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Phytochemical analysis and salivary amylase inhibition activities of *Carica papaya* leaf and *Garcinia mangostana* pericarp extracts and partially purified fractions

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ABSTRACT

Plant-derived digestive enzyme inhibitors particularly those targeted to carbohydrate metabolism has been the focus of recent studies as natural supplements for weight control and diabetes. The present study explores the salivary amylase inhibition activity of Garcinia mangostana (Linn.) pericarp extracts and Carica papaya (Linn.) leaf extracts and fractions, as well as perform phytochemical screening and quantification, and thin layer – and high performance liquid chromatographic profiling. Results show that crude extracts and purified fractions were able to inhibit salivary amylase, with C. papaya fraction 1 being the most active at 30.89% inhibition. Phytochemical screening of all extracts tested positive for tannins, glycosides, phenolics, flavonoids and alkaloids. Quantification of phenolics showed that extracts contained high levels of phenolics, with C. papaya crude extract having the highest content with 219.0±12.7 mg GAE/g extract followed by G. mangostana crude extract with 247.1±18.0 mg GAE/g extract. Quantification of total flavonoids also showed C. papaya crude extract to contain the highest content with 55.12±0.679 mg QE/g extract. All extracts contained negligible alkaloid content, though. HPLC and TLC profiling showed several peaks and bands, when viewed in 210 nm and UV light, respectively. These results demonstrate in vitro the salivary amylase inhibitory activity of both plants and their potential as antidiabetic drug candidates; however, further studies need to be done, like isolation and structure elucidation of active components and toxicity assays.

Keywords: Amylase inhibition, phytochemical quantification, *Carica papaya, Garcinia mangostana*
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INTRODUCTION

Diabetes mellitus, or simply diabetes, is a group of metabolic disorders characterized by heightened blood glucose levels and caused either by the pancreas' failure to produce enough insulin or the body's cells responding abnormally to insulin [1]. In addition to hyperglycemia, complications like cardiovascular diseases, retinopathy, nephropathy and peripheral gangrene could occur when diabetes is left unchecked [2]. Also, it increases infections like mucormycosis, cystitis, urinary tract infections, intra renal abscesses, pneumonia and malignant otitis externa have been linked to diabetes [3]. Affecting 366 million worldwide (8.30% of the world population) and 4.2 million in the Philippines (8.2% prevalence), diabetes is the eighth leading cause of mortality in the Philippines [2].

Current drugs used in diabetes treatment include sulfonylureas, biguanides, thioazolidinedione, and α-glucosidase inhibitors, sodium-glucose co-transporter inhibitors, glucagon-like peptide-1 analogues and dipeptidyl peptidase-IV inhibitors; however, these are costly and not without side effects [2,4-7]. To circumvent these problems, scientists are exploring natural products as potential sources of diabetes treatments. Currently, more than 400 natural

compounds have been shown to possess antidiabetic effects, both *in vitro* and *in vivo* [8-12]. Several of these are found in the Philippines*, e.g. Momordica charantia* and *Andrographis paniculatac* [13]. Moreover, isolating phytochemicals that inhibit carbohydrate degradation enzymes, such as α-glucosidase and α-amylase, have been the focus of recent studies as a source of potential antidiabetic drugs [14-15].

Carica papaya is an erect, fast growing unbranched tree that is native to Central America, but is now being cultivated in many tropical countries [16]. Traditionally, its latex is used in the treatment for jaundice, diabetes, food poisoning and dog bites [17].

On the other hand, *Garcinia mangostana* is a slow growing tropical evergreen tree mainly found in India, Myanmar, Sri Lanka and Thailand [18]. Traditionally, it is used for its anti-inflammatory, antibacterial, antiparasitic and wound healing properties, as well as for the treatment of diahhrea, eczema and skin infection [19].

The present study aims to demonstrate the salivary amylase inhibitory activities of *C. papaya* leaf and *G. mangostana* pericarp crude extracts and partially purified fractions, as well as screen them for phytochemical constituents. Total flavonoids, phenolics and alkaloid content of the extracts were determined, and thin layer- and high-performance liquid chromatography profiles were also made.

MATERIALS AND METHODS

2.1 Plant sample collection and preparation

C. papaya leaves were collected from Bulacan, Philippines while *G. mangostana* pericarp were collected from fruits from Las Pinas, Metro Manila, Philippines. Voucher samples of the leaves and fruits were authenticated by and stored at the Botany Division, National Museum, Philippines. These were air-dried for a week and then powdered using a homogenizer.

2.2 Extraction and Partial Purification

Extraction was taken out via maceration. Plant materials were soaked in methanol at a ratio of 20 g: 500 mL solvent for 48-72 hours. These were then filtered, and the filtrate collected into clean amber bottles.

Partial purification was done using Silica gel G60 (Merck, Philippines) as the stationary phase and ethyl acetate as the mobile phases. Samples were loaded by loading approximately 10 mL of the filtrates into the column and elution was done isocratically with ethyl acetate. The fractions were pooled based on TLC profile, using chloroform: methanol (4:1) as solvent system.

For the crude extracts and partially purified fractions, the solvents were removed by rotary evaporation to dryness. Residues were stored in 4°C until further use.

2.3 Salivary amylase inhibition

Amylase inhibition assay was performed using DNS assay based on the protocol by Gusakov *et al* (2011) [20]. Saliva was collected using the passive drool technique, then diluted to 1:100 in 0.05 M Tris-EDTA buffer (pH 6.7). Plant treatments – crude and partially purified *C. papaya* and *G. mangostana* extracts – were resolubilized in 0.05 M Tris-EDTA buffer (pH 6.7) to make 2.5 mg/mL samples. 1% starch was prepared from soluble starch (Merck, Philippines).

3,5 – Dinitrosalicylate (DNS) reagent was prepared by dissolving 5 g 3,5 – dinitrosalicylic acid (Sigma-Aldrich, Philippines) in 100 mL 2 M NaOH. One hundred and fifty grams (150 g) sodium potassium tartrate (Sigma-Aldrich, Philippines) was then dissolved in 250 mL distilled water. The two solutions were mixed and diluted to 500 mL to make the DNS reagent.

For each sample tested, a corresponding "blank" without the enzyme was also performed, for use in computation of amylase inhibition. Each sample was performed in triplicates for subsequent statistical analysis.

Hydrolysis was allowed to continue at room temperature (25°C) for 15 minutes. Then, the reaction was stopped by adding 0.5 mL DNS and immediately placing in a boiling water bath for 5 minutes, taking care to cover the tubes to prevent evaporation. The test tubes were cooled and diluted to 10 mL, then read at 540 nm using a FluoStar fluorometer (BMG-LabTech, Philippines).

DNS assay for each plant sample was performed in triplicates. Amylase inhibition activity was calculated using **Equation 1**:

$$\% \text{ inhibition} = (A_{540,\ neg} - A_{540,\ neg,\ blank}) - (A_{540,\ sample} - A_{540,\ sample,\ blank}) \ / \ (A_{540,\ neg} - A_{540,\ neg,\ blank}) * 100\%$$

Equation 1. Calculations for the salivary amylase inhibition assay.

Where, $A_{540,neg}$ is the absorbance of the negative control, $A_{540,\ neg,blank}$ is the absorbance of the negative control – blank, $A_{540,sample}$ is the absorbance of the sample treatment and $A_{540,sample,blank}$ is the absorbance of the sample treatment – blank.

2.4 Thin layer chromatography profiling

Only plant extracts and fractions that tested positive in the amylase inhibition assay had its TLC profiled. Each of these were spotted on thin-layer silica gel plates G60 (Merck, Philippines) and then developed using varying solvents – for the methanolic crude extracts, hexane: ethyl acetate: acetic acid (2:2:1) was used while for the ethyl acetate fractions, hexane: ethyl acetate: acetic acid (3:6:1) was used. After drying, the plates were visualized using long – wave ultraviolet light, as well as in visible light after staining with 5% sulfuric acid in methanol.

2.5 High-performance liquid chromatography profiling

High-performance liquid chromatography was performed on crude extracts only. The HPLC system used was SPD-$10A_{VP}$/$10AV_{VP}$ (Shimadzu, Philippines). These were carried out using normal phase a C_{18} column as the stationary phase and methanol: water:ortho-phosphoric acid (20:79.9:0.1) as the mobile phase, at a flow rate of 1 mL/minute and pressure of 139 kgF/cm^2 and temperature 40°C. Elutions were visualized by UV-Vis detection at 210 nm to detect phenolic compounds [21].

2.6 Phytochemical screening

Phytochemical screening was performed based on the protocols by Aguinaldo et al (2005) [22]. Each extract and crude fraction were screened for tannins, alkaloids, cardiac glycosides, flavonoids, anthraquinones and phenols.

2.7 Total phenolic content

A volume of 15.4 µL of crude extracts and gallic acid standard (at different concentrations, for the standard curve) were mixed with 61.5 µL of Folin Ciocalteu reagent (diluted 1:10 with de-ionized water) and were neutralized with 123µL of 7.5% sodium carbonate. The mixtures were allowed to stand at room temperature for 30 min. The absorbance was measured at 765 nm and total phenolic content was calculated in terms of gallic acid equivalents (GAE).

2.8 Total flavonoid content

One hundred microliter (100µL) of crude extracts and quercetin standard (at different concentrations, for the standard curve) were mixed with 100 µL of 2% $AlCl_3$. The mixtures were allowed to stand at room temperature for 10 min with intermittent shaking. The absorbance was measured at 415 nm and total flavonoid content was calculated in terms of quercetin equivalents (QE).

2.9 Total alkaloid content

Two hundred microliter (200µL) of reserpine standard and crude extracts at different concentrations were mixed with 100 µL of $FeCl_3$ solution and 100 µL of 1,10-phenanthroline solution. The reaction mixture was diluted to 1 mL volume using deionized water. The mixtures were placed in a water bath at 70°C for 30 min. The absorbance was measured at 510 nm and total alkaloid content was calculated in terms of reserpine equivalents (RE).

RESULTS AND DISCUSSION

3.1 Partial Purification

Partial purification with column chromatography yielded several fractions each for *C. papaya* and *G. mangostana* crude extracts; however, after DNS assay only one fraction each (*C. papaya* fraction 1 and *G. mangostana* fraction 1) had inhibition activities. Hence, only the crude extracts and these active fractions were subjected to the other assays.

3.2 Salivary Inhibition Activity

Table 1 summarizes the results of the DNS assay. As observed, *C. papaya* Fraction 1 was the most potent inhibitor, followed by *G. mangostana* Fraction 1 and *G. mangostana* Crude extract. It should be noted that *C. papaya* Crude extract showed negligible inhibition but its partially purified fraction showed the highest. Since purification essentially increases the concentration of the active component and may remove other inhibitor-blockers that could prevent inhibitor-enzyme interactions, then partial purification was successful in increasing the activity of the *C. papaya* extract.

Table 1: Salivary amylase inhibition activities of the crude extracts and partially purified fractions
'NI'=No Inhibition

Plant sample	Extract	% Inhibition
C. papaya	Crude extract	NI
	Fraction 1	30.89%
G. mangostana	Crude extract	8.92%
	Fraction 1	24.32%

3.3 Thin Layer Chromatography Profiling

The solvent system used were optimized for each extract. **Table 2** shows the Rf values for each band observed after staining and under UV light. As observed, several bands can be observed for the TLC profiles of both crude extracts and partially purified fractions. There are more bands observed in the crude extract of *C. papaya* than in the partially purified fraction; thus, purification was successful in removing unwanted phytochemicals. However, the number of spots in the crude extract and partially purified fraction of *G. mangostana* was the same; however, their Rf values were not the same. Since the amylase inhibition activity increased nonetheless, the extract was still purified. Thus, the same number of bands could have been due to similar mobilities of different phytochemicals in the crude extract, congregating into larger bands containing different components.

Table 2: Rf values of all phytochemicals present viewed after sulfuric acid staining (counted as yellow bands) and long-wave UV light (counted as fluorescent bands)

Plant samples	Rf values of phytochemicals present			
	Number of bands	Crude extract	Number of bands	Fraction 1
C. papaya	6	0.092, 0.185, 0.308, 0.431, 0.785, 0.877	2	0.0769, 0.846
G. mangostana	7	0.108, 0.169, 0.215, 0.431, 0.738, 0.846, 0.923	7	0.154, 0.185, 0.385, 0.646, 0.831, 0.892, 0.938

3.4 High-Performance Liquid Chromatography Profiling

Figures 1 and 2 shows the HPLC profiles of *C. papaya* and *G. mangostana* crude extracts. Elutions were detected at 210 nm, corresponding to the maximum absorbance of phenolics[21]. Several peaks can be observed from both profiles, implying that multiple types of phenolic compounds are present in each crude extract. However, there are more peaks observed from the *G. mangostana* profile compared to the *C. papaya* profile. These corresponds to the phytochemical screening and total phenolic quantification results below – both contain high levels of phenolic compounds, but *G. mangostana* crude extract has higher phenolic content.

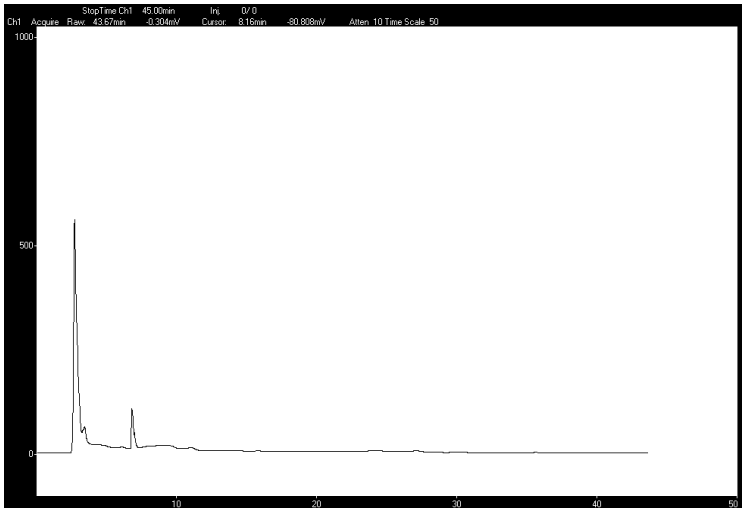

Figure 1: HPLC profile of C. papaya crude methanolic extract (x-axis: retention time; y-axis: absorbance at 210 nm). Two prominent peaks can be observed

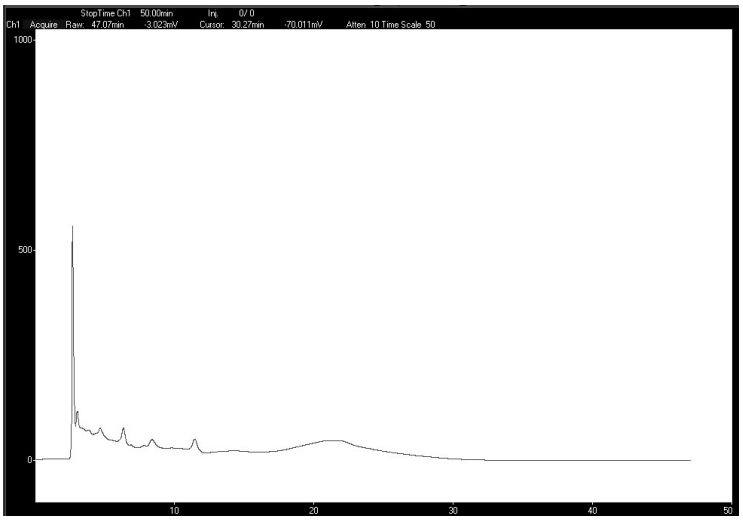

Figure 2: HPLC profile of G. mangostana crude methanolic extract (x-axis: retention time; y-axis: absorbance at 210 nm). Two prominent peaks and several other less prominent peaks can be observed

3.5 Phytochemical Screening

Table 3 shows the phytochemical screening results of all crude extracts and fractions. *C. papaya* and *G. mangostana* crude extracts and fractions all contained tannins and phenols. On the other hand, all of these extracts and fractions except *C. papaya* fraction 1 contain glycosides and flavonoids. Only *G. mangostana* crude extract and fraction contain anthraquinones and all of them contain negligible amounts of alkaloids. It should be noted that *C. papaya* crude extract contains glycosides and flavonoids whereas the fraction does not; this is a consequence of the purification process, removing the glycosides and flavonoids from the fraction.

Table 3: Phytochemical screening results of the crude extracts and partially purified fractions

Legend: '+' = Present, '-' = Absent.

Plant sample		Tannins	Alkaloids	Glycosides	Flavonoids	Anthra-quinones	Phenolics
C. papaya	Crude extract	+	-	+	+	-	+
	Fraction 1	+	-	-	-	-	+
G. mangostana	Crude extract	+	-	+	+	+	+
	Fraction 1	+	-	+	+	+	+

3.6 Total phenolics, flavonoids and alkaloids quantification

Only crude extracts had total phenolics, flavonoids and alkaloids quantified (**Table 4**). Based on the phytochemical screening results, both extracts were positive for flavonoids and phenolics and negative for alkaloids. The results of the quantification correlates with the screening results – both extracts had high phenolic concentrations (more than 200 mg GAE/g extract) and moderately high flavonoid concentration (more than 10 mg QE/g extract). Since both extracts had negligible alkaloid content (less than 5 mg RE/g extract), these were not detected in the screening.

Table 4: Total phenolic, flavonoid and alkaloid contents of the crude *C. papaya* and *G. mangostana* methanolic extracts

Plant sample	Total phenolics content (mg GAE/g extract)	Total flavonoids content (mg QE/g extract)	Total alkaloids content (mg RE/g extract)
C. papaya crude extract	219.0±12.7	55.12±0.679	3.439±0.0919
G. mangostana crude extract	247.1±18.0	16.88±2.08	2.951±0.0391

DISCUSSION

The results above demonstrate the salivary amylase inhibition activities of *C. papaya* and *G. mangostana in vitro*, in addition to their known hypoglycemic activities *in vivo*. The aqueous extract of *C. papaya* has been demonstrated previously to possess hypoglycemic activity in streptozotocin-induced diabetic Winstar rats [23-24], as well as its unripe mature fruit aqueous extracts in diabetic Winstar rats [24]. Also, the aqueous extracts of *C. papaya* leaves have antihyperglycemic and hypolipidemic activities in alloxan-induced albino rats [25]. On the other hand, *G. mangostana* rind has been previously reported to possess antihyperglycemic activity, antioxidant activity and high total phenolic content [26].

The phytochemical profiles of both extracts and fractions show relatively high concentrations of either flavonoids, phenolics, or both. Wongsa *et al.* (2011) screened thirty herbs for total phenolic content and α-amylase inhibition activities, and correlated these to one another [27]. Their results show significant correlation (Pearson correlation matrix, significant at $p<0.05$, *p-value* = 0.01) between α-amylase inhibition and total phenolic content; hence, at higher total phenolic activity, we expect higher α-amylase inhibition activity. Moreover, other literature suggest that phenolic-rich extracts have high propensity for α-amylase or α-glucosidase inhibition activity [28-29]. Flavonoids have also been shown to possess α-amylase inhibition activity, both *in vitro* using IC_{50} values and percent inhibitions as basis for comparisons and *in silico* using docking scores [30]. Since all the crude extracts and fractions have high flavonoid content, phenolics content, or both, then the salivary amylase activities of these extracts may have been due to these phytochemicals.

CONCLUSION

The present study demonstrated the salivary inhibition activities of *G. mangostana* and *C. papaya* methanol extracts, in correlation to their phytochemical content and TLC and HPLC profiles. The most active fractions, *C. papaya* fraction 1 and *G. mangostana* fraction 1, could be candidates for potential antidiabetic therapy. However, further studies involving isolation of the active components, *in vivo* assays and toxicity assays need to be addressed first.

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