

ANTIBACTERIAL, ANTIOXIDANT AND TOPOISOMERASE-I INHIBITOR ACTIVITIES OF THE COASTAL ETHNOMEDICINAL PLANT *PEMPHIS ACIDULA*

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ABSTRACT

Pemphis acidula stem bark had been used traditionally in Indonesia to treat Recurrent Aphthous Stomatitis. This research was conducted to examine the biological activities of its extract as antibacterial, antioxidant and topoisomerase-I inhibitor. The latter mentioned is one of the target molecules for anticancer drug. The antibacterial activity was examined using disc diffusion assay against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Streptococcus mutans*. The antioxidant activity was measured using superoxide radical scavenging activity assay. Topoisomerase-I inhibitor activity was determined using the method reported by TopoGEN. The chloroform extract did not show antibacterial activity and it has low antioxidant activity (48.5%). The ethyl acetate and methanol extracts positively inhibited the growth of target bacteria at concentration of 0.3-mg/paper disk. It also showed high antioxidant activity. At concentration of 250 µg/ml both extracts were able to scavenge superoxide free radicals which was 94.8% and 83.4% for ethyl acetate and methanol extract, respectively. The methanol extract also inhibited topoisomerase-I activity at concentration of 7.5 µg/ml. The results suggest that there is a correlation between antibacterial, antioxidant and topoisomerase-I inhibitor activity. The stem bark extracts contain biologically active compounds that could be potential for nutraceutical or pharmaceutical development.

Key words: *Pemphis acidula*, ethnomedicinal plant, antibacterial, antioxidant, topoisomerase-I inhibitor

INTRODUCTION

Pemphis acidula is an ethno-medicinal coastal plant of Southeast Asia and Australia. Aborigin people use *Pemphis acidula* to treat toothache. In Indonesia, especially in Pari Island, the stem bark of this plant is used to treat Recurrent Aphthous Stomatitis. The root is used for cosmetics; the leaves and flowers are used for other medicinal purposes (Bourdy *et al.* 1996). Based on the traditional usage, the possible biological activity contained in this plant includes antimicrobial, pain relief, antiviral, anti-inflammation and antioxidant.

Previous studies reported that methanol extract of *Pemphis acidula* leaf contained antioxidant compound known as galloyl flavonol glycosides (Masuda *et al.* 2001). Oku *et al.* (2003) reported that lipid composition of *Pemphis acidula* leaf consisted of

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sterol, triacyl glycerols, *wax ester* and sterol ester. Sterol ester is the most constituent of its lipid comprising 17.6-33.7% of total lipids. Sterol ester consisted of tri-terpenoid alcohol that increases by increasing the salinity.

Previous studies focused on the leaf of *P. acidula*, none of those studies described the bioactive compounds of stem bark or other parts of the plant. Antimicrobial and antioxidant are considered as important bioactive compounds that relate to the traditional usage. Antimicrobial activity may involve in the treatment of toothache and Recurrent Aphthous Stomatitis, while antioxidant act as a major defence against radical-mediated toxicity that is an important factor in the etiology and pathophysiology of human diseases such as inflammation and viral infections (Jose and Janardhanan 2000; Bafna and Balaraman 2004; Kumar and Shanmugasundaram 2004). Free radicals have been known causing cancer, diabetes and cardiovascular diseases. It was reported that antioxidant could protect from those diseases (Anderson *et al.* 2004; Stanner *et al.* 2004).

Topoisomerase-I is an essential nuclear enzyme for DNA replication. It consists of Topoisomerase-I which generates single-stranded breaks and topoisomerase-II that introduces double-stranded breaks. Topoisomerase-I inhibitors stabilize the transient enzyme-DNA complexes, resulting in an inhibition of transcription and replication that ultimately leads to DNA damage and cell death. Due to the fast growth of cancer cells, topoisomerase is found in abundant concentration in cancer cells compared to normal cells. According to the report of The Association of the British Pharmaceutical Industry, in 2005 there were eight anticancer drugs having mechanisms as topoisomerase inhibitors. Thus topoisomerase is one of the important targets in anticancer.

Topoisomerase-I is also found in pathogenic microorganisms such as *E. coli* (Lima *et al.* 1994) and *Candida albicans* (Jiang *et al.* 1997), so topoisomerase-inhibitor is also an important target for antimicrobial agent. Due to those reasons, it has been assumed that there is a correlation between antibacterial, antioxidant and topoisomerase-inhibitor activities as reported in this paper. Specifically, it focused on the examination of *Pemphis acidula* stem bark extracts concerning their biological activity as antibacterial, antioxidant and topoisomerase-I inhibitor.

MATERIALS AND METHODS

Plant material and preparation of extracts

Stem bark of *Pemphis acidula* was collected from Pari Island. It was sun dried for 24 hours and powdered. The sun-dried and finely ground sample of 25 g was extracted overnight sequentially in 100 ml using chloroform, ethyl acetate and methanol. The filtrates were concentrated and evaporated to dryness under vacuum condition. The extracts were kept in the dark at $\pm 4^\circ\text{C}$ until tested.

Antibacterial Assay

The extracts were tested for their antibacterial activities using well-established disc diffusion method against target bacteria included *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* and *Streptococcus mutants*. The inocula were grown in liquid media containing peptone (10 g/l), yeast extract (5 g/l) and NaCl (15 g/l). Mueller-Hinton plates were incubated at 37 °C after inoculation with organisms (20 µl aliquot having OD_{600 nm} of 0.7) and placed in discs (6 mm) containing extract equivalent to 0.30 mg extracts. The extracts were dissolved in the extracting solvents at a concentration of 15 mg/ml and 20 µl of each aliquot was dispensed on a 6 mm sterile paper disc. Chloramphenicol was used as a positive control at concentration of 60 µg/ml. Antibacterial activity was measured as the inhibition zone of bacterial growth (mm) produced by the plant extract after 24 hours incubation

Antioxidant Assay

The activity of antioxidant was measured using superoxide radical scavenging activity (Sun *et al.* 1988; Chung *et al.* 2004). It measures the ability of compounds to scavenge superoxide radicals using colorimetric enzymatic techniques. The superoxide radical was generated by xanthine/xanthine oxidase (XO) and measured by the nitroblue tetrazolium (NBT) reduction method. A test sample (250 µg/ml) was mixed in a 100 mM phosphate buffer solution (pH 7.5) containing XO (1.65×10^{-2} units/ml), NBT (133 µM), xanthine (164 µM) at 37 °C for 20 minutes. The reaction was stopped by adding 500 µl SDS (69 mM). The presence of superoxide radical was measured spectrophotometrically at 560 nm. The superoxide scavenging activity was expressed as the percentage inhibition compared to the blank (without extract) and calculated according to the following formula:

Topoisomerase-I inhibitor Assay

$$\text{superoxide scavenging activity (\%)} = \frac{\text{absorbance}_{\text{control}} - \text{absorbance}_{\text{sample}}}{\text{absorbance}_{\text{control}}} \times 100\%$$

Topoisomerase-I and all chemicals required for its inhibitor assay were provided such as Topo I Drug Screening Kit purchased from TopoGEN, USA. It includes topoisomerase-I, supercoiled DNA, relax DNA, TGS buffer, camptothecin. Electrophoresis grade agarose, Sodium Dodecyl Sulfate (SDS), proteinase K and other chemicals were purchased from Sigma. Topoisomerase-I inhibitor assay was conducted as described by TopoGEN. The method allows the detection of the compound that either stabilizes the complex DNA/enzyme or otherwise inhibits catalytic activity of topoisomerase-I. The positive control of inhibitor is camptothecin, which stabilizes the cleaved intermediate complex. The standard topoisomerase-I inhibitor assay mixture was 20 µl. It contained 10x TGS (*assay/cleavage*) buffer (100 µl/ml), Supercoiled DNA (7.5 µg/ml), topoisomerase-I 200 unit/ml. Extracts were applied at concentration of 0.25 µg/ml, 7.5 µg/ml, 37.5 µg/ml and 75 µg/ml, while camptothecin was applied at 34.84

µg/ml. The solvent used to dissolve extract or camptothecin is 10% dimethylsulfoxide (DMSO). Reaction was carried out at 37°C for 30 minutes and then terminated by adding SDS 1% (100 µl/ml). Furthermore, the mixture was added with proteinase K (50 µg/ml) and incubated for 30 minutes at 37°C. The samples were electrophoresed in a horizontal 1% agarose gel in Tris-acetate/EDTA buffer. The gel was stained with ethidium bromide and destained in water and photographed under UV illumination.

The activity of topoisomerase-I is determined by the formation of relax DNA. The topoisomerase-I inhibitor has two mechanisms that are catalytic and poison. The first is observed if the substrate supercoiled DNA remains unchanged after reaction, while the second is indicated by the formation of complex DNA/Enzyme containing open circular DNA.

RESULTS AND DISCUSSION

Antibacterial activity

The extraction of stem bark of *Pemphis acidula* using chloroform, ethyl acetate and methanol resulted in the yield of 0.31%, 0.61%, 20.09%, and with yellow, orange-red and red colour, respectively. The results indicated that the bark of *Pemphis acidula* contains significant amount of polar extract.

Antibacterial assay indicated that the chloroform extract did not inhibit the growth of target bacteria, while the other extracts have a wide spectrum of antibacterial activity which inhibited weakly the growth of *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. The ethyl acetate and methanol extracts applied in each disc (6 mm) were 0.3 mg, resulting in the inhibition zone diameter of 3.0 mm.

The stem bark of *Pemphis acidula* is traditionally used in Indonesia to treat toothache and Recurrent Aphthous Stomatitis by chewing the bark. To investigate the rational use of *Pemphis acidula* further study was carried out on methanol extract. Antibacterial activity was re-tested by increasing the extract concentration. Another bacterium, *Streptococcus mutans* was added as target bacteria as it is known as bacterium causing plaque formation (Pai *et al.* 2004). The results indicated that the extract inhibited the growth of Gram positive and Gram-negative bacteria. It inhibited the growth of *S. mutans* at higher concentration compared to other bacteria. The higher the extract concentration increased the zone inhibition as presented in Table 1. The results suggest that the stem bark extract contains antimicrobial compound which supports the local application to treat toothache and Recurrent Aphthous Stomatitis. The use of bark extract as antiplaque preparation should be considered.

Table 1. The growth inhibition of *Pemphis acidula* methanol extract against bacteria

Extract (mg/paper disc)	Inhibition Zone (mm)/Target bacteria			
	<i>S. aureus</i>	<i>P. aeruginosa</i>	<i>E. coli</i>	<i>S. mutants</i>
0.3	4.0	4.0	4.0	-
0.6	4.5	4.5	4.5	-
1.2	5.0	5.0	5.0	4.0
2.4	6.0	6.0	6.0	7.3
4.8	6.8	7.1	6.6	7.8
Chloramphenicol (15 µg)	10.0	10.0	10.0	10.0

Antioxidant activity

The chloroform, ethyl acetate and methanol extracts at concentration of 250 µg/ml were able to scavenge superoxide radicals of 48.50 %, 94.78 % and 83.34%, respectively. It is clear that ethyl acetate and methanol extracts showed high scavenging activity compared to chloroform extract. It has been recognized that many natural substances in plants have antioxidant activities. Of these substances, the phenolics which are widely distributed have the ability to scavenge free radicals (Cui *et al.* 2005). Previous studies reported that the leaf extract of *Pemphis acidula* contained galloyl flavonol glycosides as antioxidant (Masuda *et al.* 2001). At this stage, the antioxidant compound contained in the stem bark of *Pemphis acidula* has not been identified. Purification and structure elucidation of ethyl acetate and methanol extracts are necessary to prove whether the antioxidant compound of leaf extract is the same as that of stem bark extract.

Reactive oxygen species such as superoxide anion radical ($O_2^{\cdot-}$) hydroxyl radical ($OH^{\cdot}$) and hydrogen peroxide (H_2O_2) are considered to be important factors in the etiology of several pathological conditions such as inflammation, viral infection (Jose and Janardhanan 2000; Bafna and Balaraman 2004; Kumar and Shanmugasundaram 2004). Antioxidant contained in the stem bark extract of *Pemphis acidula* might act as a major defence against inflammation, infections in the treatment of toothache and Recurrent Aphthous Stomatitis.

Topoisomerase-I Inhibitor

Topoisomerase-I inhibitor is one of the target molecules for anticancer drug discovery and it is known that cancer development is closely related to free radicals damaging DNA (Anderson *et al.* 2004; Stanner *et al.* 2004). Topoisomerase-I is also found in prokaryotic microorganisms such as *E. coli* (Lima *et al.* 1994) and pathogenic fungi *Candida albicans* (Jiang *et al.* 1997) so topoisomerase-I inhibitor is also an important target for antimicrobial agent. Due to those reasons, it is interesting to investigate the correlation between topoisomerase-I inhibitor, antibacterial and antioxidant potencies of *Pemphis* extract.

The topoisomerase-I inhibitor activities of methanol extract at various concentrations are presented in Figure 1. The results showed that methanol extract inhibits

catalytic reaction of topoisomerase-I at concentration of 75 $\mu\text{g/ml}$. This activity is indicated by unchanged supercoiled DNA after reaction (lane 1). At concentration of 7.5 $\mu\text{g/ml}$ and 37.5 $\mu\text{g/ml}$, the extract showed poison activity described by the formation of complex DNA/enzyme (lane 2 and lane 4), whereas at concentration of 0.25 $\mu\text{g/ml}$, it did not inhibit topoisomerase-I activity, indicating by the formation of relax DNA as a results of reaction of topoisomerase-I and supercoiled DNA.

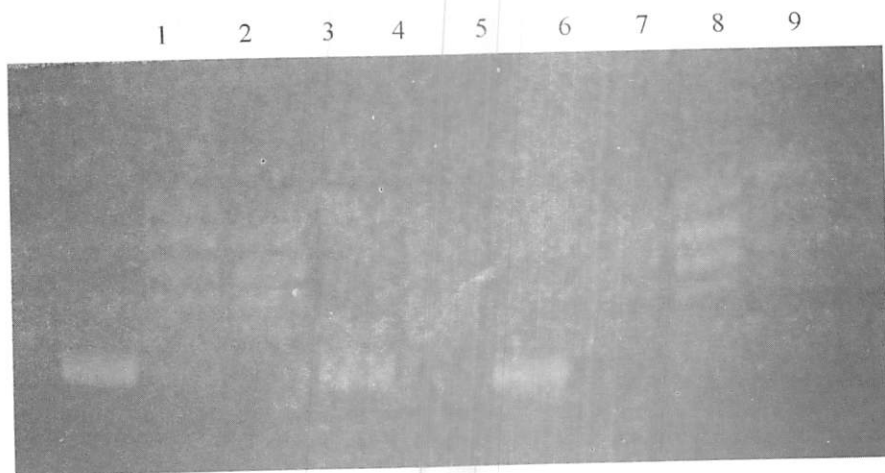


Figure 1. Catalytic inhibition of topoisomerase-I by methanol extract of stem bark *Pemphis acidula* at various concentrations. Lanes 1-3; the inhibition of topoisomerase-I in the presence of 75 $\mu\text{g/ml}$, 7.5 $\mu\text{g/ml}$, 0.25 $\mu\text{g/ml}$ methanol extracts. Lane 4: the inhibition of topoisomerase-I in the presence of 37.5- $\mu\text{g/ml}$ -methanol extract. Lane 5: supercoil DNA added with topoisomerase-I. Lane 6: supercoil DNA added with DMSO 10%. Lane 7: supercoil DNA, added simultaneously with solvent (DMSO 10%), and topoisomerase-I enzyme. Lane 8: relax DNA marker. Lane 9: supercoil DNA added simultaneously with camptothecin at concentration of 34.84 $\mu\text{g/ml}$ and topoisomerase-I.

The finding describes that methanol extract of *Pemphis* stem bark inhibits topoisomerase-I activity by interacting directly with the enzyme (catalytic) and also by forming complex compound between enzyme and DNA. The minimum inhibitory concentration of methanol extract against topoisomerase-I was 7.5 $\mu\text{g/ml}$, while camptothecin (used as standard in this investigation) describes inhibitory effect at concentration of 34.84 $\mu\text{g/ml}$. Camptothecin was first isolated from the bark of a Chinese tree, *Camptotheca acuminata*. It was developed by NCI and has been commercially available as anticancer drug manufactured by Aventis and Merck. It also has two derivatives

approved by the FDA: Camptosar® (Irinotecan hydrochloride; CPT-11) for advanced colo-rectal carcinomas and Hycamtin® (Topotecan) for ovarian cancers.

Another inhibitor was reported by Chowdhury *et al.* (2002) explaining that betulinic acid inhibited eukaryotic topoisomerase-I with IC_{50} of 0.5 μ M. Betulinic acid is a pentacyclic triterpenoid and its derivative, dihydrobetulinic acid has been identified as anti-HIV agents for their inhibitory activity against HIV-1 replication in acutely infected H9 cells. They are also reported to be melanoma specific cytotoxicity agent against cell lines MEL-1, -2, -3 and -4. These compounds have been shown to completely inhibit tumor growth in athymic mice carrying human melanoma. This information supported that topoisomerase-I inhibitor can be exploited as a strong candidate for anti-tumor drug.

Compared to the study reported previously, this paper discusses applied human topoisomerase-I as target enzyme in order to mimicked the traditional usage of *Pemphis acidula*. In addition, the methanol extract, though it is crude one, is a very potent inhibitor. The minimum inhibitory concentration of methanol crude extract against topoisomerase-I was 7.5 μ g/ml, while camptothecin demonstrates an inhibitory effect at concentration of 34.84 μ g/ml. Though, these data cannot be compared directly, it gives a general idea about the potency of *Pemphis* extract. Furthermore, it was reported that topoisomerase inhibitor is closely related to anticancer drug (Chowdhury *et al.* 2002), so it is necessary to confirm the methanol extract activity against cancer cell line. Further study is still in progress.

The methanol extract of *Pemphis acidula* could be easily dissolved in water resulting to acid solution. It is assumed that the extract contains acid or phenolic compound which needs further confirmation. Purification and structure elucidation of the compound is in progress. This investigation concluded that stem bark extract of *Pemphis acidula* contained topoisomerase-I inhibitor that could be potential for anticancer development which is beyond the traditional usage. This possible application is supported by the evidence that it contained the antioxidant compound that has been reported to have significant role to scavenge free radicals causing cancer (Anderson *et al.* 2004; Stanner *et al.* 2004).

CONCLUSIONS

This study provides preliminary data on biological activities of stem bark. *Pemphis acidula* extract. The investigation concluded that the methanol extract contains antimicrobial, antioxidant and topoisomerase-I inhibitor. It shows a good correlation with the traditional medical uses of this plant reported in Indonesia especially in Pari Island. The plant is potential to be developed as nutraceutical or pharmaceutical preparations and requires further investigation.

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