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In Vitro ACE Inhibitory Activity and Bioactive Compounds of Aqueous Extract of *Citrus amblycarpa*

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ABSTRACT

Citrus amblycarpa contain flavonoid-rich compounds and play important role in suppressing the conversion of angiotensin-converting enzyme 1 (ACE 1) to angiotensin-enzyme 2 (ACE 2). This study aimed to determine the bioactive compounds in the lime peel extract and their ability as in vitro ACE inhibitor activity. The lime peel extract was obtained by boiling the dried peel and dried leaves for 7 min at 70^{o}C. The bioactive compound of the peel and the leaves were compared. The total phenolic, quercetin, rutin, and GABA were further quantified using spectrophotometer UV vis. The aqueous extract of *C. ambylcarpa* peel showed a high concentration of phenolic, quercetin, rutin, and GABA than that of the leaves extract. Furthermore, the peel extract at low concentration (0.0001 g/mL) has high efficiency in inhibiting ACE activity up to 133%. It can be concluded that the peel of *C. amblycarpa* is a good candidate for the management of hypertension.
Keywords: *Citrus amblycarpa*; ACE inhibitory; Flavonoid; ɣ-aminobutyric acid

INTRODUCTION

Hypertension is recognized as one of the diseases of metabolic syndrome and contributes to global health problems (Balasuriya and Rupasinghe, 2011). Hypertension is a condition where the blood pressure is consistently raised (Oparil *et al.,* 2018) and is initiated by the conversion of Angiotensin-converting enzyme 1 to Angiotensin-converting enzyme 2 (Messerli *et al.,* 2018). Hypertension is one of the risk factors and may lead to the development of several chronic diseases such as strokes, heart disease, cardiovascular disease, chronic renal disease, and atherosclerosis (Saputri *et al.,* 2015). Angiotensin-converting enzymes (ACE, EC 3.4.15.1) are an important enzyme of the renin-angiotensin system (ARS) which plays a significant role in regulating cardiac blood pressure and cardiac output. Angiotensin I is a decapeptide amino acid, produced on angiotensinogen by the action of renin. Subsequently, angiotensin I is converted to angiotensin II by removing two residues of the C-terminal (Ferrarlo, 2011). Moreover, ACE is also able to degrade bradykinin, a vasodilator, into an inactive fragment through the action of kinase II. In this way, will lead to a decrease in vasodilation (Cangiano *et al.,* 2012). Thus, inhibition of ACE is crucial and considered an effective strategy in the prevention and treatment of hypertension.

Synthetic ACE inhibitors have been developed based on snake venom peptide scaffolds (Daskaya-Dikmen *et al.,* 2017). Several synthetic ACE inhibitors such as captopril, lisinopril, and enalapril have been used for clinical purposes. However, the effectiveness of those inhibitors was 40-50% when used as monotherapy. Numbers of side effects were developed in the long term of usage such as dry cough, taste disturbances, and skin rashes were reported (Gu and Wu, 2013). Considering the side effects of the inhibitors, the development of natural ACE inhibitors from biological resources has been raised. In addition, ACE inhibitors derived from plants show no side effects, are safe, and have less cost. Plants rich in flavonoid and phenolic have the potential for ACE-inhibitory activity. Flavonoid constituents such as quercetin can also act as ACE-inhibitor. ACE is important in regulating the renin-angiotensin-aldosterone system and plays a significant role in regulating blood pressure. The activity of ACE can convert angiotensin I to powerful vasoconstrictor angiotensin II in the blood vessel (Balasuriya and Rupasinghe, 2011). The study by Men *et al.,* (2013) reported that *S. physophora* plant extracted with ethanol and butanol show significant ACE inhibitory activities. Another study that water extract of *Blumea balsamifera* leaves showed ACE inhibitory activity on rabbit lung ACE. In addition, the inhibitory activity of water extract was 211.30% (See, *et al.,* 2016).

Utilization of various plants and different plant's part to prepare traditional herbal beverage have been used by Balinese people. The herbal beverage is considered more convenient as a traditional healing practice. Citrus herbal remedies

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are one of the most widely used prepared from the citrus extract and exhibit beneficial effects to cure various diseases. In addition, Flavonoid content in citrus has been reported to protect against oxidative stress (Mahmoud *et al.,* 2019). Balinese people frequently used the juice of kefir lime as a condiment and as a part of spices to make chili paste due to its distinctive taste and aroma. However, the lime peel is often discarded and cannot be utilized. Several studies have shown that the part of kefir lime such as the leaves contains vitamin E, phytosterols, fatty acids, and terpenes. In addition, the pulp contains 6-octadecenoic acid, palmitate, sinensal, alfa limonene, beta citronellal, citronellol, and sabinene (Budiarto *et al.,* 2017). Furthermore, Stevenie *et al.,* (2019) reported that the ethanolic extract of the lime peels exhibit high antiaging activity than its seeds. Flavonoid and phenolic compounds derived from citrus plants have a potential activity as ACE-inhibitory activities (Siti *et al.,* 2017). In addition, the hypotensive activity of citrus plants is linked to the flavonoid-rich compounds (Siti *et al.,* 2017). However, ACE inhibitory activities of the lime peel have not yet been determined as far as we concern. Since kefir lime is essential to use as a condiment and herbal remedy, it is of great interest to determine the ACE inhibitory activities of the lime peel.

METHODOLOGY

Materials

The reagents used were rutin, quercetin, Hip-His-Leu substrate, boric acid buffer, NaCl, Angiotensin converting-enzyme, HCl, ethyl acetate, n-hexane, DPPH, sulfate acid, methanol, GABA standard. All reagents were purchased from *Sigma Aldrich* (Singapore). All chemicals were of analytical grade.

Methods

Extraction of the plants

The fresh fruits and leaves of *C. amblycarpa* were collected from Denpasar, Bali in September 2020. The peels of fruit were washed with tap water and separated from the pulp manually. The peels and leaves were cut into 1x1 cm slices. All the samples were air-dried for two weeks in the shade. The samples were ground to a fine powder and then the powder was passed through the sieve of mesh size 40. One gram of sample was boiled in 100 mL water at 70°C. The extracts were kept at 4°C for further analysis.

Identification of phenolic and ɣ-aminobutyric acid (GABA) compound by Thin Layer Chromatography (TLC)

The phenolic was identified according to Sathishkumar *et al.,* (2013) with slight modification and GABA was identified according to Agung Yogeswara *et al.,* (2018). Silica plates 60 F_{254} were dried and activated at 90°C for 30 min. The activated plates were spotted by 20 µl of the extracts and the standards and leave it to dry. A 30 ml of mobile phase for phenolic detection consists of ethyl acetate: formic acid: toluene: water (6:1,5:3:0,5) and n-butanol, acetic acid, and water (5:3:2) for GABA detection. The plates were sprayed with 0.5% ninhydrin and the spots were visualized under UV light at 366 nm.

Quantification of Phenolic Compounds

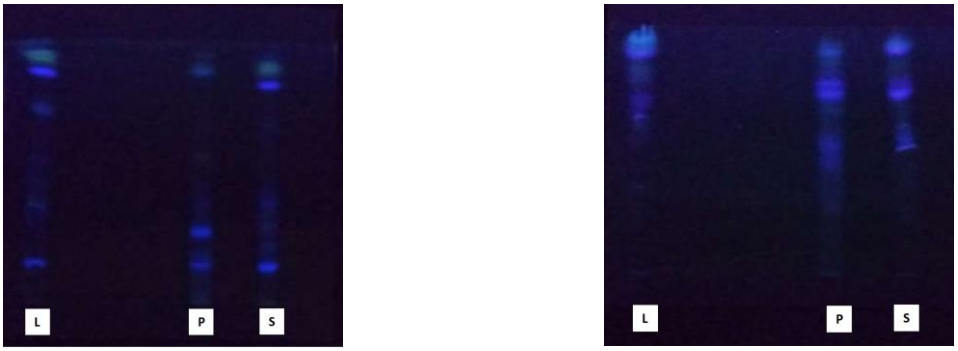
The total phenolic was measured according to Ammar, *et al.,* (2014). A 100 µl of samples were mixed with 6 µl of distilled water and 500 µl of Folin-Ciocalteu. The reaction mixtures were mixed well for 1 min. subsequently, 1.5 ml 20% sodium carbonate was added to the reaction mixtures and incubate at room temperature for 30 min. Distilled water was used as blank and various concentration of gallic acid was prepared to construct the calibration curve. The absorbance was measured at 760 nm and TPC was expressed as milligram of gallic acid equivalents (GAE)/g sample extract.

Quantification of Flavonoid Compounds

The total flavonoid of the extracts was measured according to Chang *et al.,* (2002). Briefly, 2 ml of extract were added to 2 ml 2% of $AlCl_3$ (in ethanol). The reaction mixtures were vortexed for 20 min and incubated at room temperature for 25 min. Distilled water was used as blank and the calibration curve was prepared with various concentrations of quercetin and rutin. The absorbance was measured at 415 nm. The total flavonoid was expressed as mg of quercetin /g sample (mg QE/g) and as mg of rutin/g sample (mg RE/g).

Quantification of GABA Contents

GABA concentrations in extracts were determined by pre-staining paper chromatography Yogeswara *et al.,* (2018). A 2 µl of supernatants were spotted onto silica plates and conducted using n-butanol-acetic acid-water (5:3:2) containing 1.2% ninhydrin for color development. After development, GABA spots were scratched out from the paper and were extracted



(A) (B)

Figure 1. Identification of phenolic (A) and GABA (B) in aqueous extract of *C. amblycarpa* peel using TLC. L; leaves, P; peel, S; Standard, respectively. The spots were visualized under UV light at 366 nm.

Table I. The phenolic, quercetin, rutin and GABA content of *C. amblycarpa* extracts

Contents	Peel	Leave
Total phenolic (mg GAE/g)	5.7	3
Total quercetin (mg QE/g)	22.2	9.3
Total rutin (mg RE/g)	52.5	9.5
Total GABA (mg GABAE/g)	3.8	0.05

with 5 ml of 75% alcohol (v/v):0.6% cupric sulfate (w/v) (38:2) at 40° C. The absorbance was read using a spectrophotometer at 512 nm. The GABA concentrations were calculated based on the standard curve.

Determination of ACE-inhibitory

The inhibitor ACE assay of the plant extract was measured according to Saputri *et al.,* (2015) with slight modification. The solutions of peel extract were prepare in various concentration of 0,0001 g/mL; 0,001 g/mL and 1 g/mL respectively. Briefly, 20 µL of the sample solution was added to 50 µL of 8 mM HHL as substrate and 10 µL of ACE solution (0.25 U/mL). The mixtures were mixed and incubated for 1 hour at 37^0C. The reaction mixtures were stopped by adding 62.5 µL HCl 1M. The hippuric acid formed was extracted with 375 µL of ethyl acetate. Subsequently, the solution was added to 4 mL of distilled water and the absorbance of hippuric acid was measure by using a UV-visible spectrophotometer at 228 nm. The ACE inhibitor was calculated based on inhibition percentage versus ACE activity, by using the formula:

$$\%inhibitor = \frac{(A-B)}{(A-C)} x100\%$$

A = absorbance of ACE + substrate; B = absorbance of sample + ACE + substrate; C = absorbance of substrate + sample

RESULT AND DISCUSSION

TLC was performed to identify phenolic compound and GABA in the extract of lime peel and the leaves extract (Figure 1). Phenolic and GABA were detected on TLC plates after visualization under the UV light at 366 nm. The results of phenolic and GABA were also confirmed by the TLC chromatogram which showed a similar residence time to that of the standard (data not shown). The concentration of phenolic compounds, flavonoid, and GABA was further determined using spectrophotometer UV-vis. The total phenolic, rutin, quercetin, and GABA in the aqueous extract of *C. amblycarpa* peel were higher than the leaves. In terms of flavonoid contents, rutin showed a higher concentration (52.2 mg RE/g) compared to quercetin (22.2 mg QE/g) (Table I), suggesting that rutin is the predominant flavonoid in the peel of *C. amblycarpa.*

Various concentrations of aqueous extract of *C. amblycarpa* peel were employed to determine the ACE inhibitory activity by using a UV-visible spectrophotometer at 228 nm. The solutions of peel extract were prepare in various concentration of 0,0001 g/mL; 0,001 g/mL and 1 g/mL. At the concentration of 0.0001 mg/mL of the extract showed high ACE inhibitory activity (133.33±0.00%). Whereas, 1 mg/mL of the extract has low ACE inhibitory activity (91.98±0.21%) (Table II). This result indicating that a low concentration of the extract was more efficient in

Table II. ACE inhibitory activity of peel *C. amblycarpa* extracts

Concentration (g/mL)	ACE inhibitory (%)
0.0001	133.33±0.00
0.01	115.15±5.25
1	91.98±0.21

reducing ACE activity.

This study showed that the lime peel extract has higher phenolic content than that of lime leaves extract. Generally, plants have phenolic compounds that are widely distributed in the leaves and bark of the plants. in addition, phenolic can also act as a vitamin which serves as a protection against cancer. Each plant organ has different phenolic content. A recent study by (Bakhouche *et al.,* 2021) reported that the aqueous extract of *Limonium delicatum* root has higher phenolic content than the aqueous extract of the leaves. Flavonoids are ubiquitously found in citrus plants. Citrus leaf extract has offer hypotensive effects by modulating vasoactive mediators and prevent vascular damage (Siti *et al.,* 2017). A recently, hesperidin and hesperitin have been detected in citrus waste and exhibit high ACE inhibitory activity (Ruviaro *et al.,* 2020).

Quercetin and rutin content was reported to be higher in the lime peel extract than that of the lime leaves extract. The flavonoid in plants can serve as an anti-inflammation to humans. Flavonoid is widely distributed in fruits and vegetables. Some studies reported that quercetin and rutin is a constituent of flavonoid that is ubiquitous in the *Citrus sp.* While rutin is the predominant flavonoid in the *Citrus sinensis* species. Quercetin and rutin have different concentrations in each plant organ. Bakhouche *et al.,* (2021) reported that the flavonoid in the leaves extracts were higher than the root extract. Whereas, the flavonoid in unripe seed and stem bark was reported to be higher than that of the flavonoid in the leaves and the peel of *Malus domestica* and *Ferulago angulata* respectively. (Hazrati *et al.,* (2019); Pandey *et al.,* (2020).

GABA is an inhibitor neurotransmitter that has physiological functions such as relaxation, sleeplessness, anti-depression, and antihypertensive (Diana, *et al.,* 2014). GABA is ubiquitous in fermented foods and plants. GABA has been detected in *Ziziphus jujuba Mill* fruits (0.15-3.33 mg/g DW) and water extract of the leaves of *Morus alba L* (3.8 mg/g DW) **(**Pu, *et al.,* 2019); Yang, *et al.,* (2012). The levels of GABA in plants were affected by harvest, drying, storage, and temperature. GABA in the fresh fruit of *Z. Jujube* was much higher than that of the dried fruit of *Z. Jujube.* In addition, GABA content in dried fruit of Jujube was decreasing during storage at ambient temperature (Pu *et al.,* 2019).

Inhibition of ACE prevents the conversion of angiotensin I to angiotensin II. Hence, reduce the vasoconstriction of the blood vessels which results in stabilizing blood pressure. The ACE inhibitor can improve the function of the arterial vessels and stabilized the plaque caused by atherosclerosis. In addition, ACE inhibitors also improve insulin sensitivity in humans. A study by Balasuriya and Rupasinghe, (2011) revealed that apple peel showed high ACE inhibitory activity. Furthermore, Yang, *et al* (2012) showed that *Morus alba* L. contains GABA and ACE inhibition that be used for antihypertensive. This study suggests that the extract of lime peel has the potential to be used for hypertension management.

CONCLUSION

The present work confirmed that *C. amblycarpa* contains phenolic content and GABA based on TLC detection. Aqueous extract of *C. amblycarpa* peel showed higher phenolic, quercetin, rutin, and GABA. Whereas, leaves extract showed a lower concentration of phenolic, quercetin, and GABA. Rutin was reported to be the predominant flavonoid in the peel of *C. amblycarpa.* In addition, a low concentration of aqueous extract of the peel showed high efficiency in inhibiting ACE activity. The results of this study showed that the peel of *C. amblycarpa* can be developed as a natural ACE inhibitory activity.

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