

# Potency of Lobak Leaves (*Raphanus sativus* L. var. *hortensis* Back) as Anticancer and Antimicrobial Candidates

RITA RAKHMAWATI<sup>♥</sup>, ENDANG ANGGARWULAN, ESTU RETNANINGTYAS

Biology Department, Faculty of Mathematics and Natural Sciences, Sebelas Maret University (UNS), Surakarta 57126.

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## ABSTRACT

One of vegetables can preventive cancer and have been used traditionally to cure infection, such as lobak (*Raphanus sativus* L.). Ineffectiveness antibiotics against microbial infections were still problem until now. Types of antibiotics and anticancer agents from natural resources should be explored and developed. This study was aimed to know toxicity effect and antimicrobial activity of active fractions from lobak leaves. Toxicity study was conducted using Brine Shrimp Lethality Test (BST). Samples were prepared at the concentration of 100, 500, and 1000 g/mL. Antibacterial study against *Staphylococcus aureus* was conducted using agar-well diffusion method at concentration 30, 40, 50, 60, 70, 80, 100%. Ethyl acetate fraction from methanol extract is the most active that had larger clear zone in *S. aureus* culture (10,64 mm) and insoluble ethyl acetate fraction from methanol extract is the most active against *A. salina* (84% death *A. salina* at 100 µg/mL). Bioactive compounds at active fraction were identified to contain polar compounds.

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**Key words:** *Raphanus sativus* L., BST, *Staphylococcus aureus*, active fraction.

## INTRODUCTION

Based on epidemiological evidences, consumption of vegetables, belonging to the Brassicaceae family, has been associated with a decreased incidence of various cancers (Duthie et al., 2000; O'Hare al., 2006). On the basis of this information, researchers recently have estimated that vegetable consumption prevent 20% to 50% of all cases of cancer (Nestle, 1997). Lobak (*Raphanus sativus* L.) is not only vegetables but in many countries, lobak is used in traditional medicines (Shoeb, 2006). There were three species of lobak variety in Java, var. *hortensis* Back., var. *niger* Willd. and var. *radicula* Pers. Lobak var. *hortensis* have white sprouts and long cylinder. Lobak var. *niger* have black sprouts, meanwhile var. *radicula* with red skins or pure white throughout (Backer and Bakhuizen v.d. Brink, 1968). In this study, we focus on lobak var. *hortensis*.

Lobak is reported as good remedy used for treatment of anti tumor, anti infection, chemoprevention for breast cancer, and immunomodulator (Wijayakusuma, 2005). Lobak has been traditionally used for medicinal purposed, therefore further research is needed. The current study was carried out to determine the toxicity of

lobak against *A. salina*, and the antimicrobial activity of lobak leaves against *S. aureus*.

## MATERIALS AND METHODS

### Material

Lobak leaves (*Raphanus sativus* L.) were collected from Tawangmangu in August 2008. Chemicals such as silica gel GF<sub>254</sub>, ethanol, n-hexane, methanol, chloroform, and ethyl acetate were purchased from Merck (Darmstadt, Germany), aquadest, sea water, *Artemia salina* egg, yeast (Fermipan®), cerium (IV) sulfate, *Staphylococcus aureus* (Rosenbach) colony, Nutrient Agar (NA) and Muller Hinton (MH), amoxicillin, rotary evaporator (Heidolph vv 2000, Germany), micropipette, lamp 5 watt, aerator, micro syringe, oven, spray, laminar air flow and UV detector.

### Extraction

Extraction was carried out by simple maceration process. The powdered leaves (600 g) were initially macerated with 1700 mL chloroform (24 hours x 3) at room temperature. Macerate were filtered and distilled in rotary evaporator and concentrated to obtain the crude chloroform extract. Residue were remacerated (24 hours x 3) with methanol (1700 mL) for 24 hours, then filtered. Procedure was done as above mentioned and the crude methanol extract obtained. Sample was prepared by dissolving 50 mg

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### ♥ Alamat korespondensi:

Jl. Ir. Sutami 36A Surakarta 57126  
Tel. & Fax.: +62-271-663375  
e-mail: oktia@yahoo.com

of each sample in 5 mL of chloroform: methanol (1:1) v/v and obtained stock solution 10 µg/mL. The extract of chloroform and methanol were examined their toxicity and antimicrobial activity against *S. aureus*. The most active extract was partitioned with ethyl acetate and obtained fraction to form insoluble and soluble ethyl acetate fraction. Both of them were further examined with reduced concentration.

**Larvae *A. salina* hatching**

*Artemia salina* eggs were hatched in a container filled with aerated sea water and illuminated with 5 watt light source. The container was compartmentalized into dark compartment and lightened one with several holes. After 24 hours, brine shrimp larvae were collected by pipette from the lightened side through the holes; then 48 hours of *A. salina* larvae were ready for toxicity test.

**Assay for antibacterial activity *S. aureus* with agar-well diffusion method**

All the stock cultures were obtained from Faculty of Medicine, Sebelas Maret University, Surakarta, Central Java. Stock cultures were regenerated on nutrient agar (NA) slants and incubated at 37°C for 24 hours for bacterial proliferation. Muller Hilton Medium was sterilized at 121°C for 15 minutes, then cool down until 50°C. After that MH medium were inoculated with cultures (1-2 ose) and homogenized. Active cultures used for experiments were prepared by transferring a loopful of culture to 10 mL of nutrient agar in petri dish.

The medium seeded with test organism were punctured with sterile cork borer to make wells. Methanol extract and chloroform extract at 60, 70, 80,100%; insoluble and soluble ethyl acetate fraction at 30, 40, 50% were transferred to each well under aseptic condition and incubated for 24 hours. The antimicrobial activity was detected as clear zone of inhibition around wells and it was measured in millimeter (mm). Each experiment was replicated three times. Amoxicillin was used as control.

**Brine Shrimp Lethality Test (BST)**

From this stock solution, 1000, 500 and 100 g/mL was transferred to 5 vials and 5 vials were kept as control having chloroform: methanol (1:1) v/v. Brine shrimp (*A. salina* larvae) eggs were hatched in a shallow rectangular plastic dish, filled with sea water. An unequal partition was made in the plastic dish with the help of a perforated device. Eggs were sprinkled into larger compartment, which was placed under the dark condition while the smaller compartment was opened to ordinary light. After two days naupils were collected. A sample of the test extract and fraction were prepared and transferred to vials. Some vials were kept as control having solvent only. The solvent was allowed to evaporate overnight. When shrimp Larvae were ready, 1 mL of sea water was added to each vial along with 10 shrimps and the volume was

adjusted with sea water to 5 mL per vial and give yeast 1 pipettes. After 24 hours the number of surviving shrimps was counted. Each experiment was replicated thrice.

**Thin Layer Chromatography (TLC)**

Extract and fraction obtained then speckled on plate TLC silica gel GF<sub>254</sub> and developed with appropriate mobile phase. Detection with UV<sub>254</sub>, UV<sub>366</sub>, and cerium (IV) sulphate was conducted to monitoring bioactive compounds.

**RESULTS AND DISCUSSION**

**Extraction and activity test with BST**

The fresh lobak leaves were dried under sunlight and covered with black cloth. This was carried out to protect compounds from oxidation or enzymatic reactions such as decomposition, change on the pH stimulate hydrolysis of iridoid and glycoside flavonoid compounds (Cannell, 1998). Drying process stopped when the leaves can be easily broken. There was effort to maintain the water level in raw material to 5-10%. It was expected at that water level, most fungi can not grow. The raw material then powdered to easily assist the penetration of solvent onto cellular structure of plant, in other to help secondary metabolite dissolution and broaden extraction field (Cannell, 1998). *Bioassay guided extraction* using BST was aimed to determine the activity of chloroform and methanol extract. BST was simple, fast, reliable, inexpensive, reproducible and can be used to gain depiction of toxicity from one compound or substance (Carballo et al, 2002) by counting the total death of *A. salina* (Meyer et al., 1982). Brine shrimp bioassay results (Table 1) clearly indicated that methanol extract has more toxic effect than chloroform extract.

**Table 1.** Results of BST using chloroform extract and methanol extract of lobak leaves.

| Sample             | Concentration (µg/mL) | Replication (% death) |    |    |
|--------------------|-----------------------|-----------------------|----|----|
|                    |                       | 1                     | 2  | 3  |
| Methanol extract   | 1000                  | 80                    | 84 | 80 |
|                    | 500                   | 64                    | 70 | 64 |
|                    | 100                   | 10                    | 0  | 34 |
| Chloroform extract | 1000                  | 6                     | 28 | 14 |
|                    | 500                   | 10                    | 26 | 8  |
|                    | 100                   | 12                    | 20 | 4  |
| Control            |                       | 0                     | 8  | 6  |

According to Lee et al. (2006), the activity of a methanol extract of radish (*R. sativus*) sprouts for the induction of nicotinamide adenine dinucleotide (phosphate) NAD(P)H/quinone reductase (QR), which plays critical roles in protection against chemical carcinogens and other toxic xenobiotics, was

examined in murine Hepa1c1c7 cells. Active substance *indole-3 carbinole* in lobak can be use as anti tumor, preventing carcinogenesis against *cell line estrogen responsive*, serves as immunomodulator and increase TNF (*tumor necrosis factor*) (Irma and Gilang, 2005). The methanol extract was found to be the most active extract, and then partitioned using liquid-solid partition with ethyl acetate, and result in two fractions. The two fractions obtained were then tested with BST. TLC was conducted on every stages of partition process to monitor the content of compounds and to ensure that there is no overlapping compound between the two extracts or fractions. The result shows that insoluble ethyl acetate fraction caused a higher percent death than soluble ethyl acetate fraction (Table 2.).

**Table 2.** Results of BST from soluble ethyl acetate fraction and insoluble ethyl acetate fraction of lobak leaves.

| Concentration<br>( $\mu\text{g/mL}$ ) | Percent death (%)                    |                                     |
|---------------------------------------|--------------------------------------|-------------------------------------|
|                                       | Soluble ethyl<br>acetate<br>fraction | Insoluble ethyl<br>acetate fraction |
| 100                                   | 10                                   | 84                                  |

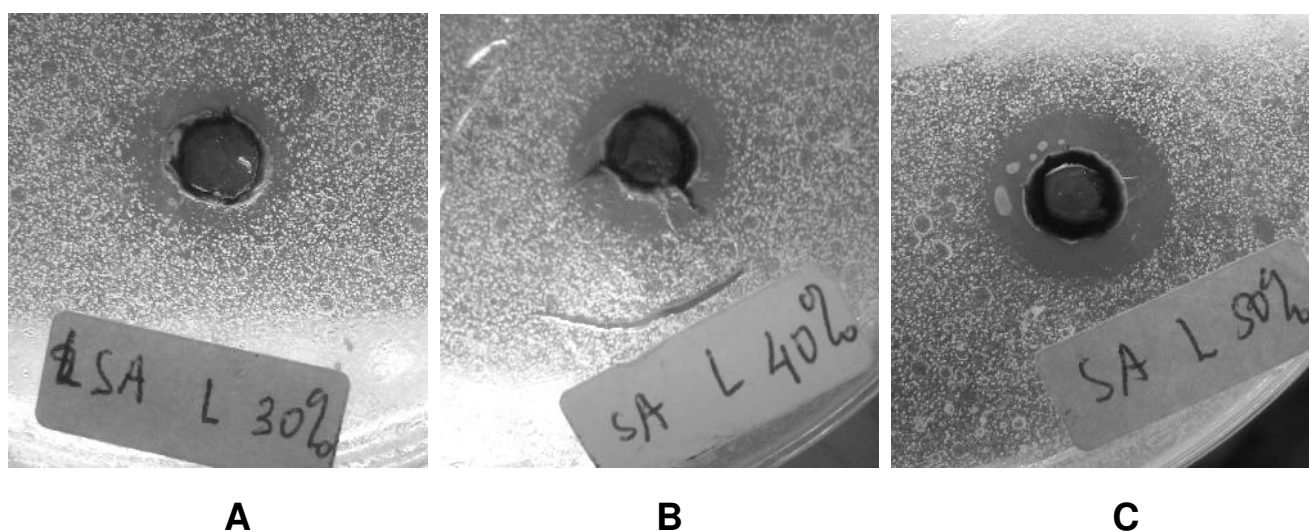
According to Meyer et al., (1982) several naturally extracted products which had  $\text{LC}_{50} < 1000 \mu\text{g/mL}$  using brine shrimp bioassay were known to contain physiologically active principles. Although toxicity test using BST does not give a clear depiction on cytotoxicity against cancer cell, however this method has been reported useful for screening anticancer test. Seventy plants collected from Central Kalimantan shown that 17 plants were potential as bioactive compound resources (Wahyuningsih et al., 2008). This result showed that the toxicity activity of the insoluble ethyl acetate fraction of lobak leaves

(84% death) was higher than that of the other fractions, so this fraction could be potential as anticancer candidate. The antioxidant properties of radish (*Raphanus sativus* L.) sprouts (Kaiware Daikon) extract, in which the glucosinolate glucoraphasatin (GRH), showing some antioxidant activity, is present at 10.5% w/w. (Barillari et al., 2006; Salah-Abbès, et al., 2007; Yee et al., 2007). Sulforaphan (SFN) from Brassicaceae family especially broccoli (*Brassica oleracea italica*), was reported could induce apoptosis in cancer cell. SFN has been identified as potent inducers of phase 2 enzymes in human (Alessio, 2008).

Duthie et al. (2000) reported that free radicals cause damage to the DNA and other molecules. Over time, such damage may become irreversible and lead to disease including cancer. Antioxidants have been used as important protective agents for human health (Poon et al., 2004). Because antioxidants can neutralize free radicals as the natural by-product of normal cell processes, daily consumption antioxidant was suggested to prevent cancer.

#### Antimicrobial bioassay

*Staphylococcus aureus* local strain was used to test antibacterial activity. Cultures for experiments were regenerated into Nutrient agar (NA) slants and incubated at  $37^{\circ}\text{C}$  for 24 hours for bacterial proliferation. Mueller Hinton (MH) was used to test antibacterial activity because it has complete nutrition. Agar-well bioassay was employed for testing antibacterial activity of lobak leaves. The medium seeded with test organism were punctured with sterile cork borer to make wells (6 mm diameter) (Ali et al., 2006). Each extracts were made to a final concentration of 60, 70, 80, and 100%. Antimicrobial activity of chloroform and methanol extract lobak leaves as shown in Table 3.



**Figure 1.** Result of zone of inhibition of soluble ethyl acetate fraction against *S. aureus* at (A) 30 %, (B) 40%, (C) 50%.

**Table 3.** Antimicrobial activity of chloroform and methanol extract lobak leaves.

| Sample             | Zone of inhibition (mm) |      |      |      |       |
|--------------------|-------------------------|------|------|------|-------|
|                    | 0,1%                    | 60%  | 70%  | 80%  | 100%  |
| Chloroform extract | -                       | 0    | 0    | 0    | 0     |
| Methanol extract   | -                       | 7,78 | 7,29 | 9,86 | 12,68 |
| DMSO control       | -                       | -    | -    | -    | 0     |
| CMC control        | 0                       | -    | -    | -    | -     |

Methanol extract shown to be more toxic than chloroform extract. The polarity from methanol extract showed by growth inhibition against positive-gram bacteria (Hartini, 2006). Table 3 shows increase zone of inhibition by methanol extract from various concentration. Antibacterial compound in lobak leaves tend to be polar. This can be shown from Table 3 that the inhibitory activity (measured by zone of inhibition) of chloroform extract was not pronounced against *S. aureus*. Saponin, flavonoid and polyphenol in lobak leaves and sprouts are potential as antimicrobial. Methanol extract of lobak sprouts contain isothiocyanate compound which can inhibit bacterial activity in mouth (Ervina et al., 2007). Several researchers have reported that methanol extracts have potential activity against bacteria. Methanol extract of *Tectona grandis*, *Asphaltum punjabianum*, and *Valeriana wallichii* have been reported inhibit *Alternaria cajani*, *Curvularia lunata*, *Fusarium* sp., *Bipolaris* sp. and *Helminthosporium* sp. in various concentration (1000, 2000, 3000, 4000, 5000 g/mL) (Shalini and Srivastava, 2008). The methanol extract of *Piper ribesoides* root was effective on *S. aureus*. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of the methanol extract of *P. ribesoides* were 3.125 mg/mL and 6.250 mg/mL, respectively (Zakaria et al., 2007). Methanol extract of *Cassia nigricans* Vahl. is also reported potential as antimicrobial candidate (Ayo and Amupitan, 2004).

Furthermore, methanol extract was partitioned with ethyl acetate to yield 2 fractions, soluble and insoluble ethyl acetate fraction. Soluble ethyl acetate has more polar compounds. On the other hand, insoluble ethyl acetate has semi polar compounds. Each extracts were made to a final concentration of 30, 40 and 50% (Figure 1 and Table 4).

**Table 4.** Antimicrobial activity (Zone of inhibition) of soluble and non-soluble ethyl acetate lobak leaves methanol extract.

| Sample                  | Zone of inhibition (mm) |      |       |
|-------------------------|-------------------------|------|-------|
|                         | 30%                     | 40%  | 50%   |
| Insoluble ethyl acetate | 0                       | 0    | 0     |
| Soluble ethyl acetate   | 5,21                    | 7,58 | 10,64 |
| CMC control             | -                       | -    | 0     |
| Ethyl acetate control   | -                       | -    | 0     |

The current work has shown that soluble ethyl acetate is a potential source of antimicrobial agents and it is active against *S. aureus* (gram-positive bacteria). We assumed that this due to the existence of polar compounds in soluble ethyl acetate fraction. Based on the cell wall structure; gram-negative bacteria have an outer cell wall that is rich in lipopolysaccharides, preventing the polar compounds from penetrating the membrane causing cell lyses, whereas gram-positive bacteria do not have lipopolysaccharides that decorate the membrane, therefore the compound can easily destroy the protein porins and cause cell lyses (Jawetz et al., 2005; More, 2007). This finding demonstrated that the higher concentration of sample, result in larger zone of inhibition due to higher amount of bioactive compound to be applied.

Amoxicillin is a member of the penicillin antibiotic group, used as positive control, which is effective against gram-positive bacteria. Amoxicillin inhibits transpeptidase, preventing cross-linking of bacterial cell wall and leading to cell death. Amoxicillin is sufficiently lipophilic to cross through the membranes of gram positive bacteria. The presence of the polar side chain on the 6 position also confers amoxicillin suitable for entrance into gram negative bacteria through their polar porins (Siswandono and Soekardjo, 2000). This result showed that zone of inhibition amoxicillin is 26.7 mm.

This research is opened up the potential use of lobak leaves as anticancer and antimicrobial agents which to our knowledge, is the first report. Bioactive compounds at both of fractions were identified to contain polar compounds. In our study, lobak leaves are not only vegetables, but it can be exploited as used medicinal plant so it can be considered as nutraceuticals which have a nutritional role in the diet and phytochemical constituents of this plant have long term health promoting due to long term use in the daily diet.

## CONCLUSION

Insoluble ethyl acetate fraction from methanol extract was the most active (100 µg/mL, 84% death) against *A. salina*. On the other hand, the soluble ethyl acetate fraction from methanol extract exhibited strong inhibitory activity against *S. aureus* (10.64 mm).

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