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A STUDY ON BIOLOGICAL ACTIVITIES OF *BOUEA MACROPHYLLA* GRIFF LEAF EXTRACT

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SUMMARY

Bouea macrophylla in the family Anacardiaceae is a prominent fruit in Southeast Asia, especially in Vietnam with several prospects. This study focused on the bioactive components and biological effects of *B. macrophylla* ethanol leaf extract. Phytochemical analysis revealed various compounds including polyphenol, tannin, saponin, reducing agent, flavonoid, and essential oil in the extract. The DPPH assay showed the remarkable antioxidant capacity of the extract (IC_{50} = 6.4 ± 0.08 µg/ml), which is stronger than ascorbic acid (IC_{50} = 9.35 ± 0.12 µg/ml). The disc diffusion method and minimal inhibitory concentration test displayed the significant antibacterial ability of the extract against *Listeria monocytogenes*, *Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium,* and *Bacillus cereus* at the range from 10 - 15 mg/ml, but no activity against *Staphylococcus aureus*. Moreover, the MTT assay was performed the noticeable anticancer activity of the extract in HepG2 (IC_{50} = 72.33 ± 0.68 µg/ml), and MCF-7 cells (IC_{50} = 50.25 ± 1.36 µg/ml). Taken together, this study indicated the effects of *B. macrophylla* ethanol leaf extract from Vinh Long Province, Vietnam on antibacterial, antioxidant, and anticancer properties. Therefore, *B. macrophylla* possibly is used in the food industry and medicine.

Keywords: antibacterial, anticancer, antioxidant, bioactive compounds, Bouea macrophylla.

INTRODUCTION

Bouea macrophylla Griff, 1841 (*B. macrophylla*) belongs to Anacardiaceae (Kochummen, 1996) is a wet tropical plant in Southeast Asia (Indonesia, Myanmar, Malaysia, and Thailand…). In fork medicine, *B. macrophylla* plays vital roles in various disease prevention and treatment, such as sore throat and thirst (Sasitorn Chusri *et al.,* 2014), boils, tumor, fever, and heart disease (Nuning Rahmawati *et al.,* 2020). Several research have reported

flavonoids, saponins, and triterpenoids of *B. macrophylla* root (Lutfi *et al.,* 2018), which had strong antioxidant effect and potential diabetes treatment (Zainah *et al.,* 2016). The components were also applied in dietary supplements, cosmetic productions, disease prevention and treatment (Lutfi *et al.,* 2018). *B. macrophylla* seed extract effectively inhibited human squamous carcinoma FaDu HTB43, breast cancer MCF-7, and MDA-MB-231 cells as well (Arapoc *et al.,* 2016; Zainah *et al.,* 2015). *B. macrophylla* seed extract additionally suppressed chemoresistance of some human metastatic cancer cells, such as breast cancer MCF-7/IR6 (Siwaphon paksee *et al.,* 2019), leukemia K562/adr, and lung cancer GLC4/adr cells (Wipob Suttana *et al.,* 2013).

In Vietnam, *B. macrophylla* is a flavored fruit with plenty of vitamins (including vitamin C, vitamin A, carotenoids, vitamin B1, B2, B3, and minerals,...), and variety of bioactivities (such as antioxidant, antibacterial, and anticancer abilities) (To Nguyen Phuoc Mai *et al.,* 2017; Ngoc Hong Nguyen *et al.,* 2010); however, there are currently not many phytochemistry and bioactivity analyses of this plant. Accordingly, more research of *B. macrophylla* may be essential for new drug applications.

MATERIALS AND METHODS

B. macrophylla leaf extraction

B. macrophylla leaves were collected in Vinh Long Province, Vietnam during dry season (from February to April). Botanical identification was confirmed by Assoc. Prof. Tran Hop at University of Science, VNUHCM. The leaves were washed and their weight was recorded. Additionally, they were subjected to slicing, drying, and grinding into fine powder prior to extraction.

The ethanolic leaf extract of *B. macrophylla* was carried out by twice soaking the fine powder in ethanol (EtOH) with the mass/volume ratio of 1:10 at 50ºC. The mixture was shaken for about 24 - 48 h at the speed of 150 rounds/min and repeated three times. The mixture was then gone through several steps of filtration to gather the crude extract, which was stored at 4ºC for further experiments and fractional extractions.

Qualitative phytochemical analysis

Phytochemical groups were identified in the sample using standard procedures (Ciulei *et al.,* 1982; Sarla *et al.,* 2012) improved by the Department of Pharmacology, Faculty of Pharmacy, University of Medicine and Pharmacy, Ho Chi Minh City (2015).

Essential oil: 2 ml of *B. macrophylla* ethanol leaf extract was added into a ceramic bowl, evaporated to scum and added a few drops of alcohol. The presence of essential oil was detected with gentle smell.

Reducing agent: 1 ml of Benedict reagent was added into a test tube with 2 ml of *B. macrophylla* ethanol leaf extract. This mixture was heated directly over flame for 5 min until CuO red precipitation.

Alkaloid: 2 ml of Mayer reagent was added into a test tube with 2 ml of *B. macrophylla* ethanol leaf extract until the record of opaque phenomenon. 2 ml of Dragendorff reagent was dropped into 2 ml of *B. macrophylla* ethanol leaf extract for the observation of yellowish orange precipitation. Wagner experiment was performed until the reorganization of brownish red precipitation.

Saponin: A test tube with 5 ml of *B. macrophylla* ethanol leaf extract was strongly shaken and examined the formation of a stable white foam layer.

Anthraquinone glycosides: Bontrager assay was done by adding 2 ml of H_2SO_4 into a test tube with 2 ml *B. macrophylla* ethanol leaf extract, boiling the mixture for 30 min, then adding a few drops of $FeCl_3$. The new solution was filtered and cooled. 3 ml of benzene was added and the mixture was shaken evenly. The organic layer was then discarded. 10 ml of ammonia 10% was added into the mixture, then the mixture was boiled for 30 min to observe

ammonia layer. The red precipitation was used to indicate for anthraquinone.

Polyphenol: A few drops of $FeCl_3$ 5% was added into 0.5 ml of *B. macrophylla* ethanol leaf extract, then shaken evenly. Navy color of the mixture was used to identify the presence of polyphenol.

Tannin: 2 ml of NaCl 10% was added into a test tube with 2 ml of *B. macrophylla* ethanol leaf extract. Lead acetate was then dropped to observe yellow precipitation. In another experiment, gelatin was dropped into 2 ml of *B. macrophylla* ethanol leaf extract and the mixture was shaken evenly. The white precipitation was used to specify the presence of tannin.

Flavonoid: NaOH 10% was dropped with 2 ml of *B. macrophylla* ethanol leaf extract. The mixture turned yellow when NaOH was added and then lost color when HCl was added. Shinoda assay was also performed the presence of flavonoid, by adding a few drops of HCl, Mg, and 5 ml of alcohol 95% into a test tube. If the color of the mixture was orange, pink, red, and purple, then there would be flavonoid.

Steroid: Salkowski assay was done by previously adding 2 ml of chloroform then 2 ml of concentrated H_2SO_4 into 2 ml of *B. macrophylla* ethanol leaf extract. The mixture was strongly shaken and left aside until two layers separation. Red color of the bottom layer was used to indicate sterol. Meanwhile, yellow color of the bottom layer would specify triterpenoid.

Triterpenoid: Liebermann-Burchard assay was examined by adding 2 ml of acetic anhydride, and 2 ml of concentrated H_2SO_4. The steroid was proven by the presence of dark blue or green color. Meanwhile, the triterpenoid was performed by the presence of reddish brown.

Antioxidant activity

The ability of the extract in scavenging the free radical could be analyzed by DPPH assay (Yen *et al.,* 1994; Formagio *et al.,* 2014). At room temperature, the DPPH reagent (2,2-diphenyl-1-picrylhydrazyl) is a stable radical with a strong absorption band centered at about 517 nm. It has a deep violet color in solution and becomes colorless or pale yellow when neutralized by radical scavengers. It thus offers a quick and easy method to measure the radical scavenging activity.

B. macrophylla ethanol leaf extract was prepared in a concentration-dependent manner (0 - 12 µg/ml) to develop the linear equation representing the antioxidant capacity. Ascorbic acid (as positive control) was also prepared at the following concentrations (0, 1, 5, 10, 20 µg/ml). Blank sample without DPPH solution was set. All the samples were added into the prepared DPPH solution, and then kept in the dark at room temperature for 60 min. After incubation, the absorbance was measured at 517 nm using a spectrophotometer (JenWay Genova Plus). The experiments were done in triplicate.

DPPH free radical scavenging capability was determined by percent (%) based on the formula:

$$I\,\% = \frac{A_1 - (A_2 - A_3)}{A_1} \times 100\%$$

I: radical scavenging activity (%)

A_1: absorbance without the tested sample

A_2: absorbance of the extract

A_3: absorbance of the samples without DPPH solution.

IC_{50} value was defined as the concentration of the extract which is capable of scavenging 50% of free radical.

Antibacterial activity

Antibacterial screening of *B. macrophylla* ethanol leaf extract was performed by the disc diffusion method and minimal inhibitory concentration assay (Srinivasan *et al.,* 2001), as previously described.

Disc diffusion method: Bacterial strains (American Tissue Culture Collection, ATCC; Manassas, VA, USA) were inoculated in nutrient-enriched Brain Heart Infusion (BHI)

culture medium so that the turbidity of broth culture was adjusted to 0.5 MacFarland Standard (~1.5×10^8 CFU/ml). The bacterial suspension was streaked into MHA agar plate following Kirby-Bauer method (disc diffusion method). Three 6 mm diameter holes were punched on the agar plate, then added with the extract. Positive control was amoxicillin (Sigma Aldrich), and negative control was DMSO. The experiments were done in triplicate.

The antimicrobial effect of *B. macrophylla* ethanol leaf extract was evaluated by measuring the inhibition zone diameter, according to the following formula:

$$I\ (mm) = D - d$$

D: diameter of zone of inhibition

D: diameter of agar hole

Minimal Inhibitory Concentration (MIC): Bacteria were inoculated in BHI culture at 37ºC so that the turbidity of broth culture was adjusted to 0.5 MacFarland Standard (~1.5×10^8 CFU/ml). The bacterial suspension was then diluted to reach 1.5×10^6 CFU/ml. *B. macrophylla* ethanol leaf extract with different concentrations were also added to the bacterial culture. Negative controls were DMSO diluted in the bacterial media into differing concentrations. Positive controls (blank) were *B. macrophylla* ethanol leaf extract diluted in the culture media into differing concentrations. After 24 h of incubation, 20 µl resazurin 0.1% (Sigma Aldrich) was added into each well. The experiments were done in triplicate.

MIC concentration was determined at the well containing the lowest concentration of *B. macrophylla* ethanol leaf extract in which there was no visible bacterial growth (while blue color of resazurin does not change). The changing color of resazurin from blue to pink indicated that the bacteria were still alive.

Cytotoxic activity

MTT assay based on the principle that MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromid) involved in the oxidative-reductive reaction with the cell mitochondria and generated crystalized formazan. MTT was plausible to use a few different solutions to both destroy the cell membrane and dissolve the formazan crystals. Then the absorbance of these solutions was then measured at 595 nm. This method evaluated cell viability through their respiratory activities.

Cancer cell lines HepG2 (human hepatocyte carcinoma cancer, ATCC) and MCF-7 (human breast cancer, ATCC) were cultured with the density of 5×10^4 cells/ml. The cell culture medium consisted of high glucose Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco), 10% Fetal Bovine Serum (FBS, Gibco), and 1% penicillin/streptomycin. The cells were then incubated at 37ºC and 5% CO_2.

Following overnight incubation, cells are treated with various concentrations of B. macrophylla ethanol leaf extract and Doxorubicin (DOX, as positive control) in 96-well plates. After the treatment, 10 µl of MTT is added to each well and incubated for an additional 4 h. Subsequently, 100 µl DMSO is added to each well, resultant optical densities are measured at 595 nm in an ELISA microplate reader (Biotek) and graphed by Prism v5.0 software. The percent cell death (I %) is then calculated based on the formula:

$$I\ (\%) = 100 - 100*(A_{sample} - A_{blank})/(A_{DMSO} - A_{blank})$$

I (%): % cell death

A_{sample}: the OD value measuring at 595 nm of the wells which the cells were treated with the extract.

A_{blank}: the OD value measuring at 595 nm of the blank wells.

A_{DMSO}: the OD value measuring at 595 nm of the wells which the cells were treated with DMSO.

IC_{50} value was defined as the concentration of compounds which is capable of 50% of anticancer viability. The result was represented as mean ± standard deviation (SD).

RESULTS AND DISCUSSION

Phytochemical Screening

The qualitative analysis of *B. macrophylla* ethanol leaf extract was presented in Table 1, with six active components, in of which prevalently polyphenol, tannin, saponin, reducing agent, flavonoid, and essential oil. Similar to the previous findings, saponin, polyphenol, tannin, and flavonoids were also exhibited in the study of Ngoc Hong Nguyen *et al.* (2020).

Polyphenol, tannin, saponin, reducing agent, flavonoid, and essential oil that this study reported in *B. macrophylla* ethanol leaf extract could possess many beneficial bioactivities, such as antioxidant, anti-inflammatory, anticancer, antihyperglycemic, and antidiabetic activities (Hoensch *et al.,* 2015). In addition, alkaloid provided the underlying structure for the development of several antibiotics with a diverse range of action (Othman *et al.,* 2019). Phenolic compound served as reducing agents, donors of hydrogen, and chelators of metals. Flavonoid possessed scavenging or chelating antioxidant activity (Baccarin *et al.,* 2015). On the basis of the above findings, further experiments were then carried out to determine the bioactivities of *B. macrophylla* leaf extract.

Table 1. Phytochemical screening of *B. macrophylla* leaf extract.

No.	Compound	Recognizing reaction	Positive reaction	Result
1	Essential oil	Evaporation until precipitate appeared	Mild aroma	+
2	Reducing agent	Benedict's solution	Red precipitation CuO	+ +
3	Alkaloid	Mayer/ Dragendorff / Hager/ Wager solution	Opaque/ orange-yellow/ yellow/reddish brown precipitation, respectively	_
4	Saponin	Bubbling	A stable white foam layer	+ +
5	Anthraquinone glycoside	H_2SO_4, $FeCl_3$, benzene, ammonia	Red solution	_
6	Polyphenol	$FeCl_3$ solution	Navy or green	+ + +
7	Terpenoid	Chloroforms and HCl solution	Brown and two layers separation	_
8	Tannin	Lead acetate/ Gelatin	Yellow and white precipitation, respectively	++
9	Flavonoid	NaOH and HCl Mg, HCl, and 95% alcohol	The solution turned yellow when NaOH was added and lost its color when HCl was added. Orange, pink, read, and purple solution	+
10	Steroid	Chloroform and H_2SO_4	The red below layer	_
11	Triterpenoid	Acetic anhydride and H_2SO_4	Dark reddish-brown solution	_

Note: -: negative; +: positive; ++: many; +++: high amount

Antioxidant activity

The percentage of DPPH free radical scavenging calculated the antioxidant potential of *B. macrophylla* ethanol leaf extract. As shown in Figure 1, the straight linear line had positive slope, indicating the directly proportion between the concentration-dependent manner of extract and the percentage of free radical scavenging. To be more specific, *B. macrophylla* leaf extract exhibited significant free-radical scavenging activity, which was higher than that of ascorbic acid at same concentration of 12 µg/ml (91% and 62%, respectively). Hence, the *B. macrophylla* leaf extract showed strong antioxidant potential.

As shown in Table 2, the IC_{50} value of *B. macrophylla* leaf extract (6.4 µg/ml) was lower than that of ascorbic acid (9.35 µg/ml), revealing *B. macrophylla* leaf extract showed strong antioxidant property. These results were suitable to previously reports with IC_{50} = 6.04 µg/ml of *B. macrophylla* bark extract (Tarso Rudiana et al., 2018) or IC_{50} = 4.73 µg/ml of *B. macrophylla* seed extract (Zainah Adam et al., 2016). So the data obtained in this analysis determined that the ethanolic leaf extract of *B. macrophylla* was a valuable repository of bioactive compounds of critical medicinal value.

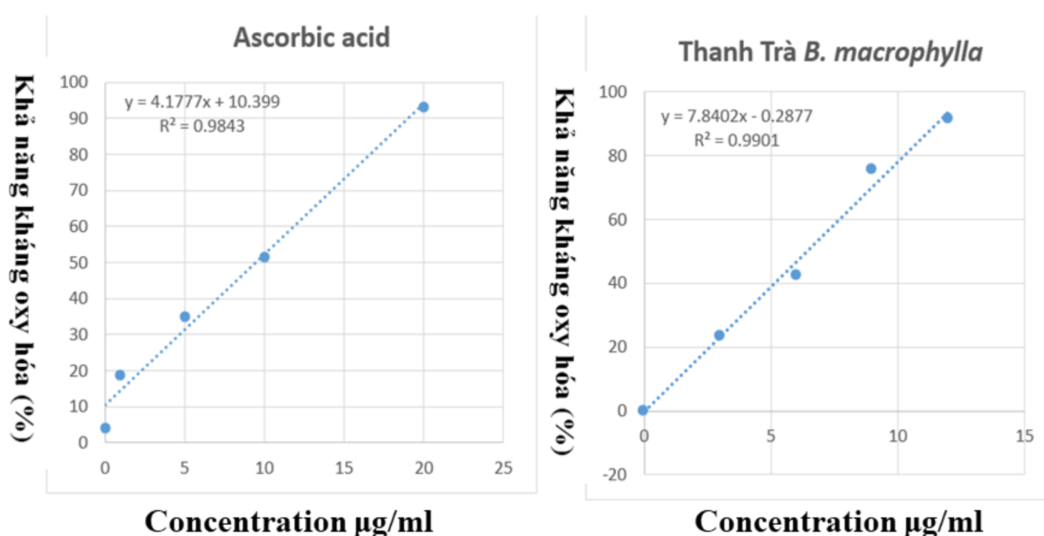

Figure 1. Antioxidant capacity of *B. macrophylla* leaf extract.

Table 2. DPPH radical scavenging activity (IC_{50} value) of *B. macrophylla* leaf extract.

Samples	IC_{50} (µg/ml)
B. macrophylla extract	6.4 ± 0.08
Ascorbic acid	9.35 ± 0.12

Antibacterial activity

Disc diffusion method

B. macrophylla ethanol leaf extract at the concentration 100 mg/ml was subjected to the antibacterial analysis (*Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium, Bacillus cereus, Staphylococcus aureus,* and *Listeria monocytogenes*), using disc diffusion method. As shown in Table 3, the bactericidal activity of *B. macrophylla* leaf extract against *E. coli* (d = 20 mm) and *B. cereus* (d = 18mm) was stronger than that of amoxicillin (d = 19 mm and 16 mm, respectively). Regarding *P. aeruginosa* and S. *typhimurium*, the zone of inhibition were both d = 20 mm, which did not

show much difference when compared with that of amoxicillin (d = 25 mm and 28 mm, respectively). However, *B. macrophylla* leaf extract's effect against *L. monocytogenes* was not strong (d = 13 mm). Especially *B. macrophylla* leaf extract did not show the antibacterial effect against *S. aureus*.

In general, the results demonstrated the diameter of zone of inhibition ranged from 13 - 20 mm regarding five bacterial strains *L. monocytogenes*, *E. coli, P. aeruginosa, S. typhimurium, B. cereus,* but did not appear on testing plates of *S. aureus*, similar to what previously reported by Ngoc Hong Nguyen et al. (2020). *B. macrophylla* leaf extract possibly displayed stronger antibacterial effect on gram negative bacteria than gram positive bacteria.

MIC-dilution method

Based on Figure 2, the antibacterial property of *B. macrophylla* ethanol leaf extract was pertinent to concentration-related manner, in which the higher the extract's concentration the stronger its antibacterial effect. This phenomenon was also shown through the changing of colors in the wells.

At the concentration 12 - 15 mg/ml, *B. macrophylla* extract sufficiently inhibited gram positive bacteria *L. monocytogenes* and *B. cereus*, as presenting blue color of resazurin which was similar to the color of positive control (blank). 10 mg/ml of *B. macrophylla* extract did notlimit *B. cereus*. 15 mg/ml of *B. macrophylla* extract was capable of quite repressing *B. cereus* with blue color of resazurin. Similarly the extract at the concentration 12 mg/ml restrained *L. monocytogenes* with blue color of resazurin. *B. macrophylla* leaf extract fully suppressed gram negative bacteria *P. aeruginosa, E. coli, S. typhimurium* at the concentration 10 - 12 mg/ml with blue color of resazurin. 5 mg/ml of *B. macrophylla* leaf extract did not inhibit *P. aeruginosa* and *E.coli* with dark pink color of resazurin. 10 mg/ml of *B. macrophylla* leaf extract completely repressed bacterial growth with blue color of resazurin. At higher concentration of 12 mg/ml, *B. macrophylla* leaf extract completely restricted *S. typhimurium*.

Table 3. Antibacterial ability of *B. macrophylla* leaf extract.

Bacterial strains	Diameter of zone of inhibition (mm)		
	***B. macrophylla* extract**	**Amoxicillin**	**DMSO**
Escherichia coli	20 ± 0.5	19 ± 0.6	-
Pseudomonas aeruginosa	20 ± 0.5	25 ± 0.5	-
Salmonella typhimurium	20 ± 0.6	28 ± 0.3	-
Bacillus cereus	18 ± 1.0	16 ± 0.1	-
Staphylococcus aureus	-	30 ± 0.5	-
Listeria monocytogenes	13 ± 0.6	33 ± 0.3	-

Note: -: negative.

Generally, the ethanolic leaf extract obtained from *B. macrophylla* revealed strong activity against most of the tested bacterial strains. Among the different microorganisms tested, *E. coli* was proved to be the most sensitive to the extract, followed by *B. cereus*, *P. aeruginosa*, *S. typhimurium*, and *L. monocytogenes*, while no zone of inhibition was observed for *S. aureus*. Previous studies revealed that various components of plant extracts, such as terpenoids, alkaloids, and phenolic compounds, could inhibit the growth of foodborne and spoilage bacteria, disrupting bacteria enzymatic activity and damaging proteins of the microbial cell membrane (Burt *et al.,* 2004). The components of B. *macrophylla* here identified which were believed to be the most important for the extract's biological activity were polyphenol,

tannin, saponin, reducing agent, flavonoid, and essential oil, as reported in Table 1. On the basis of the data given above, the ethanol extract of *B. macrophylla* leaves could be a good candidate in the search of natural antimicrobial agents against infections or diseases caused by the tested microorganisms. Further studies should be intensively explored to isolate and characterize the extract's bioactive compounds for the development of new antibacterial drugs.

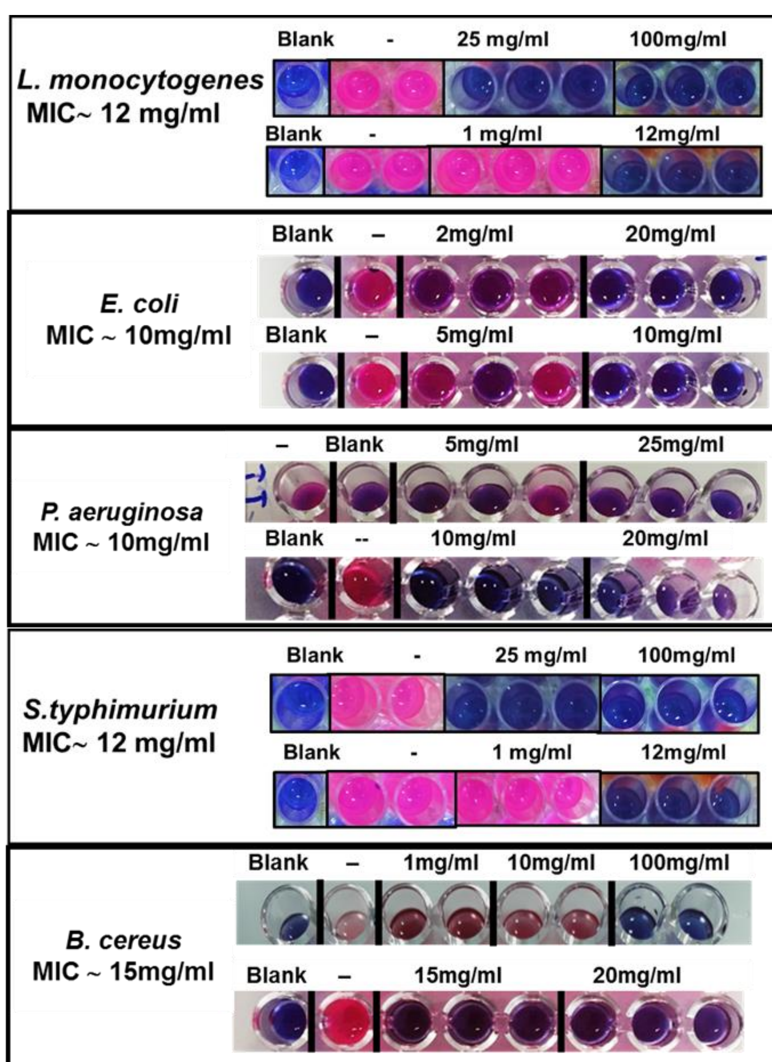

Figure 2. MIC assay of *B. macrophylla* leaf extract using various bacterial strains.

Cytotoxic activity

Plant-based medicinal therapeutics are drawing the attention of researchers to develop natural products as potential anticancer drugs (Cragg *et al.,* 2016). To evaluate the potential anticancer activity, this study first compared *B. macrophylla* ethanol leaf extract's *in vitro* cytotoxicity to that of DOX in HepG2 and MCF-7 cells. As a standard therapeutic drug, DOX was used in this experiment and showed great toxicity towards the tested cell lines.

Figure 3 showed that *B. macrophylla* extract had cytotoxicity at 100 µg/ml, corresponding to the ability of DOX at the concentration 12.5 µg/ml against

HepG2 cells. Similarly, the extract also suppressed MCF-7 cells at 100 µg/ml, corresponding to the ability of DOX at the concentration 50 µg/ml. Meanwhile, DMSO at 1.0% corresponding to the concentration used to dissolve the extract at 100 µg/ml did not show any cytotoxic activities against both HepG2 and MCF-7 cells. Thus, *B. macrophylla* extract possessed moderate cytotoxicity.

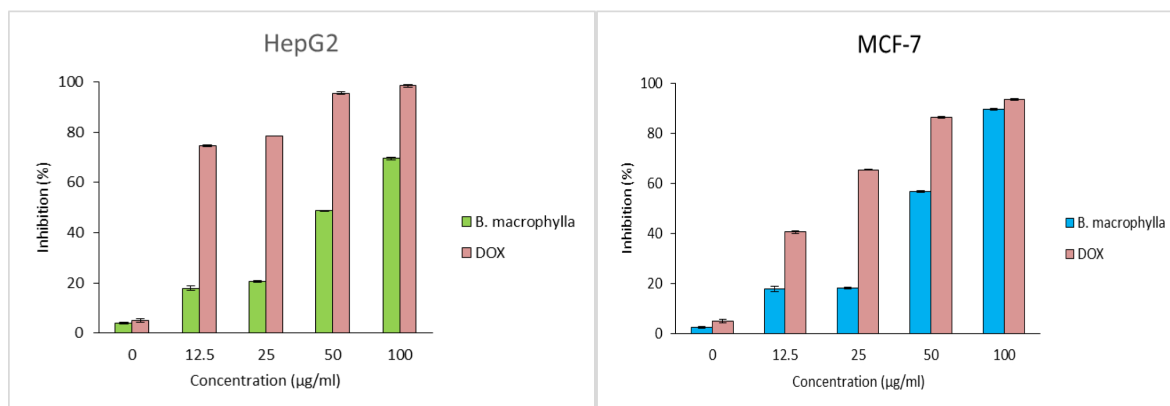

Figure 3. Cytotoxic activity of *B. macrophylla* leaf extract in human hepatocyte carcinoma cells HepG2 and human breast cancer cells MCF-7.

According to Table 4, the IC_{50} value demonstrating the cytotoxic activities of *B. macrophylla* leaf extract against HepG2 and MCF-7 cells to be rather high. *B. macrophylla* leaf extract namely exhibited cytotoxicity about 5 times weaker than DOX for HepG2 cells (IC_{50} = 72.33 ± 0.68 µg/ml) and 2.5 times for MCF-7 cells (IC_{50} = 50.25 ± 1.36 µg/ml). Although the extract did not show the cytotoxic activity to be as effective as the current DOX, this experiment was the first time showing the stronger cytotoxicity of *B. macrophylla* leaf extract against MCF-7 cells than against HepG2 cells. The findings of this study generally were consistent with previous anticancer studies of Laila *et al.* (2018) and Ngoc Hong Nguyen *et al.* (2020). The cytotoxic or antiproliferative activity of *B. macrophylla* leaf extract might be mediated by its bioactive constituents.

Furthermore, since the cytotoxic activity of the plant extract against both cell lines was demonstrated, it is necessary to carry out in the future a bioassay guided study to isolate and characterize the bioactive compounds responsible for this effect and to evaluate their mechanism of action in order to further understand the medicinal effects on this plant against cancer. In the future, the evaluation of protein signaling pathways as well as of the specific activity of the active compounds of this natural extract could be carried out to assuredly understand its potential anticancer properties.

Table 4. IC_{50} values of different cancer cell lines-cytotoxicity assays of *B. macrophylla* leaf extract.

Sample	IC_{50} (µg/ml)	
	HepG2	MCF-7
B. macrophylla extract	72.33 ± 0.68	50.25 ± 1.36
DOX	13.15 ± 0.57	20.23 ± 0.18

CONCLUSIONS

Taken together, the bioactive compounds and biological effects of *B. macrophylla* leaf from Vinh Long Province, Vietnam were evaluated in this study. The ethanolic leaf extract

obtained from *B. macrophylla* revealed a broad-spectrum activity against almost all the tested microorganisms and effectively inhibited cancer cell viability. Further investigation is necessary to discover the mechanisms mediating these antimicrobial and anticancer activities *in vitro* and identify pathways to be targeted for therapeutic applications. Moreover, the application of the extract in several fields (such asfood processing, the production of cosmetics and the pharmaceutical industry) will become a promising field in the future, contributing to economic development of the native *B. macrophylla* in Vietnam.

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