Original Research Article

Inhibition of Angiotensin-converting Enzyme by the Fractions of Onion (*Allium cepa*) and Shallot Bulb (*Allium ascalonicum*) Extracts

ABSTRACT

The impact of hypertension as being the leading cause of death in industrialized societies pressed the importance of low-cost and accessible therapeutic agents present in food sources. This study was undertaken to evaluate the in-vitro Angiotensin-converting Enzyme (ACE) inhibitory activity of flavonoid-rich Onion and Shallot bulbs. Ethyl acetate, Butanol and Aqueous fractions of onion and shallot bulbs at varying concentrations were obtained through purification by solvent extraction. Positive control was the prototype ACE-inhibitor, Captopril. ACE activity was determined using the ACE kit commercially manufactured by Dojindo (Japan). All fractions of onion and shallot bulb extracts exhibited greater than 50% inhibitory activity towards ACE. The aqueous fractions of both bulbs showed the highest ACE-inhibitory activity exceeding the flavonoid standard, quercetin, and were comparable with the prototype ACE-I, captopril. Based on the study findings, onion and shallot bulbs can be potential sources of bioactive compounds that can inhibit the activity of ACE.

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INTRODUCTION

Hypertension is a chronic disease that has become one of the major health problems worldwide. In both developing and developed countries, hypertension is the leading cause of mortality. Kearney et al. (2005) estimated that more than 1.56 billion people worldwide are expected to have hypertension by 2025, making the disease more alarming to healthcare providers.

Hypertension is defined clinically as an average of at least 2 measurements of resting systolic blood pressure (SBP) of ≥140 mmHg and/or a diastolic blood pressure (DBP) of ≥90 mmHg).In the great majority of cases, hypertension is the result of dysfunction of the mechanisms used by the circulation for the long-term rather than short term control of arterial pressure (Ahmed et al. 2011). There is a strong positive and continuous correlation between blood pressure and the risk of target organ damage that in the long-term results in diseases such as coronary artery disease, stroke, myocardial infarction, heart failure, and renal disease, and thus mortality (Bazzano et al. 2008).Furthermore, chronic hypertension can lead to dysfunction of the endothelium and damage to the endothelial cells that produces a number of proliferative responses, including arteriosclerosis. The challenges of managing hypertension and preventing the development of these latter outcomes are unlikely to relent; the global burden of hypertension is projected to increase by 60% to affect approximately 1.6 billion adults worldwide by 2025 (Saputri et al. 2017).

According to the World Health Organization (WHO), the leading causes of death among Filipinos are ischemic heart disease (15.4%) and stroke (11.1%). Both of these non-communicable diseases are rooted on the uncontrolled elevation of blood pressure and blood glucose levels. Additionally, the Department of Health

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(DOH) stated that about eight out of ten people who had their first stroke are diagnosed with hypertension which is responsible for worsening the quality of lives of some 14 million Filipinos. The DOH also revealed that more than 276 Filipinos die of heart disease on a daily basis and at least one Filipino suffers from stroke every nine minutes.

The renin-angiotensin-aldosterone system (RAAS) has an important contribution in the maintenance of vascular tone and involved in controlling blood pressure. The key enzyme in the RAAS is angiotensin converting enzyme (ACE). ACE is a cell membrane peptidase that plays a central role in the regulation of blood pressure through the production of angiotensin II, the potent vasoconstrictor(Antonios andMacGregor 1995). The use of an angiotensin-I-converting enzyme inhibitor (ACEI) is well established as one of the primary therapeutic agents for the treatment of hypertension. However, it's quite costly to maintain taking these drugs every day. These drugs are also associated with adverse effects like cough and angioedema which can be intolerable to some patients. A good ACE-I alternative can be obtained from natural products and it can be isolated from various plants. Some of edible plants and traditional medicines are known for containing compounds that have the same functions and actions with ACE-I that are present in the marketplace. Phenolic compounds, such as flavonoids, in some plants were reported to lower blood pressure through inhibition and decrease the expression of ACE (Rumiyati et al. 2016). These flavonoids can be easily found in the most edible plants, and two examples of edible plants which contain abundant flavonoids are onion bulbs (*Allium cepa*) and shallot bulbs (*Allium ascalonicum*). These plants are ubiquitous and present in what most people eat every day (Slimestad et al. 2007). Both onion and shallot bulbs are notoriously

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abundant with high concentrations of the flavonoid compound quercetin (Fattorusso et al. 2002). Studies of Guerrero et al. (2012) on the inhibition of ACE activity show that Quercetin has inhibitory activity against ACE with Inhibitory concentration 50 (IC_{50})of 43. Therefore, the investigation of new, natural product-based ACE inhibitors could greatly benefit hypertensive patients.

Hence, with the alarming increase in the number of hypertensive individuals in the Philippines and in the world, with expensive hypertensive medications present in the market nowadays, intolerable adverse effects of hypertensive drugs, and poor lifestyle choices, this study aimed to provide a natural, perhaps safer, cost-effective alternative by using onion bulbs and shallot bulbs in reducing blood pressure through the determination of its inhibitory activity on Angiotensin-converting enzyme using ACE kit-WST and comparing it with the positive control which is an ACE-inhibitor available in the market, Captopril.

Thus, this study determined the inhibitory activity of the fractions of onion (*Allium cepa*) and shallot bulb (*Allium ascalonicum*) extracts on Angiotensin-converting enzyme (ACE).Moreover, the study also characterized the organic compounds and functional groups present in both the onion and shallot bulbs including the quantification of quercetin, the standard flavonoid, touted to be responsible for its ACE-inhibitory activity.

MATERIALS AND METHODS

Plant Collection and Authentication

The plants, bulbs of onion (*Allium cepa)* and shallot (*Allium ascalonicum)*, were obtained from an exclusive supplier. Samples of the plants from the same supplier were obtained for verification. Plant verification was done by a certified taxonomist in Department of Agriculture Region XI office, Philippines.

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Plant Extraction, Fractionation and Lyophilization

The bulbs of onion (*Allium cepa*) and shallot (*Allium ascalonicum*) were cleaned, peeled, air-dried and minced. They were macerated with concentrated methanol (99%) for 24 hours, filtered, and macerated again for another 24 hours to ensure exhaustive extraction. A polar organic solvent (methanol) was employed in an attempt to extract as many compounds as possible. This is based on the ability of alcoholic solvents to increase cell wall permeability, facilitating the efficient extraction of large amounts of polar and medium- to low-polarity constituents (Sarker and Nahar 2012). The methanolic extracts obtained were then concentrated using rotary vacuum evaporator. An analytical grade methanol was used in the study and was purchased from Scharlau Laboratories (Barcelona, Spain).

Fractionation process then commenced after concentrating the methanolic extract of the two plants. Purification by solvent extraction using partition coefficient was utilized in getting all the fractions. A total of five (5) liters of hexane (96%) was utilized to “defat” the concentrated methanolic extracts 6 times to give a fraction containing nonpolar compounds, such as lipids, chlorophylls, and so on. The methanolic layer was then again concentrated using rotary vacuum evaporator. After which, it was dissolved in equal volume of distilled water and then successively extracted with chloroform (99%), ethyl acetate (99%), and butanol (99%) obtaining the different fractions. The fractions obtained were then subjected under rotary vacuum evaporator to remove the solvents utilized. Analytical grade solvents were used in the fractionation process and were purchased from Scharlau Laboratories (Barcelona, Spain). A table top type Freeze Dryer from Biobase(Shandong, China) was used in lyophilizing the fractions obtained.

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Chromatographic Analysis and Quantification of Quercetin

Onion bulb and Shallot bulb extracts were characterized using Reversed Phase-HPLC Analysis. This equipment is used to determine and quantify the quercetin content of the onion bulb and shallot bulb extracts. An HPLC instrument (Prominence Shimadzu) with a CBM-20A System Controller, LC-20AP preparative pump, SIL-10AP autosampler, and a SPD-M20A Photodiode Array Detector was used. An LC stop time was set at 15 minutes. The total flow was 1 ml/min and the mobile phase was 1% orthophosphoric acid with 65% methanol HPLC grade. The detection was carried out using a PDA detector set at 250-400 nm.

a. Preparation of the sample

1. Onion bulb and shallot bulb extracts were dissolved in methanol HPLC grade to prepare the stock solution. 0.5 mL from the stock was withdrawn and added to 4.5 mL of HPLC grade methanol to create the 0.05% dilution and this method was repeated another time to create the 0.005% dilution.

2. Aliquot portions from the stock, 0.05% and 0.005% were filtered via 0.45 µmisodisk before injection.

b. Preparation of the standard

1. Quercetin standard was obtained from Sigma-Aldrich Corporation (Missouri, USA).

2. The standard was diluted in methanol HPLC grade.

3. The solution was then filtered three times via a 0.45µm isodisk.

c. High Performance Liquid Chromatographic Analysis

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1. To ensure that no foreign debris were present which may hamper the process, the sample underwent isodisk filtration for three times prior to making the injections.

2. The mobile phase underwent the process of filtration and degassing for three times

3. A syringe was used to withdraw the solvent. Afterwards, all air bubbles were removed.

4. To facilitate and increase the flow rate, the silica gel C-18 column was moved in an upward and downward motion for 45 minutes. The eluent system consisted of a gradient program from 1% orthophosphoric acid with 65% methanol HPLC grade. Monobasic sodium phosphate and concentrated phosphoric acid served as buffer solutions. This was observed at a flow rate of 1.0 ml/min and a column temperature of 40°C.

5. 20uL filtered samples was then injected. Care was taken to not inject large volumes of samples as it may cause damage to the column, and subsequently affect the chromatogram's resolution.

6. Analysis conditions for the aforementioned were as follows:

i. Column- (ODS C18, 5µ, 150mm × 4.6mm)

ii. Detection- 250-400nm

iii. Flow Rate- 1ml/minute

iv. Injection Volume-20uL

v. Mobile Phase- 1% orthophosphoric acid, 65% HPLC grade methanol

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vi. Retention time of shallot bulbs: 11.754 min

vii. Retention time of onion bulbs: 11.737 min

viii. Run time: 15 min

Identification of Organic Compounds using Fourier-transform infrared spectroscopy

The onion and shallot bulb extracts were placed on the sample chamber of FT-IR spectrophotometerto identify organic, polymeric, and inorganic materials.Spectra was recorded in the range of 3600–600 cm^{-1} on IRAffinity-1S spectrometer. An Attenuated Total Reflection (ATR) accessory was used because it is ideal for strongly absorbing thick samples which often produce intense peaks when measured by transmission. Important absorption frequencies that appeared in functional group region as well as fingerprint region of the spectra were then noted using a He-Ne laser.

Measurement of ACE-inhibitory Activity

ACEI activity was measured by a fluorimetric assay following the method of Lam and Shimamura using the ACE kit-WST commercially manufactured by Dojindo Molecular Technologies, Inc. (Japan). The procedure provided by Dojindo was the one that was followed in the determination of percent inhibitory rate. Borate buffer pH 8.3 containing 380 mMNaCl was used as a buffer. Absorbance measurements were carried out at a wavelength of 450 nm and utilized a filter-based microplate reader for determination.

a. Preparation of Enzyme working solution ·

1. 2 mL of deionized water was added to enzyme B. ·

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2. 1.5 mL of Enzyme B solution was added to Enzyme A to prepare the Enzyme working solution.

Since inside of each vial of Enzyme A and Enzyme B was under reduced pressure, deionized water or Enzyme B solution was added to the vial with a syringe in order to avoid the dispersal of powder.

b. Preparation of Indicator working solution

1. 3 mL of deionized water was added to each vial of Enzyme C and Coenzyme.

2. 2.8 mL of Enzyme C solution and Coenzyme solution was added to Indicator solution to prepare Indicator working solution.

Since inside of the vial of Enzyme C and Coenzyme was under reduced pressure, deionized water was added to the vial with a syringe in order to avoid the dispersal of powder.

c. Preparation of Sample solution

1. Sample solution was prepared based on the concentration for each fraction which were 100 µg/mL, 300 µg/mL, 500 µg/mL.

d. General procedure for the assay

1) 20 µl of sample solution was added to a sample well and 20 µl of deionized water was added to blank 1 and blank 2 wells.

2) 20 µl of Substrate buffer was added to each well.

3) 20 µl of deionized water was added to blank 2 well.

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4) 20 µl of Enzyme working solution was added to each sample well and blank 1 well.

5) The 96 well microplate with the solutions mixed was incubated at 37^{o}C for 1 hour.

6) 200 µl of Indicator working solution was added to each well.

7) The well was again incubated at room temperature for 10 minutes.

8) The absorbance was read at 450 nm using a microplate reader.

The activity of each sample was tested in technical and biological triplicate. The ACEI activity was calculated using the following formula:

ACE inhibitory activity (inhibition rate %) = [(A- C) / (A- B)] X 100

where A is the fluorescence without the flavonoid solution, B is the fluorescence without ACE and C is the fluorescence in the presence of both ACE and the flavonoid solution.

Determination of Acute Oral Toxicity

Acute oral toxicity testing was done in accordance to OECD Guidelines for Testing of Chemicals (OECD 423). Determination of acute toxicity was carried out using 3 female, 3 months old, nulliparous and non-pregnant albino rats with weights not more than 20% deviation from each other.A certification from the Institutional Animal Care and Use Committee was obtained to ensure proper and humane handling of the test animals.

The animals were kept in their cages for 2 weeks prior to dosing to allow for acclimatization to the laboratory conditions (temperature of 25 ºC, artificial lighting

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with 12 hours light then 12 hours dark). The rats were fed with conventional laboratory diets with an unlimited supply of drinking water. The rats were caged individually so as not to interfere with clear observations of each animal.

The rats were fasted prior to dosing. Food but not water was withheld. The rats were weighed and extracts of onion bulb and shallot bulb were administered orally via gavage at a dose of 2000 mg/kg of body weight. Single doses of 286 mg, 324 mg, 314 mg were administered to rats in the Shallots group while single doses of 364 mg, 374 mg, 358 mg were administered to rats in the Onion group. The rats were observed during the first 30 minutes, periodically during the first 24 hours, with special attention given during the first 4 hours, and daily thereafter, for a total of 14 days for the presence of any alterations on the animal's behavior, nervous manifestations and appearance of any signs of physical discomfort as stipulated in the Humane Endpoints Guidance Document (OECD guideline 19) which include the following: changes in physical appearance (coat texture, hair soiled with urine or feces), changes in clinical signs (respiration rate; posture), changes in unprovoked behavior (self-mutilation, compulsive behavior), behavioral changes in response to external stimuli (excitability, righting reflex), changes in body weight, and related changes in food and water consumption.

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RESULTS

The percent yield of ethyl acetate, butanol, and aqueous fractions of onion and shallots is shown in Table 1. The percent yield of quercetin was less than the total percent yield.

Table 1. Total percent yield of onion and shallot bulb extracts and amount of quercetin extracted.

Plant	Weight before extraction (dry wt, g)	Weight after extraction (dry wt, g)	Total % Yield	% Quercetin yield
Onion	1,200	2.346	0.196	0.113
Shallots	1,200	2.567	0.214	0.155

The ACE Inhibitory activity of the ethyl acetate, butanol, and water-soluble fractions of Onion (*Allium cepa*) and Shallot Bulb (*Allium ascalonicum*) extracts was compared using ACE-Kit WST in this study. The percent inhibition rate was computed following the equation presented on the manual of the ACE-Kit. As shown in Table 2, all of the fractions of onion and shallot bulb extracts showed greater than 50% inhibitory rate. As the doses of the fractions increased from 100 ug/ml to 500 ug/ml, the percent inhibitory rate also increased showing a linear relationship. Among the three fractions, the water-soluble fraction of both Onion Bulb and Shallot Bulb extracts showed the highest ACE Inhibitory activity. Moreover, all of the fractions of the Onion Bulb (*Allium cepa*) extract were noted to have higher ACE Inhibitory activity than that of the same fractions of Shallot Bulb (*Allium ascalonicum*) extracts except for the water-soluble fraction at 100 µg/mL and 500 µg/mL concentration.

Table 2.Percent inhibition rate of the fractions of Onion and Shallot Bulb Extracts with its corresponding concentrations.

Plant Fractions	Percent Inhibition Rate (%)		
	100 µg/mL	300 µg/mL	500 µg/mL

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Onion bulb			
Ethyl Acetate	88.49	88.95	91.77
Butanol	81.27	84.88	86.13
Water	92.39	96.56	97.29
Shallot bulbs			
Ethyl Acetate	62.38	63.11	74.40
Butanol	63.23	64.41	68.87
Water	94.42	95.94	96.22

The summary for the percent inhibition activity of the fractions of onion bulb and shallot bulb extracts comparing it with Captopril and Quercetin are shown in Figures 1 and 2, respectively. As being the positive control, Captopril showed the highest ACE inhibitory activity. The water-soluble fractions of shallot and onion bulb extracts were comparable to captopril in all three concentrations. The percent inhibitory rate of the Quercetin standard was noted to be lower compared to the water-soluble fractions of both onion and shallot bulbs.

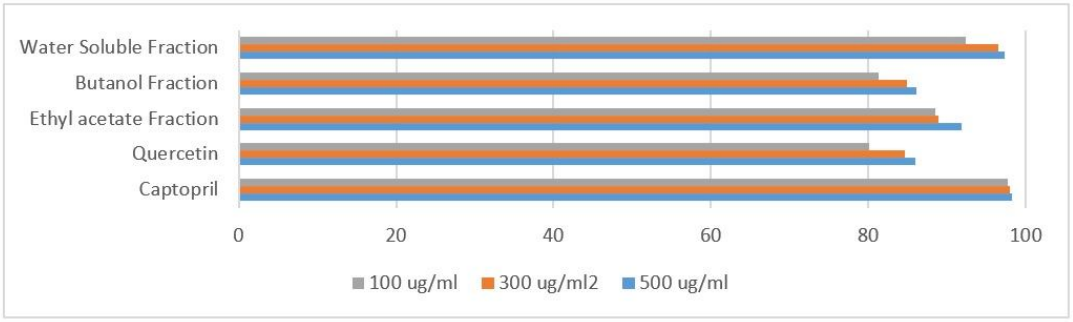

Figure 1. Comparison of the Percent Inhibition Rate of the Onion Bulb Fractions with Captopril and Quercetin

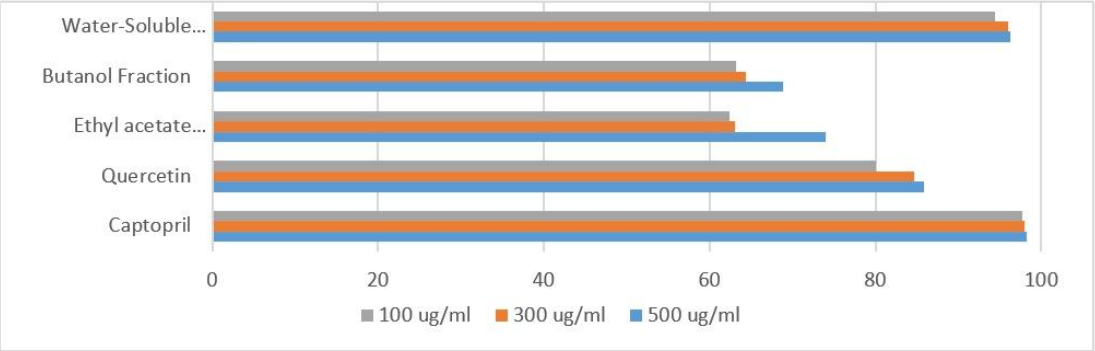

Figure 2. Comparison of the Percent Inhibition Rate of the Shallot Bulb Fractions with Captopril and Quercetin

During HPLC analysis, the peak for quercetin was detected at 11.737 minutes retention time using a standard calibrated curve for quercetin. The amount of quercetin present in the Onion Bulb extract was 2.643 mg/ml while the amount of quercetin present in the Shallot Bulb extract was 3.993 mg/ml. Table 1 shows the percentquercetin yield, computed as the amount of HPLC-detected quercetin over the amount of crude extract.

Table 3 shows the functional groups present in the onion bulb and shallot bulb extracts using the IRAffinity-1S machine. All peaks below 1400 were not included as they are in the fingerprint vibration region, and hence not relevant.

Table 3.FTIR analysis of onion and shallot bulb extracts.

Signal to Noise Ratio (S/N)	Peak cm^{-1}	Bond	Functional Group
Onion Bulb Extract			
1	3332.99	OH	Alcohol and Phenol
2	2924.09	C-H	Aliphatic Group
3	2358.94	C≡C, C≡N	Alkyne Group
4	1651.07	C=C	Alkene Group
Shallot Bulb Extract			

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1	3307.92	OH	Alcohol and Phenol
2	3290.56	OH	Alcohol and Phenol
3	1645.28	C-H	Aliphatic Group

For Acute Oral Toxicity Testing, the rats were observed immediately after administration, after 30 minutes, after 1 hour and 30 mins., after 4, 16, 20, and 24 hours after the administration of a single dose of 2,000 mg/kg. There were no nervous manifestations and mortality noted. Also, no signs of alteration in the behavior of rats were observed.

DISCUSSION

Elbl and Wagner in 1991 developed one of the earliest assays for ACE inhibition and stated that an extract is considered active if it is able to inhibit the enzyme by more than 50%. Based on the results gathered, there were pronounced activities for all fractions (ethyl acetate,butanol, and water-soluble) of both onion and shallot bulbs which were all greater than 50%. The water-soluble fraction showed the highest percent-inhibition rate with 97. 29% inhibition at 500 ug/ml for onion bulb and 96.22% inhibition at 500 ug/ml for shallot bulb. These activities were comparable with the activity of the positive control, captopril, which had a percent inhibition of 98.20% at 500 ug/ml. A study conducted in 2016 byKharadiet al. notedthat aqueous extract of onion (*Allium cepa*) bulb showed high amounts of total flavonoids, total phenolics, and a high DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging activity which could be the reason why in the experiment, aqueous fraction of both onion and shallot bulbs showed the highest inhibitory activity on Angiotensin-converting enzyme compared to all other fractions because it does not contain only one

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flavonoid but a combination of phenolics suggesting a possibility of synergism between the active constituents.However, further identification of these proposed active biochemicals was not done which serves as the limitation of the study in terms of chemical characterization.

Quercetin standard, on the other hand, showed a lower percent-inhibitory rate at 86.01% at 500 ug/ml. The reason for the increased inhibitory activity of the fractions of onion and shallot bulbs extracts compared to Quercetin standard could be attributed to the synergism of active constituents present in the fractions. Synergistic effect is a phenomenon in which activity is lost in purified fractions (Elbl and Wagner 1991).Additionally, HPLC Analysis of Quercetin as a standard showed a peak time at 11.74 minutes retention time validating the supplier's claim of the identity of the standard via linearity method. The HPLC Analysis showed that the onion and shallot bulbs contain quercetin, which is the standard flavonoid that has been shown to have in-vitro ACE-inhibitory activity. Abilities of different flavonoids to inhibit the activity of ACE confirmed that the principal structural features for their inhibitory activity are as follows: (a) the double bond between C2 and C3 at the C-ring; (b) the catechol group in the B-ring (3′,4′-dihydroxy); and (c) the ketone group at the C4 carbon on the C-ring which is a functional group that has been observed to be essential for inhibiting ACE. It was confirmed that a distinguishing feature for ACE inhibition by flavonoids is the presence of an unsaturated 2–3 bond conjugated with a 4-oxo- function, aside from the 3′,4′-catechol B-ring pattern as what is seen in quercetin (Guerrero et al. 2012). However, the key molecular flavonoid sub-structures that dictate effective ACE inhibition activity have not yet been characterized.

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Fourier Transform Infrared Spectroscopy showed the functional groups present in peaks greater than 1400 cm^{-1}. This signified that indeed hydrogen bonding is present in the extracts, which could be because of the presence of flavonols since flavonols are rich in phenolic -OH groups (Panche et al. 2016). The presence of several hydroxyl groups in the flavonoids seem be important for the extent of inhibition of the zinc metalloproteinases like ACE. Moreover, Guerrero at al. (2012) theorized that the exact position of this group has been revealed to be very important for ACE inhibition. Hydroxylation at the 4′-position of the B ring seems to be of particular relevance, and in addition, the presence of a catechol group in the B ring (3′,4′-dihydroxy) appears to be fundamental to achieving an increased ACEI activity, as occurred in flavonoids like luteolin (as well as quercetin and rutin), which presented the highest ACEI efficiency.A number of other functional groups were also detected in the FT-IR analysis which proves that onion and shallot bulbs contain numerous other active biochemicals, not just flavonols.

There has been an established positive correlation between the consumption of foods and supplements rich in flavonoids and protection against cardiovascular diseases, notably hypertension (Loizo et al. 2008).However, this study is only limited in exploring the potential of onions in inhibiting ACE to modulate vascular function and blood pressure. The potential of the said plants appears to be related with the action of nitric oxide and its inhibition of the key component of the RAAS which is the enzyme ACE. There are three parts in the active site of ACE: a carboxylate binding functionality such as guanidinium group of arginine, a sieve that accommodates a hydrophobic side chain of C- terminal amino acid residues and lastly, a zinc ion. The carbonyl of the penultimate peptide bond of the substrate coordinates to the zinc ion making it polarized and vulnerable to nucleophilic attack. Thus, flavonoids are able to

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inhibit the activity of ACE through generating chelate complexes within the active center of ACE (Wagner at al. 1991). Thus, through its ACE inhibition, blood pressure is reduced and hypertension is prevented.

Results of Acute oral toxicity testing showed that the onion and shallot bulb extracts are not toxic if taken orally at 2,000mg/kg weight of laboratory rats showing that these extracts can be formulated in nutritional supplements or in oral pharmaceutical formulations that can be studied further to better assess its in-vivo anti-hypertensive ability through ACE inhibition.

This preliminary evaluation of the fractions of Onion and Shallot bulb extracts showed that these plants are potential sources of bioactive compounds that can inhibit the activity of ACE.

CONCLUSION AND RECOMMENDATIONS

Conclusion

In this study, the water-soluble fraction of both onion and shallot bulbs showed a promising ACE inhibitory activity which was comparable with the activity of the prototype ACE-inhibitor present in the market, Captopril.Nevertheless, the remaining fractions of onion and shallots bulb extracts showed greater than 50% inhibitory activity which signified that the extracts are all active in inhibiting ACE. HPLC analysis showed there was indeed quercetin present in the extracts. Furthermore, FT-IR analysis supported the claim by elucidating abundance of phenolic compounds. Hence, onion and shallot bulbs are potential sources of phenolic compounds and can be good candidates for developing alternative ACE-inhibiting drugs for hypertension.

Recommendations

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To further enhance researches pertaining to this study, the researchers recommend the following:

1. Conduct an in-vivo ACE inhibition analysis using the onion and shallot bulb extracts
2. Develop and/or manufacture a capsule or preparation out of the onion and shallot bulb fractions
3. Identify other flavonoids or bioactive compounds in onion and shallot bulbs that may have ACE-inhibiting and blood pressure lowering activity
4. Add lower concentrations and compute for the IC_{50} of the fractions obtained.

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