

Antioxidant Anti-Cancer and Antimicrobial Activities of Ethanol *Pandanus amaryllifolius* Roxb. leaf extract (In Vitro) - A potential medical application

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Abstract

Pandanus amaryllifolius (Roxb.) is a Southeast Asia tropical plant used in culinary and traditional medicine for century. It is a source of phytochemical constituents. The aim of the study was to evaluate the antimicrobial activity, antioxidant, and cytotoxic effect of the ethanol *Pandanus amaryllifolius* (Roxb.) leaf extracts for therapeutic potential in oral applications. To determine the amount of total phenolic contents and to evaluate the antioxidant activity of 70% ethanol leaf crude extracts of *Pandanus amaryllifolius* Roxb. the Folin-Ciocalteu method and the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay were used. The ascorbic acid (vitamin C) and butylated hydroxytoluene (BHT) were used as standard positive controls. A broth serial micro dilution method was used to determine the minimum inhibitory concentration (MIC) against oral pathogens i.e. *Porphyromonas gingivalis*, *Streptococcus mutans*, *Streptococcus sanguinis* and *Streptococcus salivarius* compared to the standard antibiotics and 0.2% (w/v) chlorhexidine gluconate which were positive controls. The 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide reduction assay (MTT assay) was used for cytotoxicity effect tested. Results showed that the extracts contained 57.25 ± 0.02 mgGAE/g of total phenolic contents. The antioxidant activities of all tested substrates were calculated and exhibited as (IC₅₀) the half maximum inhibitory concentration; is the concentration of antioxidant which reduces the free radical DPPH about 50%. The value of IC₅₀ of ascorbic acid, the leaf extracts and BHT were 7.79 ± 1.25 , 93.16 ± 10.46 and 110.57 ± 36.42 µg/mL, respectively. *Pandanus amaryllifolius* (Roxb.) leaf extracts had antimicrobial activities (MICs ranging from 32 to 125 µg/mL) µg/mL against tested oral bacteria. The extract exhibited the cytotoxicity effect on head and neck squamous cell carcinoma cell line (HNSCC cell line: HN31) with 50% lethal concentration (LC₅₀) of 75.19 ± 3.48 µg/mL. Collective data from this study suggested the therapeutic potentials of the *Pandanus amaryllifolius* (Roxb.) for the future research and development health applications.

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Introduction

Recently the emergence of degenerative disorders including head and neck cancers are risen and most of them are known that associated with free radicals¹⁻⁴. These free radicals are reactive oxygen species (ROS) including superoxide ion, hydroxyl ion hydrogen peroxide

which are produced by cellular metabolisms⁵. Elevated intracellular levels of reactive oxygen species causing lipids, proteins and DNA damages associated with several degenerative pathogenesis and developments⁶⁻⁷. These disease for examples are cancers⁸⁻⁹, neurodegenerative disorders and inflammatory conditions including periodontitis⁹⁻¹⁰.

Plants are rich sources of many natural constituents such as phenolic, flavonoids, tannins and proanthocyanidin, these components are protective and curative several diseases in folk medicines¹¹. Therefore, much attentions are giving rise to research and study the natural antioxidative biological properties and pharmacological

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activities of plants in order to develop a safe efficient and cheap anti-oxidative agent in prevention and therapy¹²⁻¹⁷.

Pandanus amaryllifolius (Roxb.) is a tropical plant of Southeast Asia countries including Thailand, Malaysia and Indonesia¹⁸. The plants are used as a food flavoring agent in many traditional recipes¹⁹. Its leaves and other parts are also used in medicine such as antidiabetic, anti-diuretics and cardioprotective agents and folk medicines²⁰. Its components are composed of a variety of alkaloids and unglycosylated pandamin protein which have antiviral activity against a number of human viruses²¹. Some secondary metabolites such as quercetin, carotenoids, tocopherols, tocotrienols, and essential oils are also contained in their leaves²². The ethanol extract of the leaves cultivated in Malaysia demonstrated a good scavenging activity¹⁸. A few studies of pharmaceutical activity and none of works on dental applications¹⁷. Thus, the objective of this study was to determine the total phenolic content and the antioxidant, anti-cancer and antimicrobial activity of ethanol extract of leaves of *Pandanus amaryllifolius* (Roxb.).

Methodology

Reagents and Chemicals

All chemicals were of analytical grade and were purchased from Sigma- Aldrich, Singapore. All chemicals and reagents were used without any further purification.

Plant Material and Extraction

Fresh and healthy leaves of *Pandanus amaryllifolius* (Roxb.) were collected in Ta Po, Muang Phitsanulok, Thailand during October to November 2017. The identity of plant was confirmed at the Faculty of Pharmaceutical Sciences, Ubon Ratchathani University, Thailand. Collected leaves were dried at room temperature in a well-ventilated room and ground to fine powder in a mixer grinder. The powder obtained was weighed and extracted in ethanol/water solvents (70:30) for 72 hours. The extract was filtered through a Whatmann no. 1 filter paper thrice and filtrate was evaporated to dryness at 40° C under reduced pressure by using a rotary evaporator obtain the crude extract. The extracts were kept at 4° C until further use.

Determination of total phenolic content

The total phenolic content of the extract was determined by the Folin–Ciocalteu method. Briefly, 200 µL of crude extract (1 mg/mL) were made up to 3 mL with distilled water, mixed thoroughly with 0.5 mL of Folin–Ciocalteu reagent for 3 min, followed by the addition of 2 mL of 20% (w/v) sodium carbonate. The mixture was allowed to stand for a further 60 min in the dark, and absorbance was measured at 765 nm. The total phenolic content was calculated from the calibration curve, and the results were expressed as mg of Gallic acid equivalent per g dry weight.

Determination of Antioxidant Activity

DPPH free radical scavenging assay

The ability of the *Pandanus amaryllifolius* (Roxb.) extract to scavenge the DPPH free radical was determined by using the stable 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) (Re et al., 1999). An aliquot of 50 µL (of varying concentrations) was placed in 96-well microplate, and 200 µL of 0.1 mM DPPH dissolved in methanol was added and allowed to react at room temperature in the dark. The reduction of DPPH concentration was recorded by a decrease in absorbance at 515 nm till the absorbance stabilized (30 min). Ascorbic acid was used as positive control, methanol as negative control and extract without DPPH as blank. IC50 which represents the amount of antioxidant necessary to produce a 50% reduction of the DPPH was calculated with the calibration curve by linear regression. Results were expressed as a percentage reduction of DPPH absorption compared to control.

Analysis of antimicrobial activities

Test organisms

Bacterial strains of *Porphyromonas gingivalis* (ATCC33277), *Streptococcus mutans* (ATCC 35668), *Streptococcus sanguinis* and *Streptococcus salivarius* (ATCC 9222) were used in this present study. All microorganisms were grown in Brain Heart Infusion (BHI) Broth, Tryptic Soy Broth (TSB), Blood Agar and Mitis Salivarius Agar (MSA) at 37°C in the atmosphere containing 5% CO₂ was used as the culture medium to support the growth. This culture was

transferred into the tubes containing 2 mL of the selective medium for each strain to get culture suspension. The selected test bacterial strains were adjusted for 0.5 McFarland turbidity standards (10^8 colony forming units/mL) to determine the MIC of tested substrates that against them.

Determination of Minimum Inhibitory Concentration (MIC) of Pandanus amaryllifolius (Roxb.) extract

To determine the minimum inhibitory activity of the extracts, the broth micro dilution technique was used. The bacterial suspension was adjusted to a final concentration of 1.0×10^4 CFU/mL ($OD_{600} \sim 0.4-0.5$). The *Pandanus amaryllifolius* extract was added at different concentrations in 96-well microtiter plate containing a bacterial culture as test. Various concentration of the extract was used individually as specific controls. Chlorhexidine 0.12%, and Metronidazole along with bacterial culture were used as positive controls. Solvent DMSO was used as negative control containing a bacterial culture. The MIC value of the extract was determined as the lowest concentration that completely inhibited bacterial growth after 24 and/or 48 hours of incubation at 37°C.

Cytotoxicity Activity

Cell Lines and Culture Medium

The Human Neck and Neck Squamous Cell Carcinoma (HNSCC) cell line; HN31 was kindly provided by Associate Professor Dr. Prasit Pavasat (Faculty of Dentistry, Chulalongkorn University, and Bangkok, Thailand). HN31 is a metastatic lymph node squamous cell carcinoma of the pharynx. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with L-glutamine (Gibco, UK) supplemented with 10% fetal bovine serum (FBS), (Gibco, UK), 100 IU/mL penicillin, and 100 mg/mL streptomycin. The cells were cultured at 37°C in a humidified atmosphere with 5% CO₂. Cells were detached from the culture flask by treatment with 0.02% EDTA or 0.25% trypsin for 10 min. at 37°C and then subcultured when 70–80% confluence was reached, approximately every 2-3 days.

MTT assay

The cytotoxicity of the extracts was tested against HNSCC cell line HN31 by the MTT reduction assay. HN31 cell monolayer was trypsinized and seeded on 96-well microtiter plates with a cell density of approximately 10×10^4 cells per 100 μ L of media in each well. The plates were incubated at 37°C for 24 h in 5% CO₂ atmosphere. After incubation, the cells were treated with eight different concentrations (1, 10, 25, 50, 75, 100, 125, and 150 μ g/mL) of crude leaf extracts of *Pandanus amaryllifolius*. Cells were incubated with 0.5% of methanol used as blank and untreated cells as a control was included for each sample. Each sample was performed in triplicate and cells were incubated for 72 h. After incubation, the culture medium was removed from each well by aspiration and 20 μ L of MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide, Sigma Chemical Co., USA, 5 mg/mL in PBS) was added to each well. After 4 h of incubation, DMSO was added to dissolve the purple formazan of MTT. The absorbance was measured by a microplate reader at a wavelength of 570 nm. The cell viability (%) was calculated using the formula.

$$\text{Cell viability (\%)} = (\text{OD sample} - \text{OD blank}) / (\text{OD control} - \text{OD blank}) \times 100\%$$

Where OD sample is the absorbance of the samples, OD blank is the absorbance of the blank (with the respective concentration).

Statistical Analysis

The results were expressed in mean \pm SD of the values obtained in triplicates from three independent experiments and analyzed by one way analysis of variance (ANOVA). All statistical calculations were performed by using SPSS software version 17.0.

Results

Total Phenolic content assay

The study revealed that 1 mg of ethanol extract of *Pandanus amaryllifolius* (Roxb.) contains 57.02 ± 0.02 mg/gm GAE (Gallic acid equivalent). The standard graph of Gallic acid was shown in Figure 1.

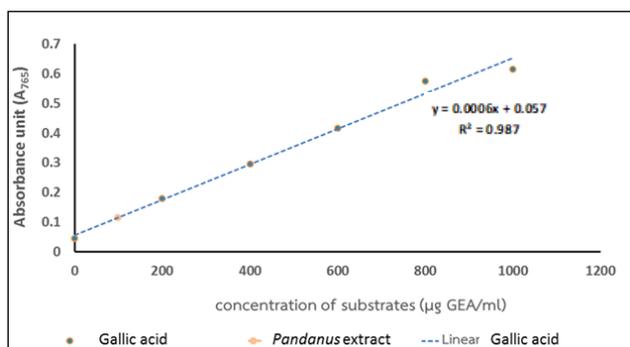


Figure 1. Total phenolic content of Gallic acid extract at different concentrations.

A linear calibration curve of gallic acid with r^2 value of 0.987 was obtained Figure 3 shows mean TPC of the plants' leave extracts measured using the GAE equation of $y = 0.0006x + 0.057$ ($R^2 = 0.987$), whereby $y =$ absorbance at 765nm and $x =$ concentration of gallic acid

DPPH Free Radical Scavenging Activity

DPPH, a relatively stable organic radical has been widely used in the determination of antioxidant activity of single compounds, as well as of different plant extracts. The IC₅₀ results of the DPPH scavenging activity of *Pandanus amaryllifolius* (Roxb.) are presented in Table 1.

Extract	Total phenolic content(GAE) mg/g	IC ₅₀ µg/mL
<i>Pandanus amaryllifolius</i> ethanol extract	57.25 ± 0.02	110.57 ± 36.42
BHT	-	93.16 ± 10.46
Ascorbic acid (Vit.C)	-	7.79 ± 1.25

Table 1. The DPPH IC₅₀ of *Pandanus amaryllifolius* (Roxb.) leaf extract. Table 1 Values are mean ± standard deviation; n = 3; *P<0.05, IC₅₀: half maximal inhibitory concentration for a 50 % inhibition ± SD; Standard deviation. DPPH: diphenyl-picryl hydrazyl; GAE: gallic acid equivalent.

The scavenging ability of all the extracts was compared with the standards ascorbic acid and BHT in Figure 2. *Pandanus amaryllifolius* (Roxb.) ethanol leaf extract exhibited significant DPPH radical scavenging activity with IC₅₀ values 110.57 ± 36.42 µg/mL compared to

vitamin C (IC₅₀ 7.79 ± 1.25 µg/mL) and BHT (IC₅₀ 93.16 ± 10.46 µg/mL). When the extracts were compared with the standards ascorbic acid and BHT, the ethanol leaf extracts showed significant lower ability of scavenging than those of ascorbic acid at 10 and 100 µg/mL, however the ability was not significantly different from the BHT at these concentrations (P>0.05) as shown in Figure 2.

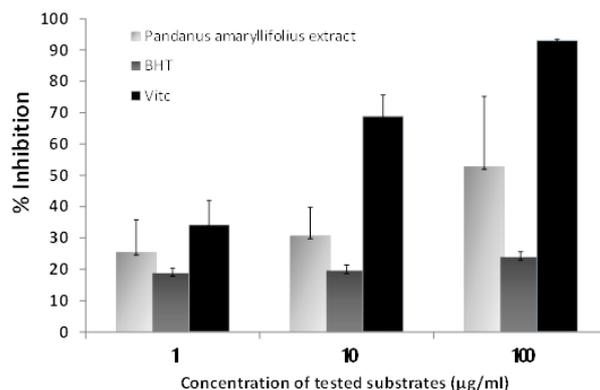


Figure 2. DPPH radical scavenging activity of ascorbic acid (Vitamin C), BHT and ethanol extract of the leaves of *Pandanus amaryllifolius*. Values are the average of triplicate experiments and represented as mean ± standard deviation.

Antibacterial activity of the extract

The MIC values of the extracts that against oral bacteria were summarized in Table 2. The extract of *Pandanus amaryllifolius* (Roxb.) exhibited the highest antibacterial activity against all the tested bacteria with MIC ranging from 3.9 to 125 µg/mL respectively. Although the extract has antibacterial activities against both cariogenic and periodontitis pathogens but the MIC values were higher than those positive controls (chlorhexidine gluconate and metronidazole).

Cytotoxicity effect of the extract

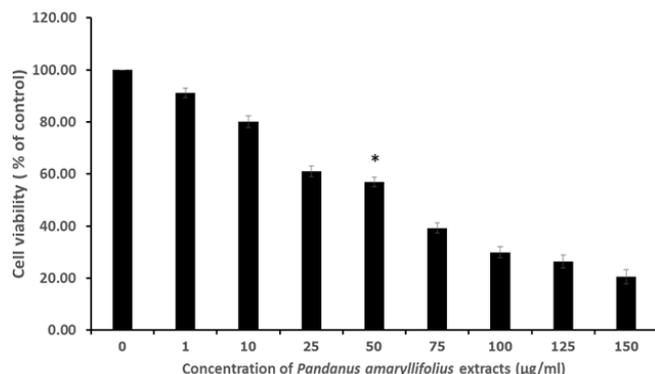
The cytotoxicity of *Pandanus amaryllifolius* (Roxb.) extract on HNSCC cell line (HN31) was evaluated by MTT assay based on percentage of cell viability. The cytotoxic effect ethanol leaf extract against HN31 was observed, showing cell proliferation inhibition in a concentration-dependent manner. The IC₅₀ for the crude extract was approximately 75.19 ± 3.48 µg/mL (Figure 3).

Microorganisms	MIC ₅₀ (µg/mL)* of tested substrates		
	leaf extract	Mz.	Chx.
<i>Streptococcus sanguinis</i>	31.25	-	0.12
<i>Streptococcus salivarius</i>	62.5	-	0.39
<i>Streptococcus mutans</i>	3.9	-	0.09
<i>Porphyromonas gingivalis</i>	125	0.01	0.64

Table 2. Antimicrobial activity (MIC) of *Pandanus amaryllifolius* (Roxb.) leaf extract compared with commercial antibiotic metronidazole and antiseptic chlorhexidine (0.12% w/v).

* Broth dilution method, mean value n = 3, P < 0.05.

Mz = Metronidazole, Chx. = Chlorhexidine gluconate.



Figures 3. HNSCC cell line (HN31) cell viability (%) in response to various concentrations of *Pandanus amaryllifolius* extracts after 24 hour-exposure determined by the MTT assay. The dose dependent manner of proliferative inhibition was observed. The represent data were the average of three independent experiments performed triplicately. * indicated significant differences versus the control (p < 0.05).

Discussion

Pandanus amaryllifolius (Roxb.) biological properties were assessed in the present study. The DPPH radical scavenging activities of the *Pandanus amaryllifolius* (Roxb.) leaf ethanol extracts and the total amount of phenolic contents were evaluated. Total phenolic content (TPC) is considered an important indicator of the antioxidant potential of *Pandanus amaryllifolius*

(Roxb.) extracts. The correlation between total phenolic content, and DPPH scavenging activities found here suggests that the phenolic compounds of the plant extracts contributed more than 50% to its antioxidant activities. Similar results of positive correlations between phenolic content and antioxidant activities of several *Pandanus amaryllifolius* (Roxb.) extracts have been documented in previous reports²³⁻²⁴.

The present study used the standard gallic acid equivalent to find out the total phenol contents (TPC) of *Pandanus amaryllifolius* (Roxb.) leaf extract. Gallic acid is used as the representor of phenolic compound in the reaction and calculation the TPC in the natural extracts from plants²⁵⁻²⁷. A previous study investigated the TPC ethanol extracts of leaves and of roots parts of *Pandanus amaryllifolius* (Roxb.), results from that study showed the higher TPC from the leaves part than those of the root part²⁴. Our results showed the slight greater TPC of leaf extract than that study, however we had not studied the root parts of *Pandanus amaryllifolius* (Roxb.). Another experiment used ethanol to extract the leaves of *Pandanus amaryllifolius* (Roxb.) and demonstrated the highest level of TPC and the free radical scavenging activities compared to water or methanol extracts²⁸. The stable DPPH radical model is a widely used, relatively quick and precise method for the evaluation of free radical scavenging activity. The reduction capacity of DPPH radical is determined by the decrease in its absorbance at 517 nm induced by antioxidants. The decrease in absorbance of DPPH radical caused by antioxidants because of the reaction between antioxidant molecules and radical progress which results in the scavenging of the radical by hydrogen donation. It is visually noticeable as a change in color from purple to yellow. Hence DPPH is usually used as a substance to evaluate the antioxidant activity²⁹⁻³⁰.

Jiamthaisong et al. (2013) showed the IC₅₀ of Vitamin C, BHT, *Pandanus amaryllifolius* (Roxb.) leaf extract and root extract were 0.012 ± 0.001, 0.290 ± 0.007, 0.810 ± 0.009 and 2.340 ± 0.040 mg/mL respectively²⁴ while Yan & Asmah (2010) reported methanol *Pandanus amaryllifolius* (Roxb.) leaf extract at 40 µg/mL equal to 65.09 ± 0.74 % inhibition²⁸. In another study inhibition of free radical ability was 93.97 ± 0.30% at 500 µg/mL of BHT and the *Pandanus amaryllifolius* (Roxb.) leaf extract were

48.60±0.13 and 39.04 ± 0.27 at 1 mg/mL in 80% ethanol and 99% ethanol respectively²⁴. Many variables such as extraction period, method of extraction, temperature, and solvents were explained the differences³¹.

In this present study the total phenolic was evaluated in order to find out main phytochemistry constituents in the extracts of *Pandanus amaryllifolius* (Roxb.) leaves. Phenol is bioactive compound that responsible for several biological activities and pharmacological properties of plants³¹. Our results showed that the extracts and the two-standard free radical scavengers (ascorbic acid and BHT) are able to reduce the reactivity. The ability was a dose dependent manner of all three tested materials. Jiamthaisong et al. (2013) performed DPPH method to assay the free radical scavenging activities of leaves and root parts of *Pandanus amaryllifolius* (Roxb.) leaf extract by 95% methanol and polyethylene glycol (PEG)²⁴. The authors reported higher activity among the other solvents and ethyl acetate displayed lower activity compared to ethanol, water and acetone.

The antibacterial activity of *Pandanus amaryllifolius* (Roxb.) leaf extract was evaluated against a number of oral bacteria (*S.sanguinis*, *S.mutans*, *S.salivarius* and *P.gingivalis*). *Pandanus amaryllifolius* (Roxb.) leaf extract showed varying degrees of antibacterial activity against most of oral bacteria tested after 24-48 hours exposure period at different concentrations. The reason for the difference in sensitivity among the bacteria might be ascribed to the differences in morphological constitutions between these microorganisms³². The antibacterial activity of ethanol extract of *Pandanus amaryllifolius* (Roxb.) leaf extract was apparently related to its phenolic and flavonoid components, respectively³³. These results support the fact that *Pandanus amaryllifolius* (Roxb.) leaf extract, investigated in this study displayed antibacterial activity.

The results obtained from this study showed that *Pandanus amaryllifolius* (Roxb.) leaves had a moderate potent anticancer activity against the HNSCC which was supported by a number of studies showing cytotoxicity effect against cancer cell lines i.e MDA-MB-231 cell via apoptosis¹⁸ and MCF-7 cancer cell line with 78.3%, 70.5% and 67.4% inhibition rate, respectively²⁶. However, the insight mechanism required more studies.

Conclusion

Results from this study revealed that *Pandanus amaryllifolius* (Roxb.) leaf extract contains a substantial phenolic content, which was suggested to be the major contributor to their antioxidant and antimicrobial activities against oral bacteria including its cytotoxicity to head and neck carcinoma cell lines. Future research work will be focused on anti-inflammatory and the cytotoxicity to human gingival fibroblasts. The effectiveness of extracts of the plant studied, should be further elucidated through additional toxicity and phytochemical analyses to discover effective pharmacological agents.

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