Antibacterial activity of organic solvent fraction from *Euphorbia supina*

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In this study, the value of *Euphorbia supina* antibiotics and disinfectants was investigated. *E. supina* was cultivated in Wonju, Gangwon-do, Republic of Korea, until August to November, 2013. After the harvest, *E. supina* was extracted with methanol. Extracts of MeOH were partitioned with different polaric organic solvents (n-hexane, CHCl₃, EtOAc, n-BUOH and distilled water fractions). The antibacterial activity of each fraction used disk diffusion. First, all fractions of *E. supina* extracts showed antibacterial activity against Gram positive bacteria. Especially, EtOAc fractions were 14±8 mm against *Staphylococcus aureus* and 10±0 mm against *Bacillus subtilis*. EtOAc fractions were partitioned into 6 sub-fractions by high performance liquid chromatography (HPLC). Each subfraction investigated the antibacterial activity by minimum inhibitory concentration (MIC). As the second result of subfraction, number 2 subfraction showed 2.5 mg/ml against *S. aureus*, 0.62 mg/ml against *B. subtilis* and number 5 subfraction showed 2.5 mg/ml against *S. aureus*, 0.62mg/ml against *B. subtilis*. Through this study, *E. supina* has utility as a natural pharmacological raw material for natural antibiotics and disinfectants.

**Key words:** *Euphorbia supina*, antibacterial activity, antibiotics, disinfectant.

INTRODUCTION

*Euphorbia supina* is an annual prostrate herb that grows in fields. Its main stream spreads on the ground and its length is about 10 to 25 cm, stem soft, curved, branched, white pilose, containing leaves and red-colored hair. On the center of it, there is red-colored mucule with white latex. Fern fruit prismatic spherical, diameter 2 mm was red, white pilose; seed ovoid and angled, florescence 3 to 5 months, fruit period is from 6 to 9 month (The State Administration of Traditional Chinese Medicine “Chinese Herbal Medicine” Editorial Board, 1999; Lee, 1989).

The Dictionary of Common Chinese Herbal Medicines reported that *E. supina* can treat hemostasis, diuretic, stomachic, invigorate the circulation of blood, detoxification, jaundice, and dysentery (Tanaka et al., 1990). Also, the plant affects diarrhea, urinary tract infection, hematochezia, hematuria, uterine bleeding, bleeding...
hemo-orrhoids, plot rickets in children, traumatic swelling, snake bites, head sore, skin Chuangdu, trauma hemorrhage. In addition, in vitro has been reported to have an anti-cancer effect (Tanaka et al 1990). So far, however, there have been no reports on antibacterial activity of subfraction about antibacterial activity fraction of E. supina.

Chemical composition of E. supina has been reported as non-polar, triterpenoid derivatives (Tanaka and Matsunaga, 1991, 1989, 1988; Tanaka et al., 1990, 1989, 1987; Matsunaga and Morita 1983), monoterpenic lactone (Tanaka and Matsunaga, 1989), tannins, flavonoids (Lee et al 1991), and phenol. In addition, polar compound supinaonodies A and B have been recently revealed (Cai et al., 2009.

Existing antibacterial agents were consistently raised regarding safety and anti-methanogenic properties (Olajuyigbe and Afolayan, 2012), antibacterial activity of honey has been attributed to hydrogen peroxide, osmolarity acidity, aromatic acids and phenolic compounds, a lot of researches were conducted using natural chemical compound about antibacterial agent.

Among the pathogenic bacteria, millions of dollars annually for food processing and health care were caused by Escherichia coli, a food-borne pathogen in North America (Liu et al., 2013), Salmonella species can be classified as a potential microorganism for bioterrorism and there are more than 2500 serovars of Salmonella spp. and all are potential pathogens (Chattopadhyay et al., 2013). Staphylococcus aureus is one of the important causes of nosocomial and cause life-threatening diseases, such as pneumonia, osteomyelitis, septicaemia and endocarditis (Soromou et al., 2013). Bacillus subtilis have been used for the fermentation of soybean as a non-pathogenic microorganism that produced psbutilin, surfactin, fengycin, gramicidins, tyrocidine, iturine, and bacitracin. Taste of food can improve the natural antibacterial agent about B. subtilis for decision of fermentation period.

Here we report that E. supina extract, fraction, and subfraction have antibacterial activity against Gram positive and negative bacteria. This study investigated E. supina utility value of antibiotics and disinfectants about pathogenic bacteria and B. subtilis.

MATERIALS AND METHODS

Plant collection
E. supina was cultivated in Dongsu Farm, Wonju, Gangwon-do, Republic of Korea on autumn season August to November in 2012 and were harvested.

Test organism
Four bacterial strains were used to assess the antibacterial properties of the test samples, two Gram-positive and two Gram-negative bacteria.

The Gram-positive bacterial strains (2) used were S. aureus (KCCM 11335), B. subtilis (ATCC 11774) and Gram-negative bacterial strains (2) used were E. coli (ATCC 8739), Salmonella typhimurium (KCCM 11862).

Before use, all bacteria were cultivated in Mueller-Hinton Broth (MHB) (Difco Laboratories, Sparks, MD, USA) and then stored in 15% glycerol and frozen at -70°C.

Preparation of E. supina extract and fraction

The plants was dried at room temperature until extraction. Dried plant was ground to a powder. The plants of 3,000 g were extracted with 60,000 ml methanol. The plant was macerated three times at room temperature using fresh methanol every 24 h. The plant extracts were filtered through filter paper (Whatman, 47 mm, USA) and then evaporated to dryness using a rotary vacuum evaporator at 45°C on water bath. The methanol extract of plant was partitioned with organic solvents of different polarities to yield n-hexane, CHCl₃, EtOAc, n-BUOH and water fractions, in sequence. E. supina fractions were concentrated by rotary evaporation. The concentrate was recovered with a small volume of solvent and kept open at room temperature until all the residual solvent had evaporated. The dried crude extracts was dissolved in dimethyl sulfoxide (DMSO, Biosesang, Korea, >99%) at final concentration 20 mg/ml. The samples were stored at 4°C (Figure 1).

Paper disk diffusion assay

The stored pathogenic microorganisms were cultured in Mueller-Hinton agar (MHA) at 37°C at 24 h. After 24 h of culture, selected colony from cultured MHA plate inoculated in Mueller-Hinton broth (MHB) and then cultured at 37°C with shaking. The bacterial cultures were adjusted to 1⁰ CFU/ml and inoculated onto MHA plates by streaking the swab (Wikler, 2006).

For the determination of antibacterial activity from E. supina, 10 µl of each extract and fraction solution absorbed sterilized paper disk (Whatman, 6 min diameter). The disks were placed on the surface of each inoculated plate. Amoxicillin (10 µg/ml) was used as positive control (P.C) of Gram positive, and colistin sulfate (10 µg/ml) were used as positive control of Gram negative bacteria. Negative control (N.C) prepared DMSO. The plates were incubated at 37°C for 18 h. The inhibition zone diameter around each of the disks was measured and recorded at the end of the incubation period.

Preparation of E. supina subfraction

The ethyl acetate fraction showed the highest antibacterial activity against S. aureus and B. subtilis. The fraction was partitioned by High Performance Liquid Chromatography (Donglishimaz, Japan), Zeopect C₁₈, 45 to 60 mm, 7 × 50 cm, step-gradient elution of water/methanol (10 to 100%, 200 min), UV detector: 245 mm. Solvent in each subfraction was evaporated to dryness under vacuum and freeze drying.

Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) was determined by microdilution in a 96-well microtitre plate (Coyle, 2005). Mueller Hinton broth (100 µl) was pipetted into each required well
and plant extracts was added serial two fold dilutions (10 mg to 0.075 mg/ml).
Bacteria were inoculated into each well (10⁶ cells/ml) except for negative control. Positive control inoculated only bacteria and negative control pipetted only Mueller Hinton broth. The plates were incubated at 37°C for 24 h. During incubation, absorbance was measured at 600 nm with an Eon Microplate Spectrophotometer (BioTek Instruments, Winooski, VT, USA) after 4, 8, 12, 16, 20, and 24 h.

RESULTS

Plant extract

*E. supina* was isolated with different polaritic organic solvents (n-hexane, CHCl₃, EtOAc, n-BUOH and Distilled water fractions). Mass of each fraction was MeOH extract 285.5 g, n-Hexane fraction 63.1 g, CHCl₃ fraction 3.8 g, EtOAc fraction 50 g, n-Butyl alcohol fraction 65.6 g, dry weight fraction 34.9 g (Table 1).

**Table 1. Euphorbia supina Rafinesque extract mass of each solvent.**

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Mass of extracts (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl alcohol</td>
<td>285.5</td>
</tr>
<tr>
<td>n-Hexane</td>
<td>63.1</td>
</tr>
<tr>
<td>Chloroform</td>
<td>3.8</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>50</td>
</tr>
<tr>
<td>n-Butylalcohol</td>
<td>65.6</td>
</tr>
<tr>
<td>Distilled water</td>
<td>34.9</td>
</tr>
</tbody>
</table>

Antibacterial activity of each fraction was MeOH against *S. aureus* 0.62 mg/ml against *S. aureus*, 0.62 mg/ml against *B. subtilis* (Table 3).

**DISCUSSION**

Nowadays, the spread of multidrug resistance (MDR) pathogenic bacteria is a threat to our future, leading to an urgent requirement for new antibacterial compounds (Bassetti et al., 2013). Therefore, in this study we studied the antibacterial activity of multidrug-resistant, β-lactamase-producing methicillin-resistant *S. aureus* (MRSA) strains and β-lactamase producing methicillin sensitive *S. aureus* (MSSA) strain (Aqil et al., 2006), antibacterial activity against MDR pathogenic bacteria of local herbs collected from Murre, Pakistan (Mansoor et al., 2013) and volatile oils of plant was used as the antibacterial agents (Dorman and Deans, 2000). However, despite these studies, scarcely natural product was used as the natural material of antibiotics and disinfectants (Olajuyigbe and Afolayan, 2012; Khan et al., 2011). Thus, development of antibiotics and disinfectants by natural material is an urgent situation. Antibacterial activity of *E. supina* was reported as an antibacterial activity conforming to light intensity (Joung et al., 2011), but was not reported to be an antibacterial activity of the subtraction of antibacterial activity fraction.

Thus, this study investigated the antibacterial activity of *E. supina* fraction and subtraction of pathogenic bacteria. MeOH extract of *E. supina* was partitioned on n-hexane, CHCl₃, EtOAc, n-BUOH and Distilled water fractions. Antibacterial activity of each fraction used disk diffusion assay. First, *E.supina* did not have antibacterial activity against Gram negative bacteria, but all fraction of *E. supina* was against *S. aureus*, *B. subtilis* of Gram positive bacteria. Among the fraction, inhibition zone of EtOAc fraction was 14±8 and 10±0 mm most excellent and at least, the fraction was partitioned into 6 chemical compounds. Antibacterial activity against Gram positive bacteria of subtraction were used as MIC. The result of subtraction number 2 was 2.5 mg/ml against *S. aureus* and *B. subtilis*, and for subtraction number 5 was 0.62 mg/ml against *S. aureus* and *B. subtilis*.

**Antibacterial effect of *E. supina* organic solvent fraction**

Antibacterial activity of *E. supina* used disk diffusion assay. First, *E. supina* had no activity against *S. typhimurium* and *E. coli* of Gram negative. But all fraction of *E. supina* was against Gram positive bacteria. Clean zone of each fraction was MeOH 8.8±1.6 mm, n-Hexane 8±0.9 mm, chloroform 8.8±2.8 mm, ethyl acetate 14±8 mm, n-Butylalcohol 12±4.4 mm for *S. aureus* and MeOH 8.5±0.7 mm, n-Hexane 8.5±0.2 mm, chloroform 8.0±0 mm, ethyl acetate 10±0 mm, n-Butylalcohol 9±1.4 mm for *B. subtilis*. Especially, EtOAc fraction showed the best antibacterial activity about *S. aureus* and *B. subtilis* (Table 2).

**Isolation of ethyl acetate layer**

EtOAc fraction of *E. supina* was isolated by High Performance Liquid Chromatography (Dongilshimaz, Japan), Zeoprep C₁₈, 45 to 60 mm, 7×50 cm, step-gradient elution of water/methanol (10 to 100%, 200 min), UV detector: 245 mm. The EtOAc fraction result was partitioned into six subfractions (Fraction 1 to 6). Each subfraction configured chemical compound (Figure 2).

**Antibacterial activity of EtOAc subfraction fraction (1 to 6)**

Antibacterial activity of EtOAc subfraction 1 to 6 conducted MIC. The result of subfraction 2 was 2.5 mg/ml against *S. aureus*, 2.5 mg/ml against *B. subtilis* and subfraction number 5 was 0.62 mg/ml against *S. aureus*, 0.62 mg/ml against *B. subtilis* (Table 3).
The paper disk diffusion assay of organic solvent fraction (MeOH, n-hexane, CHCl₃, EtOAc, n-BuOH and H2O) from E. supina.

<table>
<thead>
<tr>
<th>Classification</th>
<th>Strain</th>
<th>Methyl alcohol</th>
<th>n-Hexane</th>
<th>Chloroform</th>
<th>Ethyl acetate</th>
<th>n-BuOH</th>
<th>PC*</th>
<th>NC*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram (-)</td>
<td>S. Typhimurium</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>30±2</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>E. coli</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>24±4</td>
<td>-</td>
</tr>
<tr>
<td>Gram (+)</td>
<td>S. aureus</td>
<td>8.8±1.6</td>
<td>8±0.9</td>
<td>8.8±2.8</td>
<td>14±8</td>
<td>12±4.4</td>
<td>42±2</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>B. subtilis</td>
<td>8.5±0.7</td>
<td>8.5±2.1</td>
<td>8±2</td>
<td>10±0</td>
<td>9±1.4</td>
<td>35±0</td>
<td>-</td>
</tr>
</tbody>
</table>

*P.C: Positive control (gram positive: amoxicillin, gram negative: colistin sulfate 10ug/ml), N.C: Negative control (99%, DMSO). Na: None antibacterial activity at the highest concentration (20 mg/ml) test in this study.

The Minimum Inhibitory Concentration (MIC) of the subfraction at EtOAc fractions from MeOH extracts of E. supina.

<table>
<thead>
<tr>
<th>Classification</th>
<th>Microorganism</th>
<th>Fraction 1</th>
<th>Fraction 2</th>
<th>Fraction 3</th>
<th>Fraction 4</th>
<th>Fraction 5</th>
<th>Fraction 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram (+)</td>
<td>S. aureus</td>
<td>10</td>
<td>2.5</td>
<td>10</td>
<td>5</td>
<td>2.5</td>
<td>&gt;10</td>
</tr>
<tr>
<td></td>
<td>B. subtilis</td>
<td>10</td>
<td>0.62</td>
<td>5</td>
<td>&gt;10</td>
<td>0.62</td>
<td>&gt;10</td>
</tr>
</tbody>
</table>

**Euphorbia supina** Rafinesque

extract with Methyl alcohol

Methyl alcohol extract

extract with n-Hexane

Evaporation

n-Hexane extract

H₂O layer

extract with Chloroform

Evaporation

Chloroform extract

H₂O layer

extract with Ethyl acetate

Evaporation

Ethyl acetate extract

H₂O layer

extract with n-Butyl acetate

Evaporation

n-Butyl acetate extract

H₂O layer

**Figure 1.** Fraction of extraction of *Euphorbia supina* Rafinesque extract.
Figure 2. Qualitative analysis of subfraction number 1 to 6 about EtOAc fraction of *E. supina.*
Many studies of medicinal herbal plants have reported the antibacterial activity of pathogenic bacteria. Stem bark extracts from Jatropha curcas was 5 mg/ml against S. aureus and B. subtilis (Igbinosa et al., 2009), ethanol extracts of Tamarindus indica was 20 mg/ml against S. aureus and 18 mg/ml against B. subtilis (Doughari, 2007). Depending on the result of this study, the antibacterial activity of subfraction from EtOAc fraction of E. supina showed relatively excellent effect. For future study, effective single compound of E. supina was isolated and then each single compound was examined for every kind of in vitro and in vivo test. Depending on the result of this study, E. supina was expected to be natural pharmacological raw material antibiotics and disinfectants.

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REFERENCES


