Research Paper

In Vitro Cytotoxic activity of Rhinacanthin Enriched Extract from Leaves of *Rhinacanthus nasutus* (L.) Kurz., (Acanthaceae) against Neuroblastoma Cell Line

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Priyadarsini *et al.*: Effect of Rhinacanthin Enriched Extract against Neuroblastoma

In the present study, the shade dried powdered leaves of *Rhinacanthus nasutus* (L.) Kurz., was subjected to maceration with ethyl acetate, concentrated and evaporated to dryness. The ethyl acetate extract was subjected to anion exchange column chromatography and the isolated rhinacanthin enriched extract was analyzed by thin layer chromatography and fourier-transform infrared studies. In the present study, the *in vitro* cytotoxic activity of the rhinacanthin rich extract of *Rhinacanthus nasutus* (L) Kurz., over SH-SY5Y human neuroblastoma cell line was assessed by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide assay and compared with the standard drug doxorubicin. The preliminary phytochemical screening revealed the presence of alkaloids, quinones, glycosides, carbohydrates, amino acids and tannins. The thin layer chromatography profiling showed the presence of secondary metabolites in various mobile phase systems. Fourier-Transform Infrared analysis of rhinacanthin enriched extract confirmed the presence of functional groups including 1, 4-quinone carbonyl component. The results of *in vitro* cell line studies revealed a dose dependent cytotoxic effect on human neuroblastoma cell lines with an IC_{50} value of 88.9 µg/ml. Thus the results validate the potential of rhinacanthin enriched extract against neuroblastoma cancer and based on this initial screening, further studies at elucidation of its molecular mechanism as cancer therapeutics can be undertaken.

Key words: Anion exchange chromatography, fourier-transform infrared analysis, naphthoquinones, neuroblastoma

Neuroblastoma stands as the common malignant tumor in infants with tumor invasion and metastasis being the two major causes[1,2]. It is found to be derived from the primitive sympathetic neural precursor cells of peripheral nervous system[3]. Most neuroblastoma develop in adrenal medulla but also a few from Para spinal sympathetic ganglia of neck, chest, abdomen or pelvis[3,4]. Early detection and diagnosis followed by drugs targeting at molecular level play a major role in the survival and quality of life of patients with neuroblastoma[5].

Rhinacanthus nasutus (*R. nasutus*) (L.) Kurz, commonly known as 'Nagamalli' in Tamil holds a promising and proven traditional claims which was found due to the presence of varied active metabolites including naphthoquinones, flavonoids, coumarins and phytosterols[6,7]. The plant leaves has been traditionally used in the treatment of cancer[6]. Rhinacanthins were the naphthoquinones reported in *R. nasutus* (L.) Kurz with potential cytotoxic activity[8,9]. Studies report the neuroprotective role of *R. nasutus* (L.) Kurz which was attributed to the antioxidant activity of the plant extracts[10,11]. Hence the present study was aimed at investigating the antitumor effect of rhinacanthin enriched extract from the leaves of *R. nasutus* (L.) Kurz., in SH-SY5Y neuroblastoma cell lines.

Leaves of *R. nasutus* (L.) Kurz were collected from Chengalpattu District, Tamil Nadu in a fine dry weather during October 2019. The plant was identified and authenticated by Plant Anatomy Research Centre, Chennai, No. PARC/2020/4280. Amberlite IRA-67 and Doxorubicin HCl used in this study were purchased from Sigma-Aldrich. All the chemicals and

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reagents were purchased from certified suppliers and were of highest analytical grade. SH-SY5Y human neuroblastoma cell lines were obtained from National Centre for Cell Sciences, Pune, India (NCCS). The cells were maintained in Dulbecco Modified Eagle Medium (DMEM) supplemented with 10 % inactivated Fetal Bovine Serum (FBS), penicillin (100 U/ml) and streptomycin (100 µg/ml) in a humidified atmosphere of 5 % Carbon dioxide (CO_2) at 37°.

The plant material was dried in a hot air oven at 50° and were coarsely powdered and stored in an air tight container for further studies[12]. Extraction was performed by cold maceration of coarsely powdered leaves of *R. nasutus* (L.) Kurz with the solvent ethyl acetate. The extract obtained was then concentrated by distilling the solvent and evaporated to dryness. The percentage yield of the ethyl acetate extract was found to be 3.78 % w/w (Table 1).

Fractionation of ethyl acetate extract was carried out by anion resin exchange chromatography using the method followed by Panichayupakaranant *et al.*[12] with some modifications. The resin Amber lite IRA-67 (100 gm) was treated with 50 ml methanol, gently stirred and allowed to settle for 15 min. The mixture was then decanted and the treated resin was washed twice with distilled water. The slurry was allowed to stand in methanol for further 10 min. A glass column (5×35 cm) was packed with the treated resin slurry and the excess methanol was drained followed by subsequent addition of fresh solvent. The ethyl acetate extract was treated with methanol and filtered. This is loaded on the top of the resin and the column was allowed to run at a flow rate of 1.5 ml/min. After the green pigments if any, were eluted, the 10 % acetic acid in methanol was added to the column at a flow rate 2 ml/min[12]. These fractions were collected and subjected to 'sulphuric acid test' for quinones[13] followed by Thin Layer Chromatography (TLC) using Methanol:Acetic acid (9:1) solvent system. The fractions with positive response for quinones giving a red color with concentrated sulphuric acid were pooled together, evaporated to dryness which yielded 0.6 % w/w of rhinacanthin enriched extract and was subjected to further studies (Tables 1 and 2).

Qualitative preliminary phytochemical analysis of the leaf powder, ethyl acetate extract and rhinacanthin enriched extract was carried out with various chemical detecting agents and their chemical nature was recorded[13,14]. TLC profiling of ethyl acetate extract of *R. nasutus* (L.) Kurz and rhinacanthin enriched extract was carried out on TLC plates (20×20) precoated with silica gel G. TLC was analyzed with various solvent systems in the literature at different ratios by trial and error method. The plates were visualized both under normal and Ultraviolet (UV) lamp and the spots were observed and Retardation Factor (Rf) values were calculated[14].

Fourier-Transform Infrared (FT-IR) spectroscopy was carried out using FT-IR spectrometer (Version 7.0 Bruker Optic). The rhinacanthin enriched extract obtained by column fractionation was mixed with 200 mg Potassium bromide (KBr), pressed into a pellet and FT-IR spectra were recorded.

TABLE 1: THE PERCENTAGE YIELD OF ETHYL ACETATE EXTRACT AND RHINACANTHIN ENRICHED EXTRACT OF LEAVES OF *R. nasutus*

Extract	Method of extraction/ isolation	Physical nature	Colour	Yield
Ethyl acetate extract	Cold maceration	Sticky	Greenish black	3.78 % w/w
Rhinacanthin enriched extract	Anion resin exchange column chromatography	Semisolid mass	Reddish brown	0.6 % w/w

TABLE 2: ISOLATION OF RHINACANTHIN RICH EXTRACT BY ANION EXCHANGE COLUMN CHROMATOGRAPHY

Eluent	Fraction	Appearance	Phytoconstituents
Methanol	1	Colourless	Negative for quinones
	2-11	Slight green	Negative for quinones
10 % acetic acid in methanol	12-17 (F1)	Yellow sticky mass	Positive for quinones
	18-22 (F2)	Brown semisolid mass	Positive for quinones
	23-37 (F3)	Bright red semisolid mass	Positive for quinones

Cytotoxicity studies of rhinacanthin enriched extract on neuroblastoma cell lines were carried out using 3-(4,5-Dimethyl-2-Thiazolyl)-2,5-Diphenyl-Tetrazolium Bromide (MTT) assay. The assay is based on the ability of the metabolically active cells to convert yellow tetrazolium salt MTT to purple formazan crystals. Cells (1×10^5/100 µl) were seeded in a 96-well flat bottomed plate and incubated with various concentrations of rhinacanthin enriched extract of *R. nasutus* (L.) Kurz. After 24 h, the sample was treated with phosphate-buffered saline (pH 7.4). Then 100 µl/well (5 mg/ml) of 0.5 % MTT was added to each well and incubated for 4 h at 37°. The formazan crystals were then solubilized by adding 100 µl of Dimethyl Sulfoxide (DMSO). UV-Spectrophotometer was used to measure the absorbance at 570 nm with DMSO as control blank and the percentage cell viability was calculated. Graphs were plotted using the percentage cell viability at Y-axis and concentration of the sample in X-axis and the concentration required for a 50 % Inhibition (IC_{50}) was determined graphically[15-17].

All data were presented as the mean±Standard Deviations (SD). The experiment was repeated at least in triplicates. Unpaired t-test was included to compare the difference between the groups using Graph pad prism. Any value of $p<0.01$ was considered as statistically significant. The results of the qualitative phytochemical screening revealed that the powdered plant material showed the presence of carbohydrates, amino acids, steroids, glycosides, tannins and quinones. Carbohydrates, amino acids, quinones were present in the ethyl acetate extract and with quinones and carbohydrates in the rhinacanthin enriched extract.

TLC was run one dimensionally in the mobile phase solvent systems, methanol:Acetic acid (6:4) and toluene:ethyl acetate (6:4) and the R_f values were tabulated in Table 3. 2 spots were found to be active in ethyl acetate extract (0.82, 0.93) whereas rhinacanthin enriched extract exhibited a single spot of Rf values 0.94 in the solvent system methanol:acetic acid (6:4). TLC run in the mobile phase system, toluene:ethylacetate (6:4) revealed the presence of 4 spots with R_f values, 0.96, 0.93, 0.72, 0.67 whereas the rhinacanthin enriched extract showed a single spot with similar R_f value of 0.96 in correlation with that obtained in ethyl acetate extract (fig. 1).

FT-IR analysis was based on the vibrations of the functional groups present in the sample at specific wave numbers. The Infrared (IR) absorption bands at 1644 cm^{-1} showed the presence of 1, 4-quinone carbonyl group. The presence of a broad alcoholic hydroxyl group was indicated by an IR absorption band between 3200 cm^{-1} and 3400 cm^{-1} (fig. 2), thereby confirming the rhinacanthins in rhinacanthin enriched extract based on the previous literature studies[8,18].

The anti-tumor effect of rhinacanthin enriched extract from the ethyl acetate leaf extract of *R. nasutus* (L) Kurz., on neuroblastoma cell lines (SH-SY-5Y) was tabulated in Table 4. SH-SY5Y human neuroblastoma cells were used to assess cytotoxic potential by MTT assay (fig. 3). Doxorubicin HCl was used as the standard drug. The results of the *in vitro* cytotoxicity studies of rhinacanthin enriched extract revealed a dose dependent decline in cell viability at the end of 24 h with IC_{50} value of 88.9 µg/ml in SH-SY5Y cells. At the concentration level of 100 µg/ml, the rhinacanthin enriched extract exhibited 45 % of cytotoxic activity against SH-SY5Y cells (Table 4). The standard drug Doxorubicin showed an IC_{50} value of 6.5 µg/ml. Even though doxorubicin exhibited higher cytotoxicity compared to the rhinacanthin enriched extract, the development of resistance resulting in increased malignancy remains as a major concern[19].

TABLE 3: TLC OF ETHYL ACETATE EXTRACT AND RHINACANTHIN ENRICHED EXTRACT OF LEAVES OF *R. nasutus*

Solvent system	Extract	Number of spots	UV detection R_f value	
			Near UV	Far UV
Methanol:acetic acid (6:4)	Ethyl acetate extract	2	No UV active compounds	0.82, 0.93
	Rhinacanthin Rich extract	1	No UV active compounds	0.94
Toluene:ethylacetate	Ethyl acetate extract	4	No UV active compounds	0.96, 0.93, 0.72, 0.67
(6:4)	Rhinacanthin Rich extract	1	No UV active compounds	0.96

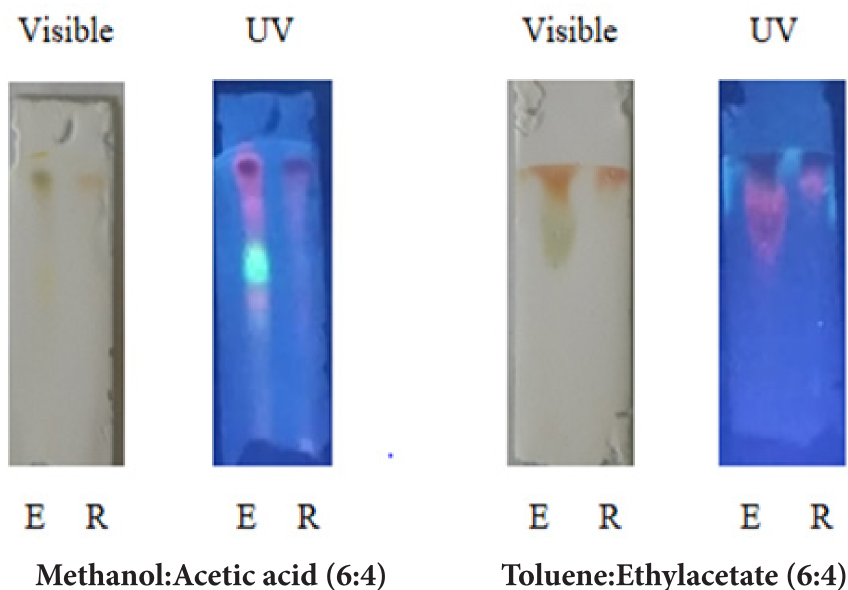

Fig. 1: TLC of Ethyl acetate extract (E) and Rhinacanthin enriched extract (R) under Visible light and UV lamp

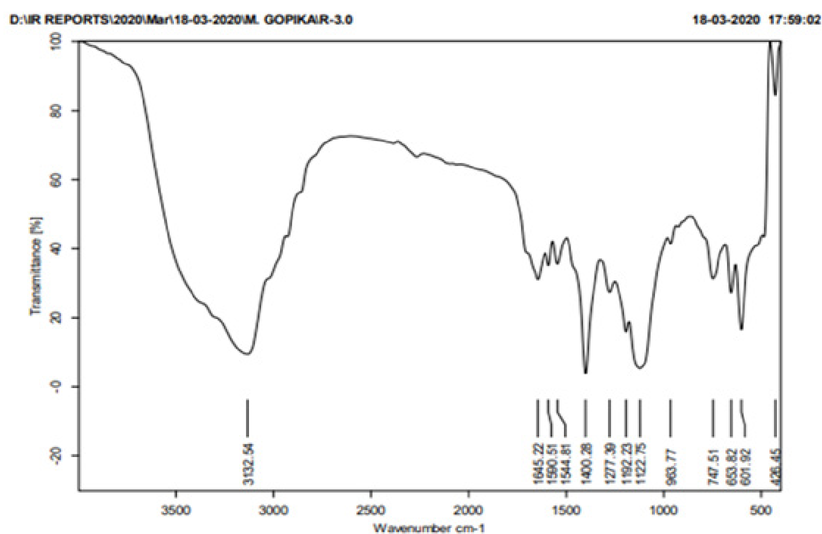

Fig. 2: FT-IR spectra of rhinacanthin enriched extract from *R. nasutus*

TABLE 4: CYTOTOXIC ACTIVITY OF RHINACANTHIN ENRICHED EXTRACT AND DOXORUBICIN ON SH-SY5Y HUMAN NEUROBLASTOMA CELL LINE

Concentration (µg/ml)	Percentage cell viability	
	Rhinacanthin enriched extract	Doxorubicin
Control (DMSO)	100	100
3.12	98.16±0.02**	78.23±0.01**
6.25	95.21±0.01**	46.92±0.01**
12.5	91.40±0.01**	24.85±0.02**
25	86.51±0.01**	16.84±0.01**
50	74.05±0.02**	10.73±0.01**
100	44.58±0.02**	4.21±0.02**

Note: All data were presented as the mean±SD. The experiment was repeated at least in triplicates. Unpaired t-test was included to compare the difference between the groups using Graph pad prism. **Any value of $p<0.01$ was considered as statistically significant

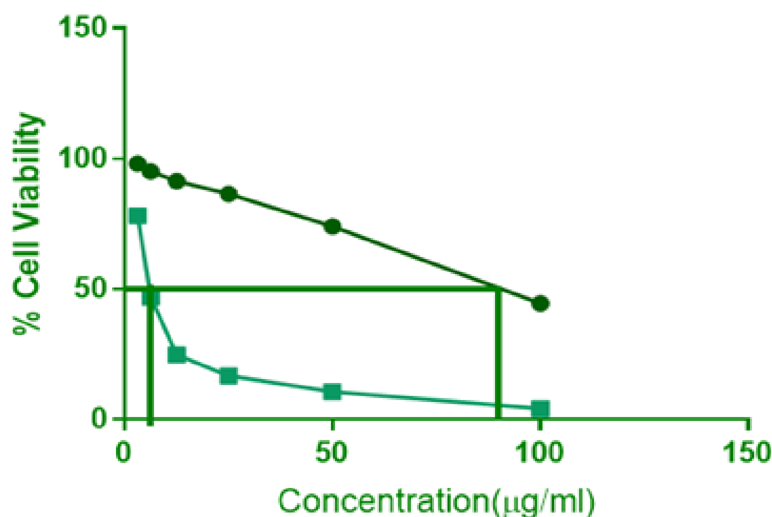

Fig. 3: Effect Rhinacanthin enriched extract of *R. nasutus* and Doxorubicin on SH-SY5Y cell lines using MTT assay
Note: ( ) RRE and ( ) DOXORUBICIN

A varied array of chemical constituents including naphthoquinones, lignans, benzenoids, anthroquinones, triterpenoids, flavonoids, sterols, coumarins, glycosides were reported in *R. nasutus*[20]. Several literatures were reported, investigating the cytotoxic potential and the underlying mechanisms exhibited by the bioactive constituents of *R. nasutus*, particularly, the rhinacanthins, on different cancerous cell lines. In a study carried out by Wu *et al.*[8], among the two isolated naphthaquinones, rhinacanthin-A and rhinacanthin-B from the methanolic root extracts of *R. nasutus*, the rhinacanthin-B was found to exhibit cytotoxic potential with an ED_{50} value of 3.0 µg/ml in KB human epidermis carcinoma cell lines.

In another study carried out by Wu *et al.*[21], rhinacanthin-Q along with several known compounds from the methanolic root extracts of *R. nasutus* were investigated for the antiplatelet and cytotoxic effect against KB, P388, A549, HT29 and HL60 cell lines. Gotoh *et al.*[9] reported the anti-proliferative activity of the ethanolic root extract and aqueous leaf extracts in comparison to the chemically synthesized rhinacanthin C against human cervical carcinoma HeLa cell lines, Hyr100-6, multidrug resistant sub line of HeLa, PC-3 human prostate carcinoma cell line and T24 human bladder cell lines. The key findings revealed the bioactive potential of rhinacanthin C, particularly in *R. nasutus* roots. Further *in vivo* anti-proliferative study on sarcoma-180 bearing Institute of Cancer Research (ICR) mice showed a significant tumor inhibition exhibited by both the extracts[9]. In a study by Siripong *et al.*[22], yet another isolated bioactive compound, rhinacanthone showed an apoptotic cell death in HeLa cervical cancer cell lines by multiple pathways, primarily, through mitochondria-dependent signaling pathway. Moreover, antimetastatic activity of liposomal rhinacanthin-N, isolated from the roots of *R. nasutus* was reported on B16F10 melanoma cells induced pulmonary metastasis in C57BL/6 mice[23]. Horii *et al.*[24] reported the diversified biological activities of rhinacanthin C. Upon investigation of five isolated components rhinacanthin C, G, N, Q and rhinacanthone from the ethyl acetate fraction of methanolic extract of *R. nasutus*, the rhinacanthin C proved to be highly tumor specific and induced non-apoptotic cell death. Additionally, rhinacanthin C also inhibited RANKL-stimulated osteoclast formation in RAW 2647 cells[24]. Moreover, synergistic approach ensuring the phytoconstituents to combat in a coordinated manner could help in further phytotherapeutic research against neuroblastoma. Thus the current study concludes that rhinacanthins present in the enriched extract may possess cytotoxic potential against malignant neuroblastoma cells and further *in vivo* studies can be considered to focus on the exact molecular mechanisms involved in the anticancer activity.

Conflict of interests:

The authors declared no conflict of interest.

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