Antioxidant and antitumor activities of polysaccharides and oils extracted from *Laurus* growing in Lebanon

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**Abstract:**

*Laurus* (Lauraceae) grows in many warm regions of the world, particularly in southern Europe and around the shores of the Mediterranean Sea, including Lebanon. The aim of the present study was to extract and study, for the first time, the antioxidant and antitumor activities of different types of polysaccharides and oils from *Laurus*. In order to elucidate their structures, H¹NMR spectroscopy was performed as well as infrared spectroscopy which allowed us to reveal their functional groups. Antioxidant effects were also tested, using DPD and DPPH methods that showed a great antioxidant activity. Furthermore, we investigated the antitumor activity of the polysaccharides, essential oil, and fatty oil obtained from *Laurus* against MCF-7 and MDA breast cancer cell lines, by the XTT assay with serial dilutions. At final concentration of 200µg/mL, the XTT results showed a strong activity for the fatty oil against MCF7 and MDA cell lines. That activity was higher than the other extracts, where 72% and 88% of MCF7 cells and 86% and 91% of MDA cells were dead after 24h and 48h respectively.

**Key Words:** *Laurus*, polysaccharides, essential oil, fatty oil, antioxidant, antitumor, MCF-7 and MDA breast cancer cell lines.

**I. Introduction**

Medicinal and aromatic plants were used in pharmaceutical preparations many years ago (Dias *et al.*, 2014 a). Nowadays, and due to the potential negative health effects of the synthetic chemical compounds as antitumor agents, it was important to seek new agents as plants; sources of natural compounds with less side effects (Dias *et al.*, 2014 b).

*Laurus*, commonly known as sweet bay, bay laurel, Grecian laurel, true bay, and bay tree, can be found either as an evergreen shrub or as small trees. It belongs to the family Lauraceae which comprises numerous aromatic and medicinal plants (Hogg *et al.*, 1974). Laurel usually grows 20 to 30 feet in height in many warm regions of the world, particularly in southern Europe and around the shores of the Mediterranean Sea (Lewis, 1984) including Lebanon in which it has been there for long time. Different extracts of laurel showed *in vitro* and *in vivo* antioxidant activities (Kaurinovic *et al.*, 2010). *Laurus* essential oil is used for relieving haemorrhoid and rheumatic pains and it is also used as a diuretic (Kivcak *et al.*, 2002). It has antibacterial and antifungal properties as well (Erturk, 2006). Also *Laurus* contains costunolide, an important sesquiterpene lactone (Ferrari *et al.*, 2005), and anticancer agent (Rasul *et al.*, 2012). Moreover, some *Laurus* constituents as gazanilide, spirafolide, reynosin, and santamarine possess neuroprotective activities (Patrakar *et al.*, 2012). Pharmacological studies have demonstrated the anesthetic, hypothermic, muscle relaxant and anticonvulsant activity of eugenol and methyleugenol (Sayyah *et al.*, 2002). Laurel has been used to treat epilepsy, neuralgia and Parkinsonism (Ham *et al.*, 2010). Given the scarcity of local studies on Laurel widespread in Lebanon, the aim of the current study was to isolate new polysaccharides (Fucoidane, Laminaran, Alginate) and different oils from the leaves or fruits to study their antioxidant and antitumor activities.
II. Materials and methods
II.1. Plant material
The samples of Laurus leaves and fruits were collected from Lebanon, in year 2013. The samples were air-dried at room temperature in the shade for few weeks. Their final moisture content was 10.0%. Before using them, the dried samples were milled in a blender so the size of the particles ranged between 0.8-0.9mm.

II.2. Volatile oil extraction
The volatile oil of Laurus leaves or fruits were obtained by hydrodistillation process in the clevenger apparatus. One hundred grams of Laurus (leaves or fruits), were placed in a flask (2.5L) and hydrodistilled for 2.5h. The oil samples were dried over anhydrous sodium sulphate and stored at 4°C in the dark.

II.3. Fatty oil extraction
Fatty oil from fruits was extracted twice by petroleum ether in ultrasonic bath for 15 min. Then the samples were filtered, and the fractions were evaporated under reduced pressure using a rotary evaporator at 50°C. The fatty oil was dried and stored in the dark at room temperature until use.

II.3. Isolation of the polysaccharides from Laurus
Fifty grams of dry Laurus leaves were extracted twice by ethanol (96%) for 3 hours at 40°C (Laurus: ethanol 1:0.8 w/w) to remove compounds of low molecular weight. The samples were centrifuged (3000 rpm, 20 minutes). The supernatant was discarded while the leaves were dried and extracted for 3 hours, 2 times by 150mL HCl (pH 2.0-2.3) at 60°C. Again the samples were centrifuged so that the supernatant 1 was used to extract fucoidan and laminaran while pellet 1 was used to extract alginate. Pellet 1 was soaked successively in aqueous solutions (100mL) of 3% and 1.5 % Na2CO3 for 8 hours at 60°C and rinsed by water. The extracts were dialyzed for 24 hours using a membrane with a retention limit of 100KDa, and then precipitated with absolute ethanol. Then, the precipitate was dissolved in water with pH=2 adjusted using HCl (12%). The obtained precipitate of alginic acid was then dissolved in a little amount of water with addition of NaOH so that the pH=8.6. The final solution was lyophilized to obtain alginate powder. However, the supernatant 1 was chromatographed over a column (polytetrafluoroethylene, 15 x 6.5) to extract the polysaccharides (fucoidan and laminaran). The fractions were eluted with water, followed by 5% and 15% aqueous ethanol/water. The elution continued until the phenol-sulfuric acid test showed an absence of carbohydrates in the eluate. The fraction eluted with ethanol, contained laminaran, and that eluted with water contained fucoidan.

II.4. H1 NMR and C13 NMR spectra
Nuclear magnetic resonance (NMR) spectra of alginate and fucoidan were recorded using Ultrashield 300 Bruker spectrometer at room temperature, with a frequency of 300MHz, an acquisition time of 5.29s and duration of impulse of 11µs. Three milligrams of the sample was dissolved in 0.5mL of 99% D2O. Tetramethylsilane (TMS) was used as internal standard.

II.5. FT-IR spectroscopy analysis
The Fourier transform infrared (FT-IR) spectra were recorded on a JASCO FT/IR6300 spectrometer. The resolution was 4cm⁻¹. Data were collected in the range of 4000-400cm⁻¹. All the alginate samples were prepared for measurement in the form of KBr pellets.

II.6. Antioxidant activity
II.6.1. DPD method
The antioxidant activity of Laurus extracts were measured using DPD (N, N-diethyl-P-phenylenedialanine) test. DPD method depends on electrolysis technique; a process by which water splits into hydrogen and oxygen via electricity. Water molecules near the cathode are split up into a positively charged hydrogen ion (H⁺) and a negatively charged hydroxide ion (OH⁻). The physiological tyrode solution was first prepared from: NaCl (137.0mM), KCl (2.7mM), MgCl₂ (1.0mM), CaCl₂ (1.5mM), NaH₂PO₄ (0.4mM), and NaHCO₃ (12.0mM). The electrolysis of physiological solution in the absence of the extracts was carried out in a bath containing 20mL of the prepared solution provided with two platinum electrodes maintained at distance of 2 cm from each other, and connected by electric wires to a stimulator adjusted to 10mA by a sensitive multimeter. As a result, a cascade of free radicals will be generated by electrolysis in the tyrode solution. A magnetic stirrer was used to speed up the mixing and homogenizing of the medium (Chahine et al., 1998). Each minute, 1mL from the electrolyzed physiological solution and 2mL of DPD (25mg/mL) were taken and vortexed. Then, the absorbance of the mixture was measured using spectrophotometer at 515nm (control). Similarly, the electrolysis of the physiological solution was performed in the presence of different concentrations (100, 500 and 1000µg/ml) of the isolated polysaccharides, and then the absorbance was measured. Trolox was used as standard of comparison. The values are expressed as mean ± SD and the experiment was done in triplicate.
II.6.2. DPPH method
The procedure described by (Braca et al., 2001) with minor modifications has been used for the scavenging ability of DPPH (1,1-Diphenyl-2-picrylhydrazyl) antioxidant test. At first 7.8mg of DPPH powder was dissolved in 100mL of absolute methanol which is equivalent to 0.2mM of DPPH. Then, 10 concentrations of each polysaccharide were prepared by serial dilution in methanol. In a tube, 1mL of each concentration was mixed with 1mL of DPPH, agitated and kept in dark for 30 minutes at room temperature, so that the free radical of DPPH reacts with antioxidants in extracts. Then the absorbance of each mixture was measured using spectrophotometer at 517nm. In fact, the free radical DPPH with an odd electron gives a maximum absorption at 517nm (purple colour) in which 1mL of methanol was mixed with 1mL of DPPH (control). When antioxidants react with DPPH, it paired off in the presence of a hydrogen donor and reduced to DPPHH. As a consequence, the absorbance of the solution decreases. The reaction results in a decolourization (yellow colour) of the solution. The blank used was absolute methanol. The DPPH free radical scavenging activity was calculated as follows: scavenging % = [(Absorbance of control - Absorbance of test sample)/Absorbance of control] × 100. The ascorbic acid was used as a standard, and for comparing the IC50 of each polysaccharide with the IC50 of the standard. The values are expressed as mean ± SD and the experiment was done in triplicate.

II.7. Antitumor activity
The cell lines used in this study were breast cancer cell lines MCF7 and MDA according to (Scudiero et al., 1988). The cells were grown in plastic bottles (75cm2) containing DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10.0% fetal calf serum (Gibco 16000-044), 1% penicillin (10000 IU/mL), and streptomycin (10mg/mL) (Gibco 15070) at 37°C in humid air with 5% CO2.

The cytotoxicity of Laurus extracts (polysaccharides and oils) on these cell lines was determined by the XTT assay using cell proliferation kit II. Incubation of cells with various concentrations of each of Laurus extract was carried out over 24h and 48h following the addition of sodium 3’-[1-(phenylaminocarbonyl)-3,4-tetrazolium]- bis (4-methoxy-6-nitro) benzene sulphonic acid hydrate (XTT) and N-methyl dibenzopyrazine methyl sulphate. Cells were incubated for another 2 hours. Viable cells with active mitochondrial dehydrogenase metabolize XTT to a coloured formazan, and their amount accumulating in growth medium was assessed spectrophotometrically. The results were expressed as percentage inhibition relative to control cells (considered as 100%).

Evaluation of cytotoxicity: MCF7 and MDA cell lines grown in DMEM medium containing 2mM L-glutamine supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin was maintained at a density of 5x10^5 cells/mL in a standard cell culture incubator at 37°C, 100% relative humidity, and 5% CO2 atmosphere. Cytotoxic assays and determination of IC50 (drug concentration that inhibits cell proliferation 50% when compared to untreated controls) dose of in MCF7 and MDA cells were performed by using XTT assay as indicated in manufacturer’s instruction. Cytotoxicity of Laurus extracts by XTT-based cytotoxicity assay was determined as follows: Cells (2x10^5) were seeded in 96-well tissue culture plates and incubated for 24h without drug. After addition of drug, cells were incubated for 24h, and cell viability was assessed using XTT-PMS mixture (XTT sodium salt; [2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt], phenazinemethosulfate (N-methyl[phenazonium methyl sulfate salt]), as recommended by supplier. Formazan formation was quantified spectrophotometrically at 490nm using the microplate reader (Bio-Rad, Coda, Richmond, CA).

II.8. Statistical results
Statistical results are expressed as mean ± standard error of mean (SEM). Statistical significance was determined. The differences were considered significant when P< 0.05.

III. Results
III.1. Isolation of the polysaccharides from Laurus
According to the method of (Imbs et al., 2009), the amount of alginate obtained from 50g of Laurus leaves was 0.26g with a yield of 0.52%, while the fucoidan amount was 0.63g and the laminaran amount was 0.21g.

III.2. H1 NMR spectroscopy of alginates
H1 NMR spectroscopy is considered to be the most reliable method to determine the composition and the detailed structure of alginates (Heyraud et al., 1996), which are typically described by their mannuronic / guluronic (M/G) ratio. Usually the ratio M/G is calculated using the method proposed by (Grasdalen et al., 1979).

The results from H1 NMR spectra of Alginate isolated from Laurus showed:

<table>
<thead>
<tr>
<th>FG</th>
<th>1A / (IB + IC)</th>
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<td>0.52</td>
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<table>
<thead>
<tr>
<th>FM</th>
<th>1- FG</th>
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<tr>
<td>0.48</td>
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</table>

M/G = FM/FG = 0.48/0.52 = 0.92

This ratio was between 0.2-1.4, thus the alginate isolated from Laurus is a good elastic gelling agent.
III.3. FT-IR spectroscopy analysis of alginate
The FT-IR spectrum of alginate isolated from Laurus is presented in figure 1. In the region between 3615.6 -1600.63 cm⁻¹ three bands appeared; a broad band centred at 3438.46 cm⁻¹ assigned to hydrogen bounded O-H stretching vibrations, the weak signal at 2920.66 cm⁻¹ is due to CH stretching vibrations, and the asymmetric stretching vibration of O-C-O is centered at 1600.63 cm⁻¹. The band at 1423.21 cm⁻¹ may be due to the C-OH bending vibration with contribution of carboxylic acid group O-C-O (Mathlouthi et al., 2001; Silverstein et al., 1991). Or the weak bands at 1097.3 cm⁻¹ may be assigned to O-C-H deformation. The band at 1022.09 cm⁻¹ may be also due to C-O stretching vibration. Moreover, the bands at 703.89 cm⁻¹, which is a weak band assigned to the C-I-H deformation vibration of α-L-guluronic acid (Mackie, 1971). While the bands 538.042 cm⁻¹ may be due to C-H Alkenes.

III.4. C¹³ NMR study of fucoidan
The C13 NMR spectrum of fucoidan fractions was complex, with the main signals of L-fucan sulphates appearing at 93.78-107 ppm (C1) and 15-16.7 ppm (C6). The signals 18-20 ppm, CH3, 170-179 ppm, C = O indicated the presence of acetyl groups and signal 16, 75 ppm refer to α-L-Fu canes.

III.5. FT-IR spectroscopy analysis of fucoidan
The FT-IR spectrum of fucoidan isolated from Laurus is presented in figure 2. A broad band centred at 3305.39cm⁻¹ is assigned to the hydrogen bonded O-H stretching vibration; a weak band at 2932.23 cm⁻¹ is assigned to a C-H stretching vibration. The band centred at 1608.34 cm⁻¹ is assigned to the carbonyl group C=O, the one at1521.56 cm⁻¹ is assigned to the C=O bond of the carboxylate group. The band at 1282.43 cm⁻¹ corresponds to thebonds=O, and band 822.491 cm⁻¹ and 773.915 cm⁻¹ S-O-C group Sulphate, the fingerprint in the region 674.963-466.082 cm⁻¹ was difficult to analyze since no previous work on fucoidan has been done nor its structure has been identified yet.

III.6. Antioxidant activity
III.6.1. DPPH method
The electrolysis of the tyrode solution generated FR (free chlorine, hypochlorous acid, and different reactive oxygen species) which react instantaneously with the DPD reagent generating a red colour whose absorbance is proportional to the generated FR. For the control curve without adding any active molecules, absorbance increased with time reaching a maximal value of 1.2 at the fifth minute of electrolysis for the same initial concentration 4mg/mL. While the concentration of alginate increased from 100µg/mL to 1000µg/mL, the absorption decreased dramatically and reaches a maximal value at the fifth minute of electrolysis about 0.71±0.02µg/mL to 0.28±0.08µg/mL(Figure 3. B). However, laminaran decreased from 0.51±0.06µg/mL to 0.08±0.02µg/mL(Figure 3. C). Fucoidan decreased from 0.39±0.04µg/mL to 0.07±0.02µg/mL(Figure 3. D). Thus, the increased concentration of polysaccharides allowed more FR trapping, and high antioxidant activity. We can conclude that the antioxidant activity of fucoidan extract from Laurus is higher than the that of laminaran which is higher than that of alginate. By comparing the antioxidant effect of trolox, alginate, laminaran and fucoidan at the concentration 100µg/ml it is clear that alginate and trolox are closed to each other according to their antioxidant activity, and laminaran presented higher antioxidant properties than them while fucoidan has the highest antioxidant activity according to this method (Figure 3. A).

III.6.2. DPPH method
In the DPPH free radical scavenging activity, the three polysaccharides compounds of alginate, laminaran, and fucoidan were evaluated for their free radical scavenging activity with ascorbic acid as standard compound. The IC₅₀ was calculated for each polysaccharide as well as ascorbic acid as standard and the results are summarized graphically in figure 4. The scavenging effect increased with the increasing concentrations of the tested compounds. The IC₅₀ values for polysaccharides were 3.26±0.11µg/mL, 2.23±0.82µg/mL and 1.62±0.04µg/mL for alginate, laminaran, and fucoidan respectively. They were comparatively lower than the IC₅₀ (1.40±0.76µg/mL) of ascorbic acid. These results indicated that fucoidan is equally effective antioxidant agent compared to ascorbic acid.

III.7. Antitumor activity
The polysaccharides, in addition to the essential and fatty oil obtained from Lebanese Laurus had shown variations in their biological effects. The effects of these materials on the growth of MCF7 and MDA breast cancer cell lines are shown in figure 5 and figure 6 respectively. Cells were treated with different concentrations of these materials (5, 25, 50, 100, 200µg/mL) for different periods of time (24h and 48h). Following this treatment, the proliferation of the cells was studied by XTT colorimetric assay. At a final concentration of 200µg/mL, the fatty oil showed a strong activity against MCF7 and MDA cell lines where 72% and 88% of MCF7 cells and 86% and 91% of MDA cells were dead after 24, 48h respectively. The antitumor activity of Laurus fatty oil was higher than those of the other extracts. The XTT results are shown in table 1.
Figure 1. Infrared spectrum of alginate isolated from the leaves of *Laurus*.

Figure 2. Infrared spectrum of fucoidan isolated from the leaves of *Laurus*.
Figure 3. Variation of the antioxidant activity of the polysaccharides isolated from *Laurus*. Trolox and the Polysaccharides (A), Alginate (B), Laminaran (C), Fucoidan (D).

Figure 4. The antioxidant activity of the polysaccharides against DPPH.
Figure 5. The activity of *Laurus* extracts against MCF7, A: Fucoidan for 24h, B: Fucoidan for 48h, C: Laminaran for 24h, D: Laminaran for 48h, E: Alginate for 24h, F: Alginate for 48h, G: Essential oil for 24h, H: Essential oil for 48h, I: Fatty oil for 24h, J: Fatty oil for 48h.
48h, C: Laminaran for 24h, D: Laminaran for 48h, E: Alginate for 24h, F: Alginate for 48h, G: Essential oil for 24h, H: Essential oil for 48h, I: Fatty oil for 24h, J: Fatty oil for 48h

Figure 6. The activity of *Laurus* extracts against MDA, A: Fucoidan for 24h, B: Fucoidan for 48h, C: Laminaran for 24h, D: Laminaran for 48h, E: Alginate for 24h, F: Alginate for 48h, G: Essential oil for 24h, H: Essential oil for 48h, I: Fatty oil for 24h, J: Fatty oil for 48h
Figure 6. The activity of *Laurus* extracts against MDA, A: Fucoidan for 24h, B: Fucoidan for 48h, C: Laminaran for 24h, D: Laminaran for 48h, E: Alginate for 24h, F: Alginate for 48h, G: Essential oil for 24h, H: Essential oil for 48h, I: Fatty oil for 24h, J: Fatty oil for 48h
Table 1. Antitumor activity of different concentrations of *Laurus* extracts against MCF7 and MDA breast cancer cell lines.

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>% of viable MCF7</th>
<th>% of viable MDA</th>
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<tr>
<td></td>
<td>24h</td>
<td>48h</td>
</tr>
<tr>
<td>Fucoidan</td>
<td>56</td>
<td>48</td>
</tr>
<tr>
<td>Laminaran</td>
<td>75</td>
<td>79</td>
</tr>
<tr>
<td>Alginate</td>
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<td>28</td>
</tr>
<tr>
<td>Essential oil</td>
<td>58</td>
<td>58</td>
</tr>
<tr>
<td>Fatty oil</td>
<td>58</td>
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</tr>
<tr>
<td>Fucoidan</td>
<td>84</td>
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<tr>
<td>Laminaran</td>
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<td>85</td>
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<tr>
<td>Alginate</td>
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<td>58</td>
</tr>
<tr>
<td>Essential oil</td>
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<td>Fatty oil</td>
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<tr>
<td>Fucoidan</td>
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<td>Laminaran</td>
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IV. Discussion

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced normally in our body as a result of normal cell metabolism, however, the imbalance between their generation and the cell capacity to detoxify them can lead to oxidative stress. This contributes to many pathological problems such as cancer, cataracts, and heart diseases. In fact, scientists are interested in finding natural antioxidant agents to replace synthetic ones and avoid their adverse side effects (Emam et al., 2010).

In the present study, three polysaccharides extracted from *Laurus* were examined for their antioxidant activities. DPD test showed that the three extracted polysaccharides possess antioxidant activities with fucoidan having the highest effect, followed by laminaran and then alginate. DPPH test indicated that fucoidan antioxidant capacity is equivalent to that of ascorbic acid. Antioxidants detoxify free radicals via different mechanisms: either by hydrogen donation (Farhan et al., 2013) or by chelating metals preventing their reactions with ROS (Flora, 2009). The hydrogen donating hydroxyl group revealed by the FT-IR spectroscopy analysis of fucoidan and Alginate may contribute to the antioxidant power of these compounds. The presence of alkenes and carboxylate groups in both polysaccharides as well as the acetyl group of fucoidan revealed by C13 NMR study may contribute to the delocalization stable system that enhances the antioxidant activity by delocalizing the resulting free radicals. In fact, further studies should be done to elucidate the structure of these polysaccharides and their vital role as therapeutic agents.

The XTT assay exhibited the antitumor activity of *Laurus* extracts against MCF7 and MDA cell lines. These results together demonstrate the significant role of *Laurus* as a potential source of antioxidant and antitumor components, thus being a wealth natural source to be used in medicine or health care.

V. Conclusion

The polysaccharides (alginate, fucoidan, and laminaran) were isolated for the first time from *Laurus* leaves. These substances in addition to the essential and fatty oils were investigated for their antioxidant and antitumor activities against MCF7 and MDA breast cancer cells. The obtained results in this study showed that they have the ability to reduce the risk of cancer through their antioxidant, and antitumor activities. In conclusion, the study demonstrated the vital role of *Laurus* active substances that can be available as powder form and have a great role in the fields of medicine, pharmacy, and food industry.
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VI. References


