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Research Article

Phytochemical Profiling of *Hemidesmus indicus* (L.) R. Br. ex Schult and its Antioxidant, Anti-Inflammatory and Neuroprotection Linked Enzyme Inhibitory Properties

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ABSTRACT

Introduction: Exponential expansion in the usage of herbal medicines was observed in recent decades due to the increasing importance of the traditionally used natural remedies. In order to identify bioactive components of medicinal value, in the present study, we aimed to screen different extracts of *Hemidesmus indicus* (L.) R. Br. ex Schult for health beneficial effect by exploring its biological properties and phytochemical profile. **Methods:** By using sequential extraction method, *H. indicus* roots were extracted with various solvents based on low to high polarity. Subsequently, quantitative phytochemical profiling, antioxidant and enzyme inhibitory activities were tested by using standard protocols. The MTT assay was carried out in SHSY-5Y cell lines to evaluate anti-inflammatory effect. **Results:** Methanol extract displayed highest phytochemical content with high concentration of terpenoid (59.82±0.97 mg LE/g of extract) and saponin (15.03±0.45 mg DE/g of extract). All the extracts exhibited concentration dependent pharmacological activities. In comparison, methanol extract produced highest activities with IC_{50} of 15.21±0.31 and 11.36±0.39 µg/ml against NO and DPPH radical scavenging assays respectively. Also, methanol extract showed maximum inhibition against acetylcholinesterase (IC_{50}=17.46±0.49 µg/ml) and butyrylcholinesterase (IC_{50}=31.05±0.39 µg/ml), however, aqueous extract displayed highest potency against monoamine oxidase-B inhibition (IC_{50}=24.60±0.45 µg/ml). At 12.5-100 µg/mL concentrations, methanol and aqueous extracts did not show any cytotoxic effect on SH-SY5Y cells and dose dependently suppressed TNF-α and IL-6 production. **Conclusion:** Collectively, *H. indicus* could act as a disease modifying therapeutic in pharmaceutical industries by utilizing it as alternative therapy for the management of oxidative stress and its related disorders.

Key words: *Hemidesmus indicus*, Antioxidant, Acetylcholinesterase, Butyrylcholinesterase, Monoamine oxidase.

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INTRODUCTION

Herbal medicine has been widely embraced since the prehistoric times and was used as dominant form of medical care by the ancient practitioner. Despite a long history of effective use, western medicine took over the world due to lack of scientific evidences in the context of contemporary medicines.[1] In recent years, a surge of acceptance of natural therapies and public interest was observed because of serious side effects associated with long term usage of conventional medicines. Besides, absence of curatives for chronic diseases encourages the advent of natural remedies.[2] As a result, global use of herbal medicinal products and nutraceuticals has been expanded across the developing and industrialized countries and often been viewed as a balanced approach of healing.[3-5]

Growing environmental stress results in accumulation of free radicals, which becomes the reason for the generation of lethal diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), cancer etc.[6] Restoration of redox homeostasis serves a great deal in maintenance of health and prevention of various diseases.[7] With increasing evidence of a wider range of safety, naturally derived antioxidants have gained its popularity over the decades. Plant derived antioxidant preserves active physiological state by efficiently scavenging the free radical generated in the cells and strengthen the defence system.[8] Recently, exogenous antioxidants from dietary sources with enzyme inhibitory properties has been considered as a promising strategy to suppress oxidative stress and its associated disorders.[9] Hence, in the quest of searching for the suitable natural antioxidant, we investigated the antioxidant potential of *Hemidesmus indicus* (L.) R. Br. ex Schult, in order to explore its value as a suitable traditional medicine or as a source of pharmaceutical compounds.

Belonging to a phylogenetic genus, *Hemidesmus indicus* (L.) R. Br. ex Schult (Apocynaceae), commonly known as Anantmula or Indian sarsaparilla, is an indigenous plant of India and Sri Lanka. This popularly ingested aromatic shrub has been served as sharbat (drink) and as a flavouring agent for the preparation of soft drink and as perfumery in cosmetic industry.[10] Apart from consumption as food, *H. indicus* is an integral part of Ayurveda and Siddha medicinal system and known

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to have numerous health profitable benefits such as anti-inflammatory, anti-asthmatic, antibacterial, antinoniceptive, anti-hyperglycemic, anti-atherogenic, anti-spasmodic anti-hepatotoxic, antipyretic, anti-epileptic, antidiarrhoeal, immunopotentiating and antiulcerogenic properties. Moreover, it was also documented to be used as a traditional remedy for leucorrhoea, gonorrhea, syphilis, impotence, gastrointestinal disorders, fever, cough, jaundice, blood disorders, bronchitis, and poisoning.[11-15] Earlier reports represent the presence of a mixture of therapeutic phytocompounds such as terpenoids, tannins, phenols, flavonoids and saponins in the *H. indicus* roots, which could provide synergistic action towards its pharmacological properties.[16] Majority of the studies conducted previously, involved the qualitative aspects of the plant and meagre attempt has been made to understand the varying impact of solvents on quantification of the phytochemical profile of this species.[17] Therefore, our objective is to deal with quantitative insights of the phytoconstituents present with respect to different polarities of solvent, which might offer a great help in understanding its biological potential.

Over the last centuries, several epidemiological, preclinical and clinical studies have demonstrated that there is a close relationship between oxidative stress, neurodegenerative disorders and inflammation.[18,19] Enzyme inhibition is considered to be the most effective strategy for the discovery of bioactive molecules for cognitive enhancement. For instance, inhibition of cholinesterase and monoamine oxidase enzymes assists as a primary treatment for the management of AD and PD respectively.[20] Similarly, inhibition of uncontrolled response of inflammatory mediators to the tissue injury leads to protection against oxidative stress and prevent augmentation of its related disorders including neurodegenerative diseases.[21] Hence, the current study is designed to evaluate the effect of different extraction solvent on the phytochemical profile of Anantmula and to investigate its antioxidant, anti-inflammatory and enzyme inhibitory capacity for the development of natural antioxidant of nutraceuticals and pharmaceutical importance.

MATERIALS AND METHODS

Plant material

Dried roots of *H. indicus* were gathered from local markets of Nilgiris district, India and was authenticated by Dr. S. Rajan, field botanist, Central Council for Research in Homeopathy, Department of AYUSH, The Nilgiris, Tamilnadu, India. The voucher specimen was deposited at Department of Pharmacognosy and Phytopharmacy, JSS College of Pharmacy, Udhagamandalam 643001, India.

Chemicals

The chemicals and solvents utilized for the experiments were procured from Sigma-Aldrich.

Preparation of root extracts

Fresh roots were air dried and coarsely powdered. Obtained powder was soaked in a conical flask with pet ether, chloroform, ethyl acetate, methanol and water on the basis of increasing polarity of solvents and kept in a mechanical shaker (100 rpm, at room temperature) for 72 h followed by filtration with Whatman filter paper. Later on, under high pressure, each filtered extract was concentrated by a rotary evaporator at a maximal temperature of 35 °C. All the extracts were protected from light and stored at 4 °C for further use.[22]

Phytochemical profiling

Determination of the total phenol content (TPC)

Phenolic content in the different extracts of *H. indicus* was determined by adopting the method described by Singleton and Rossi.[23] A mixture of 10% Folin–Ciocalteau's reagent (2.5 mL) and 2% sodium carbonate solution (2 mL) was added to different extracts (0.5 mL) of *H. indicus* (1 mg/mL). After incubation for 15 min at 45 °C, absorbance was measured at 765 nm. Based on the calibration curve prepared using gallic acid as standard, total phenol content was calculated.

Determination of total flavonoid contents (TFC)

Aluminium chloride ($AlCl_3$) colorimetric method was used in order to determine total flavonoid content. Different extracts of *H. indicus* (1mL) was added to the mixture of methanol (3 mL), 10% $AlCl_3$ (0.2 mL), potassium acetate (1 M) (0.2 mL), and distilled water (5.6 mL). Absorbance was read at 420 nm after 30 min. Based on a standard curve prepared using rutin as standard, total flavonoid content was measured.[24]

Determination of total tannin contents (TTC)

By using the method developed by Sun (1998), total tannin content was measured where a mixture of extract (1 mL) and acidic methanol (3 mL) was allowed to stand for 10 min at room temperature followed by addition of Vanillin HCl reagent (6 mL). Absorbance was measured at 500 nm. Quantification of total tannin was done on the basis of standard curve of catechin as standard.[25]

Determination of total terpenoid contents (TTRC)

Total terpenoid content in the different extracts of *H. indicus* was quantified based on the method described by Narayan Ghorai et al.[26] To the assay mixture of extract (160 µL) and chloroform (1.2 mL), conc. H_2SO_4 was slowly added and incubated for 2 h at room temperature. The supernatant of reaction mixture was decanted and 95% methanol (1.5 mL) was added to dissolve the resultant reddish brown precipitate and absorbance was recorded at 538 nm. Total terpenoid content was determined with the aid of standard curve of linalool as standard.

Determination of total saponin contents (TSC)

For estimation of total saponin content in the extract, vanillin-sulphuric acid assay was incorporated. Extracts (0.25mL) were mixed with 8% (w/v) vanillin in ethanol (0.25 mL) and 72% (v/v) sulphuric acid in water (2.5 mL) and incubated at 60 °C in water bath for 15 min. Absorbance was then measured at 560 nm after cooling at room temperature for 5 min.[27]

Antioxidant activity

Nitric oxide (NO) scavenging activity

To sodium nitroprusside solution (1mL of 25 mM), 4 mL of different concentrations (6.25-100 µg/mL) of extracts was added and incubated for 3 h at 37° C. Griess reagent (0.3 mL) was added to the solution (0.5mL) and the absorbance of the chromophore formed was measured at 570 nm. Control was prepared in a similar way, but without the extract. Butylated hydroxytoluene (BHT) was used as a reference standard.[28]

1, 2-diphenyl-2-picryl hydrazyl (DPPH) scavenging activity

Effect of *H. indicus* extracts on DPPH radical has been carried out by employing the method described earlier by Mary et al.[29] 2mL of various concentrations (2 mL) of extracts (6.25-100 µg/mL) was mixed with DPPH (0.5 mL of 0.01 M) in methanol. Absorbance of the solution was analysed at 517 nm. Ascorbic acid was used as reference standard.

Neuroprotective potential

Evaluation of Acetylcholinesterase (AChE) and Butyrylcholinesterase (BuChE) inhibition assay

Both AChE and BuChE inhibitory ability of the extracts was evaluated by a modified colorimetric method of Ellman et al.[30] Reaction

mixture consisting of 5,5-dithiobis-2-nitrobenzoate (125 µL of 3mM), acetylthiocholine iodide (25 µLof 15mM), different concentrations (6.25-100 µg/mL) of extracts and phosphate buffer pH 8.0 (50 µL of 0.1 M) was incubated at 25 °C. After 20 min, AChE (25 µL of 0.22 U/mL) or BuChE (25 µL of 0.22 U/mL) was added to initiate the reaction and the activity was determined by measuring absorbance at 412 nm. Galantamine was used as standard.

Evaluation of monoamine oxidase (MAO) inhibition assay

Monoamine oxidase (MAO) inhibitory activity was performed according to the fluorescence method as described by Diermen et al., and Novaroli et al.[31,32] For the inhibition assay, reaction was carried out in black 96-well microtitre plates, where each well contained kynuramine (8 µL of 0.75M), MAO-A (0.015 mg/mL) or MAO-B enzyme (0.015 mg/mL) (50µL) and 2µL of various concentrations of extract (6.25-100 µg/mL) prepared in dimethylsulfoxide (DMSO) (final concentration of 1% DMSO). To the mixture, 140 µL of 0.1M potassium phosphate buffer (pH 7.4) was added and incubated at 37°C for 20 min. Reaction was terminated by addition of NaOH (75 µL of 2N) followed by centrifugation at 12000 rpm for 10 min. Fluorescence reading was measured at 400 nm. Clorgyline and pargyline were chosen as standard inhibitors of MAO-A and MAO-B, respectively.

Cell viability assay

By using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay, cytotoxic effect of *H. indicus* extracts on SH-SY5Y cells was determined. All cell culture experiments were performed in a humidified atmosphere containing 5% CO_2 at 37°C, in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Predetermined number of cells (2 x 10^4 /well) were seeded in 96-well plate, cultured with or without 1 µg/mL lipopolysaccharide (LPS) and incubated for 24 h at 37 °C. Different concentrations of methanol and aqueous extracts, prepared in DMSO, were dispensed into the plates containing LPS. The cells cultured for 24 h without adding LPS under the same conditions were used as a control. After 24 h, fresh medium was added with 0.5% MTT solution and the plates were incubated for 3 h. Further, 10% sodium lauryl sulphate dissolved in 0.1 mol/L HCl was added to terminate the reaction and absorbance was measured at 550 nm with a microplate reader[33]

Determination of tumor necrosis factor (TNF-α) and interleukin 6 (IL-6) levels

Different concentrations of methanol and aqueous extracts of *H. indicus* were incubated overnight in the 96-well plate containing 1 µL/mL LPS treated SH-SY5Y cells at 4 °C. Based on the ELISA kit manual, concentration of tumor necrosis factor (TNF-α) and interleukin 6 (IL-6) was estimated with an ELISA reader.

Statistical analysis

All experiments were done in triplicates and were analysed using SPSS for Windows (version 17.0, SPSS Inc., Chicago, IL, USA). All values were expressed as mean ± standard deviation (SD). Correlation analyses were performed using Pearson's Correlation Coefficient. Mean values were compared using the one-way analysis of variance (ANOVA) and $P<0.05$ was considered statistically significant.

RESULTS

Phytochemical analysis

The percentage yield of different extracts of *H. indicus* roots obtained after successive maceration extraction with polar and non-polar solvents varied from 4.49% to 33.23% (mass of extract/mass of roots). The highest yield was obtained from aqueous extract followed by methanol extract, suggesting presence of high amount of polar compounds soluble in methanol and water solvents. Total yield obtained with regards to the different solvents used for extraction was as follows: water> methanol> ethyl acetate> chloroform> pet ether (Table 1). Results from the quantitative phytochemical analysis indicated that terpenoids and phenols constituted the major fraction ranging from 13.51 to 59.82 mg LE/g and 3.57 to 45.40 mg GAE/g of extract respectively. In addition, based on the data expressed in Table 2, all the crude extracts showed a substantial amount of flavonoid and saponin content ranging from 0.88 to 23.22 mg RE/g and 1.39 to 15.03 mg DE/g of extract respectively, whereas tannin content was quantified in the least amount in the plant roots. As evident from our study, in comparison, methanol and aqueous extracts showed maximum amount of phytochemical content where methanol extract exhibited relatively high concentration of terpenoids (59.82±0.97 mg LE/g of extract) and saponins (15.03±0.45 mg DE/g of extract), whereas aqueous extract displayed adequate amount of phenols (45.40±0.08 mg GAE/g of extract) and flavonoids (23.22±0.37 mg RE/g of extract) (Table 2).

Antioxidant activity

In the present study, we have validated antioxidant activity of *H. indicus* by using NO scavenging test and DPPH reducing capacity. We detected that all the five extracts of *H. indicus* roots displayed radical scavenging activity in a concentration dependent manner. Amid all, methanolic extract predominantly produced potent NO (IC_{50} =15.21±0.31 µg/ml) and DPPH (IC_{50}= 11.36±0.39 µg/ml) scavenging activity when compared to reference drug BHT (IC_{50} =1.30±0.21µg/ml) and ascorbic acid (IC_{50}= 0.99±0.42 µg/ml) respectively (Table 3). Surprisingly, aqueous extract also displayed strong radical scavenging activity and exhibited IC_{50} of 19.14±0.67 and 28.91±0.22 µg/ml against NO and DPPH scavenging assays. However, on the contrary, pet ether and chloroform extracts revealed lowest antioxidant activity. Further, in order to evaluate the relation between phytoconstituents and antioxidant activity, we have performed correlation studies by calculating Pearson's correlation coefficients which indicated TPC, TTRC and TFC have a significant correlation with both the antioxidant assays (Table 5).

Neuroprotective potential

Acetylcholinesterase (AChE) and Butyrylcholinesterase (BuChE) inhibition assays

Based on our exploratory study, we can convey that, methanol extract of *H. indicus* demonstrated the highest AChE and BuChE inhibitory activity with IC_{50} of (17.46±0.49 µg/mL) and (31.05±0.39 µg/mL) respectively. Likewise, aqueous extracts too showed equally satisfactory results against AChE (IC_{50} =21.19±0.4 µg/mL) and BuChE (IC_{50} =39.23±0.11 µg/mL), while, pet ether extract displayed marginal inhibition potential (Table 4). Interestingly, a significant correlation was observed between AChE (R=-0.888) and BuChE (R=-0.915) inhibitory activities and concentration of TTRC (Table 5).

Monoamine oxidase inhibition assays

The effect of the extracts on the MAO inhibition is presented in the Table 4. From our experiment, we can conclude that all the extracts have displayed dose dependent enzyme inhibitory activity against MAO-B. However, none of the extracts showed any remarkable MAO-A inhibitory effect. Results from MAO-A enzyme inhibition analysis is contradictory to our antioxidant and anti-cholinesterase studies. However, the reason behind the conflict is unknown. Surprisingly, unlike other assays, aqueous extract (IC_{50} = 24.60±0.45 µg/mL) manifested potent inhibitory activity against MAO-B than methanol extract (IC_{50} = 55.01±0.56 µg/mL). Correlation coefficient studies indicated a significant relation between MAO-B inhibition and concentration of TPC (R=-0.881) and TFC (R= -0.850) (Table 5).

Table 1: Extraction yield of different extracts of *H. indicus* roots.

	Pet ether	Chloroform	Ethyl acetate	Methanol	Water
% yield	4.49	6.45	11.11	27.87	33.23

Table 2: Quantitative phytochemical analysis of different extracts of *H. indicus* roots.

	Pet ether	Chloroform	Ethyl acetate	Methanol	Water
TPC (mg GAE/g)	3.57 ± 0.22^{a}	9.93 ± 0.1^{a}	16.29 ± 0.16^{b}	40.95 ± 0.6^{c}	45.40 ± 0.08^{c}
TFC (mg RE/g)	0.88 ± 0.38^{a}	2.19 ± 0.73^{a}	6.58 ± 0.55^{b}	19.09 ± 0.6^{b}	23.22 ± 0.37^{c}
TTC (mg CE/g)	0.22 ± 0.09^{a}	0.86 ± 0.15^{a}	1.36 ± 0.15^{a}	4.61 ± 0.2^{b}	2.33 ± 0.22^{a}
TTRC (mg LE/g)	13.51 ± 0.78^{a}	18.13 ± 0.64^{a}	25.97 ± 0.16^{b}	59.82 ± 0.97^{d}	48.94 ± 0.82^{c}
TSC (mg DE/g)	1.39 ± 0.37^{a}	2.63 ± 0.15^{a}	6.59 ± 0.47^{b}	15.03 ± 0.45^{d}	10.33 ± 0.33^{c}

Each value represents average of three analysis (mean ± SD). Values with different letters (a-d) in the same row indicated significant difference (P<0.05). TPC-total phenolic content, TFC-total flavonoid content, TTC- total tannin content, TTRC-total terpenoid content, TSC-total saponin content, GAE-Gallic acid equivalents, RE-Rutin equivalents, CE-Catechin equivalents, LE-Linalool equivalents, DE- Diosgenin equivalents.

Table 3: IC_{50} values of different extracts of *H. indicus* roots for radical scavenging and anti-inflammatory assays.

Extracts	NO IC_{50}(µg/mL)	DPPH IC_{50}(µg/mL)
Pet ether	87.62 ± 0.54^{a}	106.23 ± 0.44^{a}
chloroform	67.40 ± 0.47^{b}	94.23 ± 0.75^{a}
ethyl acetate	51.89 ± 0.22^{b}	78.81 ± 0.66^{b}
methanol	15.21 ± 0.31^{c}	11.36 ± 0.39^{d}
water	19.14 ± 0.67^{c}	28.91 ± 0.22^{c}
BHT	1.30 ± 0.21^{d}	-
Ascorbic acid	-	0.99 ± 0.42^{e}
Diclofenac	-	-

The values are expressed as mean ± SD of three independent experiments. Values with different letters (a-e) in the same column indicated significant difference (P<0.05).

Table 4: IC_{50} values of different extracts of *H. indicus* for cholinesterase and monoamine oxidase inhibition assays.

Extracts	AChE	BuChE	MAO-A	MAO-B
Pet ether	111.01 ± 0.67^{a}	146.20 ± 0.55^{a}	239.59 ± 0.45^{a}	108.92 ± 0.22^{a}
chloroform	59.65 ± 0.33^{b}	85.51 ± 0.21^{c}	215.83 ± 0.41^{a}	113.96 ± 0. 78^{a}
ethyl acetate	90.78 ± 0.45^{a}	104.09 ± 0.23^{b}	217.38 ± 0. 67^{a}	57.91 ± 0.86^{b}
methanol	17.46 ± 0.49^{c}	31.05 ± 0.39^{d}	205.68 ± 0.48^{a}	55.01 ± 0.56^{b}
water	21.19 ± 0.45^{c}	39.23 ± 0.11^{d}	154.78 ± 0.23^{b}	24.60 ± 0.45^{c}
Galantamine	0.18 ± 0.84^{d}	3.23 ± 0.67^{e}	-	-
clorgyline	-	-	5.91 ± 0.33^{c}	-
L-deprenil	-	-	-	2.02 ± 0.39^{d}

The values are expressed as mean ± SD of three independent experiments. Values with different letters (a-e) in the same column indicated significant difference (P<0.05).

Table 5: Correlation coefficient between phytochemical compounds and antioxidant, anti-inflammatory, cholinesterase and MAO-B inhibitory activities of *H. indicus* roots.

Assays	TPC		TTRC		TFC	
	r	P	r	P	r	P
NO	-0.976	0.033*	-0.969	0.289	-0.979	0.043*
DPPH	-0.973	0.675	-0.999	0.0321*	-0.987	0.343
Anti-inflammatory	-0.973	0.043*	-0.999	0.039*	-0.986	0.028*
AChE	-0.906	0.677	-0.888	0.0028**	-0.839	0.989
BuChE	-0.925	0.578	-0.915	0.0443*	-0.890	0.879
MAO-B	-0.881	0.036*	-0.796	0.698	-0.850	0.048*

** indicates P<0.01 and *P<0.05. TPC-total phenolic content, TFC-total flavonoid content, TTRC-total terpenoid content

Cell viability assay

Since from all the aforementioned assays, best result was exhibited by methanol and aqueous extracts, hence both of the extracts were further subjected to subsequent experiments. Results from the cell viability assay displayed that at concentrations of 12.5-100 µg/mL, both the extract showed no cytotoxic effect on SH-SY5Y cells and displayed >90% of viability. However, at the concentration of 200 µg/mL, slight reduction in cell viability was observed for both the extracts. Based on the outcome from cell viability assay, we have used 25-100 µg/mL concentrations of both methanol and aqueous extracts for other experiments on SH-SY5Y cells (Figure 1).

Determination of TNF-α and IL-6 levels

Treated SH-SY5Y cells with only LPS significantly increased the levels of TNF- α (1111.67± 29.67 µg/mL) and IL-6 (11548.67±57.4 µg/mL) when compared to control. As expected, both the extracts showed concentration dependent suppression of cytokines production. However, on comparison, highest inhibitory activities against TNF- α (351.33±71.49 µg/mL) and IL-6 (2639±36.51 µg/mL) in LPS treated SH-SY5Y cells was displayed by 100 µg/mL of methanol extract (Figure 2, 3).

DISCUSSION

A Plethora of studies has witnessed the protective role of herbals against several chronic diseases. Based on the epidemiological and clinical evidences, phytochemicals have shown to affect cell integrity and redox status by modulating the cellular metabolism and antioxidant enzyme expression.[34] In this context, the objective of the present study intended to explore the phytochemical profile of *H. indicus* quantitatively and validate its radical scavenging, anti-inflammatory and enzyme inhibitory capacity linked to neuroprotection.

Interaction between bioactive phytoconstituents depends upon its solubility, chemical structures and polarities of solvents used during extraction.[17] Hence, to investigate the medicinal value of *H. indicus*, we have analyzed the effect of extracting solvents on its phytochemical content. Although, there were several documents available on qualitative identification of phytochemicals in *H. indicus* but information on quantitative data was limited. Polyphenols and terpenoids are the major phytoconstituents isolated by various researchers from the roots, which is in correspondence to our findings.[35-38] Results from our phytochemical profiling studies indicated appreciable amount of polyphenols, terpenoids, flavonoids and saponins. Similar results were observed previously, but with the variable amount.[39] The probable reason behind

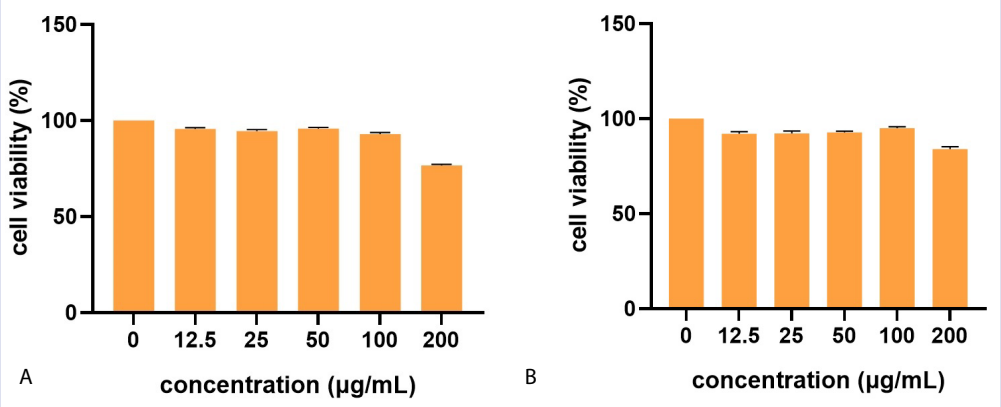

Figure 1: (A) Effect of methanol extract of *H. indicus* on the viabilities of SH-SY5Y neuroblastoma cells. (B) Effect of aqueous extract of *H. indicus* on the viabilities of SH-SY5Y neuroblastoma cells.

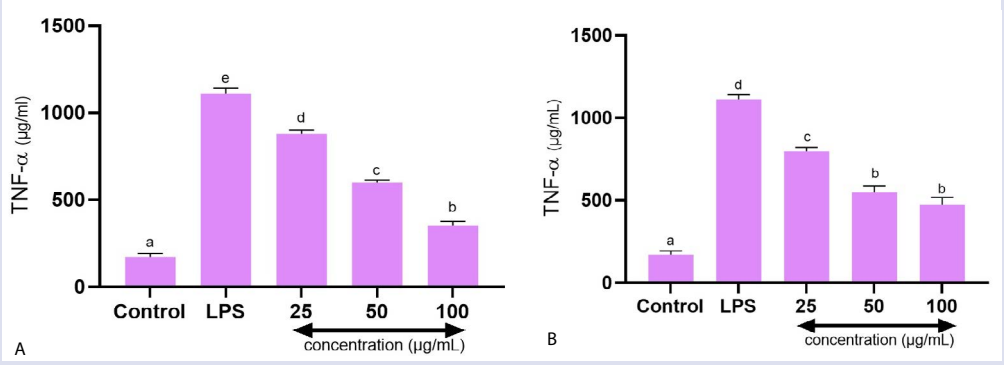

Figure 2: (A) Effect of methanol extract of *H. indicus* on TNF-α in lipopolysaccharide (LPS)-stimulated SH-SY5Y neuroblastoma cells. Values with different letters (a-e) indicate a significant difference (P<0.05). (B) Effect of aqueous extract of *H. indicus* on TNF-α in lipopolysaccharide (LPS)-stimulated SH-SY5Y neuroblastoma cells. Values with different letters (a-d) indicate a significant difference (P<0.05).

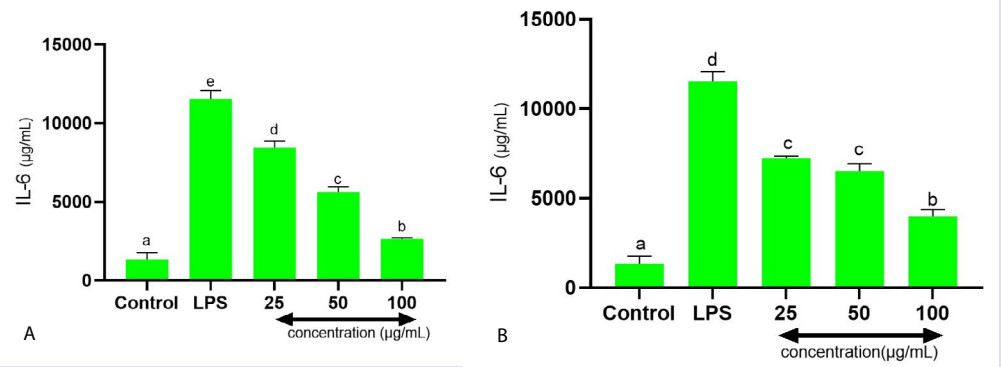

Figure 3: (A) Effect of methanol extract of *H. indicus* on IL-6 in lipopolysaccharide (LPS)-stimulated SH-SY5Y neuroblastoma cells. Values with different letters (a-e) indicate a significant difference (P<0.05). (B) Effect of aqueous extract of *H. indicus* on IL-6 in lipopolysaccharide (LPS)-stimulated SH-SY5Y neuroblastoma cells. Values with different letters (a-d) indicate a significant difference (P<0.05).

the variation in content could be due to edafoclimatic effect and/or use of different extraction and analytical techniques. Presence of mixture of phytochemicals in *H. indicus* roots, when acting in combination, could be accountable for its health beneficial properties.

The generation of free radicals *in-vivo* is a constant phenomenon either due to physiological metabolism or pathological alterations. Aberrant accumulation and generation of free radicals or reactive oxygen species (ROS) contributes to the damage of intracellular and extracellular matrix components such as proteins, lipids, proteoglycans and collagens, which leads to the development of oxidative stress and its related diseases.[40] Antioxidants potentially counteract this deleterious effects of ROS, providing protection against oxidative stress and maintain ion homeostasis, thereby, sustain the physiological functions of cells.[41] Therefore, we checked the antioxidant activity of *H. indicus* and noticed that the polar extracts have manifested good free radical neutralization ability. Both methanol and aqueous extracts displayed strong radical scavenging and reducing property against NO and DPPH tests, followed by ethyl acetate extract. However, almost no antioxidant effect was observed in the pet ether extract. Our results are in accordance with the previously accomplished studies, suggesting presence of quantifiable amount of terpenoid and phenol compounds in *H. indicus,* synergistically could attribute to the potent antioxidant activity due to their redox properties, possibly through hydrogen atom, electron-proton and sequential proton loss-electron transfer mechanisms.[42-49]

Natural antioxidant protects from a wide range of lethal diseases such as AD by inhibiting hyper accumulation of ROS. Oxidative stress triggers neuronal cell apoptosis and creates neurotoxic environment which hinders the brain functions, supporting neurodegeneration.[50,51] Loss of cholinergic synapses results in decrease of ACh and BuCh levels in the brain regions, which appeared to be the major element in the development of AD and the first thing experienced by the patients suffering from the disease.[52] Restoration of these neurotransmitter levels by inhibiting AChE and BuChE enzymes is the current therapy accepted worldwide. However, side effects associated with currently available AChE and BuChE inhibitors encouraged scientific investigation for identification of potent inhibitors from herbal sources in an effort to discover new AD drugs.[53,54] Although, very few studies were conducted previously on the choline esterase activity of *H. indicus* indicating some extent of neuroprotective property by its methanol and hydroalcoholic extracts, but no attempt has been made to measure these activities of the plant with a combination of solvents.[10,37] Therefore, we investigated the effect of different solvents on cholinesterase inhibitory properties of *H. indicus* by employing AChE and BuChE inhibitory assays. From our exploratory study, we found that methanol and aqueous extracts of *H. indicus* have demonstrated the highest AChE and BuChE inhibitory activities followed by chloroform extract, while pet ether and ethyl acetate extracts showed minimal effect. As suggested by Mukherjee et al., terpenes possess a carbon skeleton which is susceptible to hydrophobic interactions with the hydrophobic active site of AChE and BuChE and thus contribute to the anti-cholinesterase activities.[55] Results from our Pearson's Correlation Coefficient study and the presence of ample quantity of terpenoids in methanol extract found from our quantitative phytochemical experiments, support the aforementioned findings. In addition, outcomes of our cholinesterase inhibition test are in agreement with our antioxidant studies, suggesting that the extracts with higher scavenging potential have a direct impact on AChE and BuChE inhibition activity. Although more research is needed to understand the underlying mechanism.

Excessive production of monoamine oxidase (MAO) lead to the neuronal injury and aggravates the oxidative stress condition in the cells.[56] Earlier studies have demonstrated that selective inhibition of two isoforms of MAO i.e., MAO-A and MAO-B could be a potential therapeutic strategy in the management of neurological disorders, where MAO-A inhibitors are helpful in the treatment of depression and mood disorders and MAO-B inhibitors provide protection against AD and PD.[57] In our experiment, different root extracts have shown dose dependent MAO-B inhibition, but highest was exhibited by aqueous extract followed by methanol extract. However, no remarkable activity was produced by any extract against MAO-A inhibition. Previously, several reports have indicated that phenols are potent reversible MAO-B inhibitors with different mode of inhibition such as non-competitive, competitive, or mixed-type inhibition.[57] Besides, flavonoids have been widely reported for its MAO enzyme inhibition activity due to its structural similarities with synthetic MAO inhibitors.[58] Thus, the highest inhibitory effect of aqueous extract correlates with the high phenolic and flavonoid content when compared to the methanol extract. Altogether, methanol and aqueous extracts of *H. indicus* could be promising candidate for the drug development against neurodegenerative disorders and its related complications.

Evaluation of cytotoxicity potential of the extracts act as an important tool for identification of bioactive molecule. Hence, in order to exclude any probable cytotoxic effects, methanol and aqueous extracts of *H. indicus* were subjected to cell viability assay on SH-SY5Y neuroblastoma cells which indicated no cytotoxicity at the 12.5-100 µg/mL concentrations.

Inflammation functions as doubled edged sword, as it provide protection against many harmful stimuli and helps repair tissue

damage, while on other hand, excessive secretion of inflammatory mediators such as TNF –α and IL-6 can exacerbate the oxidative stress and its related disorders.[59] Results from the present study demonstrated that both methanol and aqueous extracts of *H. indicus* dose-dependently reduced TNF –α and IL-6 levels in LPS treated SH-SY5Y cells, however, on comparison, best result was exhibited by methanol extract. These results are in accordance to the previously conducted studies and supported that both the extracts could be potential target for the development of anti-inflammatory drugs.[60-62] Studies conducted earlier demonstrated that terpenoids are capable of producing anti-inflammatory properties by suppressing nuclear transcription factor κB (NF-κB) pathway, which is considered to be a major regulator of the immune system and inflammatory response. Reason behind the inhibitory effect of terpenoids is due to the presence of α-β-unsaturated carbonyl functions which act as NF-κB inhibitory pharmacophore and thereby either acts directly on IκB kinase (IKKs), on the proteosome machinery, or on the NF- κB -DNA binding.[63] Likewise, few researchers have reported that phenolic compounds along with flavonoids have shown desirable anti-inflammatory property by inhibiting COX activity or its gene expression or by up/downregulating crucial inflammatory transcriptional factors, like NF- κB or nuclear factor erythroid 2–related factor 2 (Nrf-2).[64] Our results are in agreement with these reports and signified that presence of the rich amount of secondary metabolites, especially phenols and terpenoids, as reported from our quantitative phytochemical investigations, could be responsible for the anti-inflammatory activity of the extracts. However, the compounds present in the extracts and their mechanism behind these advantageous effects of the extracts need to be explored.

CONCLUSION

The present study, for the first time, reveals the effect of different extracting solvents on phytochemical content, antioxidant, anti-inflammatory and neuroprotection linked enzymes (MOA-B, AChE, BuChE) inhibitory properties of *H. indicus*. In the light of our findings, it can be concluded that based on our phytochemical profiling studies, terpenoids and phenols were identified as a major class of secondary metabolites present in the plant roots. Besides, amid all, methanol and aqueous extracts exhibited highest amount of these phytochemical compounds which strongly correlates with the performed pharmacological activities. However, further investigation is required to find active components from these extracts. Collectively, results from our study represents the dietary and medicinal importance of *H. indicus* and thus could be a promising source of natural antioxidants for the utilization in the food, pharmaceutical and cosmetic industries.

DECLARATION OF COMPETING INTEREST

None.

ABBREVIATIONS

AD: Alzheimer's disease (AD); PD: Parkinson's disease (PD); MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; NO: Nitric oxide; DPPH: 1, 2-diphenyl-2-picryl hydrazyl; ROS: Reactive oxygen species; TPC: Total phenol content; TFC: total flavonoid contents; TTC: Total tannin contents; TTRC: Total terpenoid contents; TSC: Total saponin contents; AChE: Acetylcholinesterase; BuChE: Butyrylcholinesterase; MAO: Monoamine oxidase; LPS: lipopolysaccharide; TNF-α: Tumor necrosis factor; IL-6: interleukin 6; ELISA: Enzyme-linked Immune Sorbent Assay; ANOVA: Analysis of variance.

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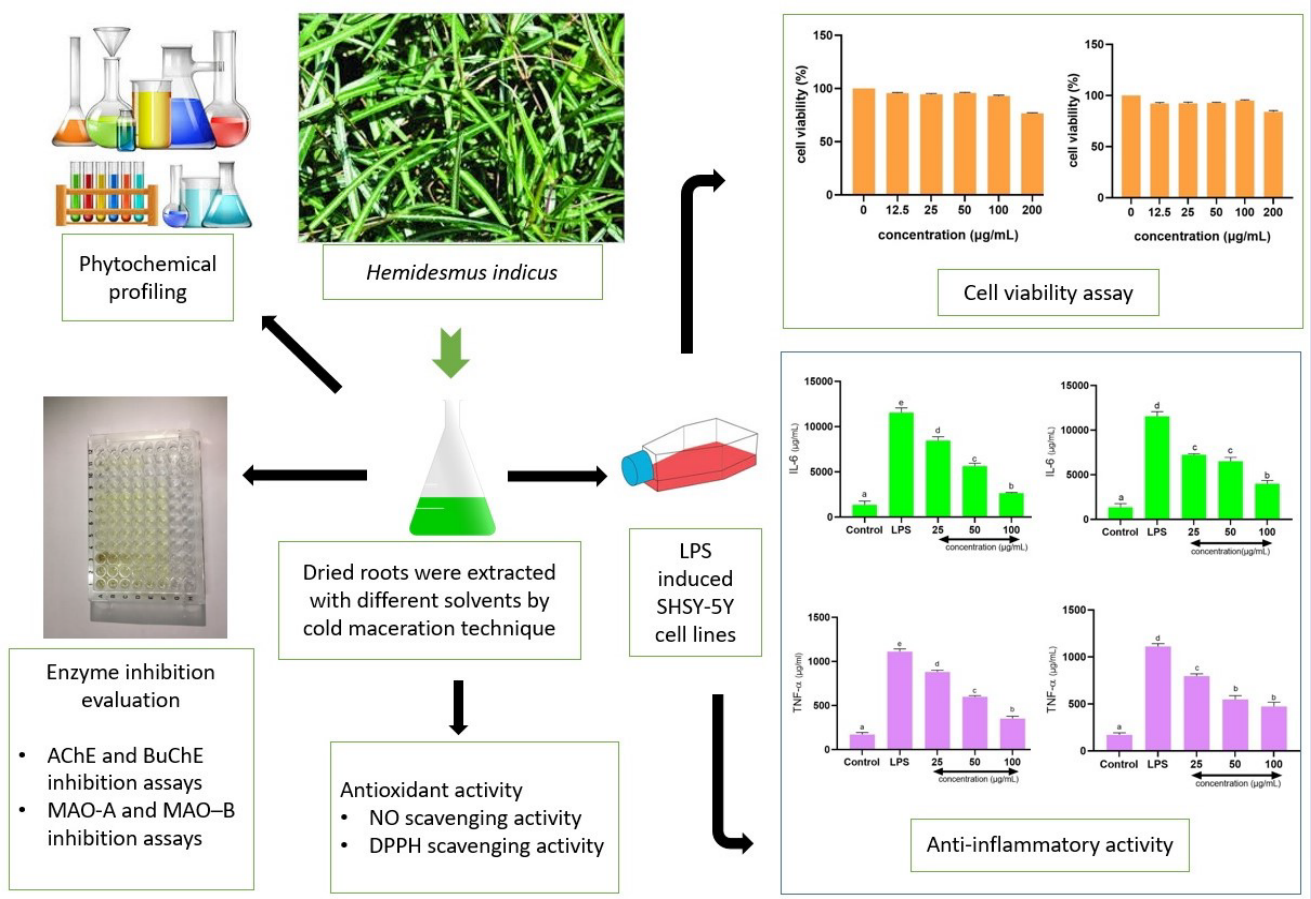
GRAPHICAL ABSTRACT



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