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ORIGINAL ARTICLE

Phytochemical Analysis and *In Vitro* Biological Assessment of *Gyrocarpus americanus* for its Potential Effectiveness in Treating Alzheimer's Disease and Diabetes Mellitus Simultaneously

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ABSTRACT

As Alzheimer's disease (AD) and Diabetes Mellitus (DM) are most likely to appear together in aged people, many studies have been recorded over the past few years on the search for agents to treat AD and DM simultaneously. In this study, methanolic extract of Gyrocarpus americanus and derived $CHCl_3$, n-BuOH and H_2O fractions were assessed for their capacities to inhibit target enzymes Acetylcholinesterase (AChE), Butyrylcholinesterase (BuChE) and α-& β–glucosidases. The methanolic extract and derived chloroform fraction were found to be most prominent in inhibition with IC_{50} values range of 47.6±4.17–19.7±2.48 µg/mL. The most active chloroform fraction exerted remarkable DPPH (10.4 ± 0.3 mg AAE/g) and ABTS (241.36 ± 3.89 mg TE/g) radical scavenging activity. In MTT cell viability assay, these fractions were nontoxic and neuroprotective against induced SK-N-SH cell injury. The phytochemical analysis revealed that these activities could be attributed, at least in part, to the presence of large quantities of phenolics and flavonoids in active chloroform fraction. In conclusion, the aforementioned results provided valuable evidence for the potential of chloroform fraction of methanolic extract of G. americanus as prospective material for further development of multifunctional agents to control both DM and AD simultaneously.

Keywords: *Gyrocarpus americanus, phytochemical analysis, antioxidant activity, anticholinesterase activity, antidiabetic activity.*

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INTRODUCTION

Diabetes mellitus (DM) is a complex and chronic disorder of metabolism that affects all genders, in all ages worldwide. Over the last few decades, the increasing worldwide prevalence of DM is also of global public health concern, especially because DM accelerates ageing in organ systems which in turn lead to premature morbidity and mortality [1]. At present, 387 million individuals live with DM around the world, and it is projected to achieve 592 million by 2035 [2]. DM is characterized by postprandial hyperglycemia (PPHG) and abnormalities in metabolism of carbohydrate, protein and fat owing to insulin resistance, insulin deficiency or both [3]. Elevated PPHG has been linked to the onset of diabetic complications such as nephropathy, neuropathy, retinopathy and coronary heart diseases. Decreasing the

absorption of glucose through inhibition of α-& β–glucosidases play a prominent role in managing PPHG in diabetic patients [3]. Moreover, glucosidase inhibitors reported to be effective against HIV, cancer, hepatitis, and heart disorders [3]. Extensive research efforts have proved DM as a major risk factor for dementia by almost 2-fold or one in ten cases [4].

Alzheimer's disease (AD), the most common cause of dementia contributes 60 to 80 percent of all dementia. AD is the most prevalent age-related progressive neurodegenerative brain disorder with symptoms of memory loss, language deterioration, and poor judgment, and functional impairment, loss of independence, emotional distress, and behavioral impairment. The key histopathological hallmarks seen in AD brains are extracellular senile plaques, intraneuronal neurofibrillary tangles and dysfunction of cholinergic neurons [5-8]. Dysfunction of basal forebrain cholinergic neurons results in remarkable depletion and lead to cognitive decline in AD. Currently, the main and putative therapeutic method to alleviate the AD symptoms is increasing the acetylcholine (ACh) concentration in the synaptic cleft by inhibition of acetylcholinesterase (AChE), an enzyme that plays a key role in the hydrolysis of ACh in the healthy brain. Recent discoveries indicated that the levels of AChE is very low in AD advance cases, while in such instance, butyrylcholinesterase (BuChE) takes over the hydrolysis and helps to continue AD progression. Therefore, it is of essential importance to search for dual inhibitors of AChE and BuChE as viable therapeutic strategy to treat cognitive dysfunction in AD [9,10]. In addition, numerous studious side effects were noted with FDA approved synthetic drugs such as tacrine, donepezil, rivastigmine and galantamine due to their peripheral inhibitory activities [11-15]. At this juncture, a great attention has been intended to explore natural AChE and BuChE dual inhibitors with a improved safety profile.

Over production of free radicals and progressive decline of the cellular antioxidant defense with aging, impose oxidative stress. The excessive oxidative stress causes neuronal degeneration through damaging cellular lipids, proteins, or DNA. Since, oxidative stress promotes the appearance of pathological hallmarks it has been implicated in the pathogenesis and progression of degenerative disorders such as AD and DM [16]. Consequently, averting the formation or scavenging the free radicals could be considered as potential in both prevention and treatment of AD and DM [17]. Against a background of growing concerns about the toxicity and side effects from currently available therapeutic options for AD and DM, the World Health Organization has also recommended the development of improved and safer herbal medicines. The growing relevance of herbal medicine as an alternative form of health care is often based on their traditional use in Ayurvedic, Chinese and African medicinal practices. However, there is a dearth of scientific data on the folkloric applications of medicinal plants. Therefore, pharmacological screening of medicinal plants has become a renewed interest globally.

In the present investigation, methanolic extract and derived $CHCl_3$, n-BuOH and H_2O fractions from leaves of *Gyrocarpus americanus* were evaluated for phytochemicals, AChE, BuChE, α-& β–glucosidases inhibitory and antioxidant properties as an attempt to find the potential of titled plant in dual therapy of AD and DM. *G. americanus* belonging to Hernandiaceae family is a slender, conspicuous deciduous tree with large leaves, smooth, grey bark and winged fruits, growing up to 18 meters tall with a wide pantropical distribution. Current knowledge about *G. americanus* is limited to the ethnobotanical uses and pharmacological applications as noted in Table 1. However, no scientific reports on its phytochemicals.

According to data from literature, neither enzyme inhibitory nor antioxidant activities and quantitative phytochemical screening of the titled plant has earlier been reported elsewhere. In order to fill this gap, the present study was conducted to evaluate AChE, BuChE, α-& β–glucosidases inhibitory, DPPH, ABTS radical scavenging, neuroprotective activities, kinetic analysis of inhibition as well as phytochemical contents of crude extract and subsequent fractions of *G. americanus* as original contribution in the field of AD and DM dual therapy.

Table1. Traditional uses and other activities of *G. americanus.*

Plant name	Regional name	Traditional uses	Activities
Gyrocarpus americanus	Kumaaripoliki, Nallaponaku, Papurapukaaya, Tanuku and Tanakur	Wound healing, to treat diarrhoea and scabies [18], treat cancer [19].	Antimicrobial, anticancer and antiprotozoal [18, 20, 21].

MATERIAL AND METHODS

Chemicals and materials

Human neuroblastoma cells (SK-N-SH) were obtained from National Centre for Cell Sciences (Pune, India). *Ee*AChE from Electric eel (EC 3.1.1.7), *Eq*BuChE from Horse serum (EC 3.1.1.8), *α*–glucosidase from *Saccharomyces cerevisiae* (EC 3.2.1.20), and β–glucosidase from almonds (EC 3.2.1.21), ABTS, DPPH,

DTNB, pNPG-α, pNPG-β, ATCI, BTCI, MTT, Folin-Ciocalteu reagent, galantamine, acarbose, D-Glucono-δ-lactone, trolox and ascorbic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents used in this study were of analytical grade or better and were purchased from Merck.

Plant material

The leaves of *G. americanus* were collected from Seshachalam hills in Andhra Pradesh, India in September 2013. The voucher specimens vide No. YVU 43 AGD were identified by Prof. A. Madhusudana Reddy and deposited in the herbarium of Yogi Vemana University. The collected leaves were air dried in the shade for two weeks at RT, and ground into fine powder. The smooth powder was stored in an air-tight container and kept in darkness at –20 °C until further use.

Extraction and fractionation

For extract preparation, powdered material (150 g each) was exhaustively extracted with 90% methanol at 35 °C for 48 h. After filtration, the combined extracts were concentrated using rotary vacuum evaporator (Heidolph, Germany). The methanolic extract was suspended in water (50 mL) and sequentially partitioned with chloroform and n-butanol. The residual water solubles were concentrated using rotary vacuum evaporator to get water fraction. Percentage yields of extracts and fractions were calculated (Table 2).

Cholinesterase inhibition assay

The methanolic extract (GAM) and subsequent fractions (GAC, GAB, and GAW) were assayed for their AChE and BuChE inhibitory activities by Ellman's method using ATCI and BTCI, respectively as substrates [22].

Glucosidase inhibition assay

The inhibitory properties of extract: GAM and fractions: GAC, GAB, and GAW against *α*– and β–glucosidases were determined as described by Shibano et al. [23] using *p*NPG-α and *p*NPG-β as substrates for α- and β-glucosidases, respectively.

Kinetic study on enzyme inhibition

Kinetic studies for inhibition of enzymes were carried out by measuring enzyme activity at different concentrations of relevant substrate, various concentrations of inhibitor and fixed concentration of enzyme using concerned method as described above. Lineweaver Burk (double reciprocal) plots of 1/V versus 1/[S] and secondary plots of slopes Vs inhibitor concentration were constructed using Graph pad prism (version 5) software.

Assays to evaluate antioxidant potential

ABTS free radical scavenging assay

ABTS radical scavenging potential of active fraction was estimated by the method of Re et al. with some modifications [24] using Trolox as a positive control and results were expressed in Trolox equivalents.

DPPH radical scavenging assay

As previously reported by Sarikurkcu et al., the radical scavenging activity of test fraction was measured using DPPH radical with Ascorbic acid as reference and results were expressed in Ascorbic acid equivalents [25].

Cell culture and treatment

The SK-N-SH, human neuroblastoma cells were cultured in MEM to reach approximately 80% confluence as reported earlier [26] and used for the following *in vitro* experiments.

Cell viability by MTT assay

The cell viability in SK-N-SH cells was determined using MTT assay to predict cytotoxicity of active fraction as disclosed in our recent studies [27].

Protection against H_2O_2 induced oxidative injury in SK-N-SH cells

Neuroprotectivity of active fraction on H_2O_2 induced cell death in SK-N-SH cells was determined using MTT colorimetric method [27].

Phytochemical analysis

Determination of total phenolic contents

Determination of total phenolic contents (TPC) was performed by Folin-Ciocalteu (FC) reagent method with slight modifications [28] and the contents were expressed as mg of gallic acid equivalents (mg GAE/g).

Determination of total flavonoid contents

The total flavonoid content was quantified according to the aluminum chloride colorimetric method [29, 30] and expressed as mg of rutin equivalent (mg RE/g).

Determination of total tannins contents

Total tannins were estimated using Vanillin HCl method [31] and quantities were expressed as mg of Catechin equivalents.

Determination of total terpenoids

Quantification of Total terpenoids was analyzed by an assay of Narayan Ghorai et al. (Narayan et al., 2012) [32] and noted as linalool equivalents (mg LE/g extract).

Total alkaloid content

Quantitative analysis of alkaloids was carried out by spectrophotometric method using BCG solution with slight changes [33] and recorded as atropin equivalents (mg AE/g extract).

Statistical analysis

All the tests were done in triplicate and results were expressed as means ± Standard deviation (SD). Percentage inhibition values were log-transformed before being subjected to statistical analysis. Statistical analysis of data was performed using one-way analysis of variance (ANOVA) with the aid of SPSS version 10 software (SPSS Inc., Chicago, USA) and differences were considered statistically significant at $p < 0.05$. Calculation of the IC_{50} values from the logarithmic non-linear regression curves was carried out using Graph Pad Prism 5.0 software (San Diego, CA, USA).

RESULTS AND DISCUSSION

Extraction yields

The shade dried, powdered leaves of *G. americanus* was extracted with 90% methanol in water. The crude methanolic extract obtained was subjected to fractionation using successive solvent-solvent extraction process starting from chloroform, n-butanol and water. Yields of methanolic extracts of *G. americanus* (GAM) and its derived chloroform (GAC), n-butanol (GAB) and water (GAW) fractions were calculated as given in Table 2. As per the data, the extractive capacities are in the decreasing order of residual aqueous > n-butanol > chloroform fraction.

Biological evaluation

In order to assess the potential capabilities of the methanolic extracts (GAM) and various fractions (GAC, GAB and GAW) of the *G. americanus* as multifunctional agents against AD and DM, investigations were carried towards the inhibition of target enzymes like AChE, BuChE and α–& β–glucosidases that are linked to pathophysiological symptoms. In addition, kinetic analysis of enzyme inhibition and *in vitro* radical scavenging capacities are also assayed for active fractions.

Cholinesterase inhibitory activity

Both AChE and BuChE enzymes are responsible for lowering the level of acetylcholine in the synaptic cleft of the brain leading to neurodegenerative disorders. Thus, searching for inhibitors of these enzymes should remain perennial since no cure has yet been registered for the aforementioned pathologies. In recent years, plants have proven to be an important source for the inhibitors of cholinesterases (AChE and BuChE) that are useful for the symptomatic treatment of AD.

In the present study, the methanolic extract of *G. americanus* (GAM) and its derived chloroform (GAC), n-butanol (GAB) and water (GAW) fractions were evaluated for their inhibitory activity toward AChE and BuChE according to the modified Ellman's method in a 96-well microplate reader using Galantamine as reference (Ellman et al. 1961). All the screened samples exhibited dose-dependent inhibitory abilities on AChE and BuChE activities at different concentrations (15, 30, 90 and 150 µg/mL) (Figure 1). The IC_{50} values for AChE and BuChE inhibition were summarized in Table 2. The methanolic extract GAM showed strong inhibition abilities against AChE and BuChE with IC_{50} values in micrograms range that to below 35 µg/mL. Among the fractions tested the chloroform fraction GAC showed the best inhibitory capacity with IC_{50} values of 28.76±5.6 and 47.6±4.17 µg/mL towards AChE and BuChE enzymes, respectively. Thus, *G. americanus* can be considered as dual inhibitor of AChE and BuChE enzymes. Therefore, it is identical to our aim of investigation to find multifunctional agents. Regarding n-butanol and water fractions, moderate to low activity against both the enzymes was recorded.

Inhibition of α– and β–glucosidase enzymes

In the present study, inhibitory activities toward α– & β–glucosidases of the methanolic extracts and subsequent fractions of titled plants were evaluated using a method described by Shibano et al. with minor modifications [23]. The enzyme reaction was performed under *in vitro* using p-nitrophenyl-D-glucopyranoside (pNPG- α– or pNPG- β) as the substrate, which was hydrolyzed by glucosidases to release p-nitrophenol, a color agent that can be monitored at 415 nm. Acarbose and D-Glucono-δ-lactone were used as reference for *α*– & β–glucosidases, respectively. The calculated IC_{50} values of extracts and their fractions on α– & β–glucosidases are showed in Table 2 (Figure 2).

The *in vitro* inhibitory studies demonstrated that the methanolic extract GAM have strong α– & β–glucosidase inhibitory activities with IC_{50} values less than 40 µg/mL. Among all the fractions screened, GAC found to be the most potent with IC_{50} values of 38.663±5.9 and 26.84±4.2 against α–glucosidase and β–glucosidase, respectively. Therefore, GAC was regarded as dual inhibitor. Dual α– and β–glucosidase

inhibitors may have better clinical potency without remarkable side effects. Consequently, most potent fraction GAC that exhibited good balance of α– & β–glucosidases inhibitory activity may represent better therapeutic option to treat DM. It is also interesting that the GAM and GAC provided stronger inhibitory activities on *α*–glucosidase than reference compound acarbose. According to data, n-butanol fraction GAB and water fraction GAW were considered to be moderate inhibitors of *α*– & β–glucosidases.

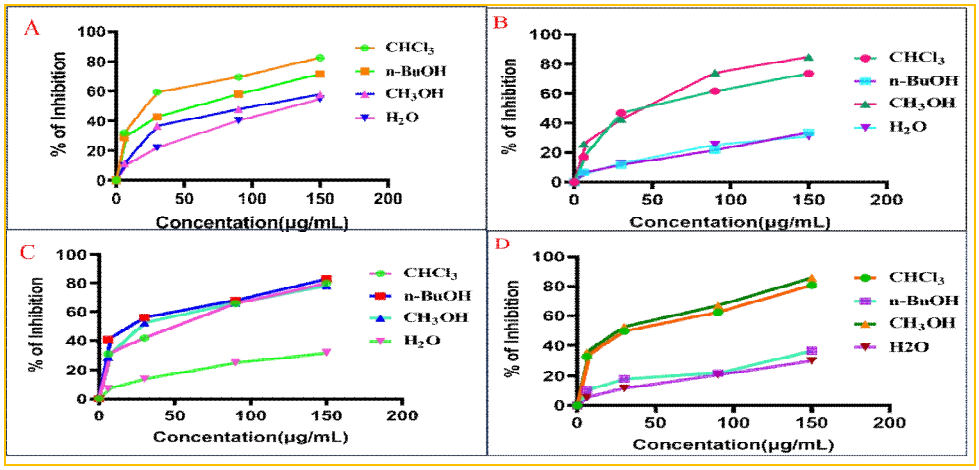

Figure 1. The percentage of inhibition exerted by *G. americanus* on the activity of cholinesterases (AChE & BuChE) and α– & β–glucosidases at 15, 30, 90 and 150 µg/mL concentrations.

Table 2. % of yield and IC_{50} Values of 90% methanolic plant extracts and its derived fractions in AChE, BuChE, α– & β–glucosidases inhibition assays.

Plant	Extract	% of yield	IC_{50} Values (µg/mL)			
			AChE	BuChE	α-Glcosidase	β-Glcosidase
G. americanus	Methanol	21.58	34.67±3.64	27.69±1.01	36.03±5.48	19.7±2.48
	Chloroform	2.1	28.76±5.6	47.6±4.17	38.663±5.9	26.84±4.2
	n-Butanol	3.7	146.28±12.09	156.18±2.9	119.63±5.2	224.3±12.9
	Water	12.69	116.71±1409	156.03±7.16	233.47±25.09	235.67±28.04
Galantamine	-		0.77 ± 0.09	8.1± 0.02	-	-
Acarbose	-		-	-	117.20±0.017	-
D-Glucono-δ-lactone	-		-	-	-	10.68±0.0058

Kinetic study of inhibition

To study the mode of inhibition towards all target enzymes, the most active fraction GAC was chosen for further enzyme kinetic analysis. First, the initial velocity of each enzyme was monitored at 15 µg, 30 µg and 90 µg concentrations of fraction using different concentrations (0.1–0.5 mM) of the corresponding substrate. The Lineweaver-Burke plots (Figure 2), double reciprocal plots of initial velocity (1/v) and substrates concentration (1/s) revealed the pattern of increasing slopes and intercepts with increase of inhibitor concentration and the falling intersect points in the upper left quadrant indicating a mixed-type inhibitory behavior of active fraction. In this mode, inhibitor impedes catalytic process of enzyme without preventing substrate binding, thus, it is neither pure competitive nor pure non-competetive inhibition. Here, inhibitor binds either to the free enzyme (E) or the enzyme-substrate complex (E+S), accordingly, two inhibition constants; Ki_1 and Ki_2 were determined from secondary plots as shown in Table 3. The values indicating strong potency of active fractions to bind to the free enzyme.

Table 3. Kinetic study of most active fraction GAC on AChE, BuChE and α–Glc., β–Glc. enzyme inhibition.

Plant	Extract	Enzymes	Type of inhibition	Inhibition constant (ki)	
				Ki_1	Ki_2
G. americanus	Chloroform	AChE	Mixed	49.74	82.25
		BuChE	Mixed	44.94	82.47
		α-Glu	Mixed	66.92	96.28
		β-Glu	Mixed	46.32	78.96

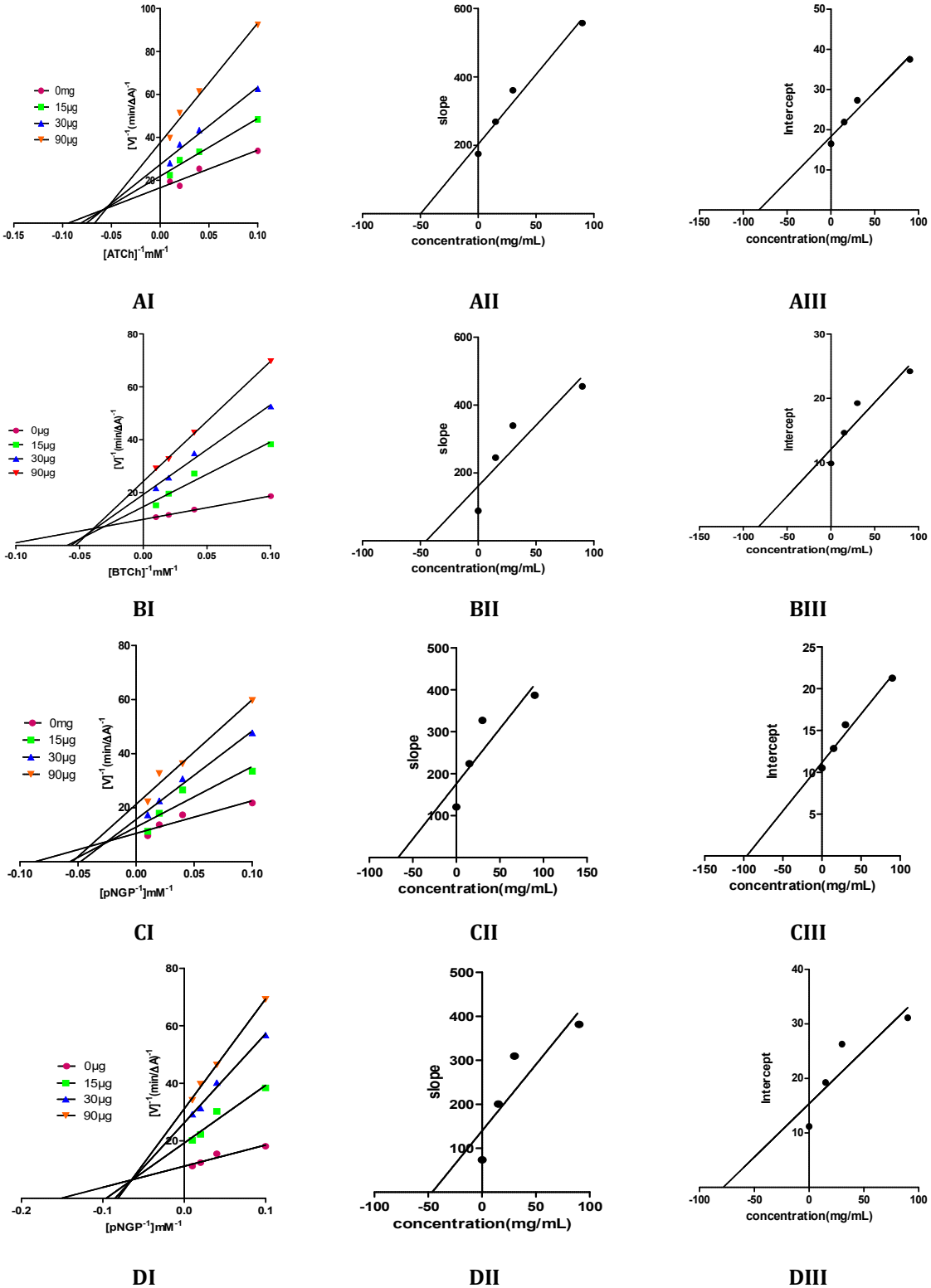

Figure 2. Steady-state inhibition of AChE (A), BuChE (B), α–Glucosidase (C) and β–Glucosidase (D) by most active chloroform fraction (GAC) from *G. americanus* (Left); Lineweaver Burk plot of reciprocal of initial velocities versus reciprocal of substrate concentrations (0.1–0.5 mM) in the absence and presence of GAC at 15 µg, 30 µg and 90 µg; (right) secondary plots of the Lineweaver Burk plot, slope versus various concentrations of GAC (I) regarding inhibition constant Ki_1 and intercept versus various concentrations of GAC (II) regarding inhibition constant Ki_2.

Antioxidant activity

Imbalance in oxidative defense mechanism plays a special role in processes of degenerative diseases such as AD and DM [34]. Recently, natural sources like plants and plant products with better biocompatibility and lower side effects have gained ample recognition as antioxidants.

Among various *in vitro* antioxidant assays, the DPPH and ABTS radical scavenging assays were regarded as rapid and cheap methods to measure the antioxidant potencies of plant extracts. Therefore, ABTS and DPPH radical scavenging abilities of fraction GAC was assessed using trolox and ascorbic acid, respectively as reference and results were expressed in mg Trolox and Ascorbic acid Equivalents as shown in Table 4.

In ABTS radical scavenging assay, the tested fraction GAC exhibited strong radical scavenging capacity with 241.36 ± 3.89 mg Trolox Equivalents per gram. Regarding DPPH radical scavenging activity, GAC disclosed modest scavenging abilities with mg Ascorbic acid Equivalents per gram of 10.4 ± 0.3. These observations inferred that the fraction GAC may contain principles capable of donating hydrogens to ABTS free radical.

Table 4. ABTS and DPPH Free Radical scavenging activity of chloroform fraction.

Plant	ABTS mg TE /g	DPPH mg AAE /g
G. americanus	241.36 ± 3.89	10.4 ± 0.3

All values are mean ± standard deviation of triplicates; TE: Trolox Equivalents, AAE: Ascorbic Acid Equivalents.

Cell viability in SK-N-SH cells

The cytotoxic effect of fraction GAC was measured in human neuroblastoma cell line, SK-N-SH cells. After treating the SK-N-SH cells with different concentrations (50, 100, 200 and 400 µg) of fraction, they were incubated for 24 h and measured cell viability by MTT assay. Figure 3 represents cell viability (%) after treatment of the fraction GAC comparing with control cells. GAC showed concentration dependent cell viability. The higher cell viability by fraction at 400 µg than control cells indicated that fraction stimulated the growth and proliferation of cells with increasing concentration. Similar results were reported in the literature and concluded that it is due to the presence of phytochemicals which delay the natural death of neuronal cells in culture medium [26]. Based on the interesting result, it was concluded that the chloroform fraction is nontoxic to SK-N-SH cells.

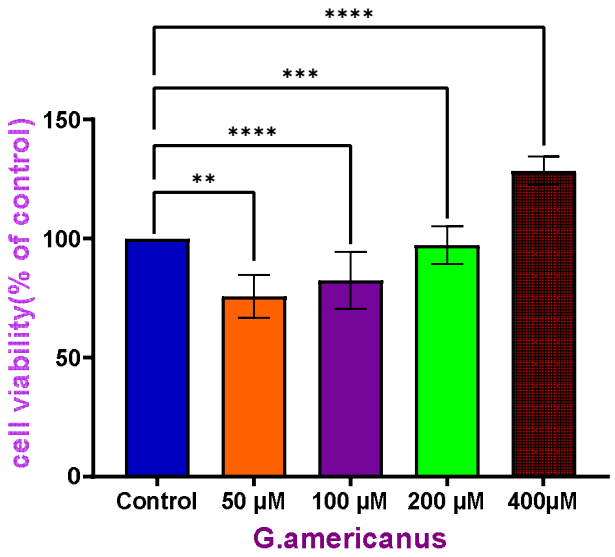

Figure 3. Neurotoxic effects of chloroform fraction of methanolic extract of *G. americanus* on SK-N-SH cells (human neuroblastoma cell line). Bar chart show the percentage of cell viability in the presence or absence (control) of indicated concentrations of *G. americanus*. Cell viability corresponding to control cells was considered as 100%. *P < 0.05 versus 100% cell viability (one-way ANOVA test).

Neuroprotective activities against H_2O_2 induced cell death in SK-N-SH cells

The neuroprotective capacity of the fraction GAC against H_2O_2 induced cell death in SK-N-SH cells were investigated by MTT assay. H_2O_2 was used as oxidant to induce oxidative cell injury in SK-N-SH cells. After

exposure to 1.0 mM of H_2O_2 for 8 h, the cell survival rate of SK-N-SH cells was markedly reduced to 44.65 %. The sensitivity towards H_2O_2 was noticed in concentration and time-dependent manner. The protective efficiency of fraction GAC at different concentrations of 50, 100, 200 and 400 µg against H_2O_2 induced oxidative stress was expressed in the form of corresponding cell viability in Figure 4. Tested fraction GAC excelled remarkable protection capability with cell viabilities in dose response way. Very interestingly, GAC showed strong neuroprotective effects (49.14 – 130.73 %) at 100, 200 and 400 µg concentrations. To our surprise, both GAC displayed significantly higher neuroprotective effect than control at higher concentrations. It is reasonable assumption that phytoconstituents of tested fraction may likely to promote cell survival or delay the natural death of neuronal cells. Taken together, the increase in cell viability by averting H_2O_2 induced oxidative cell death indicating that the fraction GAC might be potential oxidative suppressor.

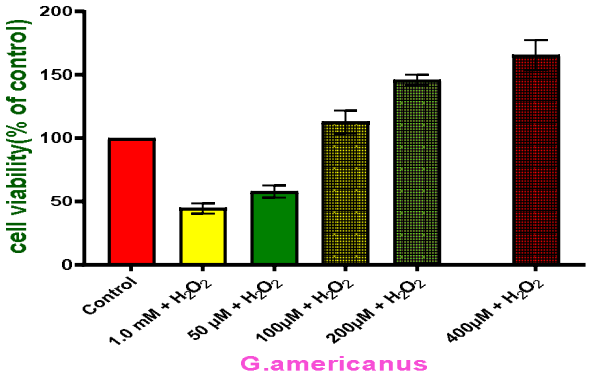

Figure 4. Neuroprotective activity of chloroform fraction from methanolic extract of *M. pubescence* on SK-N-SH cells (human neuroblastoma cell line) against H_2O_2 induced cell death in SK-N-SH cells in MTT assay. *P < 0.05 versus H_2O_2 treatment.

Phytochemical analysis

Natural phytochemicals usually protect plants from disease, pathogenic attack and damage from environmental hazards. Bioactivities exhibited by plants usually owing to the interactions of their constituents with specific metabolic pathways. Hence, phytochemical analysis is very important to find new sources of therapeutically valuable classes of compounds within plant extracts in order to make the best use of available natural wealth. Therefore, in the present study, an attempt was made to screen the phytochemicals present in the active chloroform fraction of tiled plant using standard procedures.

The results of phytochemical analysis (Table 5) revealed the presence of phenolics, flavonoids, tannins and alkaloids in GAC. However, the total flavonoid and total phenolic contents were significantly higher in GAC. Total phenolic contents (TPC) quantified using Folin-Ciocalteu reagent was expressed as milligram gallic acid equivalents per gram (GAE/g extract). The total flavonoids (TFC) estimated was expressed as milligram rutin equivalents per gram (RE/g). As can be seen from the Table 5, the highest amounts of flavonoids (228.5 ± 6.1 mg RE/g dry matter) and phenolics (194.2 ± 1.39 mg GAE/g dry matter) were found in GAC. However, moderate quantities of tannins (18.369±2.69 mg CE/g) and alkaloids (12.4 ± 0.87 mg AE/gm) were noticed in GAC while lowest in terpenoid (1.16 ± 0.09 mg LE/gm) content.

The flavonoids being a prominent group of secondary metabolites have broad spectrum of biological activities such as anti-cancer, anti-diabetic, antioxidant, antimicrobial, anti-inflammatory, anti-cholesterolemic etc. Flavonoids were also reported to be robust free radical scavengers due to their hydrogen or electron donating abilities and chelating nature with transition metals [35]. Flavonoids were also found to inhibit target enzymes involved in the treatment of AD and DM.

Plant phenolics were also regarded as powerful antioxidants through multiple mechanisms like scavenging, adsorbing, quenching, decomposing and neutralizing free radicals. Hence, many of the natural polyphenols were considered to have therapeutic potential against AD and DM. In plants, phenolic have also found to act as plant defense mechanisms against pathogens, parasites, and predators [36]. In this investigation, the chloroform fraction GAC was found to have significantly higher total flavonoid and total phenolic contents. Thses results suggested that phenolics and flavonoids alone or in combination may be the major contributors for the antioxidant and enzyme inhibitory activities of *G. americanus*.

Table 5. Quantitative phytochemical analysis of the active chloroform fractions from *G. americanus*.

Plant	TPC mg GAE/g	TFC mg RE/g	TTC mg CE/g	TTRC mg LE/g	TAC mg AE/g
G. americanus	194.2 ± 1.39	228.5 ± 6.1	18.369±2.69	1.16 ± 0.09	12.4 ± 0.87

TPC: Total Phenolic Content, TFC: Total Flavonoid Content, TTC: Total tannin content, TTRC: Total Terpenoid Content, TAC: Total Alkoloid Content, GAE: Gallic Acid Equivalents, RE: Rutin Equivalents, CE: Catechin Equivalents, LE: Linalool Equivalents, AE: Atropin equivalents.

CONCLUSION

From the best of our knowledge, minor attention has been handed out to the bioactivity and phytochemical screening of the extracts of *G. americanus*. As part of our ongoing research on finding possible applications of the Indian flora, methanolic extract and its derived $CHCl_3$, n-BuOH and H_2O fractions of *G. americanus* were evaluated for their enzyme inhibitory and antioxidant properties. From the results of the present study, it is evident that methanol extract and its chloroform fraction are very strong mixed type inhibitors of AChE, BuChE, α–Glc and β–Glc enzymes, important pathological targets of AD and DM. Results also strongly suggest that the chloroform fraction could be considered as a significant source of natural antioxidants with potent neuroprotective abilities. According to phytochemical analysis, the tested chloroform fraction have a rich mixture of phenolics and flavonoids that offer a remarkable enzyme inhibitory, antioxidant and neuroprotective properties. Therefore, taking into account these observations, chloroform fraction of methanolic extract of *G. americanus* could be proposed as attractive, nontoxic agents with consistent multipotent therapeutic potential as neuroprotectors in the dual therapy of AD and DM. Further investigations are warranted for bioactivity guided isolation and structural elucidation of compounds responsible for these activities from the active fraction of *G. americanus* that may help to develop new drug candidates or at least a template for lead generation for AD and DM dual therapy.

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CONFLICT OF INTERESTS

The authors declare that this article content has no financial or commercial conflict of interests regarding the publication.

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