Assessing the effects of Roots and Rhizomes Extracts of *Acorus Calamus* on SH-SY5Y Cell Survival Using MTT, SRB Assay- An *Invitro* Study

Kousalya Mariyappan[1], Muralidharan Palayyan [2*]

[1]Department of Pharmacology, The Tamil Nadu Dr. M.G.R. Medical University, Chennai, India, 600097. 0000-0002-9155-7677

2* Professor and Head, Department of Pharmacology, The Tamil Nadu Dr. M.G.R. Medical University, Chennai, India, 600097. 0000-0003-0495-236x

Corresponding Author e-mail: Clbmpharmacology@hotmail.com

Mobile no.: +919789087826

Abstract

Among many neurodevelopmental disorders, Attention Deficient Hyperactivity Disorder (ADHD) is recognized as the most commonly occurring disorder in childhood. Nature-derived compounds with neuroblastoma inhibitory effects shall improve the medical condition of ADHD patients with minimal adverse effects. The aim of the study is to evaluate the inhibitory effect of Acorus calamus extracts on the neuroblastoma SH-SY5Y cell line using MTT and SRB assays. The hexane and ethanol extracts of dried Acorus calamus roots and rhizomes were extracted using the Soxhlet, following the evaporation of the solvent. The neuroblastoma cell line (SH-SY5Y) was cultured and then MTT and SRB assay was performed using the hexane and ethanol extract. The in-vitro cell viability studies revealed that both hexane and ethanol extract exhibited a dose-dependent anti-neuroblastoma effect. The ethanol extract inhibited the SH-SY5Y cell line to a greater extent, with an IC_{50} value of 126.87 µg/mL (MTT) and 110.88 µg/mL (SRB).

Keywords: *Antipsychotic, Percentage viability, NAD(P)H-dependent cellular oxidoreductase, ANOVA, Neuroblastoma cell line.*

Introduction

Among 8% to 10% of all paediatric malignancies are extracranial solid tumors in children called neuroblastoma (NB). A wide range of clinical behaviours is displayed by this diverse disease in children. The SH-SY5Y cell line was used in this study [*Sharma V, 2020*]. The original clone was known as SH-SY, and it was then subcloned into SH-SY5 and then SH-SH5Y. As they can be differentiated into a more mature neuron-like phenotype with neuroblast-like morphology that is characterised by neuronal markers like tyrosine hydroxylase (TH) and D-H, characteristics of catecholaminergic neurons, the SH-SY5Y and SK-N-SH are widely used for the study of neuronal diseases. [Campos Cogo, 2020]

Acorus calamus, an antioxidant and anti-inflammatory herb is commonly used in traditional medical systems like Ayurveda, Unani, Chinese, and Siddha for the treatment of ailments such as nervous disorders, pain, cramps, diarrhoea, indigestion, bronchitis, and vascular disorders [Bae, 2019; Admin, 2021]. The roots and rhizomes of the *Acorus calamus* are rich in volatile oils including β-asarone and α-asarone, saponins, lectins, sesquiterpenoids, ligands, and steroids [Ascher, 1989; Radusiene, 2007]. Having α- asarone and β-asarone as predominantly promising active components, the *Acorus calamus* has various pharmacological activities such as tranquiliser, antispasmodic, antioxidant, cardiovascular, anticonvulsant, CNS Depressants, antidiabetic, immunosuppressive, hypolipidemic, cryoprotective, antioxidant, antidiarrheal, anticancer, and antimicrobial [Schmid, 1990; Lee, 2011].

In this study, an attempt was made to understand the neuroblastoma (*SH-SY5Y*) inhibitory effect of *Acorus calamus* extracts of roots and rhizomes via *in-vitro* studies. The physiological difference between the viable cell and the dying cell is the base of all cell viability assays. The viable cells have the potential to convert the substrate into a product that can be detected with a plate reader [Wubet, 2009]. Though MTT assay is commonly employed in various studies for the determination of cell viability, the confounding variable such as cell number, MTT concentration, incubation time, supernatant removal, and wavelength of optical density measurement affect the reduction of dye during *in-vitro* analysis and lead to inconsistencies [Berridge, 1993; Ghasemi, 2021]. Thus, in this study, the neuroblastoma inhibitory activity of the *Acorus calamus* was further confirmed using an SRB assay, which has a stable endpoint, superior linearity, and higher sensitivity [Kuete, 2017; *Schmidt, 2010*]. This is one of the methods to validate neurodevelopmental disorders like ADHD, Autism Spectrum Disorder, etc. Here, the study was done to prove the neuroprotective effect of the plant *Acorus calamus* in the neurodevelopmental disorder Attention Deficit Hyperactive Disorder, with a prevalence of roughly 4-12% in children.

Materials and Method

The roots and rhizomes of the *Acorus calamus* were collected from the Coimbatore district and authenticated (GSMC/MB-442/21) by Botanist, at the Government Siddha Medical College, Arumbakkam. The extractives from the dried roots and rhizomes of the *Acorus calamus* were obtained using the Soxhlet apparatus. *In-vitro* evaluation of the neuroblastoma inhibitory effect of the *Acorus calamus* was conducted by obtaining the neuroblastoma (SH-SY5Y) cell line stock culture from "The National Centre for Cell Science" – Pune, that was used in the investigation. Fetal Bovine Serum (FBS, Himedia), Streptomycin (Pen-strep,

Himedia), Trypsin (Himedia), MTT (Thermo Fisher Scientific), and Sulforhodamine B were the materials utilised in the investigation (SRB, Sigma Aldrich). The other chemicals and solvents such as Dimethyl Sulfoxide, ethanol, Hexane, and trichloroacetic acid used in the study were procured from Sisco Research Laboratories, India.

Extraction of Bioactive compounds

The authenticated roots and rhizomes of the *Acorus calamus* were chopped into fine pieces and shade dried. The dried material was milled into a fine powder. The dry powdered material was extracted with solvents of increasing order of polarity using Soxhlet assembly [Autor, 2022]. Herein, 500 g of dry powdered roots and rhizomes of *Acorus calamus* was extracted initially with a non-polar solvent n-Hexane through continuous percolation until the discoloration of solvent, using Soxhlet apparatus. The n-hexane soluble extractives were concentrated through the evaporation of solvent to obtain a dry mass. Further, the powder for extraction was collected and dried to remove the n-hexane residual solvent. The extraction process was repeated with polar solvent ethanol (95%) using a Soxhlet assembly, wherein the powder for extraction was closely packed. The extracts obtained was dried at 45°C in a hot air oven A dark red color residue of hexane and ethanol extractive was obtained.

Neuroblastoma Cell culture

Neuroblastoma (SH-SY5Y) cells were cultured in a mixture containing 10% inactivated FBS, 100 IU/mL penicillin, and 100 µg/mL streptomycin until confluence was reached.

***Screening of* Cytotoxicity activity**

Using appropriate media containing 10% FBS, the monolayer cell culture was collected by trypsinization (0.03% w/v EDTA solution and 0.25% w/v trypsin). A 100 µL portion of the diluted cell suspension (1 x 10^4 cells/well) was put into each well of the 96-well microtiter plate. After 24 hours, a partial monolayer of cells was created, and the supernatant was flicked off and rinsed with medium [Lee, 2018; Kamiloglu, 2020]. 100 µL of test samples (hexane and ethanol extract) at concentrations (3.125 µg/mL, 6.25 µg/mL, 12.5 µg/mL, 25 µg/mL, 50 µg/mL, and 100 µg/mL) were applied to the partial monolayer on microtiter plates and incubated at 37 °C for 24 h in a 5% CO_2 environment. Following treatment, the medium was washed in phosphate buffer to remove it. MTT and SRB assays were used to measure cell viability [*Shipley MM, 2016*].

MTT Assay:

The test media was washed with phosphate buffer as a post-treatment to detach it for the MTT assay. Each well treated with the test solutions received 20 µL of MTT (2 mg/1 mL of MTT in PBS), which was then added and incubated for 4 hours at 37ºC with 5% CO_2. MTT was carefully removed after the incubation period using 100µL of DMSO. The amount of formazan formation in each well with the help of a microplate reader at 570 nm gives the viable cell amount. The following formula was used to determine the percentage of viable cells [McKim, 2010; Balakrishnan,2022].

$$Cell\ Viability\ (\%) = \frac{Sample\ Absorbance}{Control\ Absorbance} \times 100 \qquad Eq\ 1$$

Mean ($\bar{x}$), ± standard deviation (SD) were used to express the data.

SRB Assay:

In order to prepare the cells for the SRB test, they were post-treated with ice-cold trichloroacetic acid (100 µL/well, 10% w/v) for an hour at 4°C. Each test-treated well fixed with ice-cold trichloroacetic acid received 100 µL of SRB solution (0.057% w/v in 1% aqueous acetic acid) and was then left at room temperature for 30 minutes. To get rid of the unbound SRB solution, the plates were washed five times with 1% v/v acetic acid. After that, they were dried. 200 µL of 10 mM Tris Base (pH 10.5) was then added to each well, and the bound SRB was solubilized using a vortex shaker for 5 minutes. With the use of a microplate reader, the absorbance at 570 nm was read to determine cell viability [Chellian, 2017; Omer, 2022].

Equation 2 was used to calculate the percentage of viable cells.

$$Cell\ Viability\ (\%) = \frac{Sample\ Absorbance}{Control\ Absorbance} \times 100 \qquad Eq\ 2$$

Mean ($\bar{x}$), ± standard deviation (SD) were used to express the data.

Statistical analysis

The Data are displayed and reported as a percentage of the corresponding controls. Student T-test, One way -ANOVA analysis, and Regression analysis were employed to assess the impacts. The inhibitory potential of the extracts was determined using the IC_{50} value. Regression R^2 value, standard error, probability, and mean were calculated.

Results and Discussion

Acorus calamus was said to have anti-tumorigenic properties since it contains bioactive substances including α-asarone and β-asarone [Heliyon, 2019]. The bioactive compounds from roots and rhizomes of the *Acorus calamus* were extracted with solvents of increasing order of polarity. The inhibitory effects of extracts of hexane and ethanol were studied in the neuroblastoma (SH-SY5Y) cell line using the MTT and SRB assay. According to number of studies, cell viability was a crucial endpoint in the assessment of the toxicity and safety of pharmacological molecules. Also, cell viability is considered a marker for quantifying the effectiveness of antineoplastic or antipsychotic agents. [McKim, 2010]

By indirectly measuring the metabolic activity of cells, the MTT assay was used to detect the vitality of neuroblastoma cells. The effect of various concentrations of hexane extract on *SH-SY5Y* cell survival was shown in figure 1. It was clearly evident from figure 1, that *SH-SY5Y* cell inhibitory activity was dose-dependent, and a significant change in the cell morphology was observed with 100µg/mL of hexane extract. Also, the optical density recorded for the wells treated with hexane extract was found to be decreased with increased concentration of extract, confirming their significant neuroblastoma activity.

Further, figure 2 shows the inhibitory effect of various concentrations of ethanol extract on *SH-SY5Y* cells. A significant change in the cell morphology was observed with an increased dosage of ethanolic extract, indicating its dose-dependent inhibitory activity on neuroblastoma cells. Also, the optical density curve confirmed the significant neuroblastoma activity of the ethanolic extract.

Table 1: **Analysis of MTT assay using Hexane and Ethanol extracts**

SAMPLE	CON.(µg/ml)	Mean OD-Blank OD	Standard deviation	%Cell Viability
Hexane extract	3.125	0.922	0.0032	98.9624
	6.25	0.914	0.0045	98.1037
	12.5	0.8796	0.005	94.4186
	25	0.8383	0.0025	89.9821
	50	0.8143	0.0030	87.4060
	100	0.7716	0.0036	73.5241
Ethanol extract	3.125	0.9143	0.0045	98.1395
	6.25	0.899	0.0035	96.4937
	12.5	0.862	0.0045	92.5223
	25	0.7423	0.0035	79.6779
	50	0.666	0.0040	71.4847
	100	0.5946	0.0045	63.8282
Control	0	0.9316	0.004	100

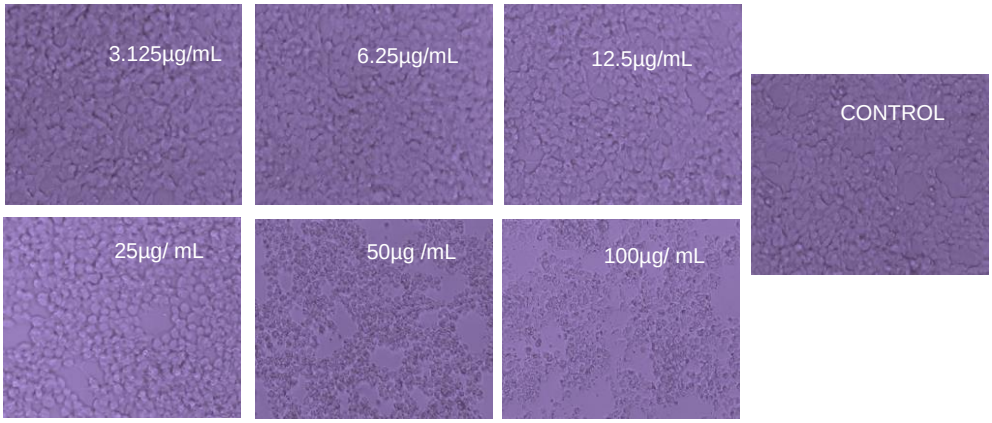

Figure 1: shows the microscopical representation of neuroblastomal cell inhibition by hexane extract of *Acorus calamus* at various concentrations.

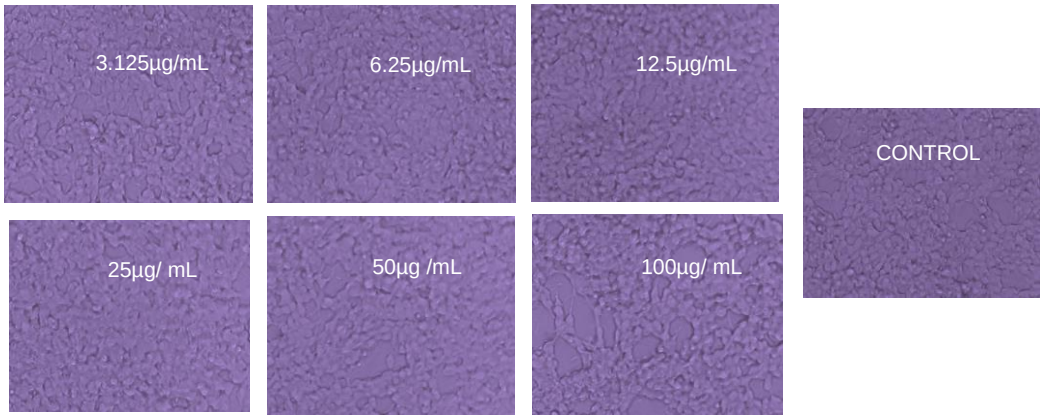

Figure 2: **Inhibitory effect of ethanolic extract of *Acorus calamus* at various concentration on Neuroblastomal cell line**

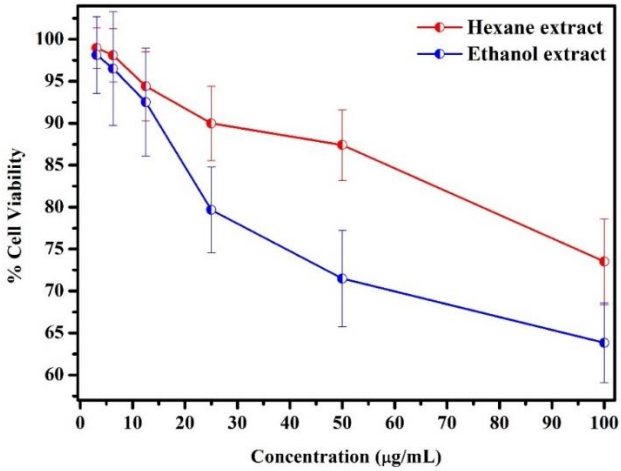

Figure 3 depicts the comparison of cell viability of hexane and ethanol extract-treated Neuroblastoma *SH-SY5Y* cells. The treatment of 100µg/mL of hexane and ethanol extract showed a cell viability of about 73.52% and 63.83% respectively.

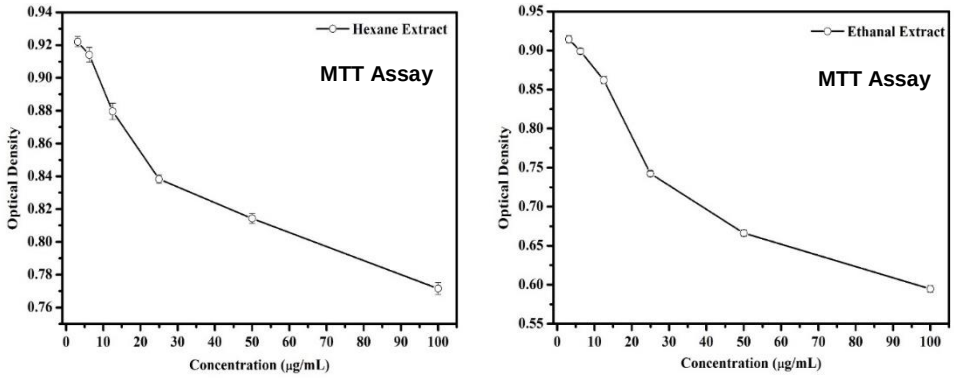

Figure 4 shows the data of Concentration and Optical Density curve of Hexane and Ethanolic extract of MTT Assay.

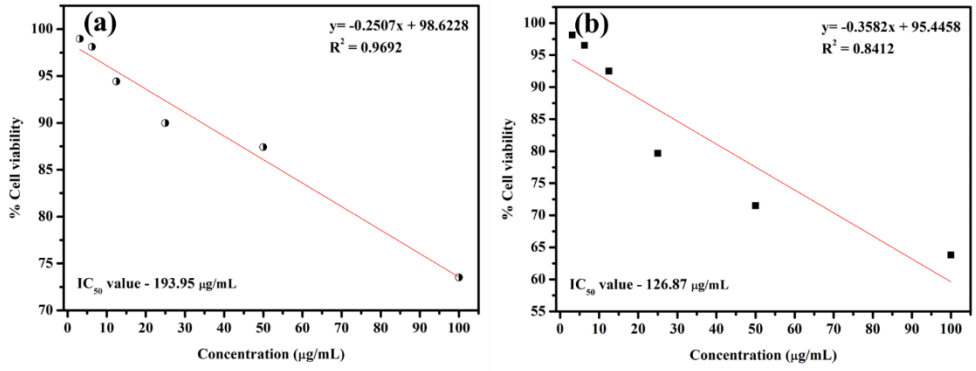

Figure 5: IC_{50} value of a) Hexane Extract, b) Ethanol extract using MTT assay

Further, linear regression analysis (Figures 5a and 5b) was performed for the plot to identify the IC_{50} value of the hexane and ethanol extracts. The IC_{50} value of the hexane and ethanol extracts were found to be 193.95 µg/mL and 126.87 µg/mL respectively. The values indicate that the neuroblastoma inhibitory activity of ethanol extract possessed higher activity than hexane extract. This higher activity could be mainly due to the presence of both α-asarone and β-asarone in ethanol extract in higher concentrations than the hexane extract [Balakrishnan, 2022; Chellian, 2017]. Further, this can also be concluded based on the higher solubility of β-asarone in ethanol than in non-polar solvents [Omer, 2022].

In addition to the MTT assay, an SRB assay was performed for the hexane and ethanol extracts to confirm the neuroblastoma inhibitory activity of the *Acorus calamus*. Sulforhodamine B's capacity to bind to fixed cellular protein components on culture plates is the basis for the SRB assay. Figure 6 clearly indicates a significant dose-dependent inhibitory activity of hexane extract and, was confirmed by the optical density measurements. Further, figure 7 confirms the substantial neuroblastoma activity of various concentrations of ethanol extract on *SH-SY5Y* cells. A comparison chart of cell viability of hexane and ethanol extract treated Neuroblastoma *SH-SY5Y* cells was shown in figure 10. The treatment of 100µg/mL of hexane and ethanol extract showed a cell viability of about 83.16% and 59.79% respectively. Further, The IC_{50} value of hexane and ethanol extracts calculated using linear regression (figures 10a and 10b) analysis were found to be 303.91 µg/mL and 110.88 µg/mL respectively.

The neuroblastoma inhibitory activity of ethanol extract was found to be higher than the hexane extract, due to the presence of both bioactive α-asarone and β-asarone. Overall, The MTT and SRB assay results indicate that extracts of the *Acorus calamus* significantly reduced the viability of the neuroblastoma *SH-SY5Y* cell line.

Table 2: **Analysis of SRB assay using Hexane and Ethanol extracts**

SAMPLE	CON.(µg/ml)	Mean OD - Blank OD	Standard deviation	%Cell Viability
Hexane extract	3.125	0.496	0.0023	99.0679
	6.25	0.491	0.0035	98.0692
	12.5	0.4703	0.0041	93.9414
	25	0.4516	0.003	90.2130
	50	0.439	0.0045	87.6830
	100	0.4163	0.0040	83.1557
Ethanol extract	3.125	0.488	0.0035	97.4700
	6.25	0.4706	0.004	94.0079
	12.5	0.4383	0.0032	87.5499
	25	0.3766	0.003	75.2330
	50	0.325	0.0030	64.9134
	100	0.2993	0.0030	59.7869
Control	0	0.5006	0.0036	100

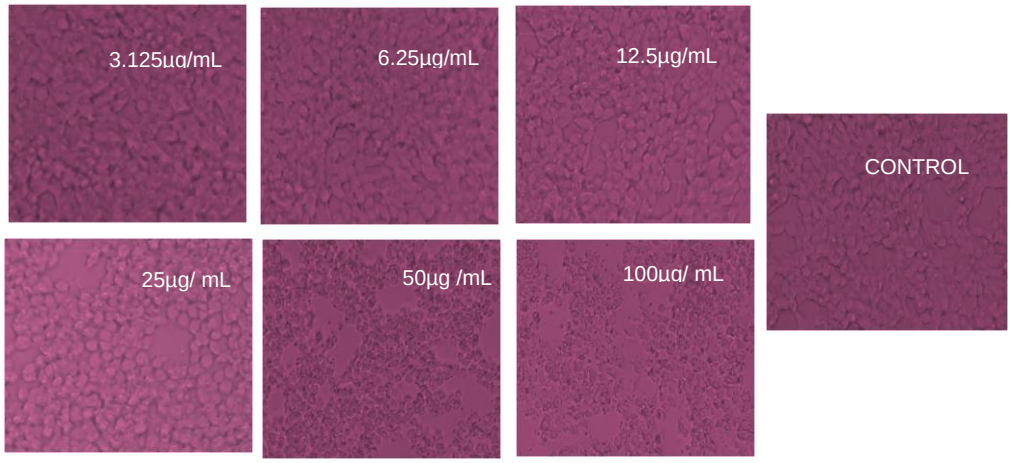

Figure 6: **Effect of *Acorus calamus* hexane extracts on *SH-SY5Y* cell line at different concentrations using SRB assay**

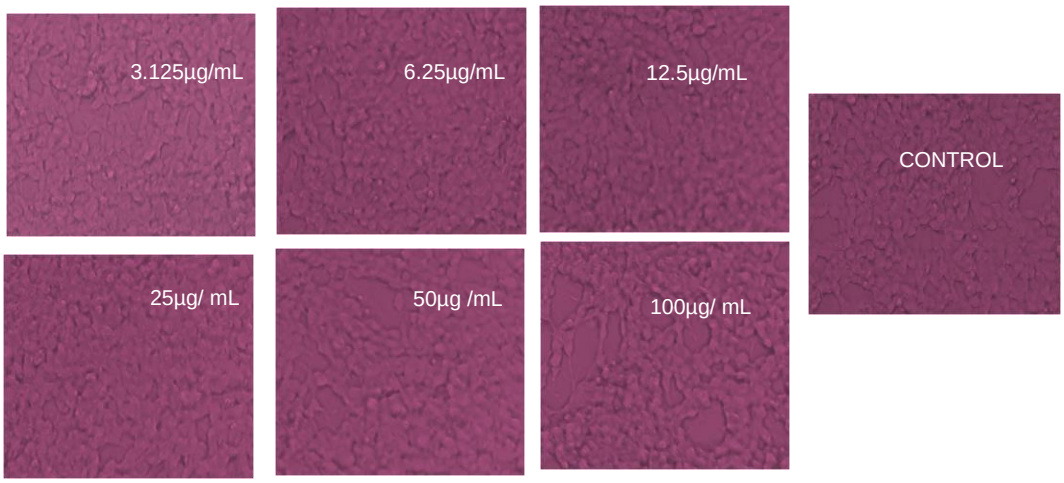

Figure 7: Effect of *Acorus calamus* Ethanol extracts on *SH-SY5Y* cell line at different concentrations using SRB assay

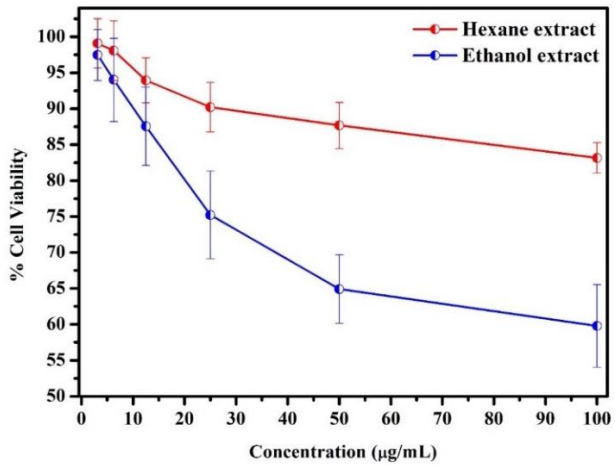

Figure 8: Comparison of cell viability between Hexane extract and Ethanol extract (SRB)

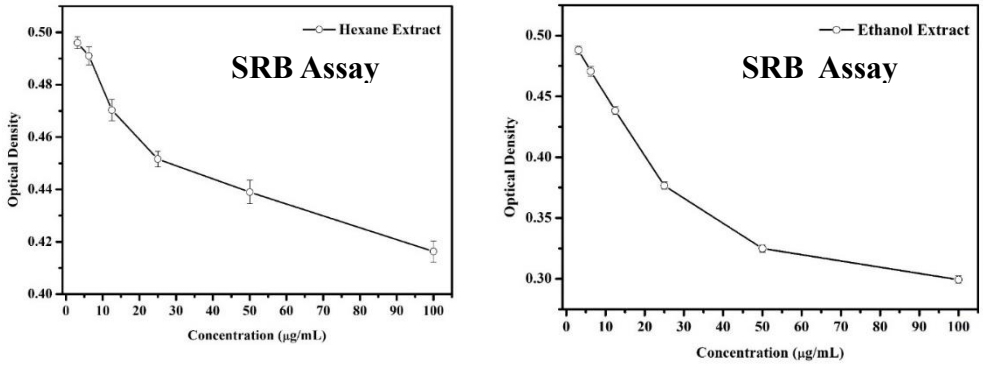

Figure 9: reveals the Concentration – Response (Optical Density) of Hexane and Ethanolic extract of *Acorus calamus* in inhibiting the Neuroblastomal cell line.

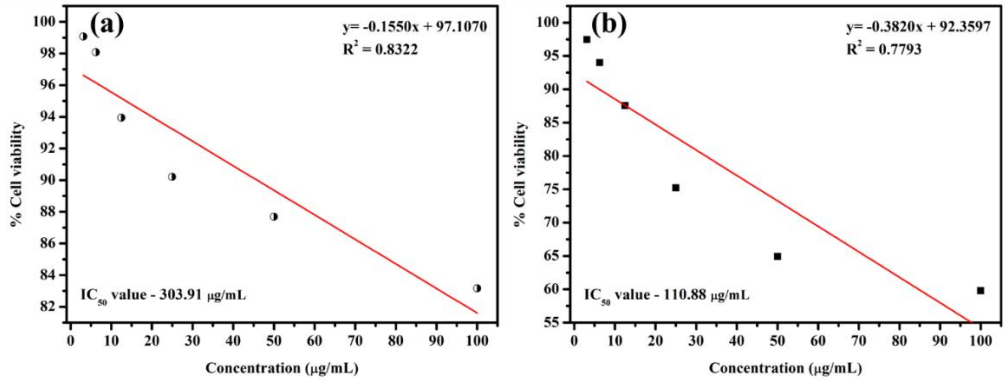

Figure 10: IC_{50} value of a) Hexane Extract, b) Ethanol extract using SRB assay

Conclusion

Acorus calamus, an antipsychotic drug being used for the treatment of ADHD, Bipolar disorder, etc. Though the plant is having many pharmacological activities such as gastrointestinal, respiratory, metabolic, kidney, and liver dsorders, neurological benefits overcome the other benefits of it. *Acorus calamus* has been tested intensively to understand its effects on the SH-SY5Y cell line. The study reported that hexane and ethanolic extracts of *Acorus calamus* possess an excellent neuroblastomal cell inhibitory effect. Further, a dose-dependent effect on cell viability was observed for both hexane and ethanol extract. In both MTT and SRB assay, it was observed that ethanol extract showed significantly higher activity than hexane extract. Based on MTT and SRB assay, the IC_{50} value of ethanol extract was found to be 126.87 µg/mL and 110.88 µg/mL respectively. Preclinical studies are also needed to confirm the pharmacokinetic and pharmacodynamic properties of *Acorus calamus'* ethanolic extract against ADHD. To establish the pharmacological action and toxicity of the *Acorus calamus,* dose-dependent *in-vivo* investigations are needed in addition to the ethanol extracts' *in-vitro* neuroblastoma inhibitory effect. From this study, we arrived that the possible mechanism of action may be due to a few mechanisms like neutralizing the free radical formation in the brain (which seems to be neuroprotective) or reducing inflammatory mediators

like cytokines and interleukins. Also, the proven antipsychotic activity [*Menon MK, 1967*] of this plant shows that the plant has effective in modulating the neurotransmitter level in brain. The current analysis is anticipated to have widespread knowledge and may offer guidance to researchers studying immunopharmacology and neuropharmacology. In future, this concept may be carried forward to treat various neurological disorders like Parkinson's disease, ADHD, OCD, anxiety, intracranial tumor etc. Our future aim is to extract the chemical components from this plant and prove the same neuroprotective action by *in-vivo* studies.

Abbreviations

ADHD – Attention Deficit Hyperactive Disorder
FBS - Fetal Bovine Serum
MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
SRB - Sulforhodamine B
PBS - Phosphate Buffer Saline
OCD- Obsessive-compulsive disorder

Acknowledgments

- My sincere thanks to the management of the Institution, C.L.Baid Metha College of Pharmacy managed and owned by Tamil Nadu Pharmacist Welfare Association Trust for the facilities availed to carry out the research.
- Also, I would like to express my deepest gratitude to "The National Centre for Cell Science" from where the cell line was purchased to carry the research.
- Thanks to Green Med laboratories for availing the facilities to carry out the research.
- My sincere thanks to the laboratories, Thermo Fisher Scientific laboratory and Sigma Aldrich for providing chemicals for this research.

References

1. *Admin. La Liste Rouge de la Flore Vasculaire de la Réunion, "UICN France", UICN France.* ***2021***.
2. *Ascher KR, Schmidt GH, Neumann WP," Vapor pressure and volatility of β-asarone, the main ingredient of an indigenous stored-product insecticide, Acorus calamus oil", Phytoparasitica. vol. 17, no. 4,* ***(1989)****, pp. 299-313.*
3. *Autor, E.; Cornejo, A.; Bimbela, F.; Maisterra, M.; Gandía, L.M.; Martínez-Merino, V, "Extraction of Phenolic Compounds from Populus Salicaceae Bark", Biomolecules. vol. 12,* ***(2022),*** *pp. 539.*
4. *Balakrishnan R, Cho DY, Kim IS, Seol SH, Choi DK, "Molecular Mechanisms and Therapeutic Potential of α- and β-Asarone in the Treatment of Neurological Disorders", Antioxidants (Basel). vol. 11, no.2,* ***(2022)****, pp. 281.*
5. *Berridge MV, Tan AS, "Characterization of the cellular reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT): subcellular localization, substrate dependence,*

and involvement of mitochondrial electron transport in MTT reduction", Archives of biochemistry and biophysics. vol. 303, no. 2, ***(1993)****, pp. 474-82.*

6. *Campos Cogo, S., Gradowski Farias da Costa do Nascimento, T., de Almeida Brehm Pinhatti, F., de Franca Junior, N., Santos Rodrigues, B., Cavalli, L.R. and Elifio-Esposito, S., "An overview of neuroblastoma cell lineage phenotypes and in vitro models", Experimental Biology and Medicine. vol. 245, no. 18,* ***(2020)****, pp.1637-1647.*
7. *Castellanos FX, Lee PP, Sharp W, Jeffries NO, Greenstein DK, Clasen LS, Blumenthal JD, James RS, Ebens CL, Walter JM, Zijdenbos A, "Developmental trajectories of brain volume abnormalities in children and adolescents with attention-deficit/hyperactivity disorder" Jama. vol. 9, no.14,* ***(2002)****, pp. 1740-8.*
8. *Chellian, R., Pandy, V., & Mohamed, Z, "Pharmacology and toxicology of α- and β-Asarone: A review of preclinical evidence", Phytomedicine. vol. 32,* ***(2017)****, pp. 41–58.*
9. *Menon MK, Dandiya PC, "The mechanism of the tranquillizing action of asarone from Acorus calamus Linn",. Journal of Pharmacy and Pharmacology. vol. 19, no. 3,* ***(1967),*** ***pp.*** *170-175.*
10. *Ghasemi, M., Turnbull, T., Sebastian, S., and Kempson, I. "The MTT Assay: utility, limitations, pitfalls, and interpretation in bulk and single-cell analysis", International Journal of Molecular Sciences. vol. 22, no. 23,* ***(2021),*** *pp. 12827.*
11. *Kamiloglu, S., Sari, G., Ozdal, T., Capanoglu, E., "Guidelines for cell viability assays", Food Frontiers. vol. 1, no. 3,* ***(2020)****, pp. 332-349.*
12. *Kuete, V., Karaosmanoğlu, O., & Sivas, H., "Anticancer Activities of African Medicinal Spices and Vegetables", Medicinal Spices and Vegetables from Africa.* ***(2017)****, pp. 271–297.*
13. *Lee H, Park H, Noh GJ, and Lee ES., "pH-responsive hyaluronate-anchored extracellular vesicles to promote tumor-targeted drug delivery", Carbohydr. Polym. vol. 202,* ***(2018)****, pp.323-33.*
14. *Lee MH, Chen YY, Tsai JW, Wang SC, Watanabe T, Tsai YC., "Inhibitory effect of β-asarone, a component of Acorus calamus essential oil, on inhibition of adipogenesis in 3T3-L1 cells", Food chemistry. vol. 1, no. 126,* ***(2011)****, pp.1-7.*
15. *McKim JM Jr, "Building a tiered approach to in vitro predictive toxicity screening: a focus on assays with in vivo relevance", Comb Chem High Throughput Screen. vol. 13, no. 2,* ***(2010)****, pp. 188-206.*
16. *Moffitt TE, Houts R, Asherson P, Belsky DW, Corcoran DL, Hammerle M, Harrington H, Hogan S, Meier MH, Polanczyk GV, Poulton R, "Is adult ADHD a childhood-onset neurodevelopmental disorder? Evidence from a four-decade longitudinal cohort study", American Journal of Psychiatry. vol. 172, no. 10,* ***(2015)****, pp. 967-77.*
17. *Omer, N.; Choo, Y.-M.; Ashokkumar, M.; Yusof, N.S.M, "Ultrasound-Assisted Extraction of β-Asarone from Sweet Flag (Acorus calamus) Rhizome", Appl. Sci. vol. 12,* ***(2022)****, pp. 11007.*
18. *Radusiene J, Judzentiene A, Peciulyte D, Janulis V, "Essential oil composition and antimicrobial assay of Acorus calamus leaves from different wild population", Plant Genetic Resources, vol. 5, no. 1,* ***(2007)****, pp. 37-44.*
19. *Schmidt, A. J., Krieg, J.-C., Hemmeter, U. M., Kircher, T., Schulz, E., Clement, H.-W., & Heiser, P, "Impact of plant extracts tested in attention-deficit/hyperactivity disorder treatment on cell survival and energy metabolism in human neuroblastoma SH-SY5Y cells", Phytotherapy Research.; vol. 24, no.10,* ***(2010),*** *pp. 1549–1553.*

20. *Schmid R, "Floristic Inventory of Tropical Countries: The Status of Plant Systematics, Collections and Vegetation", Plus Recommendations for the Future. vol. 39, no. 3, **(1990)**, pp. 464.*
21. *Sharma V, Sharma R, Gautam DS, Kuca K, Nepovimova E, Martins N, "Role of Vacha (Acorus calamus Linn.) in neurological and metabolic disorders: evidence from ethnopharmacology, phytochemistry, pharmacology and clinical study", Journal of clinical medicine. vol. 9 no. 4, **(2020)**, pp. 1176.*
22. *Shipley MM, Mangold CA, Szpara ML, "Differentiation of the SH-SY5Y human neuroblastoma cell line", JoVE (Journal of Visualized Experiments). vol. 17, no. 10, **(2016),** e 53193.*
23. *Wu HS, Zhu DF, Zhou CX, Feng CR, Lou YJ, Yang B, He QJ, "Insulin sensitizing activity of ethyl acetate fraction of Acorus calamus L. in-vitro and in-vivo", Journal of Ethnopharmacology, vol. 123, no. 2, **(2009)**, pp. 288-92.*
24. *Xie HR, Hu LS, Li GY, "SH-SY5Y human neuroblastoma cell line: in vitro cell model of dopaminergic neurons in Parkinson's disease", Chinese medical journal. Vol. 123, no. 08, **(2010)**, pp. 1086-92.*