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1 **Original research article**

2 # Inhibition of diabetes-related enzymes by
3 # *Acanthus montanus* leaf extracts

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5
6 **ABSTRACT**
7

The objective of this study was to determine the *in-vitro* antidiabetic potentials of *Acanthus montanus*. This was done by assessing the inhibitory effect of both methanol and ethyl acetate extracts of the plant on the activities of diabetes-related enzymes (α-amylase and α-glucosidase). The results showed that of the two extracts, methanol exhibited more inhibitory action against the two enzymes. Lineweaver-Burk plot also depicted that the methanol extract inhibited both α-amylase and α-glucosidase in a non-competitive and competitive manner respectively. It can be concluded that the hypoglycemic effect of *A. montanus* may be as a result of the inhibition of these enzymes (α-amylase and α-glucosidase), which may be elicited by the presence of phytochemicals present in the extracts.

Aims: The aim of this study was to determine the *in-vitro* antidiabetic potentials of *Acanthus montanus*.
Study design: Extraction of *A. montanus* leaves with methanol and ethanol and evaluation of their hypoglycemic effect.
Place and Duration of Study: The leaves of *A. montanus* were obtained from Badagry Area of Lagos, Nigeria in December 2012. The plant was identified and authenticated by Dr. S. O. Shosanya of the Forestry Research Institute of Nigeria (FRIN), Ibadan, Nigeria.
Methodology: The powdered leaves were separately extracted with ethyl acetate and methanol for 24 h. The resulting infusions were decanted, filtered and evaporated in a rotary evaporator. Dried extracts were weighed and dissolved in dimethylsulphoxide (DMSO) to yield a stock solution from which lower concentrations were prepared. The inhibitory actions of both extracts against α-amylase and α-glucosidase were determined established procedures.
Results: The results showed that of the two extracts, methanol exhibited more inhibitory action against the two enzymes. Lineweaver-Burk plot also depicted that the methanol extract inhibited both α-amylase and α-glucosidase in a non-competitive and competitive manner respectively.
Conclusion: It can be concluded that the hypoglycemic effect of extracts of *A. montanus* may be as a result of the inhibition of these enzymes (α-amylase and α-glucosidase). This observation may be elicited by the presence of some phytochemicals present in the extracts.

8 *Keywords: Acanthus montanus,* α-amylase,α-glucosidase, antidiabetic
9
10 ## 1. INTRODUCTION
11

12 Diabetes is a metabolic disease which is as old as mankind and its incidence is considered
13 to be high (4–5%) all over the world [1]. It is also a major cause of disability and
14 hospitalization and results in significant financial burden [2]. It is considered a “modern day
15 epidemic” and is rightly recognized as a global public health issue. The number of people
16 suffering from the disease worldwide is increasing at an alarming rate with a projected 552
17 million people likely to be diabetic by the year 2035 as against 382 million estimated in 2014
18 [3]. There is need for the discovery of antidiabetic agents from natural sources due to limited
19 efficacy and serious side effects associated with synthetic drugs which include
20 hypoglycaemia, chronic tissue damage and death [4].

UNDER PEER REVIEW

21 *Acanthus montanus* (Nees) T. Anderson (Acanthaceae) is a small shrub with sparse
22 branches and soft stems. It is commonly known as Mountain Thistle or Bears Breech and is
23 believed to have originated from West Africa [5]. It is used in traditional medicine in the
24 Southern part of Nigeria under the names; 'Mafowokan omomi', 'Agamsoso' and 'Agameru'.
25 It is also used in different parts of Africa in the treatment of various illnesses such as cough,
26 epilepsy, pain, dysmenorrhoea, hypertension, false labour, syphilis, skin infections and
27 diabetes mellitus [6, 7]. Several studies have reported the pharmacological properties of this
28 plant which include hepatoprotective [8], tocolytic [9], anti-inflammatory, antimicrobial and
29 immunological properties [5]. Nana et al. [10] reported the safety of this plant in pregnant
30 rats as well as their offspring while Djami et al. [11] also stated its tolerance in female rats at
31 < 1000 mg/kg body weight. Though, there is a study on the hypoglycemic potential of the
32 methanolic extract of this plant [7], there is dearth of information on the possible mechanism
33 by which it elicits its hypoglycemic action.
34
35 It is well known that any antidiabetic agent can act by one or more of the following
36 mechanisms; pancreatic β-cells regeneration, insulin secretion, mimicking the action of
37 insulin, inhibition of carbohydrate metabolizing enzymes as well as slowing down the
38 absorption of sugars from the gut [12]. The aim of this study was to assess the effect of leaf
39 extracts of Nigerian grown *A. montanus* on diabetes-related enzymes (α-amylase and α-
40 glucosidase) as well as its mode of inhibition of these enzymes. Previously the anti-diabetic
41 potentials of some medicinal plants of Nigeria have been reported [13].
42
43
44 ## 2. MATERIAL AND METHODS
45
46 ### 2.1 Chemicals and reagents
47
48 Porcine pancreatic α-amylase, rat intestinal α-glucosidase and paranitrophenyl-
49 glucopyranoside were products of Sigma-Adrich Co., St Louis, USA while starch soluble
50 (extra pure) was obtained from J. T. Baker Inc., Phillipsburg, USA. Other chemicals and
51 reagents were of analytical grade and the water used was glass-distilled.
52
53
54 ### 2.2 Plant sample
55
56 The leaves of *Acanthus montanus* were obtained from Badagry Area of Lagos in Nigeria in
57 December 2012. It was identified and authenticated by the taxonomist; Dr. S. O. Shosanya
58 of the Forestry Research Institute of Nigeria (FRIN) and voucher specimen (FHI 109720)
59 was deposited in the Institute's herbarium. The leaves were dried, pulverized and kept in
60 airtight plastic bags.
61
62 #### 2.2.1 Preparation of extracts
63
64 The powdered leaves were divided into two portions of 10 g each and these were extracted
65 with ethyl acetate and methanol respectively. They were left to steep in covered conical
66 flasks for 24 h; the flasks were shaken and kept still to allow the plant material settle at the
67 bottom of the flask. The resulting infusions were decanted, filtered and evaporated in a
68 rotary evaporator (Cole Parmer SB 1100, Shangai, China). Dried extracts were weighed and
69 dissolved in dimethylsulphoxide (DMSO) to yield a stock solution from which lower
70 concentrations were prepared. All extracts were stored at 4 ℃ prior to analysis.
71
72 ### 2.3 α-Amylase inhibitory assay

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73 This assay was carried out using a modified procedure of McCue and Shetty [14]. A total of
74 250 µL of extract was placed in a test tube and 250 µL of 0.02 M sodium phosphate buffer
75 (pH 6.9) containing α-amylase solution was added. This solution was pre-incubated at 25 ℃
76 for 10 min, after which 250 µL of 1% starch solution in 0.02 M sodium phosphate buffer (pH
77 6.9) was added at timed intervals and then incubated at 25 ℃ for 10 min. The reaction was
78 terminated by adding 500 µL of dinitrosalicylic acid (DNS) reagent. The tubes were then
79 incubated in boiling water for 5 min and cooled to room temperature. The reaction mixture
80 was diluted with 5 mL distilled water and the absorbance was measured at 540 nm using a
81 spectrophotometer (Spectrumlab S23A, Globe Medical England). The control and blank
82 were prepared using the same procedure replacing the extract with DMSO and distilled
83 water respectively. The α-amylase inhibitory activity was calculated as percentage inhibition,
84 thus;
85 $\% \text{ Inhibition} = [(\Delta A_{control} - \Delta A_{extract})/A\Delta_{control}] \times 100$
86
87 where $\Delta A_{control} = A_{control} - A_{blank}$ and $\Delta A_{extract} = A_{extract} - A_{blank}$
88
89 Concentrations of extracts resulting in 50% inhibition of enzyme activity (IC_{50}) were
90 determined graphically.
91
92
93 **2.3.1 Mode of α-amylase inhibition**

94 The mode of inhibition of α-amylase by the leaf extract was conducted using the most potent
95 extract according to the modified method described by Ali et al. [15]. Briefly, 250 µL of the (5
96 mg/mL) extract was pre-incubated with 250 µL of α-amylase solution for 10 min at 25 ℃ in
97 one set of tubes. In another set of tubes α-amylase was pre-incubated with 250 µL of
98 phosphate buffer (pH 6.9). 250 µL of starch solution at increasing concentrations (0.3–5.0
99 mg/mL) was added to both sets of reaction mixtures to start the reaction. The mixture was
100 then incubated for 10 min at 25 ℃, and then boiled for 5 min after addition of 500 µL of DNS
101 to stop the reaction. The amount of reducing sugars released was determined
102 spectrophotometrically using a maltose standard curve and converted to reaction velocities.
103 A double reciprocal (Lineweaver-Burk) plot (1/v versus 1/[S]) where v is reaction velocity and
104 [S] is substrate concentration was plotted to determine the mode of inhibition.
105
106 **2.4 α-Glucosidase inhibitory assay**

107 The effect of the plant extracts on α-glucosidase activity was determined according to the
108 method described by Kim et al. [16]. The substrate solution, p-nitropheynyl glucopyranoside
109 (pNPG) was prepared in 20 mM phosphate buffer, pH 6.9. 100 µL of α-glucosidase (E.C.
110 3.2.1.20) was pre-incubated with 50 µL of the different concentrations of the extracts for 10
111 min. Then 50 µL of 3.0 mM pNPG dissolved in 20 mM phosphate buffer (pH 6.9) was added
112 to start the reaction. The reaction mixture was incubated at 37 ℃ for 20 min and stopped by
113 adding 2 mL of 0.1 M Na_2CO_3. The α-glucosidase activity was determined by measuring the
114 yellow coloured para-nitrophenol released from pNPG at 405 nm. The control and blank
115 were prepared using the same procedure by replacing the extract with DMSO and distilled
116 water respectively. Percentage inhibition was calculated thus;
117
118 $\% \text{ Inhibition} = [(\Delta A_{control} - \Delta A_{extract})/A\Delta_{control}] \times 100$
119 where $\Delta A_{control} = A_{control} - A_{blank}$ and $\Delta A_{extract} = A_{extract} - A_{blank}$
120
121 Concentrations of extracts resulting in 50% inhibition of enzyme activity (IC_{50}) were
122 determined graphically
123

UNDER PEER REVIEW

124 **2.4.1 Mode of α-glucosidase inhibition**
125
126 The mode of inhibition of α-glucosidase by the extracts was determined using the extract
127 with the lowest IC_{50} according to the modified method described by Ali et al. [15]. Briefly, 50
128 µL of the (5 mg/mL) extract was pre-incubated with 100 µL of α-glucosidase solution for 10
129 min at 25 ℃ in one set of tubes. In another set of tubes, α-glucosidase was pre-incubated
130 with 50 µL of phosphate buffer (pH 6.9). 50 µL of pNPG at increasing concentrations (0.63 –
131 2.0 mg/mL) was added to both sets of reaction mixtures to start the reaction. The mixture
132 was then incubated for 10 min at 25 ℃ and 500 µL of Na_2CO_3 was added to stop the
133 reaction. The amount of reducing sugars released was determined spectrophotometrically
134 using a para-nitrophenol standard curve and converted to reaction velocities. A double
135 reciprocal (Lineweaver-Burk) plot (1/v versus 1/[S]) where v is reaction velocity and [S] is
136 substrate concentration was plotted to determine the mode of inhibition.
137
138 **2.5 Statistical analysis**
139
140 Statistical analysis was performed using GraphPad Prism 5 statistical package (GraphPad
141 Software, USA). The data were analysed by one way analysis of variance (ANOVA) followed
142 by Bonferroni test. All the results were expressed as mean ± SEM for triplicate
143 determinations.
144
145 **3. RESULTS AND DISCUSSION**
146
147 Figure 1 showed the percentage inhibition of α-amylase by methanol and ethyl acetate
148 extracts of *A. montanus*. There were no significant differences between the extracts at low
149 concentrations (0.32 - 0.63 mg/mL). However at higher concentrations, ethyl acetate extract
150 exhibited significantly higher percentage inhibition of the enzyme. The higher percentage
151 inhibition displayed by the ethyl aceate extract was corroborated by its lower IC_{50} value
152 compared to that of methanol extract (Table 1).
153
154
155 Table 1: IC_{50} values for α-amylase and α-glucosidase inhibitory potential of *A. montanus* leaf
156 extracts

Extracts	**IC_{50} (mg/mL)**	
	α-Amylase	**α-Glucosidase**
Methanol	2.87 ± 0.02[a]	1.65 ± 0.02[a]
Ethyl acetate	2.39 ± 0.04[b]	7.10 ± 0.15[b]
Acarbose	2.60 ± 0.01[a]	0.63 ± 0.00[c]

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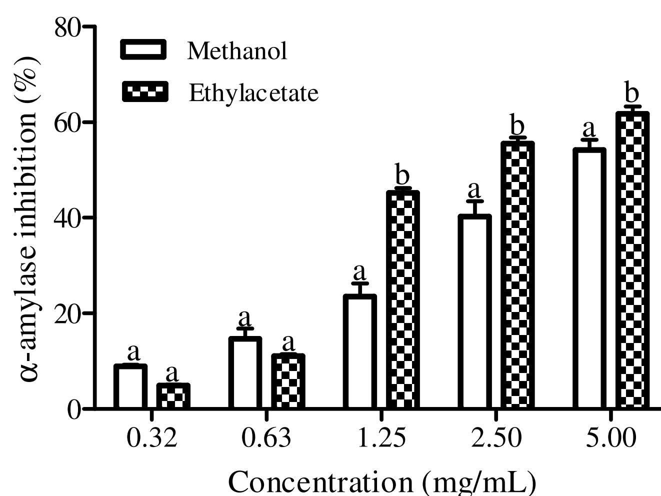
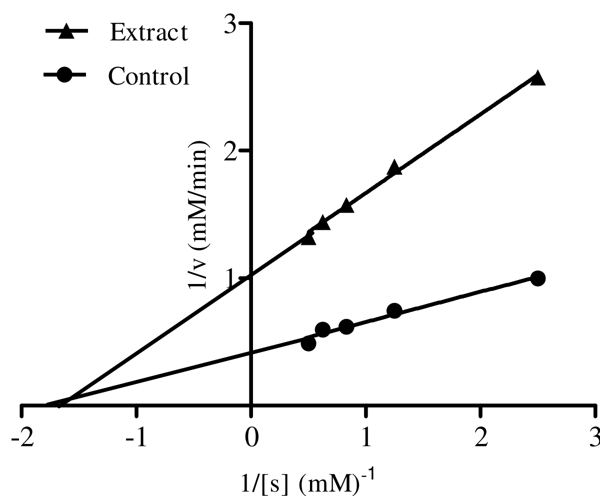


158
159 Fig 1: Inhibitory potency of *A. montanus* leaf extracts against α-amylase activity. The values are
160 expressed as means ± SEM of triplicate determinations. Means not sharing a common letter at the
161 same concentration are significantly different (P = .05)
162
163 However, the Lineweaver-Burk plot of the mode of inhibition of α-amylase by the methanol
164 extract of this plant showed that it is a non-competitive inhibitor of the enzyme (Figure 2).



165
166 Fig 2: Mode of inhibition of α-amylase by methanol extract of *A. montanus*
167 The percentage inhibition of α-glucosidase by the extracts of *A. montanus* is shown in Fig 3.
168 At all concentrations tested, methanol extract exhibited significantly higher (P=.05)
169 percentage inhibition of this enzyme compared to ethyl acetate extract. However, the
170 inhibition of the enzyme by both extract was dose-dependent. This is supported by its lower
171 IC_{50} value compared to methanol extract. Kinetic analysis of the mode of inhibition of the
172 enzyme with the aid of Lineweaver-Burk plot showed that it inhibited the enzyme in a
173 competitive manner (Figure 4).

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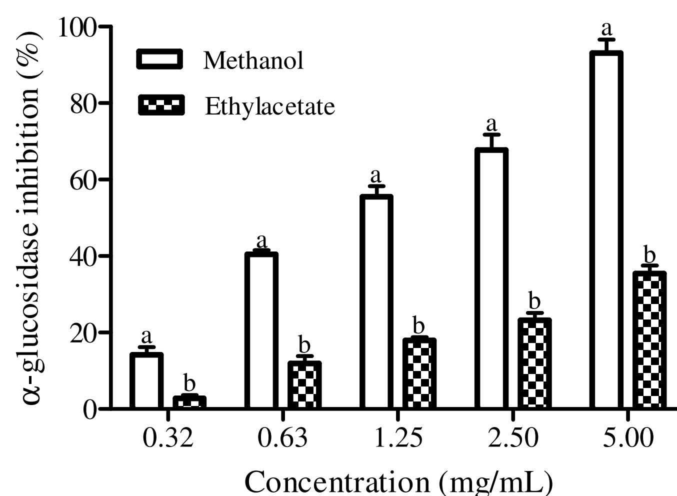
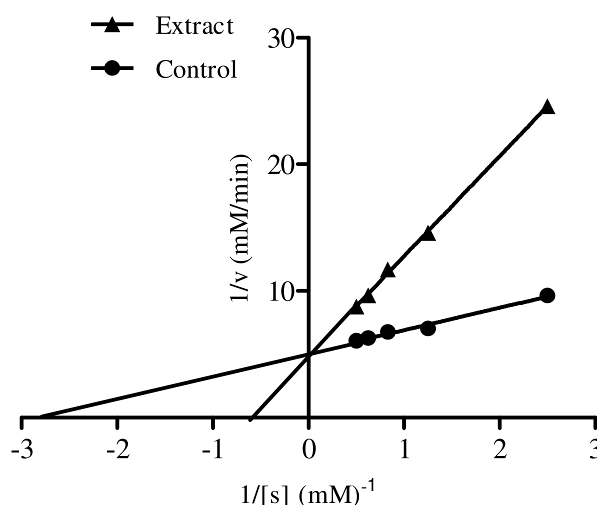


174
175 Fig 3: Inhibitory potency of *A. montanus* leaf extracts against α-glucosidase activity. The values are
176 expressed as means ± SEM of triplicate determinations. Means not sharing a common letter at the
177 same concentrations are significantly different (*P* = .05)
178



179

180 Fig 4: Mode of inhibition of α-glucosidase by ethyl acetate extract of *A. montanus*
181

182 The management of hyperglycemia is the hallmark of treatment in diabetes and one of the
183 therapeutic approaches for decreasing postprandial hyperglycemia is to retard the digestion
184 and absorption of carbohydrates. This is done through the inhibition of carbohydrate
185 hydrolyzing enzymes, α-amylase and α-glucosidase, in the digestive tract [17]. Though,
186 synthetic α-glucosidase inhibitors such as acarbose and voglibose are presently in use but
187 are bedeviled by undesirable side effects such as nausea, hypoglycaemia, diarrhoea and
188 liver failure [14], which necessitated this study.
189

190 Our study showed that ethylacetate extract of *A. montanus* produced stronger inhibition of α-
191 amylase than methanol extract. However, methanol extract will be more suitable to be used

UNDER PEER REVIEW

192 as antidiabetic agent because of its mild inhibition, having higher IC_{50} than ethylacetate
193 extract and acarbose. Previous studies have shown that any prospective antidiabetic agent
194 should be a mild inhibitor of α-amylase so as to prevent the drawback of synthetic drugs (like
195 acarbose), which occur due to the excessive inhibition of the enzyme resulting in the
196 abnormal bacterial fermentation of undigested carbohydrates in the colon [18, 19].
197 Therefore, the Lineweaver-Burk plot of the inhibition depicted that methanol extract of *A.*
198 *motanus* inhibited the enzyme in a non-competitive manner. This implies that the active
199 components in the extract binds to a site other than the active site of the enzyme and
200 combines with either free enzyme or the enzyme-substrate complex, possibly interfering with
201 the action of both [20].
202
203 The stronger inhibition of α-glucosidase by the methanol extract of *A. montanus* at all
204 concentrations tested compared to ethylacetate extract, culminated into having low IC_{50}
205 which also desirable of a good antidiabetic drug. The competitive inhibition of the enzyme by
206 methanol extract of *A. montanus* suggest that the inhibitory component(s) in the plant binds
207 reversibly to the active site of the enzyme and occupies it in a mutually exclusive manner
208 with the substrate [21, 22]. This may due to structural similarity between the inhibitor and the
209 normal substrate (disaccharides), thereby slowing down the production of glucose and
210 reducing hyperglycemia.
211
212 Tannins are phenolic compounds which have been found to induce phosphorylation of
213 insulin receptors and translocation of glucose transporter, thereby helping in the reduction of
214 blood glucose level [23]. Studies have also shown the antioxidant and antidiabetic properties
215 of saponins from different medicinal plants [24, 25]. Therefore, it is probable that the
216 inhibitory effect of *A. montanus* extracts on the activities of α-amylase and α-glucosidase
217 may be due to the presence of these kinds of phytochemicals present in the extracts.
218

219 4. CONCLUSION

220
221 This study showed that methanol extract of *A. montanus* is a more potent inhibitor of α-
222 amylase and α-glucosidase than ethyl acetate extract. However, this methanol extract
223 proved to be a non-competitive and competitive inhibitor of both α-amylase and α-
224 glucosidase respectively. It can therefore be concluded that the hypoglycemic action of this
225 plant may be due to the inhibition these diabetes-related enzymes studied.
226
227

228 REFERENCES

229

230 1. Koyuturk M, Ozsoy-Sacan O, Bolkent S, Yanardag R. Effect of glurenorm on
231 immunohistochemical changes in pancreatic β-cells of rats in experimental diabetes.
232 Indian J Exp Biol. 2005;43(3):268-7.
233 2. Nagappa AN, Thakurdesai PA, Rao NV, Singh J. Antidiabetic activity of *Terminalia*
234 *catappa* Linn fruits. J Ethnopharm. 2003;88(1):45-50.
235 3. International Diabetes Federation (IDF). Global diabetes scorecard-tracking
236 progress for action. IDF, Brussels: Belgium; 2014: p.14
237 4. Michael PK, Asim AB, Robert SB. The utility of oral diabetes medications in type 2
238 diabetes of the young. Current Diabetes Rev. 2005;1(1): 83-92.
239 5. Okoli C, Akah P, Onuoha N, Okoye T, Nwoye A, Nworu C. *Acanthus montanus*: An
240 experimental evaluation of the antimicrobial, anti-inflammatory and immunological
241 properties of a traditional remedy for furuncles. BMC Complem Alter Med. 2008;1(8):
242 27.

UNDER PEER REVIEW

243 6. Songalem EA, Foyet HS, Ekobo S, Dimo T, Kamtchouing P. Antiinflammatory, lack
244 of central analgesia and antipyretic properties of *Acanthus montanus* (Ness) T.
245 Anderson. J. Ethnopharm. 2004;95(1):63-8.
246 7. Ukwe CV, Ubaka CM. Hypoglycemic activity of leaves of *Acanthus montanus* T.
247 Anderson (Acanthaceae) in rats. Int J Diab Dev Count. 2011;31(1):32-6.
248 8. Patrick-Iwuanyanwu KC, Weghu MO. Prevention of carbon-tetrachloride (CCl_4)
249 induced liver damage in rats by *Acanthus montanus.* Asian J Biochem.
250 2008;3(4):213-20.
251 9. Foyet HS, Asongalem EA, Nana P, Folefoc GN, Kamtchouing P. Tocolytic effect of
252 *Acanthus montanus* in rat uterus. Pharmacologyonline. 2006;3:9-17.
253 10. Nana P, Asongalem EA, Foyet HS, Folefoc GN, Dimo T, Kamtchouing P. Maternal
254 and developmental toxicity evaluation of *Acanthus montanus* leaves extract
255 administered orally to Wistar pregnant rats during organogenesis. J Ethnopharm.
256 2008;116(2):228-33.
257 11. Djami TAT, Asongalem EA, Nana P, Choumessi A, Kamtchouing P, Asonganyi T.
258 Subacute toxicity study of the aqueous extract from *Acanthus montanus*. Electronic
259 J Biol. 2011;7(1): 11-5.
260 12. Cheng AYY, Fantus IG. Oral antihyperglycemic therapy for type 2 diabetes Mellitus.
261 Canadian Med Assoc J. 2005;172(2):213-26.
262 13. Ogundajo AL, Kazeem MI, Evroh JE, Avoseh MM, Ogunwande IA. Comparative
263 study on the phytochemical composition and hypoglycemic potentials of the leaves
264 extracts of *Combretum paniculatum* and *Morinda morindoides*. Eur J Med Pl.
265 2015;7(2): 77-86.
266 14. Mccue PP, Shetty K. Inhibitory effects of rosmarinic acid extracts on porcine
267 pancreatic α-amylase *in vitro.* Asia Pac J Cli Nutr. 2004;13(1):101-6.
268 15. Ali H, Houghton PJ, Soumyanath A. Alpha-amylase inhibitory activity of some
269 Malaysian plants used to treat diabetes with particular reference to *Phyllanthus*
270 *amarus*. J Ethnopharm. 2006;107(3):449-55.
271 16. Kim YM, Jeong YK, Wang MH, Lee WY, Rhee HI. Inhibitory effects of pine bark
272 extract on alpha-glucosidase activity and postprandial hyperglycemia. Nutr.
273 2005;21(6):756-61.
274 17. Kazeem MI, Adamson JO, Ogunwande IA. Modes of inhibition of α- amylase and α-
275 glucosidase by Aqueous extract of *Morinda lucida* Benth leaf. Biomed Res Int.
276 2013;1-6.
277 18. Apostolidis E, Kwon Y-I, Shetty K. Inhibitory potential of herb, fruit, and fungal-
278 enriched cheese against key enzymes linked to type 2 diabetes and hypertension.
279 Innov Food Sci Emerg Tech. 2007;8(1): 46-54.
280 19. Kwon Y-I, Apostolidis E, Shetty K. Inhibitory potential of wine and tea against α-
281 amylase and α-glucosidase for management of hyperglycemia linked to type 2
282 diabetes. J Food Biochem. 2008;32(1):15-31.
283 20. Mayur B, Sandesh S, Shruti S, Sung-Yum S. Antioxidant and α- glucosidase
284 inhibitory properties of *Carpesium abrotanoides* L. J Med Pl Res. 2010;4(15): 1547-
285 53.
286 21. Matsuda H, Morikawa T, Yoshikawa M. Antidiabetogenic constituents from several
287 natural medicines. Pure Appl Chem. 2002;74(7): 1301-8.
288 22. Kazeem MI, Ogungbe SM, Saibu GM, Aboyade OM. *In vitro* study on the
289 hypoglycemic potential of *Nicotiana tabacum* leaf extracts. Bangladesh J Pharmacol.
290 2014;9(2): 140-5.
291 23. Liu X, Kim JK, Li Y, Li J, Liu F, Chen X. Tannic acid stimulates glucose transport and
292 inhibits adipocyte differentiation in 3T3-Li cells. J Nutr. 2005;135(2): 165-71.
293 24. Yang C-Y, Wang J, Zhao Y, Shen L, Jiang X, Xie Z-G. et al. Anti-diabetic effects of
294 *Panax notoginseng* saponins and its major anti-hyperglycemic components. J
295 Ethnopharm. 2010;130(2): 231-6.

UNDER PEER REVIEW

296 25. Zheng T, Shu G, Yang Z, Mo S, Zhao Y, Mei Z. Antidiabetic effect of total saponins
297 from *Entada phaseoloides* (L.) Merr. in type 2 diabetic rats. J Ethnopharm. 2012;
298 139(3): 814-21.
299