Identification of bioactive compound from *Nigella sativa, Allium sativum, propolis* and *Oleaeuropaea* mixture as antibacterial and antifungal agent

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Identification of bioactive compound from *Nigella sativa*, *Allium sativum*, propolis and *Oleaeuropaea* mixture as antibacterial and antifungal agent

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Abstract. *Nigella sativa*, *Allium sativum*, propolis and *Oleaeuropaea*, have been used empirically as a traditional medicine. The objective of this study were to tested the antibacterial and antifungal activity of the extracts, as well as to identified the active compounds contained in the extract mixture. The antibacterial activity test was carried out against Gram positive bacteria (*Streptococcus agalactie*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Micrococcus luteus*, *Bacillus cereus*), Gram negative bacteria (*Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Eschericia coli*), and antifungi against *Candida albicans* using Kirby and Bauer diffusion disc test. The search of active compound was done by phytochemical test including alkaloid, phenolic, flavonoid and steroid-triterpenoid test, and also identification of active compound using Gas Chromatography-Mass Spectrometry (GC-MS). The results of antimicrobial activity investigation by Kirby and Bauer method showed that in general the extract had the ability to inhibit the growth of all Gram positive test bacteria and did not inhibit *C. albicans* growth, except for mixed extract A which could inhibit the growth of almost all bacteria and *C. albicans* with the average inhibitory zone diameter as much as 7.5 mm. Phytochemical tests showed that all extracts were negative against the alkaloids test (Meyer, Wagner and Dragendorf) and positive for steroid and triterpenoid tests. The identification of active compound of mixture extract A with GC-MS showed that the highest content of the compound was linoleic acid (57.87%) followed by palmitic acid (17.85%) and citronella (7.68%).

Keywords: active compound, *Allium sativum*, *Nigella sativa*, *Oleaeuropaea*, propolis

1. Introduction

Herbal medicines has been used among Asian, particularly Indonesian, for centuries. In recent years, people’s interest of herbal medicines has increased. This is supported by herbal-based research that proved the healing effects or efficacy of some herbs. Nowadays, modern lifestyle demanding instant and ready-to-consume’s product, one of them is herbal products. Herbal products are generally a combination of some herbs plant or herbs in pure extract, simplisia and others.

Some herbs and natural product that has been used for long time are *Nigella sativa* (black cumin), *Allium sativum* (garlic), propolis and *Oleaeuropaea* (olive oil). *N. sativa* (Family Ranunculaceae) is plant originated from Mediterranean. People commonly used the seed to treat some health problems. Oil seed of *N. sativa* have some biological activities such as...
hepatoprotective, antimicrobial[1], antidiabetics[2], antioxidants[3], and antidermatophic[4]. A. sativum has long been used as traditional medicine and food preservatives. A. sativum has antibacterial properties[5], antioxidants and hepatoprotector[6].

Propolis is a derivative compound from resinous plants that are collected by honeybees. Propolis is used as an adhesive and drought exuder in beehive[7]. Some biological activity of propolis includes an immunomodulator agent[8], antiinflammation[9], antidepressant and antioxidant[10] as well as antimicrobial[11]. O. europaea fruits and oil have been used among Mediterranean as food, cosmetics and medicine. O. europaea has an antioxidant[12] and antimicrobial properties[13]. This research aims to examine N. sativa, A. sativum, O. europaea, and propolis (single and mixture) against some bacteria and Candida albicans, and also identify the bioactive compounds in the mixture based on phytochemical and Gas Chromatography – Mass Spectrometry (GC-MS) assay.

2. Methodology
This study used commercial product of N. sativaoil, A. sativumoil, O. europaea, and propolis (single extract), mixture of those extract 1:1 (v/v) (mixture B), and commercial mixture of N. sativaol, A. sativumoil, O. europaea, and propolis (mixture A).

2.1. Antimicrobial assay (Kirby and Bauer)
The bacterial test was growth on Mueller Hinton Agar (MHA) media. Paper disc (contained 5 µL extract or control) is placed above the MHA media, then incubated at 37 °C for 24 hours. Diameter of inhibition zone were observed and measured.

2.2. Phytochemical assay
Alkaloid test. Sample (0.3 mL) was added to 1.5 mL of chloroform and 3 drops of ammonia. Chloroform fraction was separated and then acidified with 2 drops of sulfuric acid. The acid fraction was separated into 3 parts and added Dragendorf, Meyer and Wagner reagent for each. A positive result indicated by the formation of red deposits (Dragendorf), white deposits (Meyer), and brown deposits (Wagner).

Phenolic test. Sample (1 mL) was added to 1 mL ethanol 70% and then shed with 2 drops of 5% FeCl₃ solution. The formation of green or blue green color showed phenol in sample.

Flavonoid test. Sample (0.3 mL) was mixed with 1.5 mL of methanol and heated at 50°C for 5 minutes. Then 5 drops of the solution is transferred to the platedrops and added 5 drops of concentrated sulfuric acid. Red color formed showed flavonoid in sample.

Steroid and triterpenoid test. Sample was diluted with 2 mL of ethanol 30% and heated. The filtrate was evaporated and added with 1 mL of ether. Five drops of ether fraction was moved to plate drops and added 3 drops of acetic anhydride and 1 drop of concentrated sulfuric acid. The formation of red or purple color indicates the presence of triterpenoid compounds, and the green color indicates the presence of steroid compounds.

2.3. Identification of bioactive compounds (Shimadzu GCMS-QP 2010-Frontier Lab Single-Shot Pyrolyzer PY-2020iS)
The separation was effected using silica capillary column with 60 m x 0.25 mm, 0.25 µm film thickness and 0.85 mL/min for column flow. The GC oven temperature was programmed from 50°C to 280 °C at the rate of 5°C/min. Helium was used as a carrier gas with the inlet pressure was 101 kPa, linear velocity 23.7 cm/Sec. Injection temperature : 280°C; injection mode : split with 1:50 ratio. Pyrolyzer temperature : 300°C. MS ion source temperature : 200°C; MS interface temperature : 280°C. The identification of components based on the retention index and compared with the reference spectra (Wiley and PubChem databases).
3. Result and discussion

This study used commercial product of *N. sativa* oil, *A. sativum* oil, *O. europaea*, and propolis. The performed test including antimicrobial assay, phytochemical assay and identification of bioactive compounds using GC-MS pyrolysis. Table 1 showed that mixture A has antibacterial activity against all bacteria (Gram positive and Gram negative) and *C. albicans*. Sample *N. sativa* and mixture B showed inhibition against Gram positive bacteria and *C. albicans*. Beside *N. sativa*, propolis also showed positive result against *C. albicans*. It can be concluded that antimicrobial activity of mixture B likely caused due to presence of *N. sativa* and propolis. According to Gerige et al.[13], *N. sativa* oil has ability to inhibit bacteria genus *Staphylococcus*, *Streptococcus*, *Micrococcus*, and *Bacillus*, and also *C. albicans*. Extract of *N. sativa* also showed inhibitory effect against *B. cereus*, *M. luteus* and *S. aureus*[14]. Freires et al.also stated that propolis has antifungal properties[15].

The phytochemical assay showed as Table 2, a type of bioactive compound that identified in all samples, either single extract or mixture, was triterpenoid. Phenolic and flavonoid also showed positive result in some samples. Triterpenoid is secondary metabolite which commonly found in plant’s essential oil. Saponin, steroid and glycoside are type of triterpenoid. Phenolic and flavonoid are group of secondary metabolite that have biological activity as an antioxidant and antimicrobial. The oil of *N. sativa*’s seed contain steroid[15] and pinene[16]. Those compounds also found in *A. sativum*[17], β-sitosterol and stigmasterol (steroid) had identified in *O. europaea*[18] and propolis[19]. Phenolic and flavonoid were also found in *N. sativa* and propolis. Erkanet al.[3] showed that essential oil of *N. sativa*’s seed have antioxidant property due to flavonoid compound, thymoquinone, and phenolic compound, hydroquinones. Cuesta-Rubio et al.[19] had identified flavonoid compound in propolis namely sakuranetin as anti-leishmania. Phenolic compound were also found in ethanol extract of propolis, there areemaric acid, kaempferol and quercetin[16].

### Table 1. Inhibition zone of *N. sativa*, *A. sativum*, *O. europaea*, and propolis extract against some bacteria and *C. albicans*.

| Sample       | S. agalactiae | S. aureus | S. epidermidis | M. luteus | B. cereus | P. aeruginosa | S. typhimurium | E. coli | C. albicans |
|--------------|---------------|-----------|----------------|-----------|-----------|---------------|----------------|---------|------------|
| *N. sativa*  | 60            | 60        | 60             | 52.5      | 40        | 0             | 0              | 0       | 24.5       |
| *O. europaea*| 15            | 0         | 0              | 0         | 0         | 0             | 0              | 0       | 0          |
| *A. sativum* | 15            | 0         | 0              | 0         | 0         | 0             | 0              | 0       | 0          |
| Propolis     | 0             | 14.5      | 18             | 0         | 0         | 0             | 0              | 0       | 16         |
| Mixture A    | 7             | 14        | 11             | 8.5       | 25        | 6             | 6              | 7.5     | 7.5        |
| Mixture B    | 37.5          | 40        | 55             | 17.5      | 24.5      | 0             | 0              | 0       | 17         |

*tetracycline used as positive control*

### Table 2. Phytochemical profile of *N. sativa*, *A. sativum*, *O. europaea*, and propolis extract.

| Sample       | *N. sativa* | *O. europaea* | *A. sativum* | Propolis | Mixture B | Mixture A |
|--------------|-------------|---------------|--------------|----------|-----------|-----------|
| Alkaloid     | -           | -             | -            | -        | -         | -         |
| Meyer        | -           | -             | -            | -        | -         | -         |
| Drangelorp   | -           | -             | -            | -        | -         | -         |
| Wagner       | -           | -             | -            | -        | -         | -         |
| Phenolic     | +           | -             | -            | +        | +         | -         |
| Flavonoid    | +           | -             | -            | +        | +         | +         |
| Triterpenoid | +           | +             | +            | +        | +         | +         |
Based on GC-MS analysis in figure 1 and table 3, linoleic acid was the compound with the highest relative concentration (57.87%), followed by palmitic acid (17.85%) and citronella (7.68%). Linoleic and palmitic acid are fatty acids contained in oil of *N. sativa* [15] and *O. europaea* [18]. Palmitic acid also found in propolis [19]. Linoleic acid is saturated fatty acid which belongs to polyunsaturated fatty acids (PUFA) and omega-6 group. According to Dilika et al. [20], linoleic acid has bactericidal activity against Gram-positive bacteria (*S. aureus* and *M. kristinae*) and had no activity against Gram-negative bacteria. According to Agoramoorthy et al. [21], Gram-positive bacteria is more sensitive to fatty acid than Gram-negative. This is due to the presence of lipopolysaccharide in Gram-negative bacteria’s cell wall which effectively protect cell against hydrophobic compounds.

![Figure 1. GC-MS pyrolysis chromatogram of *N. sativa*, *O. europeae*, *A. sativum* and propolis mixture.](image)

| Compound                        | Relative Concentration (%) | Compound                        | Relative Concentration (%) |
|--------------------------------|----------------------------|--------------------------------|----------------------------|
| p-Cymene                        | 0.39                       | Palmitic acid                   | 8.49                       |
| 4-tert-Butylpyrocatechol         | 3.29                       | Linoleic acid                   | 19.27                      |
| Thymol                          | 0.08                       | Citronella                      | 3.39                       |
| Junipene (Longifolene)          | 0.14                       | Linoleic acid                   | 38.60                      |
| Octadecenyl aldehyde            | 0.10                       | Octadecenyl aldehyde            | 2.99                       |
| 2(3H)-Benzo[1,4]furanone        | 0.43                       | nerolidol Z and E               | 0.45                       |
| Methyl 11-cyclopentylundecanoate| 0.10                       | Stearaldehyde                   | 0.41                       |
| Silane, trichloroecysyl-        | 0.07                       | cis-11-Tetradecenyl acetate     | 1.76                       |
| 5-tetradecenyl acetate          | 0.35                       | Undecane, 6,6-dideutero-5-methyl-| 0.14                       |
| Palmitic acid                   | 9.36                       | Octadecenyl aldehyde            | 0.75                       |
| 3-Decyn-2-ol                    | 0.17                       | Epoxycyclododecane              | 0.39                       |
| Allyheptanoate                  | 0.19                       | trans-Farnesol                  | 0.87                       |
| Citronella                      | 0.47                       | Squalene                        | 1.88                       |
| Methyl petroselinate            | 0.60                       | 17-(1,5-dimethyl-hexyl)-10,13-dimethyl- | 0.33                       |
| Citronella                      | 3.82                       | 17-(1,5-dimethyl-hexyl)-10,13-dimethyl- | 0.72                       |
Palmitic acid belongs to saturated fatty acid’s group which consist of 16 carbon atoms. Ester of palmitic acid was known has inhibition activity against some Gram-positive bacteria (B. subtilis, B. pumilus, M. luteus), some Gram-negative bacteria (P. aeruginosa, S. aureus, K. pneumoniae, E. coli) and some species of Candida, although Gram-positive bacteria were more susceptible than Gram-negative bacteria[22]. Citronella is essential oil which commonly found in genus Cymbopogon (Lemongrass). According to Silva et al.[22], there are more than 80 active compounds in citronella oil, including geranial, citronellal and limonene. Citronella was known to have antibacterial properties, antifungal and act as mosquitoes repellent. Some studies showed, limonene was identified in N. sativa oil with relative concentration as much as 1.7%[4] and 4.3%[14].

In addition to three compounds, there are some compounds which has been identified although it has small relative concentration. Those are p-cymene (0.39%), junipene (longifolene) (0.14%) and thymol (0.08%). These compounds were also identified in N. sativa oil with relative concentration 14.1%, 6.1% and 1.2% respectively[4]. A similar result also stated that P-cymene and junipene (longifolene) was identified in N. sativa oil with relative concentration as much as 9.0% and 5.7%[14].

![Figure 2](image2.png)

**Figure 2.** Linoleic acid (a), palmitic acid (b), citronellal (c), geranial (d), limonene (e).

![Figure 3](image3.png)

**Figure 3.** p-cymene(a), junipene (longifolene) (b), dannthymol (c).
Based on the results, it can be conclude that the sample (commercial product of *N. sativa*, *A. sativum*, *O. europaea*, and propolis mixture) have broad antibacterial spectrum and antifungal against *C. albicans*. These properties mainly caused by *N. sativa*and *O. europaea*. This result supported with phytochemical assay and GC-MS chromatogram which showed that the most abundant compounds was dominated by linoleic acid, palmitic acid and citronella which also identified in *N. sativa*and *O. europaea*. This indicates that the commercial product is dominated by extract of *N. sativa* and *O. europaea*. The other extract, *A. sativum*and propolis, probably in the sample but has a very few in number.

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