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Contributed Paper

Anti-oxidative Effects and Toxicity of *Bauhinia penicilliloba* Ethanolic Leaf Extracts

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ABSTRACT

This study aimed to investigate the anti-oxidative effect and toxicity of *Bauhinia penicilliloba* ethanolic leaf extracts (EEBP). The EEBP was 23.45% from dried weight which yielded active purified fraction (AFBP) at 1.84%. The AFBP was phytochemical screened and found to be flavonoid which identified as rutin, quercetin and kaempferol by using HPLC. The scavenging activity of EEBP and AFBP was examined by DPPH (IC_{50}=12.91±0.11 and 5.55±0.23 µg/mL) and ABTS assays (TEAC=0.60±0.02 and 4.93±0.15 mM). The reducing power of EEBP and AFBP which were conducted by FRAP assay, was 0.61±0.09 and 8.68±0.15 mM/mg. The EEBP and AFBP also inhibited TBARS formation in brain tissue (IC_{50}=13.58±3.25 and 6.07±0.74 µg/mL). The phenolic contents of EEBP and AFBP were 77.71±1.43 and 4,162.59±81.90 µg/mg of gallic acid equivalents. LD_{50} values for acute toxicity of a single intra-gastric (i.g.) route administered to adult male and female mice were >6.00 g/kg BW, whereas, LD_{50} values of intra-peritoneal (i.p.) route were >4.00 g/kg BW and 2.77 g/kg BW. Sub-chronic toxicity was conducted by given orally of the EEBP for 8 weeks. The body weight, organ weight, AST, ALT, ALP, Cr and BUN serum levels were not altered. Our study suggests that *B. penicilliloba* may have a considerable potential for further development as a new anti-oxidative agent.

Keywords: *Bauhinia penicilliloba*, anti-oxidative activity, acute toxicity, sub-chronic toxicity

1. INTRODUCTION

Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) have been reported to play an important role in the initiation and/or progression of various diseases such as atherosclerosis, inflammatory injury, cancer and cardiovascular disease [1]. For managing with these conditions, the researches have investigated to a new agent and focused on the natural sources which have a high potency and less toxic for using as an alternative approach. A number of recent studies have demonstrated the potential of plant products which were used as an anti-oxidative agent for treating and protecting from various diseases that were induced by free radicals [2]. Among the various medicinal

and culinary plants, some endemic species are of particular interested because they can be used for producing raw materials or preparations that contain phytochemicals with have significant anti-oxidative capacities and health benefits [3]. Additionally, it has been determined that the anti-oxidative effect of plant products is mainly attributed to phenolic compounds such as flavonoids, phenolic acids, tannins and phenolic diterpenes [4].

Sieo Daeng (*Bauhinia penicilliloba* Pierre ex Gagnep) is a tendrillar shrub plant of Fabaceae family (Figure 1) which is found in Thailand, Cambodia, Western Laos and Vietnam [5]. It has been used in Northeastern Thai folk medicine for treating of various diseases, and some conditions including mouth sores, fungal infection of the tongue, tonic and increasing appetite [6,7]. From the previous study, it was found that the methanolic extract of *Bauhinia purpurea*, which belongs to the same family, has shown anti-oxidative and hepatoprotective effects in mice [8]. Moreover, the aqueous extract of *B. penicilliloba* has shown the immunomodulating, antimicrobial and anti-oxidative activities [7]. The screening of biologically active compounds from indigenous plants has been an interesting approach for finding and developing a new drug. However, it is essential to evaluate the toxicity and safety of the compounds. This study aimed to evaluate both of the anti-oxidative activity and acute and sub-chronic toxicities of ethanolic leaf extracts from *B. penicilliloba* (EEBP).

(a) (b) (c)

Figure 1. The pictures of *B. penicilliloba* (a) flowers, (b) stem and leave, and (c) fruits.

2. MATERIALS AND METHODS

2.1 Chemicals

2-Thiobarbituric acid (TBA), L-(+)-ascorbic acid, and 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) were obtained from Sigma-Aldrich (Germany). Potassium persulfate and glacial acetic acid were purchased from Carlo (Italy). Trolox, Vitamin E (a-tocopherol) and TPTZ (2, 4, 6-tripyridyl-s-triazine) were supplied by Acros (Belgium). Folin-Ciocalteu reagents, tannic acid and ferric chloride hexahydrate ($FeCl_3.6H_2O$) were purchased from Fluka (Switzerland). 2, 2-Azino-bis (3-ethylbenzothialine-6-sulfonic acid) or ABTS was supplied by Wako (Japan). The commercial kits for plasma analysis were obtained from CPT diagnostics (Spain) and analytical grade solvents for extraction and chromatography were purchased from Merck (Germany).

2.2 Animals

Adult male and female albino mice (25-30 g) were obtained from the National Laboratory Animal Centre, Mahidol University, Salaya campus, Nakornprathom. They were maintained under the standard laboratory conditions: 12 h dark/12 h light cycle at 25°C and given regular rat chow (Pokphand Animal Feed Co., Ltd.; Bangkok, Thailand) and taped water *ad libitum*. All experiments were performed during daytime. Prior to each experiment, these animals were not fed for 8 hours, but they were given free access to water. All animals were carefully monitored and maintained in accordance with the ethical recommendations of the Ubon Ratchathani University-Animal Care and Use Committee (UBU-ACUC).

2.3 Plant Material

The leaves of *B. penicilliloba*, which used in this study were collected in 2007 in a forest in Ubon Ratchathani province. The species was identified by comparing with voucher specimen of this plant which is deposited in The Pharmacognosy Laboratory, Faculty of Pharmaceutical Sciences, Ubon Ratchathani University.

2.4 Preparation of *B. penicilliloba* Leaf Extracts

The leaves of *B. penicilliloba* were washed, dried and then ground into dried powder which was then kept in an airtight container until use. The dried powder was soaked in 95% ethanol and remained at 25°C for 24 hours. The extract was decanted and filtered under a vacuum and then concentrated by using rotary evaporator. The crude extract (EEBP) was kept in a sealed container at 4°C until use. Separation of EEBP was completed through the column chromatography with various ratios of solvents. Hexane: ethyl acetate: methanol (100: 0: 0 to 0: 0: 100) was used as an eluted solvent system or mobile phase. Each 250 mL of eluted extracts were collected and further elucidated in the TLC fluorescent pattern under UV light. The fractions that showed a similar pattern were combined and studied for anti-oxidative effect by DPPH assay in order to determine the most active fraction (AFBP). The EEBP and AFBP were kept in light protective bottles at 4°C. They were dissolved in 95% ethanol up to 5 mg/mL and appropriately diluted before use.

2.4.1 Phytochemical screening for major constituents

The phytochemical screening of the EEBP and AFBP were performed by using general alkaloidal reagents, Shinoda test, ferric chloride reagent [9-10] and flavonoid assays, including the Pew test and the ammonia test [10].

2.4.2 Analysis of total phenolic in extracts

The total phenolic content of EEBP and AFBP was analyzed by followed the Folin-Ciocalteu method which described by Skerget et al. [11]. In brief, the test compounds were dissolved in distilled water at various concentrations. 0.5 mL of each compound was mixed with 0.25 mL of the 1N Folin-Ciocalteu reagent. The mixtures were left alone for 5 min, then 20% Na_2CO_3 1.25 mL was added to produce of final volume 2 mL. The solution was then incubated at 25°C in complete darkness for 30 min. The mixture absorbance was spectrophotometrically measured at a wavelength of 725 nm. The total phenolic content was expressed in microgram of tannic acid equivalent (TAE) per milligram of sample.

2.4.3 High performance liquid chromatography analysis

The AFBP was analyzed by a Waters liquid chromatography system (Waters Corp., Milford, MA, USA). The instruments consisted of a Waters degasser in-line AF, a Waters 1525 binary HPLC pump, Waters 717 plus auto-sample, and a Waters 2487 UV absorbance dual λ detector. The chromatography separation was achieved using a reverse phase Nova-Pak®C-18 (3.9 × 150 mm, i.d. 4 μm) at 25°C (Waters Corp, Ireland) with a gradient mobile phase of 1% phosphoric acid solution (solvent A) and methanol (solvent B) as follows: 20% B (3 min), 30% B (5 min), 35% B (40 min), 40% B (45 min), 60% B (50 min), and 20% B (60 min). The injected volume for the ultraviolet-visible spectrophotometer (Shimadzu, Japan) was 10 μL for each assay with a flow rate at 1 mL/min and a detection wavelength between 270 to 356 nm.

2.5 Anti-oxidative Studies

2.5.1 DPPH (1, 1-diphenyl-2 picrylhydrazyl) free radical scavenging activity assay

DPPH radical scavenging activity was determined. Briefly, the 0.075 mM DPPH radical solution was mixed with various concentrations of standards including vitamin C (ascorbic acid), vitamin E (α-tocopherol), trolox (the water-soluble α-tocopherol analogue) and other test solutions to produce the final concentration at 1-100 μg/mL. The mixture was then vortexed vigorously and left for 30 min at 25°C under protection from light. The absorbance at 517 nm of ethanolic solution of DPPH radical was recorded at different concentrations of the extract. Triplicates were made for each test sample. The lower absorbance of the reaction mixture showed the presence of a higher free radical scavenging activity. The inhibition ratio (%) of the DPPH radical by the test compounds were calculated as follows: % inhibition = [(absorbance of control - absorbance of test compound)/ absorbance of control] × 100. The anti-oxidative activity was expressed as the concentration of test compounds inhibiting the formation of DPPH radicals by 50% or IC_{50}. The IC_{50}values were derived from the inhibition curves [12].

2.5.2 ABTS (2, 2'-azinobis-[3-ethylbenzthiazoline-6-sulfonic acid]) scavenging activity assay

The scavenging activity of ABTS was measured according to the method described by Lo and Cheung [13] with some modifications. The ABTS (Wako, Japan) assay was employed to measure the anti-oxidative activity of the extracts. ABTS was dissolved in deionized water to 7 mM concentration and potassium persulphate was added to a concentration of 2.45 mM. The reaction mixture remained in the dark and at 25°C overnight (12 to 16 h) before used. The resultant intensely colored ABTS radical cation was diluted with deionized water to give an absorbance value of ~0.70±0.02 at 734 nm. The test compounds were diluted 100 times with the $ABTS^{\bullet+}$ solution to a total volume of 2 mL. They were then incubated for 6 min and an absorbance was measured at 734 nm. The absorbance of 1 mg/mL extracts in methanol was prepared and calculated absorbance in the range of the standard curve. The assay was performed at least in triplicates. Controls without $ABTS^{\bullet+}$ were used to allow for any absorbance of the extracts themselves and deionized water was added to these control samples instead. Fresh stocks of $ABTS^{\bullet+}$ solution were prepared every two days due to self-degradation of the radical. The assay was first carried out on trolox which served

as the standard. The results of the assay were expressed relative to trolox in terms of TEAC (Trolox Equivalent Antioxidant Capacity).

2.5.3 Ferric reducing ability power (FRAP) assay

According to previously published methods [14], FRAP was conducted with some modifications. In this assay, three reagents were used: 1) sodium acetate and acetic acid buffer (pH 3.6); 2)10 mM solution of 2,4,6-tripyridyl-s-triazine in a 40 mM solution of hydrochloric acid; and 3) 20 mM solution of ferric chloride hexahydrate, prepared in deionized water. The FRAP reagent was prepared daily with 25 mL of reagent one and 2.5 mL of reagent two and three, which were warmed to 37°C before used. The various concentrations (50-1,000 mg/mL) of standard $FeSO_4$ or test compounds were added to the FRAP reagent. The absorbance was determined at 593 nm for 4 min through the use of a spectrophotometer. The FRAP values were determined from a five point curve using the $FeSO_4$ standard and also represent the concentrations of antioxidants which having a ferric ability equivalent to the standard $FeSO_4$ solution.

2.5.4 Lipid peroxidation assay using mouse brain homogenate

Inhibition of lipid peroxidation was assessed as described by Shimazawa et al. [15]. The supernatant fraction of mouse brain homogenate of adult male mice which weighed between 20-25 g, was prepared as described by Hara and Kogure [16]. Brain tissues were homogenized in 4 vols. of ice-cold phosphate saline buffer (50 mM, pH 7.4), within a glass-Teflon homogenizer and was stored at -80°C. The stock brain homogenate was diluted 10-folds with the same buffer. After that, 2 mL of the diluted homogenate were added to 10 µL of the test compound and incubated at 37°C for 30 min. The reaction was stopped by adding 400 µL of 35% $HClO_4$, and then centrifuging it at 2,800 r.p.m. for 10 min. The supernatant (1 mL) was heated with 0.5 mL of thiobarbituric acid (TBA) solution (5 g/L in 50% acetic acid) for 15 min at 100°C. Absorbance was then measured at 532 nm.

2.6 Toxicity Study

2.6.1 Acute toxicity

The acute toxicity of *B. penicilliloba* was investigated in adult male and female mice and showed the results as LD_{50} value. This value is defined as dose when administered in an acute toxicity test, and expected to cause death in 50% of the treated animals in a given period [17]. A single dose of EEBP was either intra-gastrically (i.g.) or intra-peritoneally (i.p.) administered or the LD_{50} value for each route of administration was calculated. Various doses of EEBP with a range between 0.50-6.00 g/kg BW and dissolved in water, were administered via i.g. or i.p. All mice were monitored closely during the first 3 hours after administration and occasionally thereafter for 14 days. They were observed for only signs of toxicity or symptom of illness or death. Tissues of various organs from the dead animals and survivors which were sacrificed at the end of 14 days period were examined for gross changes.

2.6.2 Sub-chronic toxicity

The sub-chronic toxicity of EEBP was investigated in the adult male and female mice. These animals were randomly assigned to cages and each individual mouse was marked. Mice were assigned to treatment groups of 10 males and females. The EEBP

which was dissolved in water, was given through intra-gastric route to mice with free access to food and water. They received EEBP at doses 250, 500 and 1,000 mg/kg BW once a day for 8 weeks. At the end of the experimental period, the mice were fasted overnight, weighed and then anesthetized with diethyl ether. Blood samples were collected from the inferior vena cava. The plasma was prepared for further analysis of alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN) and creatinine (Cr). In addition, major organs as liver, kidney, spleen and heart, were collected, weighed and also inspected.

2.7 Statistical Analysis

The values were expressed as mean±S.D. or as mean±S.E.M. Statistical significance was determined by one-way analysis of variance (ANOVA) and the post hoc least-significant difference (LSD) test. The significant difference was accepted with p-value less than 0.05.

3. RESULTS AND DISCUSSION

3.1 Plant Extract, Phytochemical Screening and HPLC Analysis

The yields of EEBP and AFBP are shown in Table 1. The EEBP was obtained at 23.45% from leaf dried powder weight. The column chromatography was used to separate the EEBP and obtained the AFBP. The yield of the AFBP was 1.84% from EEBP weight. The phytochemical screening of AFBP was performed and given the results as shown in Table 2. The alkaloids did not find in this extract. However, the Shinoda, Pew, and ammonia tests gave positive results which indicated the presenting of flavonoids. These flavonoids were continually identified by HPLC analysis and found to be rutin, quercetin and kaempferol. Our findings are consistent with previous studies which showed that *B. penicilliloba* is composed of three flavonoid glycosides such as kaempferin, quercitrin and myricitrin [18]. A previous study of *B. racemose* has shown its stem bark contains flavonoids, triterpenoids and steroids as major compounds [19]. Ferric chloride reagent test indicated a positive result of the phenolic compounds. The total phenolic content of EEBP and AFBP were 77.71±1.43 and 4,162.59±81.90 mg/mg of gallic acid equivalents. While, this value studied in aqueous extract from root of *B. penicilliloba* was 249.96 mg/mg of gallic acid equivalents [7].

Table 1. Physical characteristics of *B. penicilliloba* leaf extracts.

Extracts	Color	Physical property	Yield
B. penicilliloba:			
EEBP	dark green	sticky	23.45% of dried powder
AFBP	greenish black	solid	1.84% of crude extract

Table 2. Phytochemical screening of the active fraction of *B. penicilliloba* leaf extract (AFBP).

Compound groups	Results
Flavonoids	
- Shinoda test	+++
- Pew test	+++
- Reaction with NH_3	+++
Alkaloids	
- General test	-
Phenolic compounds	
- Ferric chloride	+

- = absent
+ = slightly presence
++ = moderately presence
+++ = highly presence

3.2 Anti-oxidative Activity

3.2.1 Scavenging activity

The DPPH assay was performed to obtain preliminary results which identified the scavenging power of the leaf ethanol extract (EEBP) and its partial purified fractions (AFBP). EEBP demonstrated a scavenging power with IC_{50} was 12.91±0.11 mg/mL. Prasitpuriprecha et al. [7] had studied the aqueous extracts from the root of *B. penicilliloba*, and showed the IC_{50} of DPPH assay at 11.34 mg/mL which was similar to IC_{50} of DPPH assay of EEBP. Therefore, both of the extracts exhibited the strong DPPH scavenging activity. These finding results supported that *B. penicilliloba* was the good source for finding free radical scavenging substances. After the DPPH assay, EEBP was isolated to get the most active fraction (AFBP). AFBP was also tested for DPPH scavenging activity and showed a scavenging power with IC_{50} was 5.55±0.23 mg/mL. This level was comparable to vitamin C, vitamin E and trolox (IC_{50} = 3.48±0.09, 8.57±0.18 and 4.33±0.24 mg/mL, respectively). The scavenging power of EEBP and AFBP were further investigated by using ABTS assay and reported as trolox equivalent antioxidant capacity (TEAC). The results showed that the TEAC of EEBP was 0.60±0.02 mM and AFBP was 4.93±0.1 mM, whereas the TEAC value of vitamin E was 3.64±0.20 mM. These higher values indicated the greater anti-oxidative activity of this plant part. From the results, suggested that *B. penicilliloba* has a potent of scavenging power which equal to standard antioxidants as shown in Table 3.

Table 3. Anti-oxidative activity of *B. penicilliloba* leaf extracts using DPPH, ABTS, FRAP and TBA assays.

Compounds	IC_{50} of DPPH assay (µg/mL) (mean±S.D.)	TEAC value of ABTS assay (mM trolox) (mean±S.D.)	FRAP value (mM/mg) (mean±S.D.)	IC_{50} of TBA assay (µg/mL) (mean±S.D.)
Vitamin C	3.48±0.09	5.72±0.46	NT	NT
Vitamin E	8.57±0.18	3.64±0.20	5.79±0.36	NT
Trolox	4.33±0.24	-	9.92±0.08	10.47±1.44
EEBP	12.91±0.11	0.60±0.02	0.61±0.09	13.58±3.25
AFBP	5.55±0.23	4.93±0.15	8.68±0.15	6.07±0.74

TEAC = trolox equivalent antioxidant capacity
The values were expressed as mean±S.D., n=3.
NT = not tested

3.2.2 Reducing power

Since the reducing power of a compound serves as a significant indicator of its potential anti-oxidative activity, the FRAP assay was performed on the plant. The reducing power of EEBP and AFBP were of 0.61±0.09 and 8.68±0.15 mM per mg of leaf dried weight, whereas the FRAP values of vitamin E and trolox were 5.80±0.36 and 9.92±0.08 mM per mg, respectively as shown in Table 3. However, from a previous study showed 2.16 mM of FRAP value from aqueous root extract of *B. penicilliloba* [7]. These results, may be indicated that root extract have a reducing power higher than EEBP but lower than AFBP, the partial purified fraction of EEBP.

3.2.3 Inhibition of lipid peroxidation by using thiobarbituric acid reactive substances (TBARS)

The inhibitory effect of EEBP, AFBP and trolox on TBARS production in mice brain homogenates are shown in Table 3. The results showed the inhibition of TBARS formation was dose dependent of EEBP, AFBP and trolox with IC_{50} values were 13.58±3.25, 6.07±0.74 and 10.47±1.44 mg/mL, respectively.

The results of anti-oxidative study found that *B. penicilliloba* has a potent naturally occurring antioxidant. Both of the crude extract and active fraction of *B. penicilliloba* scavenged DPPH and ABTS free radical and donated free electrons as shown in FRAP assay. Moreover, it also inhibited MDA formation during the lipid peroxidation reaction. All of reactions indicated that the anti-oxidative power of *B. penicilliloba* was comparable to those of the standard antioxidants such as vitamin E, vitamin C and trolox. Our result showed that active fraction of *B. penicilliloba* contains substantial amounts of total phenolic compounds which indicate a powerful of anti-oxidative activity. Takeoka and Dao [20] have reported that phenolic compounds such as phenolic acids and flavonoids have anti-oxidative properties. In addition, large amounts of phenolic content exhibit high level of anti-oxidative activity [21]. Therefore, it is possible that the anti-oxidative compounds of *B. penicilliloba* are one of these flavonoids. Our findings are consistent with previous studies of *B. racemose* which reported to be a strong antioxidant [19]. A methanol extract of *B. racemose* stem bark exhibited anti-oxidative and hepatoprotective effects in Wistar albino rats which were induced by paracetamol and carbon tetrachloride [22]. In fact, this anti-oxidative activity was detected in various systems including DPPH radical, superoxide anion radical, nitric oxide radical and hydroxyl radical scavenging assays [23]. A previous study also showed *B. penicilliloba* from root extracts exhibit various pharmacological properties including immunomodulatory, antibacterial [8], antifungal and antimalarial effects. *B. penicilliloba* has been used in Northeastern Thai folk medicine as a tonic, increasing appetite as well as a remedy for mount sore. The success of herbs in Thai remedies may due to its ability to prevent oxidative stress in the body. In addition, it also has immunomodulatory and antimicrobial activities.

3.3 Toxicity

3.3.1 Acute toxicity

Study of the acute toxicity, the LD_{50} value was used for evaluating of toxicity when administered in an acute toxicity test and was expected to cause death in 50% of the treated animals in a given period. The LD_{50} values for a single dose of EEBP by intra-gastric administered to adult male and female mice were >6,000.00 mg/kg BW. Therefore, *B. penicilliloba* did not induce any

lethality. When administered through the peritoneum and the LD_{50} values were lower than the i.g. route. The LD_{50} values in adult male and female mice were >4,000.00 and 2,765.52 mg/kg BW, respectively. The physical signs of toxicity such as an initial decline in motor activity, an increase in respiratory rate and followed by restlessness, seizure and death. All of the mice increased weight and have no adverse physical signs when they were observed over 14 days. Additionally, there were no gross lesions found in any of the organs upon necropsy (data not showed). These results showed that EEBP did not have acute toxicity.

3.3.2 Sub-chronic toxicity

The sub-chronic toxicity of EEBP was evaluated by feeding in both of adult male and female mice at doses of 250, 500 and 1,000 mg/kg BW once daily for 8 consecutive weeks. The body weights of mice were recorded weekly throughout the experiment. At the end of the experiment, the internal organs were harvested, recorded and reported in g/100 g BW for their weight and gross appearance. The body weight and weight increased in both of male and female treated groups were not statistically different compared with control group as shown in Table 4. Furthermore, there were no changes in animal behavior and no toxic signs such as diarrhea, salivation, tremors and coma were detected in the treated mice. There were no macroscopic changes when observed in the internal organs of the treated mice following necropsy. As shown in Table 5, the major organ weights were also not statistically different between the treated groups compared with the control group. The clinical blood chemistry was examined in both of adult male and female mice and found increased levels of ALT and AST which indicated liver cell injuries as well as an increased level of ALP which showed a hepatobiliary dysfunction. Plasma BUN and creatinine were used to assess renal function. From the results as shown in Table 6, there were no significant differences in ALT, AST, ALP, BUN and Cr levels in EEBP treated group compared with control group.

Table 4. Body weight of adult male and female mice in the sub-chronic toxicity study of *B. penicilliloba* ethanolic leaf extracts (EEBP).

	Body weight (g)			
	Week 0	Week 4	Week 8	Weight increased in 8 weeks
Male				
Control	36.30±0.50	38.79±0.71	39.98±0.61	3.68±0.54
EEBP 250 mg/kg	38.69±0.57	40.22±0.68	42.42±0.67	3.50±0.67
EEBP 500 mg/kg	37.43±0.71	38.94±0.80	40.20±0.77	3.25±0.55
EEBP1,000mg/kg	38.14±0.66	39.09±0.45	39.84±0.84	2.90±0.59
Female				
Control	26.83±0.48	29.02±0.54	30.39±0.50	3.32±0.52
EEBP 250 mg/kg	27.96±0.52	29.98±0.59	32.07±0.89	3.89±0.88
EEBP 500 mg/kg	28.03±0.55	28.84±0.64	30.16±0.58	2.13±0.37
EEBP1,000mg/kg	27.39±0.69	29.48±0.67	30.56±0.75	3.56±0.75

The values were expressed as mean±S.E.M., n=10.
There were no significant differences at $p< 0.05$ (ANOVA).

Table 5. Organ weight of adult male and female mice in the sub-chronic toxicity study of *B. penicilliloba* leaf extracts (EEBP).

Organ weight (g/100g BW)	*B .penicilliloba* leaf extracts			
	Control	250 mg/kg	500 mg/kg	1,000 mg/kg
Male				
Liver	4.66±0.13	4.89±0.18	4.55±0.15	4.32±0.11
Kidney	2.07±0.08	1.78±0.07	1.82±0.07	1.90±0.06
Heart	0.33±0.02	0.59±0.14	0.39±0.04	0.41±0.04
Spleen	0.49±0.01	0.45±0.02	0.50±0.02	0.50±0.02
Female				
Liver	4.76±0.09	4.91±0.20	4.78±0.11	4.57±0.09
Kidney	1.31±0.03	1.30±0.03	1.31±0.03	1.34±0.05
Heart	0.35±0.02	0.70±0.27	0.46±0.08	0.36±0.02
Spleen	0.46±0.01	0.43±0.01	0.44±0.01	0.44±0.01

The values were expressed as mean±S.E.M., n=10.
There were no significant differences at $p< 0.05$ (ANOVA).

Table 6. Clinical blood chemistry of adult male and female mice after administered of the *B. penicilliloba* leaf extract.

	B .penicilliloba leaf extracts			
	Control	250 mg/kg	500 mg/kg	1,000 mg/kg
Male				
ALP (IU/L)	42.84±1.89	34.97±4.83	40.80±3.54	35.36±2.22
ALT (IU/L)	12.50±1.49	24.07±6.81	20.26±4.16	15.39±3.76
AST (IU/L)	50.62±6.05	62.78±8.02	60.72±8.85	46.86±10.72
BUN (mg/dL)	18.12±0.92	17.74±1.09	16.10±1.02	15.42±1.17
Creatinine (mg/dL)	0.26±0.03	0.33±0.06	0.24±0.03	0.26±0.02
Female				
ALP (IU/L)	45.03±3.14	41.77±3.82	36.31±2.99	41.34±3.83
ALT (IU/L)	12.22±2.47	10.84±2.24	17.11±3.46	12.35±2.24
AST (IU/L)	59.34±6.70	57.85±11.52	68.79±5.87	57.25±9.53
BUN (mg/dL)	14.20±0.96	14.21±1.96	12.62±1.27	11.55±0.90
Creatinine (mg/dL)	0.36±0.03	0.34±0.05	0.30±0.06	0.32±0.04

The values were expressed as mean±S.E.M., n=10.
There were no significant differences at $p< 0.05$ (ANOVA).

This study was conducted to investigate the toxicity of *B. penicilliloba* in the acute and sub-chronic phase in both of adult male and female mice. In the process of drug development, it is essential to investigate the toxicity and safety of the compounds while they are undergoing pharmacological testing. Oral administration of *B. penicilliloba* was reported as non-toxic. The LD_{50} values of oral administration were greater than 6.00 g/kg BW in both sexes. However, i.p. administration of *B. penicilliloba* was more toxic than oral administration, especially in female mice. The reason for the toxicity of

B. penicilliloba through the i.p. route may be due to a more rapid absorption of the extract in the peritoneum than in the gastrointestinal tract. The toxicity of *B. penicilliloba* in females were higher than in males. This result may be due to females usually have the higher activities of liver enzyme especially CYP3A4, the enzyme that metabolism over 50% of therapeutic drugs than in males [24]. Sex differences in drug metabolism and elimination are mainly related to steroid hormone levels. Sex-related differences in responses to toxic chemicals have also been observed in rats and mice [25]. Animal deaths were related to fatigue of both limbs, loss of righting reflex, lack of a responsible to stimuli, gasping, respiratory paralysis, and convulsion. The cause of death could be linked to its toxicity to the neuromuscular system [26].

The result of sub-chronic toxicity test showed that *B. penicilliloba* did not affect animal growth, body and organ weight, in 8 weeks. Since previous studies demonstrated that the organ weight increased may occur in the absence of any morphological changes [27] and the alterations of body or organ weight from the control would reflect the toxicity of the substance [28], therefore *B. penicilliloba* revealed less toxicity to the mice in this study. There were no significant differences between the control and treated group of plasma ALT, AST, and ALP levels. Consequently, the plants did not affect the liver and hepatobiliary function. Similarly, levels of plasma BUN and Cr were not altered by *B. penicilliloba* treatment, indicated that this plants did not influence the renal function. However, the toxicity testing should be extended to different species and ages to establish the safety profile of the compound before continuing pharmacological and clinical investigation.

4. CONCLUSIONS

The results of this study found that *B. penicilliloba* has a potent naturally occurring antioxidant and can be a potential source of natural anti-oxidative agents. Furthermore, the toxicity study of this plant extract did not show toxicity when given either acutely or sub-chronically. Our study suggests that *B. penicilliloba* may have a considerable potential for further development as a new anti-oxidative agent.

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