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Research Article

Functional food components and activities of *Pinus nigra* and *Pinus sylvestris* barks as food supplements

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ABSTRACT

Nowadays, the need for new natural origins medicinal sources has increased because of their low-harmful potential and can be developed at low cost. The Pine plants have excellent natural and economic benefits. In addition, they have numerous bioactive chemical compounds. In this work, we aimed to determine the chemical composition and the bioactivity of *Pinus sylvestris* bark (PSB: Sarı Çam) and *Pinus nigra* bark (PNB: Kara Çam) extracts and also compared a commercial product. The PSB extract was fractionated by a colon chomatography. The PSB-FR-2 fraction of PSB extract had a high phenolic content of 157.62±1.06 mg GAE g^{-1} extract. The inhibitory activities of PNB and PSB extracts against α-amylase and α-glucosidase showed twice the activity of acarbose compared to acarbose. Moreover, the qPCR test showed that PSB extract could reduce the expression of the ACE2 gene in lung cells even at the lowest concentration of 50 µg mL^{-1}. The MIC test indicated that extracts could potentially have antibacterial effects. The results reveal that PNB, PSB extracts and PBS fractions had various bioactivity besides the high phenolic and flavonoid metabolites content. So, it could be a promising model of natural medicinal products for forwarding studies.

Keywords: *Pinus nigra*, *Pinus sylvestris*, phytochemical component, cytotoxic activity, antibacterial activity, enzyme inhibition, DNA protective activity.

1. INTRODUCTION

Nowadays, researchers are more interested in discovering novel bioactive substances, might be used for several purposes. Phytochemicals are represented by phenolics, the most widespread compound group of secondary metabolites formed in plants.[1] Secondary metabolites are not produced continuously, and they can be produced under certain specific conditions to play a vital role in defending plant' cells against environmental riskiness; contamination, oxidative-stress, dehydration, UV radiation and pathogenic contraventions, in addition to the protection the plant from disease and damage also contribute to being volatile attractant agent, coloring agent and provide structural support for plants. [2-6] Phytochemicals such as phenolics, flavonoids, and other categories have different biological properties. Their effects have been intensely studied on human health as an antioxidant activity. Diabetes, arthritis, diabetes-related complications, and respiratory, cardiovascular, and neurological disorders may all be slowed down by polyphenols[7-10], detoxification which can affect by preventing the formation of reactive oxygen species, prohibiting damage caused by reactive oxygen species or extend the detoxification. [11-13]

Moreover, on top of owning antioxidant effects, phenolic derivatives have various biological activities too, such as anti-ulcer, anti-inflammatory, antidiabetic, antiviral, cytotoxic, and antitumor activity. [14] Farther phenolics can inhibit enzymes by linking to an enzyme and reducing its activity, killing the pathogen that blocks enzyme activity, or correcting metabolic imbalance. [15, 16] Furthermore, antitumoral potential against various cell lines and the

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synergistic effects with conventional cancer treatments were identified. [17-19]

Pinus (Pinaceae) species have more than 200 species representing the major genus of *Pinopsida*.[20] Economically, They are among the significant trees valued for ornamentals, food (seeds), wood, charcoal, and paper.[21] Pines trees are considered an essential source of bioactive composites, structurally varied, and provide an association to detect medicinal agents and other biomedical applications.[22] The turpentine (oil of turpentine) that distillates from resin harvested from *Pinus* trees has been utilized in Traditional Turkish medicine for years because of its antiseptic effect on the respiratory system and urinary infections. Moreover, it is used as plaster and dermatological, stomachical, and analgesic medicines for pain.[23] Also, some previous studies suggested the possibility that these plants could act as a cancer-fighting agent.[24] *Pinus nigra* also known as the black pine, is a taxon belonging to the collection of Mediterranean pines with a comprehensive chemical component such as essential oils and contains many phenolics; catechin, epicatechin, and taxifolin. [25-27]

The purpose of this study is to identify chemical profile and biological activities of the *Pinus sylvestris* L. bark (PSB: Sarı Çam) and *Pinus nigra* Arn. bark in addition to the commercial product. This work was achieved by determination of total phenol contents and total flavonoid contents, antibacterial, antidiabetic inhibition (α-amylase and α-glucosidase), DNA protective and cytotoxic effect (MTT) activities, in addition to regulation of gene expression (qPCR).

2. EXPERIMENTAL

2.1. Plant material

The *Pinus sylvestris* L. bark (PSB: Sarı Çam) and *Pinus nigra* Arn. bark (PNB: Kara Çam) extract and fraction samples were provided by ERSAG (Denizli/Türkiye).[28] In this work, we also tested a commercial product, a similar sample imported from abroad by the same supplying company (ERSAG Company).

2.2. Chemicals

All materials used in this research are supplied from reliable sources: Dulbecco's Modified Eagle Medium (DMEM, Sigma), Fetal bovine serum (FBS, Biowest), Dimethyl sulfoxide (DMSO, Carlo Erba), Trypsin-EDTA (Sigma), Gallic acid, Na_2CO_3, $CaCl_2.2H_2O$, $MgCl_2$, quercetin, acarbose, Folin–Ciocalteu reagent (FCR), CH_3COOK, K_2HPO_4, KH_2PO_4, starch, $NaCO_3$, KI, HCl, α-glucosidase and α-amylase enzymes and trypan blue (Sigma). *p*-nitrophenyl-α-D-glucopyranoside (Gelentham). I_2 (Indosaw). $AlCl_3$ (Fluka). Mueller Hinton II Broth (MHB) (Himedia). Penicillin-streptomycin (Gibco), and Cell Proliferation Kit I (MTT, BioVision). Hyperocide, Quercetin-3-glucoside, Resveratrol, Kaempferol-3-glucoside, and Kaempferol from other standards were purchased from Carl Roth (Gmbh & Co). HPLC-grade methanol, ammonium formate, and formic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultra-pure water (18 mΩ) was obtained from a Milli-Q water purification system (Millipore Co., Ltd.).

2.3. Total Phenol Determination

Total phenolic contents (TPC) for the extracts and fractions were calculated using the Folin/Ciocalteu reagent.[29] 100 µL extracts and 500 µL Folin/Ciocalteu were mixed, after 1 minute 1.5 mL 20% Na_2CO_3 added to the mixture. Mixtures were incubated at 25 ºC in the darkness for 120 minutes, and then the absorbance values were measured using a UV-Vis at 760 nm. The gallic acid calibration curves were drawn to express the TPC as mg gallic acid equivalent (GAE) g^{-1} extract.

2.4. Total flavonoid determination

The total flavonoid contents (TFC) of samples were estimated according to the modified aluminum chloride method using quercetin as standard.[30] 500 µL of the sample, 1.5 mL of methanol, 100 µL of 10% $AlCl_3$, 100 µL of 1 M CH_3COOK, and 2.8 mL of ddH_2O were added to the test tube mixed homogeneously. After the incubation for 30 minutes at room temperature, absorbance values were measured using a UV-Vis at 415 nm. By the quercetin calibration curves the TFC were expressed as mg quercetin equivalent (QE) g^{-1} extract.

2.5. LC-ESI-MS/MS analysis

2.5.1. Sample' preparation

10 mg of plant extract and fraction samples were dissolved by 2 mL methanol. To make ensure for the mixtures homogeneity, extract solutions were put in an ultrasonic bath. The homogeneous solutions were mingled with %50 methanol to be 2 mg/mL. Before performing LC-ESI-MS/MS study, the mixture processed with 0.45 m filters.

2.5.2. LC-ESI-MS/MS conditions

High-performance liquid chromatography with electrospray ionization mass spectrometry (HPLC-ESI-MS/MS) system, with the same technique utilized, conditions and parameters were applied as previously explained by Yilmaz MA (2020).[31]

2.6. Cell culture and cytotoxicity

Human lung carcinoma epithelial (A549) cell line was obtained from the European Collection of Cell Cultures ECACC). This cell line was chosen due to the strong

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expression of *ACE2* [32]. Cells were grown in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin mixture. Cells were cultured within 75 cm2 culture flask at 37 °C in a humanified atmosphere and 5% CO_2. Cells were passaged at 90% confluency. Cells were plated in a 96-well plate at a density of $2.5x10^3$ for 24 h and after incubation, cells were treated with different concentrations (12.5, 25. 50, 100, 125, and 250 µg/ml) of PSB Raw Extract. The extract was dissolved in 100% DMSO (not exceeding 0.5%). After 24 h incubation, 10 µL MTT was added to each well and incubated for 3-4 h. The formazan crystals were dissolved in DMSO, and absorbance was measured at 590 nm with a microplate reader (Epoch, BioTek) as described previously.[33]

2.7. Quantitative Real-Time PCR (RT-qPCR) analysis

Total RNA extraction from A549 cells was performed using the innuPREP RNA Mini Kit 2.0 (Analytic Jena, Germany). cDNA has been optaind by OneScript® Plus cDNA Synthesis Kit (ABM, USA). qPCR was performed using the StepOnePlus™ Real-Time PCR system (Thermo Fisher Scientific, USA) to quantify the relative mRNA expression levels of the *ACE2* gene. The mRNA level was determined using KiloGreen 2X qPCR MasterMix (ABM, USA). All data were normalized to the expression of the GAPDH using the StepOnePlus™ Software. To determine fold changes in mRNA levels, $2^{-\Delta\Delta Ct}$ method was used as described previously.[33]

2.8. α-amylase inhibition activity

The α-amylase inhibition activity of the samples has been calculated spectrophotometrically.[34, 35] 82 µL sample solution 10 µL 1 U mL^{-1} α-amylase solution (20 mM PBS, pH 6.9) was added to the 96-well plate respectively and mixed homogeneously. It was kept at 37 ºC for 10 minutes, and then 8 µL substrate (1% starch) was added. The mixtures have been held again at 37 °C for 12 minutes. The reaction was then ended with the addition of 50 µL 10% HCl, 15 µL iodine-KI (2.5 mM iodine (I_2) + 6.5 mM KI (ddH_2O)), and 50 µL H_2O. Then, mixtures were kept in boiling water for 10 minutes, and after that absorbance values were taken within 620 nm by the spectrophotometer reader (Epoch, BioTek). The α-amylase inhibition IC_{50} values of samples and acarbose were calculated as µg mL^{-1}.

2.9. α-amylase inhibition activity

The α-glucosidase inhibition effect of the samples was spectrophotometrically determined. [35-37] The sample was prepared as 10 µL, 25 µL 0.2 U mL^{-1} α-glucosidase solution, 25 µL substrate 0.5 mM *p*-nitrophenyl-α-D-glucopyranoside (PNPG), and 50 µL of 20 mM pH 6.9 phosphate buffer were mixed. Then, mixture was kept at 37 ºC for 30 minutes. Then, 100 µL of 0.2 M $NaCO_3$ was added to the sample and mixed homogeneously. The absorbance values were measured at 410 nm by the spectrophotometer reader (Epoch, BioTek). IC_{50} values for the α-glucosidase inhibition of samples and acarbose were calculated as µg mL^{-1}.

2.10. Antimicrobial activity

The antimicrobial activity for the samples was determined using the minimum inhibitory concentration (MIC), which could inhibit the growth of bacteria by the tested samples compared to a standard antibiotic.[38, 39] Sample stock solution (16384-16 µg mL^{-1}) was diluted using the cation solution ($CaCl_2$-$MgCl_2$) of Mueller Hinton II Broth (MHB) medium inside the sterile 96-well micro plate wells. Then, 10 µL of 0.5 McFarland bacterial solution was added to the mixtures in the wells. As a result of the applied processes, the plate was kept at +4 °C for 120 min. at 37 °C (*B. cereus* bacteria were left for incubation at 30 °C). MIC values were determined after 16-18 hours.

2.11. DNA protection activity

Using plasmid DNA, agarose gel electrophoresis was used to test the DNA preservation activities of all the samples and quercetin.[40-43] Samples application mixtures were prepared in a microtube by adding 4 µL of glycerol, 5 µL of the sample (1 mg/mL), 3 µL of pBR322 plasmid DNA (172 ng/µL), and 1 µL of 30% H_2O_2, respectively (positive control have only; 4 µL of glycerol, 3 µL of pBR322 plasmid DNA and 6 µL of ddH_2O, negative control have; 4 µL of glycerol, 3 µL of pBR322 plasmid DNA, 1 µL of 30% H_2O_2 and 5 µL of ddH_2O in addition to exposing to UV radiation). The mixtures were exposed to a UV (320 nm, 8000 µW/cm) light source for 20 minutes at 25 ºC. This is followed by adding 2 µL of loading dye. The solution mixtures were carefully added to the wells of the 1.5 % agarose gel (added in 1X TBE buffer + 2 µL ethidium bromide) in the electrophoresis tank. Gel electrophoresis was then applied at 90 V for 120 min. Finally, extracts' % DNA protection activity was determined using the ImageJ program on the recorded gel image by the UV transilluminator (320 nm, 8000 µW/cm).

2.12. Statistical Analysis

A one-way analysis of variance (ANOVA)), Tukey test was used to determine whether the results were statistically significant using the SPSS 22.0 (Statistical Packages for The Social Sciences) package program. The results were stated as mean±SD. For each set of assessments, the aggregated deviation was set at 5% (P 0.05), which was used to establish the statistical significance.

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3. RESULTS AND DISCUSSION

3.1. The phytochemical analysis

TPC and TFC for the extracts and fractions were determined spectrophotometrically byFolin–Ciocalteu and aluminum chloride methods. The gallic acid and quercetin calibration curves applied to express the results as mg gallic acid equivalent g^{-1} extract for phenol and mg quercetin equivalent g^{-1} extract flavonoid for flavonoid (Table 1).

Table 1. Total phenolic and flavonoid contents of extracts, fractions and commercial product.

Sample	Total phenolic Content*	Total flavonoid content**
PNB Raw Extract	122.85±0.38	14.00±0.11
PSB Raw Extract	100.18±0.61	13.56±0.19
PSB-FR-1	127.99±1.14	47.88±0.29
PSB-FR-2	157.62±1.06	13.96±0.05
PSB-FR-3	129.97±0.15	8.21±0.13
Commercial product	100.02±0.08	12.57±0.03

*mg gallic acid equivalent g^{-1} extract; **mg quercetin equivalent g^{-1} extract.
Note: Data are means of three repetitions ± standard deviation (SD).

As shwon in Table 1, the highest TPC was recorded by PSB-FR-2. High content also was determined in PSB-FR-1, PNB Raw Extract, and PSB-FR-3 (Table 1). Compared to our results, work results show that the experimental samples had higher TPC than *P. nigra* aqueous and acetonic extracts 28.6±2.1 mg g^{-1} (water extract) and 38.8±1.2 mg g^{-1} (acetone extract).[44] In another study, TPC for the *P. nigra* bark ethanolic extract has been found to be 18.46±3.2 mg GAE g^{-1} extract, a lower TPC than the studied samples.[45] In the present study, *P. nigra* has a high phenolic content of 135.0±0.22 mg GAE g^{-1} dw. Another study that may be consistent with our results shows that the *P. nigra* bark ethanol extracts collected from Serbia was 35.68±1.74 mg GAE g^{-1} dw.[46] In addition to that *P. sylvestris* hot water extracts have a total phenol content of about (4.38±0.94 - 12.33±1.48 mg GAE g^{-1} extract) which is lower than our results of TPC.[47]

In a previous study *P. sylvestris* hot water extracts have a total flavonoid content of about (5.19 1.05 - 1.75±0.62 mg QE g^{-1} extract) less comparing to our results of TFC .[47] Also, *P. nigra* bark extract samples collected from Mokra gora/Serbia were poor in flavonoid content as the maximum flavonoid content in ethanol and acetone extracts was 1.22±0.13 and 1.22±0.04 mg QE g dw^{-1}, respectively.[46] Further, the total flavonoid content of methanolic *P. nigra* resin extract was about 66.36 mg GAE g^{-1} extract which is more than our sample results .[48] Another work showed that the total flavonoid content of acetone, ethyl acetate, ethanol extracts, and essential oils of the twigs and needles of Turkish *P. nigra* was found as maximum content in *P. nigra* needles acetone extract as 44.60±2.30 mg QE g^{-1} extract. [49]

LC-MS/MS technique was applied to perform a qualitatively and quantitatively analysis of phenolics for both PNB and PSB samples (Table 2). When we examined the individual components of each extract and the commercial product (using 56 standard compounds), significant results were obtained for phenolic compounds Figure 1. In general, by evaluating the phenolic components of all extracts and commercial products, the highest compounds were citric acid, luteolin-7-glucoside apigenin-7-*o*-glikozid, respectively for the PNB Raw Extract, for the PSB Raw Extract, the highest compounds were citric acid and quercetin, while citric acid and luteolin-7-glucoside were the highest phenolics in the commercial product.

As seen in Table 2 and Figure 1, 56 compounds were quantitatively screened in the extract solution, and high amounts of compounds catechin, taxifolin, epicatechin, and morin were found in all extracts. According with relation to analysis performed using liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS/MS), catechin (102.261 mg/g), taxifolin (89.315 mg/g), epicatechin (4.559 mg/g), and morin (4.217 mg/g) in the PSB-FR-2 was mostly found. Further, similar compounds to PSB-FR-2 were found in other extracts and their quantitative amounts are given in Table 2. On the other hand, quercimeritrin, hyperocide, quercetin-3-glucoside, neohesperidin, quercetin, biochanin, and chrysin was discovered to be just a little in the composition of each extract according to the findings of the LC-ESI-MS/MS analysis.

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Table 2. Validation parameters of compounds and analysis of phenolic compounds in the extract solutions by using LC-MS/MS system

No	RT	Name	PNB Raw Extract, mg/g	PSB Raw Extract, mg/g	PSB-FR-1, mg/g	PSB-FR-2, mg/g	PSB-FR-3, mg/g	Pre I (w/z) > Prol I (w/z)	I.P.	LOD, µg/L	LOQ, µg/L	LR, µg/L	R^2	R, %
1	1.18	Shikimic acid	nd	nd	nd	nd	nd	173.0->93.1	Neg	68,25	210.24	500-8000	0,991	90.1
2	1.74	Gallic acid	0.016	nd	nd	nd	nd	169.0->125.0	Neg	4.8	15.25	31.25-1000	0.999	97.1
3	2.77	Protocatechuic acid	0.978	0.313	0243	0.046	0.055	152.9->108.9	Neg	4.62	14.77	31.25-1000	0.997	93.4
4	3.15	Gentisic acid	0.313	nd	nd	nd	0.138	153.0->109.0	Neg	9.45	32.5	125-2000	0.996	99.5
5	4.10	Catechin	42.917	202.390	316.313	94.670	102.261	288.9->245.1	Neg	28.74	69.24	250-8000	0.999	96.7
6	4.69	4Hydroxybenzoic acid	nd	nd	nd	nd	nd	137.05->93.1	Neg	19.25	54.12	250-8000	0.999	99.00
7	5.30	Chlorogenic acid	nd	nd	nd	nd	nd	353.0->191.0	Neg	23.4	74.1	125-4000	0.998	98.5
8	5.77	4-Hydroxybenzaldehyde	0.032	0.037	0.083	0.013	nd	121.0->920	Neg	8.78	26.7	62.5-2000	0.998	99.2
9	5.86	Vanillic acid	nd	nd	nd	nd	nd	167.0->151.8	Neg	22.54	52.1	125-4000	0.999	92.1
10	6.05	Caffeic acid	0.043	0.026	nd	nd	nd	178.9->135.1	Neg	2.63	10.8	31.25-1000	0.999	98.6
11	6.83	Epicatechin	34.693	82.588	29.438	7.255	4.559	290.9->138.8	Pos	8.45	19.69	62.5-4000	0.998	96.4
12	6.34	Syringic acid	nd	nd	nd	nd	nd	197.1->181.8	Neg	26.98	83.2	250-8000	0.994	94.3
13	8.50	*P*-coumaric acid	0.026	nd	0.016	nd	nd	163.0->119.0	Neg	2.25	7.8	15.625-1000	0.999	99.8
14	8.89	Salicylic acid	nd	nd	nd	nd	nd	137.0-> 93.1	Neg	15.94	47.84	125-4000	0.999	99.85
15	9.11	Taxifolin	20.259	148.287	417.333	103.005	89.315	3048->2589	Pos	39.3	139.2	500-8000	0.998	101.2
16	9.74	Polydatine	nd	nd	nd	nd	nd	390.9->228.9	Pos	0.97	2.83	31.25-1000	0.996	100.8
17	9.58	Trans-ferulic acid	0.457	nd	nd	nd	nd	193.1->133.9	Neg	12.45	35.32	62.5-4000	0.997	98.14
18	10.00	Sinapic acid	nd	nd	nd	nd	nd	223.1->208.0	Neg	25.6	90.09	250-4000	0.999	93.9
19	10.38	Quercimeritrin	0.270	2.815	0.627	0.47	0.258	464.8->302.9	Pos	3.13	10.21	31.25-2000	0.998	99.8
20	10.64	Coumarin	0.227	nd	nd	nd	nd	147.1->913	Pos	5.63	15.62	62.5-2000	0.999	99.5
21	11.20	Scutellarin	nd	nd	nd	nd	nd	462.8->286.8	Pos	2.3	6.2	12.5-800	0.999	99.6
22	11.30	*O*-coumaric acid	nd	nd	nd	nd	nd	163.0->119.1	Neg	6.06	13.36	62.5-1000	0.998	100.3
23	11.37	Cynmarin	nd	nd	nd	nd	nd	516.8->162.9	Pos	9.39	28.3	62.5-2000	0.994	98.7
24	11.34	protocatechuic ethyl este	nd	nd	nd	nd	nd	181.0->107.9	Neg	0.56	2.1	15.625-250	0.996	96.3
25	11.79	Hyperocide	0.494	0.683	0.726	0.708	0.529	464.8->3028	Pos	0.38	2.06	6.25-800	0.998	92.4
26	11.91	Quercetin-3-glucoside	0.728	3.486	1.471	0.444	0.474	464.8->302.9	Pos	1.04	3.21	12.5-800	0.999	99.8
27	11.91	Isoquercitrin	0.735	3.596	1.469	0.451	0.420	464.9->302.8	Pos	0.95	3.23	12.5-800	0.999	101.0
28	12.01	Rutin	0.052	0.291	Nd	0.038	0.084	608.9->299.4	Neg	21.3	62.5	250-8000	0.999	98.9
29	11.98	Resveratrol	nd	nd	nd	nd	nd	227.0->142.9	Neg	12.18	38.44	125-2000	0.999	98.00
30	11.97	Naringin	nd	nd	nd	nd	nd	580.7->272.8	Pos	14.68	43.8	62.5-8000	0.998	99.5
31	12.17	Rosmarinic acid	nd	nd	nd	nd	0.652	358.9->160.7	Pos	2.72	69.78	125-4000	0.998	99.00
32	12.51	Quercetin3D-xyloside	nd	nd	nd	nd	nd	432.7->299.5	Neg	45.85	12.58	500-8000	0.999	91.2
33	12.53	Hesperidine	nd	nd	nd	nd	nd	611.0->302.9	Pos	10.6	38.3	62.5-2000	0.999	99.5
34	13.90	Baicalin	nd	nd	nd	nd	nd	446.8->270.9	Pos	0.39	2.21	6.25-800	0.999	97.8
35	12.86	Neohesperidin	0.044	0.045	0.041	0.041	0.046	610.7->302.9	Pos	18.93	69.5	250-4000	0.998	95.6
36	13.35	Kaemplerol-3-glucoside	nd	nd	0.172	nd	nd	448.8->286.9	Pos	0.61	2.31	62.5-200	0.999	98.0
37	13.53	Fisetin	nd	nd	nd	nd	nd	286.8->137.1	Pos	20.8	68.5	125-4000	0.995	95.0
38	13.64	Oleuropein	nd	nd	nd	nd	nd	539.1->275.1	Neg	9.68	36.2	125-4000	0.999	100.5
39	14.35	*Trans*-cinnamic acid	nd	nd	nd	nd	nd	147.1->103.1	Neg	60.35	190.1	500-8000	0.997	100.00
40	15.25	Ellagic acid	nd	nd	nd	nd	nd	301.0->145.0	Neg	72.5	226.5	500-8000	0.999	102.00
41	15.03	Quercetin	nd	nd	nd	nd	nd	300.7->150.9	Neg	4.54	12.6	15.625-1000	0.999	101.8
42	15.26	Naringenin	nd	nd	nd	nd	nd	270.9-> 119.1	Neg	2.8	7.81	31.25-4000	0.999	95.5
43	15.93	Silibinin	nd	nd	nd	nd	nd	482.8->163.1	Pos	2.96	9.74	62.5-2000	0.999	97.2
44	16.25	Hesperefin	nd	nd	nd	nd	nd	300.9-> 164.0	Neg	15.7	33.25	62.5-4000	0.995	93.0
45	16.02	Morin	7.185	30.943	23.418	10.433	4.217	302.8->153.0	Pos	3.19	12.6	62.5-2000	0.999	100.1
46	16.62	Kaempferol	nd	nd	nd	nd	nd	284.9->116.9	Neg	37.26	128.1	500-8000	0.998	102.1
47	16.20	Tamarixetin	nd	nd	0.058	nd	nd	315.0->299.9	Neg	4.73	15.68	31.25-8000	0.999	99.8
48	18.14	Baicalein	0.187	0.170	nd	0.173	0.163	271.0->123.0	Pos	1.95	6.3	50-800	0.999	101.2
49	18.97	7-Hydroxyflavone	nd	nd	nd	nd	nd	238.9-> 137.1	Pos	2.19	5.87	6.25-200	0.994	98.00
50	19.60	6-Hydroxyflavone	nd	nd	nd	nd	nd	239.0->103.1	Pos	1.97	6.15	12.5-200	0.996	95.3
51	20.59	Biochanin A	0.343	0.359	0.283	0.274	0.350	2849->1519	Pos	2.45	7.81	62.5-2000	0.999	101.1
52	20.85	Chrysin	0.339	0.392	0.406	0.391	0.375	2549->153.0	Pos	4.84	15.63	31.25-1000	0.999	95.1
53	21.69	Flavone	nd	nd	nd	nd	nd	223.0->77.2	Pos	1.52	6.02	6.25-200	0.999	100.9
54	23.69	5-Hydroxyflavone	nd	nd	nd	nd	nd	238.9->103.1	Pos	7.81	23.76	62.5-2000	0.999	102.4
55	24.57	6.2.4-Trimetoxyflavone	nd	nd	nd	nd	nd	312.9->148.0	Pos	1.55	4.88	12.5-400	0.999	97.00
56	34.51	Diosgenin	nd	nd	nd	nd	nd	415.0->271.0	Pos	3.13	8.19	25-800	0.999	101.3

Neg: Negative, Poz: Positive, IP: Ion Polarity, Pre I.: Precursor Ions, Pro I.: Product Ions, LR: Linearity Range, R: Recovery

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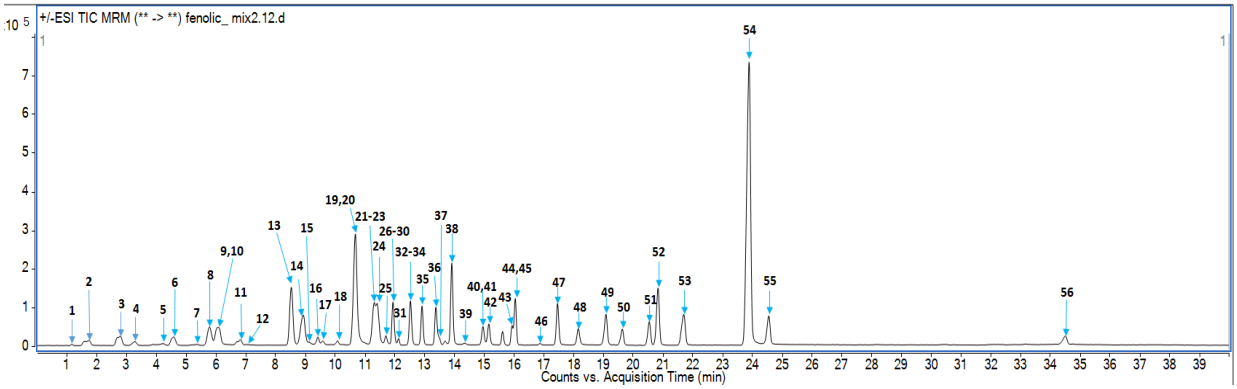

1-Shikimic acid, 2-Gallic acid, 3-Protocatechuic acid, 4-Gentisic acid, 5-Catechin, 6-4-Hydroxybenzoic acid, 7-Chlorogenic acid, 8- 4-Hydroxybenzaldehyde, 9-Vanillic acid, 10-Caffeic acid, 11-Epicatechin, 12-Syringic acid 13-P-coumaric acid, 14-Salicylic acid, 15-Taxifolin, 16-Polydatine, 17-Trans-ferulic acid, 18-Sinapic acid, 19-Quercimeritrin, 20-Coumarin, 21-Scutellarin 22-O-coumaric acid, 23-Cynarin, 24-Protocatechuic ethyl ester, 25-Hyperocide, 26-Quercetin-3-glucoside, 27-Isoquercitrin, 28-Resveratrol, 29-Naringin, 30-Rutin, 31-Rosmarinic acid, 32-Quercetin-3-D-xyloside, 34-Hesperidine, 35-Neohesperidin, 36-Kaempferol-3-glucoside, 36-Fisetin, 37-Oleuropein, 38-Baicalin, 39-Trans-cinnamic acid, 40-Ellagic acid, 41-Quercetin, 42-Naringenin, 43-Silibinin, 44-Hesperetin, 45-Morin, 46-Kaempferol, 47-Tamarixetin, 48-Baicalein, 49-7-Hydroxyflavone, 50-6-Hydroxyflavone, 51-Biochanin A, 52-Chrysin, 53-Flavone, 54-5-Hydroxyflavone, 55-6,2,4-Trimetoxyflavone and 56-Diosgenin.

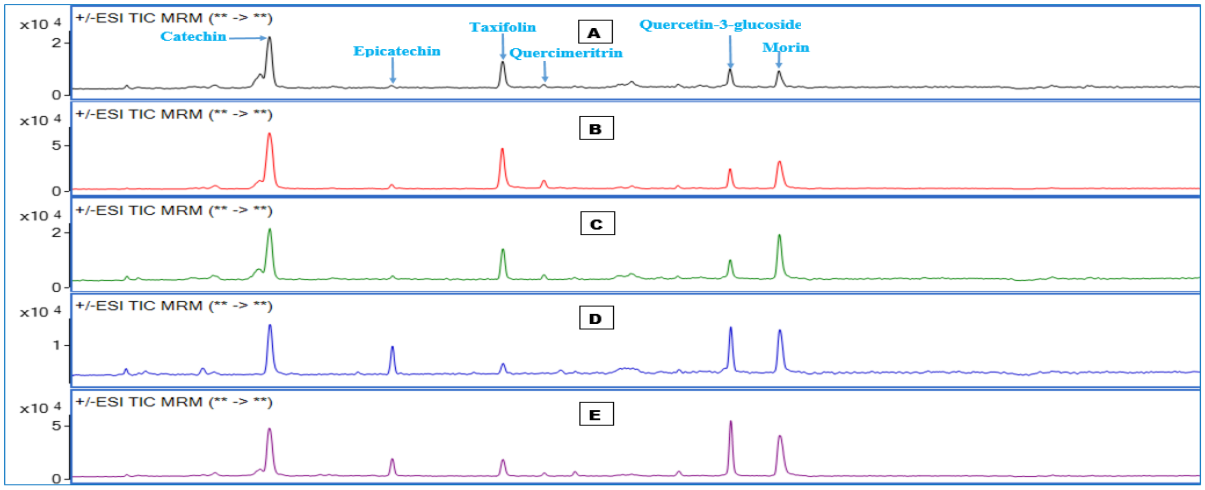

Figure 1. LC-ESI-MS/MS chromatograms of the compounds in the extract solutions (A: PSB-FR-2, B: PSB-FR-1, C: PSB-FR-3, D: PNB Raw Extract and E: PSB Raw Extract).

3.2 Cytotoxicity analysis

The cytotoxic effect of PSB Raw Extract on A549 cells was assessed with an MTT test as described in the methods part. As shown in Figure 2, the PSB Raw Extract did not show toxicity even at the highest dose (250 µg mL^{-1}). 25 and 50 µg mL^{-1} doses did not have any cytotoxic and proliferative effects. Therefore, these doses were selected for further studies.

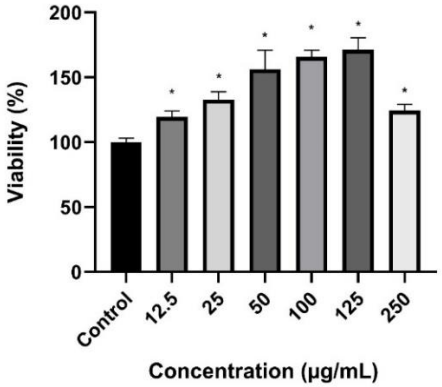

Figure 2. Cell viability of A549 cells treated with PSB Raw Extract for 24 h. Data were presented as mean ± SD, * = P < 0.05.

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3.3. *ACE2* gene expression analysis

The effects of PSB Raw Extract on *ACE2* mRNA level were determined in A549 cells by combining SYBR Green detection chemistry with qPCR. Figure 3 displays the mRNA level real-time PCR results. *ACE2* mRNA levels decreased 60% and 53% for 25 and 50 µg/mL treatment, respectively ($p<0.05$). These results showed that our extract could reduce the expression of the *ACE2* gene in A549 lung cancer cells. We observed higher inhibition in *ACE2* mRNA levels at lower concentrations of extract. These results showed that extract may prevent SARS-COV-2 binding to lung cell. But, further studies are required to test the hypothesis. Many studies have shown that natural products inhibit *ACE2* gene expression.[44-46] Our findings support the literature in that natural products reduce the level of ACE2 to treat SARS-COV-2.

3.4. α-amylase and α-glucosidase inhibition activity

It is shown that PNB Raw Extract has the highest α-glucosidase inhibitory activity (30.61±2.09) among the tested samples (Table 3). Also, PNB Raw Extract was shown to have a high activity compared to acarbose, almost twice the activity of acarbose.

Table 3. Enzyme inhibitory activities of *P. nigra* extracts, fractions and commercial product.

Sample	α-Glucosidase	α-Amylase
	IC_{50}, µg/mL	
PNB Raw Extract	30.61±2.09[a]	19.01±1.71[a]
PSB Raw Extract	36.68±0.22[b]	17.88±0.72[a]
PSB-FR-1	66.09±2.63[d]	51.50±1.62[c]
PSB-FR-2	37.99±0.74[b]	76.70±0.41[e]
PSB-FR-3	66.35±0.16[d]	69.49±1.51[d]
Commercial product	37.60±0.61[b]	38.14±2.60[b]
Acarbose	58.57±0.97[c]	38.54±1.18[b]

Note: Data are means of three repetitions ± standard deviation (SD), variance analysis: $p<0.05$

Recently, *P. pinaster* bark water extract had exhibited lower α-glucosidase inhibition activity as IC_{50}; 138.4±7.4 (µg mL^{-1}) than *P. nigra* samples. [50] Another work had shown that the inhibition properties on the α-glucosidase activity of ethanolic extracts of *P. sylvestris* reported from Kars, Gümüşhane, and Erzurum (Turkey) were found to be as IC_{50}; 43.31, 40.76 and 26.65 mg mL^{-1}, respectively which were much lower than the *P. nigra* inhibition activity. [51]

The inhibitory activity against α-amylase of the PSB Raw Extract was the highest activity with IC_{50}; 17.88±0.72 mg mL^{-1} between worked samples (Table 3). Also, PSB Raw Extract was shown to have a high activity compared to acarbose by almost twice the activity of acarbose.

In a previous study, *P. pinaster* bark 70% ethanolic extract has shown lower α-amylase inhibition activity as IC_{50}; 254.2±9.2 (µg mL^{-1}) than *P. nigra* samples. [50] Another study shows that the *P. gerardiana* methanol and ethyl acetate extracts highly inhibit the α-amylase approach to acarbose. [52]

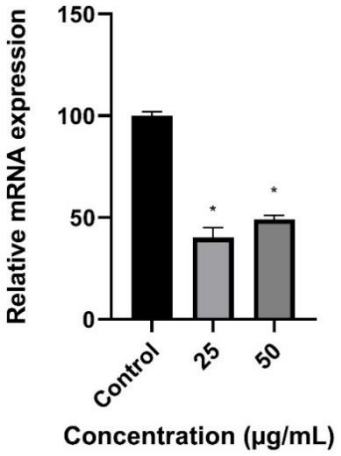

Figure 3. Effects of PSB Raw Extract on mRNA level of *ACE2* gene in human liver cancer (A549) cell line. Data were presented as mean ± SD, * = $P < 0.05$ compared to control group.

3.5 Antimicrobial activity

The data in the MIC of extracts and samples is in Table 4. In general, all tested samples and commercial product showed the maximum activity as 64 (µg mL^{-1}) against *B. cereus* bacteria. In another study, the MIC value of *P. nigra* bark was 6.25 mg mL^{-1} against *S. aureus* and >50 mg mL^{-1} against *E. coli*, *P. aeruginosa* and *K. pneumoniae* for the ethanolic ultrasound-assisted extract and 12.5 mg mL^{-1} against *S. aureus* and >100 mg mL^{-1} against *E. coli*, *P. aeruginosa* and *K. pneumoniae* for the ethanolic microwave assisted extract which is low mainly activity comparing to the tested samples.[45] Moreover, MIC against the *P. elliottii* and *P. tropicalis* resins was 200 µg mL^{-1} for *P. elliottii* and 100 µg mL^{-1} for *P. tropicalis* against *E. faecalis* bacteria.[53] Also, the MIC was investigated for the 70% methanol needles extract and ethyl acetate fraction of the Algerian *P. coulteri*, the result shows 3.1 mg mL^{-1} for *K. pneumoniae* and 6.2 mg mL^{-1} against *S. aureus* bacteria for the methanol extract and 1.5 mg mL^{-1} against *K. pneumoniae* and *S. aureus* bacteria for ethyl acetate fraction[54].

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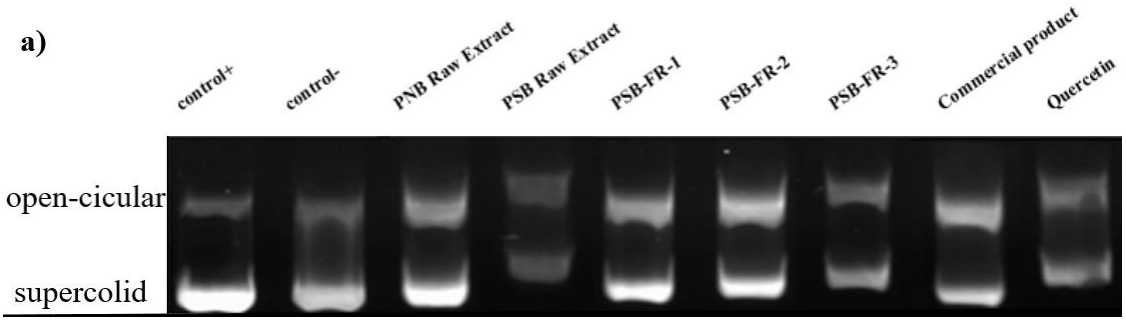
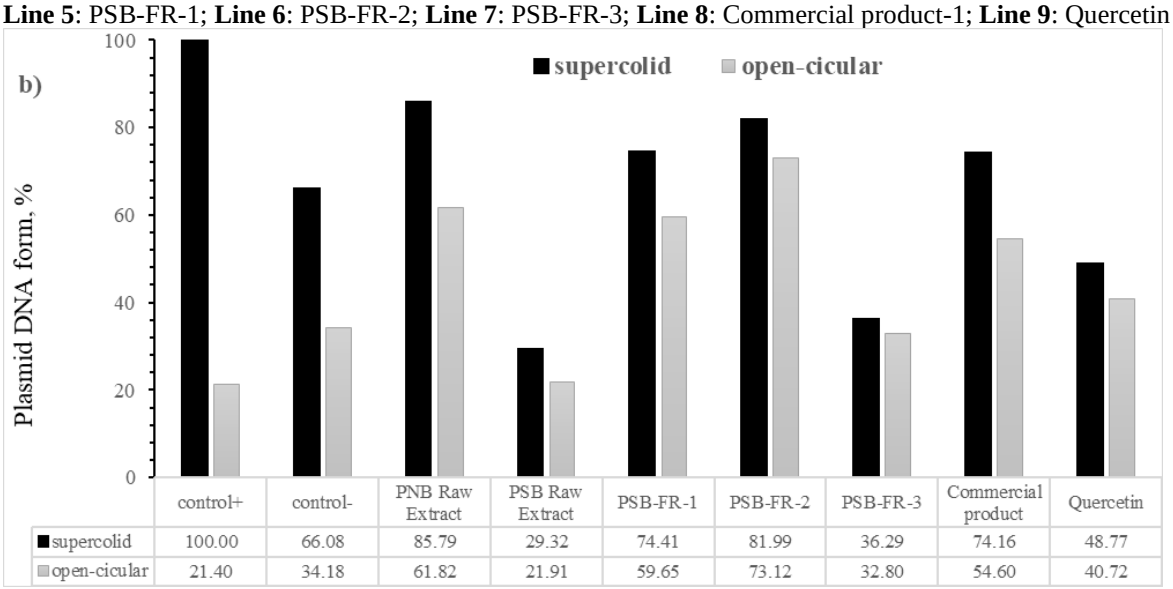
Table 4. Antimicrobial activities of *P. nigra* extracts, fractions and commercial product.

Samples/ Antibiotics	Gram-negative bacteria			Gram-positive bacteria		
	E. coli (ATCC 25922)	*P. aeruginosa* (ATCC 15442)	*K. pneumoniae* (ATCC 10031)	*E. faecalis* (ATCC 29212)	*B. cereus* (CCM 99)	*S. aureus* (ATCC 25213)
PNB Raw Extract	1024	2048	4096	2048	512	2048
PSB Raw Extract	4096	4096	8192	8192	8192	8192
PSB-FR-1	4096	2048	2048	4096	512	4096
PSB-FR-2	1024	2048	4096	2048	512	2048
PSB-FR-3	4096	8192	8192	4096	256	4096
Commercial product	512	1024	512	512	64	512
Amoxicillin	>1024	>1024	>1024	>1024	0.5<	>1024
Tetracycline	4	8	8	4	0.5<	4



Line1: Positive control; **Line 2**: Negative control; **Line 3**: PNB Raw Extract; **Line 4**: PSB Raw Extract; **Line 5**: PSB-FR-1; **Line 6**: PSB-FR-2; **Line 7**: PSB-FR-3; **Line 8**: Commercial product-1; **Line 9**: Quercetin



	control+	control-	PNB Raw Extract	PSB Raw Extract	PSB-FR-1	PSB-FR-2	PSB-FR-3	Commercial product	Quercetin
supercolid	100.00	66.08	85.79	29.32	74.41	81.99	36.29	74.16	48.77
open-cicular	21.40	34.18	61.82	21.91	59.65	73.12	32.80	54.60	40.72

a) DNA protection activity, Agarose gel electrophoresis image.
(b) Comparing chart of % density of the open-circular and supercoiled forms of plasmid DNA.
Figure 4. The results of DNA protection activities of extracts, fractions, commercial product and quercetin.

3.6. DNA protection activities

Reactive oxygen species cause modification of purine and pyrimidine bases in DNA, causing mutations. In addition, ROS lipid peroxidation while the resulting products caused the DNA breakage.[55, 56] For this purpose, DNA protection activity methods on electrophoresis gel were applied to determine the DNA-related protection activity of the tested samples that can prevent or remove DNA damage (Figure 4).

DNA protection activity on electrophoresis gel was determined using plasmid DNA. The DNA protection activity of the samples was applied within 1 mg/mL concentration, and the electrophoresis image was observed as two bands. These bands represent the fast-moving bands of the coiled circular form (supercoiled form I), slow-move bands represent the circular-form

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(open-circular form II) of the plasmid DNA (Figure 4). As a result of the applied method, electrophoresis gel images were determined. Then, effectiveness percentage values were calculated for the two forms of the plasmid DNA, the supercoiled and the open-circular forms. Results showed that PNB Raw Extract, PSB-FR-1, and PSB-FR-2 have higher protection activity than commercial product and quercetin for both forms.

4. CONCLUSION

Taken together, the study described here presented that in addition to its high natural and economic benefit, PNB and PSB extracts have a high biological capacity, corroborative by the high phenolic content, exhibit a high effect of antidiabetic inhibition enzymes and High regulation ability for the gene expression of lung cells. Additionally the cytotoxic effects against A549 cells and the antibacterial activity. In brief, our studies introduce the biological potential of PNB and PSB extracts and fractions *in vitro*, which can contribute to developing effective medicine sources as natural medicinal plant resources.

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Conflict of interests

The authors have no conflicts of interest with any person, organization, or company.

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