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Evaluation of the Volatile Oil Composition and Antiproliferative Activity of *Laurus nobilis* L. (Lauraceae) on Breast Cancer Cell Line Models

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Abstract: Volatile oil composition and antiproliferative activity of *Laurus nobilis* L. (Lauraceae) fruits and leaves grown in Jordan were investigated. GC-MS analysis of the essential oil of the fruits resulted in the identification of 45 components representing 99.7 % of the total oil content, while the leaf essential oil yielded 37 compounds representing 93.7% of the total oil content. Oxygenated monoterpene 1,8-cineole was the main component in the fruit and leaf oils. Using sulphorhodamine B assay; the crude ethanol fraction, among other solvent extracts, showed strong antiproliferative activity for both leaves and fruits, nevertheless, the fruits were more potent against both breast cancer cell models (MCF7 and T47D). At IC_{50} values; the mechanism of apoptosis was nevertheless different: where *L. nobilis* fruit proapoptotic efficacy was not regulated by either p53 or p21, *L. nobilis* leaf extract components enhanced the p53 levels substantially. In both extracts, apoptosis was not caspase-8 or Fas Ligand and sFas (Fas/APO-1) dependent. Our studies highlight *L. nobilis* as a potential natural agent for breast cancer therapy. Compared with non induced basal cells, both *L. nobilis* fruits and leaves induced a significant enrichment in the cytoplasmic mono- and oligonucleosomes after assumed induction of programmed MCF7 cell death.

Keywords: *Laurus nobilis*; Volatile oils; Anticancer; Apoptosis; Jordan.

1. Introduction

Laurus nobilis L. (Lauraceae), commonly known as bay leaf, has been used as a spice worldwide and a medicinal plant in Mediterranean countries, including Jordan. Leaves and fruits have been reported to possess aromatic, stimulant and narcotic properties [1]. The anti-convulsive and antiepileptic activities of *L. nobilis* extracts have been confirmed [2]. The leaves of *L. nobilis* are traditionally used orally to treat the symptoms of gastrointestinal problems, such as epigastric bloating, digestion, and flatulence [3]. Preliminary brine shrimp toxicity tests and few other studies related to its cytotoxic properties were carried out on the leaf extract of Turkish *L. nobilis* [4]. While the majority of the phytochemical and biological reports dealt with the leaves of *L. nobilis*, there has been very little work on its fruits. The major setbacks of most available chemotherapies are the non-selective

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cytotoxicity, severe side effects, and chemo-resistance [5]. A cornerstone of newer cancer therapy is to develop alternatives that have high specificity to induce molecular apoptosis in cancer cells.

In the last decades, newer anti-cancer drugs have been introduced, with about half of them derived from natural sources [6-7]. In fact, natural compounds from flowering plants have played a significant role in cancer chemotherapy. Some of these agents include vincristine and vinblastine from *Catharanthus roseus*, paclitaxel and taxotere from species of yew (*Taxus* spp.), etoposide derived from lignans of *Podophyllum* spp. and camptothecin analogues, such as topotecan, from *Camptotheca acuminata*. These fundamentally cytotoxic components inhibit cell proliferation through different mechanisms [8]. Apoptosis is a highly regulated mechanism by which cells undergo cell death in an active way. Accordingly, one of the challenging tasks concerning cancer therapy is to induce and augment apoptosis in malignant cells. Therefore, there is an escalating focus on natural products to modulate apoptotic signaling pathways and their emerging molecular targets [9].

Though few reports were related to *L. nobilis* antiproliferative capacity [10-12]; presently in this study, water, ethanol, butanol, ethylacetate and chloroform extracts of *L. nobilis* leaves and fruits as well as their hydrodistilled volatile oils were tested for their antiproliferative activity. To shed light on the possible anticancer mechanisms of *L. nobilis* leaves and fruits extracts, p53 and p21 levels, Fas ligand and sFas and caspase-8 activities were determined, all of which are strongly associated with the signal transduction pathway of apoptosis [13-15].

2. Materials and Methods

2.1. Plant Material and Preparation of Crude Extracts

Leaves and fruits of *L. nobilis* L., collected from the trees grown in the campus of The University of Jordan in June 2010, were identified by Prof. Barakat Abu Irmaileh (Faculty of Agriculture, The University of Jordan). Leaves and fruits were air dried at room temperature (RT) in the shade until constant weight. Voucher specimens for *L. nobilis* fruits [1LAUR FMJ] and leaves [2LAUR FMJ] have been deposited in the Department of Pharmaceutical Sciences, Faculty of Pharmacy, the University of Jordan.

Each 10 g of the dried and coarsely powdered plant material was refluxed for 30 min using different solvents, kept overnight, filtered and solvents were evaporated. The ethanol extracts of the fruits and leaves were tested for the presence of different classes of secondary metabolites using Thin Layer Chromatography (TLC) [16]. Coated analytical TLC plates were procured from Merck. For biological activity tests, 100 mg of the extracts were dissolved in 10 mL DMSO (stock solution).

2.2. Distillation of Plant Material

Air dried plants were coarsely powdered and then hydro-distilled using a Clevenger apparatus for 3 h. The distillation was repeated twice and the oils obtained were pooled separately, dried over anhydrous sodium sulfate (Na_2SO_4) and stored at 4º C in amber glass vials until analysis**.**

2.3. GC-MS and GC-FID Analysis

About 1 µL aliquot of each oil sample, appropriately diluted to 10 µL in GC grade *n*-hexane, was subjected to qualitative and quantitative GC-MS analysis. A hydrocarbon mixture of *n*-alkanes (C_8-C_{20}) was analyzed separately by GC-MS under the same chromatographic conditions using the same DP-5 column. Identification of compounds was based on the built-in libraries (NIST Co and Wiley Co, USA) and by comparing their calculated retention indices (RI) relative to (C_8-C_{20}) *n*-alkanes literature values, measured with columns of identical polarity, or in comparison with authentic samples [17]. *α*- and *β*-pinenes, *p*-cymene, limonene, linalool (Fluka, Buchs, Switzerland), eugenol, and sabinene hydrate (Sigma-Aldrich, Buchs, Switzerland) were used as reference substances in GC-MS analysis. GC-grade hexane and analytical reagent grade anhydrous Na_2SO_4 were from Scharlau (Barcelona, Spain) and UCB (Bruxelles, Belgium), respectively. Each sample was analyzed twice.

2.4. In vitro Assay for Antiproliferative Activity

Cell lines under investigation were human breast adenocarcinoma (MCF7, ATCC no. HTB-22) and human ductal carcinoma (T47D, ATCC no. HTB-133). Cells were cultured in RPMI media fortified with 10% heat inactivated bovine serum, 1% of 2 mmol/L L-glutamine, 50 IU/mL penicillin, and 50 μg/mL streptomycin. Human periodontal fibroblasts (PDL), which are a primary cell culture, were kindly provided by Dr. Suhad Al-Jundi and Dr. Nizar Mhaidat from Jordan University of Science and Technology, Irbid, Jordan.

Cells were seeded with a density of 5000 cell/well (15 000 cell/cm^2) and incubated at 37° C in a humidified atmosphere containing 5% CO_2. After 24 h, the cells were treated with the extracts, volatile oils, and controls. Test compounds were incubated with the cells for 72 h at 37º C in humidified conditions containing 5% CO_2. At the end of the exposure time, cell growth was measured using the sulphorhodamine B (SRB) assay as described earlier [18-19]. As positive controls, cisplatin and doxorubicin were used [19].

2.5. Tests for Apoptosis

Quantum Protein (Bicinchoninic Protein assay kit; EuroClone S.p.A, Siziano, Italy), Nucleosome ELISA (Roche Diagnostics GmbH; Mannheim, Germany), human total p53 ELISA, human total p21 ELISA, human Fas Ligand, human sFas immunoassay ELISA, caspase-8 colorimetric assay kits, wash buffer and substrate and stop solutions (R&D Systems Europe, Ltd; Abingdon, UK) were used.

2.6. Measurement of Apoptosis by ELISA

In our preliminary investigations, none of T47D selected extrinsic apoptosis biomarkers, namely FasL, sFas or caspase 8, were differentially or significantly up-regulated/activated in comparison to MCF7's. Thus, the decision was formulated to preferentially select for MCF7 over T47D in the antiproliferative action mechanism studies of *L. nobilis* aerial parts. In addition, it has been reported that proteins implicated in apoptosis regulation are more strongly expressed in MCF7 as compared to T47D [20]. The induction of apoptosis was assayed using the nucleosome ELISA kit. As described in the manufacturer's protocol, this kit is a photometric enzyme-immunoassay for the determination of cytoplasmic histone-associated-DNA-fragments after induced cell death. MCF7 cells were incubated with vehicle alone (0.1% DMSO) and with the obtained IC_{50}'s of the extracts under investigation for 72 h compared to the untreated (non-induced) basal cell incubations.

2.7. Assays of the Levels of p53, p21/WAF1, FasL (Fas Ligand) and sFas (Fas/APO-1)

MCF7 cells were treated as above and as described in the manufacturer's protocol. The samples of cell lysate were placed in 96-wellplates coated with monoclonal antibodies, and incubated for 2 h (sFas, p53, p21, or FasL) at RT. After removing the unbound material by washing buffer, horseradish peroxidase conjugated streptavidin was added. The absorbance was measured at 450 nm, and concentrations of p53, p21, FasL and sFas were directly determined by interpolating from standard curves. Results are presented as the percentage of the change of respective non-induced control determinations.

2.8. Assay for Caspase-8 Activity

As per the manufacturer's instructions, cell lysates were incubated with peptide substrate in assay buffer (100 mM NaCl, 50 mM HEPES, 10 mM dithiothreitol, 1 mM EDTA, 10% glycerol, 0.1% CHAPS, pH 7.4) for 2 h at 37º C. The release of *p*-nitroaniline was monitored at 405 nm. Results are represented as the percentage of the change of the activity compared to the untreated control cells.

2.9. Statistical Analysis

Results were expressed means (as %control) ± S.E.M (standard error of the mean). Statistical comparisons of the results were determined by ANOVA followed by Dunnett's post-test whenever appropriate using Graphpad Prism (version 3.02 for windows; GraphPad Software, San Diego, CA,

USA). Values of the means of untreated control and treated cells were considered significantly different if $P<0.05$ and highly significantly different if $P<0.01$ and $P<0.001$.

3. Results and Discussion

3.1. Volatile Oil Composition and Phytochemical Screening of Crude Extracts by TLC

After hydrodistillation, leaves and fruits of *L. nobilis* afforded yellowish oils with a yield of moisture-free 4.3 and 2.8 % (v/w), respectively. The retention indices and relative percentages of the identified compounds are presented in Table 1.

GC-MS analysis of the hydrodistilled essential oil of the fruits resulted in the identification of 45 components representing 99.7 % of the total oil content. Hydrocarbon and oxygenated monoterpenes were found to have the highest contribution to the hydro-distilled essential oil comprising 84.9 % of the total oil content. 1,8-cineole (eucalyptol) was the main oxygenated monoterpene detected, accounting for 29.8 % of the total oil content. With the exemption of α-terpinylacetate (5.6 %), α-terpineol (1.2 %) and γ-terpineol (1.5 %), other detected oxygenated monoterpenes were found to occur in concentrations less than 1%. Sesquiterpenes were found only in low concentration (12.7 %) with ß-elemene being the main constituent (6.2 %). GC-MS analysis of the leaf essential oil resulted in the identification of 37 compounds representing 93.7 % of the total oil content. 1,8-cineole (36.8%), α-terpinylacetate (14.6 %) and terpinene-4-ol (6.4 %) were the major components of the monoterpenoid fraction (83.3 %). Sesquiterpenoids were detected only at a concentration of 1.4 %.

Crude ethanolic extracts of *L. nobilis* leaves and fruits were rich in flavonoids and terpenoids. In TLC experiments, the occurrence of the well known flavonoids quercetin, luteolin, kaempferol and apigenin were identified in both the leaf and fruit extracts.

Volatile oil composition of the fruits and leaves of *L. nobilis* has been studied by several researchers. A comparison of these reports showed differences in the concentration of the major components. In addition to the genetic factors, determining the chemotype of the species, and the season during which the plants are collected, the environmental conditions, soil characteristics, methods of drying, extraction and analytical conditions do contribute to the differences in oil composition [21]. Consistent with our findings, 1,8-cineole is the main component of the volatile fraction as in all previous studies. The leaves of *L. nobilis* collected from Lebanon yielded 35.15% 1,8- cineole while the oil obtained from young and old leaves collected from North Black Sea region of Turkey yielded 24.2 % and 32.1 %, respectively [22] . Two other studies were carried out with the leaf oil from different location in South Turkey contained higher percentages for 1,8-cineole (46.6 %, 47.6 % , 59.9 % and 44.7 %, respectively) [23-24]. The findings of the current study are quite similar to those of Loizzo et al. [25], and stands at 36.8 %, although earlier in our laboratories, using the fresh leaves, higher percentage was detected (40.9 %) [18]. The lowest concentration for 1,8-cineole was reported from Portugal (27.2%) [26]. On the other hand, the concentration of 1,8- cineole in the hydro-distilled oil from the fruits of *L. nobilis* in the present study is higher (29.8 %) compared to the studies from Lebanon (9.4 %) and Turkey (9.5 % and 18.1 %, 20. 5 % , 17.4 %, respectively) [22-23].

Fluctuations were recognized with other constituents of the volatile fractions of the leaves and fruits, all studied *L. nobilis* oils from the Mediterranean countries were rich in monoterpenoids while sesquiterpenoids were detected in very low concentrations. In the present study, monoterpene hydrocarbons and oxygenated monoterpenoids had nearly equal share in the oil of the fruits (43.6 % and 41.3 %, respectively) while the leaf oil contained predominantly oxygenated monoterpenoids (66.2 %). Still oxygenated sesquiterpenes were absent in *L. nobilis* fruit oil. The main detected sesquiterpene in the bay fruit oil was ß-elemene (6.2 %). The latter compound found in the leaf oil only at a concentration of 0.3 %. Moreover, β-caryophyllene and caryophyllene oxide were detected in both oils in concentrations below 2 %.

Table 1. Comparison of the chemical composition of the essential oils hydrodistilled from the leaves and fruits of *Laurus nobilis*. Results present the average of 2 independent trials

RI*	Compound	Dry leaves Percentage**	Fruits Percentage**
852	(*E*)-3-hexanol	1.0	-
864	(*Z*)-3-hexanol	3.8	-
926[a]/931[b]	α-thujene	0.1	0.5
934	α-pinene	4.6	10.9
937	citronellene tetrahydro	0.1	-
944	β-citronellene	0.2	-
951	camphene	0.5	1.3
974[a]/973[b]	sabinene	3.1	4.4
980	β-pinene	3.6	8.4
985	*trans*-*m*-mentha-2,8-diene,	0.1	-
989	myrecene	0.4	-
992	dehydo-1,8-cineole	0.2	-
1009	α- phellandrene	-	9.0
1010	δ-2 -carene	0.4	-
1019[a]/1018[b]	α- terpinene	0.4	0.4
1026	*o*-cymene	1.0	-
1033[a]/1027[b]	*p*-cymene	0.3	2.5
1031[a]/1030[b]	limonene	1.6	2.5
1035[a]/1034[b]	1,8-cineole	36.8	29.8
1046	(*E*)-β-ocimene	-	3.2
1059	γ-terpinene	0.6	0.4
1073[a]/1072[b]	*cis*-sabinene hydrate	0.6	0.2
1088[a]/1087[b]	terpinolene	0.1	0.1
1102[a]/1101[b]	linalool	2.6	0.4
1128	1-terpineol	0.2	0.1
1146	*cis*-sabinol	0.3	0.2
1150	camphor	-	0.2
1173	*p*-mentha-1,5-dien-8-ol	0.6	-
1175 [a]/1173 [b]	terpinene-4-ol	6.4	0.2
1177	borneol	-	0.4
1185	α-terpineol	-	1.2
1201 [a]/1198 [b]	γ-terpineol	1.8	1.5
1208	*p*-cymene 9-ol	-	0.2
1271	iso-3-thujyl acetate	0.5	-
1287[a]/1286[b]	isobornylacetate	1.1	0.8
1316[a]/1315[b]	*cis*-dihydro-α-terpinylacetate	0.3	0.3
1340	δ-elemene	0.4	-
1350[a]/1348[b]	α-terpinylacetate	14.6	5.6
1360	nerylacetate	-	0.2
1372	α-ylangene	-	0.3
1385	β-cubebene	-	0.1
1392[a]/1390[b]	β-elemene	0.3	6.2
1409[a]/1405[b]	methyleugenol	4.2	0.2
1421	2,5-dimethoxy-*p*-cymene	0.2	-
1423	β-caryophyllene	-	1.1
1438	α-guaiene	-	0.5
1444	aromadendrene	-	0.3
1452	*cis*-muurola 3,5-diene	-	0.2

1459	α-humulene	-	0.3
1484	γ-muurolene	-	0.7
1491	β-selinene	0.4	-
1493	germacrene D	-	0.5
1500	δ- selinene	-	0.6
1505	α-selinene	-	1.0
1511	germacrene A	-	0.2
1517	γ-cadinene	-	0.5
1521	δ-cadinene	-	0.2
1525	undecenoic acid-10-methyl, methyl ester	-	0.8
1586	caryophyllene oxide	0.3	-
1596	dodecenoic acid ethyl ester	-	1.1
Total identified		**93.7**	**99.7**
Monoterpenoids		83.3	**84.9**
Monoterpenoid hydrocarbons		17.1	43.6
Oxygenated monoterpenoids		66.2	41.3
Sesquiterpenoids		**1.4**	**12.7**
Sesquiterpene hydrocarbons		1.1	12.7
Oxygenated sesquiterpenes		0.3	-
Phenylpropanoids		**4.2**	**0.2**
Miscellaneous		**4.8**	**1.9**
Unidentified		**6.3**	**0.3**

* Retention indices (RI) calculated on (DB-5MS) column, ** Percentage is given as the average of two independent measurements. Compounds are listed based on their elution order on the corresponding column. [a] refers to RI of the leaves, [b] refers to RI of the fruits.

3.2. Antiproliferative Activity

The *in vitro* antiproliferative activity of the different fractions is shown in Table 2. Results present the IC_{50} values of the fractions (concentration of the extract needed to reduce proliferation by 50% after 72 h of incubation compared to control wells). IC_{50} values were determined by plotting dose response curves for the fractions under investigation in the range of 0.1 to 100 µg/mL). The IC_{50} values of reference drugs; cisplatin and doxorubicin were 7.3 ± 1. 9 µM and 0.16 ± 0.0 µM for MCF7 cells, 21.3 ± 9.7 µM and 0.2 ± 0.0 µM for T47D cells respectively.

Table 2. The antiproliferative activity of *Laurus nobilis* leaves- and fruits-extracts on MCF7 and T47D cell lines.

	MCF7		**T47D**	
	Leaves	**Fruits**	**Leaves**	**Fruits**
Ethanol	48.2±5.2	28±1.7	19.8±4.3	12.3±4.0
Chloroform	48.8±10.3	31.2±5.24	58.2±14.1	35. 6 ± 5.6
Butanol	Non toxic	72. 5±5.8	Non toxic	75.4±6.4
Ethyl acetate	61.0±18.4	20.6 ± 4.5	27.9±1.3	33.7±13.9
Aqueous	Non toxic	Non toxic	Non toxic	Non toxic
Volatile oil	93.1±4.7	41.9±3.3	64.3±3.1	142±43.6

Results present the IC_{50} in µg/mL (concentration needed to reduce cell proliferation by 50%). Results present the average of at least two determinations on two cell line passages and each is an average with standard deviation of four wells.

For both cell lines, *L. nobilis* fruit extracts showed higher antiproliferative activity compared to the leaf extracts. In both parts, aqueous and butanol fractions demonstrated weak or no reduction in

cellular viability at the concentration range under investigation. The criteria of cytotoxic activity for the crude extracts, as established by the American National Cancer Institute (NCI) is an IC_{50} less than 30 µg/mL in the preliminary assay [27]. The ethanol extract of the fruits exhibited prominent antiproliferative activity with an IC_{50} of 12.3 µg/mL for T47D cell line and 28 µg/mL for MCF7 cells, while the IC_{50} values of the leaves were higher (19.8 µg/mL for T47D cells and 48.2 µg/mL for MCF7).

Similar results were observed for *L. nobilis* grown in Turkey, where methanol extracts of leaves, fruits and seeds were evaluated for their ovarian cytotoxic activity and DNA damaging properties against three types of yeast. In this study, the most cytotoxic extract was that of the fruit and further identification led to cytotoxic sesquiterpenes isolated from the fruits rather than from the leaves or seeds [28]. In another study, *L. nobilis* leaves from Palestine were also tested for their anticancer activity against MCF7 as well as L929sA (murine fibrosarcoma cell line) and MDA-MB 231(breast cancer cell line). Dichloromethane: methanol extracts were used and here the leaves were reported as not cytotoxic against the breast cancer cell line models used and had an IC_{50} of 174 µg/mL against the murine fibrosarcoma [29]. The concentration range of the crude extracts under investigation was nevertheless not mentioned.

Cytotoxic activity has been reported for the crude bay leaf extract and for sesquiterpene lactones isolated from the leaves and fruits of *L. nobilis.* The cytotoxicity was evaluated *in vitro* using human tumor cell lines such as Jurkat, HL-60, LoVo, SH-SY5Y, MCF7 and A2780 and promising results were obtained, especially with the isolated sesquiterpene lactones [28-31].

The antiproliferative activity of the volatile oils and pure compounds was investigated. Firstly, the crude oils were tested for their antiproliferative activity and here, the activity was less than that for both the ethanol and ethylacetate fractions. Secondly, the antitumor propensities of selected pure volatile compounds of the volatile oil fraction were tested, this included, 1,8-cineole, α-pinene, β-pinene, limonene, linalool, borneol, β-caryophyllene and caryophyllene oxide. From those, with the exception of β-caryophyllene and caryophyllene oxide, no/or minimal antiproliferative activity was detected from 0.1 up to 100 µg/mL. β-Caryophyllene and caryophyllene oxide exhibited potent biological activity; 14.4 ± 1.6 µg/mL for β-caryophyllene and 8.1 ± 0.7 µg/mL for caryophyllene oxide against MCF7 cells and 14.4 ±1.3 µg/mL for β-caryophyllene and 6.1 ± 0.8 µg/mL for caryophyllene oxide against T47D cells.

Although present in low concentrations; β-caryophyllene and caryophyllene oxide, could have contributed to the antiproliferative activities observed with the volatile oil fractions and crude extracts of the tested plants. Moreover, the well known flavonoids quercetin, luteolin, kaempferol and apigenin were identified in the ethanol extracts. In earlier publications [19] the IC_{50} of luteolin was determined (5.3 ± 0.4 µg/mL for MCF7 cells and 4.3 ± 0.1 µg/mL for T47D) and it was recognized as a potent antiproliferative agent in *Eminium spiculatum.* It can be concluded that the observed antiproliferative activity of the extracts are attributed to the flavonoids in addition to the mentioned pure volatile compounds. It was postulated by Kwom et al. [32] that induction of apoptosis by kaempferol and quercetin was caspase-3 dependent. Similar caspase-3 (and caspase-7) dependent apoptotic pathways were described for luteolin [33].

β-Elemene (found in *L. nobilis* fruits at a percentage of 6.9 but only 0.3 % in its leaves) may contribute to the differences in cytotoxic activity of the crude volatile oils as well as the ethanol, chloroform and ethyl acetate extracts from the two different aerial parts. β-Elemene has been reported to have anticancer activity against cancers of brain, breast, lung and other tissues after different *in vivo* and *in vitro* experiments. The mechanism of its anticancer effect is still unclear; nevertheless, it could be due to direct cytotoxic activity, and inhibition of free radical formation. Several reports attribute this activity to induction of apoptosis and suppression of tolemerase activity [34].

In an attempt to study the safety of these extracts, the antiproliferative activity of the crude ethanol extract was tested against normal freshly excised human periodontal cells. In those, and after 72 h incubation, the IC_{50} values for both parts were higher than those determined against the selected cancer cell lines; (81.3 µg/mL for the fruits and 41.8 µg/mL for the leaves). This suggests its preferential selectivity for cancer tissues over normal ones.

3.3. Effects of Fruit and Leaf Extracts on Induction of Apoptosis as Detected and Quantified by Nucleosome ELISA

Apoptosis is governed by a complex network of effector molecules. It is characterized by DNA fragmentation, cell shrinkage and nuclear condensation, and phosphatidylserine translocation from the inner to the outer leaflet of the plasma membrane bilayer [5]. Cisplatin's potent cytotoxicity is primarily mediated by its ability to cause DNA damage and subsequent apoptotic cell death. However, it was found that its anti-tumerogenic efficacy and proapoptotic features in many cancer cell lines involves the activation of different forms of cell death, i.e. the receptor mediated apoptotic extrinsic pathway, the intrinsic pathway as in alterations in mitochondrial membrane permeability and cytochrome c release and a death process mediated by endoplasmic reticulum stress [35].

Compared with basal control wells, 28 µg/mL of *L. nobilis* fruit extract induced a significantly detectable enrichment in the cytoplasmic mono- and oligonucleosomes after assumed induction of programmed MCF7 cell death (227.3 ± 23.3%, $p<0.05$, n=4, Figure 1). More importantly, 48.2 µg/mL of *L. nobilis* leaf extract augmented highly significantly cytoplasmic enrichment following polynucleosomes disintegration and DNA fragmentation in treated MCF7 wells over 72 h (1295 ± 37.2 %, n=4, $p<0.001$ vs. basal (untreated) controls, the same figure).

3.4. Effect of L. nobilis Fruit and Leaf Extracts on Receptor-mediated Apoptosis-target Molecules

Previous reports have indicated that MCF7 cells have a normal (non-mutated) tumor suppression gene, p53. Cisplatin is a chemotherapeutic drug used for several human malignancies. Exceptionally, cisplatin 72 hours incubation induced a highly significant augmentation of p53 levels (233.8 ± 10.4%, n=4, $p<0.001$ vs. basal control MCF7 wells).

In examining the effects of *L. nobilis* fruit and leaf extracts on cell cycle regulatory molecules, including p53 and its downstream molecule p21, Figure 2 demonstrates that *L. nobilis* fruit (28 µg/mL) enhanced the expression of neither protein at the examined incubation time. Thus, *L. nobilis* fruit extract proapoptotic efficacy might not be regulated by either p53 or p21. Interestingly, our results indicate that *L. nobilis* leaf extract components enhanced the p53 levels highly substantially (140.1 ± 4.4%, n=4, $p<0.001$ vs. basal control MCF7 wells, Figure 2) less effectively than cisplatin, although mostly unlikely, *L. nobilis* leaf extract proapoptotic effect was p21-independent. To establish the sequence of events involved in *L. nobilis* induction of apoptosis, the recruitment of sFas/FasL-mediated execution of apoptosis was investigated. Over 72 h, sFas/FasL system was not selectively upregulated in either *L. nobilis*-parts mediated inhibition of proliferation in MCF7 treatments (Figure 3). We next examined the downstream caspase of sFas/FasL system, and consistent with our findings on the lack of sFas/FasL system modulation, neither of *L. nobilis* parts increased caspase 8 activity at 72 h compared to basal control wells (Figure 4).

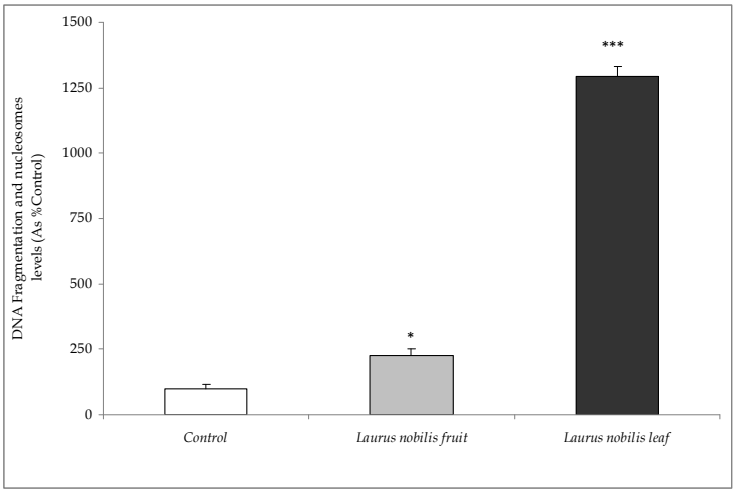

Figure 1. Effects of antiproliferative *Laurus nobilis* extracts on proapotosis DNA fragmentation.Cell lysates containing cytoplasmic oligonucleosomes of apoptotic cells were analyzed by means of nucleosome ELISA kit to investigate the induction of apoptosis in MCF7 cells by antiproliferative *Laurus* spp. Results expressed as %control are mean ± SEM (n=4 independent determinations). *P<0.05 and ***P<0.001 indicates that bioactive plants extracts had highly significant statistical differences compared to control (non-induced) wells and, as analyzed by ANOVA followed by Dunnett's test.

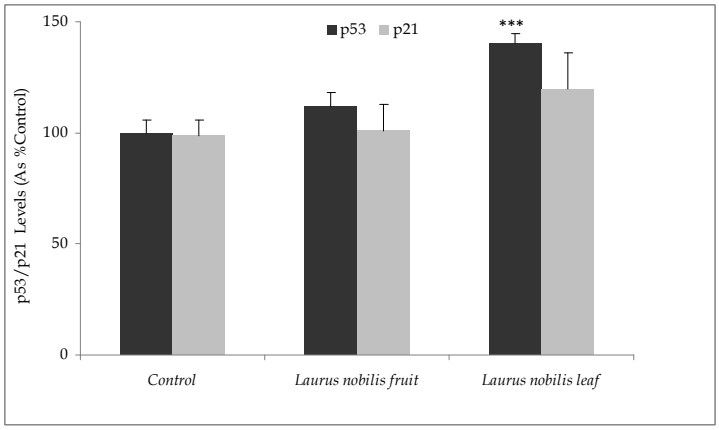

Figure 2. Effects of antiproliferative *Laurus nobilis* on protein expression of p53/p21 in Human breast adenocarcinoma MCF7 cells, as determined by p53/p21 ELISA kits. Results expressed as % control are mean ± SEM (n=4 independent replicates). ***P<0.001 indicate that plants' incubations had highly significant statistical differences *vs.* control (basal non- induced) wells, as analyzed by ANOVA followed by Dunnett's test.

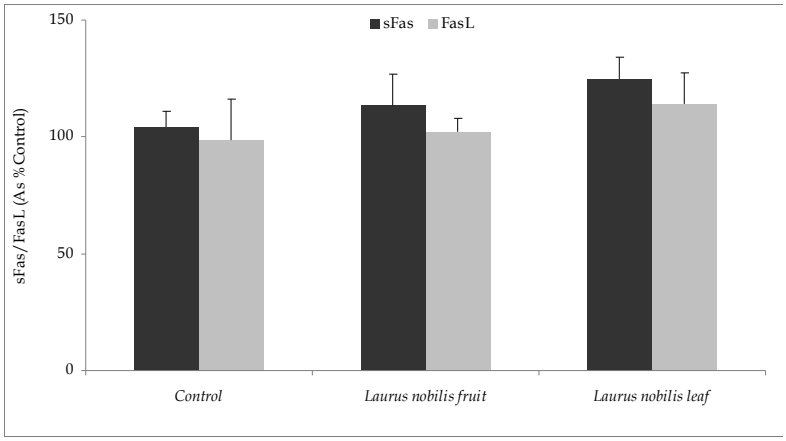

Figure 3. The lack of Fas/Fas ligand apoptotic system involvement in *Laurus nobilis* induction of apoptosis in MCF7 cells. Results expressed as %control are mean ± SEM (n=4 independent replicates). None of the bioactive plants'-treatment wells had a statistically significant difference *vs*. control (non-induced) incubations, as analyzed by ANOVA followed by Dunnett's test.

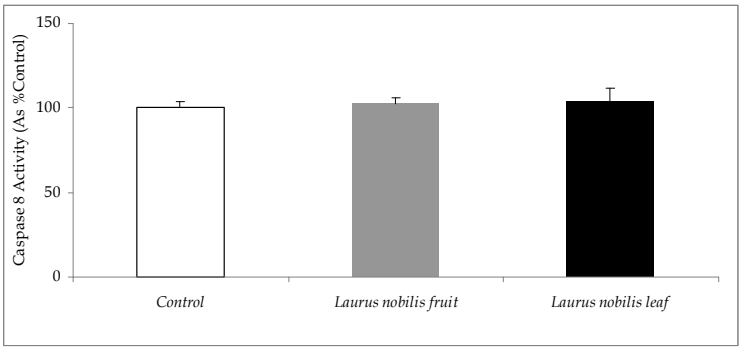

Figure 4. The lack of activation of caspase -8 in MCF7 cells by antiprolifertaive *Laurus nobilis* extracts. Results are expressed as %control are mean ± SEM (n=4 independent determinations). None of the bioactive plants'-treatment wells had a statistically significant difference *vs*. control (non-induced) incubations, as analyzed by ANOVA followed by Dunnett's test.

The findings of the present study coincide with those of Jiang et al. [36], where p53 activation was an early signal for apoptosis during cisplatin treatment [36]. Unequivocally, only *L. nobilis* leaf extract evoked a highly marked increase in p53 without a concomitant increase in the downstream effector molecule p21. The pro-apoptotic efficacy of fruits and leaf extracts was further verified and substantiated, at least in part, by DNA-fragmenting executions. Both extracts, on the other hand, were unable to augment the assembly of the MCF7 death-inducing signaling complex responsible for the activation of caspase-8 and thus unable to enhance MCF7 cells' extrinsic apoptosis cascade. This signified the caspase-8 independent action mechanism of these extracts. Importantly, caspases are very well known to act as key intermediates of apoptosis and to contribute to the apoptotic morphology through the cleavage of various cellular substrates. Consequently, it can be hypothesized that *L. nobilis* antiproliferative effectiveness may invoke other possible pathways and further studies are necessary to investigate the precise mechanisms responsible. Fruits and leaves did not seem to target succinctly the same signal transduction effectors. Hence, more elaborative investigation may verify the plant's promising multi-targeted malignancy therapeutics.

Taken together, the present data highlight *L. nobilis* as a potential natural agent for breast cancer therapy. They also present *L. nobilis* important implication as a safe and effective alternative to conventional breast cancer intervention.

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