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ANGIOTENSIN-I-CONVERTING ENZYME (ACE) INHIBITORY ACTIVITY AND NITRIC ACID PRODUCTION OF PHENOLIC COMPOUND EXTRACTED FROM *Turbinaria conoides*

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ABSTRACT
Angiotensin-I-converting enzyme (ACE) plays a vital role in regulation of blood pressure by converting angiotensin I to angiotensin II, a potent vasoconstrictor, whereas nitric acid (NO) is a vasodilator. Hence inhibition of ACE and induced NO production is considered to be a positive therapeutic approach for hypertension related ailment. Marine algae phenolic compounds possess ACE inhibitory activity, therefore, this study was carried to examine the ACE inhibitory and NO production of phenolic compounds from the marine brown seaweed *Turbinaria conoides*. The algal samples were collected from Mandapam, Tamil Nadu. Different solvent (ethanol, ethyl acetate) extracts were prepared and quantified for phenolic content and examined for ACE inhibition and NO production. Ethanol extract 83% phenolic compounds and shown strongest ACE inhibitory activity with an IC_{50} value of 0.81 mg/mL followed by ethyl acetate extract (73% and 1.24 mg/mL IC_{50} value). The phenolic compound had an inducible effect on the NO production (36%) in MCF-7 cell line without having cytotoxic effect (94% viability) when up to 100 ng of the extract applied. GS-MS data revealed the presence of four major compounds which validates above biological observations. This study found that *T. conoides* could be a potential source of phenolic compounds with ACE inhibitory activity, which may be exploited in the future to prevent hypersensitive related disease.

KEY WORDS: Brown algae; Ethanol extract, Hypertension, Seaweed, Vasodilator.

1. INTRODUCTION
Angiotensin-I-converting enzyme (EC 3.4.15.1; ACE) has an important physiological function in the regulation of blood pressure and augmenting cardiovascular and renal diseases.[1] ACE plays vital role in the renin-angiotensin system, and it catalyses the angiotensin I (decapeptide) to angiotensin II (octapeptide) which is a potent vasoconstrictor. It also catalyzes the degradation of bradykinin, which is a vasodilator.[2] Further, ACE is implicated in cell oxidative stress, generation of reactive oxygen species (ROS) and peroxynitrite, and also in thrombosis.[3] Therefore, ACE inhibiting activity leads to a decrease in concentration of angiotensin II and increase the level of angiotensin I and bradykinin resulting in reduced blood pressure, other cardiovascular and renal diseases, and oxidative stress-associated diseases.[4] The synthetic ACE inhibitors (captopril, benazepril, enalapril, and lisinopril) are efficient, yet shown undesirable side-effects (coughing, taste disturbances, hyperpotassemia, and skin rashes).[5] Therefore, search of ACE inhibitors in natural products are of great interest and a wide variety of ACE inhibitors reported from various food sources, including rapeseed,[6] seaweed,[7] antler,[8] milk,[9] cheese[10], egg white,[11] canola,[12] peanut,[13] fish muscle,[14] and tuna.[15] Nonetheless, marine brown algal hydrolyzates showed the highest ACE inhibitory activity.[16]

Marine algae become principal source of active metabolites that could be used for medicine, food, feed, and widely distributed and abundant throughout the coastal areas.[17,18] Among marine algae, brown algae have been reported to contain higher phenolic compounds as phlorotannin.[19] Phlorotannins consist of polymers of phloroglucinol units and are formed in the acetate–malonate pathway in marine algae.[16]

Furthermore these phenolic compounds are highly hydrophilic components with a wide range of molecular sizes (126–650 kDa) and have the ability to precipitate proteins.[20] Several phenolic compounds purified from brown algae have medicinal benefits and have shown strong anti-oxidant[21], anti-inflammatory[22], anti-viral[23], anti-tumor, anti-cancer[24], anti-diabetes[25], anti arthritis[26], antimicrobial[27] and anti-hypertensive effect.[28,29] Collectively, algal phenolic compounds can be used as functional ingredients in the pharmaceutical and food industries. [16]

Turbinaria conoides (Phaeophyceae), brown seaweed are found on rock surface in tropical marine water of the Indian Ocean, Korea, Japan, Indonesia and China. It has considerable amount of phenolic content, however, so far, no great attention has been given to either the qualitative or quantitative effects of these phenolic compound on ACE inhibitory activity and nitric oxide (vasodilator) production on this seaweed, Therefore, this study was to examine the above mentioned activities of phenolic compounds from the brown seaweed *T. conoides.*

2. MATERIALS AND METHODS

2.1. Algae sample collection and processing

The brown algal seaweed *T. conoides* was collected by deep sea divers at the shore of Mandapam, nearby Rameshwaram, Gulf of Mannar, Tamil Nadu, India. The collected samples were thoroughly washed in running tap water and air dried at room temperature in the shade for 7 days (Fig. 1), then powdered using a mixer grinder and stored in an air tight container. We observed grinding of this alga is very tough due to its stiffness after dry.

Fig. 1: Marine brown algae of *Turbinaria conoides* (dried).

2.2. Organic solvent extraction

Two different organic solvents (ethanol and ethyl acetate) were used to prepare algal extract. Ten gram of *T. conoides* powder was mixed with 100 mL of each solvent separately and kept in an orbital shaker at 120 rpm for 24 h at room temperature. Then the extract was filtered using a Whatman No. 1 filter paper and transferred to a petri-dish and allowed to evaporate at room temperature and weighed. Finally, all dried extract were dissolved in 1.5 mL respective solvents.

2.3. Quantification of total phenolic content (TPC)

TPC was quantified by Chandler and Dodds[30] protocol where 1 mL of algal extract was mixed in a test tube containing 1 mL of 95% ethanol, 5 ml of distilled water, and 0.5 mL of 50% Folin-Ciocalteu reagent. The mixture was allowed to react for 5 min, and 1 mL of 5% Na_2CO_3 was mixed thoroughly and placed in the dark for 1 h. Absorbance was measured at 725 nm using a UV-VIS spectrophotometer. Gallic acid standard curve was obtained for calibration of TPC.

2.4. Determination of ACE inhibitory activity

ACE inhibitory activity was analysed by Cushman and Cheung[31] protocol with slight modifications. For each assay, 50 µL of sample solution with 50 µL of ACE solution (25 mU/mL) were pre-incubated at 37ºC for 10 min, after which the mixture was subsequently incubated with 100 µL of substrate (25 mM Hipuril-His-Leu in 50 mM sodium borate buffer containing 500 mM NaCl at pH 8.3) at the same temperature for 60 min. The reaction was terminated by adding 250 µL of 1 M HCl. After that, the resulting hippuric acid was extracted with 500 µL of ethyl acetate. After centrifugation at 4000 rpm for 10 min, 200 µL of the supernatant was transferred into a glass tube and dried at 80ºC for 1 h. The residue was dissolved in 1 mL of distilled water, and the absorbance was measured at 228 nm using an UV-VIS spectrophotometer.

The inhibition was calculated as follows.

% inhibition = [(Ac-As)/Ac-Ab)] × 100

Ac - Absorbance of control solution

As - Absorbance of sample solution

Ab - Absorbance of blank solution

The IC_{50} value was defined as the concentration of inhibitor required to inhibit 50% of ACE inhibitory activity.

2.5. Determination of nitric oxide (NO) production and cell viability

Nitric oxide is a regulator of blood pressure and causes the smooth muscle cells surrounding blood vessels to relax, thereby decreasing the blood pressure. Therefore, we investigated the effect of ethanol extract (high ACE inhibitor) on the production of NO in the breast cancer cell line MCF7. Cells were obtained from NCCS (National Centre for Cell Science) Pune, and cultured in RPMI medium containing 10% heat-inactivated calf serum, with recommended AAS (Antibiotic and Antimycotic solution, Sigma-Aldrich) at 37 ºC in an incubator, under humidified atmosphere containing 5% CO_2. Cells (1.5×10^5 cells/well) in 24-well plates were pre-incubated with different concentrations of ethanol extract (in 0.1% DMSO) for 24 h, and the nitrite accumulation in the supernatant was assessed by Griess reaction.[32] Each 50 µL of culture supernatant was mixed with an equal volume of Griess reagent [0.1% N-(1-naphthyl)-ethylenediamine, 1% sulfanilamide in 5%

phosphoric acid] and incubated at room temperature for 10 min. The absorbance was measured at 550 nm by a microplate absorbance reader, and a series of known concentrations of sodium nitrite was used as a standard and 0.1% DMSO as control. Cell toxicity was estimated using MTT assay according to the method described by Mosmann.[33] MCF7 cells were seeded in a 96-well plate at a concentration of 1.0×10^5 cells/mL. After 16 h of incubation at 37 ºC, the cells were treated with 10 µL of the extract (in 0.1% DMSO) at different concentrations and incubated at 37 ºC for 24 h. After that, MTT stock solution (50 µl; 2 mg/ml) was added to each of the wells to a total reaction volume of 200 µL. After 4 h of incubation, the plates were centrifuged at 800 rpm for 5 min and removed supernatant. The formazan crystals in each well were dissolved in 150 µL of DMSO and the absorbance was measured using an enzyme linked immune sorbent assay (ELISA) reader at 540 nm. Relative cell viability was evaluated with 0.1% DMSO as control. The optical density of the formazan generated in the control cells was considered to represent 100% viability.

2.6. GC-MS analysis of the extract

Ethanol extract were chosen for the compound analysis due to high inhibition of ACE and analyzed in Agilent Technologies GC systems with GC-7890A/MS-5975C model (Agilent Technologies, Santa Clara, CA, USA) equipped with HP-5MS column (30 m in length × 250 µm in diameter × 0.25 µm in thickness of film) at Tamil Nadu Veterinary University, Chennai.

3. RESULTS

3.1. Extraction quantity and TPC

In the present study, Ethyl acetate shown the maximum quantity of extracts than the other solvent as it observed 1.36 g, from the 10 g of algal sample followed by the ethanol (1.02 g), respectively. The TPC content showed differences among the extracts, 73% in ethyl acetate and 83% in ethanol extract (Fig. 2). Considerably high TPC was obtained from the ethanol extract of *T. conoides*. Interestingly, ethyl acetate showed less TPC than ethanol though it observed more extracted quantity. The result indicates variation in extraction efficiencies among the different solvents used. Further, when considering the TPC, ethanol was found to be the most efficient extractable solvent than the ethyl acetate.

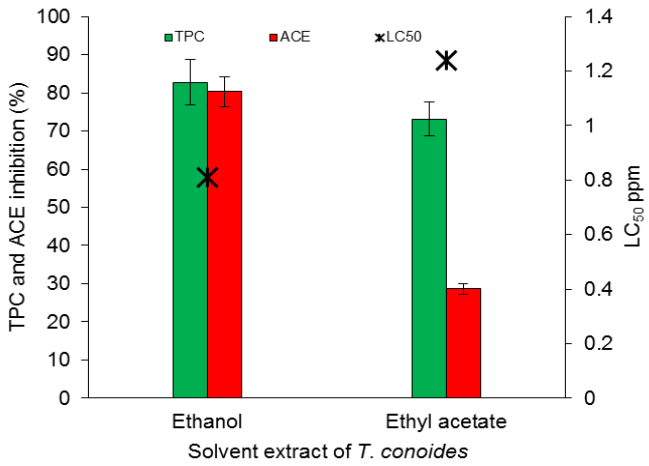

Fig. 2: Total phenolic content (TPC) and angiotensin I-converting enzyme (ACE) inhibition percentage of different organic solvent extracts from the marine brown seaweed *T. conoides*. Values are mean ± SD of seven determinations.

3.2. ACE inhibitory activity of organic extracts

Ethanol and ethyl acetate extracts of *T. conoides* were tested for their potential ACE inhibitory activities and showed varied measure according to the solvent used and compared with previous studies (Figure 2 and Table 1). Ethanol extract showed the highest ACE inhibitory activity of 80% compared to the ethyl acetate (29%). Moreover, ethanol extract exhibited the strongest ACE inhibitory activity with the lowest IC_{50} value of 0.81 mg/mL followed by the ethyl acetate (1.24 mg/mL). As we mentioned above, the highest TPC was also obtained from the ethanol extract.

Table 1: Comparative analysis of ACE inhibition activities of marine brown algae.

Marine Brown algae	IC_{50}(mg/mL)	Solvent used	ACE inhibition (%)	Reference
Turbinaria conoides	0.81	Ethanol	80	This study
	1.24	Ethyl acetate	29	
Fucus spiralis	0.23	Methanol	80	Paiva et al.[38]
Ecklonia cava	0.96	Ethanol	-	Wijesinghe et al.[29]
	1.21	Ethyl acetate	-	
	1.31	Chloroform	-	
	1.19	Diethyl ether	-	
	1.3	Hexane	-	
Ecklonia stolonifera	0.13	Ethanol	65	Jung et al.[2]
Ecklonia cava	0.05	Ethanol	167	
Undaria pinnatifida	0.15	Ethanol	53	
Pelvetia siliquosa	0.18	Ethanol	46	
Hizikia fusiforme	0.32	Ethanol	26	
Ecklonia cava	1	water	36	Athukorala and Jeon[7]

3.3. Effect of ethanol extract on nitric oxide production and cell viability

Ethanol extract had an inducible effect on production of NO in MCF7 cell line at even 1 ng amount of extract which produced 26% more NO and reached 68% on 100 µg concentration of extract, respectively (Fig. 3). The cells showed a higher than 94% survival rate at 100 ng concentrations and reduced to 57 % on 100 µg concentration (Fig. 3-4). The results indicate that ethanol extract of *T. conoides* had no cytotoxic effect on cancerous cell line up to 100 ng and more than this showed anticancer activities, whereas the NO production is reached 36% at 100 ng concentration of the extract which could be relax the blood vessel eventually reduce pressure.

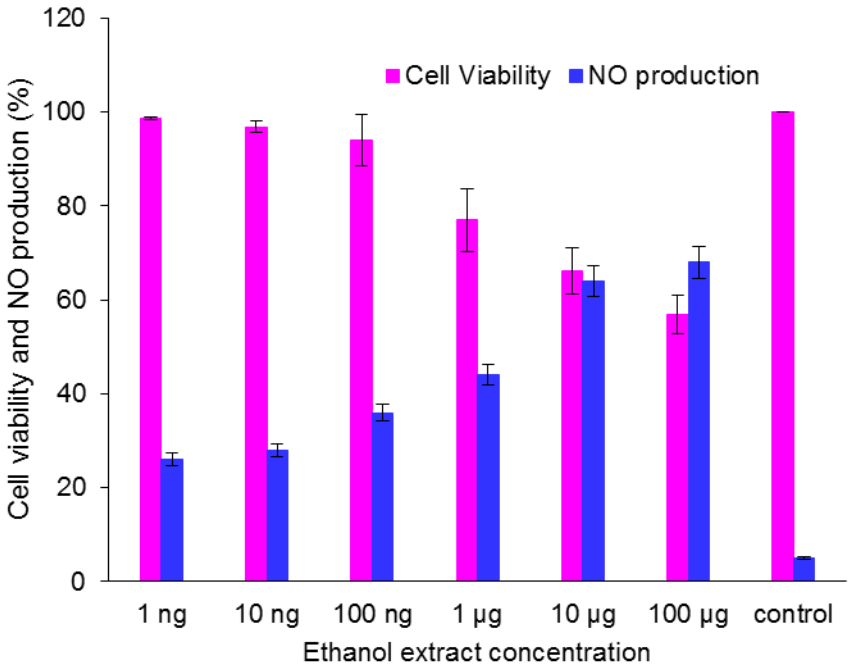

Fig. 3: Effect of ethanol extract of *T. conoides* on Nitric oxide production and cell viability in MCF7 cells lines. The viability of cells upon ethanol extract treatment was determined by MTT assay. Values are mean ± SD of three determinations.

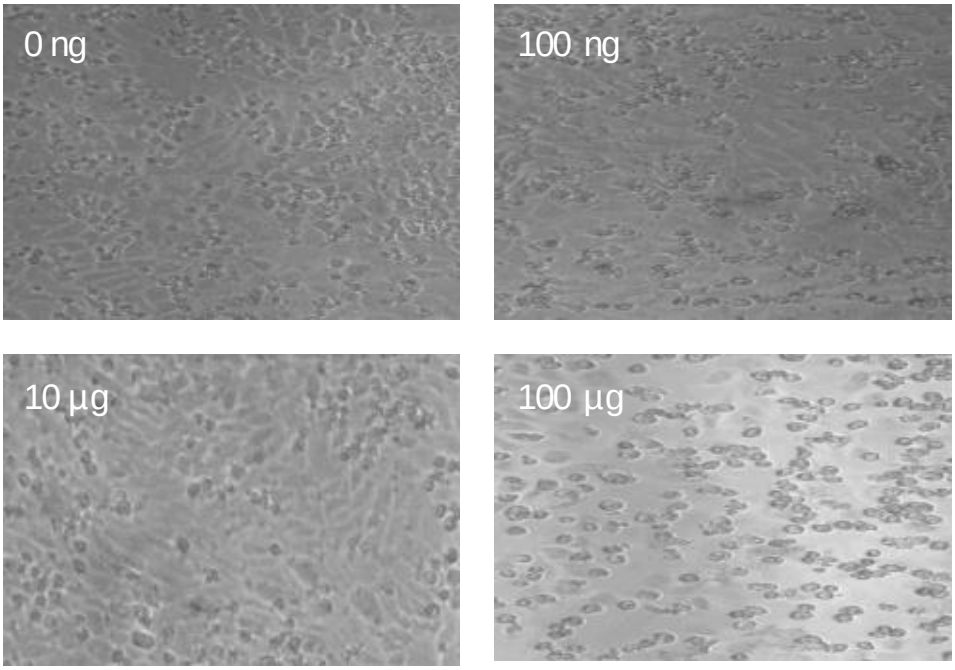

Fig. 4: Effect of ethanol extract of *T. conoides* on cell viability in MCF7 cells lines with different concentration.

3.4. Ethanol extract compound identification

Since ethanol extract of *T. conoides* contained maximum ACE inhibitory compounds, it was subjected to GC-MS in order to detect the potential ACE inhibitors (Fig. 5). We were able to identify four major peaks and its responsible compounds might liable for the ACE inhibition activity namely Pentane, 2,3,4-trimethyl- (A); benzene, 1,2,4-trimethyl- (B); 5,5-Dimethyl-1,3-dioxane-2-thione (C); Tricyclo (4,3,2,01,4) undeca-2,2,8,10-tetraen-7-one. According to the results of the present study identified that the some of these compound had strongest ACE inhibitor and IC_{50} value which will be studied in future by eluting the particular compound and assayed.

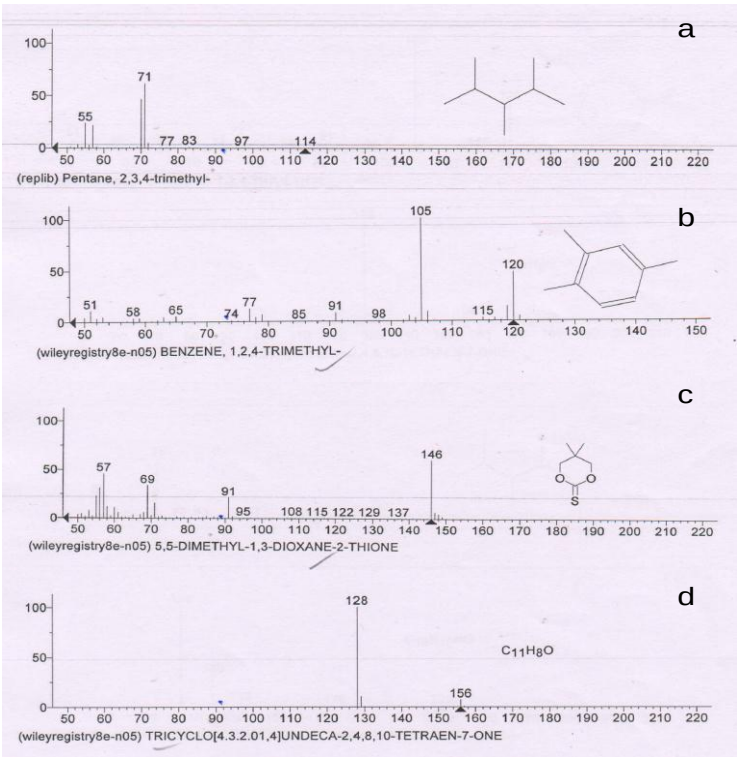

Fig. 5: GC-MS chromatogram for ethanol extracts of *T. conoides*. Four major chemical compound peaks were identified as Pentane, 2,3,4-trimethyl- (a); benzene, 1,2,4-trimethyl- (b); 5,5-Dimethyl-1,3-dioxane-2-thione (c); Tricyclo(4,3,2,01,4) undeca-2,2,8,10-tetraen-7-one (d).

4. DISCUSSION

Marine algae produce a wide variety of secondary metabolites and have different biological activities.[17] It is well established that brown algae contain polyphenolic compounds and become the focus of intense research due to their perceived beneficial effects on health, moreover these metabolites had wide range of molecular sizes and an astringent taste, and they are able to bind to metal ions and precipitate proteins.[16] Based on the increasing evidence of the importance of polyphenols, this is the first study to demonstrate ACE inhibitory activity of polyphenols of *T. conoides*, however previous studies observed only anticancer[34], antimicrobial[35] and mosquitolarvicidal.[36]

T. conoides is found in abundance in the regions Indian Ocean, Korea, Japan, Indonesia and China can be readily obtained from the coastal area of these countries. The similarity of its inherent compounds to those of other seaweeds and the convenience and economy with which it can be obtained, caused us to focus interest specifically on *T. conoides*, although the bioactive intensity were differ with other seaweeds, [2] as compared with this investigations (Table 1). It is well known that solvents with different polarities can extract different classes of compounds (polyphenols). Naturally occurring polyphenols are known to have numerous biological activities[37] and further, various solvent can be used to release soluble pholyphenols from seaweed. According to previous literature, it was reported that water extract of *Ecklonia cava* has considerable ACE inhibitory activity (around 36%) at a concentration of 1 mg/mL.[7] Nevertheless, we found that ethanol extract of *T. conoides* enhanced ACE inhibition (around 80%) and was superior compared to water extract and found IC_{50} value of 0.81 mg/mL, while in contrast Pavia *et al*. [38] observed 80% of ACE inhibition of methanolic extract of *Fucus spiralis* at IC_{50} of 0.23 mg/mL. It could have been that ethanol and methanol increased the TPC by inhibiting interactions between tannins and proteins during extraction and its algal specific.[39] In supporting of our study, the in vitro ACE inhibitory activity of ten Korean seaweeds including five Phaeophyta (*E. cava, E. stolonifera, Pelvetia siliqousa, Hizikia fusiforme,* and *Undaria pinnatifida)* have been reported[2] as ethanol extracts exhibited significant inhibitory properties against ACE at more than 50% inhibition at 164 µg/mL, whereas the eluted compounds (phlorofucofuroeckol) of ethanol extract shown maximum inhibitory activity (82.72%) at a concentration of 65.6 µg/mL. In comparison with previous studies *T. conoides* have less ACE activities due low content of phenolic compounds with other brown algae (Table 1).

Nitric oxide is an important messenger molecule involved in many physiological and pathological processes within the mammalian body with both beneficial and detrimental effects.[40] The inner lining of the blood vessels uses nitric oxide to signal relax mussels, thus resulting in vasodilation and increased blood flow in the rennin angiotensin system, ACE inactivates the vasodilator bradykinin.[41] Previous evidence suggests that nitrates may be beneficial for treatment of vasoconstriction of coronary vessels.[42] In addition to ACE inhibition the ethanol extract of polyphenols induced NO production in cell lines of MCF7 at 36% more in 100 ng of ethanol extract, while Wijesinghe *et al.* [29] stated that 0.27 mM of phenolic compound of dieckol induced NO production at 10-15%, that would reduce blood pressure eventually control hypertension. The effect of polyphenols on cell viability was measured via MTT assay, and shown insignificant toxicity (6%) upto 100 ng while previous study stated only 5% toxicity up to 0.54 mM of phenolic compound of dieckol which increase the medicinal value on this brown seaweeds.

Since all enzymes are proteins, inhibition of ACE may be closely associated with the protein-binding abilities of polyphenols, which are characteristic of all tannins. Furthermore, it has been well described that tannins have the ability to form strong complexes with proteins.[43] Therefore, we can suggest that the inhibition was due to the reduced efficiency of ACE after binding with polyphenols from *T. conoides*. Kang *et al.*[41] and Wijesinghe *et al*.[29] reported that phlorotannins components, which are mainly oligomeric polyphenols composed of phloroglucinol units, are responsible for the biological activities of *Ecklonia* sp. They identified phlorotannins such as eckol (a closed-chain trimer of phloroglucinol), 6, 6-bieckol (a hexamer), dieckol (a hexamer), and phlorofucofuroeckol (a pentamer) in *Ecklonia* species. Shibata *et al.* [44] reported that the molecular size of phlorotannins is important for strong interaction with enzyme molecules, and they have found that pentamers or hexamers of phloroglucinols act as better inhibitors. Therefore in this study ethanol extract was analyzed in GS-MS to identify the dominant compound and observed four major compounds which include dioxane group. These compounds will be checked separately for the ACE inhibition studies and further analysis.

5. CONCLUSION

We conclude, *T. conoides* have significant amount of phenolic compounds with special bioactivities, including ACE inhibitory activity and NO production with less cytotoxicity. Ethanol enhances the extraction of phenolic compounds, from the brown seaweed *T. conoides.* Based on the results of this study, we can suggest that the brown seaweed *T. conoides* could be used for the development new functional pharmaceuticals and foods and to support hypertensive related disease. Some major compounds were identified in the extract will be analyzed in future to specify the above action. Moreover, it is expected that these findings will contribute to basic research and potential applications of phenolic compounds in relevant fields.

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