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Bioactive And Cytotoxic Potentials of the Extract Fractions of *Strophanthus Hispidus* on Neuroblastoma (SH-SY5Y) Cell Line Model of Alzheimer's Disease

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ABSTRACT: Alzheimer disease (AD) is a neurodegenerative disorder that affect memory and cognitive function. Currently, the available agents used in managing this condition are associated with severe side effects and have limited efficacy in reducing or modulating events associated with AD. Hence, there is a need for an alternative therapy with fewer side effects and better efficacy. *Strophantus hispidus* (SH) is a plant product widely used in Africa for its therapeutic potential. This study was conducted to assess the bioactive constituent of the SH extract fractions, as well as the cytotoxic potential of these fractions. The root of SH was pulverized and extracted with 80% methanol. The crude extract was then fractionated with Chloroform, Hexane, and Ethylacetate. The extract and fractions were then subjected to Gas Chromatography-Mass Spectrometry (GC-MS). SH-SY5Y cell line model of AD was treated with graded concentrations (9.77 – 5000 µg/mL) of the SH extract and fractions for 24 hours. Cell viability assay was performed using WST-8 proliferation assay. The EC_{50} was determined to be 48.4 µg/mL, 15.16 µg/mL, and 151 µg/mL for cells treated with Chloroform, Ethylacetate, and Hexane fractions of *Strophantus hispidus* respectively. The selectivity index (SI) greater than 3 of the hexane and ethylacetate fractions were determined to be safe, less toxic, and effective in improving cellular growth.

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Keyword: Alzheimer Disease; *Strophantus hispidus*; Cell viability; Selectivity index.

Strophanthus hispidus (SH) is a member of the Apocynaceae family, commonly known as the dogbane family (Ishola *et al*., 2013). In several African tribes, SH have been said to constitute part of the major ingredients for making arrow poison due to the cardiac glycoside content present in its seed, which is absorbable through wounds. Other than that, it serves as traditional medicines for conditions like arthritis, rheumatism, ulcer, stroke, and heart failure (Odugbemi, 2006). Alzheimer's disease (AD) is a neurodegenerative disorder characterized by mental retardation, and neurocognitive impairment (Srivastava *et al*., 2021). It is the most common cause of dementia (WHO, 2023), and is fast becoming one of the most expensive, deadly, and challenging diseases of this century. This neuropathological condition is depicted by neurodegeneration, neural loss, low levels of neurotransmitters in the brain, associated loss of synapses and development of neurofibrillary tangles and amyloid beta (Aβ) plaques (Crews and Masliah, 2010). In the neurodegenerative progression of AD, some signaling proteins which

include fyn kinase (Chin *et al.,* 2005); glycogen synthase kinase-3β (GSK3β) (Rockenstein *et al*., 2007) and cyclin-dependent kinase-5 (CDK5) (Cruz *et al*., 2007) have been implicated. To date, there is no curative or preventive therapy for AD. The effectiveness of currently approved medication to prevent or lessen AD-related events is limited and they are associated with serious side effects (Hass, 2012). Therapies for AD require consideration of the development of compounds that target pathways implicated in neurodegeneration. Therefore, an alternative therapy with little to no side effects is needed, preferably from natural sources. The FDA approval of plant-derived Galanthamines depicts the importance of natural products in the development of AD therapy. Extensive studies are being carried out on natural products and their derived compounds for their potential to improve the memory of AD patients (Ratheesh *et al*., 2017). Pre-clinical studies on Curcumin show it exerts an effect on several AD targets, which include inhibition of inflammatory pathways, Aβ deposition, and improvements in cognitive performance (McClure *et al.,* 2017; Reddy *et al*., 2018).

Herbal remedies are thought to be more effective than currently available drugs for treating AD. As they have been perceived to have fewer side effects. Particularly intriguing are drugs based on ethnopharmacology that have a history of being safe in local populations. (Abduljawad *et al.,* 2022). The aim of this present study is to evaluate the bioactive components as well as the cytotoxic potential of the extract fractions of *Strophanthus hispidus* on Neuroblastoma (SH-SY5Y) cell line model of Alzheimer Disease.

MATERIALS AND METHODS

Plant Collection: The root of *S. hispidus* were collected from Eruwa town, Oyo North LGA, Oyo State, Nigeria. The roots of the plant, identified and authenticated by Mr. A. Odewo, of the Forestry Research Institute of Nigeria (FRIN), were allotted herbarium voucher specimen Number FHI 111955. It was then deposited for reference.

Extraction of Plant Materials: The roots of *S. hispidus* were washed, slashed into small fragments, and air dried at room temperature. The dried roots were pulverized using a mechanical grinder (Christy and Morris Limited, England). Per the extraction, 180.91 grams of the powdered plant was soaked in 80% methanol for 72hrs and subjected to intermittent agitation. Thereafter, muslin cloth and filter paper were used to filter the extract. The residue was discarded, and the filtrate was concentrated using a rotary evaporator (B-490 Buchi, Switzerland), under reduced pressure to a viscous liquid, which was left to dry in a water bath at 40°C. The crude extract was collected into an airtight McCartney bottle stored at 4ºC until use. The yield was determined to be 4.12% w/w [yield (%) = (weight of dried extract/ weight of plant starting material) × 100]. The brownish solid extract obtained was further subjected to solvent-solvent partitioning.

Solvent-Solvent Partitioning: Sixty grams of *S. hispidus* crude extract were fractionated, using the separation funnel method of fractionation. Three solvents in order of increasing polarity were used for this process. The solvents used in order of their increasing polarity are N-Hexane, chloroform and ethyl acetate.

Phytochemical Screening: Qualitative and quantitative phytochemical analyses were performed to reveal the presence of various chemical constituents. This was done by following the standard procedures described by Trease and Evans (1989).

Gas Chromatography-Mass Spectrometry (GC-MS): GC-MS analysis of the extracts or fractions was performed using an Agilent 5977B GC/MSD system coupled with Agilent 8860 auto-sampler, a Gas Chromatograph interfaced to a Mass Spectrometer (GC-MS) equipped with an Elite-5MS (5% diphenyl/95% dimethyl polysiloxane) fused a capillary column (30 × 0.25µm ID × 0.25 µm df). For GC-MS detection, an electron ionization system was operated in electron impact mode with an ionization energy of 70 eV. Helium gas (99.999%) was used as a carrier gas at a constant flow rate of 1 ml/min, and an injection volume of 1µl was employed (a split ratio of 10:1). Five (5) point serial dilution calibration standards (1.25, 2.5, 5.0, 10.0ppm) were prepared from the stock solution of 40ppm and used to calibrate the GC-MS.

The injector temperature was maintained at 300 °C, and the ion-source temperature was 250 °C, and the oven temperature was programmed from 100 °C (isothermal for 0.5 min), with an increase of 20 °C/min to 280°C (2.5 min), Mass spectra were taken at 70 eV: a scanning interval of 0.5 s and fragments from 45 to 450 Da. The solvent delay was 0 to 3 min, and the total GC/MS running time was 21.33 minutes.

Cell Culture and Conditions: The Neuroblastoma (SH-SY5Y) cells were purchased from Cell Line Services (CLS), Germany while VeroE6 cells were resuscitated from the cell bank of Lagos State Biosafety Laboratory, Nigeria. The cells were cultured

in DMEM-F12, supplemented with 10% FBS, 1% Antibiotics/Anti-mycotics in a tissue culture flask and incubated in a humidified 5% CO_2 incubator at 37 °C till confluence.

Cell Viability Assay: SH-SY5Y and VeroE6 cells were brought to suspension at a seeding concentration of 5000 cells per well in a 96 well plate. The plate was sealed and incubated for about 24 hours to allow for the cells to attach.

After 24 hours, the cells were treated with crude extract or fractions that had been redissolved in DMSO and PBS to achieve a stock concentration of 10 mg/mL and DMSO content of less than 0.5% in a 2-fold serial dilution at a start concentration of 5000 µg/mL and incubated for another 24 hours. Afterward, 10 µl of CCK-8 reagent was added to each well, incubated for 3 hours, and absorbance was measured at 450nm. The cell viability was deduced using the following equation:

$$\% \text{ Cell viability} = \frac{\text{ODTest} - \text{ODBlank}}{\text{ODControl}} \times 100 \qquad (1)$$

The half-maximal effective concentration (EC_{50}) values were calculated using GraphPad Prism (version 9.3.1 733).

RESULTS AND DISCUSSION

Phytochemical Screening: Phytochemical screening of the plant extracts and fractions revealed a variety of phytochemical constituents such as alkaloid, and phenols, tannins, saponins, steroids, phlobatanin, and cardiac glycosides as shown in Tables 1 and 2.

Table 1: Qualitative constituents of *Strophanthus hispidus* extract fractions

Qualitative screening	**RESULTS**			
Phytochemicals	**Crude**	**Chloroform**	**Ethylacetate**	**Hexane**
Alkaloids	+	+	+	-
Saponin	+	+	+	-
Reducing Sugar	+	+	+	-
Anthraquinone	-	-	-	-
Cardiac Glycosides	+	-	+	-
Terpenoids	+	+	+	+
Triterpenoids	+	+	+	+
Steroids	+	+	+	+
Phenolic Compounds	+	+	+	-
Tannins	+	+	+	-
Flavonoids	+	+	+	-

Detected: +; Not detected: -

Table 2: Quantitative cconstituents of *Strophanthus hispidus* extract fractions

Quantitative Screening	**Result (mg/g)**			
Phytochemicals	**Crude**	**Chloroform**	**Ethylacetate**	**Hexane**
Alkaloids	7.26±0.01	9.72±0.01	7.38±0.01	0.00±0.00
Saponin	2.34±0.01	0.97±0.01	1.02±0.01	0.00±0.00
Reducing Sugar	20.21±0.01	8.66±0.01	3.90±0.01	0.00±0.00
Anthraquinone	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Cardiac Glycosides	0.48±0.01	0.00±0.00	0.21±0.01	0.00±0.00
Steroids	1.65±0.01	2.53±0.01	1.68±0.02	2.71±0.01
Phenolic Compounds	24.86±0.01	7.55±0.03	9.177±0.01	0.00±0.00
Tannins	7.04±0.01	1.77±0.01	2.31±0.01	0.00±0.00
Flavonoids	40.96±0.05	18.42±0.01	13.19±0.02	0.00±0.00

Gas Chromatography-Mass Spectrometry (GC-MS): GC-MS results obtained for the plant extracts and fractions revealed the presence of several bioactive compounds, some of which have been previously identified and are of biological importance. The bioactive compounds identified from each fraction are listed in Tables 3-5.

Cell Viability: Following 24 h treatment of SH-SY5Y with the crude extract and fractions, a cell viability assay was performed, and drug potency was determined.

Table 3: Bioactive compounds of *Strophanthus hispidus*-chloroform fraction (SHCF**)**

S/N	**Bioactive Compounds**	**RT**	**Area %**
1	1,3-Dioxolane-2-pentanol	8.389	15.19
2	Acetamide	8.825	15.42
3	Hexadecanoic Acid	19.915	3.26
4	Cyclohexane	20.615	2.34
5	9,12-Octadecadienoic Acid	21.536	3.75
6	Trans-13-Octadecenoic Acid	21.585	4.6
7	11-Hexadecenoic Acid	24.391	3.7
8	4H-1,2,4-triazole-3	25.191	2.68
9	1,4-Bis(trimethylsilyl)benzene	25.951	2.8
10	2,5-Dimethyanisole	27.576	34.93
11	Carvacrol	27.748	6.24
12	2-Methyl-7-phenylindole	28.897	0.1
13	2-Ethylacridine	29.934	5

The EC_{50} for cells treated with *Strophanthus hispidus* -chloroform fraction (SHCF), -ethylacetate fraction (SHEF) and -hexane fraction (SHHF) was determined to be 48.4 µg/mL, 15.16µg/mL and 151 µg/mL respectively.

Table 4: Bioactive compounds of *Strophanthus hispidus*-ethylacetate fraction (SHEF)

S/N	Bioactive Compounds	RT	Area %
1	3-Mercaptohexyl butanoate	4.060	4.8
2	Ethylbenzene	5.233	6.55
3	p-xylene	5.393	13.03
4	Benzene, 1,3-dimethyl	5.868	7.17
5	Hexanoic acid, ethyl ester	7.962	17.22
6	Butyramide, 2,2,3,3-tetramethyl	8.391	4.88
7	3-Ethoxy-1,1,1,5,5,5-hexamethyl-3	15.224	5.17
8	Heptadecane	16.425	5.35
9	Carbonic acid, dodecyl vinyl ester	18.622	5.55
10	Phthalic acid, butyl isohexyl ester	20.328	9.94
11	D-Xylopyranose, 5-C-(acetyloxy)-2	20.597	5.21
12	Hentriacontane	20.619	5.69
13	7-Hexadecyn-1-ol	21.541	4.46
14	Sulfurous acid,2-propyl tetradecy	22.451	4.98

Table 5: Bioactive compounds of *Strophanthus hispidus*-hexane fraction (SHHF)

S/N	Bioactive Compounds	RT	Area %
1	3-Heptanol	6.251	1.37
2	2,5-Dimethyl-4-hydroxy-3-hexanone	6.572	1.28
3	Cyclopentanol, 1-methyl	7.384	2.78
4	aR-Turmerone	17.243	7.28
5	Curlone	17.661	2.99
6	Hexadecanoic acid, methyl ester	19.549	1.32
7	Hexadecanoic acid, methyl ester	19.910	17.93
8	Pentadecanoic acid	20.248	5.02
9	Didodecyl phthalate	20.322	1.81
10	9,15-Octadecadienoic acid, methyl	21.535	22.95
11	11-Octadecenoic acid, methyl ester	21.586	22.07
12	Methyl stearate	21.815	4.9
13	Linoelaidic acid	21.867	2.48
14	Pentafluoropropionic acid, tridecy	21.907	3.99
15	Z,E-2,13-Octadecadien-1-ol	22.142	1.84

Strophantus hispidus has been investigated for its therapeutic potential against several diseases. *Invivo* studies by Mensah *et al.,* (2019) yielded phytochemical interaction between methanol extract of *Strophanthus hispidus* (root) and *Aframomum meleguta* (seed) extracts to produce strong antiinflammatory activity. *S. hispidus* also possesses both *invivo* and *invitro* beneficial anti-diabetic potential, as revealed by Fageyinbo *et al.* (2019), evidenced by its significant inhibition of α-amylase and α-glucosidase enzymes on their research subject. The plant contains phenolic metabolites, such as condensed tannins (Gonzalez *et al.*, 1982), flavonoids (Leite *et al.*, 2001) and triterpenes (Shirota, 1994), which may account for its anti-inflammatory and antiulcerogenic (Ishola *et al*., 2013) potentials, anti-apoptotic (Ojo and Emoghwa, 2020) as well as its toxicological effects (Fageyinbo *et al.,* 2021). Despite all the extensive research and application of *S. hispidus*, little is known about its neuromodulatory potential and its capability to serve as a possible anti-Alzheimer's therapy. Thus, This research aims to investigate the phytochemical properties, bioactive compounds, and the efficacy of *S. hispidus* extract fractions on the Neuroblastoma (SH-SY5Y) cell line model of Alzheimer's disease.

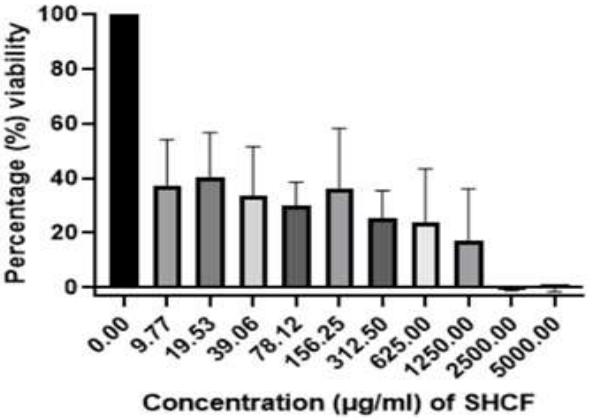

Fig 1: Viability of SH-SY5Y cells treated with *Strophanthus hispidus* chloroform fraction (SHCF)

Phytochemical analysis of the methanolic extracts of *S. hispidus* revealed the presence of Alkaloids, Saponin, reducing sugar, Cardiac glycosides, Terpenoid, Triterpenoids, Steroids, Phenolic Compounds, and Tannins, which was in tandem with results obtained by Ayoola *et al*. (2008) and Mensah *et al.* (2019).

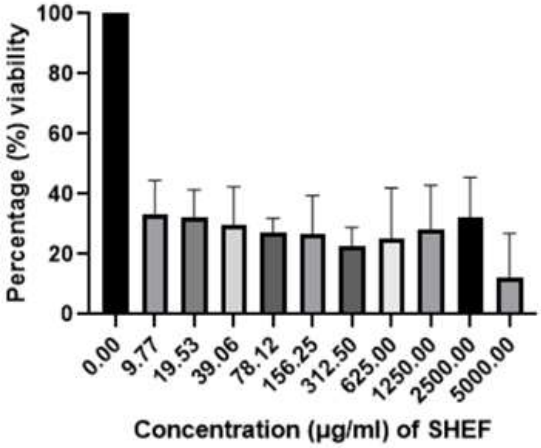

Fig 2: Viability of SH-SY5Y cells treated with *Strophanthus hispidus* ethylacetate fraction (SHEF)

Saponins are phytochemicals in several plants (Liu and Henkel, 2002). They are known to exert several pharmacological purposes, which include antioxidant, anti-neuroinflammatory and neuro-cognitive benefits (Sun *et al.*, 2015). The study by Abduljawad *et al.* (2022) concluded that Saponins have considerable efficacy against various pathological targets of neurological disorders. Based on their chemical structure, saponins are grouped into Triterpenoid saponin and Steroidal saponin (Ramos-Morales *et al*., 2017).

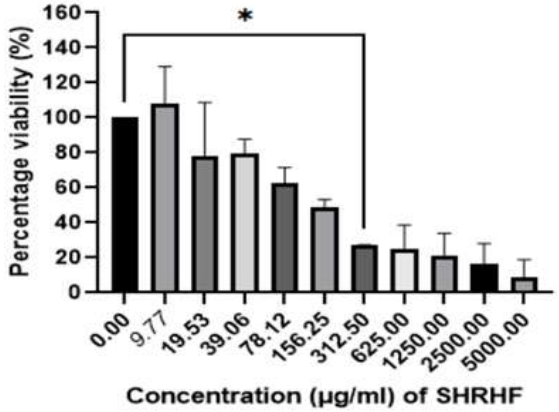

Fig 3: Viability of SH-SY5Y cells treated with *Strophanthus hispidus* hexane fraction (SHHF)

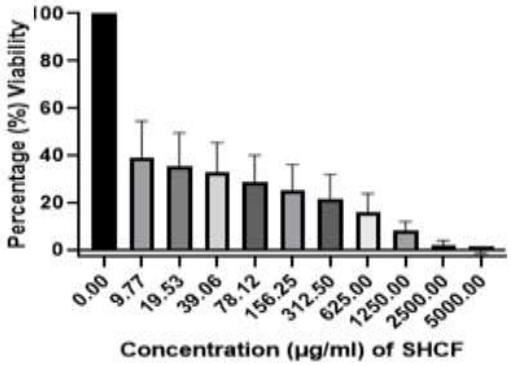

Fig. 4: Viability of VeroE6 cells treated with *Strophanthus hispidus* chloroform fraction (SHCF)

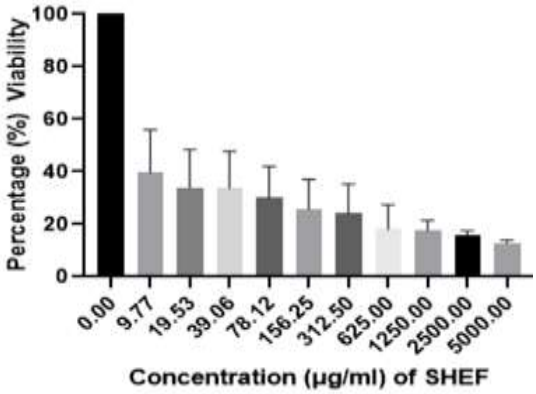

Fig 5: Viability of VeroE6 cells treated with *Strophanthus hispidus* ethylacetate fraction

GC-MS analysis of *S. hispidus* fractions showed various compounds, some previously reported for different therapeutic roles. The chloroform fraction analyzed via GC-MS identified thirteen compounds, as listed in Table 3, with 2,5-Dimethyanisole being the main compound at 34.93% and 2-methyl-7-phenylindole being the least compound at 0.10%. Carvacrol, one of the identified compounds, has been reported in a previous study by Sisti *et al. (*2021) to possess a promising neurotrophic effect by inducing neurite outgrowth. In addition,9,12-octadecadienoic acid (Z,Z) methyl ester is a potent antioxidant that helps prevent prostate cancer disease, Alzheimer's disease, and cardiovascular diseases (Yakubu *et al.*, 2018).

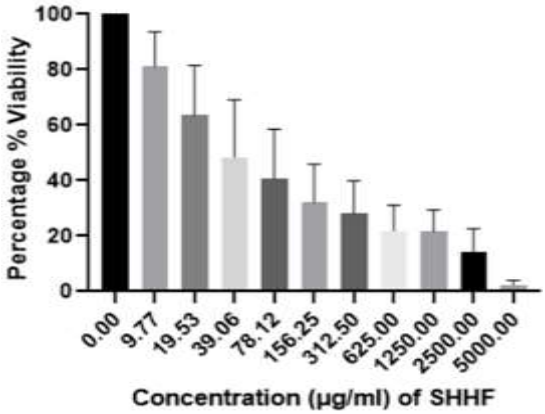

Fig 6: Viability of VeroE6 cells treated with *Strophanthus hispidus* hexane fraction (SHHF)

Table 6: Selectivity index of *Strophantus hispidus* Extracts and fractions

Extract Fractions	EC_{50}(µg/ml) SH-SY5Y	VERO E6	SI
SHCF	48.40	77.50	1.60
SHEF	15.16	116.60	7.69
SHHF	131.80	419.40	3.18

Hexadecanoic Acid has also been studied for its numerous biological effects, such as its antifungal, antioxidative, and antibacterial effects (Irulandi *et al*., 2016; Lan *et al*., 2017). Both Ethylacetate (Table 4) and Hexane (Table 5) fractions analyzed by GC-MS also showed the presence of 14 and 15 bioactive compounds, respectively. Hexadecenoic acids identified in Chloroform and Hexane fractions are found mostly in plants, animals, or micro-organisms as a form of saturated fatty acid. A study by Hucklenbroich *et al.* (2014) on the effect of ar-turmerone on neural stem cells (NSC) shows that this identified bioactive compound increased the number of NSC in both *invivo* and *invitro.* Thereby making it a potentially promising compound in neuro-regenerative pharmacology. The activity of *S. hispidus* extract fraction on the SH-SY5Y cell lines was

evaluated upon exposure to graded doses (9.77 – 5000 µg/ml) of *S. hispidus* fraction for 24 hours. The EC_{50} value, which is the approximate critical concentration at which the effect of the fractions is most effective, were determined at 48.4µg/ml, 15.16µg/ml, and 151µg/ml for the chloroform, ethylacetate, and hexane fractions of *S. hispidus*, respectively. Selectivity index (SI) is used in phytochemical research to determine a safe, non-toxic, yet bioactive extract to be used for further downstream assays. Ideally, a potential drug can exert its intended pharmacological effect on the diseased cells without being toxic to normal cells. Its effect on a non-diseased cell line is also determined to evaluate the extract's activity. Thus, the SI calculated using the ratio of the EC_{50} of the normal cell (VeroE6) to the EC_{50} of the diseased cell (SH-SY5Y) (Zwick, *et al*., 2016; Indrayanto *et al.*, 2021). An SI value greater than 3 indicates that the extract is safe and non-toxic (Masriani *et al.*, 2013).

In this study, both the hexane fraction and the ethylacetate fraction are considered safe and non-toxic having a selectivity index of 3.18 and 7.69, respectively. This makes both fractions ideal candidates to be used for further studies on their neuromodulatory potential on Alzheimer's disease.

Conclusion: This study suggests a potential in the Hexane fraction of *S. hispidus* to serve as an ideal candidate for use as a neuro-regenerative agent against AD. Therefore, further research is needed to delineate the mechanism or signaling pathway activation through which the fraction may reverse neurodegeneration.

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