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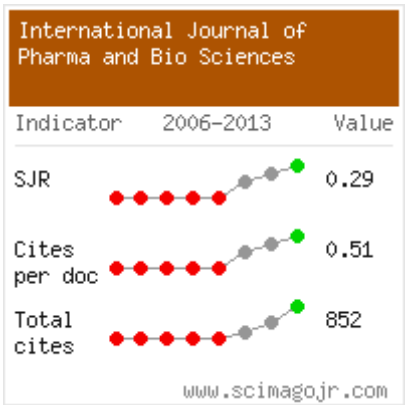

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ANTICARCINOGENIC EFFECT OF CHLOROPHYLLIN FROM *MORINDA CITRIFOLIA* L. ON HEPG2 CELLS

N. BANU[1] AND PAVITHRA. S*[2]

[1] *Associate Professor, Department of Biotechnology, Vels University, Pallavaram, Chennai, Tamil Nadu, India*
[2] *JRF, Department of Biotechnology, Vels University, Pallavaram, Chennai, Tamil Nadu, India*

ABSTRACT

Chlorophyllin (CHL), a water soluble sodium salt of Chlorophyll (Chl) is one among the family of phytochemical compounds. CHL is a promising chemopreventive agent believed to block cancer primarily. Commercial grade CHL is available and being used in most research. The idea was to obtain natural Chl derivative CHL and to study its efficiency as an anticancer agent *in vitro*. The CHL was extracted from the leaves of *Morinda citrifolia* L. (Noni) and characterized by UV-Visible spectroscopy and Infra-Red Absorption spectroscopy. The anticarcinogenic property of CHL was studied *in vitro* against two types of cell lines: HepG2 cell lines (Human Hepatocellular carcinoma) and Vero cell lines (African Green Monkey kidney). It was found that the inhibitory effect of CHL was found on cancer cell lines and absent on Vero cell lines. This is the first report on extraction of CHL from fresh leaves of *Morinda citrifolia* L. and its effect on HepG2 cell lines. The results of the present study show that the CHL extract has a higher safety ratio, which is a good indicator for use in cancer treatment as the extract inhibits only the growth of cancer cells but not normal cells. It also supports for further development of Noni leaves as a neutraceutical or drug.

PATENT No. 624/CHE/2013 PUBLISHED IN PATENT JOURNAL ON 10.10.2014. (U/S 11A) INTELLECTUAL PROPERTY INDIA. PATENTS, DESIGNS & TRADE MARKS.

KEY WORDS: **Chlorophyllin, HepG2 cell lines, Morinda citrifolia L. (Noni), Vero cell lines**

PAVITHRA. S
JRF, Department of Biotechnology, Vels University,
Pallavaram, Chennai, Tamil Nadu, India

***Corresponding author**

INTRODUCTION

Hepatocellular carcinoma (HCC) is known as a common and aggressive malignant tumor worldwide. It is a global health problem and is the fifth most common 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 risky factors of HCC are chronic hepatitis B virus and hepatitis C virus infections, chronic exposure to the mycotoxin or aflatoxin B1(AFB1), and alcoholic cirrhosis [2]. High levels of AFB1 in combination with infection with hepatitis B appear to act synergistically to increase risk of AFB1 and HCC. The liver is the primary site of biotransformation of ingested AFB1. AFB1 is involved in hepatocarcinogenesis in humans by inducing mutations in the p53 gene. AFB1 is metabolically activated to form 8,9-epoxide derivative by cytochrome P-450 and the epoxide binds to guanine residues in DNA to produce 8,9-dihydro-8-(N^7-guanyl)-9-hydroxy AFB1. AFB1 has been classified as a group 1 human carcinogen by the International Agency for Research on Cancer [3]. The development of chemotherapeutic or chemopreventive agents for HCC is important in order to help reduce the mortality caused by this disease [4]. Thus, significant research efforts have focused on novel chemotherapeutic drugs from the plant kingdom in search of cancer inhibitors and cures [5]. CHL is a water-soluble derivative of Chl in which magnesium has been replaced with copper and the phytol chains lost. CHL also act 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 [6]. CHL has been used by human population for over 50 years for medicinal purposes with no adverse effects. It is a very effective inhibitor of numerous mutagens, including AFB1, poly cyclic aromatic hydrocarbons (PAHs), heterocyclic amines, direct-acting compounds and complex mixtures [7]. CHL is a promising chemopreventive agent to block cancer primarily by inhibiting carcinogen uptake through the formation of molecular complexes with the carcinogen such as AFB1. Thus, CHL may diminish the bioavailability of dietary carcinogens by impeding their absorption and by shuttling them through the fecal stream, leading to reduced DNA adduct and tumor burden.

MATERIALS AND METHODS

COLLECTION OF MEDICINAL PLANTS

The medicinal plants were obtained from the fields of Pammal town, situated in Kanchipuram District, Tamilnadu. Six medicinal plants have been chosen for the present study. The selected medicinal plants include *Solanum trilobatum* L.*, Lawsonia inermis* L. *, Vitex negundo* L.*, Morinda citrifolia* L.*, Azadirachta indica* L., *Ocimum sanctum* L.

ESTIMATION OF Chl CONTENT

One gram of finely cut leaf sample was weighed and ground with 20ml of 80% acetone. It was then centrifuged at 5,000rpm for 5 min. The supernatant was transferred and the procedure was repeated until the residue is colorless. The absorbance of the solution was read at 645nm and 663nm against the solvent (80% acetone) blank [8]. The concentrations of Chl a, Chl b and total Chl were then calculated using the equations as follows

.

Chl a (µg/ml)= 12.72(OD 663) – 2.59(OD 645)
Chl b (µg/ml) = 22.9(OD 645) – 4.67(OD 663)
Total Chl (µg/ml) = 20.31(OD 645) + 8.05(OD 663)

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 50-100ml of acetone and filtered using filter paper and the procedure was repeated until the residue is colorless. Finally, it was washed with 100ml 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 consists of flavones. In order to remove the remaining flavones, 1% sodium carbonate was added. The ether solution was poured into a 250ml bottle. To this 10-25ml 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. 100ml of diethyl ether was added to the funnel and left for 30min.The CHL separates as a greenish layer below. The greenish layer was removed and the ether layer was washed with distilled water and dilute 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 [9].

ULTRA VIOLET-VISIBLE SPECTROSCOPIC ANALYSIS

The partially purified CHL was analysed by UV-VIS absorption by dissolving in diethyl ether and read at 405nm 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 in to discs under vaccum 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 was 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.

In Vitro STUDIES

Collection of cell line

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

Maintenance of cell line

Maintenance of cells involves the following operations: dispersion and Sub culturing (seeding) of cells, preservation of cells in repository, revival of cells from repository.

Subculturing and maintenance of cell line

The medium was brought to room temperature for thawing. The tissue culture bottles were observed for growth, cell degeneration, pH and turbidity using an inverted microscope. If the cells (HepG2 & Vero) attained 80% confluent it was taken to sub culturing process. The mouth of the bottle was wiped with cotton soaked in spirit to remove the adhering particles. The growth medium was discarded in a discarding jar (maintain distance between the jar and the flask). Then 4-5 ml of Minimum Essential Medium (MEM) without Foetal Calf Serum (FCS) was added and gently rinsed with tilting. The dead cells and excess FCS were washed out, and the medium was discarded. TPVG was added over the cells and incubated at 37º C for 5 minutes for disaggregation. The cells disaggregate and becomes individual cells and is present as suspension. 5ml of 10% MEM with FCS was added using a serological pipette. Gentle passaging was given using a serological pipette. The process was repeated if any clumps were present. Then the cells were split into 1:2, 1:3 ratio for cytotoxicity studies by plating method.

Seeding of cells

The cell suspension was taken and poured into 24 well plate. In each well 1ml of the

suspension was added and kept in a desiccator at 5% CO_2 atmosphere.

Cell observation

After 2 days of incubation the cells were observed in an inverted microscope and photographs were taken (Olympus, Japan).

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. Stock drug concentration – 100mg/ml 10µl of drug from stock was dissolved in 990µl of DMSO giving a working concentration of 1mg/ml. The working concentration was prepared fresh and filtered through 0.45µ filter before each assay. 500µl of serum free MEM was taken in 9 eppendroff tubes. Then 500µl of the working concentration was added to the first eppendroff tube and mixed well then 500µl of this volume was transferred from first to last tube by serial dilution to obtain the desired concentration of the drug. As a result the volume remains constant, but there is a change in concentration. Each dilution of the drug ranges from 1:1 to 1:64.

Sampling

Fourty eight hour monolayer culture of HEPG2 and VERO cells at a concentration of one lakh cells /well were seeded in 24 well titer plate. The plates were microscopically examined for confluent monolayer, turbidity and toxicity if the cells become confluent. The growth medium (MEM) was removed using a micropipette. Care was taken so that the tip of the pipette did not touch the cell sheet. The monolayer of cells was washed twice with serum free MEM to remove the dead cells and excess FCS. To the washed cell sheet, 1ml of serum free medium containing defined concentration of the drug was added in the respective wells. Each dilution of the drug ranges from 1:1 to 1:64 and they were added to the respective wells of the 24 well titer plates. To the cell control wells 1ml of serum free MEM was added. The plates were incubated at 37ºC in a 5% CO_2 environment and observed for cytotoxicity using inverted microscope.

MTT assay

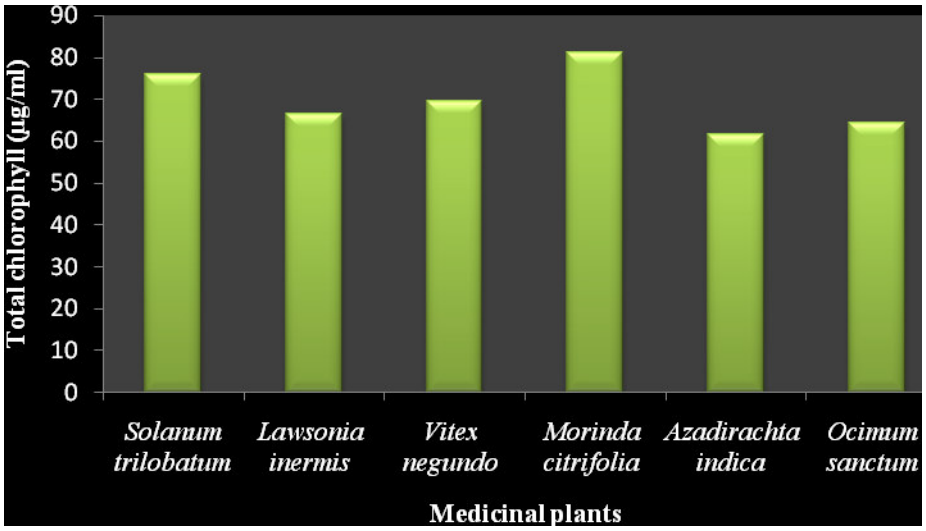
After incubation, the medium was removed from the wells carefully for MTT assay. Each well was washed with serum free MEM for 2–3 times. And 200µl of MTT (concentration 5mg/ml) was added and incubated for 6-7hrs in 5% CO_2 incubator for Cytotoxicity. After incubation 1ml of DMSO was added to each well and mixed using a pipette and left for 45sec. If any viable cells were present the formazan crystals after adding the solublizing reagent (DMSO) showed purple color formation. The suspension was then transferred to the cuvette of spectrophotometer and the OD values were read at 595nm by taking DMSO as a blank. A graph was plotted by taking concentration of the drug on X axis and relative cell viability on Y axis. The percentage of cell viability was calculated using the formula [10]:

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

RESULTS

1. Chl content of fresh leaves of medicinal plants

Graph 1
Total Chl content of six medicinal plants.



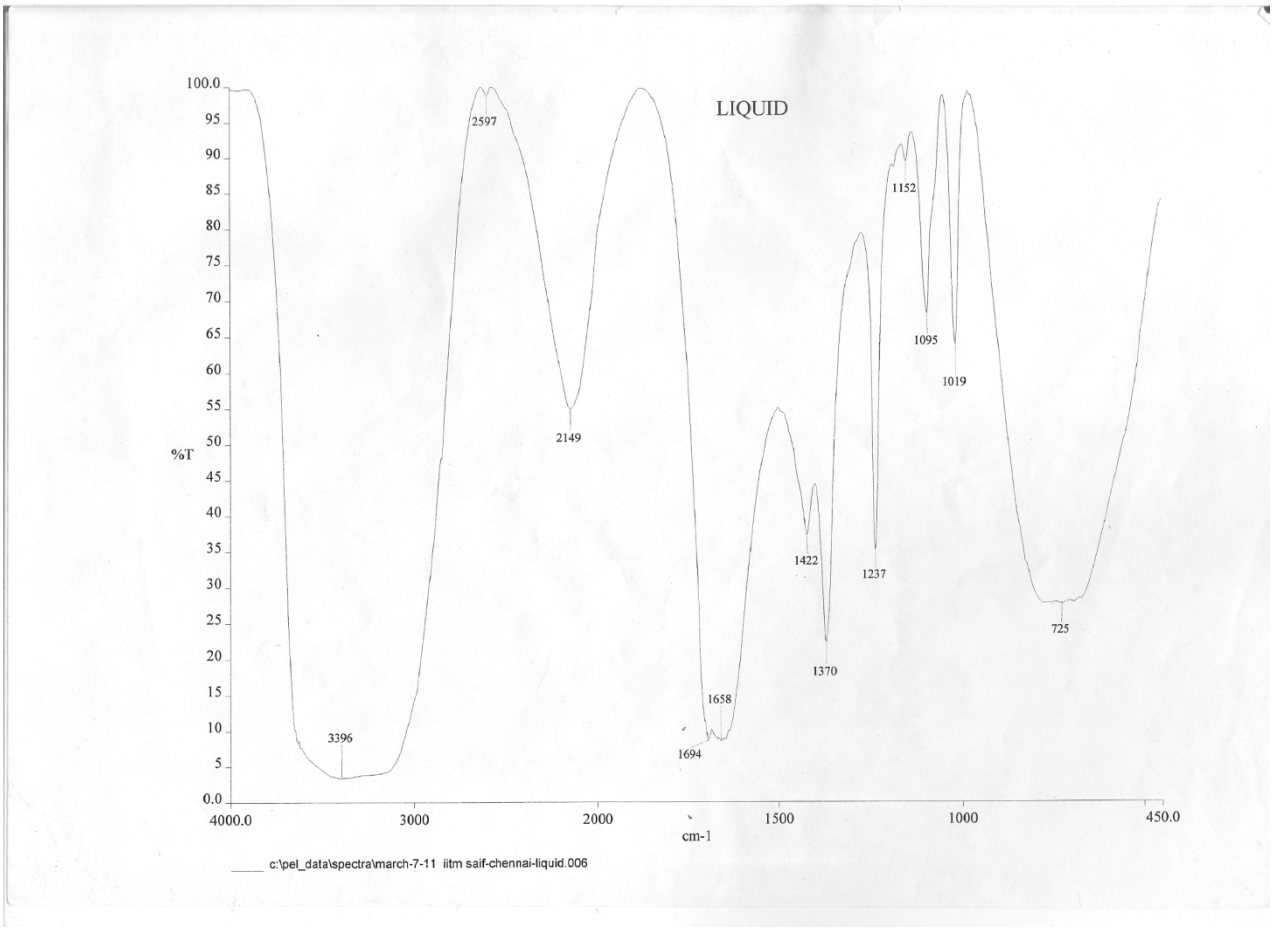
The total Chl content was estimated and was found that Morinda citrifolia L. showed more amount of total Chl (81.22µg/ml), followed by Solanum trilobatum L. (76.03 µg/ml), Vitex negundo L. (69.55 µg/ml), Lawsonia inermis L. (66.56 µg/ml), Ocimum sanctum L. (64.42 µg/ml), Azadirachta indica L. (61.49 µg/ml) (Graph 1). The leaves of Morinda citrifolia L. was selected for further studies for its higher content of Chl.

2. CHL content of Morinda citrifolia L.

CHL (Sodium-copper CHL) was extracted from Morinda citrifolia L. and was estimated as 1590µg/ml.

3 IR interpretation.

Graph 2
IR Spectra of CHL



The presence of CHL was proved by FTIR spectrum at 450-4000 cm^{-1} range (Graph 2). With reference to the sample from the spectra obtained, the following interpretation could be made. Predominant peaks are seen both in the functional group region and fingerprint region. To support for the existence of CHL, the peak observed at 3396cm^{-1} clearly reveals the existence of OH group. The peak at 1422cm^{-1} predominates the existence of COO^- group for the wet sample. The existence of aromatic system is proved by the peak at 1658cm^{-1} and 1422cm^{-1}. The peak at 2149cm^{-1} clearly indicate the existence of C-N stretching. The peak with a small twist at 1694cm^{-1} proves the existence of carbonyl group in conjugation. The peak at 1658cm^{-1} can also be accounted for non-hydrated carbonyl groups. The carbonyl group existing in the COO^- group upon chelation with a monovalent cation like Na^+, K^+, Cu^+ can be characterised by a very sharp intense peak at 1237cm^{-1}. The broad peak at 725cm^{-1} is due to OOP (Out of plane) bending vibration arrived due to the aromatic ring system or C=C system. The peaks at 1095cm^{-1} and 1019cm^{-1} is due to bending vibration, also supports the existence of carbonyl group. A small shoulder at 2597cm^{-1} may be due to C=N stretching will arrive upon conjugation. There is no evidence for existence of sp3 hybridized CH stretching and this may be due to the loss of those lengthy alkyl substituents.

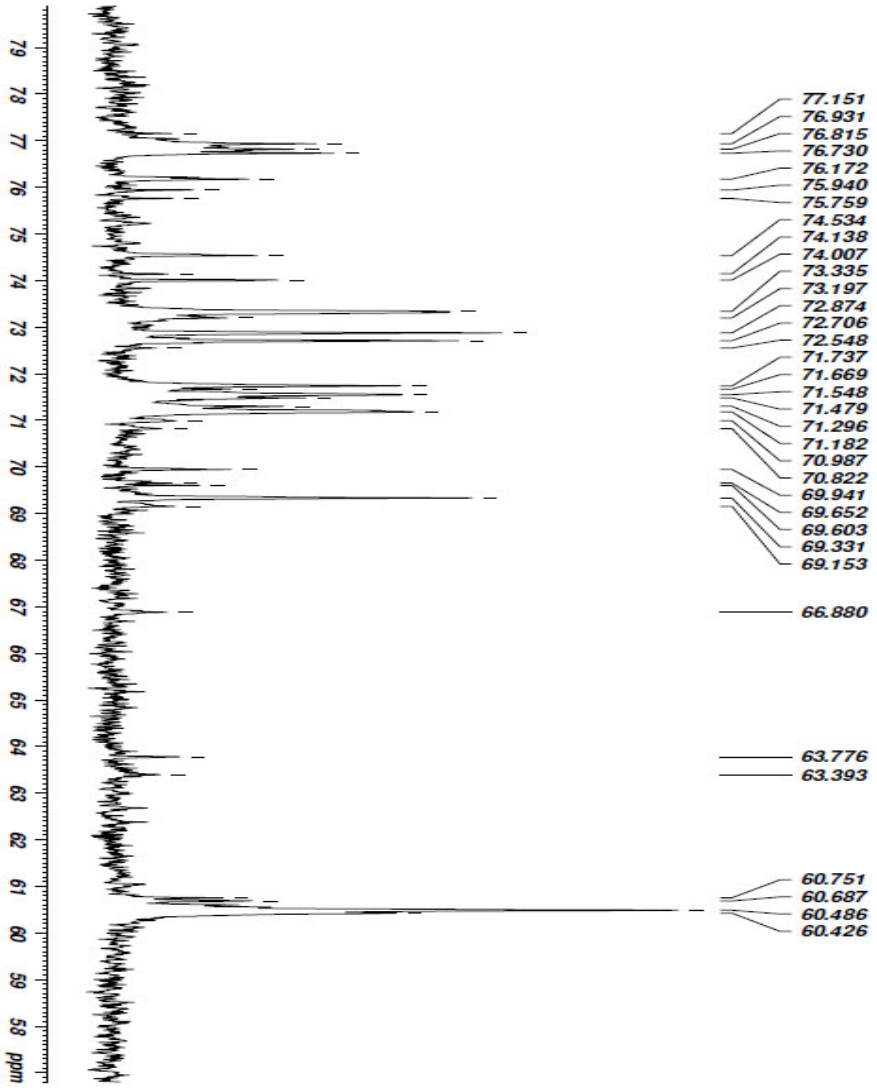
3. NMR spectroscopic analysis

The presence of CHL was further confirmed by the NMR analysis. The sample and the standard CHL were analyzed and compared.

Predominant peaks were seen both in the standard as well as the sample.

Graph 3

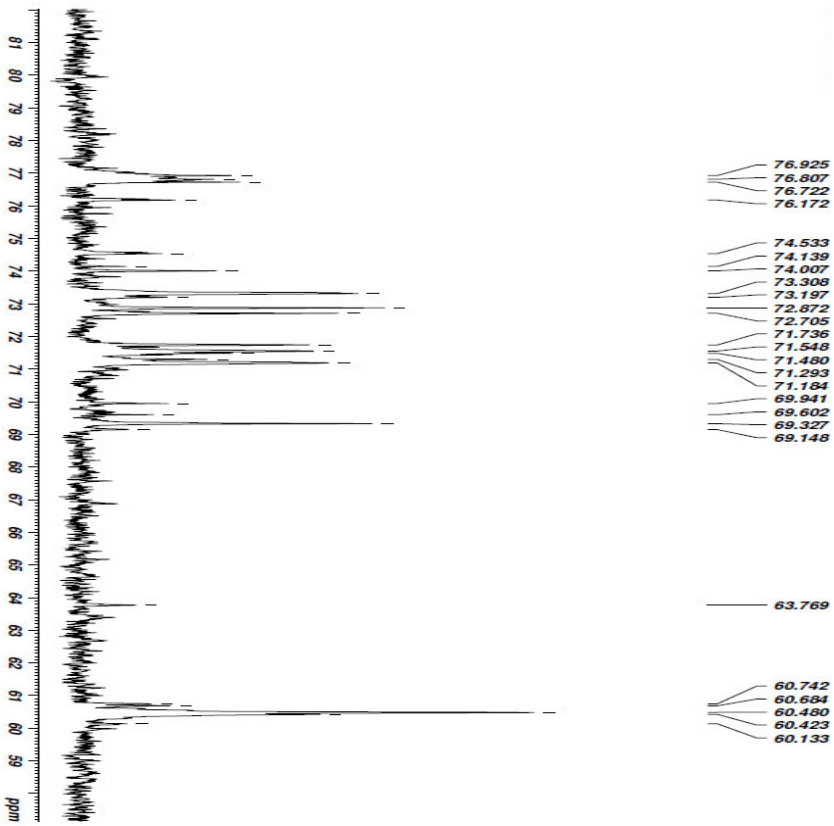
C^{13} NMR Spectrum of Standard CHL



The peaks near 77.15-74.54Δ represent C=N group and the peaks from 74.13-70.83Δ represent aromatic CH3 group. The peak at 60.6Δ corresponds to CH_3COONa (Graph 3).

Graph 4

C^{13} NMR Spectrum of sample CHL



The peaks near 77.10-74.52Δ represent C=N group and the peaks from 74.14-71.19Δ represent aromatic CH3 group. The peak at 60.14Δ corresponds to CH_3COONa (Graph 4).

4. Cytotoxic activities of CHL against HepG2 cells and Vero cells

When HepG2 cells were incubated with 7.8125-1000µg/ml CHL for 24 hours, there was a significant dose dependent reduction in cell viability. The IC_{50} value at 24 hour was 62.5µg/ml CHL (Graph 5, Table 1). Whereas the extract was devoid of cytotoxic effects on normal Vero cells, suggesting it to be selectively cytotoxic to neoplastic cells (Graph 6, Table 2). These findings indicated that CHL significantly decreased proliferation of HepG2 cells in a dose and time dependent manner.

Graph 5
MTT Assay for HepG2 cell lines.

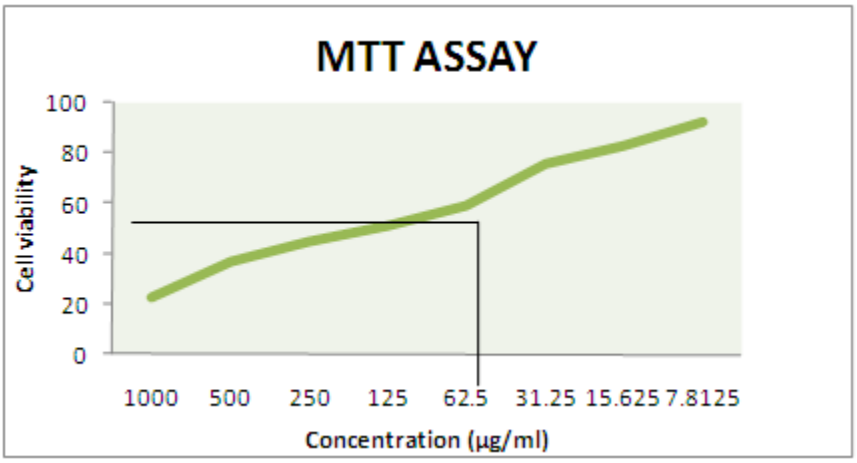


Table 1
Effect of CHL on HepG2 cell lines

S.no	Concentration (µg/ml)	Dilutions	Cell viability
1	1000	Neat	22.56
2	500	1:1	36.69
3	250	1:2	44.59
4	125	1:4	51.23
5	62.5	1:8	58.96
6	31.25	1:16	75.56
7	15.625	1:32	82.36
8	7.8125	1:64	92.15
9	Cell control	-	100

Graph 6
MTT Assay for Vero cell lines.

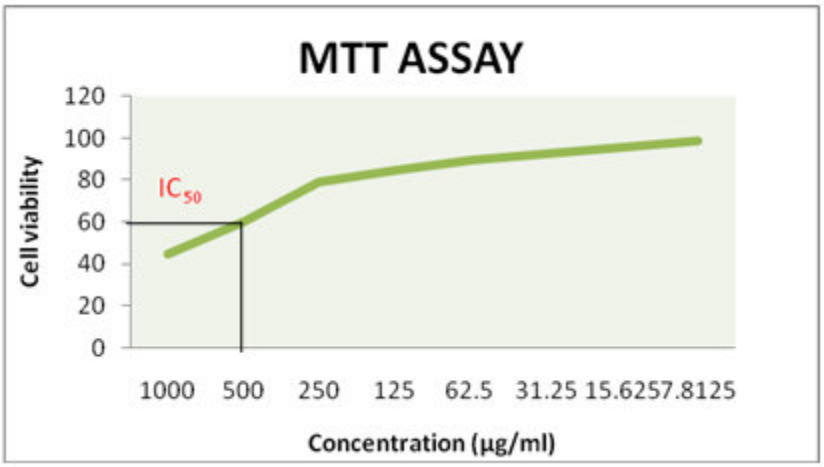


Table 2
Effect of CHL on VERO cell line

S.no	Concentration (µg/ml)	Dilutions	Cell viability
1	1000	Neat	44.56
2	500	1:1	59.69
3	250	1:2	78.85
4	125	1:4	84.43
5	62.5	1:8	89.21
6	31.25	1:16	92.23
7	15.625	1:32	95.65
8	7.8125	1:64	98.52
9	Cell control	-	100

Effects of CHL on cell morphology

After incubation with CHL at different concentrations (7.8125-1000µg/ml), the cells were examined by inverted microscope for evidence of morphological apoptosis induced by CHL (Fig 1 & 2). The Vero cells showed a typical polygonal and intact appearance, whereas the CHL treated cells displayed morphological changes with preapoptotic characteristics, such as cellular shrinkage (low toxicity), rounding (medium toxicity) and poor adherence (high toxicity) as well as round floating shapes

.

Figure 1
Cytotoxicity effect of CHL on VERO cell lines

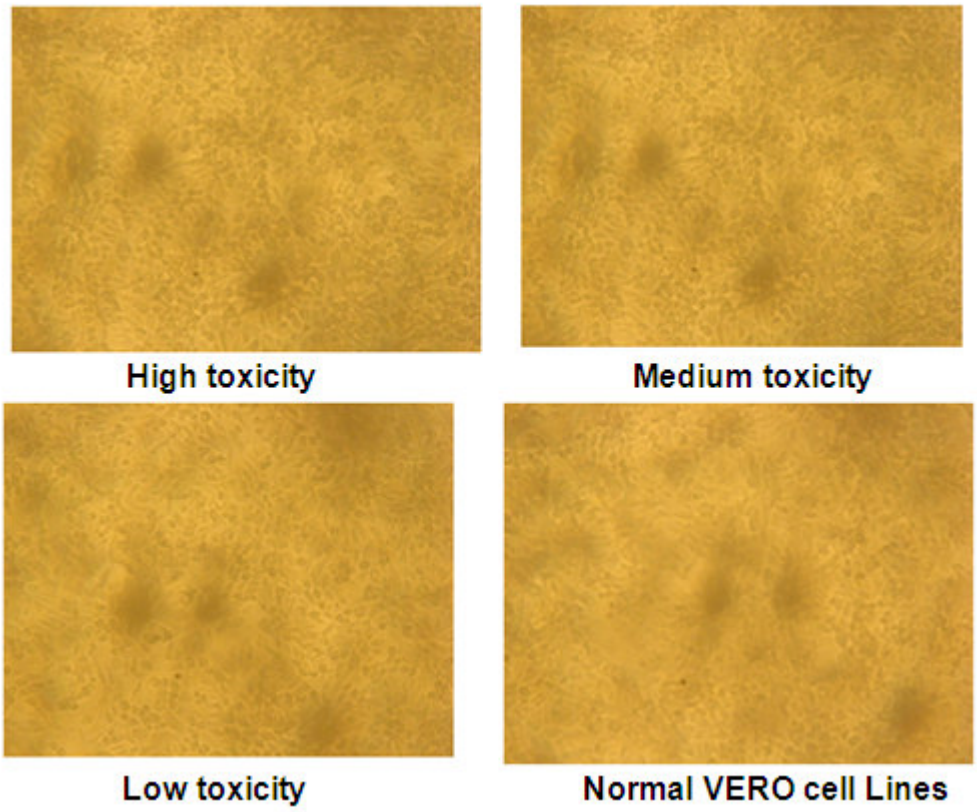
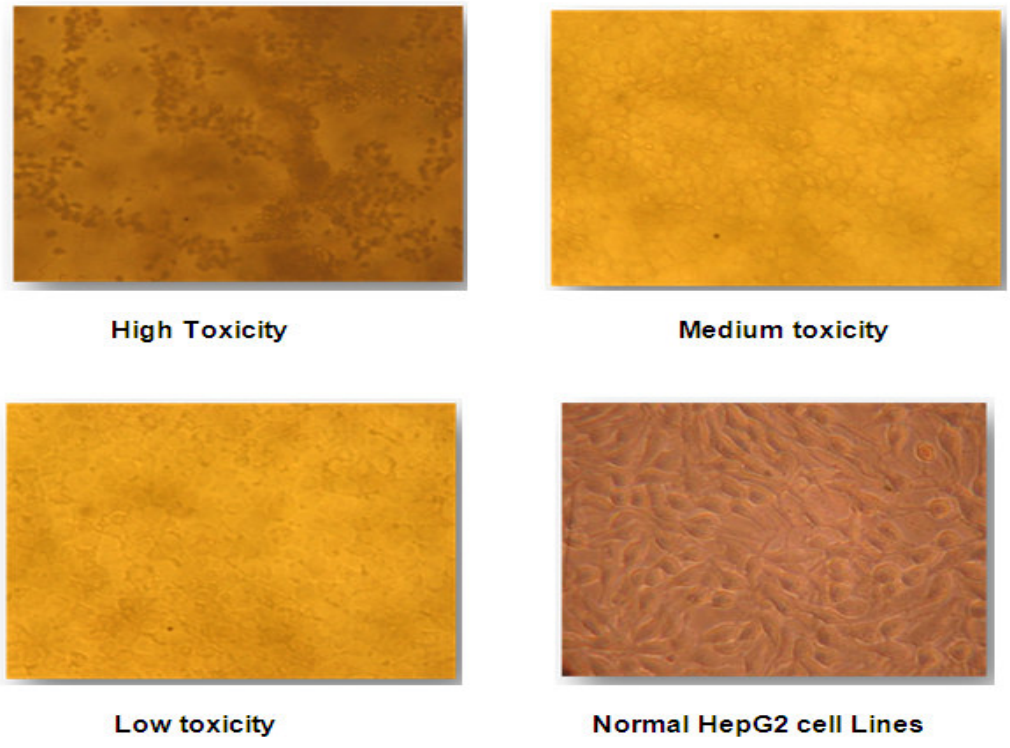


Figure 2
Cytotoxicity effect of CHL on HEPG2 cell lines



DISCUSSION

Cancer preventive effects of Chl derivatives have been extensively studied with particular emphasis on their *in vitro* antimutagenic activity against numerous dietary and environmental mutagens. Sodium copper CHL has demonstrated the ability, *in vitro*, to effectively protect against mutagenic activity of both direct and indirect acting dietary and environmental mutagens [11]. Structurally, Chl is a substituted tetrapyrole with a centrally bound Mg atom. The porphyrin macrocycle is further esterified to a diterpene alcohol, phytol to form Chl. In nature, Chl a and b predominates in higher plants. The Chl content of commonly consumed green vegetables typically exceeds the levels of other bioactive pigments, such as carotenoids by upto a 5 – fold margin 12. The inhibition of tumor cell growth without toxicity in normal cells has attracted attention as an important target in cancer therapy. 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 AFB1[13]. The CHL extracted from the leaves of *Morinda citrifolia* L. for the present study was confirmed by IR spectra (Graph 2). From the analysis, it was found that peaks were obtained at 3396cm^{-1}, 1422cm^{-1}, 1658cm^{-1}, 2149cm^{-1}, 1694cm^{-1}, 1237cm^{-1}, 1095cm^{-1}, 1019cm^{-1}, 725cm^{-1}, 597cm^{-1} which indicates the existence of OH group, COO^- group, aromatic system, C-N stretching, carbonyl group, monovalent cations like Na^+, K^+, Cu^+, C=C system and C=N stretching respectively. It clearly indicates the replacement of Mg with Na^+ or K^+ or Cu^+ as a 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. Few of the above mentioned characteristic peaks clearly indicates the existence of CHL ring system. Further, the CHL of *Morinda citrifolia* L. was characterized by NMR and it was compared with standard CHL. The peaks near 77.15Δ-74.54Δ represents C=N group and the peaks from 74.13Δ-71.19Δ represent aromatic CH3 group. The peak at 60.14Δ corresponds to CH3 COONa (Graph 3 and 4). The proliferation of human HCC MHCC97H cells were decreased by *Chrysanthemum indicum* extract (CIE) when incubated for 24, 48, 72 hours with different concentrations of CIE. A significant dose dependent reduction was found in the cell viability [14]. Aqueous extracts of some medicinal plants viz. *Artemisia vulgaris, Cichorium intybus, Smilax glabra, Solanum nigrum* and *Swertia chirayta* have been tested for its anticarcinogenic property [15]. They observed the inhibitory effect of extract of *Solanum nigrum* and *Artemisia vulgaris* on cell growth and colony formation of the prostrate, breast and colorectal cells. The dichloromethane extract of fresh leaves showed a better inhibitory effect against KB and HeLa cells with IC_{50} values of 21.67 and 68.50µg/ml respectively. The dichloromethane extract of dried leaves revealed cytotoxicity against the KB cell line with an IC_{50} value of 39.00µg/ml. Other extracts as well as rutin, scopoletin showed reduced antiproliferative effects on all cancer cell lines (IC_{50} 103 to over 600 µg/ml). But none of the extracts had inhibitory effects on MCF-7, HepG2 and non-tumorigenic vero cells [16]. In the present study, the CHL extract of *Morinda citrifolia* L. showed different anticarcinogenic properties against two types of cell lines: HepG2 cell lines and Vero cell lines. The inhibitory effect of CHL from *Morinda citrifolia* L. on human cancer cell lines HepG2 and non tumorigenic Vero cell lines were measured using a MTT colorimetric assay. The inhibitory effect of CHL was found to be present on cancer cell lines. The HepG2 cells displayed morphological changes with preapoptotic characteristics such as cellular shrinkage, rounding, poor adherence as well as round floating shapes. The greatest antiproliferative effect on HepG2 cells with IC_{50} values of 58.96% was found in the dilution of 1:8 with a respective concentration of 62.5µg/ml (Table 1, Graph 5, Fig 2). The CHL extract showed different anti-carcinogenic properties against the two types of cells: HepG2 cell lines and Vero cell lines. The inhibitory effect of CHL was found

to be absent on Vero cell lines. The cells showed a typical polygonal and intact appearance (Fig 1). The viability of these normal cells was found to be high in all the dilutions (Table 2, Graph 6). The CHL extract 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. The parts of the plant have been traditionally used for treatment of various complaints, including use of analgesia [17], antibacterial effects [18], anti-inflamatory effects [19-21], anticancer effects [22], antifungal effects, as an antidiabetic drug [23], for cancer chemoprevention [24, 25]and for immune stimulation [26, 27]. Noni fruit juice has emerged on the USA market as a safe and popular food supplement during the past decade, is supported by research in the USA [28]. The leaves were investigated and reported to have several polyphenolic compounds, including ursolic acid, quercetin, kaempferol and rutin [29, 30]. An ethanolic extract of noni leaves posses wound healing activity [31] and has been shown to be safe in acute, sub-acute and sub-chronic oral toxicity tests on mice [32]. Rutin has been reported to have antioxidant, anti-diabetic, anti-inflammatory and anticancer activity [33, 34]. Rutin appears to be a marker of the antiproliferative and antioxidant properties of noni. If these activities of Thai noni/Yor leaf extract were established, then the leaf extract would have potential as a food supplement or as chemoprevention.

CONCLUSION

The *In vitro* data presented here suggests that the consumption of the leaves of *Morinda citrifolia* L. or 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 for more effective molecules for the eventual use as cancer chemopreventive and /or therapeutic agents.

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Conflict of interest

Conflict of interest declared none.

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