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Original Article

A STUDY OF CHLOROPHYLLIN OF MEDICINAL PLANTS, ITS CHEMICAL CHARACTERIZATION AND ANTI–PROLIFERATIVE ACTIVITY WITH SPECIAL REFERENCE TO *SOLANUM TRILOBATUM* L. ON LIVER CELL LINE

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ABSTRACT

Objective: Plants are the richest source of bioactive compounds and they have been used as medicine also. Chlorophyllin (CHL) is 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 y for medicinal purposes with no adverse effects. Chlorophyllin is a promising chemo preventive agent to block cancer primarily by inhibiting carcinogen such as AFB_1. The objective was to extract the bioactive pigment chlorophyllin from medicinal plants and to study its anticarcinogenic property on liver cell lines.

Methods: In the present study the bioactive pigment, chlorophyllin was extracted and estimated from six medicinal plant leaves and characterized by IR and NMR. Further, based on the high chlorophyllin content (12.21µg/ml), *Solanum trilobatum* L. was selected for the study of anticarcinogenic property against two types of cell lines: HepG2 cell lines (Human Hepatocellular Carcinoma) and Vero cell lines (African Green Monkey kidney).

Results: It was found that the inhibitory effect of chlorophyllin was found on cancer cell lines (IC_{50} value at 48H was 62.5µg/ml) and absent on Vero cell lines. Standard chlorophyllin was used as control for all the studies.

Conclusion: This is the first report on the effect of natural chlorophyllin from the leaves of *Solanum trilobatum* L. on HepG2 cell lines. The *in vitro* data suggests that the consumption of the leaves of *Solanum trilobatum* L. or as chlorophyllin may impart anticancer effects.

Keywords: Chlorophyllin, *Solanum trilobatum* L., Hepato Cellular Carcinoma, Vero cell lines.

INTRODUCTION

Hepatocellular carcinoma (HCC) is known as a common and aggressive malignant tumour worldwide. It is a global health and is the fifth most common and aggressive cancer in the world and the fourth most common cause of cancer–associated mortality [1]. HCC is difficult to detect and in most cases is not noticed at an early stage and hence becomes chronic. The most risk factors of HCC are chronic hepatitis B virus and hepatitis C virus infections, chronic exposure to the mycotoxin or the aflatoxin B1 (AFB1). The development of chemotherapeutic or chemo preventive agents for hepatocellular carcinoma is important in order to reduce the mortality caused by this disease [2].

Medicinal plants are the rich source of harmless medicines and used for the treatment of various diseases for thousands of years. They can provide biologically active molecules and lead structures for the development of modified derivatives with enhanced activity or reduced activity. The isolation and identification of active principles and elucidation of the mechanism of action of a drug is of paramount importance. One such compound is chlorophyllin, a water soluble analogue of the ubiquitous green pigment chlorophyll.

Chlorophyllin

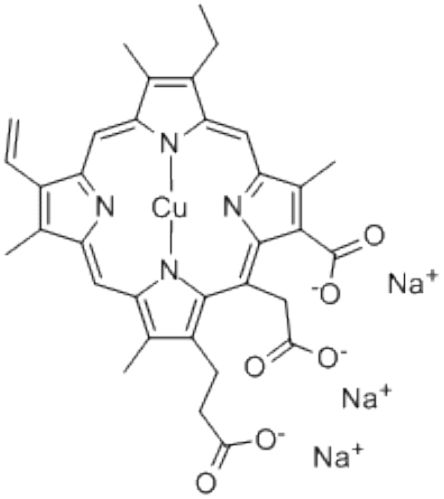

Fig. 1: Chlorophyllin

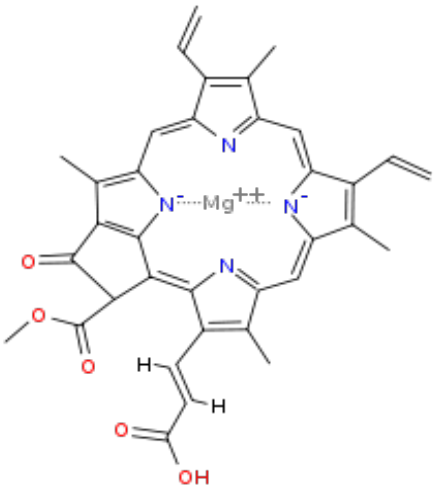

Fig. 2: Chlorophyll

Chlorophyllin is water-soluble derivative of chlorophyll in which magnesium has been replaced with copper and the phytol chains lost. Chlorophyllin is commercially available as a trisodium-copper salt with empirical formula $C_{13}H_{31}N_4Cu(CO_2Na)_3$; Molar mass 724.15 g/mol. Chlorophyllin 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 [3].

Chlorophyllin has been used by human population for over 50 y for medicinal purposes with no adverse effects. It is a very effective inhibitor of numerous mutagens, including AFB_1, polycyclic aromatic hydrocarbons (PAHs), heterocyclic amines, direct acting compounds and complex mixtures [4].

Chlorophyllin is a promising chemo preventive agent to block cancer primarily by inhibiting carcinogen such as AFB_1. Thus, chlorophyllin may diminish the bioavailability of dietary carcinogens by impeding their absorption and by shuttling them through the faecal stream, leading to reduced DNA adduct and tumor burden.

Chlorophyllin is most effective anticarcinogen in experimental models when given in large molar excess relative to the carcinogen at or around the time of carcinogen at or around the time of carcinogen exposure. It is a potent inhibitor *in vitro* of cytochrome P450 enzymes involved in the bio-activation of several carcinogens.

Thus, significant research efforts have focused on novel chemotherapeutic drugs from the plant kingdom in search of cancer inhibitors and cures [5].

MATERIALS AND METHODS

Collection of medicinal plants

The medicinal plants were obtained from the fields of Pammal town, situated at Kanchipuram District, Tamilnadu. Six medicinal plants have been chosen for the present study. The selected medicinal plants include *Scoparia dulcis* L., *Stevia rebaudiana*, *Cynodon dactylon* L., *Tamarindus indica* L., *Solanum trilobatum* L., *Carica papaya* L. The collected medicinal plants were identified by referring "Flora of India" by Alfred Byrd Graf.

Extraction of CHL

Ten grams of fresh leaves were weighed and 1g of sodium carbonate was added to neutralize the acidity. The material was ground with 50-100 ml of acetone and filtered using filter paper and the procedure was repeated until the residue is colorless. Finally, it was washed with 100 ml or more of diethyl ether to wash off acetone. The ether-acetone extract was then poured into a separating funnel and acetone was washed off using distilled water and the procedure was repeated until a yellow aqueous layer separates which consist of flavones. In order to remove the remaining flavones, 1% sodium carbonate was added.

The ether solution was poured into a 250 ml bottle. To this 10-25 ml of methanol saturated with potassium hydroxide was added and shaken thoroughly and incubated in ice box overnight. The alkaline solution of CHL salts was poured into a separating funnel.

The bottle was washed several times with distilled water and ether to remove traces of pigments. 100 ml of diethyl ether was added to the funnel and left for 30 min. The CHL separates as a greenish layer below. The greenish layer was removed and the ether layer was washed with distilled water and dilutes potassium hydroxide, to remove traces of CHL salts. The filtrate was evaporated to dryness in a rotary evaporator to give an ether extract of fresh leaves. The extracted CHL was stored in ice box [6].

Ultra violet-visible spectroscopic analysis

The partially purified CHL was analysed by UV-VIS absorption by dissolving in diethyl ether and read at 405 nm in a Beckman DU-40 Spectrophotometer and compared with authentic CHL.

Infra-red spectroscopic analysis

The partially purified CHL was ground with IR grade potassium bromide (KBr) (1:10) pressed into discs under vacuum using spectra lab Pelletier. The IR spectrum was recorded in the region 450-4000 cm^{-1} using Shimadzu FT-IR 8000 series instrument.

NMR spectroscopic analysis

The carbon–^{13}NMR spectral analyses were performed by taking the sample in NMR tubes dissolved in D2O. The NMR was recorded at 25.15MHz on a Burker AV III series instrument.

In vitro studies

Collection of cell line

The HepG2 cell lines and Vero cell lines were obtained from Life Teck Research Centre, Chennai, Tamilnadu.

Maintenance of cell line

The cells were cultured in Minimum Essential Medium (MEM) with Foetal Calf Serum (FCS) at 37 °C and 5% CO_2.

Cytotoxicity assay

In order to study the antitumor activity of a new drug, it is important to determine the cytotoxicity concentration of the drug. Cytotoxicity tests define the upper limit of the extract concentration, which is non-toxic to the cell line. The concentration at which the drug is nontoxic to the cells is chosen for antiviral assay. After the addition of the drug, cell death and cell viability was estimated. The result is confirmed by additional metabolic intervention experiment such as MTT assay [7].

The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was performed. Each well was washed with serum free MEM for 2–3 times. 200 µl of MTT (concentration 5 mg/ml) was added and incubated for 6-7H in 5% CO_2 incubator for cytotoxicity.

After incubation, 1 ml of DMSO was added to each well and mixed using a pipette and left for 45 sec. If any viable cells were present the formazan crystals after adding solubilizing reagent (DMSO) showed purple color formation. The suspension was then transferred to the cuvette of the spectrophotometer and the OD values were read at 595 nm by taking DMSO as a blank. The percentage of cell viability was calculated using the formula:

$$\text{Cell viability (\%)} = \text{Mean OD} \times 100/\text{Control OD}$$

RESULTS

CHL content of fresh leaves of medicinal plants

Table 1: Estimation of CHL of plant samples

S. No.	Plant name	Concentration (µg/ml)
1.	*Scoparia dulcis* L.	12.04
2.	*Stevia rebaudiana*	10.21
3.	*Cynodon dactylon* L.	10.43
4.	*Tamarindus indica* L.	10.22
5.	*Solanum trilobatum* L.	12.21
6.	*Carica papaya* L.	10.11

The CHL content was estimated and found that *Solanum trilobatum* L. contained more amount of CHL (12.21µg/ml).

Infrared spectroscopic analysis

The functional groups present in the chlorophyllin samples were identified and compared with the corresponding peaks obtained in the standard chlorophyllin (table 2; fig. 3-9).

Table 2: IR spectroscopy

Functional group	Peaks observed in CHL samples (cm^{-1})						
	Standard	*Scoparia dulcis* L.	*Stevia rebaudiana*	*Cynodon dactylon* L.	*Tamarindus indica* L.	*Solanum trilobatum* L.	*Carica papaya* L.
-OH -NH	3392	3401	3400	3340	3465	3409	3360
COO^-	1151	1066	1070	1070	1058	1238	-
Aromatic ring	1141 1640	1421 1456	1395	1396	1457	1410	1396
C-N	2081	-	-	-	-	-	-
C=C	761	737	695	685	666	-	688
C-H	1077	-	-	-	-	963	-
C=O	-	1066	-	1103 1175	1110	-	-
sp^3	2927	-	-	-	-	2134	-

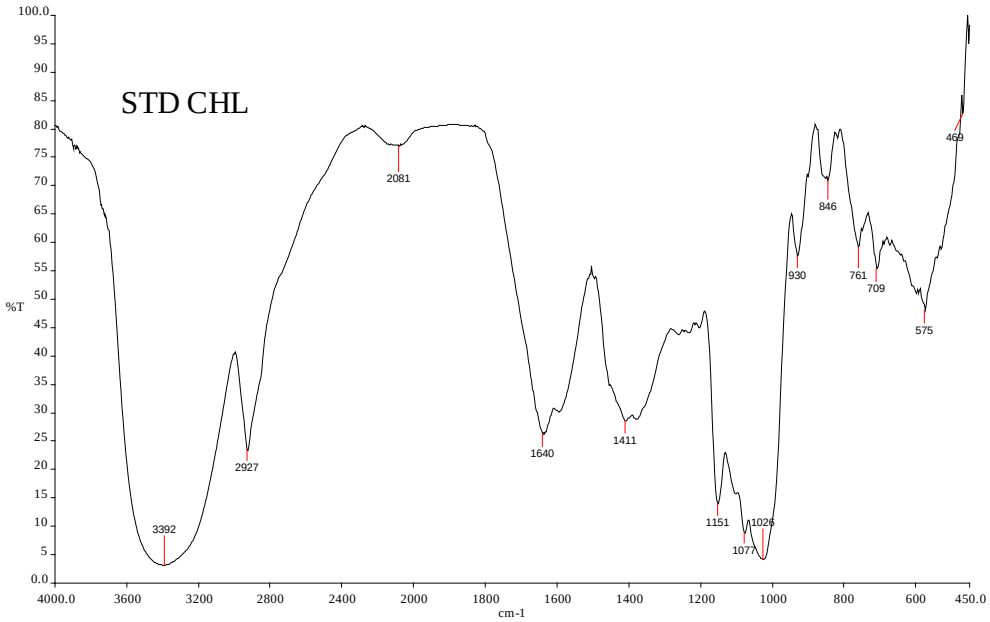

Fig. 3: IR spectrum of standard CHL

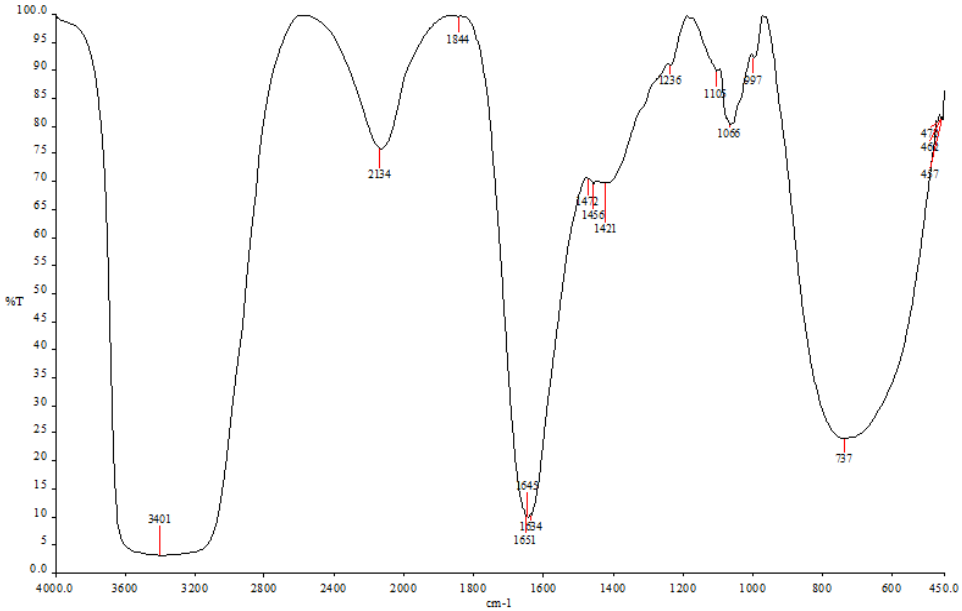

Fig. 4: IR spectrum of CHL of *Scoparia dulcis* L.

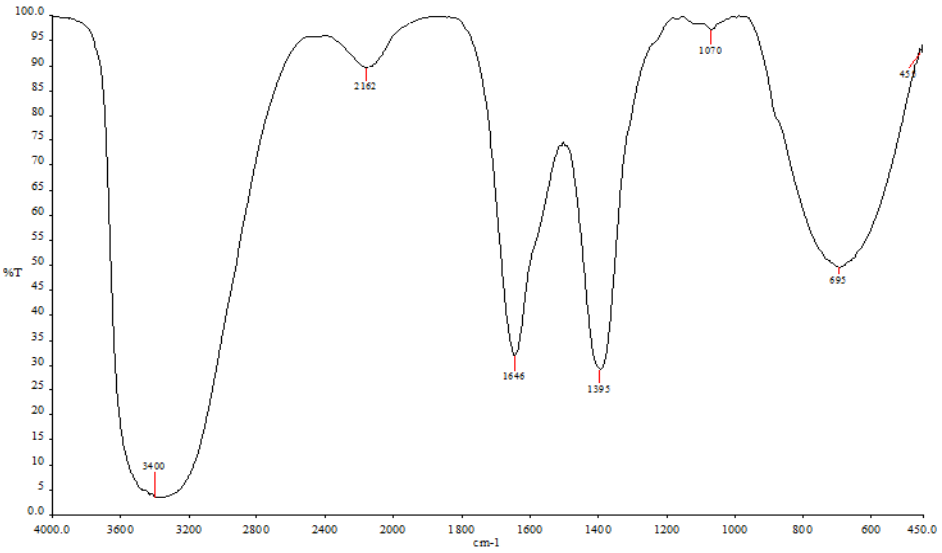

Fig. 5: IR spectrum of CHL of *Stevia rebaudiana*

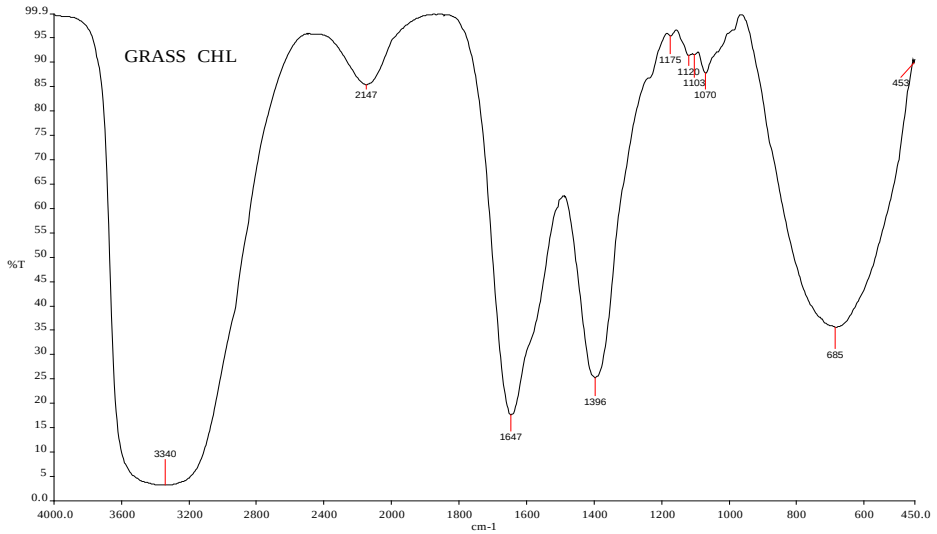

Fig. 6: IR spectrum of CHL of *Cynodon dactylon* L.

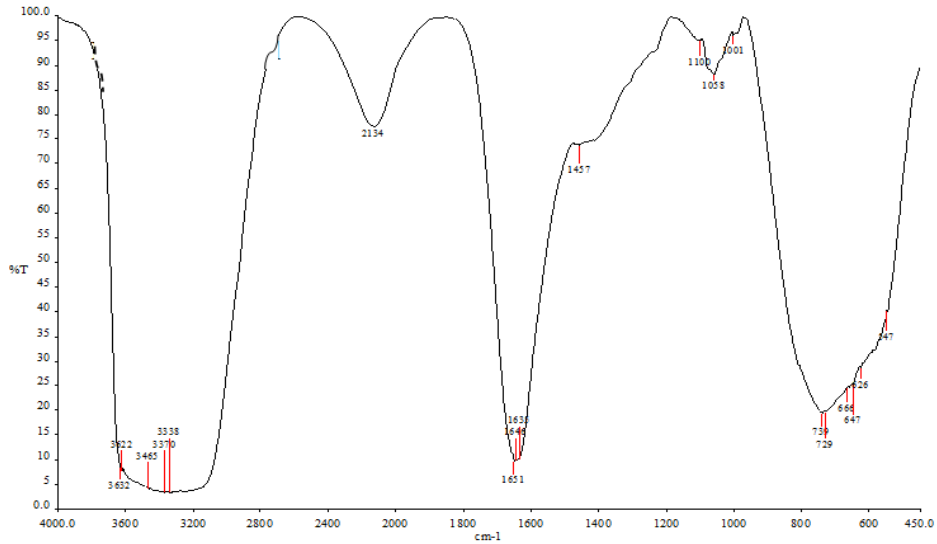

Fig. 7: IR spectrum of CHL of *Tamarindus indica* L.

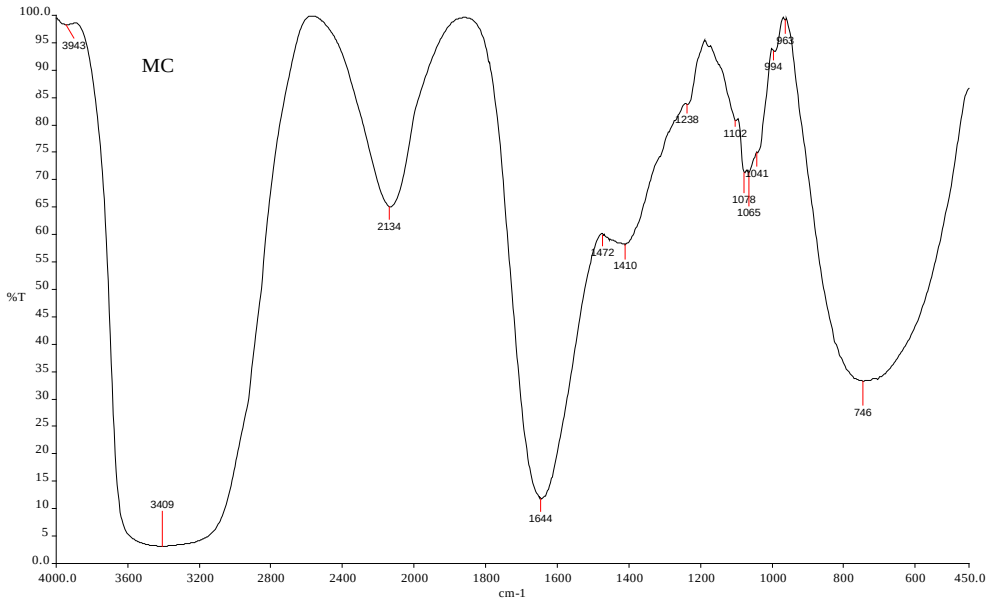

Fig. 8: IR spectrum of CHL of *Solanum trilobatum* L.

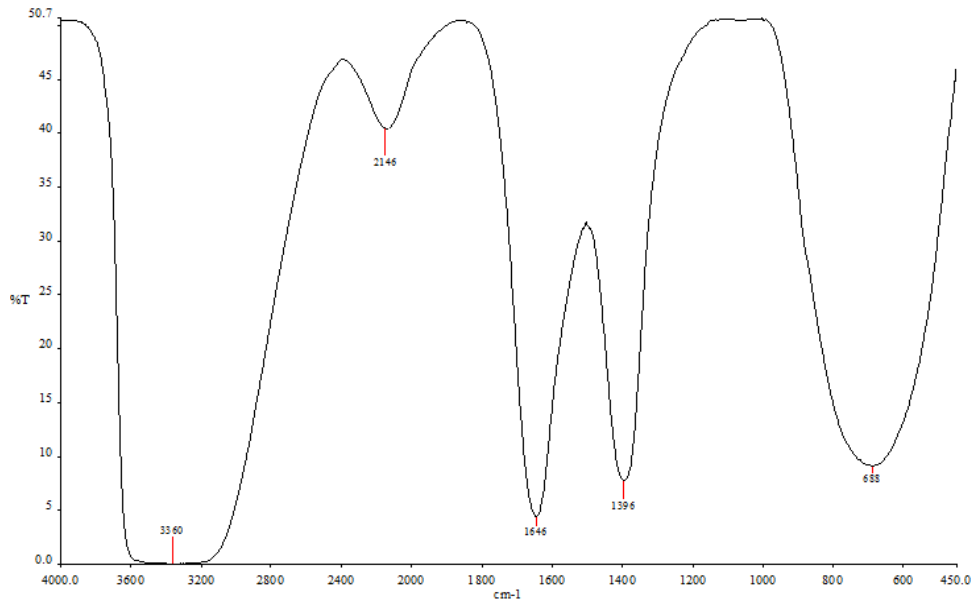

Fig. 9: IR spectrum of CHL of *Carica papaya* L.

NMR spectroscopic analysis

The presence of chlorophyllin structure of *Solanum trilobatum* was further confirmed by the NMR analysis.

The sample and the standard chlorophyllin were analyzed and compared. Predominant peaks were seen both in the standard as well as the sample (table 3; fig. 10 and 11).

Table 3: NMR Spectrum

Functional group	Peaks observed in CHL sample (Δ)	
	Standard	*Solanum trilobatum L.*
C=O	77.151	77.151
Aromatic ring	70.822-74.138	70.822-74.007
C=N	69.153-69.652	69.603
CH_3	63.010-63.776	63.010-63.776
CH_3COONa	60.486	60.486

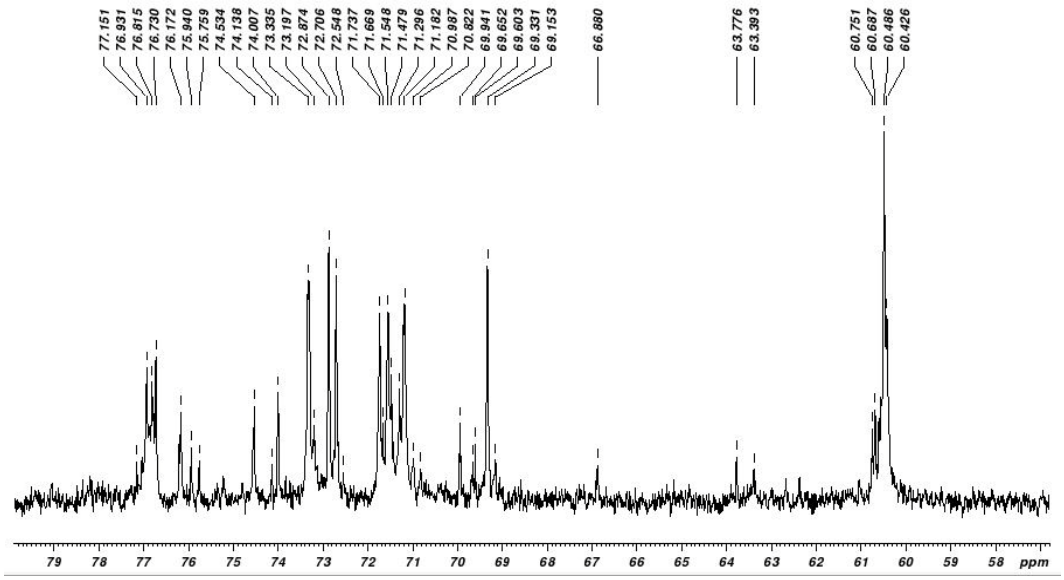

Fig. 10: NMR spectrum of standard CHL

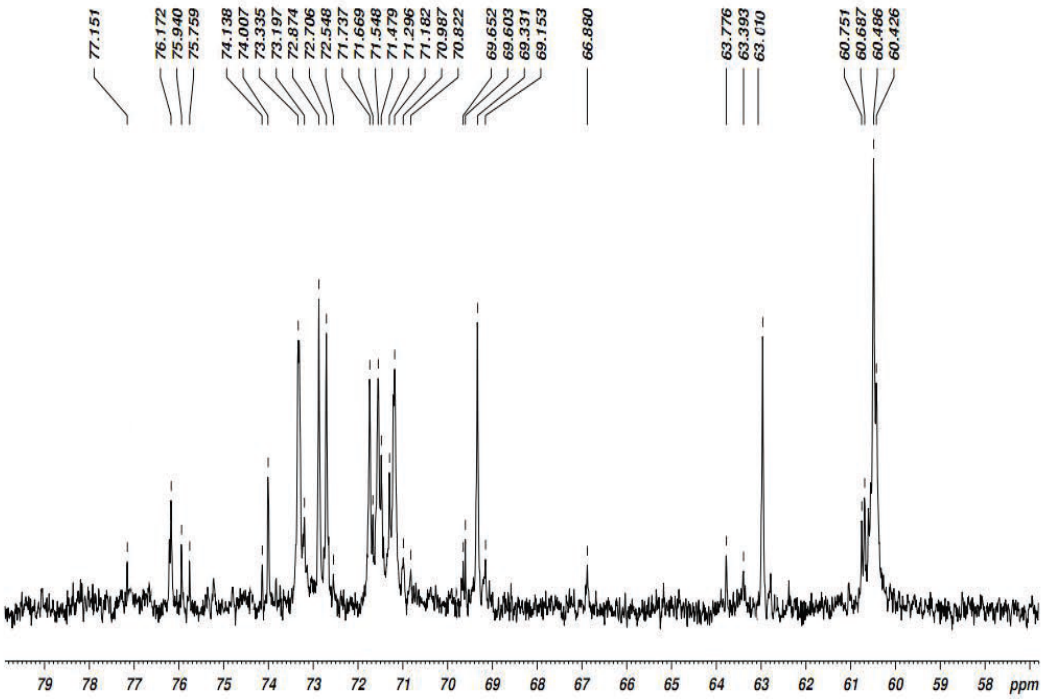

Fig. 11: NMR spectrum of CHL of *Solanum trilobatum* L.

Antiproliferative activity of CHL

Standard CHL on vero cell lines

When Vero cells were incubated with 15.6-1000µg/ml of standard CHL for 48H, there was a significant dose-dependent reduction in cell viability. The IC_{50} value at 48H was 250µg/ml. The extract was devoid of cytotoxic effects on normal vero cell line suggesting it to be selectively cytotoxic to neoplastic cells (fig. 12).

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 (low toxicity), rounding (medium toxicity) and poor adherence (high toxicity) as well as round floating shapes (fig. 13).

Standard CHL on HepG2 cell lines

When HepG2 cells were incubated with 15.6-1000µg/ml of standard CHL for 48H, there was a significant dose-dependent reduction in cell viability. The IC_{50} value at 48H was 31.2µg/ml. (fig. 14). 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 (low toxicity), rounding (medium toxicity) and poor adherence (high toxicity) as well as round floating shapes (fig. 15).

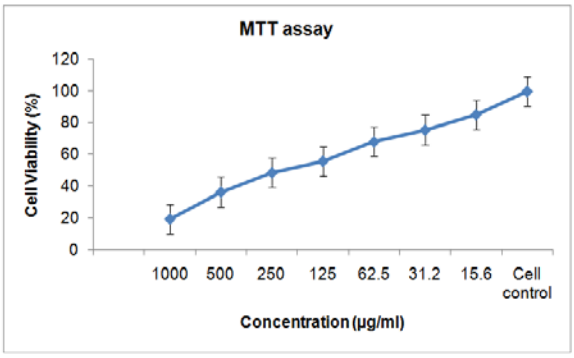

Fig. 12: MTT assay of standard chlorophyllin on VERO Cell line. The IC_{50} value at 48H was 250µg/ml

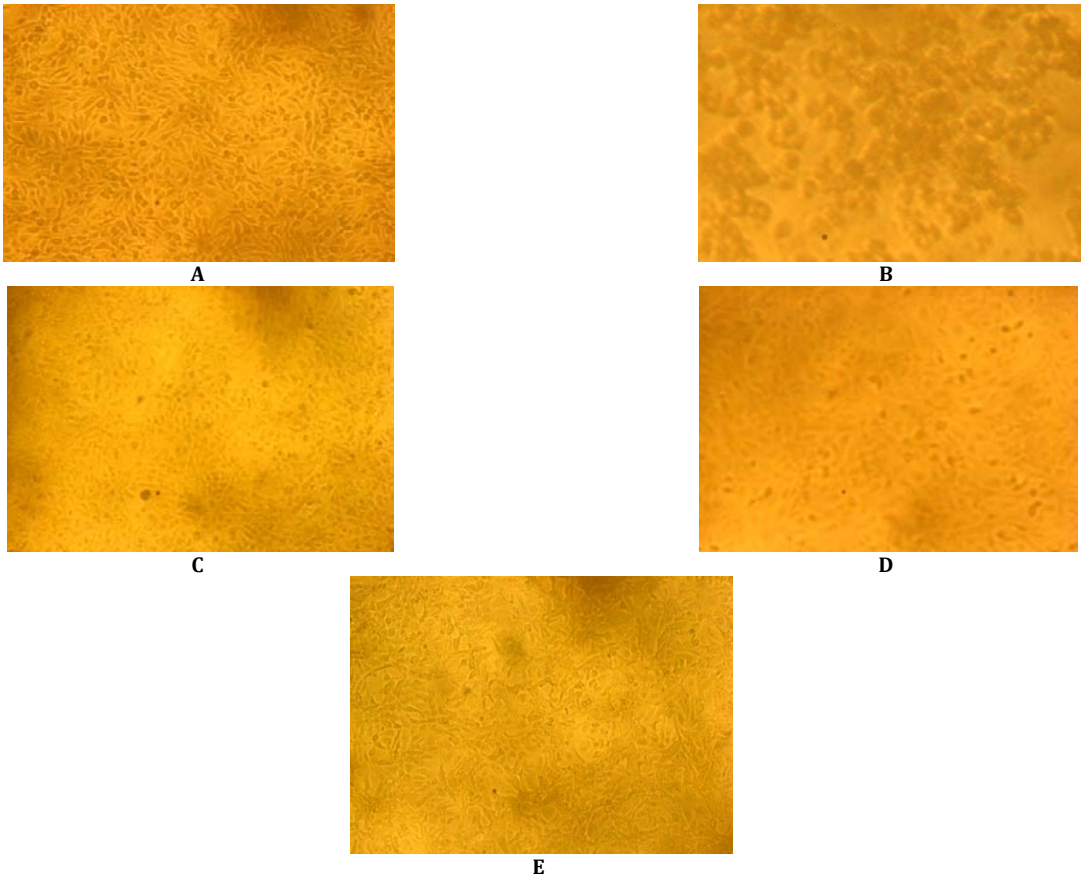

Fig. 13: Anticancer effect of standard Chlorophyllin on VERO cell line, (A): Normal VERO Cell line; (B): 1000µg/ml; (C): 125µg/ml; (D): 62.5µg/ml; (E): 31.2µg/ml

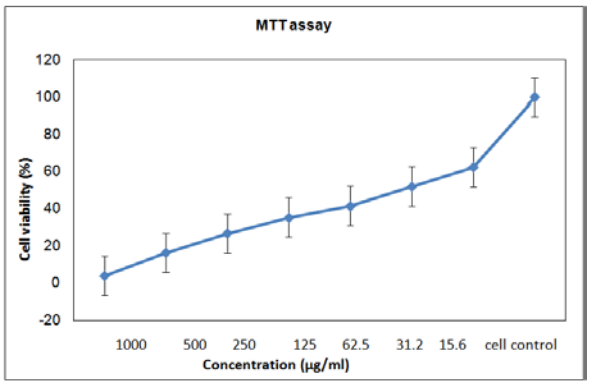

Fig. 14: MTT assay of standard chlorophyllin on HepG2 cell line. The IC_{50} value at 48H was 31.2µg/ml

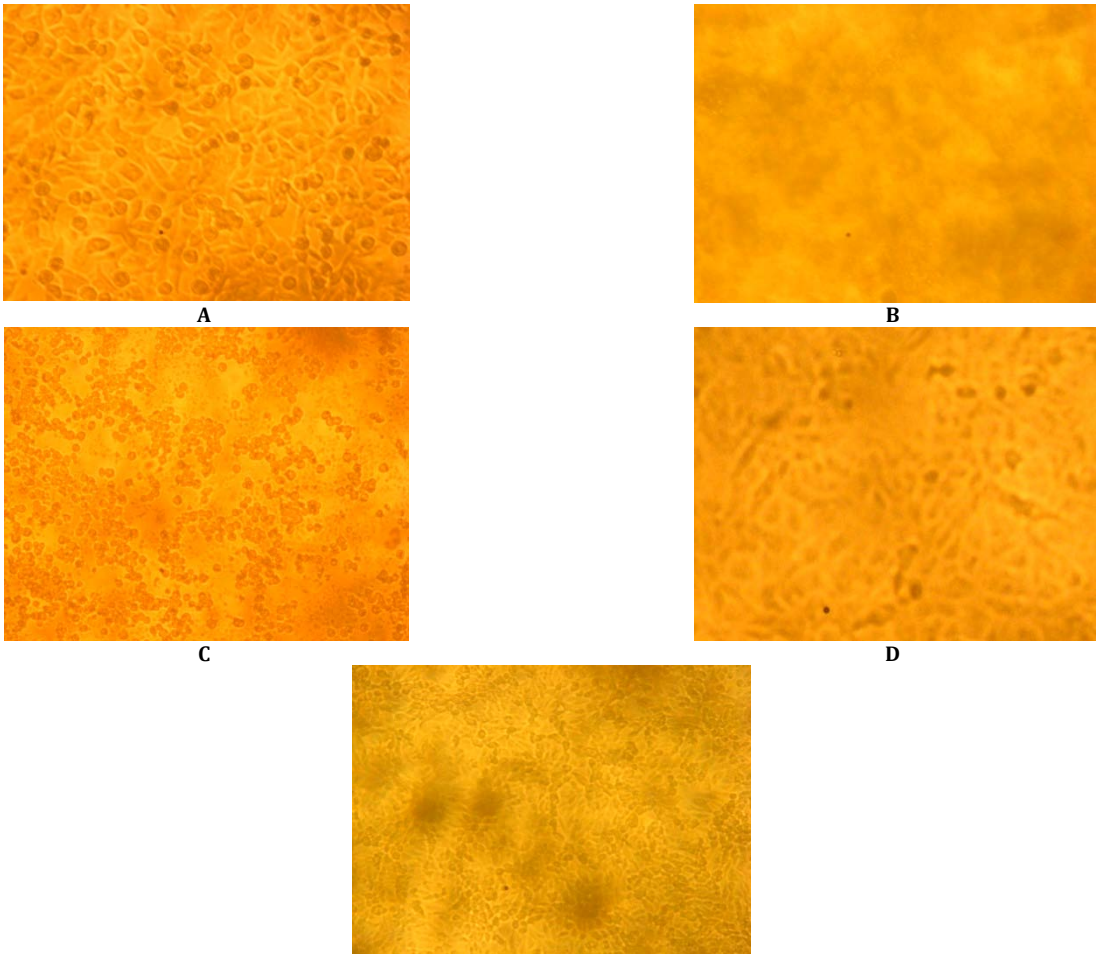

Fig. 15: Anticancer effect of Standard CHL on HepG2 cell line, (A): Normal HepG2 Cell line; (B): 1000µg/ml; (C): 125µg/ml; (D): 62.5µg/ml; (E): 31.2µg/ml

***Solanum trilobatum* L. CHL on Vero cell line**

When vero cells were incubated with 7.8-1000µg/ml of Solanum trilobatum CHL for 48H, there was a significant dose-dependent reduction in cell viability. The IC_{50} value at 48H was 125µg/ml. The extract was devoid of cytotoxic effects on normal vero cell line suggesting it to be selectively cytotoxic to neoplastic cells (fig. 16).

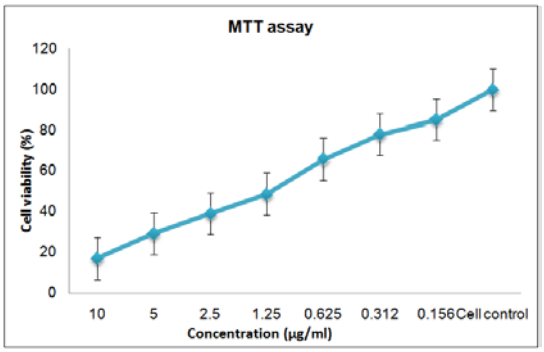

Fig. 16: MTT assay of *Solanum trilobatum* L. chlorophyllin on vero cell line. The IC_{50} value at 48H was 125µg/ml

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 (low toxicity), rounding (medium toxicity) and poor adherence (high toxicity) as well as round floating shapes (fig. 17).

***Solanum trilobatum* L. CHL on HepG2 cell line**

When HepG2 cells were incubated with 7.8-1000µg/ml of *Solanum trilobatum* CHL for 48H, there was a significant dose-dependent reduction in cell viability. The IC_{50} value at 48H was 62.5µg/ml. (fig. 18).

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 (low toxicity), rounding (medium toxicity) and poor adherence (high toxicity) as well as round floating shapes (fig. 19).

DISCUSSION

Chlorophyllin, a food-grade derivative of the green plant pigment chlorophyll has recently been shown to be a potent inhibitor *in vivo* of hepatic aflatoxin B1 (AFB1) DNA adduction and hepatocarcinogenesis [8]. They reported that CHL forms a strong non-covalent complex with AFB1 *in vitro* which may contribute to its anticarcinogenic activity.

Chlorophyll and its derivatives are believed to be among the family of phytochemicals compounds. Water soluble derivatives of chlorophyll including chlorophyllides, chlorophyllin are known to cure cancer well. Although most research has focused on commercial grade SCC, the extent to which natural chlorophyllin derivates modulate biomarkers of cancer is also being exploded.

In the present study *Scoparia dulcis* L., *Stevia rebaudiana*, *Cynodon dactylon* L., *Tamarindus indica* L., *Solanum trilobatum* L. and *Carica papaya* L. were selected for the screening of chlorophyllin.

The water-soluble derivative of chlorophyll i. e sodium copper chlorophyllin was estimated for all the plants and it ranges from 10.11-12.21µg/ml (table 1).

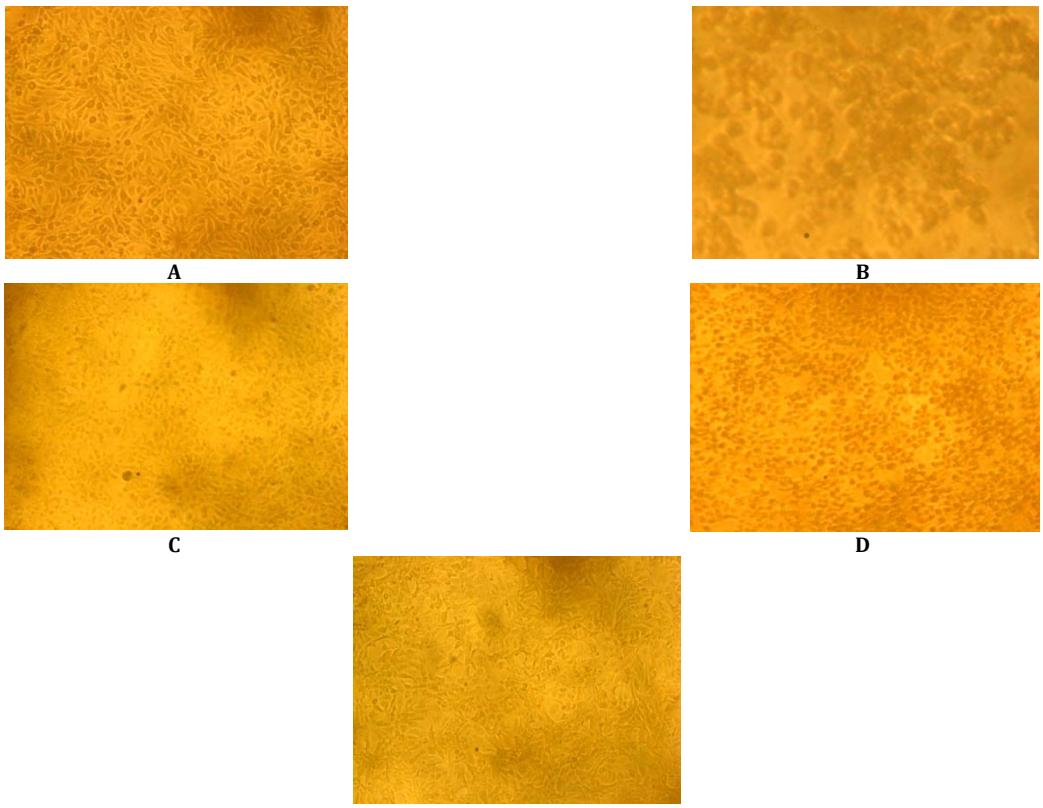

Fig. 17: Anticancer effect of *Solanum trilobatum* L. chlorophyllin on VERO cell line, (A): Normal VERO Cell line; (B): 1000µg/ml; (C): 125µg/ml; (D): 62.5µg/ml; (E): 31.2µg/ml

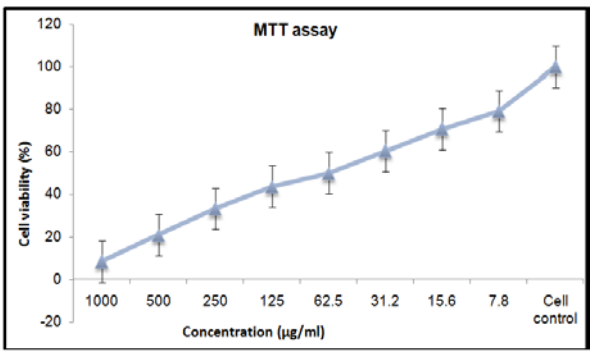

Fig. 18: MTT Assay of *Solanum trilobatum* L. Chlorophyllin on HepG2 Cell line. The IC_{50} value at 48H was 62.5µg/ml.

Structurally, chlorophyllin is a substituted tetrapyrrole with the centrally bond Mg atom. The porphyrin microcycle is further esterified to a diterpine alcohol phytol to form chlorophyll. In nature chlorophyll, a and chlorophyll b predominates in higher plants. The chlorophyll content of commonly consumed green vegetables typically exceeds the level of other bioactive pigments such as carotenoids by up to a 5 fold margin [9].

The chlorophyllin extracted from all the six plants was confirmed by IR spectra. From the analysis of standard chlorophyllin, it was found that the peak at 3392 cm-1 indicates the presence of N-H and O-H group. The peak at 2927 cm-1 indicates the existence of sp3 C-H bond. A peak at 2081 cm^{-1} indicates the presence of C-N stretching. The peaks at 1640 cm^{-1} and 1411 cm^{-1} indicate the presence of aromatic compounds. The broad peak at 1151 cm^{-1} is due to OOP(out of plane) bending vibration arrived due to aromatic ring system or C=C system. The peaks at 1077 $cm^{-1}$is due to bending vibration also supports the existence of carbonyl group. The chlorophyllin samples of the present study also showed peaks like in standard chlorophyllin. It clearly indicates the replacement of Mg with Na^+, K^+ or Cu^+on the central ion in the porphyrin ring structure.(table 2; fig. 3-9).

Hence, the IR spectrum clearly indicates the existence of monovalent substituted carboxyl group, keto group, and nitrogen substituted heterocyclic ring may be porphyrin ring system.

Further, the *Solanum trilobatum* plant leaves were selected for its high chlorophyllin content and were characterized by NMR and it was compared with standard chlorophyllin.

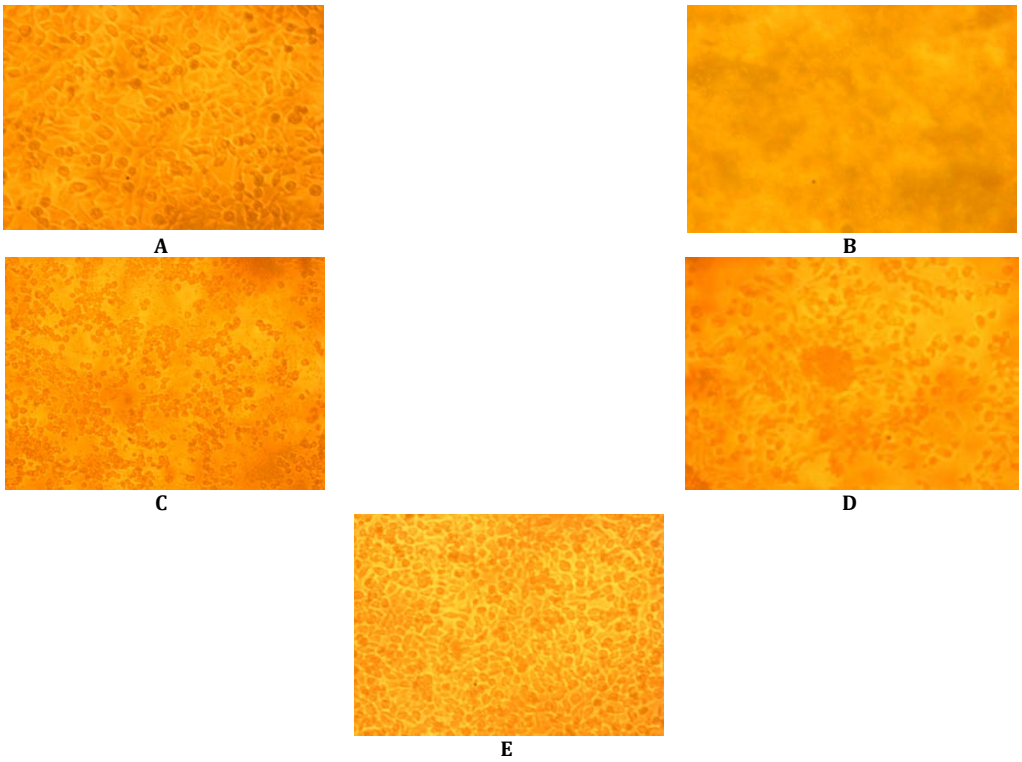

Fig. 19: Anticancer effect of *Solanum trilobatum* L. Chlorophyllin on HepG2 cell line, (A): Normal VERO Cell line; (B): 1000µg/ml; (C): 125µg/ml; (D): 62.5µg/ml; (E): 31.2µg/ml

The peak at 77.151 Δ represents C=O. The peaks from 74.138-70.822 Δ corresponds aromatic structure. The peaks near 69.153-69.652 Δ represent C=N group and the peaks from 63.010–63.776 Δ represents aromatic CH_3 group. The peak at 60.486 Δ corresponds CH_3COONa and the peak at 77.151 Δ represent C=O. The peaks from 74.007–70.182 Δ corresponds aromatic structure. The peaks near 69.603-61.331 Δ represents C=N group and the peaks from 63.010–63.776 Δ represents the aromatic CH_3 group. The peak at 60.486 D Δ represents aromatic CH_3COONa group (table 3; fig. 11).

Researchers indicate that chlorophyllin act as an inceptor molecule in order to block the absorption of aflatoxins and other cancer-causing constituents in the diet [8]. When chlorophyllin is administered along with the carcinogen, the chlorophyllin acts as an inceptor molecule forming a reversible complex with the carcinogen.

Studies show the formation of a complex known covalent bond between the carcinogen and chlorophyllin is a possible mechanism for the interceptor effects of chlorophyllin [10].

The complex formation is possibly due to planar surfaces of the compound binding with the chlorophyllin due to the hydrophobic interactions on the surface of the chlorophyllin and the compound [11].

On the basis of high chlorophyllin content, *Solanum trilobatum* were selected for the further anti-cancerous study. There were many reports related to *Solanum trilobatum* [12-15] but this is the first report of chlorophyllin from fresh leaves of *Solanum trilobatum* on HepG2. It also suggests the benefits of these natural compounds on HepG2.

In the present study, the chlorophyllin of *Solanum trilobatum* showed different anti-carcinogenic properties against two types of cell lines (HepG2 and Vero). The inhibitory effect of chlorophyllin from *Solanum trilobatum* on human cancer cell lines HepG2 (HCC) and non-tumorigenic Vero (African green monkey kidney), cell lines were measured using MTT assay (fig. 12-19).

In the present study, we have demonstrated that the chlorophyllin extract of *Solanum trilobatum* potentially inhibits the proliferation of HepG2 cells by inducing apoptosis (fig. 19) but has no cytotoxic activity in normal Vero cell (fig. 17). The morphological changes in apoptotic character such as cellular shrinkage, rounding, poor adherence and round floating shapes in chlorophyllin treated cells were also observed by phase contrast microscopy (fig. 15, 19). The induction of cancer cell apoptosis without side effect is recognized as an important target in cancer therapy. The chlorophyllin 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

The *in vitro* data presented here suggest that the consumption of the leaves of *Solanum trilobatum* L. as CHL may impart anticancer effects. Further studies are required to elucidate the precise molecular mechanisms and targets for cell growth inhibition which will allow the rationale design of more effective molecules for the eventual use as cancer chemo-preventive and/or therapeutic agents.

ACKNOWLEDGEMENT

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CONFLICT OF INTERESTS

Conflict of interest declared none.

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