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RESEARCH ARTICLE

A First Report on the Antiproliferative activity of Sodium Copper Chlorophyllin from Endangered Medicinal Plant *Rhinacanthus nasutus* on HepG2 and HeLa Cell Lines

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ABSTRACT:

Plants are the richest source of bioactive compounds and they have been used as medicine also. Chlorophyllin, a water soluble derivative of chlorophyll (chl) in which magnesium has been replaced with copper and the phytol chains lost. Chlorophyllin has been used by human population for over 50 years for medicinal purposes with no adverse effects. Chlorophyllin is a promising chemopreventive agent to block cancer primarily by inhibiting carcinogen such as AFB_1. The objective was to extract the bioactive pigment chloropyllin from *Rhinacanthus nasutus* and characterize by IR and NMR and evaluate the effect of chlorophyllin on inhibition of cell proliferation in HepG2 and HeLa cell lines. The HepG2 and HeLa cell lines was exposed with different concentrations of chlorophyllin, and found that there was a significant dose dependent reduction in cell viability. The IC_{50} value at 48Hrs was 62.5µg/ml for *Rhinacanthus nasutus* Chlorophyllin on HepG2 Cell line and HeLa cell line. This is the first report of chlorophyllin from fresh leaves of *Rhinacanthus nasutus* on HepG2 and HeLa cell lines. It also suggests the benefit of these natural compounds against HepG2 and HeLa cell lines.

KEYWORDS: Sodium Copper Chlorophyllin, *Rhinacanthus nasutus*, HepG2 Cell Lines, HeLa Cell Lines.

INTRODUCTION:

Medicinal plants are the rich source of harmless medicines, and used for the treatment of various diseases for thousands of years. The first written record on the medicinal use of plants appeared in about 2600 BC from the Sumerians and Akkaidians[1]. Documentation of the Ayurvedic system recorded in Susruta and Charaka dates from about 1000 BC[2].

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The World Health Organization (WHO) estimates that approximately 80% of the world's inhabitants rely on traditional medicine for their primary health care. The genus Rhinacanthus comprises of about 25 species confined to the old world tropics and subtropics. *Rhinacanthus nasutus* is widely distributed in some parts of sub-continent, in the region of Southeast Asia and China[3]. Different parts of *Rhinacanthus nasutus* have used in traditional medicine for the treatment in diseases such as eczema, pulmonary tuberculosis, herpes, hepatitis, diabetes, hypertension and several skin diseases[4].

Chlorophyllin belongs to a group of compounds, porphyrins that contain a chelated metal ion in the center

of the molecule. It is a man-made sodium and copper salt of chlorophyll in which magnesium has been replaced with copper, zinc, iron, tin and the phytol chains lost (Figure 1).

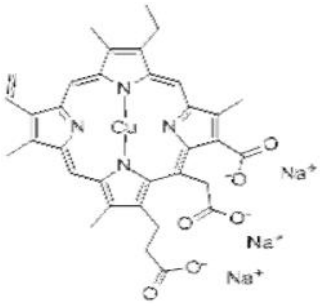


Figure1: Chlorophyllin

Chlorophyllin is the active ingredient in a number of internally taken preparations intended to reduce odors associated with incontinence, colostomies and similar procedures, as well as body odor in general. It is also available as a topical preparation, useful for both treatment and odor control of wounds, injuries and other skin conditions notably radiation burns. It also acts as an antioxidant to inhibit lipid peroxidation. It is also used extensively as a food additive for coloration. It is present in green leafy vegetables and reaching levels as high as 5.7% in spinach[5].

Chlorophyllin, a mixture of semi synthetic, water-soluble derivatives of chlorophyll that is used as a food colorant and over-the-counter medicine has been shown to be an effective inhibitor of aflatoxin hepatocarcinogenesis in animal models by blocking carcinogen bioavailability[6]. The anticarcinogenic properties of chlorophyllin, a water-soluble derivative of chlorophyll, have been demonstrated in a number of animal models[7, 8].

MATERIALS AND METHODS:

Collection of plants:

The medicinal plant *Rhinacanthus nasutus* was collected from MSME, Herbal garden in Guindy, Chennai, India.

Extraction of Chlorophyllin[9]:

10gms of fresh leaves were taken and 1gm of sodium carbonate was added to neutralize the acidity. The plant material was ground with 50 – 100ml acetone and filtered using filter paper. This procedure is repeated until the residue becomes colorless. It was then washed with 50 – 150ml of diethyl ether to wash off acetone. The mixture was poured into a separating funnel and acetone was washed off using distilled water. This was repeated until a yellow color separates off which consists of flavones. The solution was poured into a bottle and 10 – 25ml of methanol saturated with potassium hydroxide pellets was added. The solution was shaken thoroughly and kept in icebox for overnight. The alkaline solution of chlorophyllin was poured into a separating funnel and 100ml diethyl ether was added and left for 30mins. Chlorophyllin separates off greenish layer which was removed. The ether layer was washed off with dilute potassium hydroxide and distilled water, to remove traces of chlorophyllin salts. The filtrate was evaporated to dryness in rotary evaporator and the extract was stored in ice box.

Infrared spectroscopic analysis:

The partially purified Chlorophyllin was pressed into discs under vacuum. The IR spectrum recorded in the region 450 - 4500cm^{-1} using Shimadzu FT-IR 8000 series instrument.

Nuclear Magnetic Resonance Spectroscopy:

The ^{13}C NMR spectral analyses were performed by taking the sample in NMR tubes dissolved in D_2O. The NMR was recorded at 25.15MHz on a Burker AV III series instrument

Cell Culture:

HepG2 cell lines, Vero cell lines and HeLa Cell Lines were obtained from Life Tech Research Centre, Chennai, Tamil Nadu, India and routinely maintained in minimal essential medium (DMEM) with 10% FCS and antibiotics at 37^{o}c in a humidified atmosphere containing 5% CO_2.

Determination of anticancer activity[10]:

To evaluate the anticancer activity of extracts, antiproliferative activity was investigated in HepG2 and HeLa cells exposed to each extracts at range 7.8-1000µg/ml for 48h. Cell proliferation was determined using the 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay. Each cell line was plated at a density 2.5-5× 10^5 cells/well in 24-well tissue culture plate and incubated at 37^{o}C. After incubation for 48h plated cell were incubated with MTT (5mg/ml) for 6-7 hrs at 37^{o}C. After discarding all medium from the plates, 1ml of dimethyl sulfoxide was added to the each well. The plates were placed for 5min at room temperature with shaking, so that complete dissolution of formazan was achieved. The absorbance of formazan was determined at 540 nm by a reader. The % cell viability was calculated using the following formula:

% cell viability = A570 of treated cells / A570 of control cells × 100

RESULTS AND DISCUSSION:

Cancer preventive effects of chlorophyll derivatives have been extensively studied with particular emphasis on their *in vitro* antimutagenic activity against numerous

dietary and environmental mutagens. Sodium copper chlorophyllin has demonstrated the ability, *in vitro*, to effectively protect against mutagenic activity of both direct and indirect acting dietary and environmental mutagens[11].

Structurally, chlorophyll is a substituted tetrapyrole with a centrally bound Mg atom. The porphyrin macrocycle is further esterified to a diterpene alcohol, phytol to form chlorophyll. In nature, chlorophyll a and b predominates in higher plants. The chlorophyll content of commonly consumed green vegetables typically exceeds the levels of other bioactive pigments, such as carotenoids by upto a 5 – fold margin[12].

Infra red spectroscopic analysis:

The presence of Chlorophyllin was proved by FT – IR spectrum at 450 – 4500cm^{-1}. With reference to the sample from the spectra obtained it could be concluded that predominant peaks are seen in both functional region and fingerprint region (Figure 2, 3).

From the analysis of Standard Chlorophyllin, it was found out that the peak at 3418cm^{-1} clearly indicates the presence of –OH and N-H group. The peak observed at 1105cm^{-1}dominates the existence of COO^- group. The existence of aromatic rings was confirmed by the presence of peaks at 1628cm^{-1}. The bend at 2360cm^{-1} indicates the presence of C-N stretch. The peak at 710cm^{-1} was due to OOP (Out of Plane) bending vibrations arrived due to the formation of aromatic ring system or C=C system. The peak at 1399cm^{-1} was due to C-H bending vibration. The peak at 2924cm^{-1} is due to sp^3 hybridization. It clearly indicates that the replacement of Mg with Na^+ or K^+ or Cu^+ on the central ion in the porphyrin ring structure. Hence the IR spectrum clearly indicates the existence of monovalent substituted carboxyl group, keto group, nitrogen substituted heterocyclic ring may be porphyrin ring system.

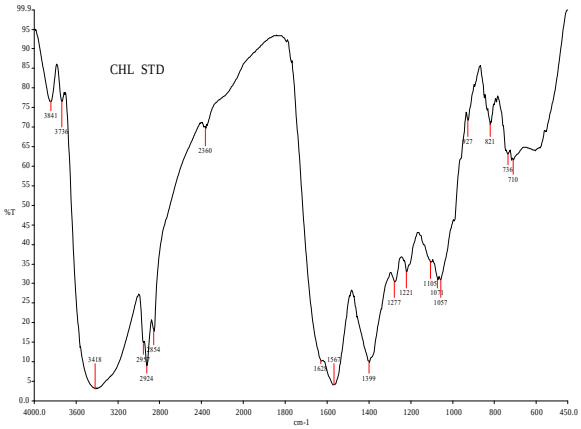


Figure.2: IR Spectrum of standard chlorophyllin

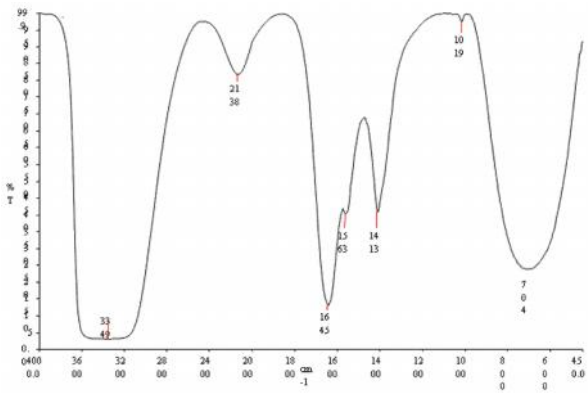


Figure.3: IR Spectrum of *Rhinacanthus nasutus* chlorophyllin.

NMR analysis:

Further the chlorophyllin *Rhinacanthus nasutus* of was characterised by NMR and it was compared with standard CHL.

Standard Chlorophyllin:

The peaks near 77.15Δ – 74.54Δ represent C=N group and the peaks from 74.13Δ – 70.83Δ represent aromatic CH_3 group. The peak at 60.06Δ corresponds to CH_3COONa (Figure 4).

***Rhinacanthus nasutus* Chlorophyllin:**

The peaks near 77.08Δ – 74.52Δ represent C=N group and the peaks from 74.14Δ – 70.83Δ represent aromatic CH_3 group. The peak at 60.49Δ corresponds to CH_3COONa (Figure 5).

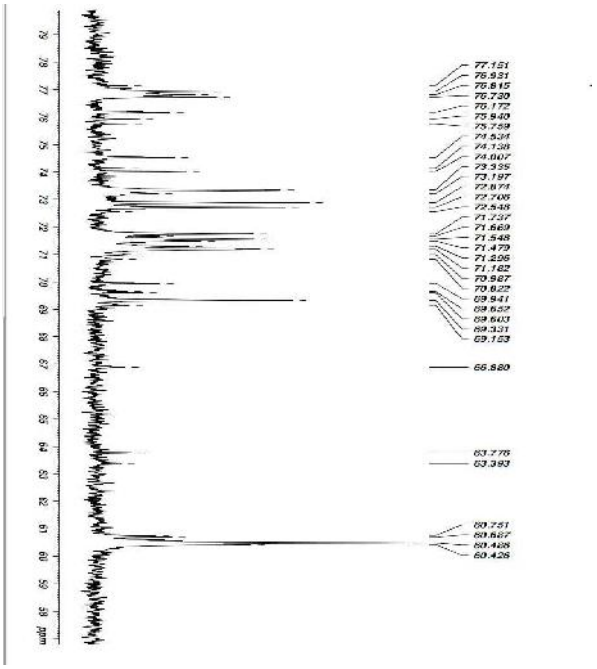


Figure.4: C^{13} NMR spectrum of standard chlorophyllin

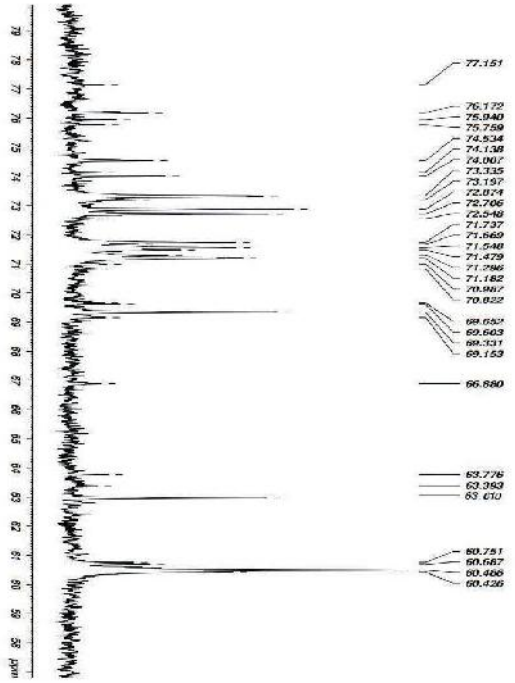


Figure.5: C^{13} NMR spectrum of *Rhinacanthus nasutus* chlorophyllin

Anti-proliferative activity of chlorophyllin:

When HepG2 and HeLa cells were incubated with 7.8-1000µg/ml of *Rhinacanthus nasutus* CHL for 48Hrs, there was a significant dose dependent reduction in cell viability. The IC_{50} value at 48Hrs was 62.5µg/ml (Table 1, 2). The cells were examined by phase contrast microscopy for evidence of morphological apoptosis. The cells showed typical polygonal intact appearance. The CHL treated cells exhibited morphological characters like cellular shrinkage at 1000µg/ml (low toxicity), rounding at 125µg/ml (medium toxicity) and poor adherence at 62.5µg/ml (high toxicity). At 31.2µg/ml the cells were observed in round floating shapes. (Figure 6, 7)

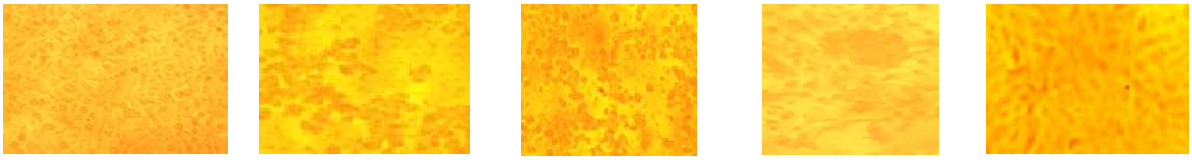
The study also concludes that anti-cancerous activity is better seen in the sample than in the standard. It is observed that IC_{50} for standard is obtained at 1:4 dilutions whereas in sample it is observed at 1:8 dilutions. In HepG2 cell line, the final cell viability was 89.45% in standard and 87.5% in sample. In HeLa cell line, the final cell viability was 93.10% in standard and 82.7% in sample.

Table 1: Cytotoxicity effect of Standard Chlorophyllin on Vero, HepG2 and HeLa cell lines.

S.No	Concentration (µg/ml)	Dilution	Absorbance (O.D)			Cell viability (%)		
			Vero	HepG2	HeLa	Vero	HepG2	HeLa
1	1000	Neat	0.10	0.09	0.14	19.23	18.75	24.13
2	500	1:1	0.18	0.13	0.21	34.61	27.08	36.20
3	250	1:2	0.22	0.19	0.26	42.30	39.58	44.82
4	125	1:4	0.25	0.23	0.30	48.07	47.91	51.72
5	62.5	1:8	0.33	0.28	0.37	63.46	58.33	63.79
6	31.2	1:16	0.40	0.32	0.43	76.92	66.66	74.13
7	15.6	1:32	0.46	0.39	0.50	88.46	81.25	86.20
8	7.8	1:64	0.49	0.43	0.54	94.23	89.58	93.10
9	Cell control	-	0.52	0.48	0.58	100	100	100

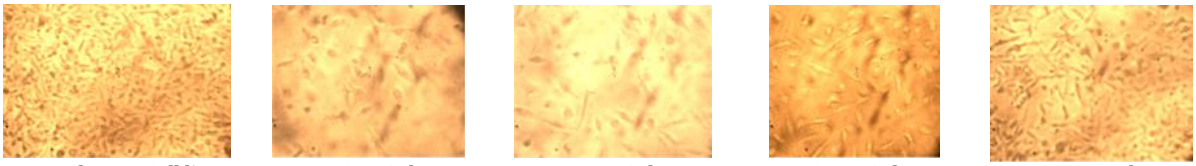
Table 2: Cytotoxicity effect of *Rhinacanthus nasutus* Chlorophyllin on Vero, HepG2 and HeLa cell lines.

S.No	Concentration (µg/ml)	Dilution	Absorbance (O.D)			Cell viability (%)		
			Vero	HepG2	HeLa	Vero	HepG2	HeLa
1	1000	Neat	0.08	0.06	0.10	15.38	12.5	17.24
2	500	1:1	0.14	0.10	0.15	26.92	20.83	25.86
3	250	1:2	0.20	0.16	0.21	38.46	33.33	36.20
4	125	1:4	0.23	0.20	0.26	44.23	41.66	44.82
5	62.5	1:8	0.27	0.24	0.30	51.92	50.0	51.72
6	31.2	1:16	0.32	0.30	0.37	61.53	62.5	63.79
7	15.6	1:32	0.41	0.37	0.44	78.84	77.08	75.86
8	7.8	1:64	0.47	0.42	0.48	90.38	87.5	82.75
9	Cell control	-	0.52	0.48	0.58	100	100	100



Normal HepG2 Cell line | 1000µg/ml | 125µg/ml | 62.5µg/ml | 31.2µg/ml

Figure.6: Cytotoxicity effect of *Rhinacanthus nasutus* Chlorophyllin on HepG2 Cell line.



Normal HeLa Cell line | 1000µg/ml | 125µg/ml | 62.5µg/ml | 31.2µg/ml

Figure.7: Cytotoxic effect of *Rhinacanthus nasutus* Chlorophyllin on HeLa cell line.

The commercially derived SCC has demonstrated the ability, *in vitro*, to effectively protect against mutagenic activity of both direct and indirect active dietary and environmental mutagens like aflatoxin B1[8]. Chlorophyllin was potent, dose responsive inhibitor of AFB1-DNA adduction and hepatocarcinogenesis in the rainbow trout model when fed with carcinogen. They found upto 77% of potent inhibition at CHL levels well within the chlorophyll content of some green leafy vegetables[7].

The CHL had a higher safety ratio which is a good indicator for use in cancer treatment i.e., the extract inhibits the growth of cancer cells but not normal cells.

CONCLUSION:

From the present study, it is concluded that cancer is the leading cause of death in developing countries like India. As there is an enormous increase in the population day by day, the alternative therapy in the market is getting its glimpse. The cheap herbal drug treatment may highly be recommended to the rural and poor people to treat effectively the cancers of various type is an ideal choice. The isolation, identification of active principles and pharmacological studies of the active phytoconstituents may be considered and studied elaborately to treat effectively for various types of cancer. The data presented here suggests that the plant, *Rhinacanthus nasutus* shows anti-cancer activity against Vero, HepG2 and HeLa cell lines. Hence the consumption of either the leaf as a whole or the chlorophyllin of this plant imparts anti-cancerous effects.

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CONFLICT OF INTEREST:

Conflict of interest declared none.

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