ORIGINAL ARTICLE

Phenolic Content and α–glucosidase Inhibitory Activity of Herbal Mixture: Effect of Processing Technique and Honey Ratio

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ABSTRACT

Introduction: Preparation of herbal mixtures from the traditional knowledge has been used for over centuries to improve and maintain health condition. Nonetheless, lack of scientific evaluations on regard to their bioactive metabolites as a mixture and their pharmacological effects have yet to be reported. Therefore, the objectives of this study are 1) to determine the effect of processing techniques (blending and juicing) on extracting polyphenols and 2) to determine the effect ratio of honey in herbal mixture (containing ginger, garlic, honey, apple cider vinegar, and lemon juice). **Methods:** Raw ingredients such as garlic, ginger, lemon and apple cider (1:1:1:1) were used as the base for this herbal mixture. The base was either blended using a blender or juiced using a juicer. The mixture was simmered (85°C - 100°C) until reduced to half of the initial volume and cooled down before being added with honey in 1:1 (rA) or 1:3 (rB) ratio. The mixtures were tested for pH, total phenolic, total flavonoid content and alpha glucosidase inhibitory activities. **Results:** Both of juiced samples in both honey ratio (rA and rB) have lower acidity compared to blended samples. Total phenolic content (TPC) and total flavonoid content (TFC) also showed significantly higher levels ($p < 0.05$) in juiced samples than blended samples especially in Juicer rB. The insignificant differences in α-glucosidase inhibitory activities among mixtures indicate both extraction and ratio did not influence α-glucosidase inhibitory activities of the mixtures. **Conclusion:** All of the results indicate that processing techniques and ratio can affect the pH and phenolic recovery.

Keywords: Phenolics, Herbal mixture, Processing techniques, Ratios, Honey

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INTRODUCTION

Traditional medicines (TM) such as herbal mixture have been used for over centuries to maintain health condition (1,2). In certain countries, the usage of traditional remedies is larger than the modern medicine. Countries such as India, Chile and Africa, is where the majority of its citizen are largely dependent on traditional medicine especially herbs, due to its easy accessibility and low in cost (3). Due to increase demand of TM, World Health Organisation have listed several guidelines that strengthen the role of traditional medicine practice in general health (1).

One of important aspect being addressed in the guidelines is the processing and the standardization of TM preparation, especially the one that involves either or mixtures of botanical (plants), animals (wholly or partly) and minerals (1). Pre- and post-harvesting processes have been known to influence the recovery of bioactive metabolites from the samples (4, 5). The pre- and post-processing method usually been applied to maintain the quality and prolong the shelf-life of the sample (6).

In this study, the extraction of juices from ingredients was done using either blender or juicer. Both of these extraction processes have been used widely in homes using household appliances, especially in extracting the juices. Mechanical process use in extracting the juices from the sample is important in recovering bioactive metabolites, since bioactive metabolites usually are scattered in the plant cell matrix (4). In addition, ratios of solvents or quantity of the mixtures have been known to influence the concentration of polyphenols in the sample (7, 8).

Herbal mixture containing ginger, garlic, honey, apple cider vinegar, and lemon juice have been consumed for ages as traditional remedies especially in preventing metabolic disease such as cardiovascular disease (9). Separately, ingredients such as ginger, garlic and honey have been reported to possess anti-oxidative effects, anti-inflammatory effects and many more (10-12). Altogether, this mixture has been reported to successfully reduce the post prandial glucose in healthy women (13). Nonetheless, their pharmacological effects have yet to be reported and there is still a need for scientific evaluations on regard to their bioactive metabolites as a mixture (13). Thus, one of the fastest, quickest and reliable to evaluate this herbal mixture preparation is by using in vitro means.

Among the in vitro assays, α-glucosidase inhibitory assay is an assay that been used to test the capabilities of the samples or compounds in inhibiting α-glucosidase enzyme (8). The enzyme is closely related to the rise of post prandial glucose in human (8, 14). Study have shown that several natural α-glucosidase inhibitor can be found in plants. This includes herbs, leafy and non-leafy vegetables, fruits and many more (14). Previously, study by Vinholes et al. (2017) and Kesavanarayanan et al. (2012) found that bioactivities and α-glucosidase inhibitory activities of combination of herbs or plants are better than the individual extracts (15, 16). This is due to the synergism effects of the metabolites in both herbs and plants (15, 16).

Since herbal preparation can influence extractable phenolic contents and the bioactivities of the sample, there is a need in investigating these factors. Thus, the objectives of this study are 1) to determine the effect of processing techniques (blending and juicing) on extracting polyphenols and 2) to determine the effect ratio of honey in herbal mixture (containing ginger, garlic, honey, apple cider vinegar, and lemon juice) on pH, phenolic contents and α-glucosidase inhibitory activity.

MATERIALS AND METHODS

Materials

The ingredients for herbal mixture such as lemon, ginger, garlic, apple cider (Heinz) and honey were bought at local market in Malaysia. Young ginger and Tualang honey were chosen for the study based on previous reports and unpublished data (10).

Chemicals and reagents

Chemical such as sodium hydroxide was obtained from Merck (Darmstadt, Germany). The α-glucosidase (EC 3.2.1.20) enzyme was purchased from Megazyme (Wicklow, Ireland) and Folin-Ciocalteau reagent, aluminum chloride, sodium nitrate and sodium carbonate, p-nitrophenyl-α-D-glucopyranoside (PNPG), quercetin and glycine were supplied by Sigma Aldrich (St. Louis, USA).

Sample preparation

The preparation of herbal mixture ingredient was according to Naseem et al. (17) and Ishak et al. (9) with modification in the honey ratio. Ingredients such as garlic, ginger, lemon and apple cider (250g each or 250 ml for liquid in 1:1:1:1 ratio) were used as base for this herbal mixture. The base was either blended using blender (National MX-895M, Kuala Lumpur, Malaysia) or juiced using juicer (ELBA EJE-A043lWH, Kuala Lumpur, Malaysia). The mixture was simmered (85°C to 100°C) until reduced to a quarter (250 ml) of the original volume (1000 ml) and cooled down before being added with honey in 1:1 (250 ml base: 250 ml honey) or 1:3 ratio (250 ml base: 750 ml honey). The ratio of herbal mixture to honey in 1:1 was labeled as rA and 1:3 was labeled as rB. Honey ratios were chosen based on previous study and also to increase the palatability (9). All the samples were prepared in triplicate. To sum up, there were four samples for this experiment: Juicer in rA ratio, Juicer in rB ratio, Blender in rA ratio and Blender in rA ratio. The mixtures were tested for pH after the preparation before being kept in 4°C. The pH was tested before storage since the temperature of the environment or storage can affected the pH.

In vitro assays

Total phenolic content

The total phenolic content was determined using Singleton and Rossi (18) method with a slight modification on the sodium carbonate percentage. In brief, 0.5 ml of each sample were added 1 ml of 10% Folin-Ciocalteu reagent. After 3 minutes, 3ml of 1% (w/v) of sodium carbonate, Na_2CO_3 was added to the mixture and incubated at the room temperature, in the dark condition for 1 hour. The absorbance was measured at 760 nm using spectrophotometer. The same procedures were used for gallic acid as control compound with different concentration (15.6, 31.25, 62.5, 125, 250 µg/mL) for the calibration curve. The total phenolic content was expressed as µg gallic acid equivalent (GAE) per ml sample (µg GAE/ml sample).

Total flavonoid content

Total flavonoid content was performed according to by Ancuceanu et al. (19) and Pekal and Pyrzynska (20) method. First, 2 ml of sample was added with 0.2 ml of 5% (w/v) sodium nitrate and incubated for 5 min at room temperature before being added with 0.2 ml of 10% (w/v) aluminum chloride. After 6 minutes, 2 ml of 1.0 M of sodium hydroxide, NaOH and 80% ethanol was added to the mixture till the final volume was 5 ml. The absorbance was read at 430 nm using spectrophotometer after 10 minutes of incubation. 80% ethanol was used as the blank, while quercetin (10-1000 mg/ml) was used as standard. The flavonoid content was expressed as µg quercetin equivalent (QE) per ml sample

(µg QE/ml sample).

Alpha-glucosidase inhibition assay

The α-glucosidase inhibition assay was carried out according to Abu Bakar Sajak et al. (21) with a slight modification in substrate and buffer volume in 96 well plates. Both enzyme and substrate were diluted and dissolved in 50 mM phosphate buffer (pH 6.5) before performing the assay. Pre-incubation was first conducted consist of 10 µL of sample, 130 µL of 30 mM of phosphate buffer solution (pH 6.5) and 15 µL of α-glucosidase solution (3 U/mL) in 96 well plates at 25°C for 5 min. 50 µL of the substrate (1 mM p-nitrophenyl-α-D-glucopyranoside) was loaded next into the well and the reaction mixture was incubated for another 15 min at 25°C. Finally, 50 µL of 2M glycine (pH 10) was added to stop the reaction. Absorbance readings were recorded at 405 nm.

Calculation for the percentage of inhibition (%):
% Inhibition = ((Δ Ac – Δ Ae)/ Δ Ac)

Δ Ac is the difference in absorbance between the control (with enzyme) and the blank control (without enzyme) while Δ Ae is the difference in absorbance between a sample (with enzyme) and the blank sample (without enzyme). The control was conducted in the same way as the experimental sample but with distilled water. For the blank control and experimental samples, the enzyme solution and substrate was replaced by 30 mM phosphate buffer solution and glycine was replaced with distilled water. The percentage inhibition was expressed as % of α-glucosidase enzyme.

Statistical analysis

The results are presented as the mean ± standard deviation. The statistical significance of the difference was evaluated using one way ANOVA with Tukey's post hoc test $p < 0.05$ is considered significant.

RESULTS

The pH values clearly indicate varying strength in acidity (from 1.85 to 3.23) of the herbal mixture obtained from blender and juicer in rA and rB (Fig. 1). No significant difference ($p > 0.05$) was found between herbal mixtures from juicer sample in rA and rB. While the lowest pH was found in herbal mixture from blender sample in rB ratio.

Fig. 2 shows the total phenolic content of herbal mixture samples from blender and juicer samples in rA and rB. As seen from the figure, the highest total phenolic content was found in juicer sample in rB, with 4.95±0.20 µg GAE/ml sample. While the least total phenolic content was found in blender sample in rB, with 3.12±0.02 µg GAE/ml sample. Overall, the total phenolic content for samples were in the order of: Juicer rB ≥ Juicer rA > Blender rA > Juicer rB.

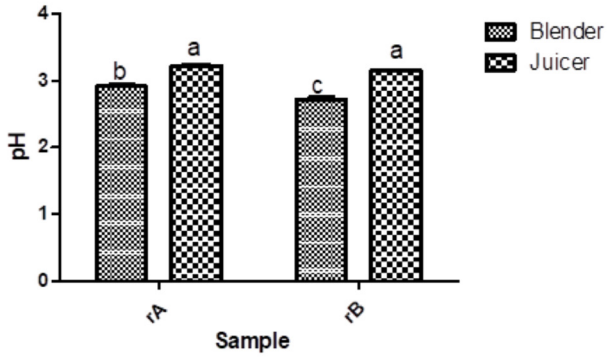

Figure 1: The pH value of herbal mixture samples prepared using blender and juicer at different honey ratios, rA (1:1) and rB (1:3).

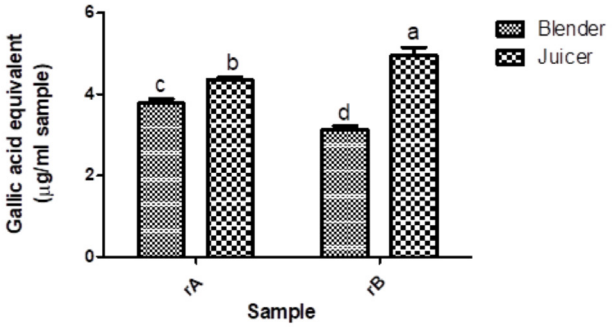

Figure 2: Total phenolic content of herbal mixture samples prepared using blender and juicer at different honey ratios, rA (1:1) and rB (1:3).

The total flavonoid content of the herbal mixtures samples were in the range of 202.84±11.66 to 106.55±15.74 µg QE/ ml sample (Fig. 3). Similarly, the highest concentration of flavonoid was found in Juicer rB. However, there was no significant difference ($p > 0.05$) in flavonoid content of the different ratios used in the juicer samples. The total flavonoid content of the samples were in the order of: Juicer rB ≥ Juicer rA > Blender rA > Juicer rB.

Alpha glucosidase inhibitory assays showed that all herbal mixtures have the capabilities to inhibit the enzyme with more than 90% (Fig. 4). Nevertheless, no significant difference ($p > 0.05$) was found between the samples. The highest percentage of inhibitory was found

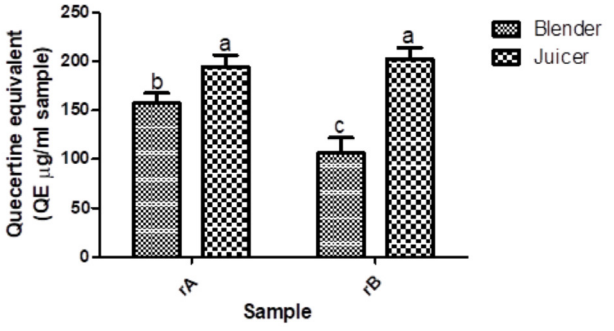

Figure 3: Total flavonoid content of herbal mixture samples prepared using blender and juicer at different honey ratios, rA (1:1) and rB (1:3).

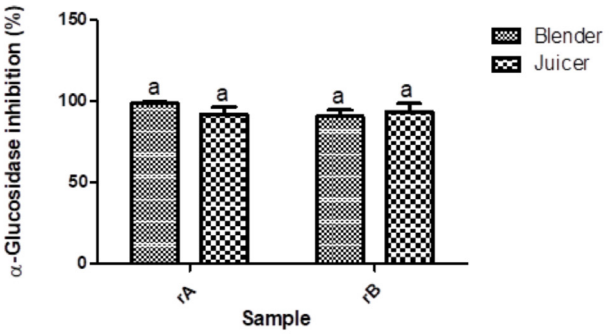

Figure 4: Percentage of alpha glucosidase inhibitory activity of 10 µl of herbal mixture samples prepared using blender and juicer at different honey ratios, rA (1:1) and rB (1:3).

in Blender rA (99.10±0.46 %) followed by Juicer rB (93.55±5.04 %), Juicer rA (92.04±4.36 %) and Blender rB (90.76±3.80 %).

DISCUSSION

The recoveries of bioactive metabolites from plants (i.e. fruits and vegetables) are largely depending on the pre and post-harvest treatment. In this study, different extraction method was used to extract of the juice from the flesh of plant tissue. In addition, different ratio of herbal mixture to honey was also studied to determine the effect of ratio to phytochemical content and bioactivity of the samples. Addition of honey in herbal mixture has been noted to poses synergistic effects in biological activities such as anti-oxidant, anti-microbial, anti-fungal and anti-inflammatory (22-24).

The pH variations in the herbal mixture samples indicate the acidity strength of the mixture (6, 25). Fig. 1 displays the acidity of sample increase as the ratio of honey increases. This is evident especially in both rB samples as the pH is lower (high acidity) compared to rA samples which contributed by the amount of Tualang honey used. Tualang honey has been noted to be more acidic (pH 3.55 – 4.00) than the other types of tropical honey such as kelulut putih, kelulut hitam and gelam (26). It also noted that the pH was lower (high acidity) in mixtures processed using blender compared to juicer. Lower pH in blender to juicer is probably due to the presence of fibers and oxidation of the samples. However these result is in contrast with the previous result by Uckoo et al. (27) conducted on grapefruit, where low level of acidity was detected in blending samples compare to juicer and hand squeezing samples. The differences between previous and current study are probably due to the type of sample itself.

It is well known that the phenolic compounds are the secondary metabolites that is form and used by plants to protect themselves, in response to the external factors such as environmental stress (i.e. drought, floods and salinity) and predators (28). From the results, the highest total phenolic content was found in juicer sample in rB which in line with total flavonoid content, where juicer in rB ratio also have the highest flavonoid content compared to others.

Mechanical process such as crushing and grinding during post processing of samples also influence the metabolites recovery (4). In this study, mechanical processes in extracting juices from the raw samples (lemon, garlic and ginger) were done using either blender or juicer. Juicing using juice extractor is a process where the juices/liquid are forced out or extract out from the sample flesh (fruits, vegetables etc.) using a mechanical press juice extractor and the presence of juice vesicles is minimized by using a strainer (29).

While blender is a process where the samples have direct contact with the rotating blade or propeller and the juices/liquid extracted out of the samples are not separated from the juice vesicles. For blender, addition of water is sometimes needed to aid the extraction process of the juices out of the flesh. All of these processes can increase the surface area in contact with the solvent, which eventually increase the recovery of phytochemical levels in the mixture (4).

Generally, mechanical process will cause the cell wall to rupture and release of phytochemicals into the juice mixture (4). Therefore, the differences in phenolic levels between mixtures using juicer and blender are due to the efficiency of the machine to maximize the juice extraction from the flesh. This result is consistent with study conducted by Pyo et al. (29), where the quantification and identification of ascorbic acid detected by using HPLC is significantly different between the fruit juices extracted with juicer and blender.

High concentration of phenolics in all herbal mixtures samples can also be contributed by food acidulants. Addition of food acidulants or weak acids such as citric acid from lemon juice and acetic acid from apple cider vinegar in the mixtures aided the extraction of phytochemicals (30). Food acidulants have been reported to improve bioaccessibility and minimizing the loss of phytochemicals such as flavonoids and β-carotene in both raw and heated of some vegetables (30, 31).

The α-glucosidase inhibitory test is a test to screen the anti-diabetic potential of samples. The α-glucosidase is an enzyme that responsible in hydrolyzing carbohydrate (such as disaccharide and oligosaccharide) to a simple sugar (i.e monosaccharide) in the digestive system (8, 32). Therefore, the inhibitors will compete with the oligosaccharides or disaccharides and prevent their cleavage to monosaccharides, thereby slowing the rise of glucose level in blood and digestion process (32). From the results there were no significant differences ($p > 0.05$) observed between the samples indicating both extraction and ratio did not influence the inhibitory activities of the mixtures.

The above results implicate that the phenolic content in the samples does not solely influence the inhibitory activity of the herbal mixture samples. The α-glucosidase inhibitory activities of herbal mixture samples are probably due to apple cider vinegar presence in the mixture. Vinegar has been reported to lower post prandial glycaemic and insulinaemic responses, in addition of increasing the satiety in healthy subjects (33-35). In addition, previous study conducted by Shahidi et al. (2008) found that fruits vinegars such as apple cider vinegar contains phytochemicals such as organic acids (i.e acetic acid and malic acids), stilbenes, flavonols, flavanols, benzoic acids derivatives, cinnamic acid derivatives and others (36). Combination of these metabolites might have a synergism effect in inhibiting the α-glucosidase enzyme. Identification and quantification of the metabolites in the herbal mixture is important for the future acute and toxicity study as well as in preparing standardize herbal mixture. Therefore, for the future study it is advice to perform identification and quantification.

CONCLUSION

In conclusion, the best processing technique in extracting the juices from ginger, garlic and lemon for the preparation herbal mixture is by using the juicer. While the best honey ratio is the 3 times honey volume to the base ingredients. Overall, herbal mixture processed with the juicer and have the higher honey ratio (rB samples), contain the highest total phenolic content (TPC) with 4.95±0.20 µg GAE/ml sample, total flavonoid (TFC) (202.84±11.66 µg QE/ ml sample) and lower acidity compared to herbal mixture processed using the blender. These results indicate that extraction techniques and ratio influences the phytochemical levels in the herbal mixtures. In addition, total phenolics and total flavonoid alone are not responsible to inhibitory effect on α-glucosidase. The effects of the α-glucosidase inhibitory activity can be due to the synergism effect of the herbal mixture as a whole.

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