Antioxidant, anti-acetylcholinesterase and anti-glycosidase properties of three species of Swertia, their xanthones and amarogentin: A comparative study

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ABSTRACT

Aim: The aim of the study was to analyze the antioxidant, anti-amylase, anti-glucosidase and antiacetylcholinesterase (anti-AChE) properties of the leafy shoots of three Indian species of *Swertia* e.g. *Swertia chirata* and its substitutes *Swertia bimaculata*, and *Swertia decussata*, their xanthones and amarogentin. **Methods:** Antioxidant activity of the methanolic extracts of leafy shoots was measured in terms of DPPH, superoxide and nitric oxide radical scavenging activities as well as metal chelating properties. Enzyme inhibitory properties were measured using AChE, α-amylase and α-glucosidase respectively. Five xanthones bellidifolin (1), swerchirin (2), decussatin (3), mangiferin (4) and 1-hydroxy-3,5,8-trimethoxy xanthone (6) and one iridoid, amarogentin (5) were isolated from *Swertia chirata*. The activities of the isolated components were compared. **Results:** *Swertia chirata* exhibited best antioxidant and anti-AChE properties than the other two species. The plants also possessed α-glucosidase inhibitory properties but weak α-amylase inhibitory activity. Highest activities were observed in *Swertia chirata*. We report here, for the first time, the antioxidant, anti-AChE and anti-glycosidase activity of 1-hydroxy-3,5,8-trimethoxy xanthone. This xanthone had strongest DPPH radical scavenging activity and anti-AChE property. **Conclusion**: The results suggest the beneficial effects of the xanthones of *Swertia chirata*. But further study should be carried out to prove the efficacy *in vivo*.

Key words: *Swertia* Sp., Xanthone, Glycosidase, Acetylcholinesterase, Antioxidant.

INTRODUCTION

Swertia chirata Buch-Ham [Syn. *Swerta chirayita* (Roxb ex Flemming) H. Karst] is considered the most important species of *Swertia* reported from India, for its medicinal properties. *S. chirata* is one of the important ingredients of many Ayurvedic / herbal formulations.[1,2] *S. chirata* is used as antipyretic, anthelmintic, hypoglycemic, febrifuge, laxative, stomachic, tonic and in asthma and leucorrhoea.[2] The plant is also used in the treatment of hepatic disorders.[3,4]

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However, populations of *S. chirata* are diminishing and the plant has been considered as critically endangered.[1,5] Other species like *S. decussata* Schult. and *S. bimaculata* (Siebold and Zuccarini.) J.D. Hooker and Thomson ex C.B. Clarke are used as substitutes of *S. chirata*.[6,7] Xanthones are the main active secondary metabolites of *Swertia* sp. But metabolites such as flavonoids, iridoid glycosides and terpenoids are also other active constituents.[8]

In the present paper, we report a comparative study of antioxidant, acetylcholinesterase, α-amylase and α-glucosidase inhibitory properties of leafy shoots of three Indian species of *Swertia* e.g. *S. bimaculata*, *S. chirata* and *S. decussata*. Activities of the xanthones and amarogentin , isolated from *S. chirata*, were also investigated.

MATERIALS AND METHODS

Plant Materials

The leafy shoots of *S. chirata* (Voucher no. Bot 332S-1), *S. bimaculata* (Voucher no. Bot 332S-2) were collected from Darjeeling Himalayas and *S. decussata* (Voucher no. Bot 332S-3) was collected from the Western Ghats, India. The plants were identified and the voucher specimens are available in the Department of Botany, Calcutta University. The extracts were made from the dried ground materials by refluxing with 100% methanol for 5 hours. The extracts were then evaporated to dryness. Different concentrations of the methanolic solutions of the extracts were used for studying the bioactivity *in vitro*. Each experiment was repeated three to five times.

Reagents and chemicals

1,1-Diphenyl-picrylhydrazyl, acetylcholinesterase from *Electrophorus electricus* (electric eel), DNSA (3,5-di-nitro salicylic acid), were procured from Sigma, USA. Nitroblue tetrazolium, 5,5'dithiobis (2 nitrobenzoic acid), acetylthiocholine iodide, ferrozine, PNPG (p - Nitrophenyl α - D - Glucopyranoside), α-glucosidase (ex microorganism), α-amylase (ex porcine pancreas) were obtained from Sisco Research Laboratories PVT. Ltd., India. Riboflavin was obtained from HiMedia Laboratories Limited. Sodium nitroprusside, ferric chloride, ammonium molybdate were obtained from Merck Specialties Private Limited. All other reagents and chemicals were of analytical grade.

Extraction and isolation of the compounds, 1 – 6.

The air dried and coarsely powdered aerial parts of *S. chirata* (2 kg) were successively extracted with hexane and ethyl acetate. These extracts were separately processed for isolation of compounds. Chemical structures of all the compounds were determined by spectral analyses (UV, IR, MS and NMR) and by comparison of the data with the reported values.[9-12] Hexane extract of the air dried plant material was concentrated and chilled in refrigerator. A yellow solid separated out. It was crystallized from rectified spirit as shining needles which was identified as 1 (1,5,8-trihydroxy-3-methoxy xanthone; bellidifolin). After separation of 1, the filtrate was concentrated and chromatographed over silica gel column. Hexane – ethyl acetate (9:1) eluents afforded 3 (1-hydroxy-3,7,8-trimethoxy xanthone; decussatin) and hexane – ethyl acetate (4:1) eluents yielded 2 (1,8-dihydroxy-3,5-dimethoxy xanthone; swerchirin; methylbelledifolin). 2 formed bright yellow fine needle shaped crystals and 3 formed a pale yellow shining crystalline solid. Further eluents in hexane – ethyl acetate (1:1) yielded 6 (1-hydroxy-3,5,8-trimethoxy xanthone) as yellow needles. Hexane defatted ethyl acetate extract of the plant was concentrated and chromatographed over silica gel. Hexane – ethyl acetate (1:1) solvent eluents separated 4 (mangiferin) as cream coloured solid which was crystallised from ethyl acetate – hexane mixture. After separation of mangiferin the bitter iridoid glucoside amarogentin (5) was separated out as amorphous white solid.

DPPH radical scavenging activity

1,1-Diphenyl-picrylhydrazyl (DPPH) free radical scavenging activities of the extracts were determined following the method described.[13] The extract (0.1 ml) was added to 0.004% MeOH solution of DPPH (3 ml). After 30 min, the absorbance was measured at 517 nm. The percentage inhibition activity was calculated as [(Ao-Ae)/Ao]x100 (Ao = absorbance without extract; Ae = absorbance without extract).

Superoxide radical ($O_2^{\cdot-}$) scavenging activity

Superoxide radical scavenging activity was measured following the method used by Banerjee and De[14] in the riboflavin-light-nitrobluetetrazolium (NBT) system. The reaction mixture contained 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, 2 μM riboflavin, 100 μM EDTA, 75 μM NBT and 1 ml extract. The production of blue formazan was followed by monitoring the increase in absorbance at 560 nm after 10 min illumination from fluorescent lamp. The entire reaction set was enclosed in a box lined with aluminium foil. Identical tubes with reaction mixture were kept in the dark and served as blanks.

Metal chelating effect (Ferrous ion)

The method[15] is based on the chelation of ferrous ions by plant extract. Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of plant extract, as the chelating agent, the complex formation is disrupted resulting in a decrease in the red colour of the complex. Metal chelating activity of the coexisting chelators was measured. Fe^{2+} chelating ability is due to antioxidant activity of the plant Extract.

Nitric oxide scavenging activity

Nitric oxide scavenging activity was measured modifying the method[16] 100 mM Sodium nitroprusside (0.2 ml) dissolved in phosphate buffer saline (pH 7.4) (PBS) and

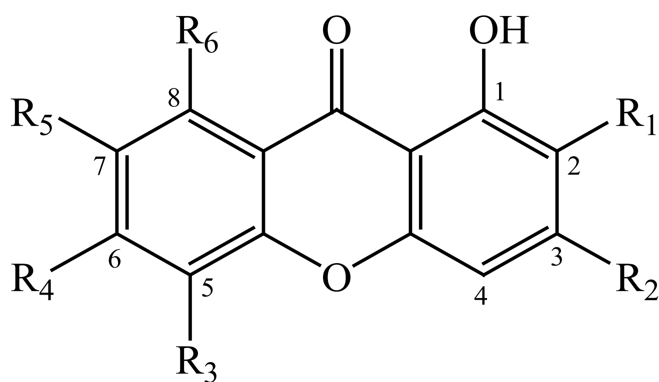

Figure 1: Xanthones isolated from *S. chirata*

	R_1	R_2	R_3	R_4	R_5	R_6
1	H	OCH_3	OH	H	H	OH
2	H	OCH_3	OCH_3	H	H	OH
3	H	OCH_3	H	H	OCH_3	OCH_3
4	Glucose	OH	H	OH	OH	H
6	H	OCH_3	OCH_3	H	H	OCH_3

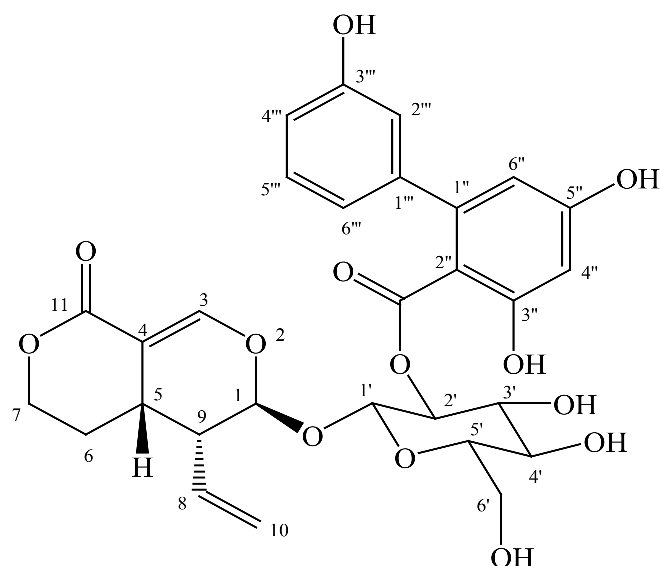

Figure 2: 5 (Amarogentin)

different concentrations of the crude extract dissolved in PBS (1.8 ml) were incubated at 25°C for 2.5 hrs. Nitric oxide generated was detected by Griess reagent (2 ml). The absorbance of the chromophore formed during diazotization of nitrite with sulphanilamide and its subsequent coupling with napthyl ethylene diamine was read at 546 nm.

Acetylcholinesterase inhibitory activity

Acetylcholinesterase (AChE) inhibitory property was measured modifying the previously reported method.[17] using AChE from the electric eel for the assay. Methanolic solution of plant extract (0.01 ml) was added to 0.02 ml AChE (19.93 unit / ml buffer, pH 8.0) and 1 ml buffer. The reaction was started by adding 0.01 ml 0f 0.5 mm 5.5' dithiobis 92 nitrobenzoic acid) (DTNB) and 0.6 mM acetylthiocholine iodide solution (0.01 ml). The reaction mixture was incubated at 37°C for 20 min. The optical density was measured at 412 nm immediately. The percentage inhibition of AChE activity by plant extract was calculated.

α-Glucosidase inhibitory property

α-Glucosidase inhibitory property was measured using *p*-nitrophenyl α-D-glucopyranoside as the substrate.[18,19] α-Glucosidase (ex microorganism) solution (0.006%) was prepared in 0.02 M phosphate buffer (pH 6.3). The enzyme solution (0.13 ml) was incubated with the extract (0.13 ml) and 0.02 M phosphate buffer (0.45 ml) for 1 hr. at 25°C. Then 2M *p*-nitrophenyl α-D-glucopyranoside (0.67 ml) was added to the reaction mixture. The mixture was incubated for 30 min at 30°C. p-Nitrophenol formed was read spectrophotometrically at 405 nm. Ther 30 min at 30°C. The reaction was terminated by adding 1 M Na_2CO_3 solution (2 ml). The percent inhibition activity was calculated as [(Ao-Ae) / Ao] x 100 (Ao = absorbance without extract; Ae = absorbance with extract).

Determination of a-amylase inhibitory activity

α-Amylase inhibitory activity was measured following the previously reported method.[19] α-Amylase (ex porcine pancreas) (0.2 ml of 0.003 % solution) dissolved in 0.02 M phosphate buffer (pH 6.9) was incubated with 0.1 ml of extract for 20 min at 37°C. Then, 0.1 ml soluble starch solution in 0.02 M phosphate buffer was added. The reaction mixture was incubated again at 37°C for 3 min. After the incubation, 0.2 ml of dinitrosalicylic acid reagent was added. The mixture was heated for 5 min in boiling water bath and then cooled. The reaction mixture was then diluted by adding 4 ml distilled water and the optical density was measured at 540 nm. The percent inhibition activity was calculated as [(Ao-Ae) / Ao] x 100 (Ao = absorbance without extract; Ae = absorbance with extract).

Statistical analysis

The results were statistically analyzed by multiple comparison analysis tests (Bonferroni method and Tukey method). P values <0.05 were considered as significant. IC_{50} value (concentration of sample required for 50% inhibitory activity) was calculated from the regression equation prepared from the concentrations of extract and percentage inhibitions due to activity.

Table 1: Spectral data of xanthones and secoiridoid glucoside

Name of the compounds. Molecular formula (M^+)	UVmax in EtOH (nm) / $[\alpha]_D^{25}$	IR in KBr (cm^{-1})	1H NMR (ppm)
Swerchirin $C_{15}H_{12}O_6$ (288)	249, 273 and 325	3150, 1660, 1620, 1600, 1550, 1420	δ (d_6-DMSO): 3.86, 3.92 (C-3, C-5-OMe), 6.29, 6.48 (d, J=3Hz each, C-2 and C-4-H), 6.65, 7.17 (d, J=9 Hz each, C-6, C-7-H), 11.30, 11.88 (C-1 and C-8-OH)
Bellidifolin $C_{14}H_{10}O_6$ (274)	255, 279, 335 and 393	3500, 3150, 1655, 1620, 1600, 1560, 1480	δ (d_6-DMSO): 3.81 (C-3-OMe), 6.27, 6.49 (d, J=2.5 Hz each, C-2 and C-4-H), 6.54, 7.15 (d, J=9 Hz each, C-6 and C-7-H), 11.1-12.0 (C-1 and C- 8-OH)
1-Hydroxy-3,5,8-trimethoxy xanthone $C_{16}H_{14}O_6$ (302)	230, 254, 275 and 330	3100, 1655, 1625, 1610, 1600, 1570, 1480	δ ($CDCl_3$): 3.81, 3.93, 3.95 (C-3, C-5, C-8-OMe), 6.28, 6.44, (d, J=2Hz each, C-2 and C-4-H), 6.65, 6.85 (d, J=9Hz each, C-5, C-6-H), 13.08 (C-1-OH)
Decussatin $C_{16}H_{14}O_6$ (302)	250, 266 and 330	2850, 1650, 1620, 1610, 1580, 1470	δ ($CDCl_3$): 4.02, 3.91, 3.80 (C-3, C-7, C-8 OMe), 6.36, 6.39 (d, J=2Hz each, C-2 and C-4-H), 6.71, 6.55 (d, J=10Hz each, C-5 and C-6-H), 11.70 (C-1-OH)
Mangiferin $C_{19}H_{18}O_{11}$ (422)	209, 241, 259, 318 and 373.5 $[\alpha]_D$ + 30° (C0.5 aq ethanol)	3370, 1650, 1485, 1340, 1100	δ (d_6-DMSO): 3.46 (br.gluc.-OH), 4.60-4.63 (gluc-protons), 6.29 (C-4-H), 6.78 (C-5-H), 7.29 (C-8-H), 9.93 (-OH), 13.84 (C-1-OH)
Amarogentin $C_{29}H_{30}O_{13}$ (586)	$[\alpha]_D^{25}$ - 108.5° (C0.5, Methanol)	3347 (br.), 1683, 1608, 1577, 1455, 1405, 1355, 1320, 1260, 1225, 1205, 1170, 1160, 1065, 1025, 986, 795	δ (d_6-Acetone): 3.80 (m, C-6'-Hz), 4.30 (d, J=7Hz, C-1'-H), 4.77 (dd, J=9.8 Hz, C-2'-H), 5.10-5.75 (m, C-1-H, C-8-H, C-10Hz), 6.26 (d, J=2Hz, C-6"-H), 6.40 (d, J=2Hz, C-4"--H), 6.80 (m, C-2'"-H, C-4'"-H, C-6'"-H), 7.25 (t, J=8Hz, C-5'"-H), 7.45 (d, J=2Hz, C-3-H); 8.40 (br.-OH), 9.30 (br.-OH), 11.76 (s, -OH)

RESULTS AND DISCUSSION

Isolation of xanthones and amarogentin

Five xanthones (Figure 1) were isolated and identified from the hexane extract of *S. chirata*. The xanthones were bellidifolin (1), swerchirin (2), decussatin (3), mangiferin (4) and 1-hydroxy-3,5,8-trimethoxy xanthone (6). The iridoid amarogentin (5) was isolated from the ethyl acetate extract and identified. The spectral data of the xanthones and amarogentin are shown in Table 1. The structures of the compounds are presented in Figures. 1 and 2.

Determination of antioxidant activity

Generation of reactive oxygen species is recognized as an important cellular process involved in numerous pathophysiological processes.[20] Antioxidants play a role in preventing such conditions. During the present study we measured antioxidant activity in different systems of assay. DPPH radical is widely used for assessing the ability of polyphenols which can transfer labile H-atoms to radicals. The radical scavenging activity of DPPH seems to depend on the position and the type of substituent attached to the aromatic part of xanthone which makes it potent antioxidant.[21] The colour of DPPH radical changes from purple to yellow in the non-radical form and its absorbance at 517 nm decreases. Methanolic extracts of the leafy shoots of different *Swertia* species quenched DPPH radical resulting in decolorization.

DPPH free radical scavenging activities of extracts of *Swertia* species were proportionate to concentration of the extracts [r in all cases being >0.93 (p=0.01)]. IC_{50} values are shown in Table 2. *S. chirata* showed highest activity. Activity of *S. bimaculata* was significantly lower than those of *S. chirata* and *S. decussata*. The xanthones isolated from *S. chirata* also scavenged DPPH radical and their IC_{50} values were compared (Table 2). The xanthone 6 showed strongest activity than the xanthones 1 and 4. DPPH radical scavenging activity of 1, 3-5 was previously mentioned.[22] But during the present study the compounds 2, 3, and 5

Table 2: Antioxidant activity of *Swertia* Species

Plant Materials	IC$_{50}$ Values*			
	DPPH radical	Superoxide radical	Metal chelation	Nitric oxide
S. chirata	64.08±6.26	193.26±1.78	1373.54±33.92	279.69±1.98
S. decussata	127.8±1.51	254.60±3.57	1505.43±12.25	300.40±2.02
S. bimaculata	167.1±3.03	289.63±2.64	1537.09 ± 6.65	322.09±6.87
1	46.16±0.91 $^{ns,\underline{ns}}$	204.35 ±8.79 ^{ns}	-	-
2	...	428.04±36.63$^{NS,\ \underline{ns}}$	-	-
3	...	527.87±9.79^{NS}	-	
4	55.72±0.88	379.58±19.49$^{\underline{ns}}$	-	-
5	...	715.25±12.56	-	-
6	30.09±0.13 ns	296.29±13.49 ^{ns}	-	-
Ascorbic acid	47.65±0.93 ns	336.34 ± 0.15 ^{ns}		-

*Results from extracts are given in μg/ml ± sd whereas data from compounds are expressed as μM ± sd; _ not done; ...not active

^{NS} Difference in superoxide radical scavenging activity between 2 vs 3 not significant

^{ns} Difference in activity between 1vs 6 not significant

$^{\underline{ns}}$ Difference in DPPH scavenging activity between 1 vs ascorbic acid and superoxide radical scavenging activity between 2 vs 3 not significant

Others significantly different

Table 3: Enzyme inhibitory properties of *Swertia* Species

Plant Materials	IC$_{50}$ values*		
	AChE inhibition	α-Amylase inhibition	α-Glucosidase
inhibition			
S. chirata	36.55 ± 1.01	1276.67 ± 2.0^{ns}	4.72 ± 0.02
S. decussata	86.50 ± 1.15	990.00 ± 1.0^{ns}	4.62 ± 0.00
S. bimaculata	149.17 ± 1.28	910.00± 8.0^{ns}	25.24 ± 0.19
1	18.47 ± 0.47	-	190.98 ± 2.65$^{\underline{ns}}$
4	43.39 ± 0.13	516.66 ± 7.15	28.05 ± 1.74
6	4.07 ± 0.06	-	154.56 ± 2.70$^{\underline{ns}}$
Physostigmine	1.42 ± 0.02	X	X
Acarbose	X	7.53 ± 0.21	0.009 ± 00

*Results from extracts are given in μg/ml ± sd whereas data from compounds are expressed as μM ± sd; _ not done; X not applicable : $^{ns,\ \underline{ns}}$Difference not significant between each other; others significantly different.

did not show any DPPH radical scavenging activity. DPPH radical scavenging activity of the xanthone 6 is reported for the first time and the activity was found to be strongest among all the xanthones reported here (Table 2). This xanthone was found to be more active than that of ascorbic acid, a well known antioxidant.

Superoxide anion radical ($O_2^{\cdot-}$) can be converted into highly active hydroxyl radical and other reactive oxygen species through formation of hydrogen peroxide.[20] $O_2^{\cdot-}$ During the present investigation it was observed that the extracts of *Swertia* species scavenged superoxide radicals in a dose dependent manner (r value being > 0.98 in all species). IC$_{50}$ values are shown in Table 2. Each species was significantly different from *S. chirata*. All the compounds isolated from *S. chirata* inhibited $O_2^{\cdot-}$. The xanthone 1 exhibited strongest activity (Table 2). The xanthone 6 also showed high activity. IC$_{50}$ values of the xanthones 1 and 6 were less than that of ascorbic acid indicating higher activity. But the differences in activities were not statistically significant.

Removal of free state iron from circulation could be a promising approach to prevent oxidative stress induced diseases.[21] Ferrous ion chelating property of different species of *Swertia* was compared. Percentage metal chelating activity was proportional to the concentration of the extract in all species (r >0.97; p=0.001). IC$_{50}$ values are shown in Table 2.

The elevation of nitric oxide (NO) within the central nervous system is known to be associated with the pathogenesis of neurodegenerative diseases such as brain ischemia, Parkinson's disease and Alzheimer's disease.[23] Extracts of *Swertia* species also scavenged NO. Extracts of the three different species of *Swertia* were also tested *in vitro* for nitric oxide scavenging activity. The extracts scavenged nitric oxide and the activity was proportional to the concentration. On the basis of IC$_{50}$ values (Table 2) highest activity was observed in *S. chirata* followed by *S. decussata*, *S. bimaculata*. The activity of each species was significantly different from the other.

Acetylcholinesterase inhibitory activity

Cholinesterase inhibitors are useful for treatment of mild to moderate Alzheimer's disease. Cholinesterase inhibitors exert three main actions: inhibit cholinesterase, increase extracellular levels of brain acetylcholine and improve cognitive process.[24] Extracts of all the species of *Swertia* exhibited anti-AChE property. IC_{50} values are shown in Table 3. Activities in different species were significantly different. Highest activity was observed in *S. chirata* and lowest in *S. bimaculata*. Xanthones (1-4, 6) isolated from *S. chirata* were tested for their AChE inhibitory properties. Compound 6 had highest AChE inhibitory activity which was proportional to the concentration. The xanthones 1 and 4 also inhibited AChE in a dose-dependent manner, 1 being more active than 4. The AChE inhibitory activity of 1[25] and 4[26] was previously reported. Compound 2 showed very little activity. The xanthone 3 was found to have no activity. The bitter iridoid 5 also showed no AChE inhibitory activity. The differences in activities between the compounds were statistically significant. Strong AChE inhibitory activity of 6 is reported for the first time. The activity of this compound was close to but less than that of physostigmine, the known AChE inhibitor.

Glycosidase inhibitory activity

Diabetes is a metabolic disorder with complications like post-prandial hyperglycemia. α-Glucosidase inhibitor and α-amylase inhibitors help in managing post-prandial hyperglycemia [27] During the present study, it was observed that the three species of *Swertia* had very little α-amylase inhibitory activity (Table 3). But the methanolic extracts of all the three species inhibited α-glucosidase activity in a dose dependant manner. High activity was observed in both *S. chirata* and *S. decussata* and lowest in *S. bimaculata* (Table 3). The xanthones 1, 4 and 6 inhibited α-glucosidase enzyme in a dose dependent manner. Highest activity was observed in 4 (Table 3). The xanthone 2 also inhibited α-glucosidase enzyme in a dose dependent manner but it was observed that this xanthone had weak activity (only 44% inhibition at concentration 160 μg/ml). Compounds 3 and 5 were found to have no activity. The xanthone 4, isolated from *Aquilaria sinensis* (Lour.) Gilg has been reported to inhibit α-glucosidase.[28] Here we report the α-glucosidase inhibitory activity of three species of *Swertia* and the xanthones 1 and 6 in addition to 4. Acarbose, the commercial α-glucosidase inhibitor had much higher activity than the isolated compounds and the crude extracts.

One of the causes of Alzheimer's disease is oxidative stress.[29] The hyperglycemia triggers several chronic diabetic complications mediated by increased oxidative stress.[30] Antioxidant and carbohydrate degrading enzyme inhibitory properties of *S. chirata* and the xanthones are probably two of the many reasons for the hypoglycemic property of the plant. The other two species of *Swertia* are also effective. But the present *in vitro* study reveals that their activity is less than that of *S. chirata*. *S. chirata* may also be helpful in preventing memory dysfunction through their antioxidant, particularly NO scavenging property, and acetylcholinesterase inhibitory properties. But further *in vivo* study is required to be carried out to prove the efficacy.

CONCLUSION

The extracts of three species of *Swertia* e. g. *S. chirata*, *S. decussata* and *S. bimaculata* showed antioxidant properties. The extracts also inhibited glycosidase and acetylcholinesterase involved in diabetes and memory function respectively. Highest activities were observed in *S. chirata*.The xanthones were found to be responsible for such activities, *in vitro*. The iridoid glucoside amarogentin did not show any such activities.

CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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