# Hepatoprotective, Gastroprotective, Antioxidant Activity and Phenolic Constituents of *Quercus robur* Leaves

**Fatma Abd-elkader Moharram[1,2*], Mohamed Soubhi Marzouk[3,4], Rabab Abdel Moneim El Dib[1,5],**

**Siham Mostafa El-Shenawy[6], Rehab F. Abdel-Rahman[6], Reham Ragaei Ibrahim[1]**

[1]*Department of Pharmacognosy, Faculty of Pharmacy,*
*Helwan University, 11795 Cairo, Egypt*
[2]*Department of Pharmacognosy and Pharmaceutical Chemistry, College of Pharmacy,*
*Taibah University, P.O. Box 344, Medina, Saudi Arabia.*
[3]*Department of Pharmaceutical Chemistry, College of Pharmacy,*
*King Saud University, P.O. Box 2457, Riyadh, 11451, Saudi Arabia.*
[4]*Chemistry of Natural Products Group, Center of Excellence for Advanced Sciences,*
*National Research Center, Dokki, 12622, Cairo, Egypt*
[5]*Department of Pharmacognosy, College of Pharmacy,*
*King Saud University, P.O. Box 22452, Riyadh 11495,*
[6]*Department of Pharmacology,*
*National Research Center, Dokki, 12622, Cairo, Egypt*

**Abstract**
Phytochemical investigation of the 80% aqueous methanol extract (**AME**) of *Quercus robur* leaves using chromatographic techniques, led to isolation of ten phenolic compounds, namely gallic acid (**1**), methyl gallate (**2**), strictinin (**3**), 4-*O*-galloyl-2,3-(*S*)-hexahydroxydiphenoyl-($\alpha/\beta$)-D-glucopyranose (**4**), 2,3-(*S*)-hexahydroxydiphenoyl-($\alpha/\beta$)-D-glucopyranose (**5**), casuarinin (**6**), casuariin (**7**), kaempferol 3-*O*-(6''-*O*-galloyl)-$\beta$-D-$^4C_1$-galactopyranoside (**8**), ellagic acid (**9**) and ellagic acid 3-*O*-methyl ether (**10**). Compounds **4**, **5** and **8** were isolated from *Quercus* genus for the first time, while **2**, **3**, **6**, **7**, **9** and **10** were isolated for the first time from *Q. robur*. Structures of the isolated compounds were established using chemical and physical methods and by comparison with compounds reported in literature. The **AME** of *Q. robur* was found to be nontoxic to mice up to 5000 mg /kg b.wt. It exhibited a significant hepatoprotective activity, as compared to paracetamol treated group and gastroprotective activity, as compared to ethanol treated group in dose dependent manner. The results are supported by histopathological data in the current study. Both **AME** and its tannin fractions exhibited marked *in vitro* antioxidant activity, as compared to pyrogallol and ascorbic acid using DPPH method.

**Key words:** Antiulcer; Antioxidant; Hepatoprotective; Fagaceae; *Quercus robur*; Tannins

## 1. INTRODUCTION

*Quercus robur* L. (Fagaceae) is commonly known as English, pedunculate or French oak. It is native to most of Europe, Anatolia, Caucasus and parts of North Africa and is cultivated as an ornamental tree in the temperate regions. Moreover, it is widely cultivated in Egypt. It is a large deciduous tree [1], and like several oak species, the bark of *Q. robur* was used for tanning of leather and dying of wool [2]. Today *Q. robur* is used for the treatment of diarrhea, anal fissures, bacterial and viral infections and has antioxidant activity [3]. Genus *Quercus* is known to contain various classes of compounds such as saponins, flavonoids and tannins [4-6]. Previous reports demonstrated that the wood and fruits of *Q. robur* are characterized by the presence of phenolic acids, hydrolysable and condensed tannins based on catechin and epicatechin nuclei [4,5]. However, there are no reports about phenolic compounds from the leaves. Moreover, the volatile oil of *Q. robur* was studied [7]. From the biological point of view, only antioxidant and antimicrobial activities of *Q. robur* were evaluated [8-9]. In this article, we report for the first time the isolation and structure elucidation of ten phenolic compounds from the **AME**, in addition to evaluation of the hepatoprotective and antiulcer activities, as well as *in vitro* study of the antioxidant effect of the **AME** and fractions containing tannins.

## 2. MATERIAL AND METHODS
### 2.1. Instruments and material
The NMR spectra were recorded using JEOL GX-500 (500 and 125 MHz for $^1$H and $^{13}$C NMR, respectively) and the results were reported as $\delta$ ppm values relative to TMS as internal reference. Negative ESI and HRESI-MS were run on LCQ deca MS and LTQ-FT-MS spectrometers for MS-analysis (Thermo Electron, Finnigan, Germany). UV analyses were recorded for pure samples in MeOH solutions and with different diagnostic UV shift reagents on JASCO (U-360) UV spectrophotometer. For column chromatography, polyamide S (Fluka Chemie AG, Switzerland), sephadex LH-20 (Sigma-Aldrich Steinheim, Germany) and microcrystalline cellulose (E. Merck-Darmstadt, Germany) were used. For paper

chromatography, Whatman No.1 chromatography paper (Whatman Ltd., Maidstone, Kent, England) was used. The pure compounds were visualized by spraying with ferric chloride, nitrous acid ($NaNO_2$/glacial AcOH) and potassium iodate spray reagents [10]. Naturstoff [11] and aluminium chloride reagents were used for visualizing the spots of flavonoids under UV light. Solvent systems $S_1$ (*n*-BuOH/HOAc/$H_2O$; 4:1:5 v/v/v, top layer), $S_2$ (15% aqueous HOAc) and $S_3$ (*n*-BuOH/isopropanol/$H_2O$; 4:1:5 v/v/v, top layer) were used. Paracetamol (Paramol, Misr Co., Egypt); silymarin (Sedico, Pharmaceutical Co., 6 October City, Egypt); Kits for alanine aminotransferase (ALT), aspartame aminotransferase (AST) and alkaline phosphatase (ALP, Biodiagnostic Co., Egypt) and DPPH (Sigma Chemical Company, USA) were used for biological evaluation.

## 2.2. Plant material
*Q. robur* L leaves were collected from Al-Zohria garden, Cairo, Egypt, during the flowering stage in April 2007. Authentication of the plant was performed by Mrs. Trease Labib, Senior Specialist of Plant Taxonomy and former Head of El Orman Botanical Garden, Giza, Egypt. A voucher specimen (No. Q.R.11) has been deposited at the herbarium of Pharmacognosy Department, Faculty of Pharmacy, Helwan University, Helwan, Cairo, Egypt.

## 2.3. Extraction and isolation
Air dried and powdered leaves of the *Q. robur* (1800 g) were exhaustively extracted with 80% aqueous MeOH under reflux (5 L x 6, 70 ℃, 4 h). The residue left after evaporation of the solvent (370 g) was pre-purified by extraction with $CHCl_3$ under reflux (2 L x 3, 50 ℃). Evaporation of the solvent resulted in 50 g dry $CHCl_3$ extract and a 301 g residue. The concentrated aqueous solution of the residue was desalted by precipitation with excess methanol (1:10) and the filtrate was evaporated to give a 200 g dry residue. The dried residue was fractionated on a polyamide S column [600 g, 125 x 10 cm] using stepwise gradient elution of $H_2O$-MeOH, (100:0 up to 0:100% v/v). The fractions were collected on the basis of comparative paper chromatography (Comp-PC), UV-light visualization and by spraying with different reagents, to give seven collective fractions (I-VII). Fraction I was found to be polyphenolic free. Fraction II was fractionated on a cellulose column using $H_2O$-MeOH (10-90%) mixtures, to give two sub-fractions, i and ii, which were further individually purified on sephadex LH-20 columns, using EtOH, to afford pure samples of **1** (10 mg) and **2** (6 mg), respectively. Fraction III was chromatographed on a sephadex column using *n*-butanol saturated with water as eluent, to reveal two sub-fractions. Final purification of both sub-fractions was done on individual sephadex LH-20 columns using EtOH, to give pure samples of **3** (20 mg) and **4** (17 mg). Fraction IV was purified on sephadex column using $S_3$, to give a semi-pure compound, which was finally purified on sephadex column using ethanol as eluent, to afford a chromatographically pure sample of **5** (21 mg). Fraction V was fractionated on a cellulose column using $S_3$, to give two major sub-fractions, i and ii. Their final purification was carried out on sephadex column using 50% ethanol/$H_2O$, which led to the isolation of two chromatographically pure compounds, **6** (14 mg) and **7** (12 mg). Fraction VI was subjected to purification on sephadex column using MeOH:$H_2O$ (50%) mixture, followed by final purification using sephadex and MeOH as eluent, which afforded a pure sample of **8** (24 mg). Fraction VII was applied on a sephadex LH-20 column using $H_2O$-MeOH mixtures (10-90%), to give two major sub-fractions. Final purification of each sub-fraction was performed using sephadex column and MeOH as eluent, to give pure samples of **9** (10 mg) and **10** (12 mg).

## 2.4. Biological activities
### 2.4.1. Animals
Wister albino rats of both sexes, weighing between 125 and 150 g and Swiss mice of both sexes of 20-30 g body weight were used throughout the experiments. Rats were used for assessment of the hepatoprotective and gastric anti-ulcerogenic activity, while mice were used for acute toxicity study. The animals were obtained from the animal house colony of the National Research Centre, Dokki, Giza, Egypt. The animals were housed in standard metal cages in an air conditioned room at $22 ± 3℃$, $55 ± 5\%$ humidity and were provided with standard laboratory diet and water *ad libitum.* Distilled water was used as a vehicle for all extracts and drugs used in the study. All animal procedures were performed in accordance with the Ethics Committee of the National Research Centre (Egypt, March 2004) and followed the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals [12].

### 2.4.2. Acute toxicity study
The **AME** was dissolved in distilled water and given orally in graded doses to mice up to 5 g/kg b. wt., while the control group received the same volume of the vehicle. The percentage mortality was recorded 24 h later. No mortality was recorded after 24 h, and according to Semler et al., 1992 [13], who reported that in the typical protocol for acute toxicity study, if just one dose level at 5g/kg is not lethal, so it is not necessary to determine the $LD_{50}$. Therefore, the experimental doses used were 1/20, 1/10 and 1/5 of 5 g/kg of **AME** (250, 500 and 1000 mg/kg, respectively).

### 2.4.3. Hepatoprotective study
The hepatic damage was induced in rats by giving a single oral paracetamol dose (1000 mg/kg), according to Silva et al., 2005 [14]. Rats were divided into six groups, each of six. The $1^{st}$ group received orally 1 ml saline/day (normal control); the $2^{nd}$ group was given a single oral dose of paracetamol (1000 mg/kg); the $3^{rd}$, $4^{th}$ and $5^{th}$ groups received daily oral dose of **AME** (250, 500 and 1000 mg/kg), alone for 10 successive days, before paracetamol oral administration. The $6^{th}$ group received a daily oral dose of silymarin as reference drug (25 mg/kg) for 10 successive days, before oral paracetamol administration. After 24 h of oral paracetamol administration, the blood was obtained from rato-orbital plexus vein from all rat groups, after being lightly anaesthetized with ether by puncturing [15]. To avoid haemolysis, the blood samples were allowed to stand for 30 min, before centrifugation for 15 min at 2500 rpm and the clear supernatant serum was separated and collected for the determination of ALT [16], AST [17] and

ALP [18]. Liver specimens of all rats were dissected immediately after being scarified for histopathological study and were fixed in 10% neutral-buffered normal saline for 72 h at least. All the specimens were washed in tap water for half an hour and then dehydrated in ascending grades of alcohol, cleared in xylene and embedded in paraffin. Serial sections of 6 μm thickness were cut and stained with haematoxylin and eosin [19] for histopathological investigation of liver tissue.

### 2.4.4. Gastric ulcerogenic effect

Gastric lesions were induced in rats by ethanol (1 ml orally) [20]. Animals were divided into four groups, each of six. The 1st group received oral dose of ethanol and served as control, while the remaining three groups received **AME** (250, 500 and 1000 mg/kg b. wt.) orally 1 h before the ethanol was given. Rats were scarified 1 h after ethanol administration by cervical dislocation, after being lightly anaesthetized with ether and the stomach was excised, opened along the greater curvature, rinsed with saline, extended on a plastic board and examined for mucosal lesions. The number and severity of mucosal lesions were noted and lesions were scaled as follows: petechial lesions = 1, lesions < 1 mm = 2, lesion between 1 and 2 mm = 3, lesions between 2 and 4 mm = 4, lesions more than 4 mm = 5. A total lesion score for each animal was calculated as the total number of lesions multiplied by the respective severity scores. Results are expressed as the severity of lesions/rat [21].

### 2.4.5 Antioxidant activity

Antioxidant activity was evaluated *in vitro* using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity method [22,23]. The methanol solution of DPPH (0.004%, 180 μl) was added to 20 μl of the different concentrations of **AME** and the fractions in a 96-well plate. Negative controls were done for colored fractions. The absorbance was measured at 520 nm. Methanol was used as blank and DPPH solution, without addition of the extract was used as control. Pyrogallol and ascorbic acid were used as positive control. $EC_{50}$ values were determined from the graph of percentage of inhibition plotted against the concentration of extract, using Graph Pad Prism Software version 5.0 ($EC_{50}$ is defined as the amount of extract needed to scavenge 50% of DPPH radicals).

### 2.4.6. Statistical analysis

The results are expressed as mean ± S.E. and all the data were statistically evaluated using Student's *t*-test [24] and one way ANOVA (Dunnnett's multiple comparison test). The P values < 0.05 were considered to be significant.

### 3. RESTLTS AND DISCUSSION

The 2D-PC screening of *Q. robur* leaves revealed the presence of a mixture of phenolic compounds (color properties under UV-light and different spray reagents). Fractionation and purification of **AME** on a polyamide, followed by cellulose and sephadex LH-20 columns, resulted in the isolation of ten compounds. Compounds **4**, **5** and **8** were isolated from *Quercus* genus for the first time, while **2**, **3**, **6**, **7**, **9** and **10** were isolated for the first time from *Q. robur*. The structures of these compounds [Figure

1] were fully elucidated on the basis of their physicochemical and spectral data and by comparison with published data [10,25-34].

### Gallic acid (1) and methyl gallate (2)

Off-white amorphous powder. Their chromatographic and physical properties, along with spectral data were completely consistent with the corresponding data in literature [25]. Final identification was achieved by Co-PC with authentic samples.

### 1-*O*-Galloyl-4,6-(*S*)-hexahydroxydiphenoyl-*β*-D-glucopyranose (Strictinin, 3)

Brown amorphous powder; $R_f$-values: 0.25 ($S_1$), 0.40 ($S_2$); it gave dark purple fluorescent spot under short UV-light, turned to indigo-red and deep blue color with nitrous acid [10] and $FeCl_3$, respectively. UV $\lambda_{max}$ (nm): 221, 246, 267 sh (MeOH). Negative HRESI-MS/MS: *m/z* 633.07410 [M-H]⁻ (cald. 633.07317), ($MS^2$) 481.06297 [M-H-gallic acid]⁻, 300.98930 [Ellagic acid-H]⁻, ($MS^3$) 257.01764 [Ellagic-H-$CO_2$]⁻, 229.02834 [Ellagic acid-H-$CO_2$-CO]⁻, ($MS^4$) 185.04608 [Ellagic acid-H- CO-2$CO_2$]⁻. ¹H NMR (500 MHz, $(CH_3)_2$CO-$d_6$): δ ppm 7.07 (2H, s, H-2'/6' G), 6.65 (1H, s, H-3‴ HHDP), 6.55 (1H, s, H-3″ HHDP), 5.34 (1H, br d, *J* = 7.7 Hz, H-1), 4.85 (1H, dd, *J* = 13, 6 Hz, H-6ₐ), 4.66 (1H, t-like, *J* = 10 Hz, H-4), 3.86 (1H, br dd, *J* = 9.9, 6.1 Hz, H-5), 3.79 (1H, br d, *J* = 13 Hz, H-6ᵦ), 3.75 (1H, t-like, *J* = 9.9 Hz, H-3), 3.58 (1H, t-like, *J* = 9.9 Hz, H-2). ¹³C NMR (125 MHz, $CH_3$OH-$d_4$): δ ppm 171.2, 170.7 (C-7‴/7″ HHDP), 170.5 (C-7' G), 146.4 (C-6″/6‴ HHDP), 145.8 (C-3'/5' G), 144.6 (C-4″/4‴ HHDP), 137.3 (C-5″/5‴ HHDP), 135.5 (C-4' G), 126.9, 126.6 (C-2″/2‴ HHDP), 118.3 (C-1' G), 115.3, 115.2 (C-1″/1‴ HHDP), 110.0 (C-2'/6' G), 108.2, 108.0 (C-3'/ 3‴ HHDP), 95.2 (C-1), 74.2 (C-3), 73.8 (C-5), 73.2 (C-4), 73.0 (C-2), 64.3 (C-6), where: G = galloyl and HHDP = hexahydroxydiphenoyl.

### 4-*O*-Galloyl-2,3-(*S*)-hexahydroxydiphenoyl-(*α/β*)-D-glucopyranose (4)

Brown amorphous powder; $R_f$-values: 0.27 ($S_1$), 0.38 ($S_2$); it gave dark purple fluorescent spot under short UV-light, turned to indigo-red and deep blue color with nitrous acid and $FeCl_3$, respectively. UV $\lambda_{max}$ (nm): 243, 278 sh (MeOH). Negative HRESI-MS/MS: *m/z* 633.07086 [M-H]⁻, 300.99893 [Ellagic acid-H]⁻. ¹H NMR (500 MHz, $(CH_3)_2$CO-$d_6$): δ ppm 7.29 (2H, s, H-2‴/6‴ G), 6.64, 6.62 (1H in total each s, H-3' HHDP α/β), 6.55 (2H in total, s, H-3″ HHDP α/β), 5.34 (1/2H, t-like, *J* = 9.2 Hz, H-3α), 5.34 (1/2H, br s, H-1α), 4.98 (1/2H, t-like, *J* = 9.6 Hz, H-3β), 4.93 (1/2H, d, *J* = 7.6 Hz, H-1β), 4.86 (1H, dd, *J* = 9.6 Hz, H-2α), 4.65 (1/2H, t-like, *J* = 9.9 Hz, H-2β), 4.29 (m, H-4α/β), (4.19-3.49 m, remaining sugar protons in α- and β-anomers). ¹³C NMR (125 MHz, $CH_3$OH-$d_4$): δ ppm 171.3, 170.7 (C-7'/7″ HHDP), 170.5 (C-7‴ G), 146.5 (C-6'/6″ HHDP α/β), 145.8 (C-3‴/5‴ G), 144.6 (C-4'/4″ HHDP α/β), 138.8 (C-5'/5″ HHDP), 135.6 (C-4‴ G), 126.2, 125.2 (C-2″ α/β), 123.7, 121.9 (C-2' HHDP α/β), 118.3, 116.8 (C-1‴ G α/β), 116.3, 116.2 (C-1' HHDP α/β), 110.0 (C-2‴/6‴ G), 107.9, 107.7 (C-3'/ 3″ HHDP α/β), 95.2 (C-1β), 91.9 (C-1α), 81.2 (C-3α), 78.9 (C-3β), 78.5 (C-2β), 78.4 (C-2α), 76.4 (C-4β), 76.1 (C-4α), 73.3 (C-5α), 68.7 (C-5β), 62.3, 62.1 (C-6α, β).
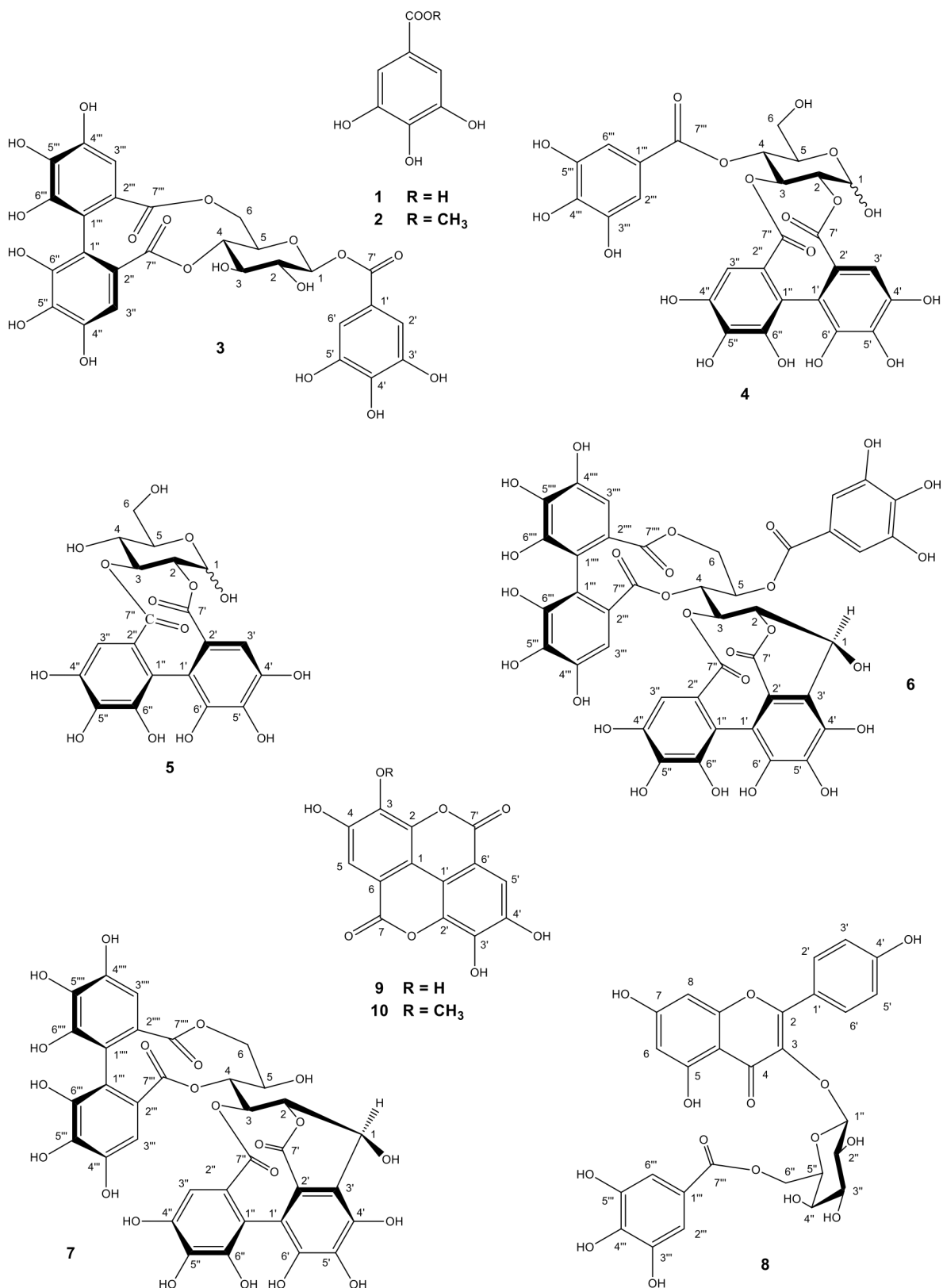
**Figure 1: Structures of phenolic compounds 1-10 isolated from *Q. robur* leaves**

**2,3-(*S*)-Hexahydroxydiphenoyl-(*α/β*)-D-glucopyranose (5)**
Brown amorphous powder; $R_f$–values: 0.12 (**S₁**), 0.76 (**S₂**). It gave dark purple spot under short UV-light that changed to blue color with FeCl₃ and indigo-red colour with nitrous acid spray reagents. UV λmax (nm): 225, 257, 287 (sh), 307 (MeOH); 222, 250, 290 (sh), 336 (+NaOH). Negative ESI-MS/MS: *m/z* 481.25 [M-H]⁻, (MS²) 301.22 [ellagic acid-H]. ¹H NMR (500 MHz, (CH₃)₂O-*d₆*): δ ppm 6.65, 6.64 (1H in total, each s, H-3″ α/ β HHDP), 6.54 (2H in total, s, H-3′ α/ β HHDP), 5.34 (1/2H, t-like, *J* = 9.2 Hz, H-3α), 5.32 (1/2H, br s, H-1α), 4.96 (1/2H, t-like, *J* = 9.9 Hz, H-3β),

4.93 (1/2H, d, $J$ = 8.4 Hz, H-1β), 4.67 (1H, dd, $J$ = 9.6 Hz, H-2α), 4.66 (1/2H, t-like, $J$ = 9.9 Hz, H-2β), 4.02-3.4 (m, H-4, 5, 6 in α- and β -anomers). ^{13}C NMR (125 MHz, CH$_3$OH-$d_4$): δ ppm 171.3 (C-7′ α, β HHDP), 170.7, 170.5 (C-7″ α and 7″ β HHDP), 145.8 (C-6′/6″ α,β HHDP), 144.6 (C-4′/4″ α, β HHDP), 137.3, 137.2 (C-5′/5″ α, β HHDP), 127.2, 126.9 (C-2″ α, β HHDP), 126.7 (C-2′ α, β HHDP), 115.4 (C-1″ α, β), 115.35 (C-1′ α, β), 108.1, 108.0, 107.7 (C-3′/3″ α, β), 95.2 (C-1β), 91.9 (C-1α), 81.8 (C-3α), 78.9 (C-3β), 78.5 (C-2β), 76.1 (C-2α), 73.3 (C-4β), 72.9 (C-5α), 68.7 (C-4α), 68.5 (C-5β), 62.3, 62.1 (C-6α, β).

**5-$O$-Galloyl-2,3:4,6-$O$-bis-[($S$)-(hexahydroxydiphenoyl)]-$\beta$-glucose (Casuarinin, 6)**
Brown amorphous powder; R$_f$-values: 0.18 (S$_1$), 0.25 (S$_2$); it gave dark purple fluorescent spot by short UV-light and dull brown under long UV-light, turned to indigo-red with nitrous acid and deep blue color with FeCl$_3$. UV λ$_{max}$ (nm): 223, 248, 275 (MeOH); 244, 277 (sh), 325 (+NaOH). ^{1}H NMR (500 MHz, (CH$_3$)$_2$O-$d_6$): δ ppm 7.13 (2H, s, H-2/6, galloyl), 6.74, 6.59, 6.46 (1H, s, H-3″″/3″/3‴ HHDP), 5.72 (1H, d, $J$ = 5.00 Hz, H-1), 5.58 (1H, dd, $J$ = 9.2, 1.3 Hz, H-4), 5.51 (1H, m, H-3), 5.10 (1H, m, H-5), 4.95 (1H, dd, $J$ = 14.5, 6.8 Hz, H-6$_a$), 4.69 (1H, dd, 4.6, 2.1, Hz, H-2), 4.02 (1H, br d, $J$ = 14.5 Hz, H-6$_b$). ^{13}C NMR (125 MHz, CH$_3$OH-$d_4$): δ ppm 171.0, 170.1, 169.0, 168.7 (C-7′,C- 7″, C-7‴, C-7″″), 167.2 (C=O G), 148.8, 146.9, 146.9, 146.7 (C-6′/6″/6‴/6″″ HHDP), 146.1, 145.9, 145.5, 144.6 (C-4′/C-4″/4‴/4″″ HHDP), 145.7 (C-3/5 G), 141.6 (C-4 G), 140.1 (2-C), 138.5, 138.4 (C-5′/5″/5‴/5″″ HHDP), 127.6 (C-2″), 127.4 (C-2″″), 126.0 (C-2‴), 122.9 (C-1 G), 122.8 (C-2′), 116.8 (C-1′), 116.6, 116.4, 116.1 (C-1″/1‴/1″″ HHDP), 116.0 (C-3′ HHDP), 110.4 (C-2/6 G), 109.0 (C-3″/3″″), 107.5 (C-3‴ HHDP), 75.8 (C-2), 75.3 (C-4), 73.7 (C-1), 70.4 (C-5), 69.9 (C-3), 66.7 (C-6), where: G = galloyl and HHDP = hexahydroxydiphenyl.

**2,3:4,6-$O$-Bis-[($S$)-hexahydroxydiphenoyl]-$\beta$-glucose (Casuariin, 7)**
Brown amorphous powder; R$_f$-values 0.15 (S$_1$), 0.30 (S$_2$): It gave dark purple fluorescent spot by short UV-light and dull brown under long UV-light, turned to indigo-red with nitrous acid and deep blue color with FeCl$_3$ spray reagent. UV λ$_{max}$ (nm): 246, 285 sh (MeOH). ^{1}H NMR (500 MHz, DMSO-$d_6$): δ ppm 6.44, 6.24, 6.15 (1H, s, H-3″″/3″/3‴ HHDP), 5.38 (1H, br s, H-1), 5.18 (1H, br s, H-3), 4.8 (1H, br s, H-4), 4.65 (1H, br s, H-2), 4.34 (1H, br d, $J$ = 9.2 Hz, H-6$_a$), 3.91 (1H, br s, H-5), 3.73 (1H, br d, $J$ = 10.7 Hz, H-6$_b$). ^{13}C NMR (75 MHz, CH$_3$OH-$d_4$): δ ppm 171.2, 168.3, 167.8 (C-7″, C-7″″, C-7‴), 167.2 (C-7′), 148.2, 148.0, 146.9, 146.9 (C-6′/6″/6‴/6″″ HHDP), 146.6, 145.9, 145.2, 144.9 (C-4′, 4″/4‴/4″″ HHDP), 139.0, 138.5, 138.4, 136.8 (C-5′, 5″/5‴/5″″ HHDP), 128.9 (C-2″), 128.8 (C-2″″), 126.0 (C-2‴, C-2′), 118.8 (C-1′), 118.2, 117.4, 117.1 (C-1″/1‴/1″″ HHDP), 114.7 (C-3′ HHDP), 109.8 (C-3″″), 107.5 (C-3″, C-3‴ HHDP), 75.8 (C-2), 75.3 (C-4), 70.8 (C-3), 68.5 (C-1), 67.7 (C-5), 66.8 (C-6).

**Kaempferol 3-$O$-(6″-$O$-galloyl)-$\beta$-D-$^4C_1$-galactopyranoside (8)**
Yellow amorphous powder; R$_f$-values: 0.47 (S$_1$), 0.36 (S$_2$); It gave dark purple spot under UV-light changed to yellowish green fluorescence and pale green color with

Naturstoff and FeCl$_3$ spray reagents, respectively. UV λ$_{max}$ (nm): 268.5, 296.5, 348 (MeOH); 275.5, 322.5, 400 (+NaOMe); 272, 300 (sh), 362 (+NaOAc); 266, 300 (sh), 355 (+NaOAc/H$_3$BO$_3$); 272, 304 (sh), 349, 396 (+AlCl$_3$); 271, 302 (sh), 350 (+AlCl$_3$/HCl). ^{1}H NMR (500 MHz, CH$_3$OH-$d_4$): δ ppm 7.89 (2H, d, $J$ = 8.4 Hz, H-2′/ 6′), 6.92 (2H, s, H-2‴/ 6‴), 6.71 (1H, d, $J$ = 8.4 Hz, H-3′/ 5′), 6.30 (1H, d, $J$ = 2.3 Hz, H-8), 6.15 (1H, d, $J$ = 2.3 Hz, H-6), 5.17 (1H, d, $J$ = 7.9 Hz, H-1″), 4.26 (1H, dd, $J$ = 9.1, 2.0 Hz, H-6″a), 3.46 (2H, m, H-2″/ 3′), 3.28 (2H, m, H-4′/ 5″). ^{13}C NMR (125 MHz, CH$_3$OH-$d_4$): δ ppm 179.3 (C-4), 168.1 (C-7‴), 165.8 (C-7), 162.8 (C-5), 161.3 (C-4'), 159.5 (C-9), 158.3 (C-2), 146.3 (C-3‴/5‴), 139.7 (C-4‴), 135.2 (C-3), 132.1 (C-2′/6′), 122.6 (C-1′), 121.2 (C-1″), 116.0 (C-2′/6′), 110.1 (C-2‴/ 6‴), 105.5 (C-10), 104.3 (C-1″), 99.9 (C-6), 94.9 (C-8), 77.9 (C-3″), 75.7 (C-5″), 75.7 (C-2″), 71.4 (C-4″), 64.3 (C-6″).

**Ellagic acid (9) and ellagic acid 3-$O$-methyl ether (10)**
Pale yellow amorphous powder. Their chromatographic and physical properties alongside spectral data were completely consistent with the corresponding data in literature [26,27]. Final confirmation of the structures was obtained by Co-PC with authentic samples.
The structures of compounds **1**, **2**, **9** and **10** were identified as gallic acid, methyl gallate, ellagic acid and ellagic acid 3-$O$-methyl ether based on their chromatographic and spectroscopic data, as well as comparison with authentic samples [25-27]. Compounds **3** and **4** exhibited chromatographic behaviour and UV-data characteristic for ellagitannins [10]. On complete acid hydrolysis, both compounds gave ellagic and gallic acids in the organic phase and glucose in the aqueous phase (PC with authentic samples and spray reagents response). Negative HRESI-MS/MS of **3** and **4** showed a molecular ion peak at $m/z$ 633.07410, corresponding to a MF of C$_{27}$H$_{22}$O$_{18}$ along with a fragment ion at $m/z$ 300.98930 for ellagic acid anion that gave an evidence for the presence of hexahydroxy-diphenoyl group (HHDP) and gallic acid moiety in their structures. ^{1}H NMR of both **3** and **4** exhibited two singlets, each for one proton at δ-range 6.6-6.5 ppm assignable to the protons of H-3″ and H-3‴ (compound **3**) or H-3′ and H-3″ (compound **4**) of an HHDP group, as well as singlet signal for two equivalent protons of galloyl moiety in the range of 7-7.3 ppm. In the aliphatic region, the sugar moiety was confirmed as β -$^4C_1$ glucose from the broad doublet signal for H-1 in case of **3**. In case of **4**, the duplication of all assigned [1]H-resonances gave an indication to a free anomeric-OH and its presence in the form of α/β-anomeric mixture. Moreover, the downfield shift of H-1, H-6 and H-4 in case of **3** gave an evidence for attachment of galloyl moiety on OH-1 and HHDP moiety at OH-4 and OH-6 [28-32]. Similarly, in case of **4**, the downfield shift of H-2 (δ ppm 4.65, 4.86) and H-3 (δ ppm 4.98, 4.93) indicated the attachment of the HHDP-group at C-2 and C-3. In addition, the downfield shift of H-4 at 4.29 ppm gave an evidence for the attachment of the galloyl moiety at OH-4. Final confirmation of the structures of **3** and **4** was done by ^{13}C NMR. In case of **3**, the downfield shift of C-1 (δ 95.2) and upfield shift of C-2 (δ 73.0), due to galloyl moiety at OH-1 and the downfield shift of C-4 (δ

73.3) and C-6 (δ 64.4) and upfield shift of C-3 (δ 74.2) and C-5 (δ 73.8) gave an evidence for the esterification of OH-4 and OH-6 by HHDP group [30]. Moreover, the duplication of all aliphatic and aromatic signals confirmed the α/β-configuration in case of **4**, especially those of the anomeric carbon at 95.20 and 91.88 for C-1β and C-1α, respectively [28]. The remaining ^{13}C-signals of both HHDP and galloyl moieties were assigned by comparison with published data of structurally related compounds [28]. Accordingly, **3** was established as 1-O-galloyl-4,6-(S)-hexahydroxydiphenoyl-β-D-gluco-pyranose (Strictinin) and **4** as 4-O-galloyl-2,3-(S)-hexahydroxydiphenoyl-(α/β)-D-glucopyranose. Like in **3** and **4**, compound **5** showed chromatographic properties and UV-data of an ellagitannin [10]. However, on complete acid hydrolysis, it gave only ellagic acid in the organic phase and glucose in the aqueous one. Its negative ESI-MS/MS demonstrated a molecular ion peak at $m/z$ 481.25 [M-H]$^-$, corresponding to a mono-hexahydroxydiphenoyl-glucose. Its ^{1}H NMR spectrum indicated the presence of HHDP group and glucose moiety. Duplication of all assigned [1]H-resonances was indicative to a free anomeric-OH and the presence of **5** in the form of α/β-anomeric mixture [28,30]. Moreover, ^{1}H and ^{13}C NMR data confirmed its structure as 2,3-(S)-hexahydroxydiphenoyl-(α/β)-D-glucopyranose, because of the characteristic downfield shift of H-2 and H-3, as well as C-2 and C-3 in the ^{1}H and ^{13}C NMR spectra. Like **5**, compounds **6** and **7** exhibited the properties of ellagitannins. Complete acid hydrolysis gave ellagic and gallic acid in case of compound **6**, and ellagic acid together with an unknown ellagitannin intermediate in the organic hydrolysate. Absence of the sugar moiety in the aqueous hydrolysate and detection of an ellagitannin intermediate gave the evidence for the C-glycosidic structure of both **6** and **7** [30]. This evidence was confirmed from the ^{1}H NMR spectrum, which exhibited in the aromatic region three singlets, each for one proton of two HHDP ester moieties with the loss of one proton (H-3′), due to oxidative coupling and formation of extra C-C linkage with the anomeric position. The galloyl moiety in **6** was indicated by the presence of a two proton signal for H-2/6. Furthermore, the evidence for C-glycosidic structure of **6** and **7** was supported by the appearance of all sugar protons in the form of broad singlets, except for CH$_2$-6 diastereomeric protons, which were assigned at δ 4.34 and 3.73 to confirm an open chain glucose structure with the other signals [30,31]. Further confirmation of the open chain structure was clearly proved due to the strong upfield location of C-1 at δ 73.7 and 68.5 ppm in comparison to that of pyranose (δ ≈ 90-95 ppm). In addition, the C-glycosidic nature was further evidenced from the typical downfield shift of C-3′ (HHDP) at 116.0 (**6**) and 114.7 (**7**) (≈ + 9 ppm), with respect to those of C-3‴ at 107.5 [30,31]. The downfield shift of C-5 at δ 70.4 ppm in **6** was an evidence for galloylation. All other ^{13}C resonances were assigned according to a comparative study with previously reported data of C-glycosidic tannins [29-32], to confirm the structures of **6** and **7** as 5-O-galloyl-2,3:4,6-O-bis-[(S)-(hexahydroxydiphenoyl)]-β-glucose (Casuarinin) and 2,3:4,6-O-bis-[(S)-(hexahydroxydiphenoyl)]-β-glucose (Casuariin), respectively.

Compound **8** exhibited the chromatographic properties and UV data of an acylated kaempferol 3-O-glycoside with three free OH groups at 5, 7 and 4′ positions [33]. It gave galactose in aqueous phase and kaempferol and gallic acid in the organic phase on complete acid hydrolysis. ^{1}H NMR of **8** showed an A$_2$X$_2$ spin coupling system of two *ortho*-doublets for H-2′/6′ and H-3′/5′ with an AM system of two *meta*-doublets describable for H-8 and H-6. The galloyl moiety was indicated by the singlet at δ 6.92 of H-2‴/6‴. In the aliphatic region, the presence of a doublet at δ 5.17, describable for H-1″, was in good agreement with a β-D-$^4C_1$-galactopyranoside. The downfield shift of C-6 of galactose and its protons gave an evidence for galloylation at OH-6″. Further confirmation of the compound was done from its ^{13}C NMR data and comparative study with published data of structurally related compounds [34].

**Table 1: Effect of oral administration of AME of *Q. robur* leaves on AST, ALT& ALP serum activity in paracetamol induced hepatotoxicity in rats, (n=6)**

| Groups | Dose (mg/kg b.wt.) | ALT(U/L) | | | AST(U/L) | | | ALP(IU/L) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | X$^-$ ± S.E. | % of change | | X$^-$ ± S.E. | % of change | | X$^-$ ± S.E. | % of change | |
| | | | From C | From P | | From C | From P | | From C | From P |
| **Control (C)** | 1ml saline | 47.5±0.98 * | -- | --- | 82.6±2.39* | -- | --- | 230.5±18.57 * | -- | --- |
| **Paracetamol (P)** | 1000 | 64.3±3.74 ‡@ | 35.37 | ---- | 98.8±6.00 ‡@ | 19.61 | --- | 294.3±14.25 ‡@ | 27.67 | --- |
| **AME** | 250 | 40.2±2.28 * | 15.37 | 37.48 | 86.7±1.97 | 4.96 | 12,25 | 257.4±12.28 @ | 11.67 | 12.54 |
| | 500 | 42.6±2.91 * | 10.32 | 33.75 | 78.2±2.86 * | 5.33 | 20.85 | 211.3±13.81 * | 8.33 | 28.20 |
| | 1000 | 42.8±1.14 * | 9.89 | 33.44 | 83.4±1.17 * | 0.97 | 15.59 | 218.0±7.40 * | 5.42 | 25.93 |
| **Silymarin** | 25 | 39.6±2.15 * | 16.63 | 38.41 | 81.5±2.97 * | 1.33 | 17.5 | 191.3±12.64 * | 17 | 34.99 |

a) Values represent the mean ± S.E. of six rats for each group.
b) ‡ P< 0.05: Statistically significant from control (Dunnett's test)
c) * P< 0.05: Statistically significant from paracetamol (Dunnett's test).
d) @ P< 0.05: Statistically significant from silymarin (Dunnett's test).
e) Percent of change in paracetamol group only was calculated as regard to saline control group (C).
f) Percent of change in other groups was calculated regard to paracetamol (P)

**Table 2: Effect of oral administration of AME of *Q. robur* leaves on gastric mucosal injury induced by oral administration of 100% ethanol in rats (n=6).**

| Treated groups | Dose (mg/ kg b.wt.) | Number of lesions/rat X ± S.E. | % change | Severity of lesions/rat X ± S.E. | % change |
|---|---|---|---|---|---|
| **Absolute ethanol control** | 1 ml | 13.7 ± 1.28 | -- | 25.2 ± 2.39 | - |
| **AME** | 250 | 2.2 ± 0.40 * | 83.9 | 3.2 ± 1.05 * | 87.3 |
| | 500 | 2.2 ± 0.48 * | 83.9 | 2.5 ± 0.62 * | 90.1 |
| | 1000 | 1.2 ± 0.17 * | 91.2 | 1.5 ± 0.50 * | 94.1 |

a) Values represent the mean ± S.E. of six rats for each group.
b) * P< 0.05: Statistically significant from control (ethanol) (Kruskal-Wallis followed by Mann-Whitney test)

In the acute cytotoxicity study, it was proved that the **AME** is non-toxic, up to 5g/kg b.wt. Administration of the **AME** exhibited a significant hepatoprotective effect, in a dose dependent manner in paracetamol induced hepatotoxicity in rats [Table 1]. Rats given a single oral dose of paracetamol (1000 mg/kg) showed significant elevation in serum level of ALT, AST and ALP, by 35.37, 19.61 and 27.67%, respectively, as compared with control group treated with a single daily oral dose of 1 ml saline. Furthermore, the **AME** exhibited significant reduction in the elevated serum levels of ALT, AST and ALP to 37.48, 12.25 and 12.54% (250 mg/kg) and to 33.75, 20.85, and 28.20% (500 mg/kg). Moreover, the dose of 1000 mg/kg reduced the elevated level by 33.44, 15.59 and 25.93 for ALT, AST and ALP respectively, as compared to paracetamol treated group**.** However, silymarin exhibited significant reduction in serum ALT, AST and ALP levels by 38.41, 17.50 and 34.99%, respectively, as compared to paracetamol treated group. Histopathological examination for liver tissue of the control group showed normal architecture [Figure 2]. Paracetamol administration showed granularity of the cytoplasm of hepatocytes, associated with focal hepatic necrosis, inflammatory cells infiltration and portal infiltration with leucocytes [Figure 3 (A-C)]. However, administration of silymarin for 10 days before paracetamol injection showed no histopathological changes [Figure 4]. Rats treated with **AME** (250, 500 and 1000 mg/kg) revealed hepatoprotective activity in a dose-dependent manner against paracetamol induced hepatotoxicity. This protective effect was more obvious in ameliorating the dilatation and congestion of blood vessels [Figures 5-7]. Oral administration of **AME** exhibited significant reduction in the number and severity of gastric mucosal lesions, being 2.2 ± 0.40 and 3.2 ± 1.05 (250 mg/ kg), 2.2 ± 0.48 and 2.5 ± 0.62 (500 mg/kg) and 1.2 ± 0.17 and 1.5 ± 0.50 (1000 mg/kg), respectively in comparison to the ethanol treated group. The number and severity of gastric mucosal lesions was reduced by 83.9 and 87.3%, 83.9 and 90.1% and 91.2 and 94.1% for 250, 500 and 1000 mg/kg, respectively, relative to the control values [Table 2]. Additionally, histopathological results revealed that normal control group, which was treated with 1 ml saline, showed normal gastric mucosa [Figure 8], while oral ethanol administration induced necrosis of apical mucosa associated with hemorrhage and submucosal edema [Figure 9 (A and B)]. Furthermore, oral administration of **AME** induced a dose-dependent gastro-protective effect in ethanol induced gastric ulcer model in rats [Figures 10-12]. Concerning the *in vitro* antioxidant examination, it is worth
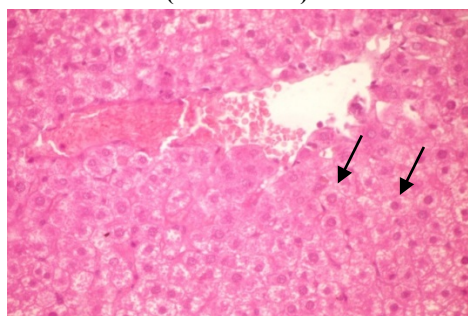
mentioning that **AME** and its fractions (II-VII) exhibited a marked scavenging activity against DPPH radicals, corresponding for $EC_{50}$-values of 270.5, 100.0, 213.0, 189.8, 205.2, 196.5 and 65.08 µg/ml, relative to 32.77 and 21.12 µg/ml for pyrogallol and ascorbic acid, respectively [Figures 13-15].
Although *Q. robur* is known to be rich in polyphenols, particularly phenolic acids and condensed tannins based on catechin and epicatechin building units [4-5], only few reports were published about hydrolysable tannins. Based on chemical and spectroscopic evidences, the present study proved that **AME** is rich in hydrolysable tannins, which gave gallic and ellagic acids as hydrolyzing degradative products. Tannins, especially hydrolysable tannins are excellent antioxidant agents, since they contains several gallic, HHDP or any other more complex phenolic acid ester groups, which possess the ability to provide protons and form stable free radicals, which enables them to be the major active groups in the molecule of tannins [35]. Moreover, it was found that HHDP groups are more potent than galloyl ones [36], because each HHDP has duplicated number of conjugated π-electrons that drastically increase the stability of free radicals. The linkage between the monomers of tannins and the existing status of the phenolic hydroxyl groups also represent important factors affecting the antioxidant properties of tannins. Furthermore, the presence of esteric and glycosidic bonds in hydrolysable tannins increase their antioxidant activity more than in the condensed ones [36]. Ellagic acid is a potent plant antioxidant [37-39], since it showed high DPPH free radical scavenging and lipid peroxidation inhibitory activities [40,41]. In addition, it activates antioxidant enzymes i.e. superoxide dismutases, catalase and glutathione peroxidase [40]. Metabolism of ellagitannins takes place in the intestine, where the pH of the small intestine causes their hydrolysis to ellagic acid, which is further metabolically converted into urolithins by intestinal microflora. The various pharmacological activities may be attributed to ellagic acid alone, its metabolites, or the combination of both. The low bioavailability of ellagic acid should be regarded as one of the major reasons of why potent biological activities reported during *in vitro* studies are not replicated *in vivo* studies [42]. Ellagitannins have multiple mechanisms of ulcer protective action, including antioxidant activity, which may be highly effective in minimizing tissue injury in human diseases. They are effective in healing experimentally induced gastric ulcers [43]. Moreover, they are associated with antiulcerogenic activity [44]. Ellagic acid is a potent competitive inhibitor
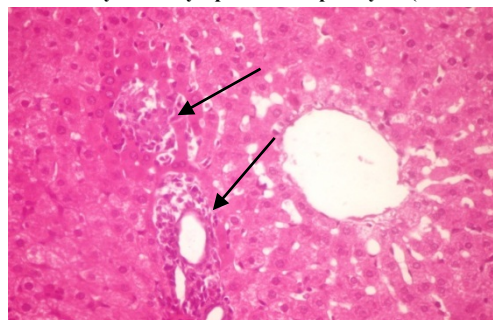
of gastric H$^+$, K$^+$-ATPase, and is proposed to compete with ATP at the ATP hydrolysis site, thus markedly inhibiting acid secretion and stress-induced gastric lesions [45]. Furthermore, another mechanism for antiulcer effect of tannins may be due to its astringent property, which enables them to bind with proteins, so they accelerate the healing of ulcers [46]. Tannins possess hepatoprotective activity, which may be attributed to their powerful antioxidant activity [47].
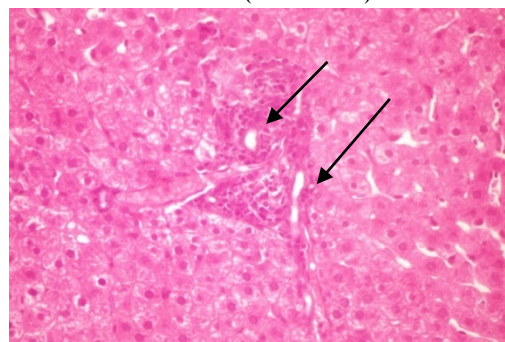


**Figure 2: A photomicrograph of the control liver tissues showing the normal histological structure of hepatic lobule (H & E x 400).**



**(A): Granularity of the cytoplasm of hepatocytes (H & E x 400).**



**(B): Focal hepatic necrosis associated with inflammatory cells infiltration (H & E x 400).**



**(C): Portal infiltration with leucocytes (H & E x 400).**

**Figure 3 (A-C): A photomicrograph of the liver tissue of paracetamol treated group (1000 mg/kg).**



**Figure 4: A photomicrograph of the liver tissue silymarin (25 mg/kg) 10 days before paracetamol administration showing no histopathological changes (H & E x 400).**



**(A): Portal infiltration with leucocytes (H & E x 400)**



**(B): Pyknosis of some hepatocytic nuclei (H & E x 400).**

**Figure 5 (A and B): A photomicrograph of the liver tissue of AME of *Q. robur* leaves (250 mg) 10 days before paracetamol administration.**
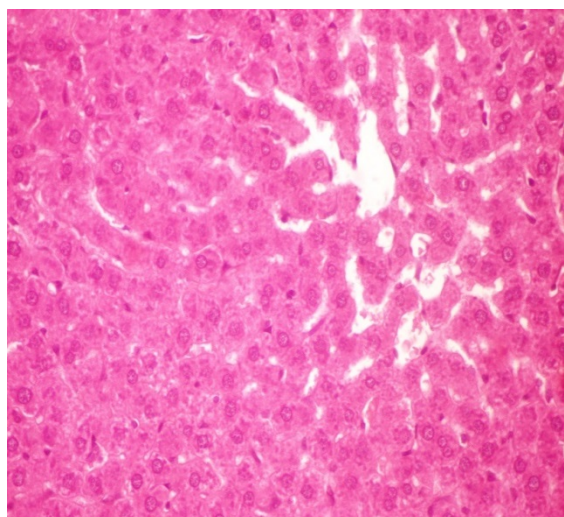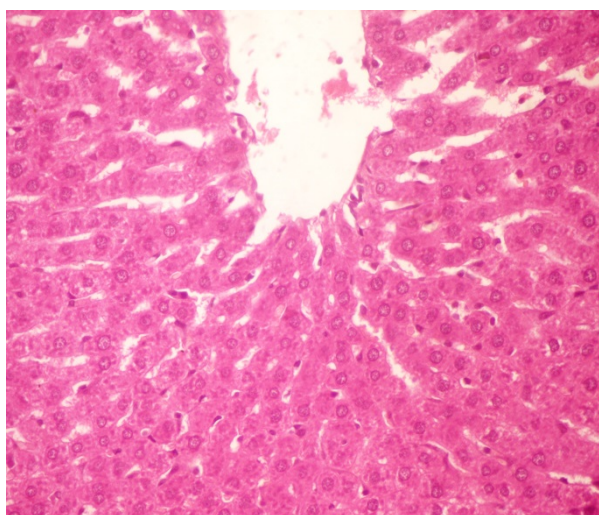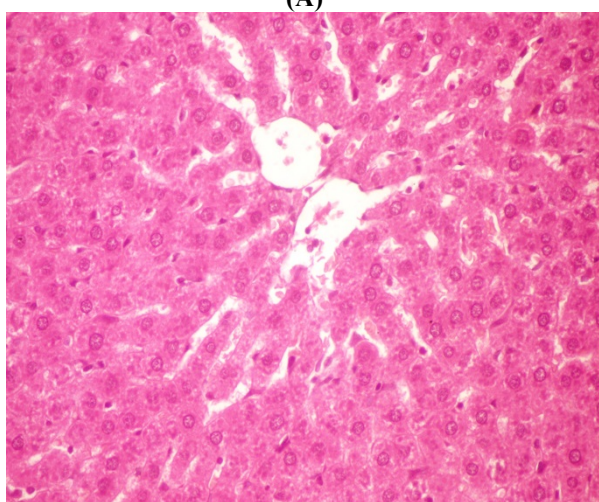
**Figure 6: A photomicrograph of the liver tissue of AME of *Q. robur* leaves treated group (500 mg) 10 days before paracetamol administration showing no histopathological changes (H & E x 400).**
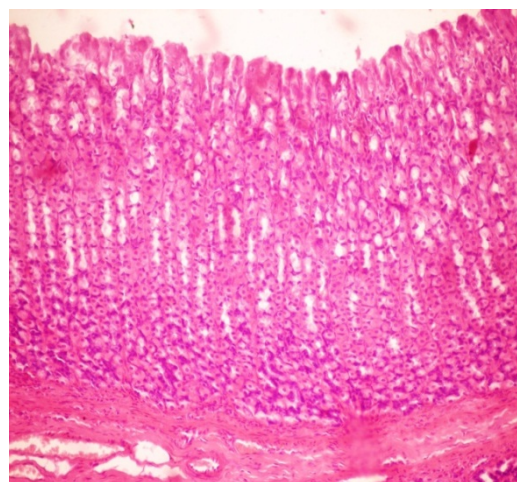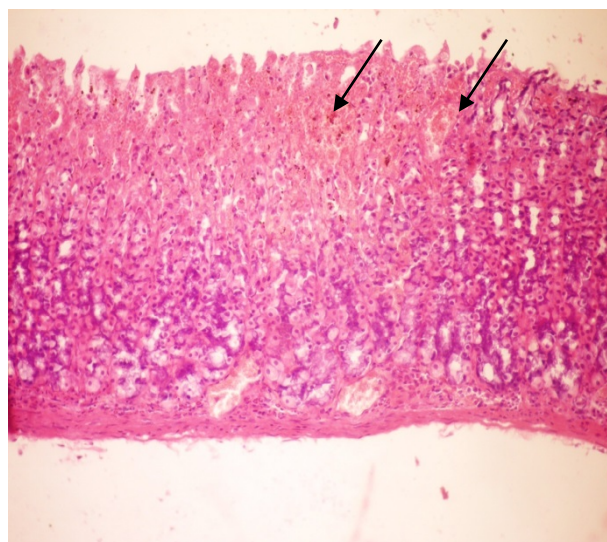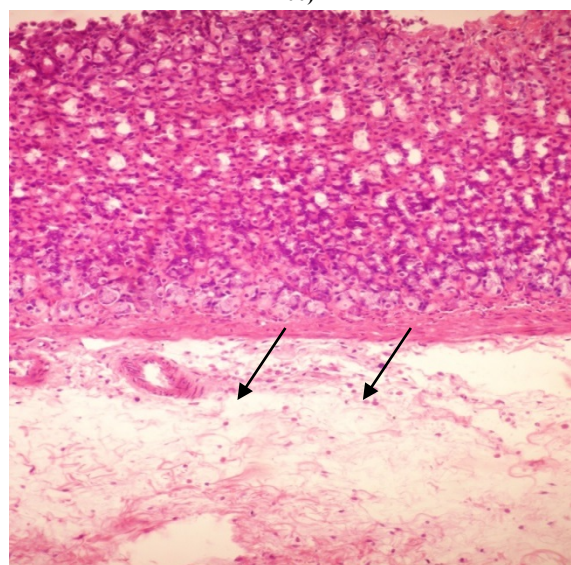


**(A)**



**(B)**

**Figure 7 (A and B): A photomicrograph of the liver tissue of AME of *Q. robur* leaves treated group (10000 mg) 10 days before paracetamol administration showing no histopathological changes (H & E x 400).**



**Figure 8: A photomicrograph of a stomach section of control saline group showing normal gastric layers (H & E x 200).**
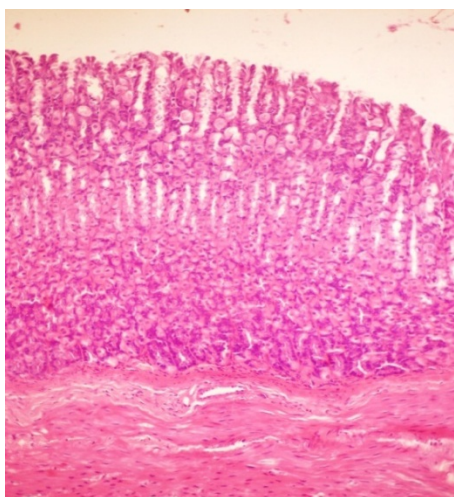


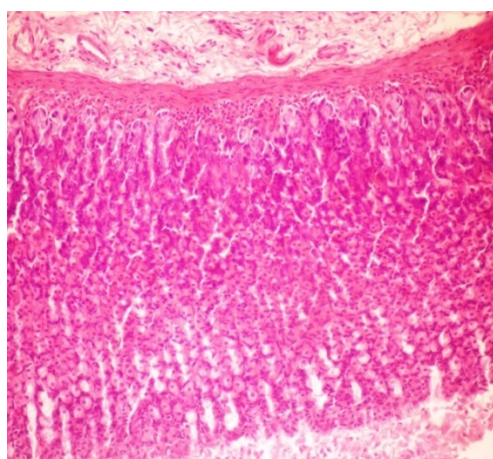**(A): Necrosis of apical mucosa associated with hemorrhage (H & E x 200)**



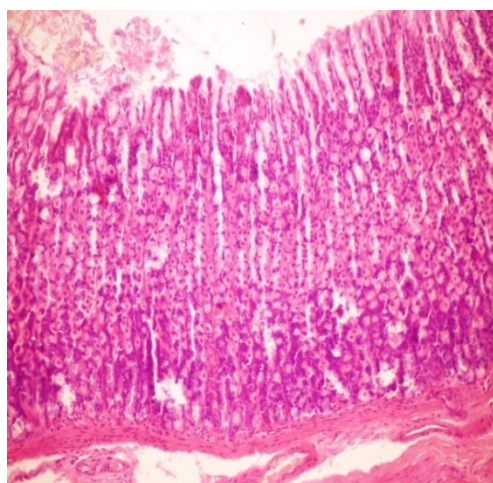**(B): Submucosal edema (H & E x 200)**

**Figure 9 (A and B): A photomicrograph of a stomach section of 100% ethanol treated group.**

**Figure 10:** A photomicrograph of the stomach section AME of *Q. robur* leaves (250 mg/kg) treated group one hour before 100% ethanol administration showing no histopathological changes (H & E x 200).



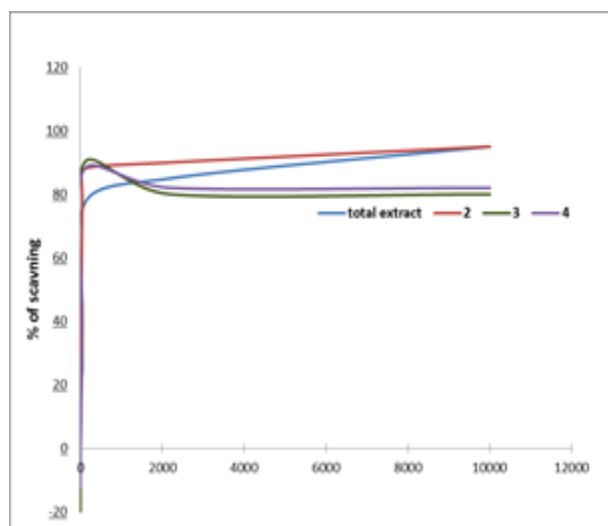**Figure 13:** Antioxidant activity of AME and fractions 2-4 = II-IV.



**Figure 11:** A photomicrograph of the stomach section AME of *Q. robur* leaves (500 mg/kg) treated group one hour before 100% ethanol administration showing no histopathological changes (H & E x 200).



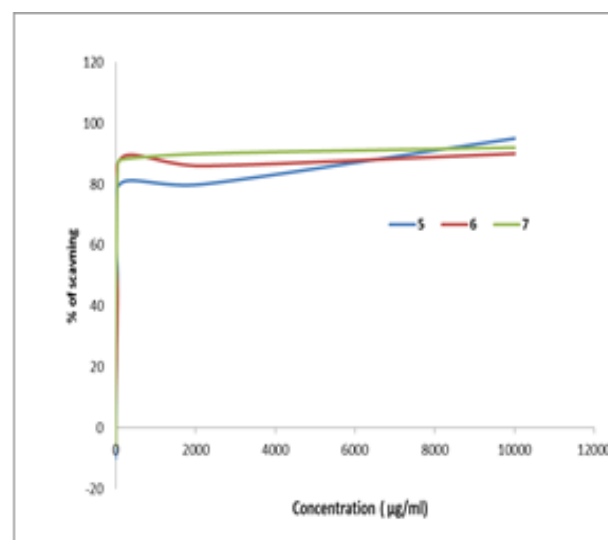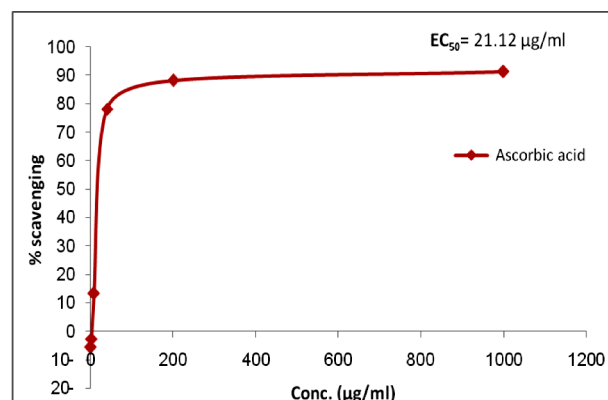**Figure 14:** Antioxidant activity of fractions 5-7 = V-VII



**Figure 12:** A photomicrograph of the stomach section AME of *Q. robur* leaves (1000 mg/kg) treated group one hour before 100% ethanol administration showing no histopathological changes (H & E x 200).



**Figure 15:** Antioxidant activity of ascorbic acid.

## 4. CONCLUSION

The current study proved the safety of **AME** and its hepato- and gastroprotective activities, in addition to the antioxidant effect of the extract and tannin fractions. This is encouraging for further phytochemical and biological investigations to confirm the possibility of their therapeutic effects, which may be important for the development of new drugs.

## CONFLICT OF INTEREST

All authors contributed to and have approved the final manuscript. The authors declare no conflict of interest

### REFERENCES

[1] Bailey, L. H., *Manual of cultivated plants*, revised edition, The Macmillan, New-York 1958, pp. 329-333.
[2] Deni, B., *Encyclopedia of herbs and their uses*, 1st American ed., A Dorling Kindersley, New-York 1995, pp. 147.
[3] Charles, W. F, Juan, R. A., *The complete guide to herbal medicines*. Springhouse Corp., Springhouse PA 1999, pp. 346-348.
[4] Şöhretoğlu, D., Sakar, M. K., *J Fac Phar Ank.* 2004, *33*, 183-215.
[5] Sakar, M. K, Sohretoglu, D., Ozalp, M., Ekizoglu, M., Piacente, S., Pizza, C., *Turk J Chem.* 2005, *29*, 555-559.
[6] Romussi, G., Barbieri, G., Caviglioli, G., *Pharmazie* 1994, *49*, 703-704.
[7] Vrkocova, P., Valterova, I., Vrkoc, J., Koutek, B., *Biochem Sys Ecol.* 2000, *28*, 933-947.
[8] Uddin, G., Rauf, A., *Middle-East J Med Pl Res.* 2012, *1*, 1-4.
[9] Almeida, I. F, Valentao, P., Andrade, P. B., Seabra, R. M., Pereira, T. M., Amaral, M. H., Costa, P. C., Bahia, M. F., *Biofactors* 2008, *33*, 267-279.
[10] Gupta, R. K., Al-Shafi, S. M., Layden, K., Haslam, E., *J Chem Soc Perkin Trans. 1*, 1982, 2525-2534.
[11] Hiermann, A., *Sci Pharm.* 1983, *51*, 158-167.
[12] NIH Guide for the Care and Use of Laboratory Animals a. DHEW Publication No. (NIH) 78-23, Revised 1978 and b. NIH Publication No. 85-23, Revised 1985: U.S. Department of Health, Education and Welfare.
[13] Semler, D. E., Gad, S.C., Chengelis, C.P. (Eds.), The rat, in: Gad, S. C., Chengelis, C. P., *Animal Models in Toxicology*, Chapter 2, Marcel Dekker, Inc. New York, USA 1992. pp. 21-162.
[14] Silva, V. M., Thibodeau, M. S., Chen, C., Manautou, J. E., *Biochem Pharmacol.* 2005, *70*, 1832-1839.
[15] Sorg, D. A., Buckner, B., *Proc Soc Exp Biol Med.* 1964; *115*, 1131-1132.
[16] Bergmeyer, H. U., Horder, M., Rej, R., *J Clin Chem Clin Biochem.* 1986, *24*, 481-495.
[17] Klauke, R., Schmidt, E., Lorentz, K., *Eur J Clin Chem Clin Biochem.* 1993, *31*, 901-909.
[18] Tietz, N. W., Shuey, D. F., *Clin Chem.* 1986, *32*, 1593-1594.
[19] Carleton, H. M., Drury, R. A. B., Wallington, E. A. *Carleton's Histological Technique*, 5th ed., Oxford University Press, New York, Pronto 1980, pp. 206.
[20] Robert, A., Nezamis, J. E., Lancaster, C., Hanchar, A. J. *Gastroenterology* 1979, *77*, 433-443.
[21] Mózsik, G. Y., Móron, F., Jávor, T., *Prosta Leukotr Med.* 1982, *9*, 71-84.
[22] Amić, D., Davidović-Amić, D., Bešlo, D., Trinajstic, N., *Croat Chem Acta.* 2003, *76*, 55-61.
[23] Phang, C. W., Abd Malek, S. N., Ibrahim, H., Abdul Wahab, N., *Afr J Pharm Pharmacol.* 2011, *5*, 842-852.
[24] Armitage, P., Berry, G., Matthews, J. N. S., *Statistical Methods in Medicinal Research.* 4th ed., Oxford, Blackwell Science 2002, pp. 14-69.
[25] Barakat, H. H, Hussein, S. A. M., Marzouk, M. S., Merfort, I., Linscheid, M., Nawwar, M. A. M., *Phytochemistry* 1997, *46*, 935-941.
[26] Hillis, W. E., Yazaki, Y., *Phytochemistry* 1973, *12*, 2963-2968.
[27] Tanaka, T., Nonaka, G., Nishioka, I., *Chem Pharm Bull.* 1986, *34*, 650-655.
[28] Okuda, T., Yoshida, T., Hatano, T., Yazaki, K., Ashida, M., *Phytochemistry* 1982, *21*, 2871-2874.
[29] Isaza, M. J. H., Ito, H., Yoshida, T., *Phytochemistry* 2004, *65*, 359-367.
[30] Okuda, T., Yoshida, T., Ashida, M., Yazaki, K., *J Chem Soc Perkin Trans. 1*, 1983, 1765-1772.
[31] Moharram, F. A., Marzouk, M. S., El-Toumy, S. A., Ahmed, A. A, Aboutabl, E. A., *Phytother Res.* 2003, *17*, 767-773.
[32] Vivas, N., Laguerre, M., Glories, Y., Bourgeois, G., Vitry, C., *Phytochemistry* 1995, *39*, 1193-1199.
[33] Mabry, T. J., Markham, K. R., Thomas, M. B., *The systematic identification of flavonoids*, Springer Verlag, New York 1970, pp. 35-40.
[34] Agrawal, P. K., Bansal, M. C., Flavonoid glycosides in: *Studies in Organic Chemistry 39, $^{13}C$ NMR of flavonoids.* Agrawal, P.K. (Ed). Elsevier, New York 1989, pp. 287-293.
[35] Wang, X. L., Xing, D. M., Ding, Y., Chen, Y. Y., Meng, Z., Du, L.J., *Chin Pharmacol Bull.* 2005, *21*, 369-372.
[36] Wei, A. C., Li, X. H., Wu, X. L., *Zhengzhou Gong Xue Yaun Xue Bao* 2000, *21*, 62-67.
[37] Atta-Ur-Rahman, Ngounou, F. N., Choudhary, M. I., Malik, S., Makhmoor, T., Nur-E-Alam, M., Zareen, S., Lontsi, D., Ayafor, J. F., Sondengam, B. L., *Planta Med.* 2001, *67*, 335-339.
[38] Festa, F., Aglitti, T., Duranti, G., Ricordy, R., Perticone, P., Cozzi, R., *Anticancer Res.* 2001, *21*, 3903-3908.
[39] Okuda, T., Structure-activity relationship of antioxidant and antitumor polyphenols, in: *Food Factors for Cancer Prevention*, (ed.) Ohigashi, H., Osawa, T., Terao, J., Watanabe, S., Yoshikawa, T., Springer-Verlag: Vienna, Austria 1997, pp. 280-285.
[40] Han, D. H., Lee, M. J., Kim, J. H., *Anticancer Res.* 2006, *26*, 3601-3606.
[41] Lee, S. E., Shin, H. T., Hwang, H. J., Kim, J. H., *Phytother Res.* 2003, *17*, 1041-1047.
[42] Bisen, P. S., Bundela, S. S., Sharma, A., *J Cancer Sci Ther* 2012, *4*, 23-30.
[43] Dhuley, J. N., *Indian J Pharmacol.* 1999, *31*, 128-132.
[44] Tan, P. V., Penlap, V. B., Nyasse, B., Nguemo, *J Ethnopharmacol.* 2000, *73*, 423-428.
[45] Sangiovanni, E., Vrhovsek, U., Rossoni, G., Colombo, E., Brunelli, C., Brembati, L., Trivulzio, S., Gasperotti, M., Mattivi, F., Bosisio, E., Dell'Agli, M., *PLoS One* 2013, *8*, e71762.
[46] Ajaikumar, K. B., Asheef, M., Babu, B. H., Padikkala, J., *J Ethnopharmacol.* 2005, *96*, 171-176.
[47] Celik, I., Temur, A., Isik, I., *Food Chem Toxicol.* 2009, *47*, 145-149.