Chemical Composition, Antimicrobial, and Cytotoxic Activities of Leaf, Fruit, and Branch Essential Oils Obtained From *Zanthoxylum nitidum* Grown in Vietnam

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**Abstract**

*Zanthoxylum nitidum* (Roxb.) DC is a traditional Vietnamese medicine to treat coughs, stomachache, toothache, blood stagnation, and sore throats. The essential oils (EOs) of the leaves, fruits, and stems of this plant were extracted by hydrodistillation and subjected to analysis by gas chromatography (GC)-flame ionization detector (FID) and GC-mass spectrometry (MS). The isolated EOs were then evaluated in terms of their antimicrobial activity by minimum inhibitory concentration (MIC) assay and in vitro cytotoxic effect against 5 human tumor cell lines. GC-MS-FID analysis showed 35, 32, and 25 compounds accounting for 97.6%, 91.7%, and 96.2% of the total EO contents from the leaves, fruits, and stems, respectively. The major compounds of the leaf EO were limonene (44.3%), β-caryophyllene (12.5%), linalool (11.0%), germacrene D (5.3%), and α-pinene (4.9%); the major compounds of the fruit EO were n-pentadecane (34.8%), sabinene (18.3%), and n-heptadecane (4.7%), and the major components of the stem EO were 2-undecanone (72.3%), β-caryophyllene (3.8%), and germacrene D (4.0%). The EOs of leaves, fruits, and stems of *Z. nitidum* exhibited antibacterial activity against *Bacillus subtilis*, *Escherichia coli*, and *Fusarium oxysporum* with MIC values of 100 µg/mL. The leaf and branch EOs exhibited cytotoxic activity against all tested cancer cell lines, especially A-549 and HepG-2. Findings from the present study provide important knowledge about the potential uses of *Z. nitidum* EOs as a natural antibacterial and antitumor agents.

**Keywords**

*Zanthoxylum nitidum* essential oil, chemical composition, antimicrobial activities, cytotoxic activities

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The genus *Zanthoxylum* (family Rutaceae) comprises around 250 species distributed worldwide in different climatic conditions.¹² Many species from this genus exhibit a wide range of inhibitory activities against microorganisms, fungi, cell proliferation, inflammation, and free radicals.³⁴ Essential oils (EOs) of various *Zanthoxylum* species have been studied, including *Z. avicennae*, *Z. rhesta*, *Z. achatophodium*, *Z. coriaceum*, *Z. limonello*, *Z. armatum*, and *Z. monogynum*.⁵⁻¹⁰ These EOs contain monoterpenes, sesquiterpenes, and straight-chain hydrocarbons. However, there is a difference in the major compounds between species. The EOs of some *Zanthoxylum* species have exhibited many interesting biological properties, such as larvicidal activity against *Aedes albopictus*,⁵ activity against the malaria mosquitoes *Anopheles anthropophagus* and *A. sinensis*,⁷ and antiallergic, anti-inflammatory,⁸ repellent,⁹ antimicrobial, and cytotoxic activities.¹⁰ *Zanthoxylum nitidum* (*Z. nitidum*) has been
commonly used as a traditional treatment for cough, stomachache, toothache, blood stagnation, and sore throat. Limonene, α-pinene, γ-terpinene, linalool, and geraniol, as well as several other monoterpenes, sesquiterpenes, and straight-chain hydrocarbons, have been identified in the leaf and fruit EOs of *Z. nitidum*, in varying quantities depending on the habitat in which the plant grows. Such diversity in the EOs necessitates extensive investigation of their potential bioactivities for successful exploitation of the plant. Foodborne microorganisms such as fungi, as well as Gram-negative and Gram-positive bacteria, are common causative agents of food contamination and spoilage, affecting food quality and consumer health, as well as raising serious concerns among the global population in recent years. Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, and Bacillus subtilis are well known as major foodborne pathogens with tremendously high resistance capability to conventional antibiotic therapies. Mycotoxins produced by *Fusarium oxysporum* and *Aspergillus niger* are also associated with a wide range of human infections. In order to prevent the growth of these foodborne pathogens, naturally occurring food preservatives have been extensively employed. In particular, the antimicrobial activity of EOs from several species of *Zanthoxylum* have been reported, such as *Z. zanthoxyloides*, *Z. bungeanum*, *Z. caribaeanum*, *Z. rhoifolium*, and *Z. armatum*. However, to our best knowledge, studies on the biological activities of *Z. nitidum* EOs have not yet been evaluated.

Therefore, the present study aimed to (1) analyze the phytochemical content of the EOs from *Z. nitidum* leaves, fruits, and stems, (2) evaluate their bacteriostatic effect against bacteria (e.g. *B. subtilis*, *E. coli*, *P. aeruginosa*, and *S. aureus*) and fungi (e.g. *A. niger* and *F. oxysporum*), and (3) determine their in vitro cytotoxicity effects against human tumor cell lines (e.g. Hep-G2, HeLa, MCF-7, A-549, and HGC-27).

Materials and Methods

Plant Materials

The leaves, fruits, and stems of *Z. nitidum* were obtained from Na Hang, Tuyen Quang Province (Vietnam). The plant was identified by Nguyen Quoc Binh, Vietnam Museum of Nature, Vietnam Academy of Science and Technology (VAST). A voucher specimen (XT-01/NaHang) was deposited at the Institute of Natural Products Chemistry, VAST. Prior to the extraction process, 500 g of leaves and branches were cut into small pieces, and 200 g of fruits were completely ground to prepare small samples.

Chemical and Reagents

Gentamycin, doxycycline, nystatin, doxorubicin, sodium sulfate, dimethyl sulfoxide (DMSO), trypic soy broth (TSB), Saboraud-2% dextrose broth (SDB), and 3-((4,5-dimethylthiazol-1-2-yl)-2,5 diphenyltetrazolium bromide (MTT) were purchased from Merck KGaA (Darmstadt, Germany). All the chemicals and reagents used in the present study were of analytical grade.

**EO Extraction**

EO extraction from *Z. nitidum* branches, leaves, and fruits was carried out by hydrodistillation using a Clevenger-type apparatus (JSOW, India) for 3 hours. The EOs were then dehydrated with anhydrous sodium sulfate and stored at 4 °C in a refrigerator until gas chromatography (GC)-flame ionization detector (FID) and GC-mass spectrometry (MS) analyses. The EO samples obtained from leaves, fruits, and branches were designated as ZN-L, ZN-F, and ZN-B, respectively.

**Phytochemical Screening of EOs**

ZN-L, ZN-F, and ZN-B EOs were analyzed by GC-MS and GC-FID methods. For GC-MS analysis, the system involved an HP7890A model GC (Agilent Technologies, Santa Clara, CA, USA) equipped with an HP5975C MS detector and an HP5 MS column (60 m × 0.25 mm, film thickness 0.25 μm) (Agilent Technologies, US). The temperature of the injector was set at 250 °C, and the injection volume of EOs was 1 μL. The temperature program began at 60 °C, then increased up to 240 °C, at 4 °C/min. Helium was selected as the carrier gas; the flow rate was 1 mL/min, and the split ratio was 100:1. The electron impact ionization voltage was 70 eV, emission current was 40 mA, and the acquisitions scan mass range was 35-450 amu. Similar conditions were applied to GC-FID analysis.

The identification of the constituents was carried out by comparing the obtained retention indices (RI) and mass spectra with HPCH1607 and W09N08 mass spectral libraries, as well as NIST Chemistry WebBook. The relative percentages of components were calculated based on the GC-FID peak areas without any correction factors.

**Antimicrobial Activity**

Six microorganisms obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) were used to evaluate the antimicrobial activity of ZN-L, ZN-F, and ZN-B EOs, including *E. coli* ATCC 8739, *B. subtilis* ATCC 27212, *P. aeruginosa* ATCC 25923, *S. aureus* ATCC 12222, *A. niger* ATCC 9763, and *F. oxysporum* ATCC 48112.

Antimicrobial activity of the samples was determined by minimum inhibitory concentration (MIC) assay against the above fungal and bacterial strains. The Gram-positive and Gram-negative bacteria were cultured in tryptic soy broth (TSB; Merck KGaA, Darmstadt, Germany), while fungi were grown in SDB (Merck, Germany) to a final inoculum size of about 150 × 10⁶ colony-forming units (CFU) per mL (or 0.5 McFarland standard at λ = 550 nm). The ZN-L, ZN-F, and ZN-B EO samples at various concentrations ranging from 12.5 to 200 μg/mL were loaded into 96-well microplates containing fresh cultures, and the plates were incubated at 37 °C for 24
The MIC was determined as the lowest sample concentration that inhibited visible microorganism growth after 24 hours. Several positive controls were employed, including gentamycin (16 IU/mg, 8 IU/mg, and 4 IU/mg) for Gram-positive bacteria, doxycycline (0.4 IU/mg, 0.2 IU/mg, and 0.1 IU/mg) for Gram-negative bacteria, and nystatin (12 IU/mg, 6 IU/mg, and 3 IU/mg) for fungi. The negative control was 5% DMSO instead of the tested samples. The experiment was performed in triplicates.

Cytotoxicity Assay

The Hep-2 (hepatocellular carcinoma), HeLa (cervical cancer), MCF-7 (human breast adenocarcinoma), A-549 (human lung adenocarcinoma epithelial), and HGC-27 (human stomach carcinoma) cell lines were acquired from ATCC (Manassas, VA, USA) and maintained at 37 °C in 5% carbon dioxide (CO₂) in suitable media (RPMI 1640, MEM, DMEM; Merck KGaA, Darmstadt, Germany) containing 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 UI/mL), streptomycin (100 mg/mL), and L-glutamine (2 mM). The cytotoxic effect of ZN-L, ZN-F, and ZN-B was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were diluted in 96-well microplates to a density of 5 × 10⁴ cells per well in 200 µL mixture. The samples (1-100 µg/mL) and positive control (ie, doxorubicin) at concentrations from 0.05 to 1.56 µg/mL were

| Chemical name | RI<sup>a/b</sup> | RI | ZN-L | ZN-F | ZN-B |
|---------------|------------------|------------------|------|------|------|
| (Z)-hex-3-en-1-ol | 854 850 | 0.57 | – | – | |
| (Z)-hex-2-en-1-ol | 855 859 | 0.50 | – | – | |
| n-Hexanol | 871 861 | 0.29 | – | – | |
| α-Pinene | 939 938 | 4.94 | 1.16 | 1.41 | 1.06 |
| Sabinene | 975 979 | – | 18.3 | – | – |
| β-Pinene | 979 985 | – | 0.90 | – | – |
| Myrcene | 991 991 | 0.90 | 0.22 | 0.14 | |
| n-Octanal | 999 1002 | 0.26 | – | – | – |
| Limonene | 1029 1034 | 44.3 | – | 1.11 | |
| (Z)−β-ocimene | 1037 1037 | 0.83 | 0.36 | 1.32 | |
| β-Pinene | 1060 1064 | – | 0.26 | – | – |
| α-Sabinene hydrate | 1070 1075 | – | 0.18 | – | – |
| 2-Nonanone | 1098 1091 | – | – | 0.38 | |
| Linalool | 1097 1101 | 11.0 | 0.64 | 0.92 | |
| Nonanal | 1101 1106 | – | 0.21 | – | – |
| Terpinen-4-ol | 1177 1187 | – | 0.13 | – | – |
| 2-Decanone | 1192 1193 | – | – | 0.10 | |
| α-Terpineol | 1189 1197 | 0.15 | – | – | – |
| Naphthalene | 1181 1198 | – | 0.25 | – | – |
| Methyl salicylate | 1192 1202 | – | 0.11 | – | – |
| Decanal | 1202 1208 | – | 0.39 | – | – |
| 2-Undecanone | 1294 1294 | 0.42 | – | 72.51 | |
| δ-Elemene | 1338 1347 | 0.46 | 0.19 | 0.46 | |
| α-Cubebene | 1351 1361 | – | 0.15 | – | – |
| α-Copaene | 1377 1388 | 0.21 | 0.89 | – | – |
| n-Tetradecane | 1400 1400 | – | 3.15 | – | – |
| β-Elemene | 1391 1402 | 0.24 | – | 0.25 | – |
| Dodecanal | 1409 1411 | – | 0.12 | – | – |
| β-Caryophyllene | 1419 1437 | 12.55 | 1.97 | 5.85 | |
| γ-Elemene | 1437 1444 | 0.18 | – | 0.33 | |
| β-Gurjunene | 1434 1445 | 0.18 | – | – | – |
| Aromadendrene | 1441 1456 | 0.53 | – | – | – |
| α-Humulene | 1455 1471 | 3.73 | 0.28 | 1.49 | |
| Dodecanol | 1471 1485 | – | 1.12 | – | – |
| γ-Murolene | 1480 1489 | 0.79 | – | 0.10 | – |
| Germacrone D | 1485 1497 | 5.35 | – | 4.02 | – |
| β-Selinene | 1490 1503 | – | 0.18 | – | – |
| n-Pentadecane | 1500 1500 | – | 34.8 | – | – |
| γ-Amorphene | 1496 1508 | 0.29 | – | – | – |
| (E,E)-α-farnesene | 1506 1511 | 0.90 | – | – | – |
| Bicyclogermacrene | 1500 1513 | 2.61 | 1.41 | 1.30 | |
| δ-Amorphene | 1512 1521 | 0.19 | – | – | – |
| γ-Cadinene | 1514 1529 | 0.70 | – | – | – |
| δ-Cadinene | 1523 1535 | 1.69 | 0.41 | 0.37 | |
| Germacrene B | 1561 1576 | 0.31 | 1.80 | 0.32 | |
| Caryophyllene oxide | 1583 1603 | 0.96 | 1.17 | – | – |
| Viridiflorol | 1593 1603 | – | – | 0.82 | |
| Cubeban-11-ol | 1591 1612 | 0.22 | – | – | – |
| Humulene epoxide II | 1608 1630 | 0.18 | – | – | – |

(Continued)
added to the cells and incubated at 37 °C for 48 hours with 5% CO₂. A total of 20 µL of MTT (Merck KGaA) was added to the wells, and incubation was continued at 37 °C for 4 hours. Absorbance was recorded at 540/720 nm using a Spark multimode reader (Tecan, Männedorf, Switzerland). The experiment was performed in triplicate. The rate of growth inhibition was calculated as: Inhibition rate (%) = (1 − OD_sampl/OD_con) × 100%, with OD_sampl and OD_con being the optical densities of the samples and the control, respectively.

Statistical Analysis

Data were expressed as mean ± SD and analyzed by two-way ANOVA at the 95% confidence level. Calculation of the half-maximal inhibitory concentration (IC₅₀) involved a Prism ANOVA at the 95% confidence level. Calculation of the half-maximal inhibitory concentration (IC₅₀) involved a Prism ANOVA at the 95% confidence level.

Results and Discussion

Chemical Composition of the EOs From Z. nitidum Leaves, Fruits, and Stems

The yields of ZN-F, ZN-I, and ZN-B obtained from the hydrodistillation process were relatively low (0.016%, 0.01%, and 0.08% w/w, fresh weight, respectively). All 3 oils were light yellow in color. The chemical compositions of ZN-F, ZN-I, and ZN-B were identified by using GC-MS and GC-FID and comparing their RI and mass spectra with HPCH1607 and W09N08 mass spectral libraries, as well as the NIST Chemistry WebBook. A total of 35, 32, and 25 compounds were detected, accounting for 97.6%, 91.7%, and 96.2% of ZN-F, ZN-I, and ZN-B, respectively (Table 1).

As shown in Table 1, monoterpens (62.1%) and sesquiterpenes (33.4%) were the main constituents of ZN-I, including limonene (44.3%), β-caryophyllene (12.5%), linalool (11.0%), germacrene D (5.3%), and α-pinene (4.9%). Alkane hydrocarbons (46.6%) and alkene hydrocarbons (12.6%) were the main components of ZN-F, including n-pentadecane (34.8%) and sabinene (18.3%). Nonterpenic acyclic ketones (72.8%) were the predominant components of ZN-B, including 2-undecanone (72.3%) and β-caryophyllene (5.8%). Of all the detected compounds, limonene and linalool were only present in ZN-I, while n-pentadecane, (Z)-8-heptadecene, and sabinene were only present in ZN-F; 2-undecanone was only detected in ZN-B.

The results of the present study were compared with those for the EOs of an Indian variety of Z. nitidum, as well as several other Zanthoxylum species. The content of linalool present in the leaf EO of Z. nitidum grown in India (33.1%) was lower than that of ZN-I (44.3%). Meanwhile, the chemical compositions of Z. acanthopodium, Z. rhesta, and Z. limoncello leaf EOs were different from ZN-I, with major constituents including estragole, eucalyptol, and β-caryophyllene for Z. acanthopodium; sabinene, α-pinene, and β-pinene for Z. rhesta; and 2-undecanone and 2-undecenal for Z. limoncello. The contents of the fruit EOs also varied between ZN-F and other Zanthoxylum species. For instance, the main constituents of ZN-F included n-pentadecane and sabinene, while those of Z. coreanum Nakai fruit EO were β-ocimene, α-pinene, 4-carvomenthenol, and sabinene.

For the stems of Z. nitidum, this is the first study on the chemical composition and biological activity of the essential oil obtained from this part of the plant. The obtained results showed 2-undecanone present in high content in this EO (72.3%), higher than in the leaf EO from Z. limoncello and Z. armatum. This compound is used as an insect and animal repellent.

The present study provides a helpful insight into the chemical profiles of Z. nitidum leaf, fruit, and branch EOs. Compared with other Zanthoxylum species, Z. nitidum EOs also possess a comparable quantity of high-value bioactive compounds whose potential activities require extensive exploitation in the future.

Antimicrobial Activity

The antimicrobial activities of ZN-I, ZN-B, and ZN-F were evaluated against 4 bacterial (E. coli, B. subtillis, P. aeruginosa, S. aureus) and 2 fungal strains (F. oxysporum, A. niger). The results are summarized in Table 2.

At the same concentration of 100 µg/mL, ZN-I was effective against F. oxysporum, ZN-F against E. coli and B. subtillis, and ZN-B was against B. subtillis and F. oxysporum. In contrast, all tested EOs showed minimal inhibitory activity against S. aureus, P. aeruginosa, and A. niger.

Table 2. Minimal Inhibitory Concentration (MIC) of ZN-I, ZN-F, and ZN-B Essential Oils Against 6 Bacterial and Fungal Strains.

| Essential oil | Escherichia coli | Pseudomonas aeruginosa | Bacillus subtillis | Staphylococcus aureus | Aspergillus niger | Fusarium oxysporum |
|--------------|-----------------|------------------------|-------------------|----------------------|------------------|-------------------|
| ZN-I         | >200            | >200                   | >200              | >200                 | >200             | >200              |
| ZN-F         | 100             | >200                   | 100               | >200                 | >200             | >200              |
| ZN-B         | >200            | >200                   | 100               | >200                 | >200             | >200              |
| Positive control¹ | 6.2          | 10.6                   | 9.0               | 18.1                 | 6.4              | 3.2               |

¹Note: Positive controls included gentamycin, doxycycline, and nystatin. The bold values indicated the antimicrobial activity.
These results add to knowledge about the antimicrobial activity of *Zanthoxylum* species, previously reported for *Z. monogynum*, *Z. zanthoxyloides*, *Z. alatum*, and *Z. tingoassuiba*. In addition to antimicrobial activity, *Zanthoxylum* EOs also exhibit a wide range of interesting biological activities such as cytotoxic, larvicidal (against malaria mosquitoes, eg, *Anopheles anthropophagus* and *A. sinensii*), repellent, antiallergic and anti-inflammatory.

The present study was the first to exploit the antimicrobial activity of *Z. nitidum* EOs. In addition, the results have great scientific significance, as they show that *Z. nitidum* promises to be a precious source of a natural herbal antibiotic.

### Cytotoxic Activity

The in vitro cytotoxic effects of ZN-L, ZN-F, and ZN-B against Hep-G2, HeLa, MCF-7, A-549, and HGC-27 were evaluated by using MTT assay (Table 3). As compared with the control, the highest cytotoxic effect against all tested cell lines was observed in ZN-L (16.2 µg/mL ≤ IC₅₀ ≤ 79.7 µg/mL), followed by ZN-B (21.6 µg/mL ≤ IC₅₀ ≤ 65.4 µg/mL) and ZN-F (69.5 µg/mL ≤ IC₅₀ ≤ 100 µg/mL). These results were comparable to other *Zanthoxylum* species. For instance, *Z. monogynum* EO exhibited significant inhibitory activity against several tumor cell lines (ie, B16F10, A2058, HeLa, HL-60, MCF-7, and T75) with IC₅₀ values ranging from 11 to 65 µg/mL. Meanwhile, *Z. avenanum* and *Z. chalybeum* EOs showed strong cytotoxicity against K-562 cells and human gingival fibroblasts, with IC₅₀ values of 1.76 µg/mL and 26 µg/mL, respectively.

In the present study, GC-MS analysis revealed that ZN-L, ZN-F, and ZN-B essential oils contained a variety of phytochemicals, including monoterpenes, sesquiterpenes, hydrocarbons, and nonterpenic acyclic ketones. Furthermore, as discussed earlier, each EO also contained unique bioactive compounds that are present either at a minimal quantity or completely undetected in other EOs. Different phytochemical profiles may have attributed to the different cytotoxicities of the EOs from different parts of *Z. nitidum*. For example, since the presence of monoterpenes has been reported to inhibit various cancer cell growth by inducing apoptosis, a high content of monoterpenes, along with sesquiterpenes, oxygenated monoterpenes, and oxygenated sesquiterpenes found in ZN-L possibly has given rise to its cytotoxic effect against Hep-G-2 and A-549 cells. Although the antitumor potentials of aliphatic ketones and hydrocarbons remain relatively unknown, these are the main constituents of ZN-B and ZN-F EOs, respectively, and the present study has evidenced a comparable inhibitory activity of ZN-B EOs against both Hep-G-2 and A-549 cells. Therefore, constituents, as well as their interaction pathways, require further studies. In general, the findings about the cytotoxic properties of *Z. nitidum* against several human cancer cell lines are essential insights for future research on promising anticancer agents.

### Conclusions

EO extraction of the leaves (ZN-L), fruits (ZN-F), and branches (ZN-B) of *Z. nitidum* by steam distillation resulted in oil yields of 0.016%, 0.01%, and 0.08%, respectively. The major compounds of ZN-L were limonene (44.3%), β-caryophyllene (12.5%), linalool (11.0%), germacrene D (5.3%), and α-pinene (4.9%). ZN-F mainly contained n-pentadecane (34.8%) and sabine (18.3%), and ZN-B 2-undecanone (72.3%) and β-caryophyllene (5.8%). The leaf EO (ZN-L) exhibited inhibitory activity against *F. oxysporum*, while the fruit EO (ZN-F) was effective against *E. coli* and *B. subtilis*; the stem EO (ZN-B) was effective against *B. subtilis* and *F. oxysporum*, with MIC values of 100 µg/mL. Of all the tested extracts, ZN-L exerted the highest cytotoxic properties against the tested human cancer cell lines, followed by ZN-B and ZN-F. The present studies provide insights into the phytochemical profile, as well as the role of *Z. nitidum* EOs as a valuable antimicrobial and anticancer agent. These insights should encourage further studies to isolate and evaluate the pharmacological value of the individual compounds, as well as other parts of *Z. nitidum*. In vivo toxicology and clinical applications should also be researched for the successful exploitation of *Z. nitidum* EOs.

### Declaration of Conflicting Interests

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