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Hepatoprotective and Antioxidant Activities of Phenolic Compounds Isolated from *Alpinia zerumbet* (Pers.) B.L. grown in Egypt.

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ABSTRACT

Three phenolic compounds: [chavicol-β-rutinoside], [1,2,di-O-β-D-glucopyranosyl-4-allylbenzene] and [(4E)-1,5-Bis(4-hydroxyphenyl)-2-(hydroxymethyl)-4-penten-1-ol], were isolated from *Alpinia zerumbet* 80% methanolic extract. The compounds were identified by physical and spectroscopic data , PC, TLC, EI/Ms and ^{1}H-NMR and comparing the obtained data with that published ones.The total methanolic extracts and the isolated compounds were subjected for hepatoprotection investigation using *In-vitro* rat hepatocytes monolayer culture. The alcoholic extract exerted the highest hepatoprotective activity followed by the ethyl acetate and the chloroform fractions.The ethyl acetate fraction, and the isolated compounds showed high antioxidant activity. Compound (3) [(4E)-1,5-Bis(4-hydroxyphenyl)-2-(hydroxymethyl)-4-penten-1-ol] showed the highest antioxidant potential.

Keywords: *Alpinia zerumbet*, antioxidant, hepatoprotection, [chavicol-β-rutinoside], [1,2,di-O-β-D-glucopyranosyl-4-allylbenzene], [(4E)-1,5-Bis(4-hydroxyphenyl)-2- (hydroxymethyl)- 4-penten-1-ol], Zingiberaceae.

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INTRODUCTION

Alpinia zerumbet (Pers.) B. L. Burtt and R. M. Smith (syn.: *Alpinia nutans* Roscoe and *Alpinia speciosa* Schum), family Zingiberaceae, is commonly known as shell flower [1, 2]. Its Arabic name is Albinyah [2]. It is an ornamental aromatic plant, grown for its attractive flowers. It is native to North-eastern India and Burma [3]. It is widely used in Brazil [4]. The rhizomes are used for treatment of rheumatism, fever and bronchial catarrh. They are also used as stomachic, stimulant, aphrodisiac, carminative and as a flavouring agent [3]. The oil is prescribed in respiratory troubles, especially in children [3, 5]. It has antispasmodic action, thus, it is useful in intestinal and biliary colics [5]. It is used in folk medicine for its anti-inflammatory, bacteriostatic and fungistatic properties [6]. It is popularly utilized by Brazilian people due to its hypotensive and diuretic properties [7, 8].

Our present work aims for the isolation and purification of phenolic compounds from the plant growing in Egypt, it deals also with the evaluation of its antioxidant and hepatoprotective activity.

MATERIALS AND METHODS

Plant material

Rhizomes of *Alpinia zerumbet* (Pers.) B. L. Burtt & R. M. Smith, used in this study were cultivated in El-Orman garden, Cairo, Egypt and were introduced to the garden of the national research center, Cairo, Egypt, where they yielded well flourished flowering plants. The rhizomes were collected during the years 2006-2011 from this place. The systematic identification of the plant material was kindly verified by Dr. Mohamed El-Gebali, Senior Botanist. The rhizomes of the plant under investigation were air dried, powdered and kept in tightly closed amber coloured glass containers and protected from light at low temperature as possible.

Experimental

General

TLC was carried out on precoated silica gel F_{254} Plates (Merck) (Darmstadt, Germany) developed with $CHCl_3$: MeOH (70:30 v/v solvent a). Whatman 3 MM Pc. Eluted by 15% AcOH (solvent b). Spots were detected using vanillin-H_2SO_4 (vanillin 1% in methanol and 5% H_2SO_4) followed by heating the plates to 110˚C for 15-20 min. Column Chromatography (CC) was performed using polyamide and Sephadex LH-20 (Pharmacia) (Darmstadt, Germany).

NMR was recorded on a NMR Jeol operating at 600 MHz for ^1H. Chemical shifts are presented in ppm down field of CD_3OD.

EI/MS was obtained on VG 70-SEQ Hybrid Mass spectrophotometer.
UV/VIS, 2401 spectrophotometer

Antioxidant activity

6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, Aldrich Chemical Co.), 1,1-Diphenyl-2-picrylhydrazyl (DPPH, Sigma Chemical Co.) and methanol HPLC.

Extraction and Isolation

About 1.5 Kg air-dried powdered rhizomes of *Alpinia zerumbet* (Pers.) were extracted by cold maceration in 80% methanol (using the sonicator agitation 2 h, then it was left overnight then agitation for 2 h, then left overnight) several times (4 × 3L) till exhaustion (no residue was obtained when a small aliquot of colourless extract was evaporated to dryness on a watch glass). The methanolic extracts were combined and evaporated under reduced pressure at a temperature not exceeding 50°C to yield dark brown residue (120 g).

The residue was suspended in distilled water (100 ml) and extracted with successive portions of n-hexane (3 × 300 ml), then chloroform (4 × 300 ml), then ethyl acetate (5 × 300 ml), and finally with n-butanol

(5 × 300 ml) till exhaustion, and all portions were combined and evaporated under reduced pressure to yield a semisolid brownish residue 0.24g, 0.62 g, 2.96 g and 6.79 g, respectively. All these fractions were subjected to antioxidant investigation as mentioned later.

The ethyl acetate fraction was subjected for column chromatography (CC) on polyamide column [120 g polyamide 30-60 mesh (Pharmacia), packed onto glass column (75 x 3.5 cm)]. Elution of the column was carried out using distilled water/ Methanol, decreasing polarity by 10%.

Fraction eluted by water: methanol (2:8 v/v) was found to contain one major fluorescent spot and minor fluorescent spots (R_f: 0.26, 0.52). It was purified on preparative paper chromatography using the solvent system 15% acetic acid (solvent b) to give **compound 1**.

Fraction eluted by methanol 100%, was found to contain a two fluorescent spots. It was purified on sephadex LH_{20} (20 x 1 cm) using 90% methanol as eluent. **Fraction (3-16)** of sephadex column gave two major fluorescent spots and minor fluorescent spots when examined under UV light (R_f: 0.36, 0.52). It was purified using preparative TLC Silica gel plates, using chloroform: methanol (70:30 v/v solvent a) to give **compound 2** and **3**.

On spoting compounds **1**, **2** and **3** on TLC plates, they were found to have the R_f values (0.41, 0.22, 0.87). They were pooled and were purified on sephadex column (solvent methanol).

Determination of total phenolic content

1.5 g of the dried powdered rhizomes of *Alpinia zerumbet* were extracted with 80% methanol. The residue obtained from 80% methanolic extract and the ethyl acetate fraction of 1.5 g of dried powdered rhizomes were transferred separately to a measuring flask 50 mL and the volume was completed with methanol. Total phenolic content (TPC) was measured as chlorogenic acid equivalents using (UV/VIS, 2401 spectrophotometer). The Folin-Ciocalteu reagent was used to determine the TPC according to the method described by Meda *et al* [9]. 1 mL of each extract was mixed with 0.5 mL of 0.2 N Folin-Ciocalteu reagent for 5 min and 1 mL of saturated solution of Na_2CO_3 (40 g/100 mL) was then added. After incubation at room temperature for 30 min, the reaction mixtures were diluted to 10 mL with deionized water. Absorbance of the clear solution was measured at 725 nm. Concentration of the total phenolic content was determined from a standard calibration curve using chlorogenic acid as standard.

Comparison between the innovated techniques and the traditional one in solvent extraction

The yield obtained from the maceration of 100 gm of the dry plant powder using 80% methanol is compared with that obtained from the microwave assisted extraction and the ultrasonic assisted extraction using the same solvent.

Free radical scavenging activity (DPPH) method [Antioxidant activity study]

An aliquot of 0.1 mL methanol solution of DPPH was mixed with methanolic solution of the samples, so that the relative concentration of the plant extract versus the stable radical in the cuvette was 0.13, then the solution with tested sample was shaken vigorously. The absorbance was monitored after 20 min. after being kept in the dark against a blank of methanol without DPPH. All tests were run in duplicate and averaged. The antioxidant activity of these samples were compared with trolox

Where

$$\% \text{ RSA} = 100 \text{ X} \frac{\text{Control absorbance} - \text{Sample absorbance}}{\text{Control absorbance}}$$

Results are expressed as radical scavenging activity (%RSA) as shown in table (3)

DPPH antioxidant test was carried on the isolated compounds from the ethyl acetate fraction. Silica thin layer chromatography (TLC) plates, were spotted with the three compounds (1, 2 and 3) isolated from the ethylacetate fraction. The solvent system (ethyl acetate: formic acid: acetic acid: water) (30:1.2:0.8:8) was used, then the TLC was sprayed with 0.2% (1,1-diphenyl-2-picryl hydrazyl) DPPH.

Rat hepatocytes monolayer culture

Isolation and preparation of rat hepatocytes monolayer culture

A primary culture of rat hepatocytes was prepared according to the Seglen method [10], which was modified by Kiso *et al.,*[11] using a Wistar male rat (250-300g). The rat was obtained from the animal house of the NRC (National Research Centre, Cairo). Animal procedures were performed in accordance with the Ethics Committee of the National Research Centre and followed the recommendations of the National Institutes of Health Guide For Care and Use of Laboratory Animals [12].

IC_{50} determination on rat hepatocytes monolayer culture

After 22-24h, the rat hepatocyte monolayer was washed twice with phosphate buffer saline (PBS). In order to determine IC_{50}, different concentrations were prepared for each sample (100-1000 µg mL^{-1}). After 2h of cells incubation with the extract, cell viability was determined using the MTT assay. The assay was performed according to the method of Mosmann, [13] modified by Carmicheal *et al*. [14] Absorbance of formasan crystals produced by viable cells was read at 540 and 630 nm dual wavelength using the Automatic Kinetic Microplate Reader (Labsystems Multiskan RC reader). Each experiment was repeated three times, and the mean absorption of each concentration was calculated. A graph plotted with X-axis showing the different concentrations of the extract used and the Y-axis showing the absorbance percentage of viable cells. The IC_{50} was graphically determined from the concentration that yielded an absorption coinciding with the 50% of cells that received no extract.

Evaluation of hepatoprotective activity

The primary rat hepatocyte monolayer was prepared as mentioned before (isolation and preparation of rat hepatocytes monolayer culture). Different concentrations were prepared from the tested samples (12.5-100 µg/mL) using the serial dilution technique by dissolving in DMSO (1% maximum concentration). For each concentration, three replicates were carried out; in addition to positive control, that was 50 µg/mL Silymarin. The plate was incubated for 2h at 37˚C and 5% CO_2, then washed twice with PBS. A 200 µL of 25 mM paracetamol was added to each well. After 1h of cells incubation with the paracetamol, cell viability was determined using the MTT assay. The concentration of the extract that was able to protect the cells from the hepatotoxic effect of paracetamol by 100% was considered hepatoprotective.

RESULTS AND DISCUSSION

Compound 1: The compound was obtained as brown wax, of R_f = 0.41 on silica gel TLC, developed by chloroform: methanol (70:30 v/v) (solvent a). **UV spectral data:** The compound showed λ_{max} (MeOH); 221, and 274 nm. **EI/MS** showed molecular ion peak at m/z = 442 which constitutes to the molecular formula ($C_{21}H_{30}O_{10}$). Fragment ion peak at 309 due to ion radical of the gluco-rhamno-pyranosyl ion ($C_{12}H_{21}O_9^+$, 3%), fragment ion peak at m/z 147 due to cleavage of the rhamnosyl radical ($C_6H_{11}O_4^+$, 21%). There is an ion peak corresponds to benzyl radical at (m/z 77, 11%) and (m/z 78, 20%). Also alkoxyl radical may be formed due to the cleavage of sugar moiety to give an ion peak at (m/z 133, 5%).

^{1}H-NMR- spectrum of compound 1

^{1}H-NMR spectrum [table (1)] showed two anomeric protons at δ (4.52, d, J = 1.38), δ (4.77 d, J = 10.2 Hz) indicating α-configuration of the rhamnosyl unit and β-D-configuration of glucopyranosyl unit, respectively.

Also there is a doublet signal at 1.26 corresponds to three protons of CH_3 group of rhamnosyl radical.

There is multiplet protons signal of aliphatic saturated methylene group (CH_2) at δ 3.28, and two (dd) doublet doublet proton signal of terminal unsaturated more deshielded vinylic methylene group at δ 4.97, J = 1.38, 16.5Hz and δ 5.03, J = 1.38, 11.5 Hz. From all these spectroscopic data the compound 1 was found to be in agreement with that reported in literature for the glycosidically bound aromatic compound 1-O-(6-O-α-L-rhamnopyranosyl-β-D-glucopyranosyl)-4-allyl benzene. (chavicol-β-rutinoside) which was previously isolated from the rhizomes of *Alpinia officinarum* [15].

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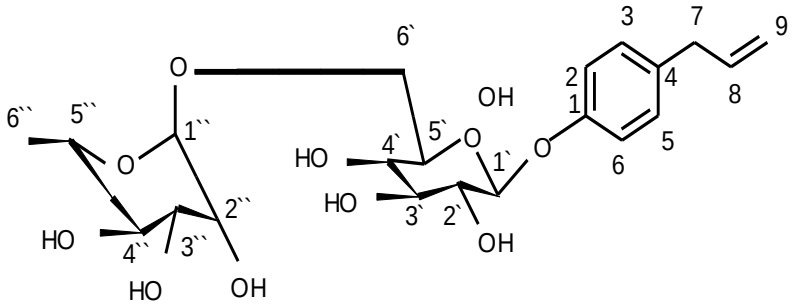

1-O-(6-O-α-L-rhamnopyranosyl-β-D-glucopyranosyl)-4-allylbenzene (chavicol-β-rutinoside)

Compound 2: The compound was obtained as yellow wax, of R_f = 0.22 on silica gel TLC, developed by chloroform: methanol (70:30 v/v). **The UV absorption spectrum** of the compound 2 showed λ_{max} in methanol at 274 nm. **The ^{1}H-NMR spectrum** indicates the presence of trisubstituted benzene protons at δ7.02 (d, J = 2.04 Hz), 6.76 (dd, J = 2.04, 4.04 Hz), 7.51 (d, J = 13.2 Hz) as illustrated in table (2) ,terminal olefinic protons at δ 5.11 (dd, J = 5.5, 7.6 Hz), δ5.24 (dd, J = 7.3, 14.4 Hz), and olefinic proton signal at δ 5.63 indicates the presence of allyl radical. The anomeric protons at δ 4.6 (d), J = 8.94 and δ 4.45 (d), J = 7.56 Hz indicates the β-conjugation of the glucose units. **EI/MS** data showed molecular ion peak at m/z (474, 3%) constitute, to molecular formula ($C_{21}H_{30}O_{12}$), with an important fragment ion peaks at m/z 335 due to the sugar radicals ($C_{12}H_{21}O_9^+$, 4%) and at m/z 162 ($C_6H_{11}O_5^+$, 2%), 118 (C_9H_{10}, 2%), 104 (C_8H_8, 2%), 91 (C_7H_7, 4%), 78, 77 (C_6H_6, $C_6H_5^+$) benzyl radicals.

From all these spectroscopic data the compound 2 was found to be in agreement with that reported in literature for the glycosidically bound aromatic compound **1,2,di-O-β-D-glucopyranosyl-4-allylbenzene** , which was previously isolated from the rhizomes of *Alpinia officinarum* [15].

Table 1: ^{1}H-NMR assignements of compounds (1) & (2) in (CD_3OD):

C.No	Compound (1)	Compound (2)
2	7.11(d), J=8.8	-------
3	6.76(d), J=8.25	7.02 (d), J = 2.04
5	6.76(d), J=8.25	6.76 (dd), J = 2.04, 4.04
6	7.11(d), J=8.8	7.51 (d), J = 13.2
7	3.28 (m)	3.11 (d), J = 7.5
8	6.28(dt)	5.63 (dd), J = 2.9, 3.5
9a	4.97(dd), J=1.38, 16.5	5.11 (dd), J = 5.5, 7.56
9b	5.03(dd), J=1.38, 11.5	5.24 (dd), J = 7.26, 14.4
1'	4.77(d), J=10.2	4.60 (d), J = 8.94
2'	2.29(m)	3.27 – 3.28 (m)
3'	3.16-3.27(m)	3.45 – 3.54 (m)
4'	3.16-3.27(m)	3.54 – 3.62 (m)
5'	3.32(ddd), J=1.38, 6.5, 11	3.61 – 3.63 (m)
6'a	3.39(dd), J= 6.8, 6.8	3.67 (dd), J = 4.14, 24.7
6'b	4.02(dd), J=1.38, 15	3.92(dd), J = 1.38, 10.98
1"	4.52(d), J=1.38	4.45 (d), J = 7.56
2"	3.38(dd), J=1.38, 2	3.27 – 3.28
3"	3.62(dd), J= 2.4, 6.8	3.45 – 3.54
4"	3.16-3.27(m)	3.54 3.62
5"	3.42(dd), J=3.04, 7.9	3.61 – 3.63
6"a	H6"(3H) 1.2(d), J=6.2	3.67(dd), J = 4.14, 24.7
6"b		3.92 (dd), J = 1.38, 10.98

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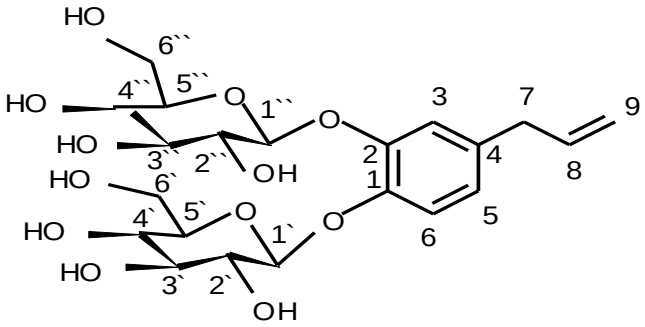
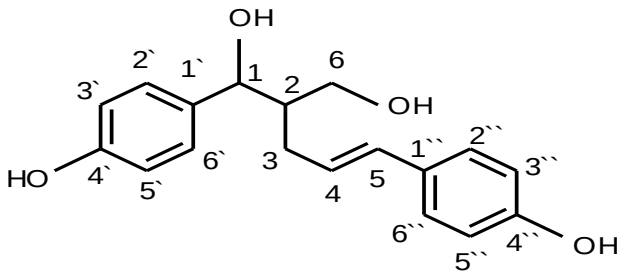


1,2,di-O-β-D-glucopyranosyl-4-allylbenzene

Compound 3: The compound was obtained as colourless wax, of R_f = 0.87 on silica gel TLC, developed by chloroform: methanol (70:30 v/v). **The UV spectrum** of the compound in methanol showed λ_{max} at 260 nm. **EI/MS** of the compound showed molecular ion peak at m/z (300, 2%) corresponds to the molecular formula $C_{18}H_{20}O_4$. With fragment ions peak at m/z = 282 due to loss of one molecule of H_2O (M-H_2O), 264 (M-2H_2O), 252 [M-(H_2O + HCHO)], 199 (3%), 166 (11%), 158 (5%), 145 (8%), 133 (10%), 123 (8%), 107 (13%), 78 (13%), 77 (17%).

The ^{1}H-NMR spectrum of the compound in (CD_3OD) table (3) indicates the presence of four doublet proton signals at δ in ppm 6.70, 6.78, 7.41, 7.53 due to two para substituted benzene rings corresponds to (H - 3", 5"), (H - 3', 5'), (H - 2", 6") and (H - 2", 6") as shown in table (). Two trans olefinic protons as two doublets at δ 5.97 (d, J = 15.75 Hz); δ 6.27 (d, J = 15.76 Hz). Also two methylene protons (ddd) as illustrated in table (2) at δ 2.07, 2.28, 3.52 and 3.89 correspond to H - 3a, H - 3B and H - 6a , H – 6b.

From all these spectroscopic data the compound 3 was found to be in agreement with that reported [16] for the phenylpropanoid compound **(4E)-1,5-Bis(4-hydroxyphenyl)-2-(hydroxymethyl)-4-penten-1-ol** which was previously isolated from the rhizomes of smaller Galanga (*Alpinia officinarum*) [16].



(4E)-1,5-Bis(4-hydroxyphenyl)-2-(hydroxymethyl)-4-penten-1-ol

Table 2: ^{1}H-NMR spectrum of compound (3) in (CD_3OD):

C. No.	Compound (3)
2	1.59 (m, 1H, H-2)
3a	2.07(dd, J = 7.5, 9.5 Hz, 1 H)
3b	2.28 (ddd, J = 7.56, 7.56, 15 Hz)
6a	3.52 (dd, J = 6.8, 6.9 Hz, 1 H, H-6a)
6b	3.66 (dd, J = 4.8, 10.98 Hz, H-6b)
1	4.58 (br.S), 1H, H-1)
4	5.79 (d), J = 12.4 Hz, H-4)
5	6.27 (d, J = 15.78 Hz, H-5)
3", 5"	6.78 (d, J = 8.2 Hz)
3', 5'	6.70 (d, J = 8.9 Hz)
2", 6"	7.41 (d, J = 10.8 Hz)
2', 6'	7.53 (d, J = 8.9 Hz)

This is the first time to isolate these compounds from the rhizomes of *Alpinia zerumbet*

Quantitative estimation of Total Phenolic Content

Total Phenolic Content of the 80% methanol extract and ethyl acetate fraction were expressed as mg of chlorogenic acid equivalents/ 1.5 g of the plant could be calculated from the following equation

$$Y=6.4X+0.065$$

Where y is the absorbance and x is the corresponding concentration mg/ml. 100 g of 80% methanolic extract contains 8.844 g chlorogenic acid equivalents. 100 g of ethyl acetate fraction contains 10.5 g chlorogenic acid equivalents.

Comparison between the innovated techniques and the traditional one in solvent extraction

Table 3: Comparison between the innovated techniques and the traditional one in solvent extraction

	Maceration	MAE	UAE
Weight of the used plant	100 gm	100 gm	100 gm
Solvent used	80% methanol	80% methanol	80% methanol
Solvent volume	500 ml x 3	500 ml	500 ml
Extraction time	3 days	20 mins	30 mins
Conditions	On cold	800 w, 90 ºC, medium stirring, stabilizing conditions 10 mins and holding conditions for 10 mins	Amplitude%: 100 cycles/ 0.5
Yield (gm)	5.1	6.26	5.32
Yield (%)	5.1	6.26	5.32

MAE: microwave assisted extraction
UAE: ultrasonic assisted extraction

From the above results we can conclude that the microwave assisted extraction (MAE) was the best technique. Since it gave the highest yield (6.26 %), at the same time it saves time and solvents

Radical scavenging activity

Table 4: The radical scavenging effect of samples on DPPH radical

Tested samples	Mean absorbance.	Radical scavenging activity %
Trolox	0.01	98.8
Aqueous extract	0.2	67
Methanolic extract	0.33	46
Chloroform fraction	0.411	42.6
Ethyl acetate fraction	0.084	86.2
Butanol fraction	0.08	56

From the obtained results in table (4), it is clear that the highest antioxidant activity was found in the ethyl acetate followed by aqueous extract followed by butanol fraction (86.2%, 67%, and 56%, respectively). This could be attributed to the presence of phenolic compounds which are known for their high antioxidant activity.

The three compounds (1, 2 and 3) isolated from the ethylacetate fraction, exerts a free radical scavenging activity with (1,1-diphenyl-2-picryl hydrazyl) DPPH, which appears as yellow spots due to the decolourization of the (1,1-diphenyl-2-picryl hydrazyl) DPPH by free radicals in their zones. Compound 3; [(4E)-1,5-Bis (4-hydroxyphenyl)-2-(hydroxymethyl-4-penten-1-ol)], have antioxidant activity higher than that of

compound 1; [1-O-(6-O-α-L-Rhamnopyranosyl-β-D-glucopyranosyl)-4-allylbenzene (chavicol-β-rutinoside)], and compound 2; [1,2-di-O-β-D-glucopyranosyl-4-allylbenzene].

This was the first time to examin the antioxidant activity of compound 1;[1-O-(6-O-α-L-Rhamnopyranosyl-β-D-glucopyranosyl)-4-allylbenzene (chavicol-β-rutinoside)] and compound 2; [1,2-di-O-β-D-glucopyranosyl-4-allylbenzene], Also Tram, Ngoc, Ly, et al, [16] reported that the compound 3; [(4E)-1,5-Bis (4-hydroxyphenyl)-2-(hydroxymethyl-4-penten-1-ol)] isolated from have antioxidant activity.

Hepatotoxicity

The assay was applied with a broad range of concentrations of the studied extracts (from 125 to 1000 µg/mL) on the monolayer of rat hepatocytes. It revealed that the methanolic extract has IC_{50} at a concentration of 500 µg/ml, while the aqueous extract, chloroform, ethyl acetate and butanol fractions showed no hepatotoxicity till a concentration of 1000 µg/ml, as shown in tables (5).

Table 5: The hepatotoxicity of different concentrations of the methanolic, the aqueous extracts, the chloroform, the ethyl acetate and butanol fractions of the rhizomes of *Alpinia zerumbet* (Pers.) B.L.

Sample concentration µg/mL.	Mean absorbance percentage (n=3)				
	Methanolic extract	Aqueous extract	Chloroform fraction	Ethyl acetate fraction	Butanol fraction
125	70	76	93	83	70
250	60	75	96	79	80
500	50	90	98	80	94
1000	40	80	99	75	97

Evaluation of the hepatoprotective activity

The assay was applied with a broad range of concentrations of the studied extracts (from 12.5-100 µg/ml) on monolayer of rat hepatocytes. It revealed that the methanolic extract exerted 100% hepatoprotection at a concentration 25-50 µg/ml, while the aqueous extract exerted 100% hepatoprotection at a concentration 100 µg/ml. The chloroform and the ethyl acetate fractions exerted 95% hepatoprotection at a concentration of 25 µg/ml, while the butanol fractions exerted 85% hepatoprotection at concentration of 25 µg/ml, as shown in tables (6).

Table 6: The hepatoprotective activity of different concentrations of the methanolic, the aqueous extracts, the chloroform, the ethyl acetate and the butanol fraction of the rhizomes of *Alpinia zerumbet* (Pers.) B.L.

Sample concentration µg/mL.	Mean absorbance percentage				
	Methanolic extract	Aqueous extract	Chloroform fraction	Ethyl acetate fraction	Butanol fraction
12.5	62	70	92	93	81
25	96	81	94	95	85
50	95	85	94	95	83
100	75	100	95	95	83

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