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In vitro anti-diabetic activity and phenolic compound profile of ethanol extracts of *Anisophyllea laurina R.* Br. ex Sabine leaves and stem bark

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Abstract:

An essential strategy in the control of diabetes mellitus and its consequences, especially in diabetes type 2, is the efficient management of postprandial hyperglycemia. Inhibitors of carbohydrate hydrolyzing enzymes, α-amylase and α-glucosidase are useful in the treatment of diabetes and most medicinal plants also serve as active principal ingredients in averting this menace. Anisophyllea laurinaR.Br.ex Sabine leaves and stem bark are considered useful for the treatment of diabetes mellitus in sub-tropical countries in Africa. In this in vitro enzyme inhibition assays, all tested concentrations of 80 % ethanol (v/v) extracts of the leaves and stem bark showed significant inhibitory activity against α-amylase and α-glucosidase. At a concentration of 4.0 mg/ml, the leaves extract exhibited α-amylase and α- glucosidase inhibitory activity of 78.5 % and 58.2 % respectively, with IC_{50} values of 2.40 mg/ml and 3.11mg/ml respectively. The stem extracts showed α-amylase and α-glucosidase inhibitory activity of 69.5 % and 63.6 % respectively, with IC50 values of 2.6 mg/ml and 3.5 mg/ml respectively. Acarbose, a drug clinically used for treatment of diabetes had an inhibition of 84 % to both of these enzymes. Digalloyl-HHDP-

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glucose isomer, Quercetin 3'- sulfate, Quercetin, Caffeoyl tarrtaric acid hexose, Quercentin 3-O-glucoside, Quercentin 3-rhamnoside, Quercentin Isorhamnetin-O-hexoside isomer and (-)-Epicatechin 3-O-gallate were the major phenolic compounds identified from leaves and stem bark extracts. Therefore, Anisophyllea laurinaR.Br.ex Sabine plant has the potential to be used as a dietary supplement or in the manufacture of drugs for the control of increased glucose level in the blood.

Key words: α-amylase inhibitory activity, Anisophyllea *laurinaR.Br.ex Sabine,* α-glucosidase inhibitory activity, phenolic compounds, type 2 diabetes

1. Introduction

Recent years have experienced a sharp increase in the incidence and prevalence of diabetes mellitus. Diabetes mellitus, an endocrine disorder characterized by high concentration of glucose in the blood (hyperglycemia) is associated with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both [1]. Diabetes mellitus is one of the common metabolic disorders and 2.8% of the population suffers from this disease throughout the world and it may reach 5.4% by the year 2025. [2]. Today, numerous therapeutic strategies for the treatment of diabetes are in use, but one useful therapeutic approach of treating diabetes is to decrease postprandial hyperglycemia. This can be achieved by delaying the process of glucose absorption through the inhibition of carbohydrate hydrolyzing enzymes α-glucosidase (EC 3.2.1.20) and α-amylase (EC 3.2.1.1) in the digestive tract. The enzymes involved in the digestion of carbohydrates, can significantly decrease the postprandial increase of blood glucose after consumption of a mixed carbohydrate diet and thereby creating a vital platform in the management of postprandial blood glucose level in type 2

diabetic patients and borderline patients [3, 4]. Recently, there has been keen interest in functional foods and plant based medicines that exert modifying physiological effects in the prevention and cure of diabetes and obesity. Hence the attractive targets like in vitro inhibition of α-glycosidase and α-amylase enzymes are currently in demand. [4]

Plant based medicines now serve the new approach of research study used in the fight against diabetes mellitus and other chronic diseases. Plant materials which are being used as traditional medicine for the treatment of diabetes are considered as one of the good sources for new drugs or a lead to make a new chemical entity (NCE). Plant extracts or different folk plant preparations are being prescribed by the traditional practitioners and have also been accepted by the users for the treatment of diabetes and any other diseases in many countries especially in the third world countries. Currently more than 400 plants are being used in different forms for hypoglycemic effects since all the claims of practitioners or users are neither baseless nor absolute. Therefore, a proper scientific evaluation and screening of plants by pharmacological tests followed by chemical investigations is of utmost importance [5].

Anisophyllea laurina R.Br.ex Sabine belongs to the Anisophylleaceae family comprising of 29–34 species of shrubs and trees occurring in lowland forests and swamps in tropical Africa, Asia, Malaysia and South America. These species are placed in four genera with distinct geographical distributions [6]. *Anisophyllea laurina R.Br.ex Sabine* commonly called "monkey apple" is a shrub or tree of 5 to 16m high which grows widely in West Africa (in Guinea-Bissau to Sierra Leone and rarely in Ivory Coast) and has been used as a traditional/herbal medicine among natives of this nations [7]. Its bark is enriched with astringents like tannin which serves as an important component in both pharmaceutical and textile industries. A decoction of the leaves is used as a mouth rinse for toothache and the leaves are said to have medicinal properties to treat

diabetes, emetics, oral treatments, pain-killers and the bark is analgesic [8]. However, the wide use of *Anisophyllea laurina R.Br.ex Sabine* as a medicinal plant has no scientific proof to attest its bioactive components as well as its usage as a medicinal source to treat diabetes. This research delves into the identification and evaluation of its leaves and stem bark extracts for bioactive compounds in relation to their anti-diabetic activity.

2. Methods and materials

All chemicals were purchased from Sigma (St. Louis, MO, USA) and were of analytical grade unless otherwise stated.

2.1. Collection of plant materials and preparation of extracts

Fresh matured whole leaves and stem-bark of *Anisophyllea laurina R. Br. ex Sabine* were collected from different farm sites at Coyah in Kindia region (Guinea, Africa). They were sun-dried for 3 days. The dried leaves of the plant were pulverized separately by means of milling. 20 grams for each of the plant powder from leaves and bark were extracted by maceration in 100mL of 80% ethanol (v/v) for 3 days with frequent agitation speed of 280 rpm at 28 °C in the dark. The supernatants were collected, filtered through Whatman No. 1 filter paper and the filtrate was then concentrated at 60°C using a rotary evaporator (Buchi Labortechnik, Flawil, Switzerland). Finally the concentrates were freeze dried (Labconco Corporation, Kansas City, MO, USA) to yield a dry powder. The yield of the powder was around 7.3%. 200mg of the powder was dissolved in 10ml 10% dimethylsulfoxide (DMSO) and kept in 4°C fridge for further use.

2.2 Alpha-glycosidase inhibition assay

The alpha-glycosidase was assayed using a method modified by Apostolidis et al [9]. Aliquot 0 - 4 mg/ml in DMSO (v/v 1:1) of 80% ethanol extract of leaves and stem bark was prepared. 50 µl of each concentration extract was mixed well with100 µl of 0.1 M phosphate buffer (pH 6.9) containing α-glucosidase solution (1.0 U/ml) and the mixtures were then incubated in 96-well plates at 25°C for 10 min. After pre-incubation, 50 µl of 5 mM p-nitrophenyl-α-D-glucopyranoside solution in 0.1 M phosphate buffer (pH 6.9) was added to each well at timed intervals. The reaction mixtures were incubated at 25°C for 5 min. Before and after incubation absorbance readings were recorded at 405 nm using a micro-plate reader (Thermomax, Molecular device Co., Virginia, USA) and compared to a control which contained 50 µl of the buffer solution instead of the extracts. The experiments were performed in triplicate and the α-glucosidase inhibitory activity was expressed as percentage inhibition. Acarbose was prepared in distilled water and used as positive controls. The percentage inhibition was calculated using the formula;

$$\% \text{ Inhibition} = \{(Ac - Ae)/Ac\}\ 100$$

Where Ac and Ae are the absorbance of the control and extract, respectively.

IC50 values (inhibitor concentration at which 50% inhibition of the enzyme activity occurs) of the plant extracts were determined by plotting graph with varying concentrations of the plant extracts against the percent inhibition.

2.3 Alpha-amylase inhibition assay

The inhibition of alpha-amylase was determined using an assay modified from the Worthington Enzyme Manual [10]. Aliquot 0 - 4 mg/ml in DMSO (v/v 1:1) of 80% ethanol extract of leaves

and stem bark was prepared and 500 µl of each concentration extract was mixed with 500 µl of 0.02 M sodium phosphate buffer (pH 6.9) containing α-amylase solution (0.5 mg/ml) and incubated at 25°C for 10min. After pre-incubation, 500 µl of a 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9) was added to each tube at timed intervals. The reaction mixtures were then incubated at 25°C for 10 min. The reaction was stopped with 1.0 ml of dinitrosalicylic acid colour reagent. The test tubes were then incubated in a boiling water bath for 5 min and cooled to room temperature. The reaction mixture was then diluted by adding 15 ml of distilled water, and the absorbance was measured at 540 nm using a micro-plate reader (Thermomax, Molecular device Co., Virginia, USA). The experiments were performed in duplicate and the absorbance of sample blanks (buffer instead of enzyme solution) and a control (buffer in place of sample extract) were also recorded. The absorbance of the final extract was obtained by subtracting its corresponding sample blank reading. Acarbose was prepared in distilled water and used as positive controls

The percentage inhibition was calculated using the formula;

$$\% \text{ Inhibition} = \{(Ac - Ae)/Ac\}\ 100$$

Where Ac and Ae are the absorbance of the control and extract, respectively

IC50 values (inhibitor concentration at which 50% inhibition of the enzyme activity occurs) of the plant extracts were determined by plotting graph with varying concentrations of the plant extracts against the percent inhibition.

2.4 Isolation and Identification of Ethanol Extracts of Leaves and Stem Bark

The ethanol extracts of *Anisophyllea laurina R. Br. ex Sabine* leaves and stem bark showed very good in vitro α-amylase

and alpha glucosidase inhibition activities. Therefore it was necessary to isolate and identify the bioactive compounds involved from the ethanol extracts. The extracts were subjected to column chromatography for separation. About 200mg of dried extracts was chromatographed over silica gel which was packed into a glass column (700 mm x100 mm) and elution was carried out from non-polar to polar solvents by gradient elution method. The column was eluted with a solvent gradient of hexane: ethyl acetate (100:0 to 0:100 v/v) at a flow rate of 1mL/min, and then eluted with 100% ethyl acetate, ethanol: ethyl acetate (1:4) and 100% ethanol. The fractions were collected and concentrated using rotary evaporator. Each fraction was freeze dried and as a result of their inhibition, ethyl acetate showed a higher inhibition percentage, hence was subjected to LC-ESI-MS mass spectra for phenolic profile.

2.5. Phenolic profile analysis by UPLC LC-ESI-MS

The LC-ESI-MS mass spectra were recorded using the Waters ACQUITY UPLC® SYNAPTTM High Definition Mass Spectrometer systems (Waters, Milford, USA) equipped with an electrospray ion source and hybrid quadrupole-time-of-flight (Q-TOF) mass spectrometer with the MSE model. The Q-TOF instrument was operated in V mode for MS experiments with the TOF data been collected between m/z 100 and 1500. The optimized condition was desolvation gas at 500 L/h at a temperature of 400◦C, cone gas at 50 L/h and source temperature at 100◦C, capillary and cone voltages at 3kv and 30v, respectively.

UPLC data were produced using the Waters ACQUITY UPLC systems (WATERS MALDI SYNAPT Q-TOF MS, Milford, USA) equipped with a binary pump, an auto sampler, a degasser, and a diode-array detector (DAD). The system was controlled with Mass Lynx V4.1 software. The DAD was monitored in the range 200–600 nm. The chromatographic column UPLC™ BEH AMIDE C18 (2.1 mm x 100 mm, 1.7µm)

was used and eluted with a linear gradient of A:100% Acetonitrile and B: 0.1 % Formic acid A+B=100% at a flow rate of 0.3 ml/min and at a temperature of 45°C: 0-5% A, 0-17min; 5-60%A, 17-20 min; 60-100%A, 20-22min; 100-5% A 22-22.1min; 22.1-25min, 5%A. The injection volume was 0.5µL. The accurate mass and composition for the precursor and fragment ions were calculated using the Mass Lynx 4.1 software.

2.6 Statistical Result

The result of three replicate experiments were pooled and expressed as mean ± standard deviation. A one-way analysis of variance (ANOVA) and positive analysis was done using Duncan multiple test. Significance was accepted at $P \leq 0.05$.

3. Results and discussion

3.1. In vitro α-glucosidase *and* α –amylase inhibitory study

Management of the blood glucose level is a critical strategy in the control of diabetes complications. Inhibitors of saccharide hydrolyzing enzymes (α-amylase and α-glucosidase) have been useful as oral hypoglycemic drugs for the control of hyperglycemia especially in patients with type-2 diabetes mellitus [11 12]. Medicinal plants or natural products involve retarding the absorption of glucose by inhibiting the carbohydrate hydrolyzing enzymes. Several α- amylase inhibitors including acarbose, voglibose and miglitol are clinically used for treatment but their prices are high and have clinical side effects [13 14]. Hence, screening of these enzymes inhibitors from plants and synthetic sources are increasing. In this study, anti-diabetic activity of the ethanol extracts from leaves and stem bark were investigated with reference to α-glucosidase and α-amylase inhibition. It was found that the plant used in this study, *Anisophyllea laurina R. Br. ex Sabine* leaves and stem bark ethanol extracts showed potential

antidiabetic activities. From the α-glucosidase and α–amylase inhibitory assays, the plant extracts showed significant antidiabetic activity.

The in vitro α-glucosidase inhibition study showed that the ethanol extract of both leaves and stem bark at concentrations of 4.0, 3.2, 2.4, 1.6 and 0.8 mg/ml inhibited α-glucosidase in a dose dependent manner. The highest concentration of 4.0 mg/ml showed a maximum inhibition of 78.53% and 69.5% for the leaves and stem bark respectively, while the lowest concentration of 0.8 mg/ml showed a minimum inhibition of 12.2% and 24.1% as well respectively (Figure 1). The IC50 values of the 80% ethanol extracts of leaves and stem bark were 2.41 and 3.1 mg/ml, respectively. Our results suggested that the leaves extract of the plant were more active than the stem back, and is comparable to acarbose activity. The standard acarbose showed a higher inhibition activity of 84.01% with IC50 1.90mg/ml value.

The ethanol extract of both leaves and stem bark showed a weaker inhibition of the α-amylase enzyme. Figure 2 shows the percentage inhibition values of the ethanol extract of the leaves, stem bark and acarbose against **α**-amylase. The maximum inhibition of the stem bark extract was 63.6 % at a concentration of 4.0 mg/ml whereas the leaves were 58.2%. At the lower concentrations, the stem bark extract did not have significant inhibition difference when compared to that of the leaves extracts Figure 2(b)). Acarbose showed a maximum percentage inhibition of 74.0% at 4.0 mg/ml with an IC50 of 2.2 mg/ml. The IC50 values for the 80% ethanol extract of leaves and stem bark were 3.1 mg/ml and 3.5 mg/ml, respectively.

For α-amylase inhibition, both the leaves and stem extracts gave good results of around 60% inhibition respectively. The results for both enzymes (α-amylase and α-glucosidase) inhibitory assays showed that ethanol extracts of the *Anisophyllea laurina R. Br. ex Sabine* plant were strong inhibitors of α-glucosidase but mild inhibitors of α-amylase

especially the leaves as shown in figure 1 and 2. It is known that dietary management of hyperglycemia linked to type 2 diabetes can be targeted through foods or botanical supplements that have high α-glucosidase and a moderate α-amylase inhibition (15).

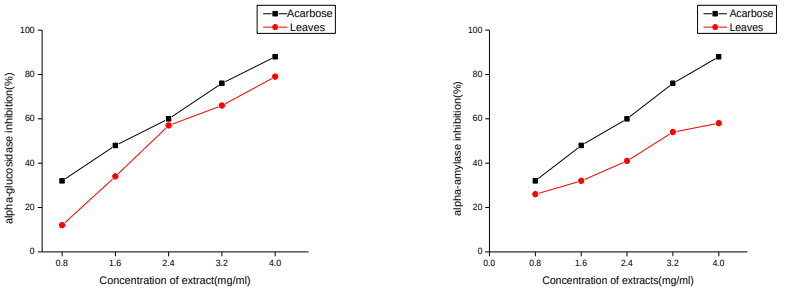

Figure 1: α -glucosidase inhibitory activities *(*a), and α -amylase inhibitory activities of leaves extracts of *Anisophyllea laurina R. Br. ex Sabine (b).*

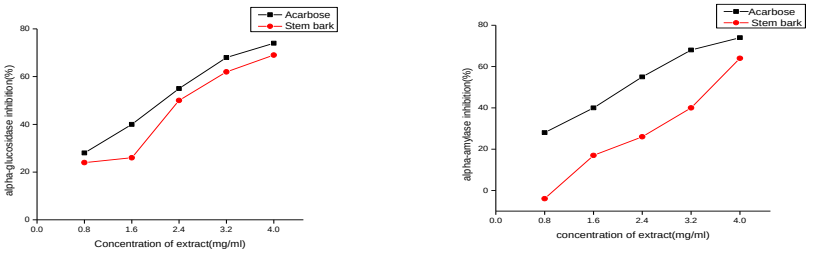

Figure 2: α-glucosidase inhibitory activities *(*a), and α -amylase inhibitory activities (b) of stem bark extracts of *Anisophyllea laurina R. Br. ex Sabine.*

3.2. Identification of phenolic compound

A total of twenty six peaks of phenolic compounds were obtained from both leaves and stem bark samples, with fifteen of them present in the leaves and eleven in the stem bark which contributed to the α-glucosidase and α-amylase inhibitory activities. The LC-ESI-MS chromatograms of the plant ethanol extracts are shown in Fig. 3 (a) and (b). Identification of the MS chromatogram peaks of phenolic compounds by detached molecular weight was done based on the search for $[M-H]^-$ ions,

using extracted ion mass chromatograms together with the interpretation of their ESI/IT/MS fragments in comparison with those found in reported literature. Characterised compounds in leaves and stem bark are summarised in Table 1.

Our extracts from both leaves and stem bark showed the presence of compounds such as, catechin, flavonoids and phenolic acids as well as others. The phenolic compounds identified from leaves extracts were: Peak 2 at RT 0.907 min revealed an ion at m/z 575 which was identified as proacnthocynaidins A type isomer with fragments m/z 418, 341 and 304 [16, 17]. At RT 1.23 mins, peak 3 at a protonated molecule of 401 m/z showed Benzyl alcohol hexose pentose as the base peak [18]. Peak 4 at RT 1.90 mins was identified as malvidin 3-0-galactoside $[M-H]^-$ at m/z 493 and fragments m/z 331, 271 and 198 are in conformity to [19 20]. Base peaks 5 and 6 at different RT 2.29 and 2.532 mins with protonated molecule ion of 783 m/z and their fragmentation pattern involved the loss of galloyl and hexahydroxydiphenoyl moieties m/z 633 and 481 respectively were detected as diagalloyl-HHDP-glucose isomer [21] However, peak 8 and 10 were characterised based on their molecular ions at m/z 463 and 431 and the presence of an intense product ion at m/z 301 was identified as quercetin 3-0-glucoside and quercetin-rhamnoside respectively [22]. The base peak 9 showed [M-H]- at m/z 447.06 with the fragment at m/z 301 ($[M-H-146]^-$, the loss of coumaroyl) was assigned as Quercetin-3-rhamnoside and has been previously reported in olives [23]. Peak 11 which eluted at RT 4.36 mins was detected at m/z 439 with its fragments at m/z 417 and 409. Thus, this compound was identified as (-)-Epicatechin 3-O-vanillate [20]. A base peak 12 identified as Quercetin $[M–H]^-$ ion at 301 m/z with fragmented ions m/z 289, 179 and 151. Syringic acid derivative of base peak 13 was detected at m/z 401 RT 5.607 mins and a base peak 14 at m/z 499 was tentatively identified as Quercetin Caffeoyl-coumaroyl hexoside [24]. At RT 5.94 peak 15 was identified as (–)-epicatechin 3-O-gallate $[M-H]^-$, at m/z 442 with

fragment ions m/z 438, 290 and 249. In leaves extracts protonated molecules at m/z 725 with base peaks 1 was identified as unknown compound.

For stem bark, the base peak 1 was identified as epigallocatechin at m/z 305 with RT 0.61mins. Syringic acid (peak 3,198 m/z) was detected at RT 1.191 mins. Base peak 4 revealed a protonated ion at 723 m/z with fragment ion at 587 m/z indicating the loss of hexose. The parent compound was identified as (Epi)afzelchine(epi)Catechin isomer. Peak 5 was identified as Procyanidin dimer B isomer $[M-H]^-$ at m/z 647, whereas peak 6 was tentatively identified as Quercetin 3'-O-sulfate $[M-H]^-$ at m/z 381. The fragmentation pattern shown by these compounds was in agreement with that reported by [25, 26]. Caffeoyl tartaric acid hexose at m/z 327 with MS^2 fragments at m/z 311, 248 and 154. Peak 9 with ion m/z 439 corresponded with the compound (-)-Epicatechin 3-O-gallate [27]. Peak 10 was tentatively identified as isorhamnetin-O-hexoside isomer ion at m/z 557 and MS^2 fragmentation on negative mode was detected at m/z 477 and base peak 11 at m/z 254 was identified as chrysin [28]. From the chromatogram results, peaks 4, 8, 9 and 12 showed a high peak area in leaves while peaks 2, 6, 7, 8, 9 and 10 in stem bark also showed the similar trend and are therefore considered to be in abundance.

Phenolic compounds are naturally occurring compounds that possess rich sources of antioxidant and free radical scavenging properties as well as medicinal properties. They have been reported to be good inhibitors of α-glucosidase and α-amylase. Additionally they are also regulators of hyperglycemia and other diabetic complications [29].

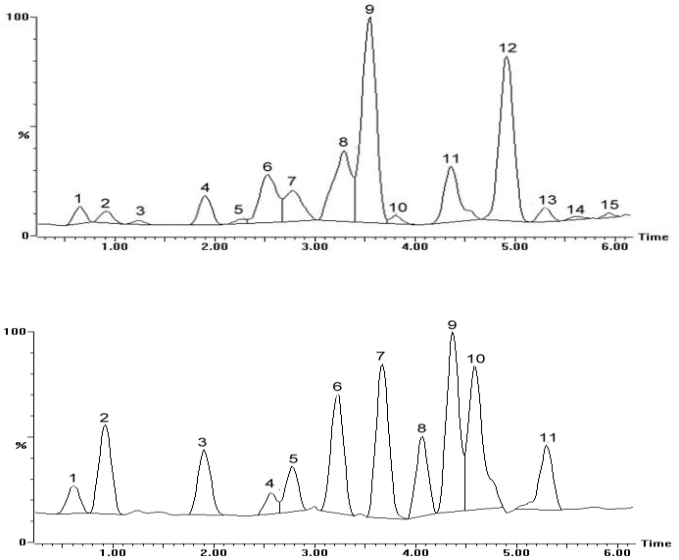

Figure 3. Phenolic profile *of ethanol* extracts of leaves (A) and stem bark (B) of Anisophyllea *laurina R. Br. ex Sabine.*

Table 1: Identification of phenolic compounds of leaves and stem bark extracts of Anisophyllea *laurina R. Br. ex Sabine.*

Peak	Retention Time	Height	Area	Area%	Mass Found [M-H]-(m/z)	Identification	Fragment
Leaves extract							
1	0.64	52	6.54	1.91	725	Unknown	657, 453,385, 317, 249
2	0.90	33	4.91	1.44	575	Proanthocyanidins A type Isomer	481, 341, 304, 268, 161
3	1.23	12	1.48	0.43	401	Benzyl alcohol hexose pentose	389, 344, 318, 296, 173
4	1.90	87	12.43	3.64	493	Malvidin 3-galactoside	331, 271, 198, 161
5	2.29	13	1.81	0.53	783	Digalloyl-HHDP-glucose isomer	633, 301, 289, 169
6	2.53	142	28.48	8.36	783	Digalloyl-HHDP-glucose isomer	633, 301, 289, 169
7	2.77	91	18.95	5.56	618	Unknown	577,301, 289,169
8	3.29	210	43.70	12.82	463	Quercentin 3-O-glucoside	301,198, 169
9	3.55	610	102.07	29.95	447	Quercentin 3-rhamnoside	301, 117
10	3.8	24	3.07	0.90	431	Kaempferol- 3-orhamnoside	286, 243, 198, 125
11	4.36	165	28.99	8.50	439	(-)-Epicatechin 3-O-vanillate	301, 198, 117
12	4.91	488	80.05	23.49	302	Quercentin	289, 179, 153
13	5.29	42	5.49	1.61	401	Syringic acid derivates	255, 198, 178, 161
14	5.60	10	1.29	0.37	499	Quercetin caffeoyl-coumaroyl hexoside	463, 301, 289, 125
15	5.93	13	1.38	0.40	442	(-) -Epicatechin 3-O-gallate	438, 291, 249, 165,139
Stem bark extract							
1	0.61	34	5.07	2.69	305	(-)-egallocatechin	289, 207, 175, 139
2	0.92	114	16.46	8.73	683	Cafferic acid hexoside dimer	377, 341, 304, 268
3	1.91	83	12.17	6.45	198	Syringic acid	161, 159
4	2.57	27	3.56	1.89	723	(Epi) afzelchine (epi) catechin isomer	647, 463, 417, 289, 196
5	2.77	58	8.34	4.42	647	Procyanidin dimer B1 isomer	577, 467, 289, 163
6	3.22	154	22.49	11.92	381	Quercetin 3'- sulfate	351, 241, 197, 162
7	3.67	197	28.80	15.27	327	Caffeoyl tarrtaric acid hexose	312, 198, 112
8	4.07	102	13.96	7.40	707	Unknown	661, 425, 287, 198, 161
9	4.36	230	33.39	17.70	439	(-) Epicatechin 3- O- vanillate	301, 198, 171
10	4.59	186	31.62	16.76	557	Isorhamnetin-O-hexoside isomer	477, 315, 301, 169
11	5.30	82	12.79	6.78	254	Chrysin	221, 198, 179, 161

4. Conclusion

Based on the results presented in this study, it can be concluded that ethanol extracts of *Anisophyllea laurina R. Br. ex Sabine* leaves and stem bark exerts an inhibitory effect on α-glycosidase and α-amylase. These could be from the phenolic compounds identified in the plant. Therefore these results suggested the potential use of this plant as a dietary supplement or in the manufacture of drugs for the control of increased blood glucose level in the body.

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