

Evaluation of Chemical Composition, Antibacterial, Antifungal, and Cytotoxic Activity of *Laurus nobilis* L Grown in Saudi Arabia

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Abstract

Present study aimed to evaluate the chemical composition, antibacterial, antifungal, and cytotoxic activity of *Laurus nobilis* grown in Tabuk region of Saudi Arabia. Dried leaves of *L. nobilis* were extracted with various solvent with increasing polarities. Solvent extracts exhibited variable inhibition zones against bacterial pathogens, however all the solvent extracts showed significant inhibition against fungal pathogens. Acetone extracts had the largest inhibition zone against *Streptococcus pneumoniae* (37.16 ± 0.23 mm) while ethanol and methanol extract showed the most efficient percentage inhibition against mycelial growth of *Alternaria alternata* (91.33 ± 0.47 ; 90.66 ± 0.94). High cytotoxicity was demonstrated by methanol and aqueous extracts (IC₅₀ 14.90 µg/ml, 24.56 µg/ml), while acetone extracts showed moderate effects on cell inhibition (IC₅₀ 41.43 µg/ml). The significant activity shown by the bay leaf extracts could be attributed to monoterpene hydrocarbons, oxygenated monoterpenes, phenylpropanoids, phenols, and other important phytoconstituents identified in GC- MS and FTIR studies. Our findings clearly show significant antifungal, antibacterial and cytotoxic activity of solvent extracts of bay leaves, which could be attributed to the presence of wide range of phytochemicals. Since plants derived natural products are less toxic, cheaper, and have negligible side effects, they would serve as an excellent alternative to antimicrobial and chemotherapeutic drugs.

Keywords: *Laurus nobilis*, solvent extracts, GCMS, cytotoxic, antibacterial, antifungal.

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INTRODUCTION

Medicinal plants are used worldwide to cure many diseases. Due to the undesirable side effects of modern synthetic pharmaceuticals and the simultaneous increase in microbes with multidrug resistance, traditional herbal approach to cure diseases is in vogue. Saudi Arabia represents one of the most biodiverse areas in the Arabian Peninsula¹. Tabuk, is located in the Northwest region of Saudi Arabia and is well known for its diverse flora and fauna². Herbal medicine (both native and non-native plants) is an ancient tradition in Saudi Arabia. Plants and their different parts possess a wide array of chemical components, which are used in ethano pharmacological research and have been explored since ages because of their immense benefits.

Laurus nobilis (family Lauraceae) is a small tree or shrub, native to the Mediterranean and European region. The leaves are called bay leaves or bay laurel and used as a spice and flavouring agent³, to treat flatulence, epigastric pain, gastrointestinal disorders⁴, epilepsy⁵, and have anti-inflammatory, analgesic⁶, antioxidant⁷, antibacterial⁸, antidiarrheal⁹, and antiproliferative¹⁰ properties.

Based on previous reports and literature collected, *L. nobilis* contains several important and potential secondary metabolites which could serve as excellent antimicrobial and anticancer agents. Hence, we explored the chemical composition, antibacterial, antifungal, and anticancer activity of *Laurus nobilis* leaves growing in Tabuk region of Saudi Arabia. To our knowledge, this is the first time leaves from Tabuk have been explored for their anticancer and antimicrobial activity.

MATERIALS AND METHODS

Plant material

The leaves of *L. nobilis* were collected from Tabuk region during the month of February (2018), and identified by Dr Najat Bukhari and Dr Mona Al Wahibi, Plant Taxonomist, Department of Botany. The leaves were thoroughly washed, dried, and grinded to fine powder, and used for the experiments throughout the research.

Preparation of Extracts

Preparation of extracts was done by following the method of Do et al.¹¹, with minor modification. Various solvents (Water, ethanol,

methanol, acetone, ethyl acetate, and chloroform) with increasing polarities were used for extraction purposes. Twenty grams of powdered leaves were added to 100 ml of water, separately in conical flasks and shaken on a rotator shaker for 24 h. Further, the crude extracts were filtered and dried using rotary evaporator, after which their extraction yields were calculated. The dried residues were reconstituted with respective mother solvents to obtain a fixed final volume of extracts.

Microorganisms

All the bacterial and fungal isolates used in the study were procured from King Khaled hospital, Riyadh, Saudi Arabia. The bacterial strains screened were *Enterococcus faecalis* (ATCC 29212), *Staphylococcus aureus* (ATCC 25923), *Streptococcus pneumoniae* (hospital isolate), and *Proteus mirabilis* (hospital isolate). Fungal strains screened were *Fusarium solani*, *Fusarium oxysporum*, *Alternaria alternata*, and *Bipolaris* sp. Nutrient agar was used to maintain all the stock cultures of bacteria, while PDA was used for fungi.

Antibacterial Activity

The antibacterial activity of the crude solvent extracts was performed by using well diffusion method¹². Sterile nutrient broth (10 ml) was inoculated with fresh bacterial cultures and incubated for 24 h at 37°C. The cultured broth was adjusted to 10⁸ CFU/ml corresponding to a turbidity of 0.5 McFarland standards. A fixed volume (10µl) of the quantified broth was evenly spread on Muller Hinton agar plates and allowed to dry. After drying, the agar was aseptically punched using cork borer (6 mm) to make wells. Each well was filled with 50µl of the crude solvent extract and incubated for 24 h at 37°C. Positive control was antibiotic disc of Tetracycline (30µg), while the extracting solvents served as negative control.

Antifungal Activity

The effect of solvent extracts on mycelial growth of test fungi was determined using poisoned food method¹³. Sterile petri plate was loaded with extract (1 ml) followed by addition of potato dextrose agar (19 ml) and the mixture was gently swirled to allow thorough mixing. Plates with amended media were allowed to solidify, after which a mycelial plug was placed centrally in the amended plate. Mycelial plugs (6 mm) were removed from the periphery of culture plates with

actively growing mycelia (10 days). Culture plates were then incubated at $25 \pm 2^\circ\text{C}$. Measurements were taken when the control plate showed growth covering the entire plate. Control plate contained media without any treatment (without extract). The assay was performed in triplicates and their mean values were noted.

The following formula was used to calculate the percentage inhibition of mycelial growth.

$$\text{PI} = (C_0 - T_e) / C_0 \times 100$$

where PI stands for percentage inhibition, T_e is the treatment and C_0 denotes the mean growth rate of control

Minimum Inhibitory Concentration (MIC)

Broth dilution method was used to determine the Minimum Inhibitory Concentration of both fungi and bacteria, by following the standard method of CLSI with slight modification^{14,15}. Muller Hinton broth (bacteria) and Potato dextrose broth (fungi) were used to prepare a double fold dilution of extracts in the range 0.125mg/ml -32mg/ml. Equal amount of broth and extract were added to a sterile test tube containing 5×10^5 CFU/ml bacterial spore suspension and 2×10^5 CFU/ml of fungal spore suspension separately. The tubes were incubated at 37°C for 24h (bacteria) and $25 \pm 2^\circ\text{C}$ for 7-9 days (fungi). The lowest concentration showing no visible growth was regarded as its MIC. The assay was performed in triplicates and their mean values were noted.

Minimum bactericidal concentration (MBC) and Minimum fungicidal concentration (MFC)

The Minimum bactericidal (MBC) concentration and Minimum fungicidal concentration (MFC) tests were conducted as an adjunct to MIC assay¹⁶. The lowest concentration inhibiting the growth of test pathogens in tubes were transferred on the respective agar plates, Muller Hinton Agar for bacteria and Potato Dextrose agar for fungi. The subcultured plates were incubated at 37°C for 24h (bacteria) and $25 \pm 2^\circ\text{C}$ for 7-9 days (fungi). Concentrations that did not show any growth on respective agar medium were regarded as their MBC and MFC.

MTT Assay and IC50 Values

Cell viability was evaluated by MTT assay (3-[4, 5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) as described by Zhi-Dong et al.¹⁷, with slight modification. To a 96

well plate, 100 μl of HeLa cells was added at a concentration of 2×10^5 cell/well and allowed to settle for 24 h. Cells were maintained in their respective medium. After 24 h incubation, various concentrations of solvent extracts were added to the wells and further incubated for 24 h. After the incubation, 100 μl (5 mg/ml) of 0.22 μm filter-sterilized 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Aldrich, UK) was added to the wells at 37°C . Cells treated with MTT solution were incubated for 4 h, and DMSO (100 μl) was later added to solubilize formazan crystals. The plate was finally read using a 96 well plate reader (Molecular Devices -SPECTRA max- PLUS384) at 570 nm. DMSO served as negative control and Doxorubicin was positive control.

GC -MS Analysis

Acetone extracts of *L. nobilis* were used for gas chromatography-mass spectrometry (GC/MSD) analysis, using Agilent technologies model 7890GC coupled with a mass detector Agilent 5975 GC/MSD. Agilent J&W HP-5MS (phenyl methyl siloxane, 30 m' 0.25 mm, 0.25 μm) analytic column was also used in the analysis, and helium (1 mL/min) was used to separate components. Following are the standardised parameters adopted during the GC extraction: oven temperature of 45°C (2 min) was increased to a temperature of 200°C at the rate of $3^\circ\text{C}/\text{min}$, solvent delay time was 5 min, inlet line temperature was 200°C , and ion source temperature was 230°C . Mass spectra were taken at 70 eV, and acquisition mode-scan was 40-550 am. Ninety minutes was the GC run time. The interpretation of mass spectrum and identification of phytocompounds present in the extracts were performed using the database of NIST libraries.

FTIR

Nicolet 6700 spectrometer (Thermo Scientific, USA) equipped with DTGS detector and beam splitter was used in studying the IR spectrum of methanol and aqueous extracts. The IR spectrum obtained was analysed (OMNIC software) and scanned in the range of 400 4000 cm^{-1} .

Statistical Analysis

All the tests in the present study were performed in triplicates and the data obtained are expressed as the mean \pm standard deviation (S.D.). Cytotoxicity graph was generated in excel

as percentage cell viability using dose dependent curves. Graph pad prism (Version 7.00 for Windows GraphPad Software Inc.) was used in calculating the IC_{50} by generating standard curve with regression analysis.

RESULTS

Extraction

Solvents with increasing polarity were used for extraction purpose. Methanol extracts gave the highest yield (52%) followed by ethanol (49%), water (27%), acetone (12%), ethyl acetate (12%) and chloroform (10%).

Antibacterial Activity

Solvent extracts of *L. nobilis* examined in the present study exhibited significant antibacterial activity except the aqueous extracts (Table 1). Acetone extracts followed by ethyl acetate and methanol displayed significant inhibition against all the bacterial isolates tested. However, aqueous extracts were least inhibitory. Maximum zone of inhibition was observed with acetone extracts (37.16 ± 0.23) and ethyl acetate extracts (25.00 ± 0.81) against *S. pneumoniae*. *Proteus mirabilis* was also strongly inhibited by acetone extracts (24.00 ± 0.81)

Antifungal Activity

Bay leaf extracts demonstrated significant inhibition against radial mycelial growth of test fungi (Table 2). It was noticed that methanol and ethanol extract effectively inhibited mycelial growth of *A. alternata*, *F. solani* and *F. oxysporum*. Highest inhibitory activity was observed by ethanol

and methanol extracts against *A. alternata* (91.33 ± 0.47 ; 90.66 ± 0.94). Aqueous extracts exhibited least antifungal activity amongst all the extracts screened.

MIC, MBC and MFC of *L. nobilis* extract against test isolates

The results of MIC, MBC and MFC are shown in Table below (Table 3, 4). The MIC and MBC values for both bacteria and fungi ranged between 8 to >32 mg/ml. All the bacterial strains were inhibited by acetone extracts with an MIC of 0.125 - 2 mg/ml. In general, aqueous extracts could not inhibited the growth of *E. coli*, *P. mirabilis* and *S. pneumoniae* at 32 mg/ml and were least inhibitory towards *S. aureus* and *E. faecalis* (>32mg/ml). The MIC values for ethanol and methanol extracts against fungi ranged between 0.125 - 0.25 mg/ml while the MFC was 0.125 - 0.5 mg/ml. *Bipolaris sp* was inhibited by all the extracts in the range of 0.125 -16 mg/ml. Aqueous extracts could not inhibit *F.solani* and *F.oxysporum* at 32 mg/ml.

Cytotoxic Activity of Extracts and IC_{50}

All the crude extracts were tested for their in vitro anticancer activity against HeLa cell through MTT assay. Fig. 1 shows the cell viability of extracts (in a dose dependent manner). All the extracts inhibited HeLa cells, except ethyl acetate extracts. Cell viability data is presented on a graph, at the highest concentration of 100µg/ml. Aqueous extracts, acetone, and methanol extracts caused 86%, 58%, and 76% inhibition respectively. The IC_{50} for methanol, aqueous, and acetone

Table 1. Inhibitory Activity of *Laurus nobilis* Solvent Extracts Against Pathogenic

Bacterial Strains	Solvent Extract Examined -Zone of Inhibition (mm)						
	Aqueous	Methanol	Ethanol	Acetone	Chloroform	Ethyl Acetate	Control
<i>Escherichia coli</i>	NI	8.66 ± 0.47	9.00 ± 0.00	23.33 ± 0.94	24.00 ± 0.81	17.00 ± 0.81	35.66 ± 0.62
<i>Proteus mirabilis</i>	12.16 ± 0.23	15.33 ± 0.47	18.33 ± 1.24	24.00 ± 0.81	NI	28.00 ± 0.00	NI
<i>Staphylococcus aureus</i>	11.33 ± 1.02	12.66 ± 0.47	10.00 ± 1.62	16.66 ± 0.94	15.00 ± 0.00	8.33 ± 0.94	34.16 ± 0.23
<i>Streptococcus pneumoniae</i>	NI	22.00 ± 0.40	17.33 ± 0.47	37.16 ± 0.23	19.00 ± 0.40	25.00 ± 0.81	NI
<i>Enterococcus faecalis</i>	10.33 ± 0.94	18.66 ± 0.62	16.00 ± 0.62	24.00 ± 0.00	11.33 ± 0.47	21.66 ± 1.24	26.00 ± 0.72

All data are expressed as mean ± standard deviation, where n = 3. Control Tetracycline. NI - not inhibited.

Table 2. Inhibitory Activity of *Laurus nobilis* Solvent Extracts Against Fungal strains.

Fungi Tested	Solvent extracts examined-Percentage Inhibition(%)						
	Aqueous	Methanol	Ethanol	Acetone	Chloroform	Ethyl Acetate	Control
<i>Fusarium solani</i>	38.33 ± 0.47	81.00± 0.81	84.66 ± 0.94	62.33 ± 0.47	57.00 ± 0.70	50.66 ± 0.62	88.00 ± 0.70
<i>Fusarium oxysporum</i>	36.00 ± 0.81	78.33 ± 1.24	80.00 ± 0.81	56.16 ± 1.02	70.66 ± 0.94	62.00 ± 1.00	86.33 ± 0.47
<i>Alternaria alternata</i>	50.16 ± 0.23	90.66 ± 0.94	91.33 ± 0.47	34.00 ± 0.81	45.00 ± 1.08	48.66 ± 0.94	90.00 ± 0.81
<i>Bipolaris sp</i>	42.66 ± 0.62	77.33 ± 0.84	76.00 ± 0.00	55.66 ± 1.02	79.00 ± 0.40	60.00 ± 1.62	84.16 ± 1.02

All data are expressed as mean ± standard deviation where n = 3. Control- Voriconazole

extracts was 14.90 µg/ml, 24.56 µg/ml, and 41.43 µg/ml respectively.

GC-MS

Crude acetone extract exhibited significant antimicrobial and cytotoxic activities, hence was subjected to GCMS analysis. Acetone extracts showed the presence of monoterpene hydrocarbons, oxygenated monoterpenes, phenylpropanoids, phenols and many others important compounds as shown in Table 5. Chemical composition of acetone extracts showed the presence of compounds such as Bicyclo (3.1.1) hept-2-ene,2,6,6-trimethyl, 6,6-Dimethyl-2-methylenebicyclo[3.1.1] heptane (α and β pinene), o-xylene, p-xylene, 3-carene, terpenyl acetate, γ terpinene, eugenol, phenol, 2-methoxy-4(2propenyl)-acetate, Camphene, and 9-Hexadecen-1-ol,(Z), Phenol,2-methoxy-4-(2-propenyl)-acetate,Cyclohexene.1-methyl-4-(1-methylethylidene) and Terpeneol acetate.

FTIR

FTIR of methanol and aqueous extract was performed, as these extracts demonstrated significant antifungal and cytotoxic activity. IR spectroscopy of methanol and aqueous extract show a broad peak at 3295 cm^{-1} and 3285 cm^{-1} , these peaks denotes the presence of phenolic compounds such as eugenol and terpineols. The two peaks in methanol extract at 2946 cm^{-1} and 2834 cm^{-1} are due to C-H asymmetric and symmetric stretching of proteins, lipids and carbohydrates, while the peaks at 2169 cm^{-1} and 2018 cm^{-1} are due to C=C stretches of alkyne molecules, similar peaks were also observed in aqueous extracts at 2026 and 2217 cm^{-1} . The peak at 1647 cm^{-1} & 1634 cm^{-1} in methanol and aqueous are related to C=C stretching of the terpenes such as pinene, cymene and terpinene. The small peaks between 1947 cm^{-1} - 1990 cm^{-1} were from C=C

Table 3. Minimum Inhibitory and Minimum Bactericidal Concentration (MIC and MFC) of *Laurus nobilis* extracts

Bacteria tested	Solvent extract examined (mg/ml)											
	Aqueous		Methanol		Ethanol		Acetone		Chloroform		Ethyl acetate	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
<i>E. coli</i>	NI	NI	NI	NI	>32	NT	0.25	1	0.25	1	2	2
<i>P. mirabilis</i>	NI	NI	8	16	4	8	0.125	0.125	NI	NT	0.125	0.25
<i>S. aureus</i>	>32	NT	32	NT	NI	NI	0.25	0.5	2	2	NI	NI
<i>S. pneumoniae</i>	NI	NI	0.125	0.5	2	8	0.125	0.125	0.5	0.5	1	2
<i>E. faecalis</i>	>32	NI	0.125	0.25	0.25	0.25	0.125	0.125	16	32	0.125	0.25

NI-Not Inhibited. *E. coli*- *Escherichia coli*, *P. mirabilis*- *Proteus mirabilis*, *S. aureus*- *Staphylococcus aureus*, *S.pneumoniae*- *Streptococcus pneumoniae*, *E. faecalis* - *Enterococcus faecalis*

asymmetric stretches of alkenes (Table 6,7 & Fig. 1,2).

DISCUSSION

Extraction is the first step by which chemical compounds are obtained from plant material. In order to maximise the extraction of biologically active compounds, various solvents

were screened in this study. Extraction yields significantly varied with different solvents. Methanol followed by ethanol and water gave higher yields than others. Therefore, the extraction yields increased with increasing polarity of the solvents, indicating that both, the extracting efficiency and extractable bioactive compounds favoured solvents with high polarity. Our findings

Table 4. Minimum Inhibitory and Minimum Fungicidal Concentration (MIC and MFC) of *Laurus nobilis* extracts

Fungi tested	Solvent extract examined (mg/ml)											
	Aqueous		Methanol		Ethanol		Acetone		Chloroform		Ethylacetate	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
<i>F. solani</i>	>32	NI	0.125	0.125	0.125	0.125	0.25	0.5	0.5	1	1	1
<i>F. oxysporum</i>	NI	NI	0.125	0.25	0.125	0.125	4	8	0.5	1	0.5	2
<i>A. alternata</i>	4	8	0.125	0.125	0.125	0.125	>32	NI	8	16	8	8
<i>Bipolaris sp.</i>	8	16	0.25	0.5	0.25	0.25	0.5	1	0.125	0.25	0.5	0.5

NI-Not Inhibited, *F. solani* -*Fusarium solani*, *F. oxysporum* -*Fusarium oxysporum*, *A. alternata* -*Alternaria alternata*

Table 5. GC-MS Analysis of *Laurus nobilis* Acetone Extract

Name of Compound	Retention time	Molecular formula	Peak area (%)
2,4-dichlorophenoxyacetic acid, methyl ester	5.061	C ₁₁ H ₁₂ Cl ₂ O ₃	1.293
2(Methoxycarbonyl)benzoic acid	5.234	C ₉ H ₈ O ₄	1.004
Bicyclo (3.1.1) hept-2-ene,2,6,6-trimethyl	6.359	C ₂₀ H ₃₂	11.914
Camphene	7.149	C ₁₀ H ₁₆	0.434
o-Xylene	7.606	C ₈ H ₁₀	1.025
p-Xylene	8.564	C ₈ H ₁₀	0.931
γ-Terpinene	31.485	C ₁₀ H ₁₆	1.422
Terpinyl acetate	44.638	C ₁₂ H ₂₀ O ₂	13.209
3 Carene	48.502	C ₁₀ H ₁₆	1.020
6,6-Dimethyl-2-methylenebicyclo[3.1.1] heptane-	54.372	C ₁₀ H ₁₆	2.380
Eugenol	56.045	C ₁₀ H ₁₂ O ₂	2.031
Phenol,2-methoxy-4-(2-propenyl)-acetate	59.421	C ₁₂ H ₁₄ O ₃	0.610
Cyclohexene.1-methyl-4-(1-methylethylidene)	74.390	C ₁₀ H ₁₆	3.010
Terpineol, acetate	75.641	C ₁₂ H ₂₀ O ₂	5.511
1,8-cineole	76.523	C ₁₀ H ₁₈ O	6.313
1,2,3,4,5,6-Hexahydro-1,1,5,5-tetramethyl-2,4a-methanonaphthalen-7(4aH)-one	77.307	C ₁₅ H ₂₂ O	35.739
4a,7-Methano-4aH-naphth[1,8a-b]oxirene, octahydro - 4,4,8,8-tetramethyl	80.223	C ₁₅ H ₂₄ O	3.638
2,5-Cyclohexadiene-1,4-dione,2,6-bis(1,1-dimethylethyl)-	81.447	C ₁₄ H ₂₀ O ₂	2.474
9-Hexadecen-1-ol, (Z)-	82.181	C ₁₆ H ₃₂ O	0.986
Oleyl alcohol	84.413	C ₁₈ H ₃₆ O	0.610
Cyclopentadecanol	86.107	C ₁₅ H ₃₀ O	1.221
Ricinoleic acid	87.324	C ₁₈ H ₃₄ O ₃	0.20

are in agreement with previous reports which show that the extraction yields increased with increasing polarity of solvents^{18,19}. It is known that plants possess a wide variety of bioactive compounds, which exhibit different solubility properties with different solvents, hence screening with different solvent helps identify the most appropriate solvent for biological studies^{19,20}.

In the present study, bacterial isolates showed varied inhibitory activities against *L. nobilis* extracts, some isolates were significantly inhibited, while others responded poorly. In general, the Gram positive bacteria were inhibited with significant inhibition zones and exhibited low MIC values when compared to Gram negative bacteria. The two gram positive bacteria *S. pneumoniae* and *E. faecalis* showed least MIC values of 0.125 mg/ml while *E. coli* was not inhibited at the highest

test concentration of 32mg/ml. Similar to our findings, Malti and Amarouch, 2009 and Ramos et al., (2012), reported that ethanolic extracts of *L. nobilis* extracts were potent in inhibiting the Gram positive bacteria than the Gram negative isolates^{21,22}.

The variation in the inhibition zones and MIC values shown by Gram positive and negative bacteria is attributed primarily to the chemical composition and morphology of the bacterial cell membrane. The pronounced antibacterial activity shown by Gram positive bacteria could be due to the peptidoglycan layer which is permeable to the hydrophobic compounds present in the extract²³. The resistance shown by Gram negative bacteria could be due to the lipopolysaccharide layer present in the cell membrane. The outer membrane of Gram negative bacteria is made

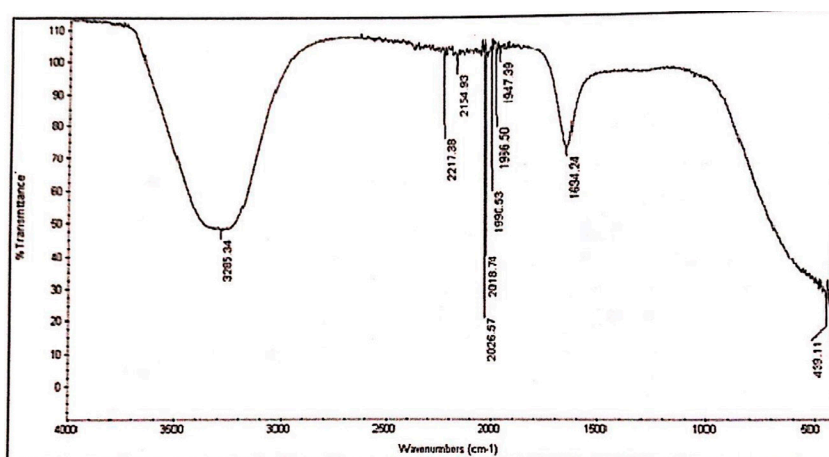


Fig. 1. IR spectrum of aqueous extracts of *L. nobilis* leaves

Table 6. FTIR spectrum of the aqueous extracts of *L. nobilis* leaves

Peak values (frequency, cm ⁻¹)	Functional group
3285	Hydrogen bond OH stretches
2217	C≡C stretching
2154	C≡C stretching
2026	C=C stretch
2018	C=C stretch
1990	C=C bending
1966	C≡C stretch
1947	C≡C stretch
1634	C=C Vibrations

Table 7. FTIR spectrum of the methanol extracts of *L. nobilis* leaves.

Peak values (frequency, cm ⁻¹)	Functional group
3295	Broad peak of OH stretch
2946, 2834	Two peaks due to CH ₂ asymmetry stretching and symmetry stretching
2169	C≡C stretch
2018	C≡C stretch
1647	C=C stretch
1449	CH ₂ stretches
1111	C-O stretches
1015	C-O stretches

up of tough and rigid lipopolysaccharide bilayer, with porins and uptake channels embedded in them, this kind of an arrangement is very efficient in resisting the entry of antimicrobial compounds^{24,25}. According to Zgurskaya et al., the lipopolysaccharide layer is rigid and impermeable to hydrophobic compounds. The porins restrict the entry of hydrophilic compounds while the multidrug transporter pumps help in extruding the bioactive compounds. Hence, together they serve as an excellent permeability barrier to antimicrobial compounds across the cell membrane²⁶.

All the fungal isolates screened interestingly exhibited strong inhibition by methanol extracts. *Alternaria alternata* showed maximum inhibition with methanol extracts, its inhibition was quite close to the antifungal voriconazole, indicating the potency of the extracts. However, there are very few reports that show the inhibition of emerging fungal pathogens by *L. nobilis* solvent extracts., especially the pathogens used in the present study (*A. alternata*, *F. solani*, *F. oxysporum* and *C. gleosporoides*).

The potent antimicrobial activity shown by acetone, methanol and ethanol extracts in a variable manner against a wide array of microorganisms in this study could be attributed to the richness of phenolic compounds along with monoterpenes hydrocarbons and oxygenated monoterpenes. Based on the antimicrobial activity we can deduce that methanol and acetone were

efficient in extracting phenols and terpenes, which was further authenticated by GCMS and FTIR findings shown in Table 5,6,7. According to Bakari et al., phenolic compounds are more soluble in polar organic solvents than in non-polar solvents. They reported acetone as an efficient solvent to extract total phenols and flavonoid compounds¹⁹. Similarly, methanol and ethanol were reported as better solvents in extracting various phenolic compounds²⁷. Recently promising antibacterial activity of *L. nobilis* extracts was reported by Fernandes et al., and they attributed it to the presence of phenolic compounds, like flavonoids²⁸. Therefore, variable inhibition of test isolates is due to their sensitivity towards the bioactive compounds present in the solvent extracts which is influenced by the polarity of the extracting solvent.

Phenolic compounds have the ability to interfere and inhibit the synthesis of peptidoglycan²⁹, modify the hydrophobicity of the membrane³⁰ and cause damage to the membrane structures³¹. Though the exact mechanism and mode of action of phytochemicals on microbes is not fully understood. However, studies indicate that phytochemicals like terpenes and terpenoids are known to alter membrane integrity by causing expansion of the cytoplasmic membrane, changing the fatty acid composition, and hyperpolarization due to lowering of pH internally, thereby destabilizing the membrane leading to cell membrane disruption^{32,33}. Furthermore, the interaction of phytocompounds causes inhibition

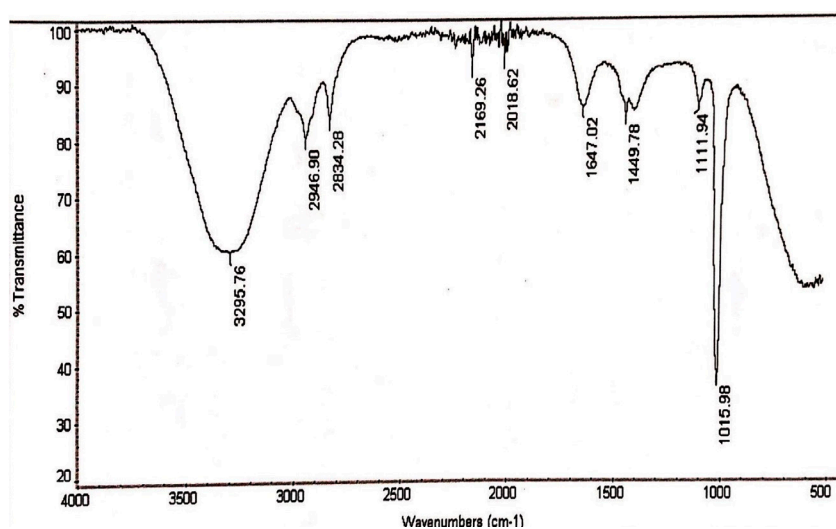


Fig. 2. IR spectrum of methanol extracts of *L. nobilis* leaves

of respiration and alters the permeability of cell membranes³¹.

GCMS analysis of acetone extracts shows presence of compounds like α pinene and β -pinene, 3 carene, terpinene, Terpinyl acetate 1,8-cineole, 1,2,3,4,5,6-Hexahydro-1,1,5,5-tetramethyl-2,4a-methanonaphthalen-7(4aH)-one and eugenol besides others. Interestingly, previous reports show the presence of all the above-mentioned compounds in various solvent extracts of *L. nobilis* and exhibited antimicrobial activity²⁸. Recently Vardapetyan *et al.*, examined bay leaves from two different origins of South Caucasus (Armenia and Georgia). The ethanol extracts of the leaves from both regions showed the presence of *o*-Cymene, λ -pinene, and D-Limonene. However, the major component was 1,8-cineole, and minor quantities of acyclic and cyclic monoterpene phellandrene, γ terpinene, and camphene were present³⁴. Similarly, 1,8-cineole, terpinyl acetate, linalool, R-pinene, and sabinene were identified as the major compounds in bay leaves growing in Spain and Italy^{35,36}. These findings are in accordance with our results as most of the above listed chemical constituents were present in the extracts evaluated in our study, denoting the richness of the extracts of plants grown in Saudi Arabia.

Some studies have reported the presence of certain sesquiterpenes and guaianolides such as phellandrene, β -eudesmol, α -tocopherol,

β -sitosterol and dehydrocostus lactone, which are missing in our findings^{37,38}. Recent studies have shown the influence of environmental factors on the presence/ absence of important bioactive components and secondary metabolites in plant extracts and oils. Among the various factors that are responsible for varied chemical composition are temperature, altitude, sunlight, soil conditions, and water^{39,40}. There are reports which show that the concentrations of certain terpenes and phenols in bay leaves vary with different drying methods³⁶.

Most of the compounds like xylene, pinene, eugenol and terpeneine have shown antibacterial, and antifungal activity in previous reports. Monoterpenes hydrocarbons and oxygenated monoterpenes like α pinene and β -pinene, 3 carene, terpinene and Terpinyl acetate possess excellent antimicrobial^{41,42} and anticancer properties⁴³. Pinenes (α pinene and β -pinene) have shown antibacterial^{43,44} antifungal⁴⁵, antiviral⁴⁶ and insecticidal properties⁴⁷. Similarly, eugenol has shown to exhibit antifungal⁴⁸, antibacterial⁴⁹, antibiofilm^{48,49}, insecticidal⁵⁰, anti-inflammatory⁵¹, and anticancer properties⁵². GCMS analysis of acetone extracts of *L. nobilis* in the present study revealed the presence of all the aforementioned compounds.

Significant cytotoxic activity recorded in this study could be attributed to the richness of phenolic compounds and terpenes present in

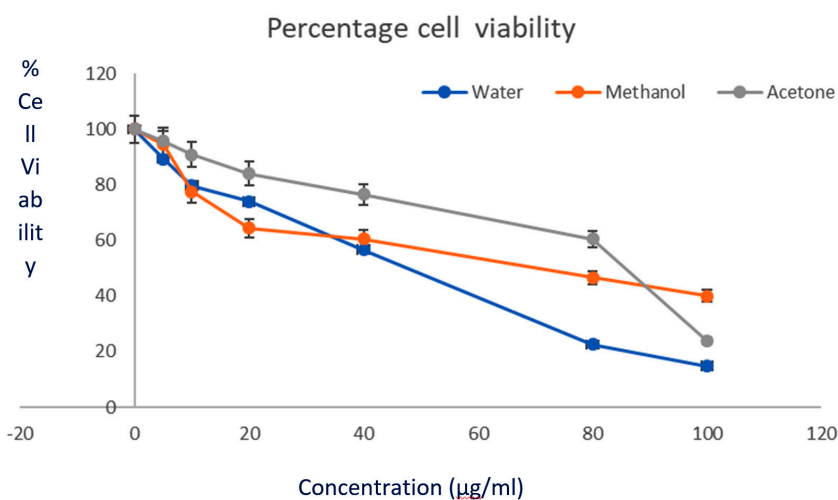


Fig. 3. Cytotoxic activity of *L. nobilis* extracts against HeLa cell line (MTT assay). The graph shows the % cell viability. All data are expressed as mean \pm standard deviation (n = 3)

methanol, ethanol and water extracts. As stated above, these solvents are excellent in extracting various phenols, flavonoids, terpenes, terpenoids and alkaloids, all of which are known for their antimicrobial, anti-inflammatory, antimutagenic and anticancer activities⁵³. In fact, the presence of diverse terpenes and phenolic compounds present in the extracts actually prompted us to investigate their cytotoxic activities.

Plant derived compounds have different modes of action; however, most of them induce apoptosis without cytotoxic effect on normal cells⁵⁴. Recently it was reported that in phenols, the number of substitutes in the OH-ring, increases its anti-proliferative and cytotoxic activities⁵⁵. Similarly, terpenes, terpenoids, alkaloids and flavonoids induce cytotoxicity of breast cancer cells by activating the intrinsic pathway resulting in apoptosis⁵⁵. Significant cytotoxic and antitumor activity of methanol and aqueous extracts of *L. nobilis* leaves towards a number of cancer cell lines, like NCI-H460-lung cancer cells, cervical carcinoma, HCT15-colon carcinoma, breast adenocarcinoma and HepG2 -hepatocellular carcinoma have been reported earlier. High amounts of phenols and flavanols were responsible for the activity⁵⁶. Similarly, Berrington and Lall, reported acetone extracts of *L. nobilis* to inhibit HeLa cells strongly with an IC₅₀ values of 34.46 ± 0.48µg/mL⁵⁷. Our results are in agreement with the all the above-mentioned findings as phenolic compounds, flavanones and terpenes were present in the extracts screened and could have contributed to the promising cytotoxic activity.

CONCLUSION

Our findings clearly show the presence of diverse phytochemicals in bay leaves grown in Saudi Arabia. The significant antimicrobial and cytotoxicity could be attributed to phenolic compounds along with Terpenes. Since drugs derived from plants are less toxic, cheaper, and with negligible side effects, they would serve as an excellent alternative to antimicrobial and chemotherapeutic drugs. Recently, several herbal extracts have been approved by FDA for clinical trials. An IC₅₀ that is lower than 30µg/ml is considered a very strong cytotoxic agent (American National Cancer Institute). The IC₅₀ of some extracts in our study was lower than this limit.

Keeping in mind their significant antibacterial, antifungal, and anticancer activities, they can serve as potential candidate for pharmaceutical industry.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

FUNDING

None.

AUTHORS' CONTRIBUTION

HR designed the experimental work. NNA and NMSM carried out the experimental work. HR, NNA and NA analyzed the GCMS and IR analysis. GA assisted in analyzing the Cytotoxicity results. HR wrote the manuscript. NA reviewed and edited the manuscript.

DATA AVAILABILITY

The data that support the findings of this study are available with the corresponding author

ETHICS STATEMENT

No animal or human subjects were involved in the work related to this manuscript.

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