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Phenolics-rich extract of guava stem bark inhibits enzymes associated with nephrolithiasis and obesity *in vitro*

Emmanuel Anyachukwu Irondi[·]
Department of Medical Biochemistry and Pharmacology, Kwara State University, Malete, P.M.B. 1530, Ilorin, Nigeria

Abstract

Aim: Inhibition of enzymes catalyzing the production of metabolites that predispose to metabolic diseases is an important index of the potential health benefits of natural products. In this study, the ability of guava (*Psidium guajava* Linn.) stem bark phenolics-rich extract to inhibit enzymes associated with nephrolithiasis (xanthine oxidase and urease) and obesity [pancreatic lipase and 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase] was evaluated *in vitro*.

Methods: The phenolics profile of the extract was determined using HPLC-DAD; while the enzymes inhibitory and anti-radical properties of the extract were tested using spectrophotometric methods.

Results: HPLC-DAD fingerprinting revealed that ellagic acid and quercetin were the most abundant phenolic acid and flavonoids, respectively, in the extract. The extract inhibited xanthine oxidase, urease, pancreatic lipase and HMG-CoA reductase, with IC_{50} values below 40 µg/mL. The extract also exhibited potent anti-radical activity by scavenging $ABTS^{*+}$, $DPPH^{*}$ and chelating F^{2+}.

Conclusions: Hence, guava stem bark may be a source of nutraceuticals for suppressing the production of uric acid, ammonium hydroxide, fatty acids and cholesterol, and mitigating oxidative stress. These bioactivities, which can be attributed to the phenolic compounds, suggest anti-nephrolithiatic and anti-obesity potentials of guava stem bark phenolics-rich extract.

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· **Contact:** Emmanuel Anyachukwu Irondi ✉ emmanuel.irondi@kwasu.edu.ng; irondi.emmanuel@yahoo.com
Tel: +2348034870657

Introduction

Several studies have consistently established a strong positive association between increased risk of nephrolithiasis (kidney stone) and obesity [1-4]. A recent report indicated that nephrolithiasis has become a highly prevalent disease globally, with a high level of both acute and chronic morbidity, and expensive care cost [5]. Nephrolithiasis is a heterogeneous disease, of which uric acid nephrolithiasis represents about 10% of all nephrolithiasis cases [6]. On its part, obesity is regarded as the most challenging public health problem of this 21st century worldwide [7], affecting 603.7 million adults and 107.7 million children globally [8].

The roles of enzymes such as xanthine oxidase and urease in the development of nephrolithiasis, and pancreatic lipase and 3-hydroxy-3-methylglutaryl coenzyme A [HMG-CoA] reductase in the development of obesity have been documented. Xanthine oxidase catalyzes the oxidation of hypoxanthine first to xanthine, and ultimately to uric acid during the degradation of purine nucleotides, with a concomitant generation of superoxide [9]. Accumulation of the uric acid so-produced precipitates the development of uric acid nephrolithiasis [10]. Urease, produced by gram-negative urea-splitting bacteria in susceptible human urinary system, is responsible for catalyzing the hydrolysis of urea to produce ammonia (NH_3) and carbon dioxide (CO_2) [11]. The ammonia produced combines with water, to form ammonium hydroxide, which results in the alkalinization of urine. The alkaline urine enhances trivalent phosphates, ammonium and carbonate ions formation, leading to precipitation of apatite and struvite crystals [12]. Thus, urease is now known to contribute to the development of several human diseases, including nephrolithiasis [11, 13], especially, struvite and calcium stones [12].

Table 1 – Phenolics profile of guava stem bark extract

Compounds	Quantity (mg/g)	t_R (min)	LOD (μg/mL)	LOQ (μg/mL)
Gallic acid	1.64 ± 0.02	13.07	0.009	0.030
Catechin	0.39 ± 0.03	15.26	0.017	0.056
Chlorogenic acid	2.81 ± 0.01	20.11	0.023	0.076
Caffeic acid	0.42 ± 0.01	24.19	0.026	0.085
Ellagic acid	3.96 ± 0.01	29.87	0.008	0.026
Rutin	1.37 ± 0.04	41.08	0.015	0.049
Quercetin	4.08 ± 0.02	49.23	0.029	0.097
Luteolin	2.83 ± 0.01	55.16	0.011	0.036

Results are expressed as average ± standard deviations (SD) of triplicate determinations. t_R – retention time; LOD - limit of detection; LOQ - limit of quantification.

Pancreatic lipase is responsible for catalyzing the hydrolysis of dietary triacylglycerol to produce fatty acids and monoacylglycerols in the small intestine [14]. Hence, delay and suppression of digestion and absorption of triacylglycerol through pancreatic lipase inhibition is a clinical approach for controlling hyperlipidemia and obesity [15]. HMG-CoA reductase catalyzes the rate limiting reaction in cholesterol and isopernoides biosynthesis, in which HMG-CoA is converted to mevalonate [16]. Elevated blood cholesterol content (hypercholesterolemia) underlies the pathogenesis of certain chronic diseases such as obesity, and precipitates generation of free radicals [17]. The catalytic roles of the above-mentioned enzymes in the production and accumulation of uric acid, fatty acids and cholesterol have made their inhibition a clinical strategy for managing uric acid nephrolithiasis and obesity. Hence, the use of urate-lowering therapies, such as allopurinol, is one of the clinical strategies currently available for the treatment uric acid nephrolithiasis. Allopurinol, a structural analogue of hypoxanthine, competitively inhibits xanthine oxidase; thereby

retarding the rate of uric acid formation and its burden to the nephrons [10]. However, the clinical use of allopurinol is associated with some side effects such as renal and hepatic impairment that interfere with its clinical use [18]. Similarly, orlistat used for the treatment of obesity, retard dietary fats digestion and absorption through inhibition of pancreatic lipase [19]. Statins mitigate hypercholesterolemia through HMG-CoA reductase inhibition [20]. However, as with allopurinol, both orlistat and HMG-CoA reductase inhibitors (statins) have some adverse effects that limit their clinical uses. Whereas orlistat is associated with gastrointestinal tract and hepatic dysfunctions [21], statins are associated with hepatic damage, myopathy and rhabdomyolysis [22]. Hence, discovery of natural inhibitors of xanthine oxidase, urease, pancreatic lipase and HMG-CoA reductase that could be useful for the treatment of nephrolithiasis and obesity is desirable.

Guava belongs to the Myrtaceae family, and is commonly found in the tropical and sub-tropical countries of the world. It serves as food, and various parts of it, including the leaf, roots and stem bark, have medicinal applications in folk medicine [23]. However, whereas several pharmacological activities such as anti-bacterial, antioxidant and anti-tumor [24], anti-hyperlipidemic and anti-diabetic [25], analgesic and anti-inflammatory [26], and anti-gout and anti-hypertensive [27] effects have been reported for the leaves, most studies on the stem bark focused on its antimicrobial effects [23, 28]. Hence, to further explore the pharmacological benefits of guava stem bark, this study evaluated phenolics profile, anti-nephrolithiasis, anti-obesity and anti-radical properties of its phenolics-rich extract *in vitro*.

Materials and method

Chemicals and reagents

HMG-CoA, HMG-CoA reductase, thiourea, jack bean urease, urea, NADPH, porcine pancreatic lipase, xanthine oxidase, allopurinol, xanthine, orlistat, Trolox, catechin, quercetin, rutin, luteolin, L-ascorbic acid, 2,2´-azino-bis-3-ethylbenzthiazoline-6-sulphonic (ABTS) and 2,2-diphenylpicrylhydrazyl (DPPH) were products of Sigma (St. Louis, USA). Caffeic, chlorogenic, ellagic, formic and gallic acids, and HPLC grade methanol were products of Merck (Darmstadt, Germany).

Collection and preparation of guava bark

Guava stem bark sample (200 g) was obtained from a guava tree at Moniya area in Ibadan, Nigeria. The sample was air-dried at room temperature for two weeks, and later ground into fine powdery form with a kitchen blender (Marlex). Powdery sample was hermetically packed in an opaque plastic vial, and stored in the refrigerator during analysis.

Preparation of guava stem bark phenolics-rich extract

Phenolics-rich extract of guava bark was prepared as per the method described by Kuo et al. [29]. A portion of 100 g bark powder was extracted with methanol (300 mL) in a soxhlet extraction apparatus at 50 ^{o}C for 3 h in three successive rounds. After each round, the extract was collected by filtering the sample through Whatman filter paper (No. 2). Afterwards, the extract was pooled and partitioned with hexane (200 mL) in a separatory funnel, to get rid of its lipophilic constituents. The aqueous phase was further extracted with ethyl acetate (180 mL) in three successive rounds, and the resulting extract was concentrated under reduced pressure in a rotary evaporator at 45 ^{o}C. The residue obtained was used for the various assays.

Quantification of compounds by HPLC-DAD

Polyphenolics profiling of the extract was performed at ambient temperature in triplicates using a reverse-phase HPLC attached with diode-array detector (DAD) (Shimadzu, Japan). The extract was injected at 12 mg/mL and separation of the phenolic constituents was achieved using reversed phase C_{18} column (4.6 mm x 150 mm) packed with particles of 5 µm diameter. The mobile phase comprised HPLC grade water with 1% formic acid (v/v) (solvent A) and methanol (solvent B) at a flow rate of 0.7 mL/min, injection volume 40 µL. The gradient system was: 13% solvent B for the first 10 min, followed by 20%, 30%, 50%, 60%, 70%, 20% and10% B at 20, 30, 40, 50, 60, 70 and 80 min, respectively [30]. Polyphenolics were quantified by integration of chromatography peaks using the external standard method; while the peaks were confirmed by matching their retention time with those of their respective reference standards and by DAD spectra (210 to 500 nm).

Enzymes Inhibition Assays

Xanthine oxidase inhibition assay

Xanthine oxidase inhibition assay was performed as per the spectrophotometric method reported by Osada et al. [31]. Xanthine was used as substrate, while allopurinol was used as reference inhibitor. Xanthine (15 mM) and XO (0.1 mU/µL) solutions were freshly prepared with Tris–HCl buffer (50 mM, pH 7.4). Afterwards, 40 µL of xanthine solution, 10 µL of xanthine oxidase solution and 1950 µL of varied concentrations (10, 20, 30, 40 µg/mL) of extract were mixed and incubated at 37 ^{o}C for 10 min. Next, 50 µL of perchloric acid solution in the same Tris–HCl buffer (3.2%, v/v) was added to terminate the XO-catalyzed hydrolysis of xanthine. Absorbance of the uric acid formed was then measured at 292 nm, and the percentage xanthine oxidase inhibition by extract was calculated.

Urease inhibition assay

Urease inhibition assay was performed as per the spectrophotometric method reported by Jaffary et al.

[32] with little modifications, using urea as substrate and thiourea as reference inhibitor. In brief, 500 µL of jack bean urease and 100 µL of varied concentrations (10, 20, 30, 40 µg/mL) of the extract was incubated at 37 °C for 30 min. Next, 1100 µL of urea was added to the reaction mixture and the mixture was incubated at 37 °C for 30 min. Afterwards, phenol reagent (1% phenol and 0.005% sodium nitroprusside, w/v) and alkali reagent (0.5% NaOH and 0.1% sodium hypochlorite, w/v) were added to the reaction mixture. Following this, the reaction mixture was incubated at 37 °C for 2 h, and the absorbance of ammonia produced by urease-catalyzed hydrolysis of urea was measured at 635 nm. The percentage inhibition of urease by the extract was calculated.

Pancreatic lipase inhibition assay

Pancreatic lipase inhibition was assayed as per the spectrophotometric method described by Eom et al. [33]. P-nitrophenyl butyrate served as substrate, while orlistat served as reference inhibitor. Enzyme solution was prepared by mixing 30 µL of pancreatic lipase (10 units) in 10 mmol/L morpholinepropane sulphonic acid and 1 mmol/L EDTA (pH 6.8), and 850 µL of Tris buffer containing (100 mmol/L Tris-HC1 and 5 mmol/L $CaCl_2$, pH 7.0). Next, 100 µL of varied concentrations (10, 20, 30, 40 µg/mL) of the extract (or orlistat) and 880 µL of the enzyme solution was incubated at 37 °C for 10 min. Following this, 20 µL of 10 mM p-nitrophenyl butyrate solution in dimethyl formamide was added to initiate hydrolytic reaction at 37 °C for 20 min. Absorbance of the p-nitrophenol produced from the hydrolytic reaction was measure at 405 nm, and percentage pancreatic lipase inhibition by the extract was calculated.

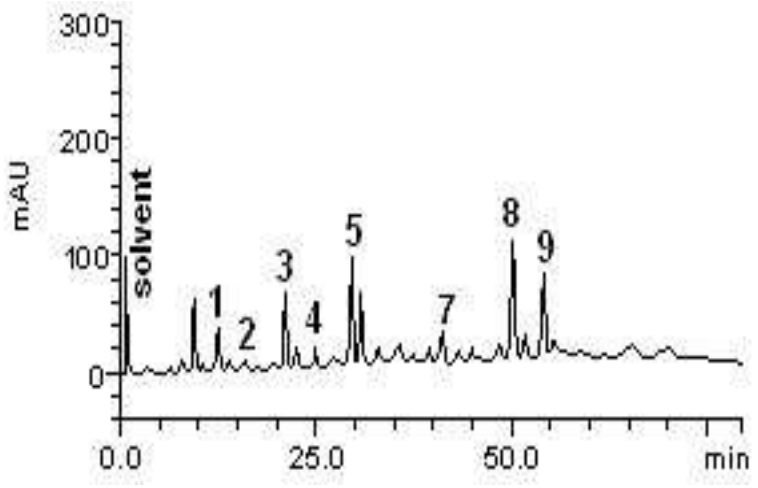

Figure 1. HPLC-DAD chromatogram of guava stem bark extract. Gallic acid (peak 1), catechin (peak 2), chlorogenic acid (peak 3), caffeic acid (peak 4), ellagic acid (peak 5), rutin (peak 7), quercetin (peak 8) and luteolin (peak 9).

Table 2. IC_{50} of guava stem bark extract on xanthine oxidase, urease, pancreatic lipase and HMG-CoA reductase

Inhibitor	Xanthine oxidase IC_{50} (µg/mL)	Urease IC_{50} (µg/mL)	Pancreatic lipase IC_{50} (µg/mL)	HMG-CoA reductase IC_{50} (µg/mL)
Extract	36.39 ± 1.68[a]	32.02 ± 1.51[a]	21.66 ± 1.47[a]	39.14 ± 1.92[a]
Allopurinol	6.85 ± 0.41[b]	-	-	-
Thiourea	-	3.89 ± 0.11[b]	-	-
Orlistat	-	-	1.08 ± 0.03[b]	-

Simvastatin	-	-	-	0.01 ± 0.00[b]

Results are mean ± SD (standard deviation) of three tests. Values with different superscript alphabets along the same column differ significantly ($p < 0.05$).

HMG-CoA reductase inhibition assay

HMG-CoA reductase inhibition was assayed as per the spectrophotometric method reported by Xie et al. [34] with slight modification, using HMG-CoA as substrate and simvastatin as reference inhibitor. In brief, 4 U/mL HMG-CoA reductase (0.1 mL), 0.15 µM HMG-CoA (0.1 mL), 0.1 mL of varied concentrations (10, 20, 30, 40 µg/mL) of the extract and 1.6 mL of 0.1 M potassium phosphate buffer (pH 7.0) containing (3.5 mM EDTA, 10 mM dithiothreitol, and 0.1 M bovine serum albumin) were mixed and incubated at 37 °C for 5 min. Next, 0.30 µM NADPH solution (0.1 mL) was added and absorbance was measured at 340 nm within 1 – 2 min. Subsequently, percentage inhibition of HMGCoA reductase by extract was calculated.

***In vitro* anti-radicals assays**

DPPH* scavenging assay was performed as per the method described by Cervato et al. [35], using ascorbic acid as reference antioxidant. ABTS^{*+} scavenging assay was carried as per the method described by Re et al. [36]. Iron (II) (Fe^{2+}) chelation assay was performed as per the method reported by Puntel et al. [37].

Table 3. DPPH* SC_{50}, ABTS^{*+} scavenging ability and Fe^{2+} chelation of guava stem bark extract

Anti-radical activity	Guava bark	Ascorbic acid
DPPH* SC_{50} (µg/mL)	15.04 ± 0.92[a]	6.07 ± 0.44[b]
ABTS^{*+} scavenging ability (mmol TEAC/g)	2.89 ± 0.11	-
Fe^{2+} chelation IC_{50} (µg/mL)	21.03 ± 1.83[a]	8.76 ± 0.39

Results are mean ± SD (standard deviation) of three tests. Values with different superscript alphabets along the same row differ significantly ($p < 0.05$).

Data analysis

The average results of triplicate determinations ($n = 3$) were analyzed using independent samples t-test at $p < 0.05$, using SPSS statistical software, version 17. IC_{50} and SC_{50} values of the extract on enzymes and free radicals, respectively, were calculated using Graphpad Prism®, version 4.0 (Sandiego, CA).

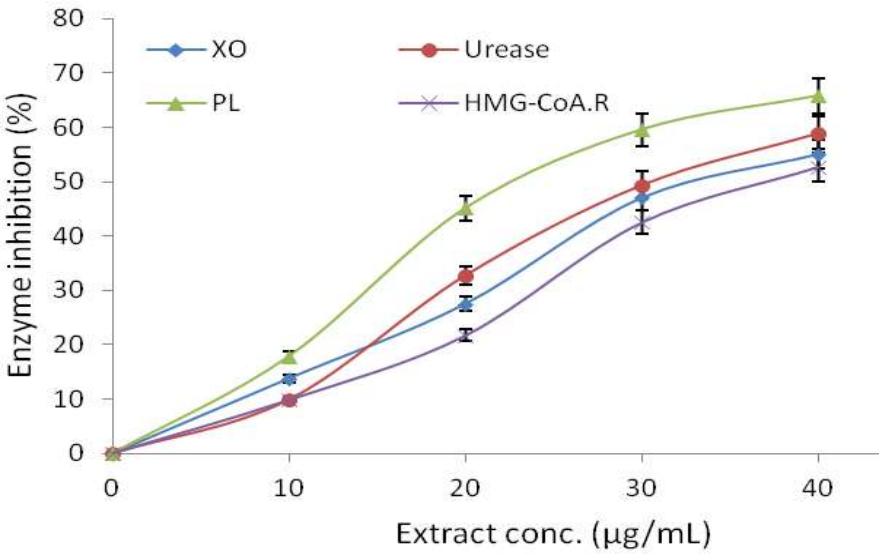

Figure 2. Dose-dependent inhibition of xanthine oxidase (XO), urease, pancreatic lipase (PL) and HMG-CoA reductase (HMG-CoA.R) by guava stem bark extract

Results and discussion

Phenolics profile

A representative chromatogram of the phenolics (phenolic acids and flavonoids) profile of guava stem bark extract, as obtained using HPLC-DAD, is depicted in Figure 1. The extract contained phenolic acids including gallic acid (peak 1), chlorogenic acid (peak 3), caffeic acid (peak 4) and ellagic acid (peak 5); and flavonoids

including catechin (peak 2), rutin (peak 7), quercetin (peak 8) and luteolin (peak 9). The quantities (mg/g) of the individual phenolic acids and flavonoids are presented in Table 1. Among the phenolic acids, ellagic acid was the most abundant followed by chlorogenic acid; while quercetin was the most abundant flavonoid followed by luteolin. Phenolic compounds are a well-known class of bioactive constituents in many natural products of plants, with diverse health benefits [38], such as anti-hypertensive, anti-obesity [18], antidiabetic and antioxidant [39] activities.

Enzymes inhibitory activity

Inhibition of enzymes catalyzing the production of metabolites whose accumulation predisposes to metabolic disorders has become an important index for testing the potential health benefits of plant natural products [40]. In this study, the extract of guava stem bark inhibited xanthine oxidase, urease, pancreatic lipase and HMG-CoA reductase (Table 2), in a dose-dependent fashion (Figure 2).

Xanthine oxidase inhibition is a clinical strategy for treating nephrolithiasis, especially uric acid stone. In this study, the inhibitory strength of the guava bark extract (IC_{50}: 36.39 ± 1.68 µg/mL) against xanthine oxidase is lower than that of allopurinol (IC_{50} of 6.85 ± 0.41 µg/mL), a reference xanthine oxidase inhibitor. However, the guava stem bark extract proved to be a stronger inhibitor of xanthine oxidase than guava leaves extract (IC_{50}: 38.24 ± 2.32 µg/mL) as earlier reported [27]. The inhibition of xanthine oxidase by the extract indicates that it may retard the conversion of hypoxanthine to xanthine and finally uric acid, thereby preventing the subsequent crystallization of the uric acid to form uric acid stone in the kidneys, as allopurinol does [41].

The inhibitory potency of the guava stem bark extract (IC_{50}: 32.02 ± 1.51 µg/mL) against urease in this study is lower than that of methanol extract of onion solid waste (IC_{50}: 18.2 ± 0.22 µg/mL), but comparable with that of chloroform extract of the same onion solid waste (IC_{50}: 32.5±0.28 µg/mL), as reported by Nile et al. [42]. However, thiourea, a reference inhibitor of urease with a lower IC_{50} of 3.89 ± 0.11 µg/mL, displayed a much stronger inhibitory effect against urease than the guava stem bark extract. By inhibiting urease, the guava bark extract may mitigate the precipitation of apatite and struvite crystals, and the subsequent development of nephrolithiasis [12].

It has earlier been stated that obesity increases the risk of nephrolithiasis [1]. Hence, the ability of the guava stem bark extract to inhibit obesity-related enzymes [pancreatic lipase and 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase] was tested. The extract inhibited pancreatic lipase, with IC_{50} value of 21.66 ± 1.47 µg/mL, compared to that of orlistat (1.08 ± 0.03 µg/mL), a reference pancreatic lipase inhibitor. When compared to other natural products that were recently reported to inhibit pancreatic lipase, the guava stem bark extract had a much stronger inhibitory effect than the ethanol extract of *Ceylon cinnamon* bark (IC_{50}: 301.09 ± 4.05 µg/mL) [43], but a weaker inhibitory effect than topical almond leaf (10.82 ±1.02 µg/mL) [18]. Through the inhibition of pancreatic lipase, the guava bark extract may help in retarding the rate of digestion of dietary fats and the subsequent uptake of fatty acids; thereby protecting against overweight and obesity [44].

The guava stem bark extract inhibited HMG-CoA reductase, with an IC_{50} value of 39.14 ± 1.92 µg/mL. Although the inhibitory strength of the guava extract is much lower than that of simvastatin, a standard drug (IC_{50}: 0.01 ± 0.00 µg/mL), it is about thrice stronger than that of *Citrus aurantium* blossoms (IC_{50}: 117.165 µg/mL) [45], and four times that of ethanol extract of *Ceylon cinnamon* bark (IC_{50}: 153.07 ± 8.38 µg/mL) [43]. As the rate-limiting enzyme in the biosynthesis of cholesterol and other isoprenoids, inhibition of HMG-CoA reductase by statins is a major clinical strategy for the treatment of hypercholesterolemia [16] in medical conditions such as obesity and CVD. Studies have confirmed that the clinical use of HMG-CoA reductase inhibitors (statins) offered protection against the formation of kidney stones [46, 47]. Given these previous findings and the established positive association between obesity and kidney stones, the guava bark extract may mitigate kidney stone formation through the inhibition of HMG-CoA reductase.

Anti-radicals activity

The reactions catalyzed by some of the enzymes tested in this study precipitate reactive oxygen species (ROS) and free radicals production [42, 48]. These ROS and free radicals, on accumulation, result in oxidative stress, which is a hallmark of many metabolic diseases, including nephrolithiasis and obesity. Hence, the anti-radical activity of the extract was evaluated, and the result is presented in Table 3. With a DPPH SC_{50} of 15.04 ± 0.92 µg/mL and $ABTS^{*+}$ scavenging ability of 2.89 ± 0.11 mmol TEAC/g, the guava stem bark extract in this study had a slightly weaker free radical scavenging potency than guava leaves extract, which was reported to have a DPPH IC_{50} of 13.38±0.86 µg/mL and $ABTS^{*+}$ scavenging ability of 3.20±0.14 mmol TEAC/g [27].

The ability of the guava stem bark extract to inhibit the enzymes and scavenge the free radicals may have derived from its phenolic constituents, especially the most abundant flavonoid (quercetin) and phenolic acid (ellagic acid) (Table 1). Generally, plant-derived phenolic compounds have affinity for proteins, via hydrogen and hydrophobic interactions, enabling them to inhibit enzymes. The interactions, made possible by the functional groups of the phenolic compounds, lead to the denaturation of the enzyme and attenuation of

their catalytic activities [18, 49]. Quercetin the most abundant flavonoid in the extract (Table 1) may have contributed more to the urease inhibitory activity of the extract, having been reported earlier to be a very potent inhibitor of urease [42]. Similar to thiourea, a reference inhibitor of urease, the dihydroxyl group at the C-ring of quercetin structure may interact with the amino acid residues around the nickel atoms within urease active site, to inhibit the enzyme [42]. In addition, pancreatic lipase [50] and xanthine oxidase [51] were reported to be strongly inhibited by quercetin.

Conclusions

In this study, phenolics-rich extract of guava stem bark had quercetin and ellagic acid as the most abundant flavonoid and phenolic acid, respectively. The extract inhibited xanthine oxidase, urease, pancreatic lipase and HMG-CoA reductase in a dose-dependent pattern. The extract also expressed anti-radicals activity by scavenging $DPPH^{*}$ and $ABTS^{*+}$, and chelating Fe^{2+}. These results suggest that the extract may suppress the production of uric acid, ammonium hydroxide, fatty acids and cholesterol, and extenuate oxidative stress, which is important for the treatment of nephrolithiasis and obesity.

Conflict of Interest

The author has no conflict of interest to declare.

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