Phytochemical Screening and Evaluation of Antioxidant, Antibacterial Activities of Ethanol Extract from *Combretum quadrangulare* Collected in Vietnam

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***Abstract*—*Combretum quadrangulare* Kurz has long been considered a precious herb, which is very useful in treating various kinds of diseases on humans and aquatic animals. The study was conducted to evaluate the antioxidant and antibacterial effects of ethanol extracts from *Combretum quadrangulare* Kurz fruit collected in Kien Giang province, Vietnam. Research results showed that ethanol extract from *Combretum quadrangulare* Kurz fruit contained bioactive compounds such as alkaloids, flavonoids, steroids and triterpenoids, tannins, and phenolics. In the ethanol extract of *Combretum quadrangulare* Kurz fruit, the content of total phenolics and total flavonoids were recorded at 230.89 ± 2.36 mg GAE/g extract and 165.35 ± 5.80 mg QE/g extract, respectively. The survey results of *in vitro* antioxidant capacity through four methods of DPPH, $ABTS^{\bullet+}$, RP and TAC indicated that the extracts of *Combretum quadrangulare* Kurz fruit showed its good activity with EC_{50} values of 103.90 ± 0.40 µg/mL, 24.18 ± 0.06 µg/mL, 128.58 ± 0.20 µg/mL and 79.30 ± 1.40 µg/mL, respectively. The investigation results of *in vivo* antioxidant activity on fruit fly *Drosophila melanogaster* model showed that the fruit flies raised in the medium supplemented with 1 mg/mL extract of *Combretum quadrangulare* Kurz fruit had *in vivo* antioxidant effect under the conditions of oxidative stress induced by Paraquat and H_2O_2. In addition, the extract from *Combretum quadrangulare* Kurz fruit also had the ability to against the bacteria causing diseases in aquatic animals such as *Edwardsiella ictaluri, Aeromonas hydrophila* and *Streptococcus agalactiae*.**

Index Terms*—antibacteria, antioxidant, phenolic, flavonoid, *Drosophila melanogaster*, *Combretum quadrangulare

I. Introduction

Combretum quadrangulare Kurz (*C. quadrangulare*) is a plant species widely distributed throughout the Southern provinces of Vietnam. Most of them are cultivated, and some grow in the wild. In addition, *C. quadrangulare* is also found in Southeast Asian and African countries. It is commonly used in traditional medicine. Research on the chemical composition and biological activity of the compounds extracted from *C. quadrangulare* has reached remarkable world achievements. According to the statistics of Roy *et al.* [1], 97 compounds were extracted from *C. quadrangulare*, in which 75 compounds belong to triterpenoids, and 19 compounds are in the group of flavonoids. These mentioned compounds belonged to the group of good antioxidant activity and antibacterial, anti-HIV, hepatoprotective and cytotoxicity abilities [2]. Seeds, leaves, and stem bark of *C. quadrangulare* were believed to have antipyretic and deworming effects [3].

Other studies have also found that the extracts from *C. quadrangulare* showed many good activities such as hepatoprotective activity [4], inhibitory ability against some bacterial species that caused diseases on aquatic animals [5]. A recent study by Dao et al. has concluded that the compounds isolated from the leaves of *C. quadrangulare* were effective against the bacterial strains of *α-glucosidase* and *Staphylococcus aureus* [6]. The study was carried out to find out the chemical composition, *in vitro* and *in vivo* antioxidant capacity, and the ability to resist pathogenic bacteria in aquatic animals of the ethanol extract of *C. quadrangulare* fruit. Thereby provide more scientific data on the biological activity of *C. quadrangulare* fruit, creating a premise for

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the development of studies on screening the biologically active compounds supporting and treating diseases in humans and aquatic animals species.

II. Experimental Methodology

A. Materials

C. quadrangulare fruit collected in Kien Giang province was identified by Dr. Nguyen Thi Kim Hue, Department of Biology, College of Natural Sciences, Can Tho University according to the Vietnamese plant taxonomy system.

Investigated bacterial strains: The research used pathogenic bacteria on aquatic species such as *Aeromonas dhakensis (A. dhakensis), Aeromonas hydrophila (A. hydrophila), Edwardsiella ictaluri (E. ictaluri),* and *Streptococcus agalactiae (S. agalactiae)*. The mentioned strains were provided by the College of Aquaculture and Fisheries, Can Tho University, Vietnam.

Experimental fruit flies: Wild fruit fly *D. melanogaster* of Canton S (CS) strain was provided by Professor Kamei Kaeko, Kyoto Institute of Technology, Japan.

Chemicals: Include ABTS (2,2'-Azinobis 3-ethylbenzthiazoline-6-sulfonic acid), DPPH (2,2-Diphenyl-1-picrylhydrazyl), $K_2S_2O_8$, $K_3[Fe(CN)_6]$, $FeCl_3$, ammonium molybdate, Folin-Ciocalte, gallic acid, quercetin, paraquat, H_2O_2, ethanol, methanol, and some other chemicals.

B. Extract Preparation

Removed the damaged parts of plant samples. After washing, the samples were dried, ground into small pieces, and soaked in ethanol 99^o 5 times for 24 hours each time. The extracts from the soaks were collected and filtered through filter paper. The extract was then subjected to evaporation to obtain the sample for analysis in further experiments.

C. Qualification of Natural Compounds

The extracts of *C. quadrangulare* fruit were qualitatively screened for their chemical components basing on their chemical properties as described by Jasuja *et al.* [7] with some adjustments. The data is presented in Table I.

TABLE I. Qualitative Screening of Chemical Composition of the Ethanol Extract from *C. quadrangulare* Fruit

Chemical compound	Reagent	Identification
Alkaloids	Mayer	Orange-brown to red precipitate
Flavonoids	Concentrated H_2SO_4	Dark yellow to orange, red precipitate
Steroids and Triterpenoids	Liebermann Burchard	Change to blue, green, orange, or red of solution
Saponins	Distilled water and ethanol	Durable foaming
Tanins	Gelatin 1%	White cotton precipitate
Phenolics	$FeCl_3$ 10%	Blue-black or red-orange precipitate

D. Quantification of Total Phenolic and Flavonoid Contents

Quantification of total phenolic content: Total phenolic content was determined according to the method described by Singleton *et al.* [8] with some adjustments. The reaction mixture of 250 µL of extract, 250 µL of distilled water, and 250 µL of Folin-Ciocalteu reagent (1:4) was shaken well and incubated for 5 min at room temperature in the dark. Then, 250 µL Na_2CO_3 10% was added to the mixture and continued to incubate for 30 min at 40ºC. The estimation of spectral absorbance of the reaction mixture was carried out at 765 nm. The total phenolic content was determined based on the gallic acid standard curve equation. Results were introduced in milligrams of gallic acid equivalent per gram of extract (mg GAE/g extract).

Quantification of total flavonoid content: The analysis of total flavonoid content was performed on the basis of the method described by Bag *et al.* [9] with some adjustments. The reaction mixture preparing with 200 µL of extract, 200 µL of distilled water, and 40 µL of $NaNO_2$ 5%, was shaken and allowed to stand for 5 min. 40 µL $AlCl_3$ 10% was then added to the mixture and kept shaking well and staying still for 6 min. Then, the reaction mixture was added 400 µL of 1M NaOH and 120 µL of distilled water. The spectral absorbance of the reaction mixture was measured at 510 nm. The total flavonoid content was determined based on the quercetin standard curve equation. Results were displayed in milligrams of quercetin equivalent per gram of extract (mg QE/g extract).

E. Evaluation of in Vitro Antioxidant Activities

The investigation by DPPH method: The antioxidant activity of the extract was performed as the description in the method of Sharma and Bhat [10] with some adjustments. The reaction mixture of 100 µL DPPH and 100 µL extract was incubated in the dark for 60 min at room temperature. The measurement of the mixture absorbance spectra was implemented at 517 nm. The positive control used was gallic acid.

The investigation by $ABTS^{\bullet+}$ method: The antioxidant activity of the extract was performed according to the method introduced by Nenadis *et al.* [11] with some adjustments. The reaction mixture consisting of 990 µL $ABTS^{\bullet+}$ and 10 µL extract was incubated for 6 min at room temperature in the dark. Measured the absorbance spectra of the reaction mixture at 734 nm. The positive control used was gallic acid.

The investigation by Reducing Power (RP): The antioxidant activity of the extract was determined by the iron reduction capacity as the method of Padma *et al.* [12] with some adjustments. The reaction mixture containing 500 µL of extract, 500 µL of phosphate buffer (0.2 M, pH = 6.6), and 500 µL of $K_3Fe(CN)_6$ 1% was incubated at 50ºC for 20 min. Then, the mixture was added 500 µL CCl_3COOH 10% and carried out the centrifugation at 3000 rpm for 10 min. Gently suck out 500 µL of the

supernatant to add to 500 µL of distilled water and 100 µL of $FeCl_3$ 0.1%. Measured the absorbance spectra of the reaction mixture at 700 nm. The positive control applied was gallic acid.

The investigation by TAC method: The antioxidant activity of the extract was performed by the method of Prieto *et al.* [13] with some adjustments. A mixture of 100 µL extract and 900 µL reagent solution was incubated at 95°C for 90 min. The absorbance measurement was performed at 695 nm. The positive control was ascorbic acid.

F. In Vivo Antioxidant Activity

CS wild-type *D. melanogaster* played the role of research model in the study. The flies were reared at 25°C under 12:12 light-dark cycle conditions. The *in vivo* antioxidant activity of the extract from *C. quadrangulare* fruit was tested in the fruit fly model as described by Etuh *et al.* [14]. The experiment was carried out by selecting 48h-eclosed male fruit flies to rear in three different food conditions, including medium supplemented with the extracts (1 mg/mL of feed), positive antioxidant medium (0.05 mg/mL gallic acid of feed), and standard medium. Fruit flies were replaced with new food every 2 days of the experiment. After 20 days, the fruit flies were kept in a fasted state for 5 hours, then transferred into test vials with 20 mM paraquat paper or H_2O_2 10%. The experiment was reiterated 5 times for each treatment of 20 fruit flies. The number of survival flies was recorded after every 4 hours of observation during the experiment. The investigating indicators in the investigation included mean lifespan, 50% survival, and maximum lifespan. The maximum lifespan was calculated as the average survival time of 10% remaining in each test vial).

G. Antibacterial Assay

The antibacterial activity of the extracts was investigated by the agar plate diffusion method as described by Das *et al.* [15]. The antibacterial ability of the extract was determined based on the formation of an inhibition zone around the agar well. Inoculum with a concentration of 10^6 CFU/mL was spread evenly on the surface of Trypto-casein Soy Agar (TSA) plates with a volume of 100 µL and allowed to dry for 15 min. Then, a small well of 6 mm in diameter was formed with a sterile perforator, and 50 µL of different extract concentrations prepared in DMSO 10% was added to the wells. The control treatment used DMSO 10%. Each extract concentration was repeated 3 times. After incubation for 24 h at 32°C, the antibacterial ring diameter was determined as the circular inhibition zone forming around each well measured in mm.

Calculation of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC): The minimum inhibitory concentration is identified as the lowest concentration that inhibits bacterial growth after 24 h of culture. The minimum inhibitory concentration was determined by the method of serial dilution of concentration in the tubes. Used a 96-well culture plate, each well contained 100 µL of inoculation with a concentration of 10^6 CFU/mL and 100 µL of the extracts at different concentrations. The wells were incubated aerobically at 32°C for 24 hours. After incubation, the lowest concentration in the concentration range that inhibited bacterial growth was recognized as the minimum inhibitory concentration [16].

III. Results and Discussion

A. Qualitative Analysis Results of Natural Compounds

The chemical constituents present in plant extracts are important for their biological activity. Therefore, determining the presence of chemical compounds is the first step of biological studies. The qualitative screening results of the chemical composition of the ethanol extract from *C. quadrangulare* fruit were presented in Table II.

TABLE II. Chemical Composition Presented in the Ethanol Extract of *C. quadrangulare* Fruit

Chemical compound	Presence
Alkaloids	+
Flavonoids	+
Steroids and Triterpenoids	+
Saponins	-
Tanins	+
Phenolics	+

Note: (+) Present; (-) Absent

The results of Table II pointed out that the chemical components in *C. quadrangulare* fruit extract were determined to contain alkaloids, flavonoids, steroids and triterpenoids, tannins, and phenolics, based on the positive expression of the chemical reactions. However, the saponin group was not detected in the extracts of *C. quadrangulare* fruit. It was possible that the presence of saponin compounds was not high enough to be identified in this study.

B. Quantification of Total Polyphenol and Flavonoid Contents

The total polyphenol content in the ethanol extract from *C. quadrangulare* fruit was determined based on the linear regression equation of gallic acid in the concentration range from 2 to 30 µg/mL with $y = 0.0822x + 0.0725$ ($R^2 = 0.9961$). The total flavonoid content in the extract was determined based on the linear regression equation of quercetin in the concentration range from 20 to 100 µg/mL with $y = 0.0064x - 0.004$ ($R^2 = 0.9995$). The survey results showed that the total phenolic and flavonoid content of the ethanol extract of *C. quadrangulare* fruit were 230.89 ± 2.36 mg GAE/g extract and 165.35 ± 5.80 mg QE/g extract, respectively. Many studies have indicated that phenolic and flavonoid compounds have good biological activities. Research by Soobrattee *et al.* [17] demonstrated that phenolic compounds had redox properties; thus, they could act as antioxidants. Flavonoids found in plants had *in vitro* antioxidant activity and acted as *in vivo* antioxidants [18]. Besides, the antibacterial activity in phenolics and flavonoids was also determined [19]. All in all, it could

be reported that the extract of *C. quadrangulare* fruit contained natural compounds that have been introduced to have antioxidant and antibacterial activities.

C. In Vitro Antioxidant Activity

Plants are capable of producing secondary compounds, including phenolics and flavonoids, which are the groups of compounds having antioxidant activity due to their redox properties and chemical structure. The *in vitro* antioxidant effect of *C. quadrangulare* fruit extract was evaluated by determining the EC_{50} value of the extract in each method and comparing it with gallic acid. The smaller the EC_{50} value, the stronger the oxidation resistance. EC_{50} values of the extracts with standards are shown in Table III.

TABLE III. EC_{50} VALUES OF THE EXTRACTS AND THE STANDARD BY METHODS

Methods	Sample	Linear regression equation	EC_{50} (µg/mL)
DPPH	Gallic acid	$y = 12.101x + 0.3985$ ($R^2 = 0.9918$)	4.10 ± 0.10
	C. quadrangulare	$y = 0.4486x + 3.4115$ ($R^2 = 0.9807$)	103.90 ± 0.40
$ABTS^{\bullet+}$	Gallic acid	$y = 90.167x + 4.8166$ ($R^2 = 0.9869$)	0.501 ± 0.002
	C. quadrangulare	$y = 1.8215x + 5.9552$ ($R^2 = 0.9807$)	24.18 ± 0.06
RP	Gallic acid	$y = 0.0508x - 0.0235$ ($R^2 = 0.9903$)	10.31 ± 0.10
	C. quadrangulare	$y = 0.0037x + 0.0243$ ($R^2 = 0.9885$)	128.58 ± 0.20
TAC	Gallic acid	$y = 0.015x + 0.1206$ ($R^2 = 0.9863$)	25.34 ± 0.15
	C. quadrangulare	$y = 0.0037x + 0.0243$ ($R^2 = 0.9885$)	79.30 ± 1.40

The results of Table III indicated that the *in vitro* antioxidant capacity of *C. quadrangulare* fruit extract in the 4 methods was lower than that of gallic acid. Specifically, the DPPH free radical neutralization effect of *C. quadrangulare* fruit extract had an EC_{50} value of 103.90 µg/mL, higher than that of the standard of gallic acid (EC_{50} = 4.10 µg/mL). Therefore, the ability to scavenge 50% of DPPH free radicals of the extract was 25.34 times lower than that of gallic acid. Similarly, the $ABTS^{\bullet+}$ free radical scavenging efficiency of *C. quadrangulare* fruit extract had an EC_{50} value of 24.18 µg/mL. In RP and TAC methods, the EC_{50} value of the extract was 128.58 µg/mL and 79.30 µg/mL, respectively. The values showed that the antioxidant activity of *C. quadrangulare* fruit extract was lower than that of the standard. Research by Nopsiri *et al.* [20] demonstrated that the extracts from *C. quadrangulare* leaves with different solvents including methanol, hexane, and dichloromethane, had the ability to neutralize DPPH and $ABTS^{\bullet+}$ free radicals. Another plant species of the same genus, *Combretum tanaense*, reported its antioxidant activity by Jared *et al.* [21] through the DPPH survey method. The lowest value of EC_{50} means the highest antioxidant activity. Sample, which had an IC_{50} or EC_{50} lower than 50 µg/mL was a very strong antioxidant, 50-100 µg/mL was a strong antioxidant and 101-150 µg/mL was a medium antioxidant while a weak antioxidant with IC_{50} or EC_{50} > 150 µg/mL [22]. Accordingly, EC_{50} values in this study ranged from 24.18 to 128.58 µg/mL, showing that *C. quadrangulare* fruit extract expressed good antioxidant activity under *in vitro* conditions.

D. The Effect of in Vivo Antioxidant Activity

The antioxidant capacity of *C. quadrangulare* fruit extract was tested *in vivo* on the fruit fly model through its effectiveness against oxygen stress under PQ- and H_2O_2-induced conditions. The *in vivo* antioxidative effect of fruit flies reared in the medium supplemented with gallic acid of 0.05 mg/mL and *C. quadrangulare* fruit extract of 1 mg/mL under oxidative stress induced by 20 mM PQ and H_2O_2 10% was introduced in Fig. 1 and Fig. 2. The three values expressed the results, including mean lifespan, 50% survival, and maximum lifespan.

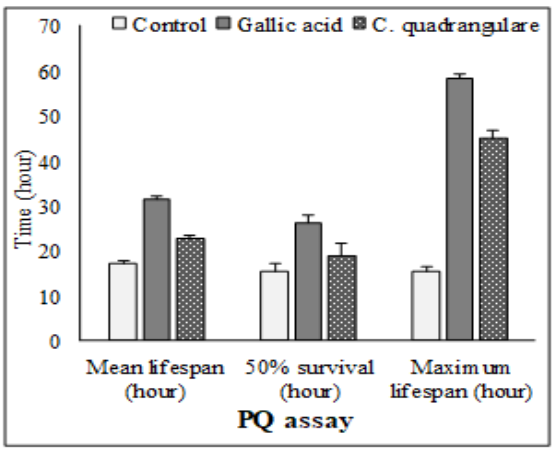

Figure 1. *In vivo* antioxidant effect of *C. quadrangulare* fruit extract under PQ condition.

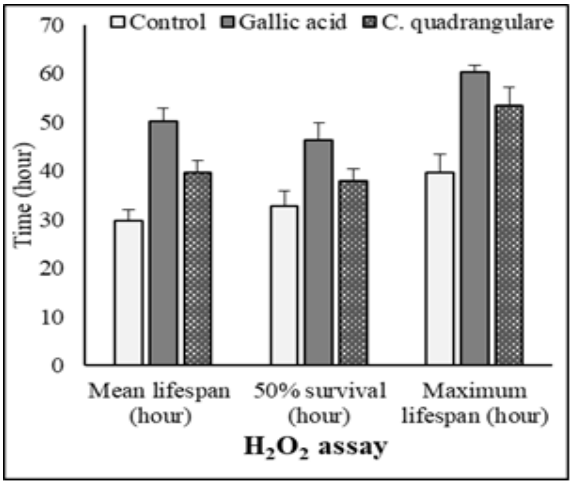

Figure 2. *In* vivo antioxidant effect of *C. quadrangulare* fruit extract under H_2O_2 condition.

The results concluded that under the conditions of oxidative stress caused by PQ 20 mM and H_2O_2 10%, the

survival ability of fruit flies reared in the medium supplemented with gallic acid and extract of *C. quadrangulare* fruit was longer than that of the fruit flies reared in standard medium. Specifically, the mean lifespan, 50% survival, and maximum lifespan of fruit flies raised in feed supplemented with extracts of *C. quadrangulare* fruit under PQ-induced oxidative stress were 22.53 h, 19.00 h, and 45.00 h, respectively, which meant higher than that of the control of 1.30 times, 1.24 times and 2.02 times, respectively (Fig. 1). Similar results were also observed in H_2O_2-induced oxidative stress, where the mean lifespan, 50% survival, and maximum lifespan were 39.67 h, 38.00 h, and 53.33 h, respectively. The data were higher than the control at 1.33, 1.16, and 1.34 times, respectively (Fig. 2). The research results once again contributed to confirming the antioxidant potential of the extract of *C. quadrangulare* fruit. This result was consistent with the qualitative, quantitative, and *in vitro* antioxidant survey results. It is known that fruit flies are the model used in many studies of *in vivo* antioxidant activity [23], [24]. Therefore, the experiment results are reliable.

E. Antibacterial Activity

The *in vitro* antibacterial activity of the ethanol extract of *C. quadrangulare* fruit is presented in Table IV. The extract had antibacterial activity against Gram-positive and Gram-negative bacteria, with MIC values ranging from 320 to 1280 mg/mL. The results in Table IV showed that *C. quadrangulare* fruit extract was resistant to three strains of pathogenic bacteria in aquatic animals, including *A. hydrophila, E. ictaluri* and *S. agalactiae*. However, *C. quadrangulare* extract was not resistant to *A. dhakensis*.

TABLE IV. ANTIBACTERIAL ACTIVITY OF ETHANOLIC EXTRACT OF *C. QUADRANGULAREIN* FRUIT

Bacteria strains	Inhibition zone (mm)						MIC (mg/mL)
	40	80	160	320	640	1280	
A. dhakensis	-	-	-	-	-	-	-
A. hydrophila	-	2.5	3.8	4.4	5.8	7.0	640 < MIC ≤ 1280
E. ictaluri	-	3.0	4.6	6.0	7.2	9.7	320 < MIC ≤ 640
S. agalactiae	-	3.1	5.2	6.3	7.6	10.3	320 < MIC ≤ 640

Note: (-) not resistant

Research results of Hang *et al.* [5] showed that the extracts of *C. quadrangulare* seeds and leaves were resistant to pathogenic bacteria on aquatic animals such as *Aeromonas hydrophyla, Edwardsiella ictaluri* and *Vibrio parahaemolyticus*. In addition, the methanol extract of *C. quadrangulare* seeds was resistant to *Staphylococcus aureus, Pseudomonas aeruginosa, Acinetobacter baumanii* and *Escherichia coli* strains with the MIC values of 1212 g/mL, 2425 g/mL, 2425 g/mL, and 9700 g/mL, respectively [25]. Phenolic and flavonoid compounds have been previously reported to have antifungal and antibacterial activities [26], [27]. Other research has also shown that flavonoids and phenolics are common secondary compounds synthesized in plants; besides that, these compounds have been studied and shown to have antibacterial effects [28]. In this study, the amount of phenolic and flavonoid identified in *C. quadrangulare* fruit extract was 230.89 ± 2.36 mg GAE/g extract and 165.35 ± 5.80 mg QE/g extract, respectively. Therefore, the antibacterial activity shown in this experiment is possibly due to the presence of phenolic and flavonoid groups in the extract.

IV. CONCLUSION

Research results showed that ethanol extract of *C. quadrangulare* fruit contained biologically active compounds such as alkaloids, flavonoids, steroids and triterpenoids, tannins, and phenolics. The investigated extract also expressed the *in vitro* and *in vivo* antioxidant activity. In addition, the activities against pathogenic bacteria strain on aquatic animals such as *A. hydrophila, E. ictaluri* and *S. agalactiae* were also recognized in the extract. The research results provided evidence that *C. quadrangulare* fruit extract was a potential herb containing many natural compounds with good biological activity. The study provided valuable scientific information for further studies in finding solutions to apply *Combretum quadrangulare* Kurz in pharmacology and disease prevention for aquatic animals.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS

Tran Thanh Men and Nguyen Trong Tuan were responsible for the study design; Tran Thanh Men was responsible for study design and manuscript writing; Huynh Kim Yen, Huynh Thi Cam Lan and Nguyen Hoang Son conducted he practical experimental work; Do Tan Khang made a statistical analysis of the experiment. All authors had approved the final version.

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