction. Both fractions showed activity against *Aspergillus flavus*. Pet-ether fraction showed weak activity against all tested organisms. These results are in accordance with that reported by Moawad et al. 2010, concerning the antimicrobial activity of other Cycadals plants. The results are presented in (Supporting Information Table S5).

2.2.7. Biological activity of isolated pure compounds

According to Ayyad et al., aromadendrin and amentoflavone showed very strong cytotoxic activity (IC_{50}= 5.87 ± 0.6, 7.49 ± 0.8, respectively) exceeds 5-Fluorouracil (IC_{50}= 9.01 ± 0.9) against VERO cell lines, sotusflavone, bilobetin and isoginkgetin showed strong cytotoxic activity with IC_{50}= 10.20 ± 1.1, 14.17 ± 1.9 and 15.94 ± 1.9, respectively. While 7,4′,7″-trimethylamentoflavone, scaidopitysin, 7,4′,7″,4‴-tetramethylamentoflavone and oliveriflavoneexhibited moderate cytotoxic activity with IC_{50}= 26.04 ± 2.8, 26.87 ± 3.7, 46.49 ± 3.1, respectively. (Supporting Information Figure S14)

There is a reverse relationship between the number of methoxy groups in biflavonoids and cytotoxic activity. Increase methoxylation decrease activity and vice versa. Amentoflavone possess no methoxy group showed higher cytotoxic activity that exceeds 5-Fluorouracil while oliveriflavone possess five methoxy group presented the lowest cytotoxic activity. Flavanol as aromadendrin showed the highest activity in comparison with biflavonoid isolated compounds.

ABTS method was carried out to evaluate antioxidant activity. Aromadendrin possessed the highest antioxidant activity (89.6% inhibition) which was comparable to the positive control (89.3% inhibition) followed by amentoflavone and sotusflavone (79.1%, 64.3% inhibition, respectively) (Supporting Information Figure S15(a)).

The results of DNA protective activity are presented in (Supporting Information Figure S15(b)). Aromadendrin presented the highest protective activity against DNA damage followed by amentoflavone as the absorbance of aromadendrin and amentoflavone was 0.085 and 0.092, respectively at a concentration of 0.1 mg/mL comparable to that of ascorbic acid (positive control) (absorbance = 0.075). Isoginkgetin possessed protective activity (absorbance = 0.104) but lower than amentoflavone. Oliveriflavone has the lowest protective activity (absorbance = 0.173). There is also reverse relationship between methoxylation level of biflavonoids and antioxidant activity. Biflavonoids have diverse biological activities; include antibacterial, antifungal, anticancer, antiviral, anti-hepatotoxic, anti-allergic and immune suppressive activities (Saponara and Bosisio

1998). Our results are in accordance to that reported concerning the activity of biflavonoids.

3. Experimental

3.1. Plant material

Leaves of *Dioon spinulosum* Dyer Ex Eichler family Zamiaceae were collected from El Abd garden in Giza city in January 2017. It was kindly identified by Dr. Esraa Ammar plant Ecology lecturer, Botany Department, Faculty of Science, Tanta University and researcher Rabea Sharawy Agronomist and palm researcher. A voucher sample No. PGG-002 was deposited at the Herbarium of Pharmacognosy Department, Faculty of Pharmacy, Tanta University.

3.2. Extraction and isolation (Supporting Information)

3.3. Spectroscopic data

Compound **15** (apigenin 7-*O*-α-D-glucopyranoside)Yellowish white powder; $[\alpha]^{25.4}_{D}$ = + 19.98° (c = 0.5, CH_3OH); UV λ_{max} (MeOH) 221, 267 and 329. IR (KBr) 3858, 3423, 2958, 2854, 1726, 1622, 1450, 1282, 1165, 1113, 1076, 877, 785, 468, 323. Positive HR-ESI-MS (*m/z*) 433.335: $[M + H]^+$ with a molecular formula $C_{21}H_{20}O_{10}$

^{1}H NMR [DMSO-d_6, 500 MHz] δ: 6.86 (1H, s, H-3), 6.43 (1H, d, *J* = 2.5 Hz, H-6), 6.82 (1H, d, *J* = 2.5 Hz, H-8), 7.95 (2H, d, *J* = 9.0 Hz, H-2′, 6′), 6.92 (2H, d, *J* = 9.0 Hz, H-3′, 5′), 5.40 (1H, d, *J* = 4.0 Hz, Glc H-1′′), 3.87 (1H, d, *J* = 10.0 Hz, Glc H-2′′), 3.16-3.74 (5H, m, H-3′′, 4′′, 5′′, 6′′), 5.15 (2′′ OH), 5.07 (3′′ OH), 5.05 (4′′ OH), 4.6 (6′′ OH). ^{13}C NMR [DMSO-d_6, 125 MHz] δ: 162.9 (C-2), 103.1 (C-3), 182.0 (C-4), 156.9 (C-5), 99.6 (C-6), 164.2 (C-7), 94.8 (C-8), 161.4 (C-9), 105.3 (C-10), 121.0 (C-1′), 128.6 (C-2′), 116.0 (C-3′), 161.1 (C-4′), 116.0 (C-5′), 128.6 (C-6′). Glucose moiety: 99.5 (C-1′′), 73.1 (C-2′′), 77.1 (C-3′′), 69.5 (C-4′′), 76.4 (C-5′′), 60.5 (C-6′′).

Compound 16 (amentoflavone- 4′-O-α-L-rhamnopyranoside)

Yellow amorphous powder; $[\alpha]^{25.4}_{D}$ = −19.98° (c = 0.5, CH_3OH); UV λ_{max} (MeOH) 333, 282 and 221 nm. IR (KBr) 3928, 3782, 3420, 2926, 2860, 2305, 1723, 1650, 1609, 1498, 1429, 1362, 1285, 1245, 1172, 1106, 948, 836, 744, 637, 560, 513. Positive HR-ESI-MS (*m/z*) 684.578: $[M + H]^+$ with a molecular formula $C_{36}H_{28}O_{14}$.

^{1}H NMR [CD_3OD, 500 MHz] δ: 6.71 (1H, s H-3), 6.18 (1H, d, *J* = 1.5 Hz, H-6), 6.41 (1H, d, *J* = 1.5 Hz, H-8), 7.95 (1H, d, *J* = 2.5 Hz, H-2′), 7.14 (1H, d, *J* = 9.5 Hz, H-5′), 7.93 (1H, dd, *J* = 9.5, 2.5 Hz, H-6′), 6.73 (1H, s, H-3′′), 6.38 (1H, s, H-6′′) , 7.56 (2H, d, *J* = 9.5 Hz, H-2′′′, 6′′′), 6.62 (2H, d, *J* = 9.5 Hz, H-3′′′, 5′′′), 4.60 (1H, s, Rha H-1), 3.3-3.65 (m, Rha H-2–Rha H-5), 1.20 (3H, d, *J* = 6.0 Hz, Rha C-5-CH_3).^{13}C NMR [CD_3OD, 125 MHz] δ: 166.0 (C-2), 103.4 (C-3), 184.3 (C-4), 163.6 (C-5), 95.9 (C-6), 166.1 (C-7), 95.1 (C-8), 159.4 (C-9), 103.9 (C-10), 123.2 (C-1′), 128.9 (C-2′), 123.2 (C-3′), 161.4 (C-4′), 117.5 (C-5′), 132.8 (C-6′), 166.0 (C-2′′), 103.3 (C-3′′), 183.9 (C-4′′), 162.2 (C-5′′), 99.9 (C-6′′), 162.5 (C-7′′), 105.5 (C-8′′), 154.9 (C-9′′), 104.0 (C-10′′), 123.1 (C-1′′′), 129.3 (C-2′′′), 116.8 (C-3′′′), 163.6 (C-4′′′), 116.8 (C-5′′′), 129.3 (C-6′′′), Rhamnose moiety: 100.1 (Rha C-1), 69.5 (Rha C-2), 70.1 (Rha C-3), 73.9 (Rha C-4), 69.5 (Rha C-5), 17.3 (Rha C-6).

Spectroscopic data of known compounds (Supporting Information).

3.4. Acid hydrolysis of the isolated glycosides (Trendafilova et al. 2011) (Supporting Information)

3.5. Biological activity

For *in vivo* hepatoprotective effect of extract (Hall et al. 1990; Bancroft and Gamble 2008; Gaertner et al. 2008; Saleh et al. 2017; Bera et al. 2018), cytotoxic, antioxidant, antimicrobial and biological activities of isolated compounds (See Supporting Information).

3.6. Statistical analysis

This analysis was carried out by Graph Pad Prism 5 Program. All measurements were performed in triplicate ($n = 3$), shown as mean ± standard deviation (*SD*) and significant difference at $p \leq .05$ by Student's test.

4. Conclusions

D. spinulosum showed promising hepatoprotective activity. *D. spinulosum* displayed potent activity against MCF-7, HepG2, VERO and HCT-116 cells. Secondary metabolites isolated from *D. spinulosum* showed the highest protective activity against DNA damage, higher than the positive control. Two new flavonoids apigenin 7-*O*-α-D-glucopyranoside (15) and amentoflavone 4′-*O*-α-L-rhamnopyranoside (16) were isolated from *D. spinulosum* leaves, in addition to fifteen known compounds. A promising future awaits *D. spinulosum* in drug discovery.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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