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In vitro Anti-inflammatory, Cytotoxic and Hepatoprotective Activities of *Laurus nobilis* L. (Lauraceae) Wood Extract and Its Constitutive Phenolics

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THE POSSIBLE anti-inflammatory, cytotoxic and hepato-protective activities of *Laurus nobilis* L. wood extract was *in vitro* evaluated. The anti-inflammatory activity was assessed by measuring Nitric Oxide (NO) production by the inflammagen lipopolysacchride. The extract was evaluated for its cytotoxic activity on six different human cancer cell lines together with the normal non-malignant melanocytes cell line (HFB4) using the SRB assay. The hepatoprotective activity against paracetamol toxicity was determined using primary cultured rat hepatocytes. The extract showed moderate anti-inflammatory activity as shown in the amount of nitric oxide produced with a level of 4.3 µM /ml (67 % inhibition), in comparison to the potent anti-inflammatory drug Dexamethasone (95 % inhibition). The extract was found to have moderate cytotoxic activity against the tumor cell lines used at the applied concentrations with IC_{50} ranging from 15.5 – 47.6 µg/ml compared to the potent cytotoxic drug doxorubicin. The wood extract showed hepatoprotective activity against paracetamol toxic effect at concentration of 20µg/ ml. The constitutive phenolics of *L. nobilis* L. wood were studied and led to the separation and identification of 12 compounds, all isolated for the first time from *L. nobilis* L. wood and were identified using chemical, conventional and advanced spectral techniques.

Keywords: *Laurus nobilis* L. (Lauraceae), Phenolics, Anti-inflammatory, Cytotoxic and Hepatoprotective.

The field of natural product biology, ethnopharmacology, as well as bio-prospecting approaches, have received renewed attention in the recent years [(1,2)] . Natural products and herbal remedies used in traditional folk medicine have been the source of many medically beneficial drugs [(3)] .

Laurus nobilis L. is an evergreen shrub up to 2.15 m height and commonly named bay laurel [(4)] . This tree belongs to the Lauraceae family and is native to the southern Mediterranean region and widely cultivated in Europe and the USA

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as an ornamental plant. It is grown commercially for its aromatic leaves. The leaves are commonly used as a spicy, aromatic flavoring for soups, fish, meats, stews, puddings, vinegars, beverages and form an essential ingredient of the herb mix "Bouquet Garni"[5]. The leaves are also widely used in folk medicine to treat gastrointestinal problems, rheumatism, diuretic, urinary problems and stones[6] . For the pharmacological properties of *L. nobilis* L., it has been reported to have anti-ulcer (seeds) and anti-diabetic (leaves)[7] effects and to enhance in liver glutathione S-transferase (GST) activity[8]. This spice is also used as an insecticide[6] , suppressing high blood sugar,[9,10] , migraine headaches [11, 12], bacterial and fungal infections[13] and gastric ulcers[14] . A survey of the literature showed that no experimental data are available regarding the biological activity and phenolic constituents of the wood extract. It was therefore, found interesting to investigate, in the present study the possible anti-inflammatory, cytotoxic and hepatoprotective effects of *L. nobilis* L. wood extract and to separate and identify its major phenolic constituents.

Experimental

Plant material

L. nobilis L. was collected from a private farm in El- Mansouria area, Giza, Egypt, in September 2010 and authenticated by Dr. M. El Gebali, National Research Centre (NRC), Cairo, Egypt.

Instruments and materials

^{1}H NMR spectra were measured by a Jeol ECA 500 MHz NMR spectrometer. ^{1}H chemical shifts (δ) were measured in ppm, relative to TMS and ^{13}C NMR chemical shifts relative to DMSO-d_6 and converted to TMS scale by adding 39.5, spectral width = 8 kHz for ^{1}H and 30 kHz for ^{13}C, 64 K data points and a flip angle of 45^{o}. UV recording was made on a Shimadzu UV–Visible-1601 spectrophotometer. Mass spectra were measured by mass spectrometer MAT 95 (Finnigan MAT) at National Research Center, Cairo. Paper chromatographic analysis was carried out on Whatman paper No. 1, using solvent systems: (1) H_2O; (2) 6 % HOAc; (3) BAW (n-BuOH : HOAc : H_2O, 4:1:5, upper layer). BAW solvent system was used for preparative paper chromatography (Prep. PC) using Whatman paper No. 3.

Extract preparation

L. nobilis L. woods (2 kg) were homogenized in a MeOH—H2O (3:1) mixture (three extractions each with 3 litres). The obtained extract was filtered and dried under reduced pressure to give a yield of 36 gm dried extract.

In vitro anti-inflammatory assay by estimation of nitric oxide production

Nitrite accumulation was used as an indicator of nitric oxide production using a microplate assay based on the Griess reaction. The Griess reaction is based on a two-step diazotization reaction in with acidified nitrites generate a nitrosating agent that reacts, which sulfanilic acid to form diazonium ion. This ion is then

coupled to N-(1- naphthyl) ethylenediamine to produce the chromophoric pink azo-derivative that can be determined spectrophotometrically at 540 nm [15] .

Reagents preparation

Griess reagent (40 mg Griess powder was dissolved in 1 ml de-ionized water). In each well of a flat bottom 96 well- microplate, 40 µl freshly prepared Griess reagent was mixed with 40 µl RAW 264.7 cells (Immortalised cell line) supernatant or different concentrations of sodium nitrite ranging from 0-50 µ mole/ml. The plate was incubated for 10 min in the dark and the absorbance of the mixture at 540 nm was determined using the microplate ELISA reader. A standard curve (Fig. 1) relating nitric oxide in µmole/ml to the absorbance is constructed, from which the nitric oxide level in the cell supernatant is computed by interpolation.

Calculation

The nitric oxide level of each of the tested cell supernatant was expressed as nitric oxide level of the tested cell supernatant x100 / nitric oxide level of the control.

Cytotoxic activity of L. nobilis L. wood extract against tumor cell lines

The cytotoxicity assay was performed at The National Cancer Institute. El-Kasr El-Aini, Cairo, Egypt. Potential cytotoxicity of the aqueous methanolic extract of *L. nobilis* L. wood was tested using the sulforhodamine B assay (SRB) based on the protein staining according to Skehan *et al.*[16] .

Human cell lines: Five different human cancer cell lines were used in this study together with the normal nonmalignant melanocytes cell line (HFB4). The human cancer cell lines included: the cervical cancer cell line (HELA), laryngeal carcinoma cell line (HEP2), breast cancer cell line (MCF7), hepatocellular cell line (HEPG2) and colonic cancer cell line (HCT116). The sensitivity of the human cancer cell lines to increasing serial concentrations equivalent to 0, 5, 12.5, 25 and 50 µg/ml of each of the standard Doxorubicin hydrochloride "DOX" (Adriamycin) and the *L. nobilis* L. wood extract.

In vitro bioassay on primary culture of rat hepatocytes monolayer

IC_{50} determination of the extract

In order to determine IC_{50}, different concentrations of the extract were prepared. The range of concentrations used started from 125 µg/ml followed by increasing concentrations in ascending order up to the concentration 1000 µg/ml that induced death of half the number of cells. *L. nobilis* L. extract was dissolved in dimethylsulfoxide (DMSO) (1% maximum concentration). For each concentration, three replicates were carried out. The plate was incubated for 2 hr, in CO_2 incubator.

After cell incubation with the extract, cell viability was determined using neutral red assay (NR) as described by Borenfreund and Puerner[17] . Evaluation of the effect of the extract on cultured hepatocytes was obtained by calculating

the absorption of the cell viability with respect to control cells. Each experiment was carried out in triplicate to confirm validity of results. A graph was plotted with x-axis showing different concentrations of extract used, y-axis showing absorbance percentage of viable cells. IC_{50} was graphically determined from the concentration that yielded an absorption coinciding with 50% absorbance (Fig. 4).

Evaluation of hepatoprotective activity

Different concentrations were prepared from *L. nobilis* L. wood extract, starting from 12.5 µg/ ml and increasing concentration in ascending order by dissolving in DMSO (1% maximum concentration). For each concentration, three replicates were carried out, in addition to controls which were: cell control (cells only), negative control (cells + paracetamol) and positive control (reference) (cells + silymarin + paracetamol). The plate was incubated for 2 hr, washed twice with PBS. Paracetamol (20 mM) was added to each well except on that of the cell control and incubated for 18 hr. Following incubation, the monolayer was washed again with PBS. 50% mortality of the hepatocyte (IC_{50}) which was determined using neutral red assay [17] .

Extraction, isolation and purification

The aqueous methanolic extract of *L. nobilis* L. wood (36 g) showed by preliminary two-dimensional paper chromatographic (2D-PC) screening to contain a phenolic mixture from which twelve compounds (1-12) were isolated and purified through fractionation on sephadex LH-20 (350 g) column (120 × 5 cm) and elution with H_2O followed by H_2O-MeOH mixtures of decreasing polarities to yield eight column fractions (I-VIII). The received fractions, were individually subjected to 2D-PC and other isolatory chromatographic techniques, thus yielding twelve phenolic compounds (1- 12) all isolated for the first time from *L. nobilis* L. wood.

Isolation and purification of phenolics from L. nobilis L. wood

Compound 1 (30 mg) was purely isolated from 0.8 g of fraction II (eluted with 10% aq. MeOH) by repeated column fractionation over polyamide 6s using H_2O for elution. Compound 2 (55 mg) was separated by Prep. PC. of 1.2 g of fraction III (eluted with 20% aq. MeOH). Compounds 3 (43 mg) and 4 (61 mg) were purely isolated from 2.7 g of fraction IV (eluted with 30% aq.MeOH) by Prep. PC, using BAW as solvent. Compound 5 (72 mg) was separated pure from (2.2 g) of from fraction V eluted with 50% aq. MeOH), using a polyamide 6s column using 50 % H_2O: MeOH for elution followed by Prep. PC. Compounds 6 (40 mg) and 7 (52 mg) were purely isolated from 1.9 g of fraction VI (eluted with 60% aq. MeOH) by applying a polyamide 6s column using 50 % H_2O: MeOH for elution followed by Prep.PC. Compounds 8 (80 mg) and 9 (72 mg) were purely isolated from 3.7 g of fraction VII (eluted with 70% aq. MeOH) through fractionation on a polyamide column using the solvent system, methanol: benzene: H_2O (60:38:2) as an eluent, followed by Prep. PC, using BAW as solvent. Compounds 10 (62 mg), 11 (70 mg) and 12 (30mg) were purely isolated from 3 g of fraction VIII (eluted with 90% aq. MeOH) by repeated Prep. PC using 6% AcOH and BAW as solvent.

Spectral data of compounds (1-12) isolated from L. nobilis L. wood

Ferulic acid (1) was isolated as colorless cubic crystals of R_f values (x 100): 42 (AcOH-6), 62 (BAW). UV spectral data λ_{max} (MeOH): 320.0, 293.1 nm sh. 232.6 nm sh. It showed a M*r* of 194 in negative ESI-MS, corresponding to a molecular ion $[M-H]^-$ at *m/z* = 193 and molecular formula $C_{10}H_{10}O_4$.^{1}H- NMR spectral data were similar to those reported in literature[18] .

p-Coumaric acid (2) was isolated as colorless prisms of R_f values (x 100): 43(H_2O), 45 (AcOH-6), 90 (BAW). UV spectral data λ_{max} (MeOH): 310.0, 226 nm. It showed a M*r* of 164 in negative ESI-MS, corresponding to a molecular ion $[M-H]^-$ at *m/z* = 163 and a molecular formula of $C_9H_8O_3$. ^{1}H- NMR spectral data were similar to those reported in literature [19] .

Quercetin 3-*O*-*β*- D-glucoside (3) was obtained as faint brown amorphous powder of R_f values (x100): 48 (H_2O), 44 (AcOH-6), 32 (BAW).UV spectral data λ_{max}(MeOH) : 256 nm, 265nm sh., 358 nm, + NaOMe: 268nm, 327nm sh., 403nm, + NaOAC : 273nm, 323nm, 387nm + NaOAC + H_3BO_3 : 262nm, 377nm, + $AlCl_3$: 273nm, 430nm. It showed a M*r* of 464 in negative ESI-MS, corresponding to a molecular ion $[M-H]^-$ at *m/z* = 463 and a molecular formula of $C_{21}H_{20}O_{12}$. ^{1}H- and ^{13}C NMR spectral data were similar to those reported in literature [12] .

Quercetin 3-*O*-β-D-galactoside (4) was obtained as yellowish white amorphous powder of R_f values (x100) : 20 (H_2O), 37 (AcOH-6), 56 (BAW). UV spectral data λ_{max} (MeOH) : 257 nm, 266nm sh., 359 nm, + NaOMe : 266nm, 227nm sh., 408 nm, + NaOAC : 274 nm, 379 nm, + NaOAC + H_3BO_3 : 268 nm, 272 nm sh., 384 nm, + $AlCl_3$: 265nm, 272 nm sh., 384nm. It showed a M*r* of 464 in negative ESI-MS, corresponding to a molecular ion $[M-H]^-$ at *m/z* = 463 and a molecular formula of $C_{21}H_{20}O_{12}$. ^{1}H- and ^{13}C NMR spectral data were similar to those reported in literature [20] .

Tamarixetin 3-*O*-α-L-rhamnoside (5) was obtained as pale yellow amorphous powder of R_f values (x 100): 12 (H_2O), 26 (AcOH-6), 62(BAW). UV spectral data λmax (MeOH): 255 nm, 265 nm sh., 348 nm, + NaOMe: 2702 nm, 400 nm, + NaOAC: 253 nm sh., 270 nm, 386 nm + NaOAC + H_3BO_3: 255 nm, 265 nm, 355 nm, +Al Cl_3: 272 nm, 400 nm. It exhibited a *Mr* of 462 in negative ESI-MS analysis $[M-H]^-$ at *m/z* = 461 and a molecular formula of $C_{22}H_{22}O_{11}$. ^{1}H- and ^{13}C NMR spectral data were similar to those reported in literature [21] .

Quercetin 3-*O*-α-L-rhamnopyranoside (6) was obtained as pale yellow amorphous powder of R_f values (x 100):22 (H_2O), 48 (AcOH-6), 68(BAW). UV spectral data λmax (MeOH): 259 nm, 297 nm sh., 348 nm, + NaOMe: 270 nm, 355 nm, 402 nm, + NaOAC : 276 nm, 372 nm, + NaOAC + H_3BO_3 : 272 nm, 383 nm, + Al Cl_3 : 268 nm, 352 nm, 408 nm. It exhibited a *Mr* of 448 in negative ESI-MS analysis $[M-H]^-$ at *m/z* = 447.1 and a molecular formula of $C_{21}H_{20}O_{11}$. ^{1}H- and ^{13}C NMR spectral data were similar to those reported in literature [22] .

Kaempferol 3-*O*- α-L-rhamnopyranoside (7) was obtained as pale yellow amorphous powder of R_f values (x 100): 24 (H_2O), 47 (HOAc-6), 76 (BAW). UV spectral data λ max (MeOH): 266 nm, 345 nm sh., + NaOMe: 271 nm, 376nm, + NaOAC: 270 nm, 346 nm, + NaOAC + H_3BO_3: 270 nm, 346 nm, 405nm, +Al Cl_3: 268 nm, 340 nm, 385 nm. It exhibited a *Mr* of 432 in negative ESI-MS analysis $[M-H]^-$ at *m/z* = 431and a molecular formula of $C_{21}H_{20}O_{10}$. ^{1}H- and ^{13}C NMR spectral data were similar to those reported in literature [22] .

Epicatechin (8) was obtained as off-white needles of R_f values (x 100): 37 (HOAc-6), 51 (BAW). UV Spectral Data λ_{max} (MeOH): 278 nm. ESI-MS [M+H] m/z 291 corresponding to a molecular weight of 290 and a molecular formula of $C_{15}H_{14}O_6$. ^{1}H- and ^{13}C NMR spectral data were similar to those reported in literature [23] .

Catechin (9) was obtained as off-white needles of R_f values (x 100): 37 (HOAc-6), 51 (BAW). UV Spectral Data λ_{max} (MeOH): 278 nm. ESI-MS [M+H] *m/z* 291 corresponding to a molecular weight of 290 and a molecular formula of $C_{15}H_{14}O_6$. ^{1}H- and ^{13}C NMR spectral data were similar to those reported in literature [23] .

Quercetin (10) was obtained as yellow powder of R_f values (x 100): 00 (H_2O), 7 (HOAc-6), 75 (BAW). UV Spectral Data λ_{max} (MeOH): 255 nm, 268 nm, 370 nm, + NaOAc: 254 nm,276 nm, 375 nm,+H_3BO_3: 272 nm, 388 nm, + $AlCl_3$: 270 nm, 360 nm, 440 nm,+ HCl :258 nm, 400 nm. It showed a *Mr* of 302 in its negative ESI-MS analysis $[M-H]^-$ at *m/z* =301 and a molecular formula of $C_{15}H_{10}O_7$. ^{1}H-NMR spectral data were similar to those reported in literature [24] .

Kaempferol (11) was obtained as yellow amorphous powder of R_f values (x 100) : 00 (H_2O), 10 (HOAc-6), 85 (BAW). UV Spectral Data λ_{max} (MeOH) : 268 nm, 369 nm, NaOAc: 270 nm, 310 nm, 375 nm,+H_3BO_3 : 270nm, 320 nm, 372 nm, + $AlCl_3$: 270 nm, 305 nm, 360 nm,+ HCl : 255 nm, 269 nm, 348 nm, 422 nm, MeONa: 278 nm, 316, 413 nm. It has a *Mr* of 286 in its negative ESI-MS analysis ($[M-H]^-$ at *m/z* =285) and a molecular formula of $C_{15}H_{10}O_6$. ^{1}H- NMR spectral data were similar to those reported in literature [25].

Tamarixetin (12) was obtained as yellow amorphous powder of R_f values (x 100): 00 (H_2O), 05 (HOAc-6), 83 (BAW). It showed a *Mr* of 316 in negative ESI-MS, corresponding to a molecular ion $[M-H]^-$ at *m/z* = 315 and a molecular formula of $C_{16}H_{12}O_7$. UV Spectral Data λ_{max} (MeOH): 254 nm, 265 nm, 365 nm NaOAc : 253 nm sh., 273 nm, 312 nm, 380 nm +H_3BO_3 : 255 nm, 265 nm sh., 368 nm, + $AlCl_3$: 268 nm, 301 nm sh., 365 nm sh., 430 nm, MeONa : 268 nm, 418 nm. ^{1}H- NMR spectral data were similar to those reported in literature [26].

Results and Discussion

Anti-inflammatory assay

Results of nitric oxide index

A standard curve, relating nitric oxide in µmole/ml of sodium nitrite to the absorbance, was constructed (Fig. 1), from which the nitric oxide level in the cell supernatant is computed by interpolation (Fig. 2).

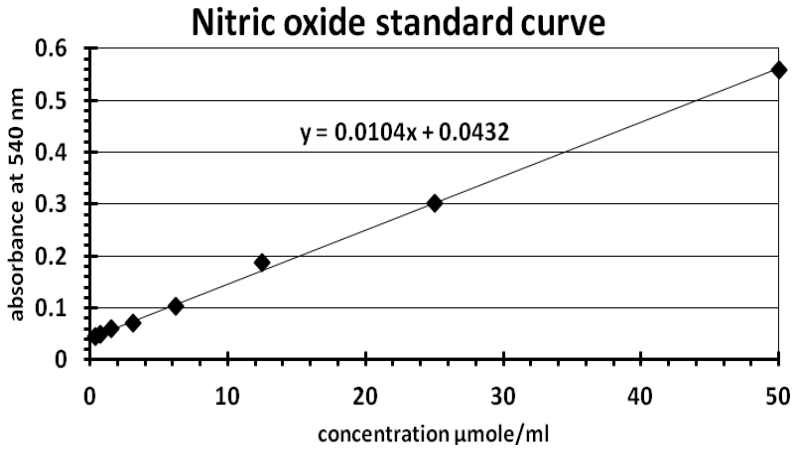


Fig. 1. A standard curve of sodium nitrite µmole/ml .

The results indicated that the inflammagen lipopolysaccahride (LPS 100 µg / ml) induced nitric oxide production up to 2 folds of the control, while that the potent anti-inflammatory Dexamethasone (50 mg / ml) inhibited nitric oxide production to 3.5 µM / ml compared to 6.5 µM / ml of that of the LPS with level of 95 % inhibition, very close to the control cells with 3.2 µM / ml nitric oxide as shown in Fig. 2 and 3.

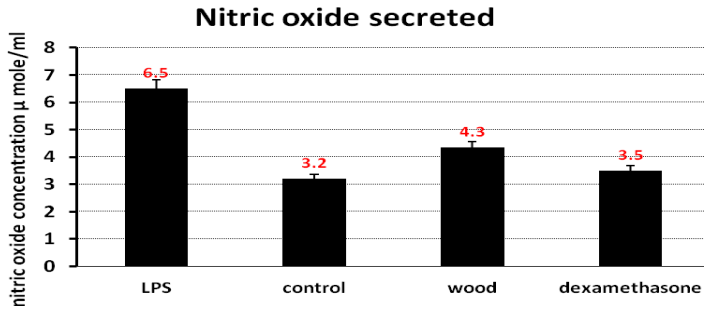


Fig. 2. The level of nitric oxide in RAW 264.7 cells supernatant after the treatment with the samples (25µg/ml) for 24 hr compared with LPS-treated cells (100µg/ml), as measured by Griess assay.

L. nobilis L. wood showed anti- inflammatory effects as shown in the amount of nitric oxide produced with a level of 4.3 µM/ml, leading to 67 % inhibition, in comparison to the potent anti-inflammatory drug Dexamethasone (95 % inhibition), (Fig. 3).

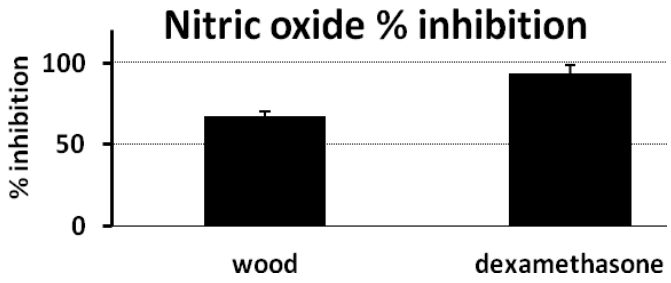


Fig. 3. The percentage of inhibition of nitric oxide in LPS-stimulated RAW 264.7 cells supernatant after the treatment with the samples (25µg/ml) for 24 hr compared with LPS treated cells, as measured by Griess assay.

Assay of cytotoxic activity of L. nobilis L. wood extract

The cytotoxic activity of the extract was determined by SRB method and proved that it possesses moderate cytotoxic activity against the human cell lines tested, with IC_{50} ranging from 15.5 – 47.6 µg/ml. (Table 1, Fig. 4).

TABLE 1. IC_{50} of *L. nobilis* L. wood extract against the cell lines tested.

Human cell lines	HEP2	HELA	HCT	HEPG2	HFB4
IC50	15.5 µg / ml	19.1 µg / ml	24 µg / ml	28.8 µg / ml	35.7 µg / ml

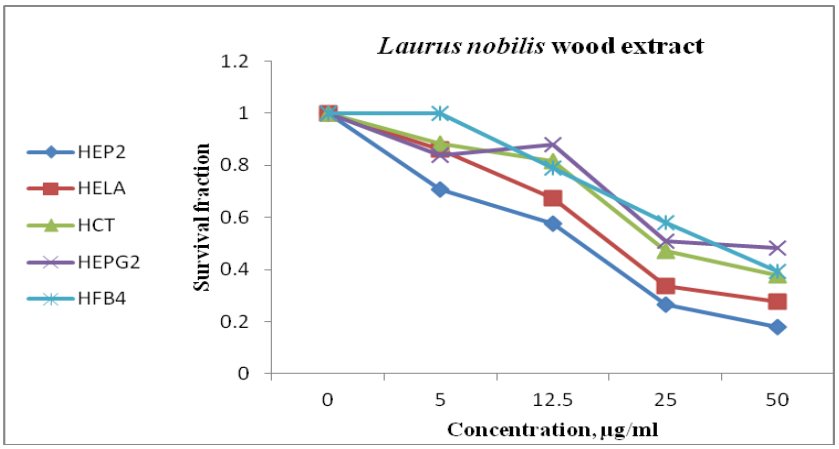
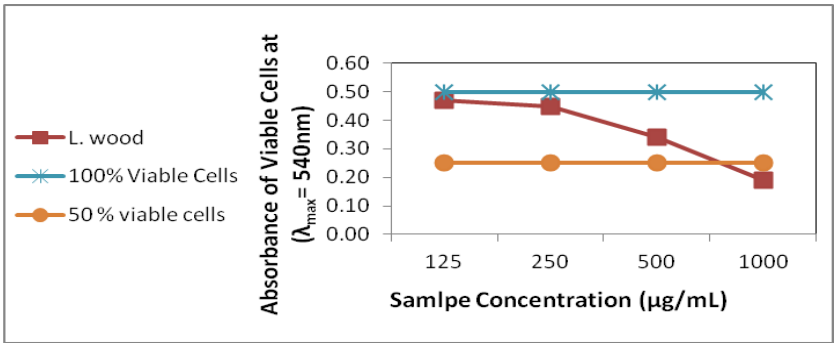


Fig. 4. Cytotoxic activity of *L. nobilis* L. wood extract.

Hepatotoxicity

The viability assay was applied with a broad range of concentrations of the studied extract of *L. nobilis* L. wood (from 125-1000µg/ml) on monolayer of rat hepatocytes. The *L. nobilis* L. wood extract showed hepatotoxic effect at a high concentration with an IC_{50} of 500 µg/ml, (Fig. 5).

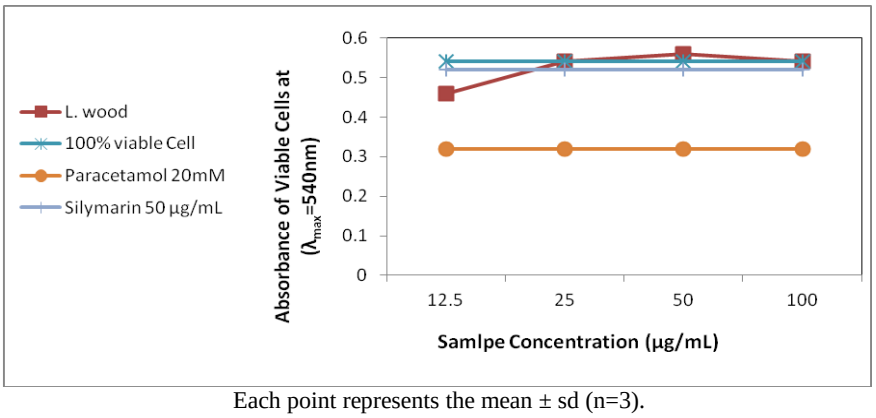


Each point represents the mean ± S.D (n=3).

Fig. 5. Viability of monolayer of rat hepatocytes after 2 hr treatment with different concentrations of the extract using NR colourimetric assay.

Evaluation of hepatoprotective activity applying rat hepatocyte monolayer

The hepatoprotective effect of the tested extract against paracetamol toxic effect could be concluded from Fig. 6.



Each point represents the mean ± sd (n=3).

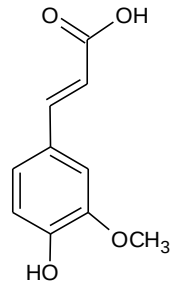
Fig. 6. Viability of monolayer of rat hepatocyte after 2 hr treatment with different concentrations of the extract followed by treatment with 20 mm paracetamol for 18 hr. In comparison with 50 µg silymarin as control using NR colourimetric assay.

The *L. nobilis* L. wood extract exhibited a hepatoprotective activity at 20µg/ ml.

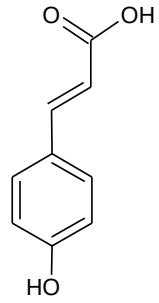
Conclusion

Laurus nobilis L. wood proved its capability of synthesizing and accumulating an appreciable number of phenolic compounds in its wood. The woods extract showed moderate anti-inflammatory activity (67 % inhibition), in comparison to the potent anti-inflammatory drug Dexamethasone (95 % inhibition). The extract was found to have moderate cytotoxic activity against the tumor cell lines used at the applied concentrations with IC_{50} ranging from 15.5 – 47.6 µg/ml. The wood extract showed hepatoprotective activity against paracetamol toxic effect at concentration of 20 µg/ ml.

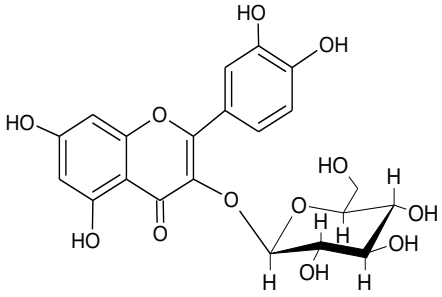
Separation and identification of constitutive 12 phenolic compounds were reported for the first time from *Laurus nobilis* L. *(Lauraceae)* wood.



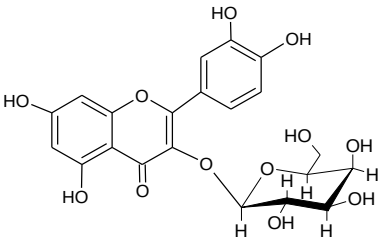
Ferulic acid (1)



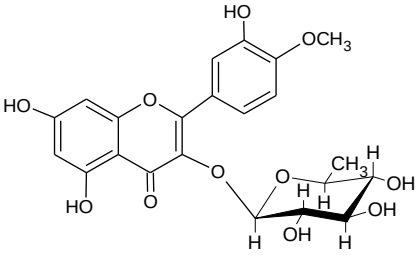
p-Coumaricacid (2)



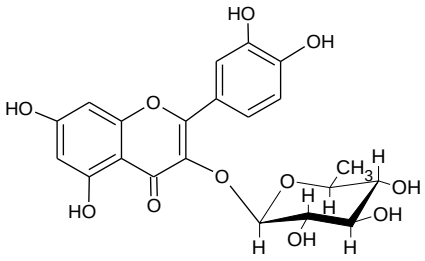
Quercetin 3-O- β- D-glucopyranoside (3)



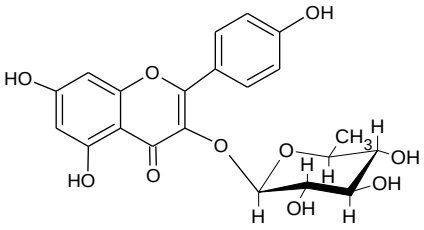
Quercetin 3-O- β-D-galactopyranoside (4)



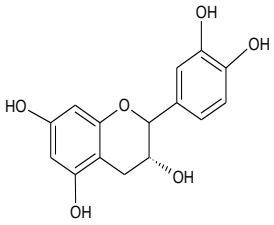
Tamarixetin 3-*O*-α -L– rhamnoside (5)



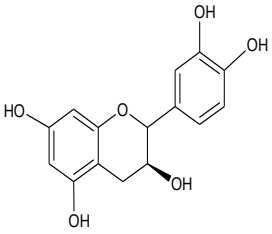
Quercetin 3-*O*-α -L – rhamnoside (6)



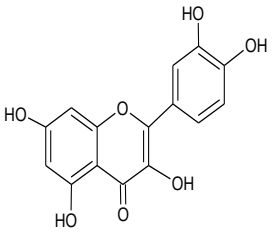
Kaempferol 3-*O*-α -L – rhamnoside (7)



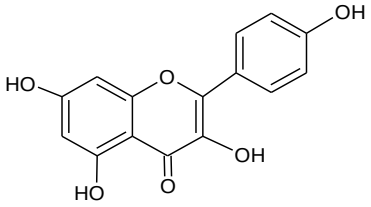
Epicatechin (8)



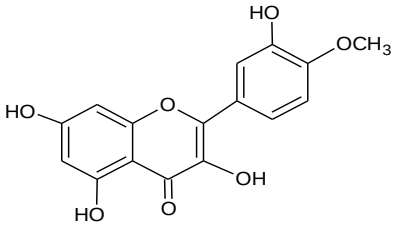
Catechin (9)



Quercetin (10)



Kaempferol (11)



Tamarexetin (12)

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دراسة فاعلية أخشاب نبات اللورس نوبيلس (لاوراسى) كمضاد للالتهابات و مضاد للأورام و فاعليته فى حماية خلايا الكبد و دراسة لمكوناته الفينولية

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تم دراسة امكانية استخدام الخلاصة الفينولية لأخشاب نبات اللورس نوبيلس كمضاد للالتهابات و للأورام و لحماية خلايا الكبد ضد التأثير السمى لعقار الباراسيتامول.

تم قياس فاعليته كمضاد للالتهابات عن طريق قياس كمية النيتريك أكسيد الناتجة عن محفز الالتهاب اليبوبوليسكاريد. و تم قياس فاعليته كمضاد للاورام على ستة أنواع مختلفة من الخلايا السرطانية بالاضافة الى تأثيره على الخلاية الطبيعية و مقارنته بالعقار دوكسيربيسين المضاد للسرطان.

و لقد أظهر النبات فاعلية متوسطة كمضاد للالتهابات (67٪) بالمقارنة بالعقار ديكساميثازون. و فاعلية متوسطة كمضاد للاورام. أما عن تأثيره لحماية خلايا الكبد، فلقد أظهر تأثير ايجابى بتركيز منخفض 20µg/ ml . و تم فصل و تعريف 12 مركب فينولى لأول مرة من أخشاب النبات .