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ORIGINAL ARTICLE

Extraction, Isolation and Identification of Kaempferol 3,7 – Diglucoside in the Leaf Extracts of *Evolvulus alsinoides* (Linn.) and its Inhibition Potency against α-Amylase, α-Glucosidase, *Acetylcholinesterase* and Amyloid Aggregation

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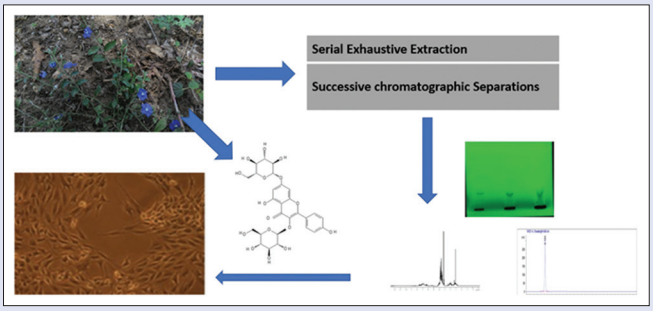
ABSTRACT

Background: *Evolvulus alsinoides* (Linn.) is an eminent *kaphahara* (balancing *kapha*) plant in Ayurvedic medicine commonly used as brain tonic. **Objectives:** To report the enzyme inhibitory potency of the plant extract and to isolate the compound of significant impact. **Materials and Methods:** The plant extracts were obtained by serial exhaustive extraction and successive chromatographic separation. Antioxidant and enzyme inhibition assays were carried out to extract the required isolate. **Results:** The current research identifies for the first time, the presence of Kaempferol 3,7 diglycoside in the leaf extracts by Spectrophotometry, Chromatography, ^{1}H and ^{13}C Nuclear Magnetic Resonance, carboxylic and hydroxylic hydrolysis. The isolated and characterized compound possesses enzyme inhibition property towards α-amylase, α-glucosidase and *Acetylcholinesterase*. **Conclusion:** The compound protects differentiating neuronal cells, SH-SY5Y from Amyloid β Peptide-induced Injury. Thus, the newly identified compound could serve as a plant-based bioactive in the management of Alzheimer's.

Key words: *Evolvulus alsinoides* (Linn.), kaempferol 3, 7-diglucoside, enzyme inhibition, neuroblastoma cell lines, spectral analysis

SUMMARY

- Pharmaceutically potent flavone compound, a Kaempferol 3,7 diglucoside derivative, was isolated for the first time from *Evolvulus alsinoides* (L.) by a method of column chromatography. Bioactivity-guided separation to screen the fractions with potent antioxidant and enzyme inhibition activity was carried out. The purified isolate was characterized using Ultra Violet, High-Performance Thin-Layer Chromatography, High-Performance Liquid Chromatography, Liquid Chromatography Mass Spectrometry, Sugar analysis and nuclear magnetic resonance. The compound was found to protect the differentiating neuroblastoma cell lines SH-SY5Y that have been injured with beta amyloids and inhibit the enzymes, alpha amylase, alpha glucosidase and *Acetylcholinesterase*.



Abbreviations used: AD: Alzheimer's disease; Aβ: Amyloid-β peptide; AChE: Acetylcholine esterase; DPPH: 2,2-Diphenyl-1-picrylhydrazyl; GCMS: Gas chromatography mass spectroscopy; HPLC: High-performance liquid chromatography; HPTLC: High performance thin layer chromatography; LCMS: Liquid chromatography mass spectrometry; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide; NIST: National Institute of Standards and Technology; NMR: Nuclear magnetic resonance; RI: Refractive index; UV: Ultra violet; VIS: Visible; VWD: Variable wavelength detector.

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INTRODUCTION

The medicinal plants aid in retarding the key metabolic pathways or inhibiting the enzymes responsible for the biochemical reactions. Moreover, herbal bioactives claim its rewards for their effectiveness, safety and acceptability.[1] The natural products are regarded to be a very productive source of lead molecules for the development of medicines.[2] *E. alsinoides* (Linn.) which belongs to the family, *Convolvulaceae* commonly called as vishnukranti is a prostrate perennial herb and native of Indian origin from tropical and sub-tropical swampy regions. In Ayurvedic medicine, the whole plant is being used in the management of neurodegenerative disorders as brain tonic,[3] amnesia and asthma,[4] epilepsy[5] and as a hepatoprotective.[6] The phytochemical analysis has reported the existence of biomolecules such as β-sitosterol, scopolin, scopoletin, umbelliferon, triacontane, shankpushpine, and betaine.[7] Extraction

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and phytochemical analysis form the key principle in isolation of therapeutically active ingredients from plant parts using serial exhaustive extraction.[8]

A dysfunction in brain cells amid distress in thinking, memory, and behavior is Alzheimer's disease (AD). There is seldom appropriate medication with a proven disease-modifying effect.[9] The current methods of therapy that slow down the fatal pathophysiological changes leading to manifestation of AD symptoms nevertheless are able to reverse the neurodegenerative process.[10] Acetylcholine esterase (AChE) is serine hydrolases present in tissues of nervous system, body muscles, blood cells and plasma. At cholinergic synapses, AChE terminates impulse transmission of acetylcholine to choline and acetate.[11] The enzyme has a quaternary structure with complex molecular polymorphism in the tissues. It has been reported through *in vitro* models that AChE induces formation of amyloid fibrils and AChE–amyloid-β peptide (Aβ) complexes.[12] These complexes get deposited in the brain as amyloid plaques resulting in AD.[13] Modern treatments targeting the plaques have not succeeded. AChE is being recognized as sensible target for suggestive development in Alzheimer's Management. The cholinergic discrepancy reports to be reliable target in the diagnosis of the disease. The inhibition of AChE is a reliable target AD treatment. Treatments with AChE Inhibitors are found to be beneficial in ameliorating the cognitive dysfunction and more specifically are most effective in improving perceptive responses.[14]

The preliminary studies and epidemiological data proposed that AD can be considered as "Type 3 Diabetes." The regulation of amyloid-β proteins and tau protein phosphorylation with insulin resistance and deficiency have been reported.[15] The effects of insulin in affecting the functions of the central nervous system and modulating cognitive function have been reported. Reduced insulin signaling and insulin resistance in the brain have been found to have a significant impact in Alzheimer's pathogenesis. Clinical trials with Humans have proven the efficacy of some oral antidiabetic medications in improvement of cognition.[16]

Ineffective insulin utilization and its resistance leads to hyperglycemia, a threat linked with increased production of reactive oxygen species damages tissues and causes diabetics mellitus, a persistent metabolic disorder. The treatment for diabetes includes control of postprandial hyperglycemia.[17] The 1,4-glycosidic linkages of polysaccharides are hydrolyzed by α-amylase and reduced into disaccharides. α-glucosidase are enzymes that act upon the disaccharides producing monosaccharides increasing glucose levels in blood.[18] Inhibition of α-amylase and α-glucosidase prevails to be significant target for diabetes treatment.[19] The inhibition of these enzymes causes delayed carbohydrate metabolism, decreased glucose retention rate and increased postprandial plasma glucose. There is wide range of biopharmaceuticals inhibiting the two key enzymes[20] and reducing blood glucose level. Acarbose and voglibose, synthetic molecules being used for maintaining blood glucose levels are reported to produce adverse effects such as abdominal pain, diabetic ketoacidosis, colonic ulcerations.[21] Hence, nutraceuticals from plants of therapeutic importance are being recognized to be efficient inhibitors of enzymes that hydrolyzes carbohydrate.[22]

In the present investigation, the presence of Kaempferol 3,7– diglucoside was reported in *E. alsinoides* (L.) and was examined for its antioxidant, neuroprotective and antidiabetic potential. The ability of the plant bioactives in inhibiting alpha amylase, alpha glucosidase and AChE were reported. The current research is the first-time report on the effect of plant extract in inhibiting the viability of neuroblastoma cell line growth.

MATERIALS AND METHODS

Plant material

E. alsinoides (L.) [Figure 1] was collected from Sathyamanglm Ghats in the months of October 2016. The plant authentication (BSI/SRC/5/23/2016/Tech/1711 Dated 27.10.2016) was done at Botanical Survey of India, Coimbatore, India. After collection, fresh plants were washed once in running water and twice using sterile water. The leaves were separated from the whole plant and dried in shade for 8 days until the moisture content becomes insignificant. Then, the raw material was grounded to powder form and stored at room temperature in an airtight dark container.[23]

The extracts were prepared by cold maceration method. The powdered raw material (100 g) was defatted twice with 500 ml pet ether.[24] The defatted sample was dried till the solvent residues were completely removed. The dried mass was successively extracted using 500 mL of ethylacetate, methanol and sterile double distilled water (aqueous).

Extraction

The extraction was carried out for 48 h at 25°C with continuous intermixing by intermittent replacement of fresh solvent every 24 h.[25] Subsequently, extracts were filtered through cheesecloth and concentrated under reduced pressure at 40°C using rotary evaporator (Make: Cyberlab, USA and Model: CR2000) and lyophilized using Freeze Dryer (Make: Martin Christ– Germany, Model: Alpha 1–2 LD Plus, P/No. 101525) and stored in amber flask at 4°C.[26] Individual metabolites were purified using vacuum-flash chromatography over silica gel followed by thin layer chromatography (TLC). The antioxidant rich fractions were further fractionated with butanol.[27] The butanol extract was further subjected to fractionation with 10%, 20%, 30%, 40%, 50% methanol.

Bioactivity guided separation

Pre-coated and preactivated Silica gel 60 F_{254} plates procured from Merck Specialities was used to detect the presence of antioxidant molecules. The extracts were loaded on the TLC plate at a distance

Figure 1: Habitat of *Evolvulus alsinoides* (Linn.) at Sathyamangalam Ghats

of 0.50 cm above its bottom using sterile glass capillary tubes. The mobile phase, 7.0:3.0:0.5 v/v Toluene: Ethyl acetate: Formic acid is used for biomoleculer elution. The plates spotted with the sample were kept in solvent saturated CAMAG twin trough glass chamber (20 cm × 10 cm).[28] After development, the plates were visualized under ultraviolet [UV]. The antioxidant molecules were detected using 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay.[29]

Radical scavenging activity

Radical scavengers are valuable dietary supplements that protect the human body from various disorders. DPPH assay is a consistent technique in validating antioxidant ability possessed by organic molecules.[30]

Gas chromatography mass spectroscopy analysis

The volatiles in the aqueous extract were determined and quantified using GC system (Agilent Technologies 6890N) coupled with Mass spectroscopy (JEOL) ported with Agilent J and W HP-5 (30 m × 0.2 mm × 0.25 µm) capillary column. System parameters used were: Injection temperature -220°C, initial oven temperature -50°C (increased up to 250°C at a rate of 10°C per min), Helium carrier gas system flow rate-1 mL/min, interface temperature -250°C. The spectrum obtained were compared with the molecular spectrum at National Institute of Standards and Technology (NIST) to identify the compounds.[31]

Acetylcholinesterase inhibition assays

Ellman method was performed with necessary modifications to estimate the cholinesterase inhibitory potential of the plant extracts.[9] Briefly, 50 µL 5,5′dithiobis nitro benzoic acid (3 mM), 50 µL AChE (0.5 mg/mL) enzyme from *Electrophorus* electricus (Type V-S), 35 µL Tris HCL (50 mM) and the extracts ((25–500 µg) were added in a microtiter plate and mixed thoroughly. Addition of 25 µL acetylthiocholineiodide (15 mM) initiates the reaction. The contents were incubated at 37°C for 10 min before and after adding acetylthiocholineiodide. Control contains all the reaction components excluding the enzyme. IC_{50} (µg/mL) for the enzyme inhibitory potential was estimated. The absorbance was read at 412 nm.

α-Amylase inhibition assay

Caraway-Somogyi iodine/potassium iodide (IKI) protocol with essential modifications was carried out for determining α-Amylase inhibitory activity.[32] Phosphate buffer (pH 6.9) is used for preparing required dilution of the enzyme and 96 well micro-titer plate is used to carry out the assay. 50 µL α-amylase (1 mg/mL) was added to the extract (25–500 µg) and incubated at 37°C for 10 min. Addition of 50 µL starch solution (0.05%) initiates the reaction. The contents were incubated at 37°C for 10 min. The control contains all the reactions reagents except the enzyme. Addition of 25 µL HCl (1M) to the reaction well terminates the reaction. 100 µL iodine-potassium iodide (0.1 M) solution was added to the reaction tubes to measure the optical density at 630 nm (iMark™ Microplate Absorbance Reader-catalog #: 168–1130). The inhibition percentage is estimated for each extract at its varying concentration and IC_{50} (µg/mL) was determined.

α-Glucosidase inhibition assay

Maltose is used as a substrate in determination of α-glucosidase inhibition potential of the plant extract. 10 mM phosphate buffer (pH 6.86) was used to dissolve the enzyme and the substrate in a micro-titer plate. 40 µL extract at varying concentration (25–500 µg) was prepared using 10 mM phosphate buffer and was mixed with 40 µL α-glucosidase (0.3U/mL). The reaction was initiated with 20 µL maltose (2 mM) after 5 min incubation at 37°C and further incubated for 15 min. The absorbance of the blank and sample was read at 695 nm after terminating the reaction with the addition of 50 µL methanol. All the reaction components without enzyme were used as blank. α-glucosidase inhibition potential was estimated as IC_{50} (µg/mL).[33]

Ultra violet spectrum

Spectral finger print was acquired for the UV region (220–400 nm). The flavonols and flavones spectra contain two absorption peaks in 240–400 nm spectral regions. These two peaks are generally reported as Band 1 (300–380 nm) and Band 2 (240–280 nm). Absorption of B-ring cinnamoyl system constitutes for Band 1 and A-ring benzoyl system absorption associates with Band 2.[34]

High performance thin layer chromatography analysis for isolated flavonoid

A chemo fingerprinting system that aids in qualitative and quantitative assessment of metabolites is high performance thin layer chromatography (HPTLC). In the present study, 10 cm × 10 cm silica-60 HPTLC plate (Merck, Darmstadt, Germany) was loaded with the purified fraction. Elution was carried out using toluene: Ethyl acetate: Formic acid (7:3:0.5 v/v) system in a CAMAG twin trough glass chamber (20 cm × 10 cm) and visualized at 254 nm.[35]

High-performance liquid chromatography analysis for isolated flavonoid

A robust and versatile technique used in natural product research for identification, quantification and purification of phytochemicals is high-performance liquid chromatography (HPLC). The analysis was performed using Agilent 1220 Infinity series (Agilent Technologies, California, USA), binary gradient system with analytical C_{18} column with UV- visible (Variable wavelength detector) and refractive index detector. The system parameters are: Mobile phase-water and acetonitrile (45:55) with 0.1% ortho-phosphoric acid, flow rate –1.0 ml/min and wavelength –340 nm.[36] HPLC grade reagents filtered with 0.45 mm Millipore filter was used.

Liquid chromatography/mass spectrometry (dextrorphan) analysis

The flavonoids can be detected by Liquid Chromatography-Tandem Mass Spectrometry Coupled with a Post-Column Derivatization Technique. UHPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with two Shimadzu UHPLC: Nexera UHPLC system Column: Shim-pack XR-ODS III (100 mm × 2 mm, 2.2 µm particle size) was used with column temperature 40°C. 0.1% formic acid in water and Acetonitrile were filtered using a 47 mm diameter cellulose nitrate filter (Sartorius, Goettingen, Germany) of pore size 0.45 µm and used as mobile phase. The system parameters are: Flow rate – 0.3 mL/min and injection volume – 5 µL.

LC-MS/MS System (Make: Shimadzu Corporation, Kyoto, Japan, Model: Liquid chromatography Mass spectrometry (LCMS) 8040, Triple Quadrupole) Ionization: ESI (positive/negative), ion spray voltage: +4.5 kV/−3.5 kV, dwell time 5 ms/pause time 1 ms ambient CDL temperature: 250°C, block temperature: 400°C, detector voltage: 1.3 kV nebulizer gas flow: 1.5 L/min drying gas: 10 L/min was used for mass detection.[37]

Sugar analysis

Acid hydrolysis of the isolate was carried out by adding 0.1 N HCl and incubating for 30 min in a boiling water bath. Diethyl ether was added to the cooled hydrolysate for fractionation. Aglycones elutes to the ether phase and the mother liquor retains the sugar. The aqueous solution was exhausted with xylene for alkaline hydrolysis using 2N NaOH. Addition of 2N HCl neutralizes the solution. The mother liquor contains deacylated compound. The absolute configurations of the glucose in the hydrolysate was determined from GC–MS analysis.[38]

Nuclear magnetic resonance

Nuclear magnetic resonance (NMR) spectra were determined in D_2O with ^{13}C NMR at 150 MHz and ^{1}H NMR at 600 MHz using AV400-Bruker High Resolution Multinuclear FT-NMR Spectrometer at Indian Institute of Science, Bengaluru. The spectrum was interpreted using ACD/Spectrus Processor from ACD Labs.[7]

Determination of neuroprotective effect

SH-SY5Y Human neuroblastoma cell lines procured from National Centre for Cell Science, India were sub-cultured on RPMI-1640 supplemented with 10% FBS at 37°C with 100 U/ml penicillin and 100 µg/ml streptomycin in a CO_2 (5%) chamber. 96-well plates were used to seed the cells at 5000 cells/well and were incubated for 48 h. 1 µM beta Amyloid was added to the cells at 60%–70% confluence and were further added with the isolate (0.01, 0.1, 1, 10, 100 µM). The cells were incubated for 24 h, followed by addition with 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) (0.5 mg/ml) for 4 h and 10% SDS solution for another 15 min at 37°C. A microplate reader was used to record the absorbance at 570 nm.[39]

RESULTS AND DISCUSSION

Antioxidant scavenging potential

DPPH assay was performed to determine antioxidant potential of *E. alsinoides* (L.) extracts. IC_{50} values are expressed as mean ± standard error of the mean of three independent experiments. Aqueous extract (IC_{50} value 52.43 µg/mL) possess strongest radical scavenging activity followed by methanolic extract (IC_{50} value 61.55 µg/mL). The aqueous crude that was fractionated with butanol and further with methanol, showed higher antioxidant potential with the order of increasing polarity. The extract fractionated with 20% methanol was found to possess radical scavenging property [Table 1] compared with other fractions. The aqueous extract was more effective than the control. A positive correlation between DPPH assay and phenolics have been reported validating the significant contributions of plant phenolics in the scavenging free radicals.[40]

DPPH assay is relatively advantageous in determining the antioxidant property of the biomolecules because DPPH reacts with all the functional groups in the sample and the incubation time adopted in the protocol is sufficient even for the weak antioxidants to react slowly with the DPPH. The assay can be performed with aqueous and non-polar extracts to examine both lipophilic and hydrophilic radical scavengers.[30] The antioxidant compounds are visualized as yellow spots on TLC plate [Figure 2]. After spraying with DPPH and visualizing at 366 nm, dark blue to light blue spots and purple spots indicates the antioxidant compounds.[41] The aqueous extract was found to be rich in antioxidant potential. Hence, the aqueous extract was used for further studies.

A similar result was found with fruit extracts of *Rhus coriaria* L. where water extracts showed higher antioxidant activities than ethanol extracts.[42] The significance of extracting solvent in altering the antioxidant property of wheat bran has been studied and recommended high polar solvents for extracting antioxidants.[43] Studies with *Limnophila aromatica* reported that the phenolic acids and flavanoides in the plant sample are consistent with each other and the antioxidant potential. Existence of plant phenolics at higher

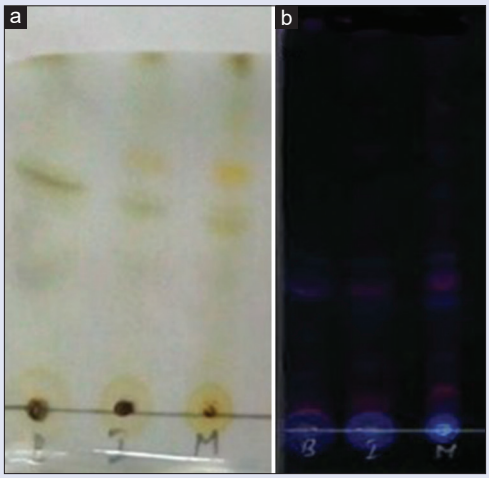

Figure 2: Flavanoid spots on thin-layer chromatography plates in the solvent system toluene: Ethylacetate: Formic acid (7:3:0.5 v/v). (a) Plate visualized using white light. (b) Plate visualized under ultraviolet lamp at 366 nm

Table 1: 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity

Fraction	DPPH assay IC_{50} µg/ml
10	48.25±0.05
20	42.06±0.07
30	68.91±0.95
40	53.48±0.12
50	72.65±0.18
Butanol	95.12±0.09
Standard[a]	68.13±0.09

[a]Butylated hydroxy toluene. DPPH: 2,2-diphenyl-1-picrylhydrazyl

Table 2: Identification of compounds in aqueous extract by gas chromatography mass spectroscopy

Compound	RT
Phenol-2-Propyl	9.23
Pyrazol-5-ol, 3-phenyl	10
1-naphthalen-1-ylbut-3-en-1-ol	14.12
1-methoxy-4-[(4-methoxyphenyl) methyl] benzene	14.87
methyl 13-methylpentadecanoate	16.02
hexadecanoic acid	16.6
10-Octadecenoic acid methyl ester	17.6
coumarine-3,(2-(1-methyl-2-imidazoylthio)-1-oxoethyl	18.25
1-ethyl-4-[2-(4-pentylphenyl) ethynyl] benzene	19.05
11-methylidenetricosane	20.28
propan-2-yl octadec-9-enoate	21.7
4-[(3,5-ditert-butyl-4-hydroxyphenyl) methyl]-2,6-dimethylphenol	23.59
2,3 dimethyl benzene, 1,4-bis (2,6 dimethyl-4 hydroxy phenyl azo)-	29.7
(2S,5E)-5-Ethylidene-2α-[3-(2-hydroxyethyl)-1H-indol-2-yl]-α-methylene-4α-piperidineacetic acid methyl ester	26.13

RT: Retention Time

concentration in the aqueous extract aids in proton donor and electron transfer ability.[44]

Gas chromatography mass spectroscopy analysis of the extract

The extract was found to be containing aromatic amines, phenols and aminophenols that are regarded to be potent antioxidants. Major peaks were found to be containing numerous aromatic aldehydes, ketones, and aliphatic compounds [Figure 3]. The mass spectra of all the phyto constituents were predicted using the NIST library as listed in the Table 2.

Enzyme inhibitory potential

An efficient strategy in Alzheimer's treatment is inhibition of cholinesterases.[11] AChE inhibitors inhibit the breakdown of Acetylcholine, thereby increasing its concentration and extending its neurotransmitter action,[45] and enhancing signal transmission at nerve synapses by prolonging the effect of acetylcholine.[46] Flavanoides with potent antioxidant activity are considered as multipotent drugs for AD treatment owing to significant AChE inhibitory activity.[47] A bioassay-guided fractionation on the plant extract will allow the isolation of anti-AChE components responsible for the activity.[48] The flavonoids and other phenolic molecules bind to peripheral anionic sites on the AChE at Tyr_{70}, Asp_{74}, Try_{121}, Trp_{279} and Tyr_{334} as non-competitive inhibitors.[49]

Shankhapushpi is being used in the Indian system of medicine to improve cognition.[50] In the present study, anti-Alzheimer's potential of *E. alsinoides* was determined. The enzyme inhibitory potential of 10% and 20% fractions [Table 3] was more active in inhibiting AChE than standard anti-Alzheimer's drug, galantamine, whereas the inhibiting property of other fractions was weak toward inhibiting the enzyme [Table 3]. AChE inhibition activity of the plant metabolites reduces neuronal dysfunctions in various parts of the brain.[51] The bioactive that acts on cholinergic function of central nervous system are significant in treating AD.[52]

Except the 40% fraction, all the extracts possess strong α-glucosidase inhibitory activity. 20% fraction showed strong α-amylase inhibitory activity whereas 10% and 30% fraction showed poor activity towards α-amylase inhibition [Table 3]. The standard antidiabetic drug, acarbose was used as a control and it has IC_{50} of 1.32 mg/mL and 6.46 mg/mL in α-amylase and α-glucosidase assays, respectively. 20% fraction showed strongest α-amylase and α-glucosidase inhibition property. Weaker activities were reported with 40% and 50% fractions. The antioxidant and antidiabetic properties of the plant extracts had a positive correlation with the flavonoids content.[53] A dose-dependent percentage inhibitory activity of the plant extract against alpha-amylase and alpha-glucosidase enzymes is being reported.

Table 3: Enzyme inhibitory activity of the extracts of *Evolvulus alsinoides* (Linn.) Linn

Extract	IC_{50} (μg/ml)		
	α - amylase[a]	α - glucosidase[b]	Acetyl cholinesterase[c]
10	1.28±0.12	4.62±0.14	2.63±0.02
20	1.21±0.13	4.12±0.15	2.60±0.05
30	1.32±0.08	4.56±0.02	2.78±0.05
40	1.35±0.08	4.63±0.08	2.96±0.03
50	1.40±0.19	5.02±0.18	3.45±0.03
Standard*	1.32±0.15	6.46±0.04	2.59±0.04

*Acarbose[a,b] and galantamine[c]

Spectral analysis of the isolated compound

The spectrum [Figure 4] obtained contains peak at 333 nm and 292 nm that corresponds to the 3-hydroxyl substituted Flavonols. Band I is highly distinct and appears at longer wavelengths for flavonols oxygenated at A and B ring.[34] The HPTLC densitogram [Figure 5a and b] shows the presence of a single spot at the Rf of 0.12–0.27. RP-HPLC was performed to identify the flavanoid compounds in the 20% Fraction. The retention time of Kampferol glycosides

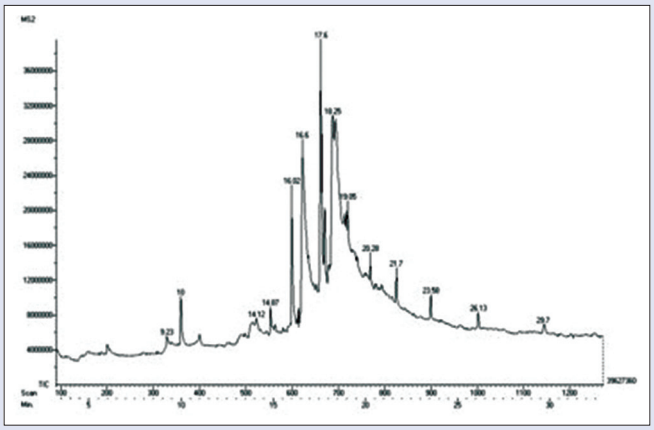

Figure 3: Chromatogram of aqueous extract through gas chromatography mass spectroscopy

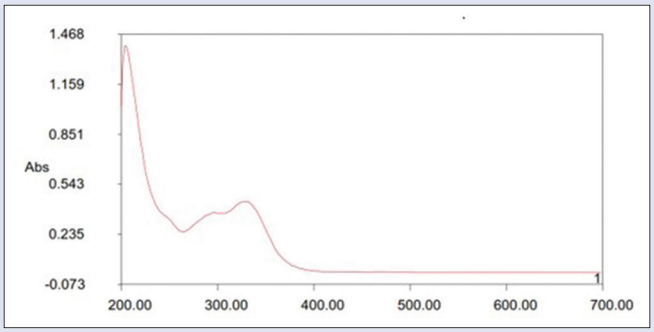

Figure 4: Spectrum of the isolated compound with Band I at 333 nm and Band II at 292 nm representing 3-hydroxyl substituted flavonols

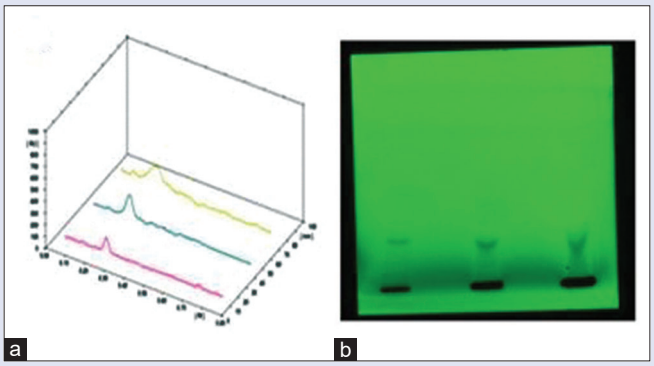

Figure 5: (a) Densitogram of purified fraction in the solvent system toluene: Ethylacetate: Formic acid (7:3:0.5 v/v) (b) thin layer chromatography plate visualized under ultra violet at 256 nm

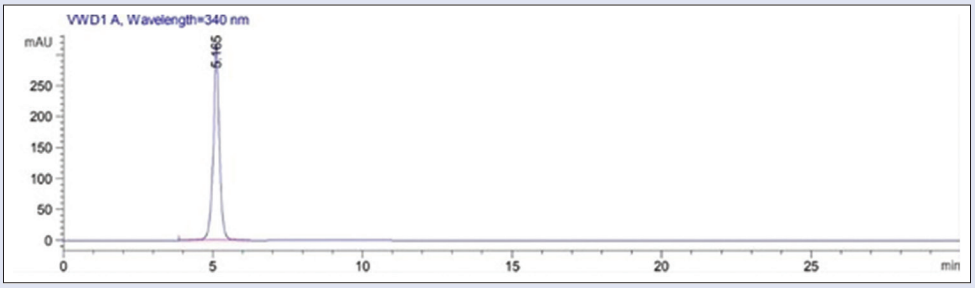

Figure 6: Chromatogram of the purified fraction through high-performance liquid chromatography

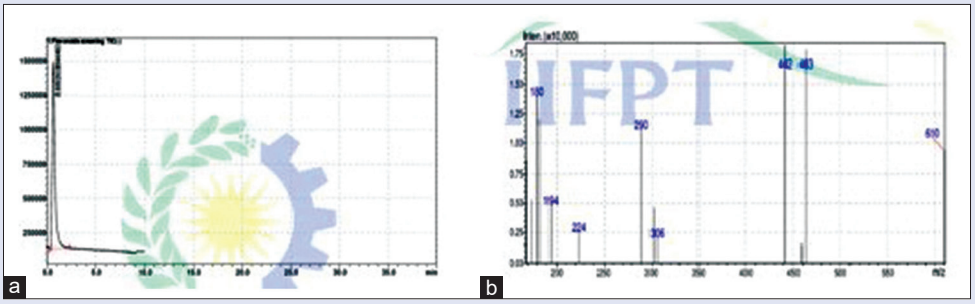

Figure 7: (a) Liquid chromatography mass spectrometry profile of the isolate (b) negative ionization chromatogram of the isolate

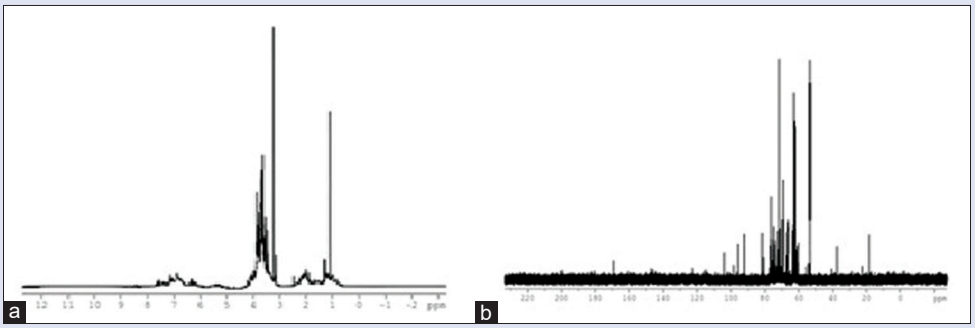

Figure 8: ^{1}H (a) and ^{13}C (b) Nuclear Magnetic Resonance Spectrum of the isolated compound

was found to be 5.165 in the chromatogram of fraction collected with significant antioxidant potential [Figure 6]. The peak eluted at 5.165 min was subjected to LC-MS analysis. In the negative ionization mode, the compound exhibited *m/z* of 609 confirming the molecule to be Kaempferol 3,7 diglucoside [Figure 7]. Purity of the isolated compound was found to be 100% with a molecular weight of 610.5 Da as determined by LC-MS method. The identity of the purified compound was established by proton NMR analysis. Acid hydrolysis liberated glucose, kaempferol and caffeic acid. Alkaline saponification yield deacylated kaempferol glycoside and caffeic acid.[54] In the ^{1}H NMR spectrum [Figure 8], peaks at δ 5.65, 5.81 and 6.61 corresponds to the anomeric protons of the glucose, H-6, H-8, H-2', 6' and H– 3', 5' corresponds to aromatic protons of kaempferol and H-2, H-5, H-6, H-α and H-β are the five protons to caffeic acid. The analysis of ^{1}H NMR spectrum of anomeric protons confirms the presence of 3 mol glucose and 1 mol caffeic acid. The HMBC correlation helps to predict the interglycosidic linkage to be 3-O-glucosyl-(1→3)-glucose, caffeic acid bonding at 6th position and kaempferol at the 7th position. The isolate was identified to be kaempferol 3,7 diglucoside (IUPAC Name: Kaempferol 3-O-β-[(6'''-E-caffeoylglucopyranosyl)-(1→3)-glucopyranoside]-7-O-β-glucopyranoside having two beta-D-glucosyl residues attached at positions O-3 and O-7. It is a dihydroxyflavone, a beta-D-glucoside, a monosaccharide derivative, a polyphenol and a kaempferol O-glucoside.

Effects of kaempferol 3,7 diglucoside on cytotoxicity of amyloid-β peptide peptide

The MTT assay reported increased percentage of SH-SY5Y Human Neuroblastoma Cells Lines [Figure 9] viability when the cells are induced cytotoxicity by Aβ1-42. The IC_{50} for Kaempferol 3,7 diglucoside is found to be 1.794 and that of control is 0.481. These data indicate effective inhibition of Aβ1-42 fibril formation in the presence of kaempferol 3,7 diglucoside reporting less neurotoxicity on the cultured SH-SY5Y cells, signifying potential effect of the compound on Aβ clearance.

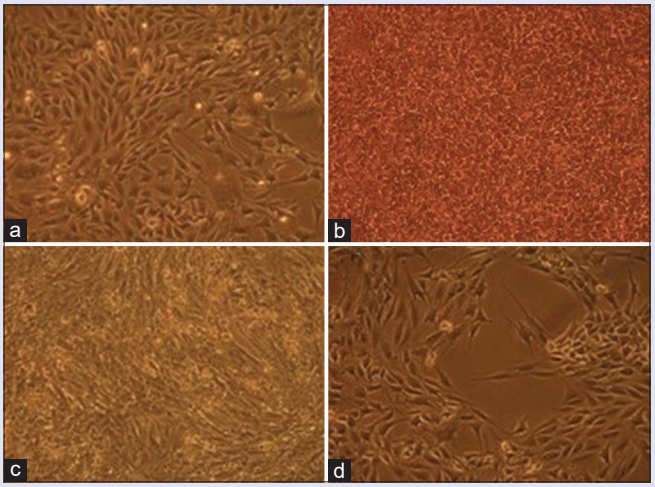

Figure 9: Morphologies of SH-SY5Y cells that are protected by the Kaempferol 3,7 diglucoside after Amyloid-β peptide incubations and examined using Electron Microscopy ([a]: Positive control-induced injury with 1 μM Amyloid-β peptide, [b]: Negative control, [c]: Amyloid-β peptide [1 μM] +0.1 μM sample B, [d]: Amyloid-β peptide [1 μM] +100 μM sample B)

CONCLUSION

The GC-MS analysis reports the presence of antioxidant rich compounds in the leaf extracts. Further studies with the extracts reported the presence of Kaempferol 3,7 diglucoside in the plant for the first time. The newly identified compound was found to possess α-amylase, α-glucosidase and AChE inhibition activity. Increased cell viability in differentiating neuronal cells, SH-SY5Y in the presence of Aβ-induced Injury have also been reported for the first time.

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Conflicts of interest

There are no conflicts of interest.

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