Enzyme Assay Guided Isolation of Alpha Amylase Inhibitor from *Manilkara hexandra* Leaves

PATEL EKTA D.*, PATEL DIVYAKANT A., PATEL YOGESH K., SHAH DHWANI A., VEGAD KUNJAL L., PATEL N. J.
Sharda school of pharmacy, Gujarat technological University,
Gandhinagar-Pethapur 382610,India

Runningtitle:α- Amylase inhibitor from leaves of *Manilkara hexandra*

*Addressforcorrespondence
E-mail:ekta.dpatel15@gmail.com

Abstract:

There has been a conspicuous resurgence of interest in the use of newer approach to treat diabetes and its complication has been immerging out because of the limitation of recently available drugs. The selection of plant was carried out on the bases of *invitro* screening of four antidiabetic plant for α- amylase activity. Methanolic extract of *Manilkara hexandra* shows good alpha amylase inhibition activity compare to other plants studied. *Manilkara hexandra* leaves traditionally known as Khirni or Rayan are considered useful for the treatment of diabetes in previous study.The alpha amylase inhibitor was separated by sequential extraction using increasing polarity of solvent such as n-Hexane, Chloroform, Ethyl acetate, n-Butanol and bioassay guided TLC fractionation. The ethyl acetate subfraction 3 shows highest inhibitory activity was further analyze by phytochemical screening, TLC, UV, Mass, IR and ^{1}HNMRspectroscopy. Spectroscopic and chromatographic data reveals that the ethyl acetate subfraction 3 is flavonoid quercetin like compound. The enzyme assay guided fractionation of methanolic extract results in isolation of quercetin from *Manilkara hexandra* leaves as an active α-amylase inhibitor.

Key words: Flavonoid, Diabetes mellitus, Quercetin, Acarbose, α- Amylase assay

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Intoduction:

Diabetes mellitus is a group of metabolic disorders of fat, carbohydrate and protein that results from defects in insulin secretion, insulin action (sensitivity), or both. The acute & chronic complications of diabetes are major cause of hospital admissions. Antidiabetic agent may exert its blood glucose lowering effect by stimulating insulin secretion from pancreatic beta cells, enhancing glucose uptake by fat and muscle cells, altering the activity of glucose metabolism enzymes or reduce absorption of sugar from gut[1,2].

Various carbohydrate metabolizing enzymes are involved in pathogenesis of diabetes as glucose 6 phosphatase, hexokinase, phosphofructokinase, glycogen phosphorylase, aldose reductase, α-amylase, glucose 6 phosphate dehydrogenase etc. Alteration in activity of enzymes leads to hyperglycemia. α-Amylase is an enzyme that hydrolyses alpha bonds of large alpha linked polysaccharides, such as starch and glycogen, yielding glucose & maltose. Researcher Olusola & Verma et al.stated that inhibition of α-amylase enzymes significantly decreases the postprandial increase of blood glucose and therefore can be an important strategy in the management of type 2 diabetic patients[3,4,5,6].

Plant based products have been known to man since ancient time[7].Thus, plant-based drugs or botanicals are emerging as the primary components of holistic approaches for diabetes management[8].Leaves of *Manilkara hexandra* (Sapotaceae) and*Ailanthus excelsa* (Simaroubaceae), peels of *Citrus medica* (Rutaceae) and rhizome of *Cyperus rotundus* (Cyperaceae) are

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used as natural antidiabetic remedies[9,10,11,12,13,14]. *Manilkara hexandra* leaves traditionally known as Khirni or Rayan [14]are considered useful for the treatment of diabetes in previous study. There are only few studies are available on antidiabetic properties of this plants. Previous In-vivo study proved that Manilkara hexandra improve hypoglycemia and hyperlipidemia in HFF-STZ induced diabetes rat, but the mechanism behind hypoglycemic control was not proven[6]. However, no study has so far been conducted concerning the activity on pancreatic *α*-amylase. So, present research was aimed to screen antidiabetic plants for α- amylase activity and to identify the possible active alpha amylase inhibitor.

MATERIALSANDMETHODS

Plant material and authentication:

Medicinal plants *Manilkara hexandra*, *Citrus medica, Cyprus rotundus* and *Ailanthus excelsa* were selected for present study based on their antidiabetic property reported in previous study and in *Ayurvedic* literature. Medicinal plants were collected from local region of Gandhinagar district, Gujarat, India. Voucher specimens were deposited in the herbarium museum of the NISCAIR, New Delhi.

Chemicals:

Alpha amylase purchased from Chemdyes corporation, India. Acarbose was procured as a gift sample from West coast pharmaceutical Ltd., India. All other chemicals were purchased from CDH Pvt. Ltd. All the chemicals used in the study wereof analytical grade.

Preparation of crude methanolic extract:

The leaves of *Manilkara hexandra* and *Ailanthus excelsa,* rhizomes of *Cyprus rotundus* and Peels of *Citrus medica* were collected, sun dried, powdered byusinggrinder, passedthrough#100 meshsizesieve and stored in room temperature for further experiment. Methanolic extract of each plant were prepared by Soxhlet extraction method, collected, vacuum dried, weight and percentage yield were calculated. Each methanolic extract were assayed for α-amylase activity.

α-Amylase inhibition assay:

The α-amylase activity was determined using the method described by Priyarani M *et al*.[15]. Briefly, appropriate dilution of 500 µl of extract (1000 µg/ml) and 500 µl of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) containing pancreatic α-amylase (0.5 mg/ ml) were incubated at 25°C for 10 min. Then, 500 µl of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) was added into tube. The reaction mixture was incubated at 25°C for 10 min and stopped with 1.0 ml of dinitro salicylic acid colour reagent. Thereafter, the mixture was incubated in a boiling water bath for 5 min, cooled to room temperature, diluted by adding 10 ml of distilled water and absorbance measured at 540 nm. Individual blanks were prepared for correcting the background absorbance. In this case, the color reagent solution was added prior to the addition of starch solution and the mixture then placed in the water bath immediately. Controls were representative of the 100% enzyme activity. Acarbose, a well-known α-amylase inhibitor, was used as positive control. A graph was plotted with concentration along the x axis and percentage inhibition along the y axis to obtain the IC_{50} value.

Equation:

$$\frac{\text{A control -- A sample}}{\text{A control}} \times 100$$

Sequential fractionation of methanolic extract:

Methanolic extract of *Manilkara hexandra*was taken for successive fractionation using increasing polarity of solvents as n-hexane, chloroform, ethyl acetate, n- butanol and residue was collected. The enzyme activity of the crude extract and all fractions were studied on pancreatic *α*-amylase.Dose dependent variation in α-amylase activity was measured using 25µg/ml to 250 µg/ml concentration of various fraction. The concentration of each test sample giving 50% inhibition (IC_{50}) will be determined by plotting log

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concentration of compound versus percentage inhibition.

Enzyme assay guided subfractionation of ethyl acetate fraction:

The fraction which showed the highest inhibitory activity was subjected to subfractionation by preparative chromatography on silica gel G (70–230 mesh size) using Toluene: Ethyl acetate: Formic acid (3.6: 1.2: 0.5) solvent system. The developed plates were air dried and visualized under UV light. For further detection of chemical compounds, the plates were derivatize with iodine vapour. All visible individual fractions were marked, scraped from the preparative TLC plate, dissolved in methanol and assayed for alpha amylase activity. Dose dependent variation in alpha amylase activity was measured using 2.5µg/ml to 15µg/ml concentration of various subfraction.

Identification and characterization of active subfraction:

The chemical constituent present in active subfraction was confirmed by phytochemical test. The active subfraction was identified on the basis of spectral and chromatographical studies. The UV-Vis spectrum was recorded on a Shimadzu UV-1800 double beam spectrophotometer in methanol. The Mass spectra was obtained on LCMS 8030. The IR spectra was obtained on Thermo IS 10 spectrometers in Kbr. The ^{1}H-NMR spectra wasrecorded on Bruker spectrometer in DMSO. The spectra were compared with the steroid and flavonoid compound reported in literatures. The active compound was further conformed by co-chromatographed along with reference quercetin using Toluene: Ethyl acetate: Acetone: Formic acid (10:5:15:1) solvent system. TLC plate was observed under UV light and develop with $FeCl_3$.

Statisticalanalysis:

The triplicate results were taken and mean ± S.E.M. were calculated. Difference between the data result were compared by one-way analysis of variance (ANOVA) using The Graph Pad PRISM version 5.00 for Windows.

RESULTSAND DISCUSSION

The % yield of methanolic extract of medicinal plants shown in Table 1.

TABLE 1: %YIELD OF METHANOLIC EXTRACT OF MEDICINAL PLANTS

Name of plant	Plant part used	Yield (%)
Ailanthus excelsa Roxb	Leaf	22.3 ± 1.54058
Cyperus rotundus L.	Rhizome	6.6 ± 0.56126
Citrus medica L.	Peel	15.96 ± 1.15682
Manilkara hexandra Roxb	Leaf	10 ± 0.75831

Each value is the mean of three observations. Values are expressed in mean ± S.E.M. n = 3.

Methanolic extract *of Manilkara hexandra* shows highest alpha amylase inhibitory activity (72.3%) amongst four medicinal plant studied (Fig. 1). Hence, the leaves of *Manilkara hexandra* was selected for further research.Ethyl acetate fraction of methanolic extract revealed highest alpha amylase inhibitory activity (IC_{50} 84.4 mcg/ml) compare to other fractions and standard alpha amylase inhibitor acarbose (Table 2).

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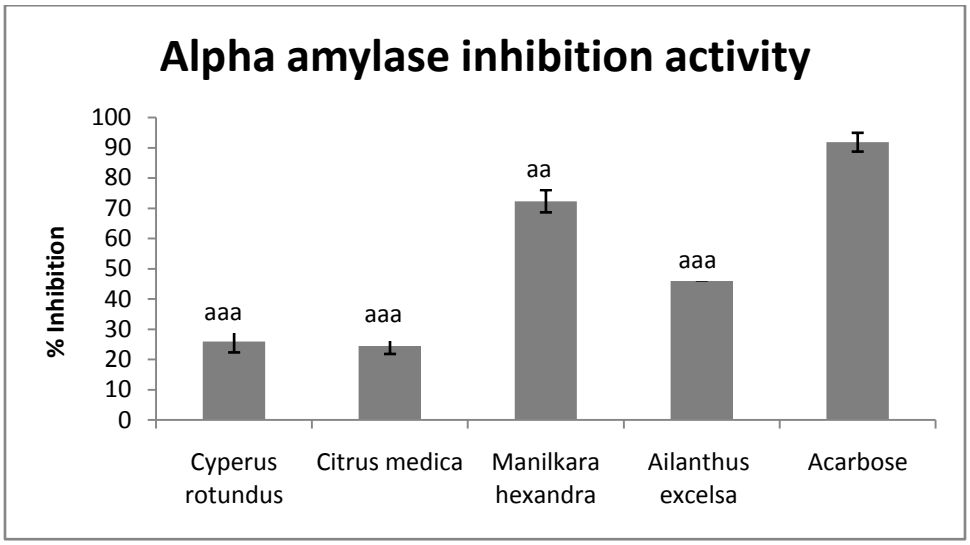

Fig. 1: Screening of α-amylase inhibition activity

Alpha amylase enzyme inhibition activity of methanolic extract of plants compared with Acarbose. Where, Values are expressed in mean ± S.E.M. n=3. aa $p < 0.01$; aaa $p < 0.001$ Vs *Acarbose.*

TABLE 2: IC_{50} OF α-AMYLASE BY VARIOUS FRACTIONS OF METHANOLIC EXTRACT OF *MANILKARA HEXANDRA* LEAVES

Name of Fraction	Concentration (µg/ml)	% Inhibition	R^2 Value	IC_{50}
Hexane	25	0	0.8967	153.5608***
	50	22.1133		
	100	23.2933		
	175	72.4933		
	250	74.7933		
Chloroform	25	29.4933	0.9388	103.0451~
	50	32.8967		
	100	47.5933		
	175	79.5933		
	250	82.9967		
Ethyl acetate	25	23.57	0.9220	84.43436***
	50	31.3933		
	100	71.2967		
	175	83.995		
	250	102.497		
Acarbose	25	11.495	0.9660	114.5768
	50	29.8967		
	100	47.9667		
	175	78.3967		
	250	91.7933		

Eachvalueisthemeanofthreeobservations. Values are expressed in mean ± S.E.M. n = 3. ***$p < 0.001$, ~$p > 0.05$ (Statistical similarity) Vs Acarbose.

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The enzyme assay guided subfraction of ethyl acetate fraction shows five different band. Subfraction 3 shows strong alpha amylase inhibition activity (IC_{50} 5.55 mcg/ml) amongst five subfraction tested (Table 3). Ethyl acetate subfraction 3 was subjected for analytical study to identify active constituent present in it.

TABLE 3: IC_{50} OF α-AMYLASE BY VARIOUS SUBFRACTIONS OF ETHYL ACETATE FRACTION OF *MANILKARA HEXANDRA* LEAVES

Name of Subfraction	Concentration (µg/ml)	% Inhibition	R^2 Value	IC_{50}
EA1	2.5	11.9967	0.9249	12.27507**
	5	32.067		
	7.5	36.4965		
	10	42.8993		
	15	56.6967		
EA2	2.5	13.187	0.9550	17.73969***
	5	18.9973		
	7.5	24.1967		
	10	32.498		
	15	42.999		
EA3	2.5	32.4983	0.9534	5.551344
	5	53.0993		
	7.5	58.8993		
	10	68.8977		
	15	84.4147		
EA4	2.5	0	NA	NA
	5	0		
	7.5	0		
	10	0		
	15	0		
EA5	2.5	0	0.9218	35.77724***
	5	0		
	7.5	7.79933		
	10	13.0967		
	15	16.9967		

Eachvalueisthemeanofthreeobservations. Values are expressed in mean ± S.E.M. n = 3. ** p < 0.01; ***p < 0.001 Vs EA3.

The Phytochemical screening shows the presence of steroid and flavonoid also evidence from reported literature (Table 4)[14]. The mass spectrum indicates the presence of a molecular ion peak at m/z (*I* %) 302 (100%) (M^+) (Fig. 2). The UV spectra analysis of active subfraction shows λmax- 369 in methanol (Fig. 3). IR (KBR, Vmax, cm^{-1}) indicate the presence of absorbance bands at 3543.23, 3446.79, 3402.43, 3257.77 (O-H stretching), 2883.58 (CH2-stretching), 1653, 1629 (c=c stretch), 1610, 1560, 1450 (Aromatic ring stretch), 1379,1309 (C-OH vibrations), 1109 (C=O) (Fig. 4). ^{1}H-NMR (400MHz in DMSO, δ ppm) data shows 6.22(1H, d, H-6), 6.44(1H, d, H-8), 6.91(1H, d, H-5'), 7.56(1H, d, H-6'), 7.72(1H, d, H-2')(Fig. 5).

TABLE 4: PHYTOCHEMICAL SCREENING OF ETHYL ACETATE SUBFRACTION 3

Plant constituent	Chemical Test	Ethyl acetate Subfraction3
Alkaloid	Dragendorff	-
	Wagner	-
Steroid	Salkowaski	+
Carbohydrate	Molish	-
Tannin	$KMnO_4$	_
	Br_2 water	-
Flavonoid	Shinoda	+
	Lead Acetate	+
Saponin	Foam Test	-
Cardiac glycoside	Keller Killani	_
Oil	Filter paper	_
	Water solubility	_

In present study methanolic extract of *Manilkara hexandra* leaves shows strong alpha amylase inhibition (72.3%) compare to all other medicinal plant tested. Researcher Bhumi et al.showed that methanolic extract of dried bark of *Manilkara hexandra* was effective in alloxan induced diabetic rat and stated that further investigations are needed to identify the exact mechanism of antihyperglycemic activity and to isolate the active constituent(s) present[10]. Reported literature showed ethyl acetate fraction of methanolic extract of Leaves of Manilkara hexandra improve diabetic and its vascular complication in HFF-STZ diabetic rats[6]. So, the present study was aimed to identify the mechanism behind antidiabetic potential of alpha amylase inhibitor from *Manilkara hexandra* leaves. Result of alpha amylase assay revealed that ethyl acetate fraction (IC_{50} 84.4 mcg/ml) and ethyl acetate subfraction 3 (IC_{50-} 5.55 mcg/ml) shows strong alpha amylase inhibition compare to other fraction in dose dependent manner.

Phytochemical screening of ethyl acetate subfraction 3 shows the presence of flavonoid and steroid and is pale yellow in colour having solubility in methanol and ethyl acetate. UV, Mass, IR and NMR data was used to identify the compound by their λ_{max}, molecular weight, functional group and position of hydrogen ion respectively in chemical structure. Based on the analytical data and literature survey, the compound identified was flavonoid quercetin like compound and all of its data were comparable with those reported in the literature [16,17,18,19,20,21].

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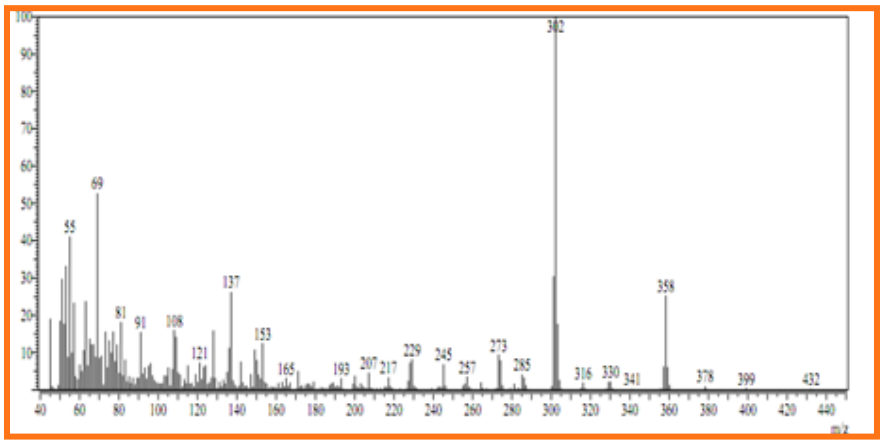

Fig. 2: Mass spectra of ethyl acetate subfraction 3

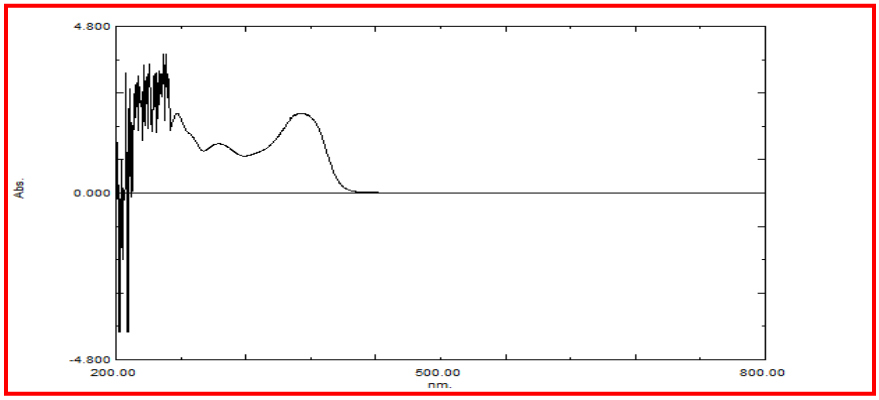

Fig. 3: UV absorption spectra of ethyl acetate subfraction 3

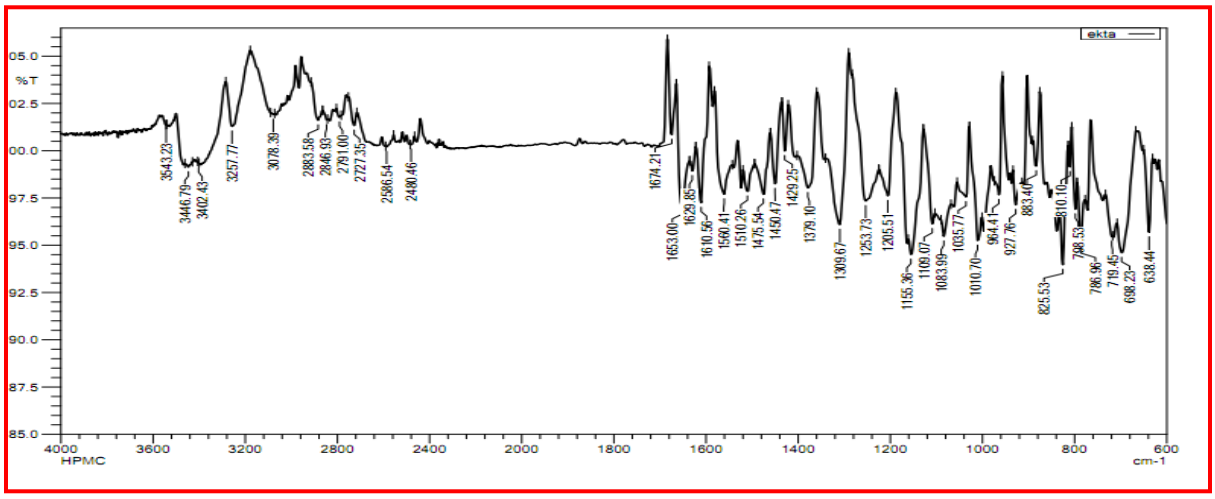

Fig. 4: IR spectra of ethyl acetate subfraction 3

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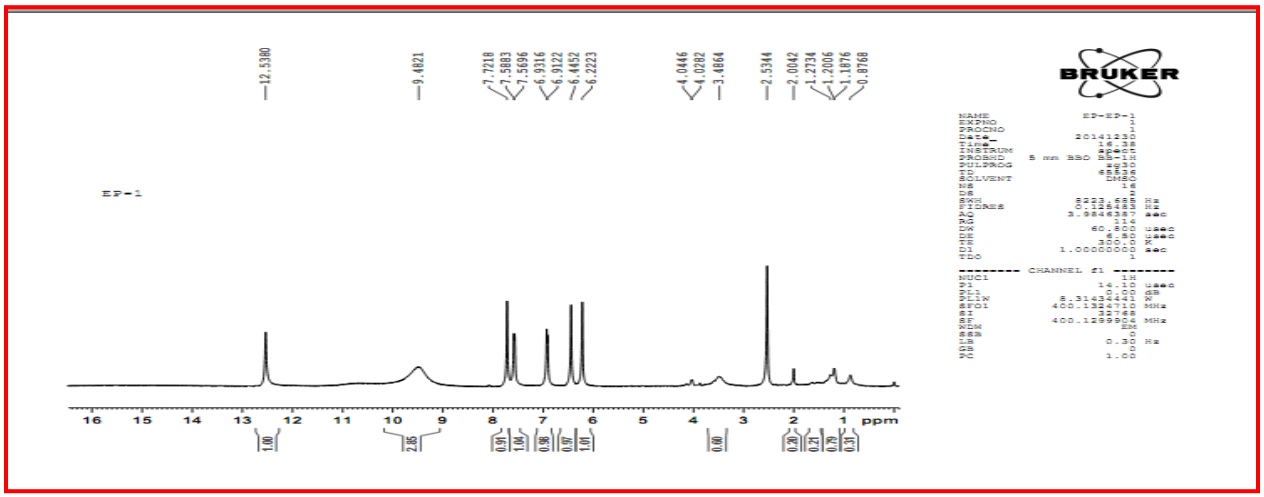

Fig. 5: NMR spectra of ethyl acetate subfraction 3

A wide range of chemical compounds including sterol, starch, terpenoid, anthraquinone glycoside, cardiac glycoside, saponin and tannin etc. have been isolated from this species[14,22]. Present of flavonoid was reported in root of *Mimusops hexandra*[23]. Three active compound such as gallic acid, myrecetin and quercetin were isolated by Eskander *et al*., from seeds of *M. hexandra*[24]. It is already reported that quercetin shows the antidiabetic activity[6,25]. However, this study is the first report on the inhibitory effect of *Manilkara hexandra* leaves on *α*-amylase and isolation of quercetin as their active constituent.

Based on these experimental results, it can be concluded that a part of antidiabetic effect observed by*Manilkara hexandra* leaves might be due to the inhibition of the *α*-amylase by quercetin. The finding of present study prove*Manilkara hexandra* lends credence to its folklore use in the management and/or control of type II diabetes.In conclusion, present study suggests that one of the targets for antidiabetic property of *Manilkara hexandra*is pancreatic α-amylase inhibition by flavonoid quercetin.

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