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Bioguided Isolation and *in-Silico* Analysis of Hep-G2 Cytotoxic Constituents From *Laurus nobilis* Linn. Cultivated In Egypt

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Abstract
The air dried leaves of *Laurus nobilis* cultivated in Egypt were extracted by petroleum ether followed by methylene chloride then ethyl acetate and finally methanol. The preliminary cytotoxic screening of these extracts against *Hep-G$_2$* using vinblastine sulfate (IC_{50}= 2.93 µg/ml) as standard showed that petroleum ether, methylene chloride and ethyl acetate extracts exhibited strong cytotoxic activity (IC_{50}= 10.6, 5.96 and 3.80 µg/ml, respectively) while the methanolic extract showed moderate activity (IC_{50}= 23.2 µg/ml). Bio-guided chromatographic isolation of *L. nobilis* extracts resulted in isolation of five compounds identified as 1-tricosanol **(1),** reynosin **(2)**, protocatechuic acid **(3)**, vincetoxicoside B **(4)** and vitexin **(5)**. The chemical structures have been established on the basis of physical, chemical and spectroscopic methods (UV, IR, MS, ^{1}H-NMR and ^{13}C-NMRand HSQC) in addition to comparison with literature data and /or authentic samples. Reynosin **(2)** showed the most potent cytotoxic activity against *Hep-G$_2$* (IC_{50}= 4.98 µg/ml) among other isolated compounds, while vitexin **(5)** showed the lowest activity (IC_{50}= 219 µg/ml). Other compounds showed moderate to weak activity. The isolated compounds were docked to caspase3 to reveal their possible cytotoxic mechanism of action on the enzymatic level. Protein fraction obtained after 0.5 hr hydrolysis with papain showed moderate cytotoxic activity (IC_{50}= 200 µg/ml) and that obtained after 3.5 hr hydrolysis exhibited strong antioxidant action (45 % inhibition at a concentration of 200 µg/ml using DPPH assay).

Keywords: Laurus nobilis; Cytotoxicity; *Hep-G$_2$*; Reynosin; Molecular modeling; Protein hydrolysis; Antioxidant.

1. Introduction

Liver cancer is the fourth leading cause of death among other cancer types with percentage of 8.2%. It is responsible for 782,000 deaths annually in 2018. Egypt has the second highest rate of liver cancer after Mongolia in 2018 with 32.2/100,000 Age-standardized rate (ASR) in both sexes. The most common type of liver cancer is hepatocellular carcinoma (HCC) [1]. Many predisposing risk factors for HCC are present including chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV), aflatoxin contaminated foodstuffs, metabolic diseases, heavy alcohol intake, obesity, smoking, and type 2 diabetes [2-4].

Nature is a rich source of biological and chemical diversity. The unique and complex structures of natural products cannot be obtained easily by chemical synthesis. At present, scientists show considerable interest among the discovery of new potential anticancer agents from plants [5, 6]. *Laurus nobilis* Linn. also known as sweet bay leaf, true bay and bay laurel, is a small aromatic evergreen tree of Lauraceae family. The plant is native to the Mediterranean region, Asia and it is cultivated in Algeria, Morocco, Turkey, Italy, Portugal, Spain and Russia [7]**.** Historically, Laurus leaves were considered as symbol for honor, victory and peace. Ancient Greek and Romans used laurel wreaths to crown their heroes and victors [8, 9]**.** Many studies on *L. nobilis* revealed the presence of sesquiterpenoids [10], flavonoids and flavonoidal glycosides [11], anthocyanins and proanthocyanidines [12, 13], phenolics [14], norisoprenoids [15], tocopherols [16], fixed oils [17], alkaloids [18], organic acids [19] and sugars [20].

Laurus nobilis showed wide variety of biological activities including antimicrobial [21], antifungal [22], analgesic and anti-inflammatory [23], antioxidant [24], neuroprotective [25] and cardioprotective [26] activity. Also, different extracts

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and oil of *L. nobilis* exhibited cytotoxic activity against breast cancer cells [27], leukemia cells [28-30], human neuroblastoma [31], cervical epithelial carcinoma [32] and colon carcinoma [33].

In the light of the previous information, we aimed in the present study for bio-guided isolation of *L. nobilis* active constituents that have cytotoxic activity against hepatocellular carcinoma (*Hep-G$_2$*).Organization of the manuscript

2. Experimental

2.1. Plant material

The dried leaves of *Laurus nobilis* Linn. were collected from El-Orman Botanical Garden, Giza, Egypt on April 2016. The plant was kindly identified by Mrs. Traes Labib, general manager and head of specialists for plant taxonomy in El-Orman Botanical Garden, Giza, Egypt. A voucher specimen (with number L.no.1) was kept in the Pharmacognosy Department, Faculty of Pharmacy, Zagazig University, Zagazig, Egypt.

2.2. Spectroscopic and Chromatographic analyses

UV spectra was measured in methanol by Shimadzu U.V.-1700 Spectrophotometer (Japan); IR was carried out on: FT/IR- 6100 type A (Jasko, Germany); EI-MS was performed by Shimadzu MS-QP5050-A, 70 ev; ^{1}H-NMR and ^{13}C-NMR spectra were recorded in $CDCl_3$, CD_3OD and DMSO at 400 and at 100 MHz, respectively using Bruker (Switzerland) spectrometer; Chemical shifts were given in ppm with the TMS as internal standard. Silica gel (Merck 60 - 230 mesh) and sephadex LH-20 (Fluka, Germany) were used for column chromatography, silica gel coated aluminum plates (Merck 60 F_{254}, Germany) and precoated cellulose sheets (Merck) were used for TLC. Developed chromatograms were visualized under UV light, spraying with anisaldehyde/sulphuric acid reagent followed by heating at 100°C for 10 min. The solvents used in this work, ammonium hydroxide, sulphuric acid, ferric chloride and glacial acetic acid were of the analytical grade. Reference compounds including sterols, flavonoids and sugars were obtained from Department of Pharmacogonosy, Faculty of Pharmacy, Zagazig University.

For the TLC analysis, the following solvent systems were used:

System I: petroleum ether: methylene chloride: methanol (5:5:0.25)

System II: methylene chloride: methanol (9.5:0.5)

System III: petroleum ether: methylene chloride: methanol (15:15:1)

System IV: ethyl acetate: formic acid: glacial acetic acid: water (10:1.1:1.1:2.7)

2.3. Extraction, preliminary cytotoxic study and bio-guided isolation

Air dried powdered leaves of *L. nobilis* (5 kg) were successively extracted with four different organic solvents, petroleum ether followed by methylene chloride then ethyl acetate and finally methanol. The different extracts were individually dried over anhydrous sodium sulphate and freed from the solvent by evaporation under reduced pressure to afford 52 g, 7 g, 15 g and 50 g, respectively. About 10 mg sample from each extract was tested for the cytotoxicity against *Hep-G$_2$* cell line using cell viability assay test and vinblastine sulfate (IC_{50}= 2.93 μg/ml) as standard.

About 15 g of petroleum ether soluble fraction was subjected to column chromatography over silica gel (370 g; 3x100 cm). The elution started with petroleum ether and the polarity was increased gradually with methylene chloride. Column fractions eluted with 50% methylene chloride in petroleum ether and 100% methylene chloride afforded **compounds 1** (25 mg).

In addition, about 7 g of methylene chloride soluble fraction was subjected to column chromatography over silica gel (175 g; 3x50 cm). The elution started with petroleum ether and the polarity was increased gradually with methylene chloride then methanol. Column fractions eluted with 100% methylene chloride were combined and concentrated to afford **compound 2** (42 mg) after crystallization from methylene chloride-methanol mixture.

Moreover, the methanol extract was suspended in water and fractionated against methylene chloride and ethyl acetate, about 10 g of this ethyl acetate soluble fraction was subjected to column chromatography over silica gel (250 g; 3x70 cm). The column elution started with methylene chloride and the polarity was increased gradually with ethyl acetate then methanol. Column fractions eluted with 50% ethyl acetate in methylene chloride (0.9 g) was re-chromatographed over Sephadex LH-20 and eluted with methanol to give **compound 3** (15 mg). Column fractions eluted with 75% ethyl acetate in methylene chloride and 100% ethyl acetate were subjected to frequent crystallization from methanol to yield **compound 4** (23 mg) and **compound 5** (20 mg), respectively.

2.4. Protein extraction, precipitation and purification

About, 30g of dried powdered leaves of *L. nobilis* were defatted using methylene chloride (0.25 L x5). The defatted dry powder was dispersed in distilled water 10% (w/v) and the pH adjusted to 7 with 1N sodium hydroxide and kept in constant stirring for 5 hours at 25˚C. Then the contents were filtered and centrifuged at 10000 xg for 15 min at 4℃. The crude supernatant was further treated with ammonium

sulphate for protein precipitation, with various saturation limits from 20 to 80%. The precipitated protein obtained using 80% saturation ammonium sulphate was centrifuged at 15000 xg for 15 min at 4℃. Supernatant was decanted and the obtained precipitate was reconstituted in distilled water and the pH adjusted to 7 with 1N sodium hydroxide. The reconstituted pellets were dispensed into dialysis bag for purification. The dialysis bag is with one end closed and the other end were clamped with clamps while keeping sufficient space above the sample and placed in a beaker containing more than ten times volume of distilled water. Occasional shaking was used to improve solute exchange. The distilled water was changed once in four hours for 24 h [34, 35]. After the purification process, the protein solution was lyophilized and stored at -20℃ for further use.

2.5. Enzymatic hydrolysis of isolated protein

Lyophilized protein isolated from *L. nobilis* was dissolved in 0.1M Na_2HPO_4-NaH_2PO_4 buffer pH 6.0 (100 g L^{-1}) and hydrolyzed by treatment with papain (E/S ratio of 1:200 (w/w)) at 37 °C and pH 6.0. The hydrolysis was allowed to proceed for 3.5 h, and the degree of hydrolysis was determined according to previously published method [36] at 30 minutes time intervals. At the end of the hydrolysis, the enzyme was inactivated by heating in a boiling water bath for 15 min. Hydrolysate was clarified by centrifugation at 4000 xg for 30 min at 4 °C to remove insoluble substrate fragments, and the supernatant was lyophilized and frozen at -20°C until further use.

2.6. Cytotoxic activity

Cytotoxicity of *L. nobilis* extracts, fractions, isolated compounds and hydrolyzed protein fractions were assessed using cell viability assay as reported before [37, 38]. The cells of *Hep-G$_2$* were seeded in 96-well plate at a cell concentration of 1×10^4 cells per well in 100µl of growth medium (Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 1% L-glutamine, HEPES buffer and 50µg/ml gentamycin). After 24 h of seeding, fresh media containing different concentrations of the tested samples (Two-fold serial dilutions dispensed into 96-well and incubated at 37ºC in a humidified incubator with 5% CO_2 for a period of 48 h) were added. Three wells were used for each concentration of the tested samples. Control cells were incubated without any tested samples and with or without DMSO. The little percentage of DMSO present in the wells (maximal 0.1%) was found not to affect the experiment. The incubation was continued for 24 h and after that, media were aspirated and crystal violet solution (1%) was added to each well for at least 30 minutes. The excess stain was removed and glacial acetic acid (30%) was then added to all wells, mixed thoroughly, and then the optical density (OD) was measured with the microplate reader (SunRise, TECAN, Inc, USA) to determine the number of viable cells and the percentage of viability was calculated as:

Cell viability% = $[1- (OD_{sample}/OD_{control})]$ x100%

Treated samples were compared with the cell control in the absence of the tested compounds. All experiments were carried out in triplicate. The cell cytotoxic effect of each tested compound was calculated. The relation between surviving cells and drug concentration was plotted to get the survival curve of tumor cell line after treatment with the specified compound. The 50% inhibitory concentration (IC_{50}) was estimated from graphic plots of the dose response curve for each concentration using Graphpad Prism software (San Diego, CA. USA). Vinblastine sulfate (IC_{50} = 2.93 µg/ml) was used as standard.

2.7. Molecular docking study

Molecular docking of isolated compounds from *L. nobilis* leaves extracts was performed using Molecular Operating Environment 2009 (MOE) as previously described [39]. Shortly, the compounds were constructed in 3D structure and their energies were minimized and saved to MDB file. Caspase3 X-ray crystallographic structure coded as 2J30 was downloaded from the Protein Data Bank (R.P.D. Bank, RCSB PDB: Homepage, 2020. https://www.rcsb.org/. The downloaded protein structure was protonated, corrections were done automatically for missed connections and their types and the potential was fixed for receptor and its atoms. The enzyme's active site was determined relying on co-downloaded natural ligand and using surfaces and maps. The constructed database of the isolated compounds was docked against caspase3 using triangle matcher for placement; London dG with ten retains for scores and forcefield for refinement. Energy, root-mean square deviation (rmsd) and formed interactions (bonds) were examined to select between the resulted poses.

2.8. Antioxidant activity of protein hydrolysate

The antioxidant activity of *L. nobilis* protein hydrolysates obtained after 0.5, 1, 1.5, 2, 2.5, 3 and 3.5 hr was evaluated using DPPH free radical scavenging activity method as reported before [40] with slight modification to select the highest activity hydrolysate. A 95% ethanolic solution of DPPH radicals was freshly prepared at a concentration of 200 µg/ml and stored at 10°C in the dark. For evaluation, one milliliter of each hydrolysate fraction was mixed with 3 ml of 0.15 mM DPPH (in 95% ethanol) and the mixture was then shaken vigorously using a mixer. After that, the reaction mixture was incubated for 30 min in the darkness at room temperature and the absorbance measurements for the resulting solutions were recorded with a UV-260 visible

spectrophotometer (Shimadzu, Kyoto, Japan) at 517 nm. Ethanol was used as a control. The radical scavenging capacity of the samples was measured as a decrease in the absorbance of DPPH radical and it was calculated using the following equation:

$$\% \, DPPH \, radical \, scavenging = \frac{Abs.control - Abs.sample}{Abs.control} \, x \, 100$$

3. Results and discussion

3.1. Preliminary cytotoxic screening and bio-guided isolation and characterization of Hep-G2 cytotoxic compounds

Cytotoxic screening of *L. nobilis* leaves extracts (Fig. 2 and Table 1) against *Hep-G*$_2$ showed that petroleum ether, methylene chloride and ethyl acetate extracts had strong cytotoxic activity (IC_{50}= 10.60, 5.96, 3.80 µg/ml, respectively), while methanolic extract showed moderate activity (IC_{50}= 23.2 µg/ml) compared to vinblastine sulfate (IC_{50} = 2.93 µg/ml) as a standard. The extracts that showed strong cytotoxic activity were subjected to further investigation and bio-guided chromatographic isolation resulting in isolation of 5 compounds. The chemical structures of the isolated compounds are shown in Fig. 1.

Compound 1 was isolated from the petroleum ether extract and eluted by 50% methylene chloride in petroleum ether. The IR spectrum showed absorption bands for hydroxyl group (3424 cm^{-}1) and long aliphatic chain (722 cm^{-1}). ES-MS showed characteristic mass fragments at *m/z* 322 (M^+-H_2O) and 209 [M^+-H_2O- $(CH_2)_7$-CH_3]. Other fragments appeared at *m/z* 153, 125 and 97 are a result of subsequent loss of CH_2 groups. The ^{1}H-NMR showed two triplet signals at δ_H 3.65 ppm (2H, J = 6.8, 6.4 Hz) corresponding to the CH_2 adjacent to the hydroxyl group and at δ_H 0.89 ppm (3H, J = 7.2, 6.4 Hz) assigned for the terminal C-23 methyl group. The other methylene protons appeared at δ_H 1.28-1.56 ppm. The ^{13}C-NMR spectra showed signal for the C-1 adjacent to the hydroxyl group at δ_C 63.12 ppm. The terminal methyl group appeared at δ_C 14.12 ppm. Other methylene carbons appeared in the range of δ_C 22.7-32.83 ppm. On the basis of the above discussion, and by comparison with the previously reported data [41-43], compound 1 was confirmed to be 1-tricosanol ($C_{23}H_{48}O$). To our knowledge, this is the first report on isolation of 1-tricosanol from *Laurus nobilis*.

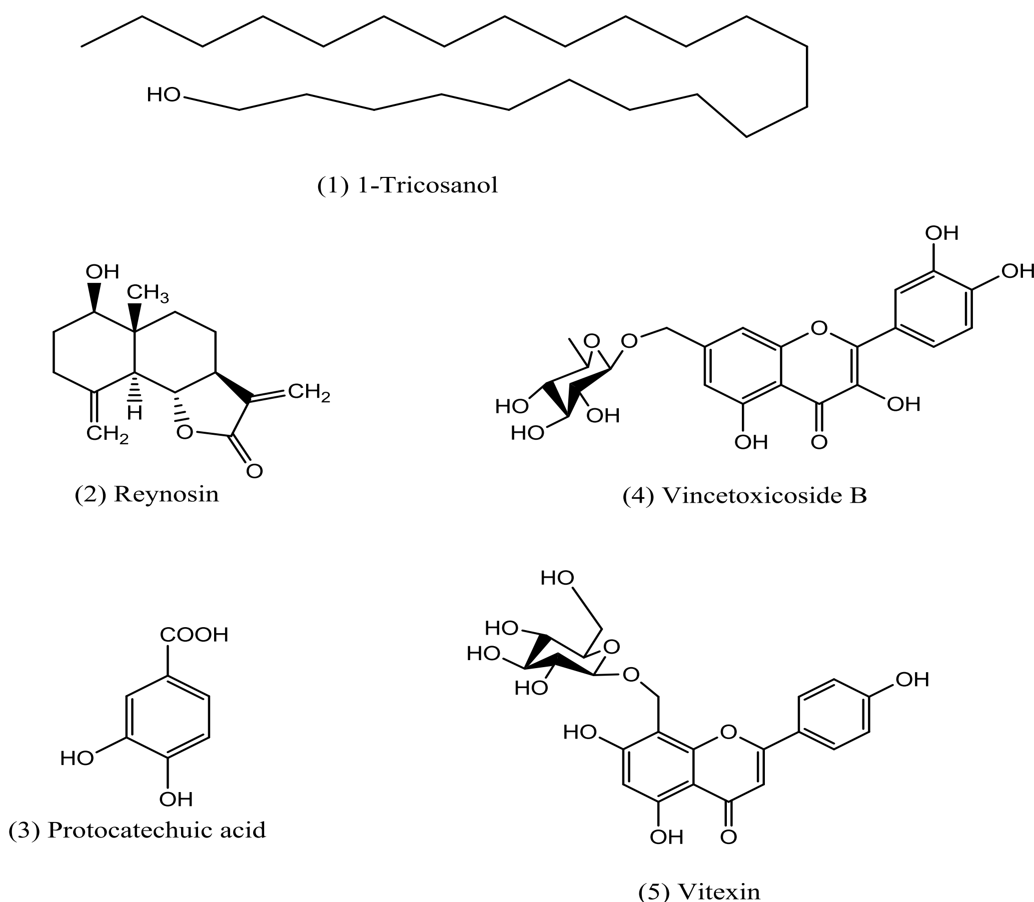


Figure 1: Chemical structures of isolated compounds from *L. nobilis* leaves' extracts.

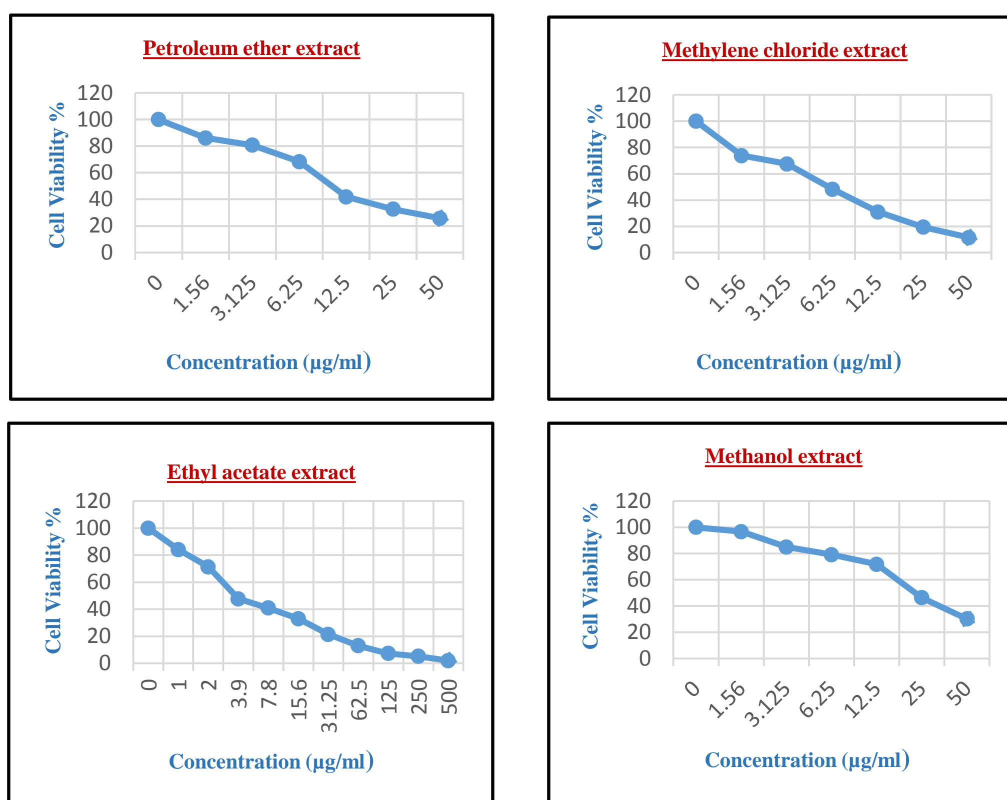


Figure 2: Percentage of cell viability of *Hep-G2* cells treated with different concentrations of *L. nobilis* extracts.

Table 1: The IC_{50} (µg/ml) of *L. nobilis* extracts, isolated compounds and hydrolyzed protein fractions.

Tested material	IC_{50} (µg/ml)	Tested material	IC_{50} (µg/ml)
Petroleum ether extract	10.60	protein fraction 1 (0 time)	< 500
Methylene chloride extract	5.96	protein fraction 2 (0.5 h)	200
Ethyl acetate extract	3.80	protein fraction 3 (1.0 h)	< 500
Methanol extract	23.20	protein fraction 4 (1.5 h)	< 500
Compound 1	35.20	protein fraction 5 (2.0 h)	< 500
Compound 2	4.98	protein fraction 6 (2.5 h)	< 500
Compound 3	96.30	protein fraction 7 (3.0 h)	< 500
Compound 4	111	protein fraction 8 (3.5 h)	< 500
Compound 5	219		

Table 2: UV data from spectral analysis of compounds 4 and 5.

Shifting reagent	Compound 4		Compound 5	
	Band II	Band I	Band II	Band I
MeOH	257	349, 327 (sh)	270	329
MeOH + NaOMe	270	394	279, 228 (sh)	392
MeOH + $AlCl_3$	273	414	271	341, 304 (sh)
MeOH + $AlCl_3$ + HCl	269	400, 356 (sh)	276	340, 303 (sh)
MeOH + NaOAc	259	348	275	340
MeOH + NaOAc + Boric acid	261	364	270	337, 300 (sh)

Compound 2 was separated from methylene chloride extract and eluted by 100% methylene chloride. The ES-MS spectrum showed a molecular ion peak $[M]^+$ at *m/z* 248 ($C_{15}H_{20}O_3$) along with other important mass fractions at *m/z* 230 (M^+-H_2O) and 215 (M^+-H_2O-CH_3). The IR spectrum showed characteristic absorption bands at 3354 (hydroxyl group), 1898 (C-H bending), 1771 (C=O of γ-lactone) and 1646 cm^{-1} (C=C stretching). The ^{1}H-NMR showed four downfield signals corresponding to two exocyclic methylene groups, two protons on CH_2-13 resonate at δ_H 6.09 and 5.41 ppm (*br s* each) and the other protons (CH_2-15) resonate at δ_H 4.99 and 4.86 ppm (both 1 H, s). Also, a sharp singlet appeared at δ_H 0.82 ppm corresponding to the methyl group on C-14. The proton on C-1 appeared with a downfield shift at δ_H 3.51 ppm (1H, *dd*, *J*=11.02, 11.6 Hz) due to its direct attachment with a hydroxyl group. The ^{13}C-NMR spectrum showed a total of fifteen signals. The carbonyl appeared at δ_C 170.78 ppm along with other signals at δ_C 139.36 ppm (C-11), 117.22 ppm (CH_2-13), 79.71 ppm (CH-6) and 49.72 ppm (CH-7). These signals in conjunction with the characteristic methylene proton resonances at δ_H 6.09 ppm (1 H, *br s*) and 5.41 ppm (1 H, *br s*) in the ^{1} H-NMR spectrum suggest the presence of a *γ*-lactone moiety with an exocyclic methylene group. Moreover, olefinic carbon signals at δ_C 142.59 ppm (C-4) and 110.80 ppm (CH_2-15), together with proton resonances at δ_H 4.99 ppm and 4.86 ppm (both 1 H, *s*), were indicative of another exo-double bond. This spectroscopic data showed that compound 2 possesses a bicyclic carbon skeleton, possibly eudesmane sesquiterpene skeleton. On the basis of the above discussion, and by comparison with the previously reported data [50-52], compound 2 was proved to be reynosin. To our knowledge, this is the first report of isolation of reynosin from *L. nobilis* growing in Egypt.

Compound 3 was isolated from ethyl acetate extract after purifying fractions eluted by 50% ethyl acetate in methylene chloride over Sephadex LH-20. It gave yellow colour with ammonium hydroxide, brown charring with 50% aqueous sulphuric acid and green colour with ferric chloride (T.S.). The ES-MS spectrum showed a molecular ion peak $[M]^+$ at *m/z* 154 ($C_7H_6O_4$). The ^{1}H-NMR exhibited three aromatic proton signals at different chemical shifts and splitting suggesting that they are not magnetically equivalent. Two of them appeared at δ_H 7.43 (1H, *br s*, H-2) and 7.41 ppm (1H, *dd*, J= 8.0, 2.0 Hz, H6) suggesting that they are subjected to the deshielding effect of carboxylic group. The other aromatic signal appeared at δ_H 6.79 (1H, *d*, J= 8.0 Hz, H-5). All that supported the 3,4- di-hydroxyl substitution. The ^{13}C-NMR spectra of this compound showed seven different, non-equivalent carbon signals at δ_C 169.03, 150.08, 144.65, 122.49, 121.89, 116.34 and 114.36 corresponding to C=O, C-4, C-3, C-6, C-1, C-2 and C-5, respectively. On the basis of the above discussion, and by comparison with the previously reported data [53-55], compound 3 was proved to be protocatechuic acid. To our knowledge, this is the first report of isolation of protocatechuic acid from the Egyptian *L. nobilis*.

Compound 4 was eluted from the ethyl acetate extract's column using 75% ethyl acetate in methylene chloride. It coloured yellow with dilute solution of alkalies and bluish green with neutral ferric chloride. A yellow colour develops after addition of few drops of 0.01 M $AlCl_3$. Compound 5 tested positive with Molisch's reagent and reduced Fehling's solution after acid hydrolysis [56]. All that suggested the presence of flavonoidal glycoside skeleton. the UV spectral analysis in methanol (Table 2) showed two absorption bands at 349 nm and 257 nm indicating its flavonol skeleton [57]. The presence of free hydroxyl group at C-4' was confirmed by bathochromic shift after the addition of NaOMe. The presence of ortho-dihydroxylation and free hydroxyl groups at C-3 and C-5 was confirmed after addition of $AlCl_3$ followed by HCl. ES-MS showed characteristic mass fragment at *m/z* 302 (M^+-sugar) in addition to other fragments at *m/z* 153 and 137 that are corresponding to ring A and ring B with two hydroxyl groups, respectively. In ^{1}H-NMR (Table 3), three signals appeared at δ_H 7.36 (1H, *d*, J= 1.6 Hz, H-2'), 7.32 (1H, *dd*, J=8.4, 1.6 Hz, H-6') and at 6.92 (1H, *d*, J= 8.4 Hz, H-5') that are characteristic for ABX system of B-ring. Other two doublet signals appeared in the aromatic region at δ_H 6.22 (1H, J=1.2 Hz, H-6) and at 6.39 (1H, J=1.6 Hz, H-8) suggesting a 5, 7, di-substituted A ring. The glycosidic nature was confirmed by the one anomeric proton signal at δ 5.37 (1H, br s) and a doublet at 0.96 (3H, d, J= 6.4 Hz) suggesting the presence of methyl hexose as one sugar unit. The ^{13}C-NMR (Table 4) exhibited 21 carbon signals. These signals include one carbonyl at δ_C 178.26 (C-4) characteristic for the flavonol skeleton [58], nine quaternary carbons at δ_C 104.62, 121.59, 136.05, 145.03, 148.41, 157.14, 157.92, 161.82 and 164.51 (C-10, C-1`, C-3, C-3`, C-4`, C-2, C-9, C-5 and C-7, respectively) and one methyl at δ_C 16.25 (C-6``). The sugar carbons ranged from δ_C 70.51-71.87 ppm. In HSQC, the anomeric proton (H-1'') was easily assigned because its peak at 5.37 ppm was attached directly to ^{13}C peak at 102.15 ppm. Moreover, the doublet proton signal resonates at δ_H 0.96 is directly attached to carbon atom at δ_C 16.25 ppm which confirmed the presence of methyl hexose (rhamnose) as one sugar unit. These data are in

agreement with previously reported data [59-62], compound 4 was proved to be quercetin-7-O-rhamnoside (syn. vincetoxicoside B). To our knowledge, this is the first report of isolation of vincetoxicoside B from *Laurus nobilis*.

Compound 5 was isolated from the ethyl acetate extract and eluted with 100% ethyl acetate. It dissolved in dilute solution of alkalies with the production of intense yellow colour, while upon addition of neutral ferric chloride solution, it gave bluish green colour. Also it developed yellow colour after addition of few drops of $AlCl_3$ suggesting the presence of flavonoidal skeleton. The UV spectral analysis in methanol (Table 2) showed two absorption bands at 329 nm and 270 nm indicating its flavone skeleton [57]. Bathochromic shift after addition of NaOMe, $AlCl_3$ and NaOAc indicated the presence of free hydroxyl groups at C-4', C-5 and C-7, respectively. In ES-MS, fragments appeared at *m/z* 324 and 312 is characteristic for the fragmentation of 8-C glycosides [63]. The presence of fragments at m/z 165 and 163 confirmed the C- glycosidic linkage of ring A. Fragment appeared at *m/z* 152 confirmed the presence of two hydroxyl groups at ring A, while mass fragment at *m/z* 118 confirmed the presence of one hydroxyl group in ring B. In ^{1}H-NMR (Table 3), two doublet signals appeared at δ_H 8.02 (2H, J= 8.0 Hz, H-2',6') and at 6.88 (2H, J= 8.0 Hz, H-3',5') that are characteristic for AB system of ring B. Also, one singlet signal in the aromatic region at δ_H 6.27 (1H, H-6) confirmed a 5, 7, 8-trisubstituted A ring. The flavone skeleton was confirmed through the presence of the singlet signal localized at 6.78 ppm assigned for H-3 olefinic proton. One anomeric proton signal appeared at δ_H 4.67 (1H, d, J= 9.6 Hz) with other signals at δ 3.26-4.60 integrated for six protons of the sugar moiety. The ^{13}C-NMR (Table 4) spectra revealed the presence of 21 carbon signals, including one carbonyl at δ_C 182.04 (C-4) characteristic for the flavone skeleton [58]. Another eight non-protonated carbons at δ_C 103.9, 104.6, 121.6, 155.99, 160.38, 161.14, 162.64 and 163.93 (C-10, C-8, C-1`, C-9, C-5, C-4`, C-7 and C-2 respectively). In HSQC, anomeric proton of sugar moiety (H-1'') was easily assigned because its doublet peak at 4.69 ppm was attached directly to ^{13}C peak at 73.51 ppm. The C-6`` signal of glucose appeared at δ_C 61.27 ppm and the rest of sugar carbons ranged from δ_C 61.83-81.84. The attachment of sugar is confirmed by the downfield shift of ^{13}C signal of C-8 (10.52 ppm) compared to apigenin [58]. On the basis of the above discussion, and by comparison with the previously reported data [63-65], compound 5 was proved to be apigenin-8-C-glucoside (syn. vitexin). To our knowledge, this is the first report of isolation of vitexin from *Laurus nobilis* growing in Egypt.

Table 3: ^{1}H-NMR data of compounds 4 and 5.

	Compounds	
H-No.	**4**	**5**
2	--	--
3	--	6.78 (1H, *s*)
4	--	--
5	--	--
6	6.22 (1H, *d*, *J*=1.2 Hz)	6.27 (1H, *s*)
7	--	--
8	6.39 (1H, *d*, *J*=1.6 Hz)	--
9	--	--
10	--	--
1'	--	--
2'	7.36 (1H, *d*, *J*=1.6 Hz)	8.02 (1H, *d*, *J*=8.0 Hz)
3'	--	6.88 (1H, *d*, *J*=8.0 Hz)
4'	--	--
5'	6.92 (1H, *d*, *J*=8.4 Hz)	6.88 (1H, *d*, *J*=8.0 Hz)
6'	7.32 (1H, *dd*, *J*=8.4, 1.6 Hz)	8.02 (1H, *d*, *J*=8.0 Hz)
3`-OCH_3	--	--
Glu-1``	--	4.67 (1H, *d*, *J*=9.6 Hz)
2``	--	3.26-4.60 (6H, *m*)
3``	--	
4``	--	
5``	--	
6``	--	
Rha-1```	5.37 (1H, *br s*)	--
2```	4.24 (1H, *s*)	--
3```	3.35-3.78 (3H, *m*)	--
4```		--
5```		--
CH_3-rha	0.95 (3H, *d*, *J*=6.4 Hz)	--

3.2. Isolated compounds and protein hydrolysate Hep-G2 cytotoxicity

The cytotoxic activity (Table 1) of *L. nobilis* extracts' isolated compounds and hydrolyzed protein fractions were tested against *Hep-G₂* cell using vinblastine sulfate (IC_{50}= 2.93 µg/ml) as standard. The IC_{50} values are represented in (Table 1). According to the American National Cancer Institute (ANCI), the modified criteria to consider a crude fraction promising for further study [66, 67] are: $IC_{50} \leq 20$ µg/ml is highly active, IC_{50} = 21 - 200 µg/ml is moderately active, IC_{50} = 201 – 500 µg/ml is weakly active and IC_{50} > 501 µg/ml is inactive. Thus, ethyl acetate extract was the most potent with IC_{50}=3.8 µg/ml, followed by methylene chloride and petroleum ether extracts (IC_{50} =5.96 µg/ml and 10.6 µg/ml, respectively), while methanol extract has moderate activity (IC_{50}< 20 µg/ml). Percentage of cell viability of *Hep-G₂* cells treated with different concentrations of *L. nobilis* extracts are shown in Fig. 2.

Among the five isolated compounds tested against *Hep-G₂*, reynosin (2) showed the highest cytotoxic activity with with IC_{50} value of 4.98 µg/ml. 1-Tricosanol (1) and protocatechuic acid (3) showed intermediate activity with IC_{50} values of 35.2 and 96.3 µg/ml, respectively. On the other hand, vitexin (5) showed the lowest cytotoxic activity (IC_{50}= 219 µg/ml). Percentage of cell viability of *Hep-G₂* cells treated with different concentrations of *L. nobilis* isolated compounds are shown in Fig. 3. These results showed that reynosin, isolated from the methylene chloride extract exhibited higher cytotoxic activity than the extract (IC_{50}= 4.98 and 5.96 µg/ml, respectively). On the other hand, compounds isolated from the petroleum ether and ethyl acetate extracts showed lower cytotoxic activity than their original extracts suggesting that the high cytotoxic activity of these extracts is attributed to the synergism between their components.

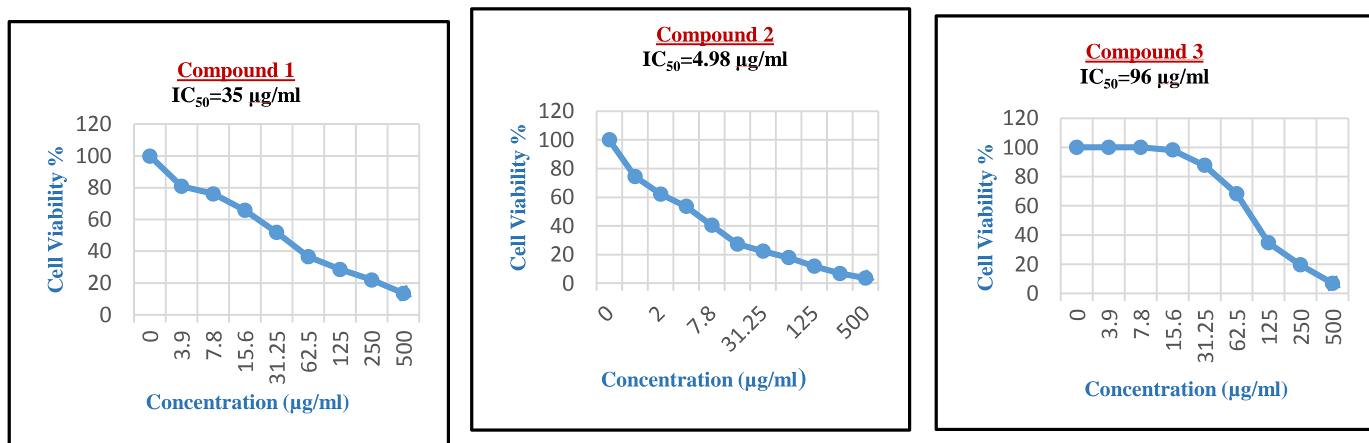
All of the protein fractions obtained after the enzymatic hydrolysis were screened for their cytotoxic activity against *Hep-G₂* and we found that fraction obtained 0.5 h after hydrolysis showed intermediate cytotoxic activity with IC_{50} value of 200 µg/ml. Other fractions were inactive (IC_{50}< 500 µg/ml). Percentage of cell viability of *Hep-G₂* cells treated with different hydrolysates of *L. nobilis* proteins are shown in Fig. 4.

3.3. Molecular docking of isolated compounds against caspase3 and their cytotoxicity against Hep-G2

Caspasae3 is considered as a main enzyme in apoptosis process of cancer cells including *Hep-G2* [68]. In this molecular modeling study, we aimed to highlight the site of interaction of the isolated compounds with caspase3 to initiate apoptosis of *Hep-G2*. The results reflected the ability of the isolated compounds to interact with caspase3 active site via formation of different kinds of bonds, like hydrogen bonds, arene-arene hydrophobic and arene-cation interactions, with amino acids responsible for caspase3 activity (Table 5, Fig. 5). From the docking results, it was clear that Arg207 is the most essential amino acid for activity which is in agreement with natural ligand binding site.

3.4. Protein hydrolysate antioxidant activity

The antioxidant activity of the hydrolyzed protein fractions (Fig. 6) showed that there is a direct relationship between the hydrolysis time, degree of hydrolysis and the antioxidant activity. Fraction obtained after 3.5 hr hydrolysis showed the highest antioxidant activity (45 % inhibition at a concentration of 200 µg/ml using DPPH assay).



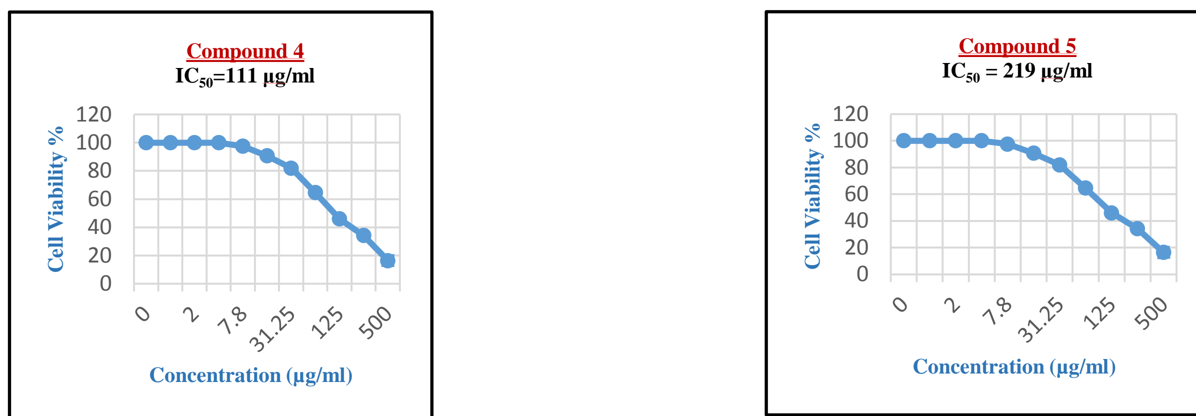


Figure 3: Percentage of cell viability of *Hep-G$_2$* cells treated with different concentrations of *L. nobilis* isolated compounds.

Table 4: ^{13}C-NMR data of compounds 4 and 5.

	Compounds	
C-No.	**4**	**5**
2	157.1	163.9
3	136.1	102.3
4	178.3	182.2
5	161.8	160.5
6	98.4	98.1
7	164.5	162.4
8	93.3	104.5
9	157.9	156.0
10	104.6	103.8
1'	121.6	121.5
2'	115.6	128.9
3'	145.0	115.8
4'	148.4	161.1
5'	115.0	115.8
6'	121.5	128.9
3`-OCH_3	--	--
Glu-1``	--	73.5
2``	--	70.9
3``	--	78.8
4``	--	70.5
5``	--	81.5
6``	--	61.4
Rha-1```	102.2	--
2```	70.6	--
3```	70.7	--
4```	71.9	--
5```	70.5	--
CH_3-rha	16.3	--

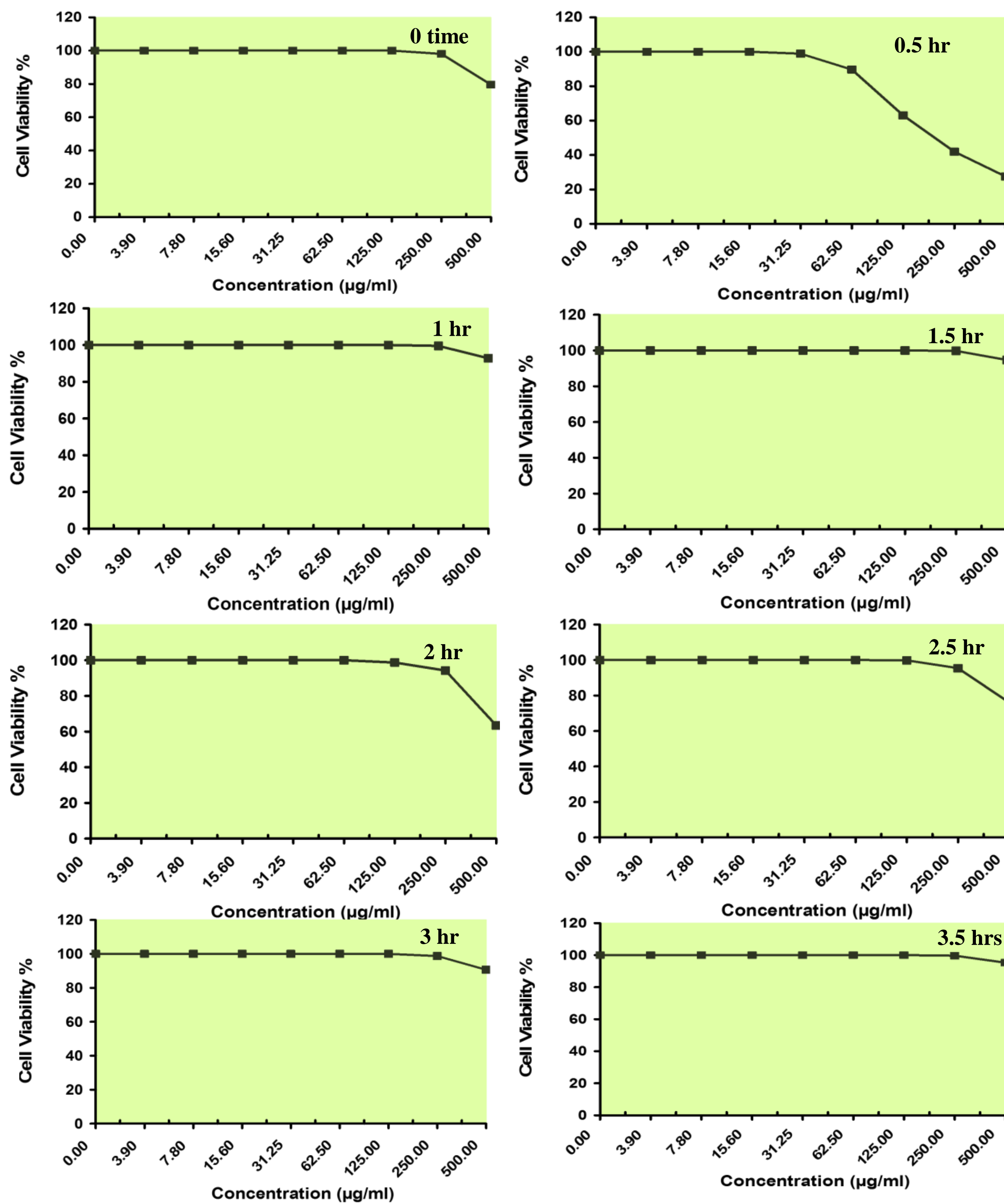


Figure 4: Percentage of cell viability of *Hep-G2* cells treated with different concentrations of hydrolyzed protein fractions from *L. nobilis* leaves.

Compound	Three dimensional	Two dimensional
Reynosin		
Vincetoxicoside B		
Vitexin		

Figure 5: Representative examples for molecular docking of *L. nobilis* isolated compounds to Caspase3 using MOE.

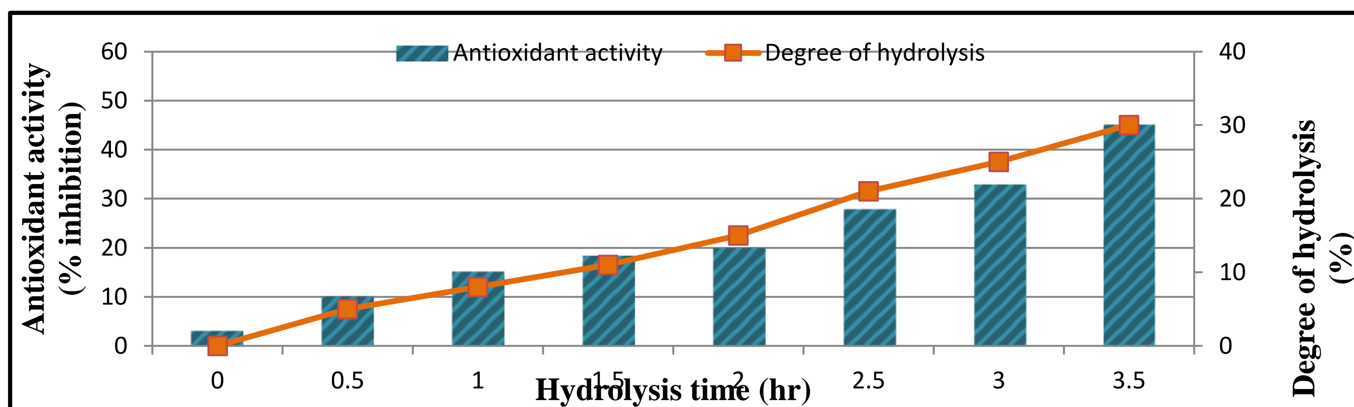


Figure 6: Antioxidant activity of L. nobilis leaves protein hydrolysates estimated by DPPH assay (% inhibition) at different degree of hydrolysis.

Table 5: Molecular docking of isolated compounds from *Laurus nobilis* leaves extracts to Caspase3.

Compound	Score	Amino acid interactions
1-tricosanol	-18.3654	Arg207
reynosin	-12.0415	Arg207
protocatechuic acid	-15.4604	Ser205, Arg207
vincetoxicoside B	-18.3651	His121, Arg207, Asn208, Phe250
vitexin	-18.4608	Arg207, Phe256

4. Conclusion

From the aforementioned study, we can say that *Laurus nobilis* leaves are potential source of *Hep-G$_2$* cytotoxic active compounds. Reynosin exhibited the most potent cytotoxic activity (IC_{50}= 4.98 μg/ml) in comparison with standard vinblastine sulfate (IC_{50}= 2.93 μg/ml), while vitexin exhibited the lowest activity (IC_{50}= 219 μg/ml). The promising cytotoxic results of reynosin make it a good candidate for further study and investigation as a treatment for hepatocellular carcinoma. Even other compounds that exhibited moderate cytotoxic activity against *Hep-G$_2$* could be tested against other types of cancer cells. Moreover, *L. nobilis* proteins showed moderate cytotoxic action against *Hep-G$_2$* (IC_{50}= 200 μg/ml) and strong antioxidant activity (45 % inhibition).

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5. Conflicts of interest

There are no conflicts to declare

6. Acknowledgment

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