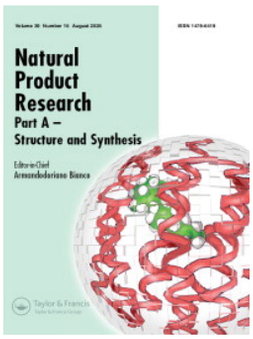

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Determination of phenolic constituents with LC-MS/MS and cytotoxicity evaluation of 'tar' from *Pinus nigra* Arnold *(Pinaceae)*

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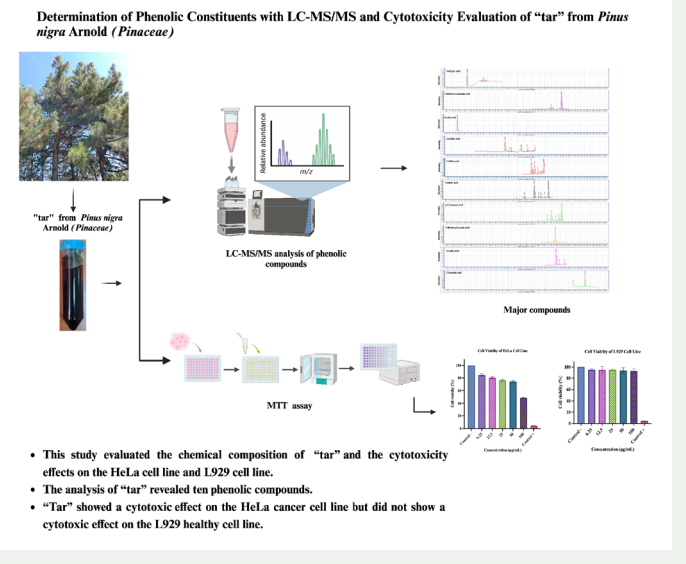
ABSTRACT

In folk medicine, 'tar' is an essential drug in every situation and is obtained from *Pinus nigra* Arnold *(Pinaceae)*, which is very common in Anatolia, especially in the Mediterranean Basin. This study aims to evaluate the chemical composition and determine cytotoxicity effects on the HeLa cell line and L929 cell line of the tar. The analysis of tar by LC-MS/MS revealed ten phenolic compounds: oxalic acid, salicylic acid, vanillic acid, ferulic acid, caffeic acid, 4-hydroxybenzoic acid, cinnamic acid, *p*-coumaric acid, and 3-hydroxycinnamic acid. Besides, total phenolic content (62.31 ± 0.31 mg GAE/g) and flavonoid content (3.43 ± 0.0006 mg QE/g) were determined. Cell viability was determined by MTT analysis. Tar has a significant cytotoxicity effect on the HeLa cell line in concentrations 100, 50, 25, 12.5, and 6.25 µg/mL. IC50 value of tar on HeLa cell was 132.9 µg/mL.



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1. Introduction

Pinus nigra Arnold *(Pinaceae)* (black pine), an essential species of the world forest ecosystem, is a tree species in the coniferous forest belonging to the *Pinaceae* family. *P. nigra* covers an area of more than 3.5 million hectares worldwide; the widest distribution is in Turkey, with more than 2.5 million hectares (Sevgi and Akkemik 2007).

Tar, also known as 'black doctor/püse/pise', is essential in Anatolian culture and has long been used ethnobotanically to treat various human and animal diseases (Ak 2017; Alptekin 2019). These diseases include respiratory diseases such as asthma, bronchitis, colds, coughs, and urinary tract diseases, cover oral wounds, calloused and cracked hands, and feet, and treat skin diseases such as eczema, acne, alopecia, and fungal diseases (Arı et al. 2014). Tar also treats smallpox, ulcers, diarrhoea, and pox (Crimaldi 1996). Tar is not only beneficial for humans, but it is also effective for animals. It has been used in the treatment of goats infected with *Mycoplasma agalactiae*, which causes a reduction in goat milk. It has also been reported to be used externally for wounds, scars, and purple spots caused by the *Tabanus bovinus* fly on animals (Arı et al. 2014).

Tar is a dark or brown, viscous liquid with a smoky odour and an acrid, slightly aromatic taste obtained from plant material by pyrolysis (Ninich et al. 2022; Derbali et al. 2024). There are reports in the literature that tar extraction is mainly done using traditional methods, but there are also reports of tar produced under laboratory conditions (Kurt et al. 2008). The most commonly used species to produce tar are Juniper (Burri et al. 2017), *Cedar* (Takci et al. 2020; Jaouadi et al. 2021), and Pin (Mazela 2007). In addition to these species, tar is produced using alternative species. Although the wood of the plant is generally used in tar production, sapwood and bark have been reported. Tar can mainly be obtained from organic materials like wood, bark, roots, branches, seeds, or a combination of both (Ninich et al. 2022).

In the study conducted by Ninich et al. (2024), a comprehensive GC-MS analysis was employed to determine the content of tar samples produced by both laboratory and traditional methods belonging to *Cedrus atlantica*. This thorough analysis revealed the presence of terpenes (sabinene, α-phellandrene, 9-*epi*-(*E*)-caryophyllene and β-cedene), monoterpenols (*cis*-carveol and α-terpinene-7-al), and sesquiterpenes (γ-gurjunene and allo-aromadendrene), and esters (cedryl acetate and *trans*-carvylacetate). Terpenes, polycyclic aromatic hydrocarbons (PAHs) compounds, and phenolic compounds have been reported in the content of tar produced by the traditional method (from *Pinus sylvestris*, *C. atlantica* Manetti, *Cedrus libani*, *Cedrus libani* A. Rich) (Egenberg et al. 2002; Kurt et al. 2008; Skanderi and Chouitah 2020; Jaouadi et al. 2021; Temiz et al. 2022).

There is currently no information in the literature regarding the analysis of phenolic compound content in tar obtained from *P. nigra* using traditional methods. Most scientific research has concentrated on plant essential oils, with only a limited number of studies on traditional tar and its sources. Existing research on tar primarily focuses on ethnohistorical and ethnopharmacological aspects. Despite this, traditionally produced tar continues to be widely utilised in local cultures. In this paper, the phenolic compound analysis of 'tar', which is obtained from *P. nigra* was achieved. The analysis was conducted using the Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

method in dynamic multiple reaction monitoring (dMRM) modes to assess the phenolic compound content in tar. Furthermore, the potential effects of tar on cell viability were examined, specifically whether it had a positive or negative impact. This was studied using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay on L929 fibroblast (Mouse C3H/An connective tissue) and HeLa (human epithelial cervical carcinoma) cell lines for the first time. Additionally, molecular docking studies between the UHRF1 protein and the major compounds (oxalic acid, salicylic acid, and vanillic acid) of 'tar' were performed. In this way, the need for more literature on this subject was tried to be eliminated.

2. Results and discussion

2.1. The total phenolic content (TPC) and the total flavonoid content (TFC) of tar

In this study, the total phenolic content and the total flavonoid content of tar was obtained as gallic acid and quercetin equivalents. The total phenolic content amount was 62.31 ± 0.31 mg GAE/g, and the total flavonoid content amount was 3.43 ± 0.0006 mg QE/g.

In the literature, no information was found on the total phenolic and flavonoid content amounts of the tar produced from *P. nigra*. In the study conducted on tar obtained by the same method from *C. libani* A. Rich, which is from the *Pinaceae* family and known as the tar tree, it was reported that the TFC and TPC amounts were 0.068 ± 0.02 mg RE/g and 0.85 ± 0.06 mg GAE/g, respectively (Takci et al. 2019). A study reported TPC for *Pinus halepensis* tar extract as 169 µg/mL GAE and TFC for *Acacia cyanoplylla* tar extract as 125 µg/mL GAE. It was also reported as 1.9 mg/mL QE for *P. halepensis* tar extract and 1.4 mg/mL for *A. cyanoplylla* tar extract (Derbali et al. 2024). In the total phenolic compound analysis of the waste tar mixture obtained from a mix of Scots pine (*P. sylvestris*) and beech (*Fagus orientalis*), the value was reported to be 842 mg CE/L (Yalçın et al. 2020). In another study, the TFC of *C. atlantica* Manetti tar was reported as 57.15 ± 0.15 mg GAE/g tar (Skanderi and Chouitah 2020).

2.2. Analysis of phenolic compounds by LC–MS/MS

The amount of phenolic compounds obtained from the LC-MS/MS analysis is given in Table S1. The LC-MS/MS chromatograms and the chemical structures of phenolic compounds (salicylic acid, 3-hydroxycinnamic acid, oxalic acid, gentisic acid, caffeic acid, vanillic acid, *p*-coumaric acid, 4-hydroxybenzoic acid, ferulic acid, cinnamic acid) are illustrated in Figure S1 and Figure S2. Based on the analysis of phenolic compounds, significant components of tar include ferulic acid, oxalic acid, salicylic acid, and vanillic acid which are essential compounds with various bioactivities. Their medical applications are summarised in Table S2.

LC-MS/MS is a highly efficient screening and verification tool generally used to detect phenolic compounds in plant samples (Wu et al. 2016). The sensitivity of the developed method was specified with the determination of the limit of detection LOD and the limit of quantification LOQ (Table S1). The acceptable linear regression

coefficients in the study ranged from 0.994 to 0.999, indicating good linearity (Table S1). The results obtained are supported by other studies in the literature (Kivrak et al. 2013; Temiz et al. 2022). When looking at the literature, the acceptable value for % RSD value is generally expected to be ≤20% (Myrtsi et al. 2021). The % RSD values calculated in this study are less than the highest % acceptable limit of 20% and show the acceptable precision of the method.

There are analysis reports on the phenolic content of the extracts obtained from the organs such as needle leaves, wood, and bark of the species member of the *Pinaceae* family. On the other hand, there is no report in the literature on the phenolic content of the tar obtained by heat treatment from resinous wood pieces of *P. nigra*. Temiz et al. (2022) reported that phenolic compounds such as coumarin, *p*-coumaric acid, vanillic acid, ferulic acid, and cinnamic acid (14,165.16, 162.02, 2988.35, 5, 27,215.06, 189.52 µg/L, respectively) were detected as a result of LC-MS/MS analysis of *C. libani* tar.

On the other hand, gas chromatography-mass spectrometry (GC-MS) was used to analyse the chemical content of tar produced from forestry trees, and compounds such as monoterpenes, sesquiterpenes, acids, aldehydes, phenols were reported (Jaouadi et al. 2021; Temiz et al. 2022; Ninich et al. 2024). In analysing the chemical components of the bark organs of *Pinus brutia*, gentisic acid, 4-hydroxy benzoic acid, vanillic acid, caffeic acid, *p*-coumaric acid, and ferulic acid were detected (Kıvrak et al. 2013). In the phenolic compound analysis of the bark organ of *Pinus eldarica*, the presence of catechin, epicatechin, vanillic acid, *o*-coumaric acid, and tyrosol was reported by Sadeghi Afjeh et al. 2014. In addition, Nisca et al. reported the presence of 8.393 mg/g bark and 0.588 mg/g bark catechin in the extracts of *P. nigra* bark using ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE) methods, respectively (Nisca et al. 2021). It has been reported that phenolic compounds such as gallic acid, *p*-hydroxybenzoic acid, catechin, vanillic acid, ellagic acid, syringic acid, chlorogenic acid, *p*-coumaric acid, and rosmarinic acid are found in the hydromethanolic extract of *Pinus coulteri* needles (Merah et al. 2018). In analysis reports of the phenolic contents of bark extracts of pine species, it has been shown that 4-hydroxybenzoic acid, caffeic acid, cinnamic acid, ferulic acid, *p*-coumaric acid, and vanillic acid are found (Ferreira-Santos et al. 2020). These compounds are known for their anti-inflammatory, antioxidant, hepatoprotective, renoprotective, anti-neurodegenerative, and antimicrobial properties. In our study, oxalic and salicylic acid were also detected in high amounts compared to other compounds (4-hydroxybenzoic acid, caffeic acid, cinnamic acid, ferulic acid, gentisic acid, *p*-coumaric acid, oxalic acid, salicylic acid, 3-hydroxycinnamic acid, vanillic acid).

Salicylic acid was detected in a study determining the essential oil content of *Pinus canariensis* Sweet ex Sprengel needles (Pfeifhofer 2000). Salicylic acid was also detected in another study conducted with Moroccan *Pinus pinaster* bark extract (Benlarbi et al. 1999). The presence of oxalic acid (3.948 µg), an organic acid, has been reported in the extracts obtained from the seedlings of *Pinus massoniana* (Yu et al. 2008). The presence of oxalic acid (1071 ± 50.4 ng/L) was reported in the study conducted by Wang et al. on the determination of the organic acid content of *P. massoniana* seeds (Wang et al. 2015). Therefore, the data obtained from the study are compatible with the literature.

2.3. Cell viability test

In the current study, the first report on the cytotoxic effects of tar in HeLa and L929 cells was demonstrated and determined by the MTT viability test. Cells were exposed to different tar concentrations (0-100 µg/mL) for 24 h. The cytotoxicity of tar on HeLa cells increased concentration-dependently, and the half-maximal inhibitory concentration (IC_{50}) value of 132,9 µg/mL has been observed (Figure S3A, B). A 1% phenol solution was used as a positive control (Srivastava et al. 2018, 2020; Cannella et al. 2019; Coco-Martin et al. 2021) and significantly inhibited the growth of both HeLa and L929 cells (percentage of cell viability: 4.50% and 4.488%, respectively).

Burri et al. (2018) investigated the cytotoxic effect of *Juniperus drupacea* and *Juniperus oxycedrus* tars on breast adenocarcinoma cells (MCF-7 and MDA-MB-231) cancer cell lines and 3A3 normal cell lines and reported a significant cytotoxic effect by inhibiting cell growth in both standard and cancer cell lines in a dose-dependent manner. In a study conducted by Temiz et al. (2022), the cytotoxic effect of *C. libani* tar prepared by traditional methods was investigated on colorectal adenocarcinoma cells (HT-29, HCT-116, DLD-1), prostate carcinoma cells (DU-145, PC-3), MDA-MB-231, MCF-7 cancer cell line, cervical adenocarcinoma cells (HeLa), endometrial adenocarcinoma cells (ECC-1), (HGC-27), human lung carcinoma cells (A549), bone osteosarcoma cells (U2OS), and five normal cells including umbilical vein endothelial normal cells (HUVEC), mammary epithelial normal cells (CRL-4010), breast epithelium normal cells (CRL-8798), kidney cells (HEK-293, PNT-1). The IC_{50} of cedar tar on HCT-116 cells among cancer cells: 30.48 µg/mL on HCT-116 cells and IC_{50} on HeLa cancer cell line: 44.40 µg/mL. IC_{50} on HUVEC, a regular cell line of 74.07 µg/mL, was calculated. Dalkiliç et al. (2024) investigated the cytotoxic effect of tar obtained from *P. brutia* and *C. libani* on MDA-MB-231, MCF 7, and hepatocellular carcinoma (HepG2) cell lines. They reported that the number of viable cells against the MDA-MB-231 cell line was between 12% and 17%, and the cytotoxic activity of these extracts against the MCF7 cell line was between 15% and 44%.

In this study, the L929 fibroblast cell line's percentage of viable cells was 92.7612%, even at the highest dose of 100 µg/mL, and the IC_{50} value for the L929 cell line was calculated to be higher than 1000 µg/mL. The percentage of viable cells in HeLa cancer cells at a concentration of 100 µg/mL was 48.65%, and this concentration was found to have a lethal effect on this cell line.

2.3. Molecular docking studies

This investigation assessed the docking results for the tested ligands with the receptor protein using the root mean square deviation values (RMSD). RMSD/UB and RMSD/LB provide information about how much the dynamic motions of a molecule can vary statistically and can be used to evaluate the reliability of molecular dynamics simulation results. As a result of the molecular docking study, more than one binding mode was predicted. Among these modes, binding energies with RMSD/LB and RMSD/UB values of 0, indicating that the molecular dynamics simulation results are the most stable and reliable, are given in Table S3. A negative binding energy indicates that the free energy of a ligand decreases when binding to a target molecule, and

therefore, the binding is thermodynamically favourable. The decrease in free energy during a binding process means the interactions between the two bound molecules create a stable binding conformation (Shamsuddin et al. 2021). Therefore, the complexes with the strongest binding affinity are between the vanillic acid (-6.2 kcal/mol) and salicylic acid (-6.2 kcal/mol) ligands and the target molecule (UHRF1) encoded by 2BP7.

UHRF1 (Ubiquitin-like with PHD and RING Finger domains 1) is a member of the UHRF protein family (Unoki and Sasaki 2022). It is an epigenetic coordinator bridging DNA methylation and histone modifications and has been involved in the regulation of a series of biological functions (Mancini et al. 2021), such as DNA replication, DNA methylation, and DNA damage repair. Aberrant overexpression of UHRF1 has been reported in more than ten cancer types, indicating that it is a typical oncogene and plays a critical role in cancer initiation and progression (Gu et al. 2024).

UHRF1 overexpression has been observed in a panel of cancer types, including lung cancer, breast cancer, gastric cancer, prostate cancer, colorectal cancer, cervical cancer, pancreatic cancer, bladder cancer, and endometrial cancer. UHRF1 plays a crucial role in cancer initiation, progression, metastasis, and recurrence and becomes an ideal drug target since UHRF1 knockdown inhibits tumour progression (Alhosin et al. 2011). Therefore, UHRF1 may be used as a diagnostic biomarker (Tu et al. 2020; Wang et al. 2023) and a potential target for developing anti-cancer drugs (Ashraf et al. 2022).

Interactions between the active site of the target protein coded 2PB7 and vanillic acid, salicylic acid, and oxalic acid ligands are shown in Figure S4. Among this group, the 2PB7-vanillic acid (Figure S4A) and 2PB7-salicylic acid (Figure S4B) complexes have the highest binding energy (-6.2 kcal/mol). According to Figure S4A (2PB7-vanillic acid), conventional hydrogen bond (between the hydrogen atom and the ASP472 residue of the target protein coded 2PB7, Length: 2.60 Å), two carbon-hydrogen (GLY541, SER545 Length: 3.21 Å, 3.41 Å respectively), Pi-sigma, and Pi-alkyl bonds were determined between the ligand (LEU462 and TYR478 residues Length: 3.42 Å, 5.08 Å, respectively) and the receptor.

As shown in Figure S4B (2PB7-salicylic acid), the conventional hydrogen bond (between the oxygen atom and the GLY464 residue of the target protein coded 2PB7, Length: 3.07 Å), two carbon-hydrogen (GLY541, SER545 Length: 3.18 Å, 3.65 Å respectively), Pi-sigma, and Pi-alkyl bonds were determined between the ligand (ASP471 and LEU462 residues Length: 3.61 Å, 4.66 Å respectively) and the receptor.

Molecular docking analysis indicates that 2PB7-oxalic acid has the binding energy (−4.5 kcal/mol) among these complexes. According to Figure S4C, four conventional hydrogen bonds and two carbon hydrogen were predicted between the oxalic acid ligand and the active sites of the 2PB7-encoded target protein.

The presence of conventional hydrogen bonds between the ligand and the receptor indicates a strong and specific interaction between these two molecules. These bonds increase the stability of the interaction between the ligand and the receptor, ensuring specific binding and initiating the biological reaction. Therefore, conventional hydrogen bonds between ligands and receptor play an important role in understanding important processes such as cellular signalling and drug interactions in biological systems (Horowitz and Trievel 2012). Carbon-hydrogen bonds can increase the binding affinity of the ligand

to the target protein. Ligands containing aromatic rings can form strong interactions *via* π-σ bonding with specific regions on the protein surface (Sagaama and Issaoui 2020; Singh et al. 2022). Pi-alkyl bonds can enhance the hydrophobic interaction of a ligand within a receptor's binding pocket, as supported by research (Arthur and Uzairu 2019).

In Sidhu and Capalash (2021) study, salicylic acid showed stronger binding to the UHRF1 protein. Therefore, the molecular interaction between vanillic acid, salicylic acid, oxalic acid, and UHRF1 conducted in the current study is consistent with the literature. In addition, *in vivo* studies are needed to evaluate the therapeutic efficacy of vanillic acid, salicylic acid, and oxalic acid against cancer.

3. Experimental

See Supplementary Material.

4. Conclusions

From the past to the present, tar has played an essential role in treating both human and animal diseases ethnomedically, and it has crucial uses in human life. Most scientific research has focused on essential oils from plants, and very few studies have covered traditional tar production, content, and biological activities. Most of the studies on tar in the literature are ethnohistorical and ethnopharmacological (Burri et al. 2017).

In this study, for the first time, phenolic compound analysis of tar obtained from P. nigra resinous wood pieces prepared by traditional methods was performed. According to the results of phenolic content analysis of tar, ten phenolic compounds were determined. In addition, the tar showed a cytotoxic effect on the HeLa cancer cell line but did not show a cytotoxic effect on the L929 healthy cell line.

Pine tar, also known as 'püse', produced by local people in Turkey, is still used to treat various human and animal diseases. The literature has confirmed that pine tar has low levels of PAHs (coal tar components hypothesised to cause human cancer). Furthermore, pine tar is non-mutagenic (Barnes and Greive 2017). Further biological and clinical studies need to be conducted to understand better the positive or negative cellular effects of tar use.

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