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Chemical Profiling of *Acacia auriculiformis* (A. Cunn.) ex Benth. Leaves Extract and its Impact on Gene Expression and Apoptotic DNA Damage in CCl_4-Induced Hepatotoxicity in Male Rats

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ABSTRACT

Acacia auriculiformis (A. Cunn.) ex Benth. is an ornamental evergreen tree with diverse medicinal importance. It was subjected to different chromatographic techniques. Eighteen known phenolics were identified from the leaves using different spectroscopic methods. Among them, twelve compounds were isolated and characterized as: three phenolic acids, one flavone, four flavonols, three isoflavones, and one flavanone. From the LC-ESI-MS profiling technique additional six flavonoids were tentatively identified. HPLC-UV chromatographic quantitation for quercetin 3-*O*-glucoside, eriodicotyl 7-*O*-glucoside, genistein and biochanin A was determined as 3.475, 2.406, 6.658 and 5.997 mg/g defatted extract, respectively. The extract genoprotective effect was assessed in 80 adult male rats (10 rats/group) against CCl_4-induced genotoxicity in liver tissues (1 mg/kg bw, twice per week), in three doses (35, 70 and 140 mg/kg bw/d, four weeks), using real-time PCR, comet assay, DNA fragmentation and flow cytometry apoptosis assay. In treated rats, the plant extract significantly down-regulated the hepatic mRNA expression levels of cytochrome P450 (CYP 450) and heat shock protein 70 (Hsp 70) genes and attenuated DNA damage rates to 19.4, 16.2 and 13.8% for the three doses, respectively compared to that of the untreated (23.8%). It also reduced apoptotic DNA fragmentation and apoptotic rates by 26.2, 19.4 and 15.2%, respectively compared to CCl_4 treated rats (37.1%). These findings indicated the potential effect of *A. auriculiformis* leaves to retain DNA structural integrity and its genomic expression against common industrial pollutant such as CCl_4 in a dose-dependent manner.

***Keywords*:** *Acacia*, LC-ESI-MS, Genotoxicity, Hsp70, CYP450.

Introduction

Acacia is a cosmopolitan genus comprising of more than 1350 species and is widely spread in almost all habitat types.[1,2] Although it is the second largest genus of the family Fabaceae, little is known about the chemical profile of most of the species.[3] *A. auriculiformis* is a vigorously growing tree native to Australia and was introduced to India.[4] It has been used in folk medicine to treat aches, skin diseases, rashes, sore eyes and rheumatism.[5] Several pharmacological effects have been accredited to *A. auriculiformis* including antimalarial and antioxidant activities of the heartwood and leaves, respectively, in addition to the hepatoprotective, antimutagenic and chemopreventive activities of the empty pods and bark.[4]

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Previous phytochemical studies on *A. auriculiformis* reported the presence of a unique tridesmoside saponins proacaciaside-I, proacaciaside-II and acaciamine from its pods.[3] From the funicles two acylated biglycoside saponins (acaciaside A and B) were isolated, they possess antimicrobial activity.[6] A Central nervous system (CNS) depressant flavan glycoside (auriculoside) was isolated from the heartwood.[7] The reported DNA protection effects of the heartwood and empty pods extracts and the previous pharmacological properties were inspiring to investigate the genoprotective effect of *A. auriculiformis* against DNA damage in carbon tetrachloride (CCl_4)-induced hepatic injury in rats.[3] Despite the well-recognized hepatic toxicological problems induced by CCL_4, its industrial uses for the production of refrigerants, propellants, and as a solvent for varnishes, lacquers, and oils have not been discontinued. However, there is great scientific advancement in the understanding of liver pathogenesis with limited hepatoprotective interventions. Because of this, herbal supplements represent the best global solution. The chemical profile on the leaves extract of *A. auriculiformis* and its DNA protective effects have not been previously investigated. The continuous exposure to environmental factors leads to genomic DNA damage, which results in a set of signalling pathways known as the DNA damage response. Aberrations of this response results in genetic expression alteration, which is a common feature in human cancer cells. Heat shock protein 70 (Hsp70) and cytochrome P450 (CYP450) genes are commonly overexpressed after DNA damage.[8] Therefore,

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this study is aimed at carrying out a phytochemical investigation on *A. auriculiformis* leaves extract using chromatographic techniques, along with evaluating its efficacy against CCl_4-induced genotoxicity in hepatic tissues. Assessment of its impact on gene expression of heat shock protein 70 (Hsp70) and cytochrome P450 (CYP450), DNA damage and apoptosis rates.

Materials and Methods

Plant material

A. auriculiformis was collected from Orman Botanic Garden, Giza, in September 2016 and identified by Dr. M. Gibali, a senior botanist and consultant at Orman Botanic Garden, Giza, Egypt). A voucher specimen (M124) was deposited in CAIRC (National Research Centre herbarium, Egypt).

General experimental procedure

The ultraviolet spectra of the isolated compounds were recorded on Shimadzu UV 240 (P/N 204-58000, Japan). Nuclear magnetic resonance (NMR) measurements were carried out using Jeol EX-500 spectrometer (Japan): 500 MHz (^{1}H-NMR) and 125 MHz (^{13}C-NMR) and Bruker Avance III spectrometer: 400 MHz (^{1}H-NMR) and 100 MHz (^{13}C-NMR). Solvents used for plant extraction were from SDFCL (industrial Estate, 248 Worli Road, Mumbai-30, India). For preparation of petroleum ether extract, a soxhlet apparatus was used. Column chromatography (CC) for isolation of phenolics was carried out on polyamide 6S (Riedel-De-Haen, AG, Sleeze Hanver, Germany) eluted with 100% H_2O and followed by H_2O/MeOH mixture with decreased polarity. Paper chromatography (PC) descending Whatman No.1 and 3 MM papers, using solvent systems: (1) H_2O, (2) 15% HOAc (water:acetic acid 85:15, v/v), (3) 50% HOAc, (4) BAW (butanol:acetic acid:water, 4:1:5, v/v, upper layer) and (5) BBWP (benzene:butanol:water:pyridine 1:5:3:3, v/v, upper layer). Solvents 4 and 5 were used for identification of the sugar. Sephadex LH-20 (Pharmazia, Sweden) was used for purification. Authentic aglycone samples of apigenin, luteolin, vitexin, quercetin, kaempferol, genistein and isorhamnetin, and sugars as glucose, rhamnose, galactose, arabinose and xylose were obtained from the Department of Phytochemistry and Plant Systematics, NRC, Giza, Egypt. Complete acid hydrolysis for the defatted extract and *O*-glycosides (2N HCl, 2 h, 100°C) was performed and followed by identification of the aglycones and sugar moieties by using paper co-chromatography with authentic samples.

Extraction and isolation of phenolics from DAAE

The air-dried powdered leaves of *A. auriculiformis* (2 kg) were extracted with 70% MeOH infusion (3 × 4L) and evaporated under reduced pressure at 60°C to yield 248 g dried *A. auriculiformis* extract (AAE). It was then defatted with petroleum ether (3 × 1 L) to afford the petroleum ether extract (32 g). The defatted AAE residue (DAAE) (125 g) was chromatographed on Polyamide column chromatography eluted with H_2O and MeOH using gradient elution. Fractions were collected and monitored by PC to give ten main fractions (A-J). CC of fraction B (12 g) on Sephadex LH-20 column using H_2O: MeOH (1:1) followed by elution with MeOH afforded compound 1 (20 mg). Further purification by CC of fraction C (4.7 g) on Sephadex LH-20 column using H_2O: MeOH (1:1) gave two major subfractions: C1 (1.4 g) and C2 (1.8 g), each was subjected to CC on Sephadex LH-20 column with MeOH used as an eluent to isolate compounds: 2 (12 mg) and 3 (10 mg). CC of Fraction D (3.8 g) on Sephadex LH-20 column, gradient elution with H_2O/MeOH gave three major subfractions: D1 (0.8 g), D2 (1.1 g) and D3 (0.6 g), each was chromatographed on Sephadex LH-20 column using MeOH as an eluent and afforded compounds: 4 (5 mg), 5 (11 mg) and 6 (5 mg). CC of fraction F (4.5 g) on polyamide column using gradient elution with H_2O/MeOH gave three major subfractions F1 (1.2 g), F2 (1.7 g) and F3 (1.1 g), each was subjected to CC on Sephadex LH-20 column using MeOH as an eluent and afforded compounds: 7 (10 mg), 8 (20 mg) and 9 (7 mg). Fraction G (2.1 g) was chromatographed on Sephadex LH-20 column using H_2O: MeOH (1:1, v/v) for elution yielding one major subfraction G1(1.3 g) then subjected to preparative paper chromatography (PPC) using butanol-acetic acid-water (BAW) and followed by purification on Sephadex LH-20 column using methanol to afford compound 10 (2 mg). CC of fraction H (2.3 g) on Sephadex LH-20 column using gradient elution with H_2O/MeOH, gave two major sub fractions H1 (0.8 g) and H2 (1 g). Repeated CC for each on Sephadex LH-20 using MeOH: H_2O (1:1, v/v), followed by PPC using BAW afforded compounds **11** (12 mg) and **12** (10 mg).

Characterization of isolated compounds

Twelve known phenolic compounds were isolated (**1-12**). They were subjected to structural elucidation through colour reaction, R_f values, chemical investigation (acid hydrolysis and $FeCl_3$ degradation) and physical examination (UV, ^{1}H-NMR, ^{13}C-NMR and MS).[9-11] Their data were compared to the available literature (the data of compounds 1-12 were recorded in the supplementary material, S3 and S4).

LC-ESI-MS analysis of DAAE

LC-ESI-MS analysis was carried out according to the parameters and method previously described by Elkhateeb *et al*.[12] The phenolic compounds isolated in the present study together with other pure flavonoids: kaempferol 7-*O*-glucoside, kaempferol 3-*O*-rutinoside and kaempferitrin obtained from the Phytochemical and Plant Systematic Department, NRC, Giza, Egypt were used as authentic. Tentative identification of the known peaks were carried out by comparing their retention times and mass spectra with those compounds. The mass fragmentation patterns of the unknown peaks were compared with the available literatures.

Quantitative analysis of major phenolic compounds of DAAE

Quantitative HPLC analyses were performed on liquid chromatograph (Agilent 1260 infinity), supplied with a quaternary pump (VL 1260, Waldbronn-Germany) with operating pressures of up to 400 bar, an Ultraviolet multiple wavelength detector model G7165 A, 1260 MWD, a rheodyne injector model 7225/7225I and 20 µL injector (Rohnert Park, CA, USA). The analyses were carried out on a Zorbax Eclipse XDB column C18 (150 x 4.6 mm, 5 µm), using gradient elution. The mobile phase comprised of water (A) and acetonitrile (B) as follows: at 0 minutes 95% A; at 5 minutes 75% A; at 15 minutes 60% A; at 20 minutes 40% A; at 25 minutes 20% A; at 30 minutes 5% and 35 minutes 100% B at 1 mL/min flow rate and column temperature was kept at 30°C. The detection wavelength was adjusted at 270 nm. Compounds quercetin 3-*O*-glucoside (5), eriodicotyl 7-*O*-glucoside (8), genistein (11) and biochanin A (12) were used as standards. Standard stock solutions containing 1 mg/mL of these compounds were prepared by dilution with methanol. The concentrations of the standard solutions were then adjusted to be 15.5, 31.25, 62.5, 125, 250, and 500 µg/mL. The defatted hydroalcoholic extract of *A. auriculiformis* was prepared in methanol at a concentration of 10 mg/mL. All samples and the mobile phase were subjected to filtration through a 0.45 µm PTFE filter membrane with 10 µL injection volume. For confirmation of peak identity, comparison of the peaks retention times was performed as revealed from the DAAE with that of isolated compounds used as standards. All samples were analyzed by triplicate injections. The calibration curves were obtained in the range of 15.5-62.5 (µg/mL) for the four flavonoids. The regression equations calculated for each standard are shown in Table 1.

Table 1: Regression equations of the four flavonoids

Compounds	Regression equation	r^2
Quercetin 3-*O*-glucoside	y = 2.6404x - 36.551	0.9927
Eriodicotyl 7-*O*-glucoside	y = 0.9273x + 0.5636	0.9938
Genistein	y = 1.9272x - 13.69	0.986
Biochanin A	y = 1.9283x - 25.42	0.9858

y: mAU: mean area under the curve, x: concentration µg/ml.

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Experimental animals

Eighty adult male albino rats (100-120 g), obtained from the Animal House Colony, National Research Centre, Giza, Egypt were maintained on standard laboratory diet (protein, 16.04%; fat, 3.63%; fiber, 4.1%; and metabolic energy, 0.012 MJ) and water *ad libitum*. After an adaptation period for 1 week, rats were then divided into eight groups (10 rats/group) and kept in a filter-top polycarbonate cages at temperature-controlled (23 ± 1°C) and artificially illuminated (12 h dark/light cycle) in chemical contamination free room. All animals received humane care according to Animal Care and Use Committee guidelines of the National Research Centre, Egypt. The recommendations of Health Guide for care and use of laboratory animals with ethical approval No. (MP1899) were strictly followed which were in accordance with the guidelines of Institutional Animal Care and Use Committee, Cairo University, Egypt.

Experimental design

The animals were classified for 4-weeks period treatments as the following: Group 1, control group: was treated orally with saline solution (0.2 mL/day); Groups (2-4) were treated orally with 35, 70 and 140 mg/kg body weight (bw)/day of the defatted *A. auriculiformis* hydroalcoholic extract (DAAE35, DAAE70 and DAAE140) which were equivalent to 0.1, 0.2 and 0.4 of its LD_{50}, respectively.[13] Group (5) was treated with CCl_4 (1 mg/kg bw twice per week); Groups (6-8) were treated with DAAE (similar to those in groups 2-4) combined with CCl_4 (1 mg/kg bw twice per week).

Tissue samples preparation

At the twenty-fourth hour after the last injection, rats in each group were anesthetized and sacrificed by decapitation. The tissue samples were collected on an ice bath and were separated to investigate the apoptosis, mRNA (for determination Hsp70 and CYP450 mRNA), DNA (for determination DNA fragmentation) and comet assay.[14]

Isolation of total RNA and cDNA synthesis

TRIzol® Reagent (Invitrogen, Germany) was used for extraction of total RNA from liver tissues of male rats following previously reported method.[14] The isolated RNA was then subjected to reverse transcription (RT) reaction to synthesize a complementary DNA (cDNA) as previously described.[15]

Real-Time polymerase chain reaction (Rt-PCR)

A 25 µL reaction mixture were applied as PCR for determining the liver tissue cDNA copy number. It was programmed in 3 steps with distilled water as control as described by Linjawi *et al*. (2017).[14] To check the used primers quality, a melting curve analysis was accomplished at 95°C at the end of each qRT-PCR. The sequences of specific primers of the genes used are listed in Table 2.

Table 2: Primers sequences used for RT-PCR

Gene	Primer sequence (5'–3')[a]	Annealing temp. (°C)
Hsp70a	F: CGG GAG TTG TAG CGA TGA GA R: CTT CCT AAA TAG CAC TGA GCCATAA	60
CYP450	F: ATC AAG CAA GGG GAC GAG TT R: GCT CGC TGA CAA TCT TTT GC	57
β-Actin	F: TGG GGC AGT ATG GCT TGT ATG R: CTC TGG CAC CCT AAT CAC CTC T	55

The calculation of gene expression was by using $2^{-\Delta\Delta CT}$ method, it was applied to ascertain the relative quantification of the target to the reference.[16]

Comet assay

The isolated liver tissues of all rat groups were subjected to a modified single-cell gel electrophoresis or comet assay.[17] Processing of the tissues were performed as described by Lu *et al*. (2002).[18] About 100 cells from each animal were examined as shown in Table 3.

DNA fragmentation assessment

The laddering pattern of nuclear DNA was used to qualitatively analyze the apoptotic DNA fragmentation, according to conditions and methods described by Lu *et al*. (2002).[18]

Apoptosis assay

The cortex of the hepatic tissue (100 mg per sample) was made into single-cell suspensions.[19] Flow cytometry (FCM) assay was used to estimate the cells apoptosis by using Annexin V/PI apoptosis detection kit as stated in the method previously described.[14]

Statistical analysis

General Liner Models (GLM) procedure followed by Scheffé-test was applied to compare significant differences between groups. The results were expressed as mean ± SEM of the mean. SAS software version 9.1 (Statsoft Inc., Tulsa, USA). All statements of significant were based on probability of $P < 0.05$.

Table 3: Rate of DNA damage in liver tissues of male rats exposed to CCl_4 and/or DAAE using comet assay

Treatment	Number of animals	No. of cells Analyzed (*)	Total comets	Class[¥] of comet 0	1	2	3	DNA damaged cells (%)
Control	5	500	28	472	23	5	0	5.6
DAAE35	5	500	27	473	21	6	0	5.4
DAAE70	5	500	29	471	22	7	0	5.8
DAAE140	5	500	31	469	21	8	2	6.2
CCl_4	5	500	119	381	34	37	48	23.8
CCl_4+DAAE35	5	500	97	403	28	33	36	19.4
CCl_4+DAAE70	5	500	81	419	28	27	26	16.2
CCl_4+DAAE140	5	500	69	431	22	24	23	13.8

[¥]: Class 0 = no tail; 1 = tail length < diameter of nucleus; 2 = tail length between 1X and 2X the diameter of nucleus; and 3= tail length > 2X the diameter of nucleus. (*): No of cells analysed were 100 per an animal.

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Results and Discussion

Isolation and characterization of compounds from DAAE

Chromatographic investigation of the defatted aqueous methanol extract of *A. auriculiformis* leaves led to the isolation of 12 phenolic compounds for the first time. The aglycones of DAAE were identified as apigenin, genistein, quercetin, kaempferol, and isorhamnetin by complete acid hydrolysis as well as the sugar moieties were identified as glucose, rhamnose, and arabinose. The structural elucidation of the isolated compounds was carried out through spectroscopic, chemical and physical examinations. They were characterized as phenolic acids: gallic acid (**1**),[20] 3,4,5 trihydroxycinnamic acid (**2**)[21] and caffeic acid (**3**),[22] flavone-*C*-glycoside: vitexin 2"-*O*-rhamnoside (**4**),[23] flavonol glycosides: quercetin 3-*O*-glucoside (**5**),[24] kaempferol 7-*O*-arabinoside (**6**),[25] and kaempferol 7-*O*-neohesperidoside (**9**),[26] isoflavone glucoside: biochanin A 7-*O*-glucoside (**7**),[27] flavanone glucoside: eriodicotyl 7-*O*-glucoside (**8**),[28] flavonol aglycone: kaempferol (**10**),[26] and isoflavones aglycones: genistein (**11**) and biochanin A (**12**) (Figure 1).[29,30] Their spectroscopic data (supplementary material, Tables S1-S3) were in accordance with the previously published ones from the available literature.

HPLC quantitative analysis of isolated phenolic compounds from DAAE

The isolated major compounds in the DAAE: quercetin 3-*O*-glucoside (**5**), eriodicotyl 7-*O*-glucoside (**8**), genistein (**11**) and biochanin A (**12**) were subjected to simple and rapid HPLC quantification. A good chromatographic separation was observed, with good linearity obtained for all standards (Table 1). The HPLC chromatograms of the standard mixture of the four compounds and in the extracted sample of *A. auriculiformis* were shown in Figures 2 and 3. The concentrations from the standard calibration curve were found to be 3.475, 2.406, 6.658 and 5.997 mg/g defatted extract, for quercetin 3-*O*-glucoside (**5**), eriodicotyl 7-*O*-glucoside (**8**), genistein (**11**) and biochanin A (**12**), respectively.

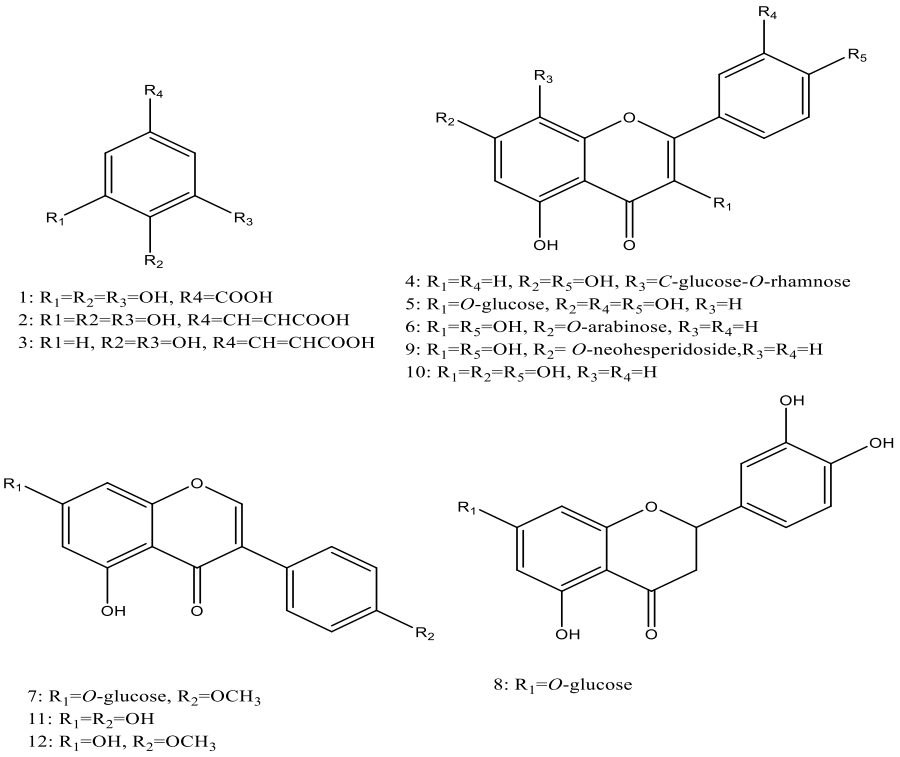

Figure 1: Compounds isolated from *A. auriculiformis* leaves

LC-ESI-MS profiling of DAAE

The LC-ESI-MS chromatogram (Figure 4) revealed a chemical profile of 23 peaks for DAAE, from which 18 phenolic compounds were ascertained by correlating their fragmentation patterns and retention times with standards and by comparison with the available literature data. The identity, retention time, observed deprotonated molecular ion and fragment ions for each compound were presented in Table 4. The chromatographic peaks were found to be eluted in a decreased polarity order, whereby phenolic acids were eluted first, followed by flavonoid glycoside and finally the free aglycones. The twelve isolated phenolics were recognized in the LC-ESI-MS chromatogram as peaks 1, 2, 3, 5, 8, 9, 13, 14, 15, 17, 18 and 19 (Figure 4 and Table 4). The Other additional six peaks were also observed and represented as peaks 6, 7, 10, 11, 12 and 16. They were tentatively identified as flavonols of kaempferol, quercetin and isorhamnetin derivatives. The molecular ion peak *m/z* 285 for a kaempferol moiety was detected in peaks 6, 7, 11, and 16. Isorhamnetin and quercetin were detected in peak 10 and 12, respectively. Peak 6 showed a deprotonated molecular ion peak at *m/z* 739 $[M-H]^-$. The fragment ion peak at *m/z* 593 $[M-H-146]^-$ is due to the loss of rhamnose moiety. For the same peak the fragment ion at *m/z* 575 $[M-H-164]^-$ indicates that rhamnose is terminal with the presence of interglycosidic linkage. In the same vain, the presence of fragment ion at *m/z* 473 $[M-H-146-120]^-$ indicated the presence of another rhamnose with interglycosidic linkage at position 6 of the hexose, this fragment ion is due to hexose internal cleavage in positions O, 2 with the presence of rhamnose at position 6. Therefore, the two rhamnoses are linked to glucose at positions 2 and 6. Together with the deprotonated ion peak of kaempferol at *m/z* 285, it was thus tentatively identified as kaempferol 3-*O*-(2-rhamnosyl) rutinoside.[31]

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Peak 7 with a deprotonated molecular ion peak at *m/z* 593 $[M-H]^-$, yielding only the aglycone ion fragment of kaempferol at *m/z* 285, indicating the loss of rhamnosylhexoside moiety $[M-H-146-162]^-$, with the presence of (1→6) interglycosidic linkage. Peak 7 was tentatively identified as kaempferol 3-*O*-rutinoside.[30] Peak 10 showed a molecular ion peak at *m/z* 755 $[M-H]^-$, the fragment ion of isorhamnetin aglycone at *m/z* 316 is due to the loss of a glucose, a rhamnose and a pentose units attached to the same position of the aglycone $[M-H-162-146-132]^-$. Therefore, it was tentatively identified as isorhamnetin 3-*O*-glucosyl-rhamnosyl-pentoside.[32] Peak 11, tentatively identified as kaempferitrin, showed a deprotonated molecular ion peak at *m/z* 577 and a fragment ion of kaempferol aglycone at *m/z* 285 due to the loss of 2 rhamnose units $[M-H-146x2]^-$, without the loss of 164 amu (146+18) indicating the absence of the interglycosidic linkage revealing that each rhamnose moiety is directly attached to the aglycone hydroxyl group.[33] Peak 12 showed the same fragmentation pattern as that of peak 6, the molecular ion peak at *m/z* 755 $[M-H]^-$ and the deprotonated ion of quercetin aglycone at *m/z* 300 suggested peak 12 to be quercetin 3-*O*-dirhamnosylhexoside and was thus tentatively identified as quercetin 3-*O*-(2-rhamnosyl)rutinoside.[32] Peak 16 showed a molecular ion peak at *m/z* 447 $[M-H]^-$. The deprotonated ion of kaempferol aglycone at *m/z* 285 is due to the loss of glucose $[M-H-162]^-$. It was tentatively identified as Kaempferol-*O*-glucoside.[34]

The LC mass fingerprinting of the leaves extract, in addition to the HPLC quantitative estimation of the major bioactive compounds (quercetin 3-*O*-glucoside, eriodicotyl 7-*O*-glucoside, genistein and biochanin A) encourage us to assess its impact on gene expression modulation and DNA protection of hepatic tissues in CCl_4 treated male rats.

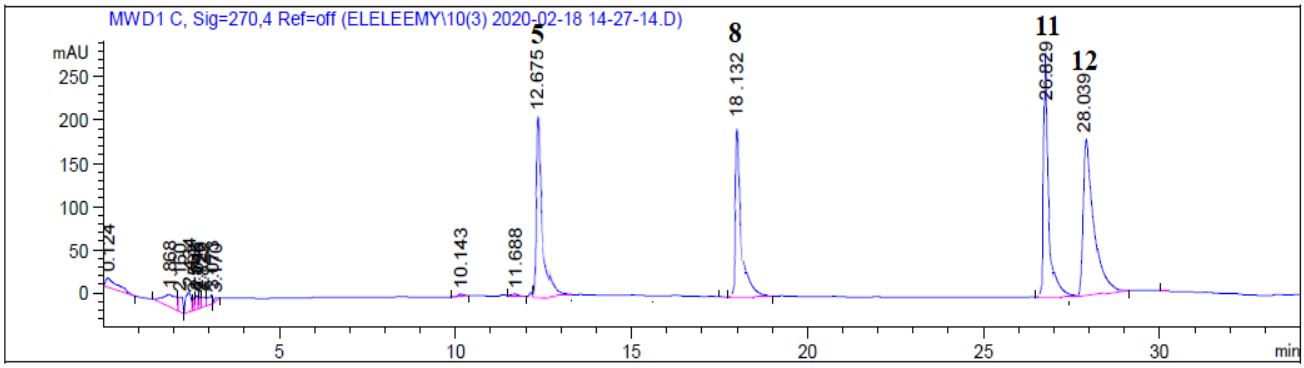

Figure 2: Chromatographic separation of a standard mixture of quercetin 3-*O*-glucoside (**5**), eriodicotyl 7-*O*-glucoside (**8**), genistein (**11**) and biochanin A (**12**)

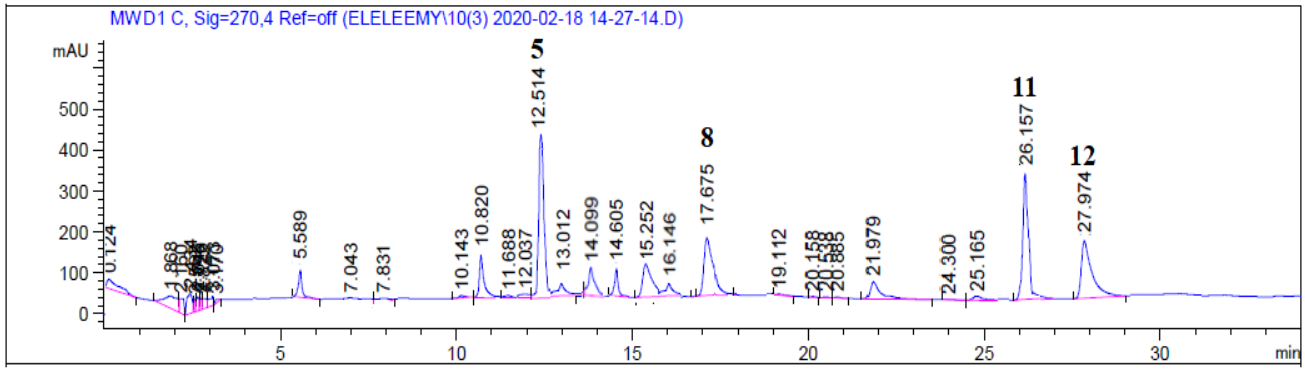

Figure 3: HPLC chromatogram of the defatted *A. auriculiformis* extract

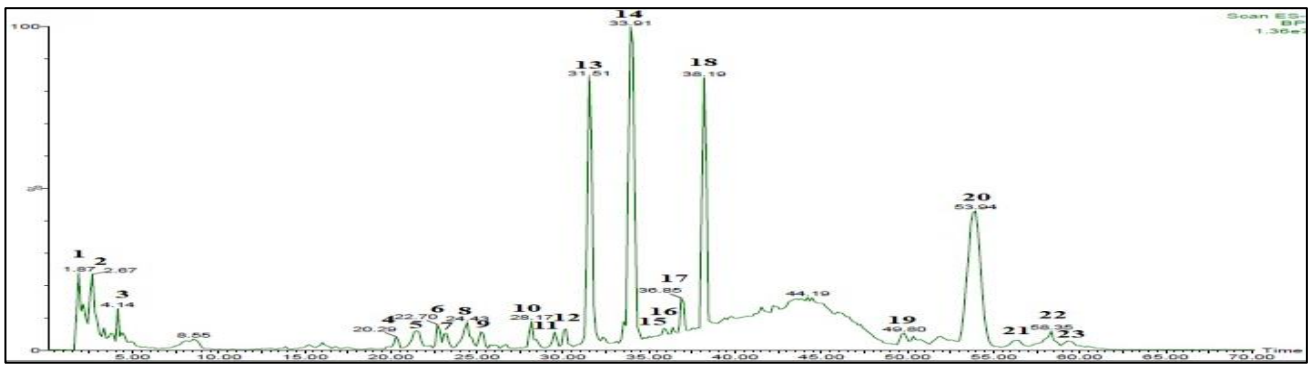

Figure 4: LC-ESI-MS chromatogram of *A. auriculiformis* leaves extract

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Table 4: Tentative identification of phenolic compounds in *A. auriculiformis* leaves

Peak no.	R_t (min.)	M	$[M-H]^-$	*m/z* fragments	Tentative identification
1	1.87	170	169	125, 79	Gallic acid*
2	2.67	180	179	135	Caffeic acid*
3	4.14	196	195	151	3,4,5-trihydroxycinnamic acid*
4	20.29	444	443	303, 179, 101	Unknown
5	21.49	594	593	447, 285, 284	Kaempferol 7-*O*-neohesperidoside*
6	22.69	740	739	593, 575, 473, 285	Kaempferol 3-*O*-(2-rhamnosyl)rutinoside[#]
7	23.23	594	593	285	Kaempferol 3-*O*-rutinoside[#]
8	24.43	418	417	285	Kaempferol 7-*O*-arabinoside*
9	25.23	578	577	457, 413, 311	Vitexin 2"-*O*-rhamnoside*
10	28.17	756	755	623, 477, 316	Isorhamnetin 3-*O*-glucosyl-rhamnosyl-pentoside[#]
11	29.50	578	577	431, 413, 285	Kaempferitrin[#]
12	30.17	756	755	609, 591, 489, 300	Quercetin 3-*O*-(2-rhamnosyl)rutinoside[#]
13	31.64	446	445	283	Biochanin A 7-*O*-glucoside*
14	33.91	464	463	300	Quercetin 3-*O*-glucoside*
15	36.12	450	449	287	Eriodicotyl 7-*O*-glucoside*
16	36.52	448	447	285	Kaempferol-*O*-glucoside[#]
17	36.85	286	285	151, 96, 62	Kaempferol*
18	38.18	270	269	241, 151, 123	Genistein*
19	49.8	284	283	269, 255, 151	Biochanin A*
20	53.94	652	651	489, 179	Unknown
21	56.31	542	541	471, 241	Unknown
22	58.35	472	471	411, 329	Unknown
23	59.21	958	957	825, 651	Unknown

*Compounds isolated in the present study. [#] Compounds detected for the first time in the plant by LC-ESI-MS technique.

Effect of DAAE on CYP450 and Hsp70 gene expressions

The effect of DAAE on the expression of cytochrome P450 (CYP450) and heat shock protein 70 (Hsp70) genes against CCl_4-induced genotoxicity in the liver tissues of male rats is summarized in Figure 5. Their expression was quantified by RT-PCR. In DAAE treated groups (DAAE35, DAAE70 and DAAE140) expression of Hsp70 and CYP450 genes exhibited relatively similar expression values in comparison to the control group (100%). However, CCl_4 treatment increased significantly the Hsp70 and CYP450 mRNA expression values by 337.9 and 434.1%, respectively compared with control group. In contrast, comparing with CCl_4 treatment alone, there was a significant decrease in the expression values of Hsp70 genes in rats treated with DAAE30, DAAE70 and DAAE 140 combined with CCl_4 by 305.2, 194.8 and 165.5%, respectively. The expression values of CYP450 genes were significantly decreased in DAAE30, DAAE70 and DAAE 140 treated groups combined with CCl_4 by 336, 214.6 and 180.5%, respectively. Moreover, the lowest expression of Hsp70 and CYP450 genes was observed in the treatment with DAAE140 combined with CCl_4 (Figure 5).

Protective effect of DAAE against DNA damage

The protective effect of DAAE against CCl_4-induced DNA damage was assessed by using comet assay. As evident from Table 3, the DNA damage corresponds to DNA extracted from different groups treated with CCl_4 and/or DAAE35, DAAE70 and DAAE140, respectively. In supplemented animal groups with DAAE35, DAAE70 and DAAE140, the DNA damage rates (5.4%, 5.8% and 6.2%, respectively) were relatively similar to that of the control group. There was a significant increase ($P < 0.01$) in DNA damage in those treated with CCl_4 (23.8%) compared to the control group (5.6%). In contrast, the damage was significantly attenuated ($P < 0.05$, $P < 0.05$ and $P < 0.01$, respectively) in groups treated with DAAE35, DAAE70 and DAAE140 combined with CCl_4 (19.4%, 16.2% and 13.8%, respectively). Moreover, the highest protective effect for DNA damage induced by CCl_4 was observed for supplemented rats treated with high dose of DAAE.

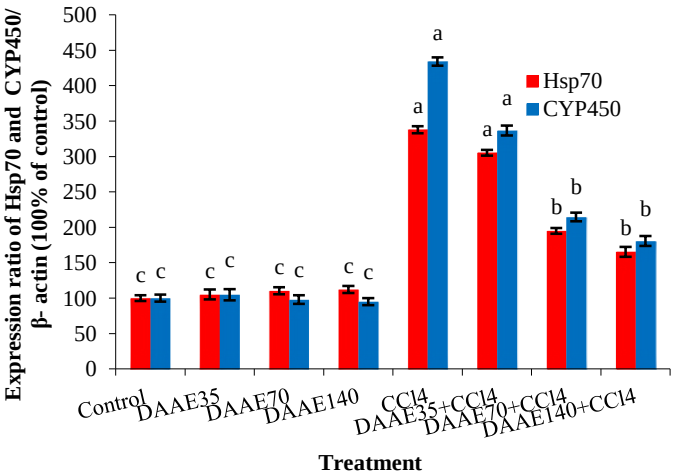

Figure 5: Relative expression of Hsp70 and CYP450 mRNAs in liver tissues of male rats with CCl_4 alone or combined with DAAE. Data are presented as mean ± SEM. [a,b,c]Mean values within tissue with unlike superscript letters were significantly different ([a]$P < 0.01$, [b,c]$P < 0.05$).

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Protective effect of DAAE against DNA fragmentation

The protective effect of DAAE against CCl_4-induced DNA fragmentation in hepatic tissues of male rats was assessed (Figure 6). The DNA fragmentation in the groups of DAAE35, DAAE70 and DAAE140 were relatively the same as that in the control group where low DNA bands were observed. While, treatment with CCl_4 (1 mg/kg bw) increased the DNA fragmentation as revealed from several DNA bands in comparison to that of the control group. In contrast, the DNA fragmentation was decreased clearly in groups treated with DAAE35, DAAE70 and DAAE140 combined with CCl_4 compared with CCl_4 treatment alone. Furthermore, the lowest DNA fragmentation induced by CCl_4 was observed in rats treated with a high dose of DAAE (140 mg/kg bw/d for 4 weeks) combined with CCl_4 (1 mg/kg bw).

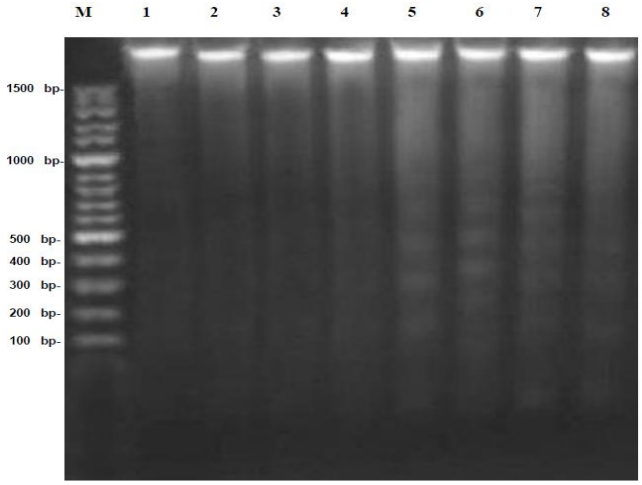

Figure 6: DNA fragmentation in liver tissues of male rats treated with CCl_4 alone or combined with DAAE.
M: represents DNA ladder. **Lane 1:** represents control rats. **Lanes 2-4:** rats were treated orally with AAE extract (35, 70 and 140 mg/kg b.wt. respectively). **Lane 5:** rats were treated with 1 mg/kg b.wt. CCl_4. **Lanes 6-8:** rats were treated with AAE (similar to those in lanes 2-4) and combined with CCl_4.

Protective effect of DAAE against apoptosis

Apoptosis analysis was fulfilled to appraise the protective effect of DAAE against CCl_4-induced apoptosis in liver tissues of male rats (Figure 7). The results revealed that CCl_4 increased apoptosis rates by 37.1% as compared to the control group (6.7%). On the other hand, low rates of apoptosis were found in rats treated with different doses of DAAE which were relatively similar to the control. Apoptosis rates declined to 26.2, 19.4 and 15.2% in rats treated with DAAE35, DAAE70 and DAAE140 combined with CCl_4, respectively, compared with those induced by CCl_4 alone (37.1%).

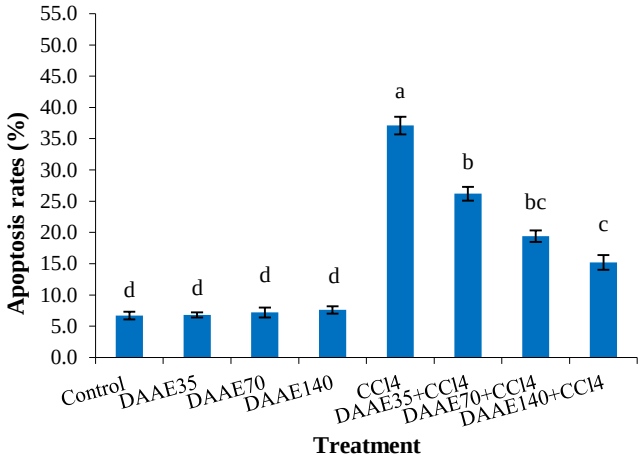

Figure 7: Alterations of apoptosis rates (%) in liver tissues of male rats treated with CCl_4 or combined with DAAE.
Data are presented as mean ± SEM. [a,b,c]Mean values within tissue with unlike superscript letters were significantly different ($P < 0.05$).

The present biological assessment revealed that the defatted *A. auriculiformis* leaves extract significantly attenuated the genotoxicity and apoptosis induced by CCl_4 in supplemented rats. The genotoxic effect of CCl_4 has been reported in various assays.[35] The mechanism by which CCl_4 induces genotoxicity is through the formation of trichloromethyl-free radical which initiates the lipid peroxidation process that is considered as a rate limiting process for its genotoxic effects.[36] The formation of such free radicals lead to oxidative stress which results in interconnected disruption of cellular metabolism, DNA fragmentation and cellular destruction.[37] The results in this study were in agreement with those findings, where DNA fragmentation and damage increased significantly in liver tissues of male rats after CCl_4 treatment as compared to the control group. In the same vain, a significant increase in the expression of cytochrome P450 (CYP450) and heat shock protein 70 (Hsp70) were detected after CCl_4 treatment as compared with the control. CCl_4 was found to alter the gene expression of 47 different genes in cultured human hepatoma cells (HEPG2) by using microarray assay.[38] Cytochrome P450 stands for a multigene family of enzymes with a crucial role in the oxidation of numerous endogenous and xenobiotic substrates.[39] It is frequently used as an environmental contaminants biomarker.[40] Whereas, Heat shock protein 70 (Hsp70) is commonly overexpressed upon exposure to high temperature or other stress conditions as oxidizing agents and chemotherapeutic drugs.[41] Several studies have shown the genotoxic effects of CCl_4, together with our study results it was revealed that it induced these effects probably due to a decrease in cellular antioxidant levels. Antioxidants play a crucial role in DNA repair maintaining genome stability, thus preventing subsequent mutagenesis and carcinogenesis.[42] The observed expression alternation of CYP450 and Hsp70 DNA damage and apoptosis after DAAE treatment were relatively similar to the control group. Moreover, their elevated levels after CCl_4 were significantly decreased by DAAE. Thus, it indicates that DAAE possessed the antioxidant potential to protect DNA from damage. The possible mechanism of DNA protection from CCl_4 toxicity is probably due to the presence of phenolic compounds that possess the capability to intercept the free radicals, preventing subsequent DNA damage.[43] The presence of bioactive molecules as genistein and biochanin A as major compounds in the DAAE contribute to its genoprotective effects, as revealed from previous studies that isoflavonoids modulates the effects of some cancer cells.[44] Genistein and biochanin A possessed a potent inhibitory effect on DNA damage, where genistein exhibited a potent scavenging effect on both hydrogen peroxide and superoxide anions free radicals.[45] Gallic acid is as a radioprotective phytochemical that reduce lipid peroxidation and DNA damage in gamma-radiated mice.[46] Quercetin suppressed DNA damage induced by hydrogen peroxide with protecting effect against oxidative base damage.[47] It also possessed DNA protecting effect against the alkylating agent ethyl methanesulfonate through hydrogen bond donation.[48] It ameliorated the oxidative stress caused by exposure to ultraviolet A irradiation in rats.[48] Thus, the antigenotoxic effect of DAAE and its ability to restore DNA damage induced by CCl_4 is probably ascribed to its phenolic compounds.

Conclusion

The study is considered the first report concerning the phytochemical investigation of *A. auriculiformis* leaves. From which, twelve known phenolic compounds were isolated and identified. The extract led to potent genoprotective activity. It possessed the capability of protecting the cells from oxidative damage induced by CCl_4 in hepatic tissue. Besides, it significantly down regulated CYP450 and Hsp70-genotoxicity related genes and protected DNA against apoptotic damage and fragmentation. Furthermore, deep research on *A. auriculiformis* leaves for its application as a herbal genoprotective supplement to reverse the toxic effects caused by industrial pollutant as CCl_4.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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