

IN VITRO STUDIES ON ANTICANCER ACTIVITY OF CAPSAICINOIDS FROM *CAPSICUM CHINENSE* AGAINST HUMAN HEPATOCELLULAR CARCINOMA CELLS

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ABSTRACT

Objective: To evaluate the antitumor potential of capsaicinoids extracted from *Capsicum chinense* Bhut Jolokia.

Methods: The capsaicin and dihydrocapsaicin extracted in acetonitrile were detected and quantified by HPLC. *In vitro* studies were performed to examine the effects of capsaicinoids on (HepG2 cells) after exposure to acetonitrile (AN) extract at different concentration. The MTT assay for cell viability and markers such as Lactate dehydrogenase (LDH), Nitric oxide (NO) and Lipid peroxidation (LPO) were predictable to confirm the cytotoxicity.

Results: Peaks of capsaicin and dihydrocapsaicin were identified by HPLC and calibrated with standard Capsaicin. The treatment of AN extract on HepG2 cells showed reduction in the cell viability through MTT assay and it also significantly suppressed the release of LDH, LPO and NO production in a dose-dependent manner.

Conclusion: Thus the study confirmed the anticancer property of the capsaicinoids in AN extract, through modulating the free radicals release and also established anti-inflammatory potential.

Keywords: Anticancer activity, Capsaicinoids, LDH, NO, LPO, HepG2 cells,

INTRODUCTION

Fruits and vegetables are natural medicines and have been used in our daily diet. Phytochemicals present in the dietary fruits and vegetables have anticancer properties [1]. Capsaicinoids are naturally occurring phenolic compounds commonly present in the genus *Capsicum* (*Solanaceae*) and having remarkable antimutagenic and antitumor property [2-4]. Among the chilli varieties *C. chinense* contains the highest concentration of capsaicinoids [5]. Capsaicin and dihydrocapsaicin are the most abundant capsaicinoids in pepper fruits [6-7]. Diverse studies have shown that capsaicin has anti-proliferative effect on several human cell lines derived from multiple myeloma [8], gastric cancer, [9], pancreatic cancer [10], breast cancer [11] and prostate cancer [12] etc.

Over the past decade, there is continuous increase in Hepatocellular carcinoma (HCC) in the world as the most common malignant diseases [13]. Shang-Pang Huang et al., (2009) has reported the elevation of intracellular Ca²⁺ production, ROS and caspase-3 activity in HepG2 cells treated with capsaicin. In recent findings, capsaicin is seen to show anti-proliferative effects on various human cancer cell lines by apoptosis mediated cell death [14]. It suppresses the expression of the inhibitor of caspase activated DNase to induce apoptosis of human melanoma cells [15].

It induces apoptosis through increased intracellular reactive oxygen species (ROS) and calcium levels in a carcinoma cell model [16]. Capsaicin has analgesic-anti-inflammatory agent targeting the nociceptive primary afferent neurons [17]. Capsaicin a promising molecule with pharmacological properties could be developed for the new generation therapeutic drug. The economic, nutritious and pharmacological importance of *Capsicum* sp is accountable for its high demand in the market.

Several phytochemical studies have been conducted to optimize techniques for purification of capsaicin to meet the high value demand. Hence the present study was conducted to find out the

effect of acetonitrile crude extract of *Capsicum chinense* Bhut Jolokia on human HepG2 cells for its anticancer activity.

MATERIAL AND METHODS

Sample collection

Capsicum chinense fruits were obtained from Manipur, North India. The morphology of the fruit shape, colour, seed colour and size of the *C. chinense* were examined following Moscone [18] and Dias [19]. The fruits were dried by traditional method i.e. sun dried for a day, ground, sieved through 20–30 mesh and kept in air tight containers until further process. The standard Capsaicin (8-methyl-*N*-vanillyl-trans-6-nonenamide) was purchased from Sigma Chemical Co, St. Louis, MO, USA. All solvents used for capsaicinoid analysis were of HPLC grade from Merck.

Extraction

The extraction and quantification of capsaicinoids in polar aprotic solvent was performed according to Collins [20] with little modifications. The chilli powder was mixed with acetonitrile in the ratio of (1:10; gram: milliliter). The mixture was placed in 120 ml glass bottles with Teflon lined lids. Bottle was capped and placed at 65°C water bath for an hour and swirled manually. Samples were removed from water bath and cooled at room temperature. The supernatant was centrifuged at 10000 rpm then filtered through Whatman No.1 filter. The filtrates were concentrated and stored at 5°C until further analysis.

Quantification of Capsaicinoids by High Performance Liquid chromatography (HPLC)

The samples were filtered through 0.45 µm (Millipore filter) using a 5 ml disposable syringe (Millipore, Bedford, MA) into a sample vial. A Shimadzu (LC-10, Shimadzu, Japan) HPLC system equipped with LC-10AS multisolvent delivery system, a SPD-10A UV-Vis detector at 280 nm and control of parameters with system controller unit (SCL-10A) was used. The analysis was carried out with the following

conditions: column temperature 30°C, flow rate of mobile phase; 1 ml/min and data acquisition was made using Class LC-10 software. All analyses were performed isocratically using degassed HPLC grade 50% acetonitrile (Merck) and 50% milli 'Q' water as a mobile phase. The reverse-phase chromatographic column (Discovery C18 (250 x 4.6 mm, 5 mm), Supelco, and Bellefonte, PA, USA) was used for the detection of capsaicin and dihydrocapsaicin [20].

Simple linear regression curve for capsaicinoids by HPLC

The simple linear regression curve was plotted using standard capsaicin purchased from Sigma Chemicals. A stock solution of one milligram capsaicin per milliliter of ethanol was dissolved and different concentrations from the stock solution namely 1000µg, 600 µg, 200 µg, 50 µg and 1 µg. Standard curves were developed using concentration versus peak area and capsaicinoids were quantified using linear equation were prepared.

Quantification of capsaicinoids in acetonitrile extracts by HPLC

A 5µl aliquot was used for each HPLC injection. Capsaicinoids were identified with reference to retention time of standards and by spiking the samples with standards. The major capsaicinoids in peppers, capsaicin and dihydrocapsaicin, were determined by comparison to external reference standards injected under the same conditions. Their identification was based on the retention times measured under identical HPLC conditions while their quantitative determination in the different peppers samples was carried out using the peak areas. The ratio between these capsaicinoids was calculated by dividing capsaicin and dihydrocapsaicin contents by the total capsaicinoids [21]. The capsaicinoids concentrations in samples are expressed as µg/g pepper.

Scoville Heat Unit Conversion

According to the commonly accepted Scoville organoleptic test, the spicy strength of the investigated samples was calculated by converting the capsaicin content expressed in grams of capsaicin per gram of pepper. This conversion to Scoville heat units was done by multiplying the capsaicin content in pepper dry weight by the coefficient corresponding to the heat value for pure capsaicin, which is 1.6×10^7 [22].

Anticancer activity

HepG2 cell lines were obtained from National Centre for Cell Sciences, Pune, India and grown on Dulbecco's modified essential medium supplemented with 10% (v/v) fetal calf serum, Penicillin (100 U/mL) and Streptomycin (100 µg/mL). Cells were seeded in 24 well plates at a concentration of 3×10^4 cells/mL of DMEM/well and incubated for 48 h at 37° C under 5% CO₂ to attain confluence. The cells were then treated with various concentrations (10, 25, 50 and 100µg/ml) of acetonitrile extract of *Capsicum chinense* fruit and positive control Cyclophosphamide (5µg). The, the cells were then incubated for 24 h and cell supernatants (100 µL) were analyzed for leakage of LDH levels, using commercial spectrophotometric kits. The cells were used to analyse viability, NO production and lipid peroxidation. The experiments were carried out in triplicates in each group.

Estimation of cell viability- MTT assay

The MTT assay is a colorimetric assay for measuring the activity of cellular enzymes that reduce the tetrazolium dye, MTT, to its insoluble formazan, giving a purple color. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole), is reduced to purple formazan in living cells. A solubilization solution (usually either dimethyl sulfoxide, an acidified ethanol solution, or a solution of the detergent sodium dodecyl sulfate in diluted hydrochloric acid) is added to dissolve the insoluble purple formazan product into a colored solution.

The absorbance of this colored solution can be quantified by measuring at a certain wavelength (usually between 500 and 600 nm) by a spectrophotometer. Cell proliferation was measured using MTT assay. After 24 hours of treatment, 20 µL of MTT solution (5 mg MTT in 1 mL PBS) was added per well and incubated at 37° C for 4 hours in 5% CO₂ atmosphere. Then, the medium was removed

and washed with PBS; 200 µL of DMSO was added to each well. The intensity of the coloured product was measured using an ELISA microplate reader at 570/620 nm. The results were expressed as the percent optical density of treated cells to that of the control cells.

Assay for Lactate dehydrogenase (LDH)

LDH is a soluble cytosolic enzyme present in most eukaryotic cells, released into culture medium upon cell death due to damage of plasma membrane. The increase of the LDH activity in culture supernatant is proportional to the number of lysed cells. LDH Cytotoxicity Assay provides a colorimetric method to measure LDH activity using a reaction cocktail containing lactate, NAD⁺, diaphrose and INT. LDH catalyses the reduction of NAD⁺ to NADH in the presence of L-lactate, while the formation of NADH can be measured in a coupled reaction in which the tetrazolium salt INT is reduced to a red formazan product.

The amount of the highly colored and soluble formazan can be measured at 490 nm spectrophotometrically. Upon completion of the incubation, 50 µL of the upper phase were collected from each well. The cells were then lysed with a cell lysis solution for 40 minutes at room temperature and the lysate was collected. LDH activity was measured using LDH release quantification (Fischer scientific, India) Cytotoxicity Assay Kit, in accordance with manufacturer's instructions. Then the percentage of LDH released from the cells was determined.

Assay for NO production

Nitric oxide (NO) plays an important role in neurotransmission, vascular regulation, immune response and apoptosis. NO is rapidly oxidized to nitrite and nitrate which are used to quantitate NO production. Nitric oxide (NO) is a reactive radical that plays an important role in many key physiological functions. NO, an oxidation product of arginine by nitric oxide synthase (NOS) is involved in host defence and development, activation of regulatory proteins and direct covalent interaction with functional biomolecules. Simple, direct and non-radioactive procedures for measuring NOS are becoming popular in Research and Drug Discovery. Nitric Oxide Synthase Assay involves two steps: a NOS reaction step during which NO is produced followed by an NO detection step. Since the NO generated by NOS is rapidly oxidized to nitrite and nitrate, the NO production is measured following reduction of nitrate to nitrite using an improved Griess method.

The procedure is reduced to as short as 40 min. This assay determines nitric oxide based on the enzymatic conversion of nitrate to nitrite by nitrate reductase. The reaction is followed by a colorimetric detection of nitrite as an azo dye product of the Griess reaction. The Griess reaction is based on the two-step diazotization reaction in which acidified NO₂⁻ produces a nitrosating agent which reacts with sulfanilic acid to produce the diazonium ion. This ion is then coupled to N-(1-naphthyl) ethylenediamine to form the chromophoric azo-derivative which absorbs light at 540 nm (Miles, 1996). After 24 hours of incubation, the level of NO production was monitored by measuring the nitrite concentration in the supernatant of cultured medium using the Griess reagent.

Estimation of lipoperoxides

Quantification of lipid peroxidation is essential to assess oxidative stress in pathophysiological processes. Lipid peroxidation forms Malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), as natural bi-products. Measuring the end products of lipid peroxidation is one of the most widely accepted assays for oxidative damage. Lipid Peroxidation Assay provides a convenient tool for sensitive detection of the MDA in a variety of samples. The MDA in the sample is reacted with Thiobarbituric Acid (TBA) to generate the MDA-TBA adduct.

The MDA-TBA adduct can be easily quantified colorimetrically ($\lambda = 532$ nm) or fluorometrically (Ex/Em = 532/553 nm). This assay detects MDA levels as low as 1 nmol/well colorimetrically and 0.1 nmol/well fluorometrically. After treatment, the cells were trypsinized, suspended in 0.5 mL of PBS and sonicated for 10

seconds. To this 0.5 mL of TCA-TBA reagent was added and heated to 100^o C for an hour and centrifuged. The extent of lipid peroxidation was quantified by estimating the levels of malondialdehyde. The absorbance was measured at 535 nm and the results were expressed as nmol/mg protein [23].

RESULTS

HPLC analysis

The chromatograms obtained for acetonitrile crude extracts of *C. chinense* fruit showed two major peaks, identified as capsaicin and dihydrocapsaicin, which registered a difference of 0.38 min between the retention periods of the capsaicin (5.18 min) and the dihydrocapsaicin (5.56 min) which corresponds to the standard Capsaicin (Fig.1). In our study the HPLC analysis of acetonitrile extracts showed 11,161,030 SHU for capsaicin and 4,830,189 SHU for dihydrocapsaicin respectively (Table.1). The total capsaicinoids present in the acetonitrile extract was found to be 15,991,219 SHU.

Anticancer activity

The anticancer activity of the extract was confirmed by MTT assay. The control HepG2 cells showed high proliferation that has been taken as 100%. In the present study, the treatment with the AN extracts suppressed the cell viability upto 50% at 50 μ g/ml concentrations when compared to the untreated cells (Fig 2A and Fig 3). To confirm the anticancer activity further, LDH leakages were measured in the treatment condition. The treatment with AN extract significantly suppressed the release of LDH at $57.95 \pm 5.1\%$ for 50 μ g/ml in HepG2 cell lines and lightened the anticancer property. The positive control of Cyclophosphamide 5 μ g/ml reduced the release of LDH upto $35.19 \pm 4.1\%$ (Fig2 B). The increased NO production ($41.99 \pm 2.4 \mu$ M) was observed in control cells and the positive Cyclophosphamide 5 μ g/ml reduced the NO level to $13.12 \pm 1.3 \mu$ M of nitrate. The extract showed significant inhibition in the NO production in a dose-dependent manner. The NO production of 32.00 ± 1.8 , 23.16 ± 2.7 , 23.16 ± 2.7 , 20.97 ± 1.5 , $18.56 \pm 2.4 \mu$ M were observed at 10, 25, 50,100 μ g/ml of the crude extract of AN respectively. Hence the crude extract reduced the NO production in the cells (Fig.2C). The treatment with AN extract also significantly reduced the release of LPO, the positive Cyclophosphamide 5 μ g/ml showed $1.71 \pm 0.2 \mu$ M with the crude AN showed $2.03 \pm 0.2 \mu$ M in the 100 μ g/ml. As the concentration of the AN extract increases from 10, 25, 50,100 μ g/ml the LPO release also reduced to 3.30 ± 0.2 , 2.67 ± 0.2 , 2.23 ± 0.3 , $2.03 \pm 0.2 \mu$ M respectively (Fig.2D).

DISCUSSION

Pungency, a commercially important characteristic of peppers, is due to the presence of capsaicinoids in the genus *Capsicum* [24]. The determination of capsaicinoid content in the *C. chinense* fruit, using HPLC a reliable method to analyze these compound. It is based only on the period of retention and on the size of the peak of each capsaicinoid present, which was identified by comparing the retention periods of the commercially available standards. The two most abundant capsaicinoids in peppers are capsaicin and dihydrocapsaicin [25]. The HPLC analysis showed ($4,249.0 \pm 190.3 \mu$ g/g) and ($4,482.2 \pm 35.6 \mu$ g/g) of capsaicin and dihydrocapsaicin respectively in hot chili which were higher than those of cayenne pepper samples which had mean capsaicin and dihydrocapsaicin contents of 1,320 and 830 μ g/g dry weight, respectively [26]. with the concentration of capsaicin $4249.0 \pm 190.3 \mu$ g/g with the highest pungency level 67984.60 SHU reported by Zeid [27]. The exercise of the SHU parameter is the recommended method for pepper evaluation as it provides a better indicator of the pungency level, but it is considered less precise [28]. Other composites with shorter retention periods in the HPLC chromatogram, which probably correspond to pigments, our results corroborate with what has been described by Attuquayefio [29] and Collins [20] detected a peak corresponding to pigments in approximately the first few minute. The results of our study showed that acetonitrile extract has extremely high pungent capsaicinoids with less impurity. The contents of capsaicin and dihydrocapsaicin found in the present work for *C. chinense* are in good agreement with those found by other authors who reported the capsaicin concentration detected in

the different peppers. In this study apoptosis activity and cytotoxicity of the acetonitrile crude extract from *Capsicum chinense* were compared with the positive standard. Acetonitrile was used for extraction of capsaicinoids because it gives a high extraction rate at the same time through minimum level of impurities [30]. Cancer has become the number one cause of death in the world [31]. In particular, chronic liver disease has become a global health problem; it causes around 20,000 deaths every year [32]. Apoptosis, cytolytic cell death, enhanced ROS generation in mitochondria, leakage of ROS, activation of Kupffer cells and collagen production by stellate cells are the major signs of the pathophysiology of the liver disease condition [33] that leads to liver cancer. Serious adverse effects and limited efficacy of the conventional therapies have led to an increase in the dependence on complementary and alternative therapies for the management of liver diseases [34]. In the present study, we aimed to screen potentiality of the (AN) acetonitrile extracts of *C. chinense* fruit for its anticancer property. HepG2 liver cancer cell line is widely used due to its relatively high steady-state functioning of the free radical production and antioxidant defenses; therefore, variations of responses at different conditions are more easily detected [35].

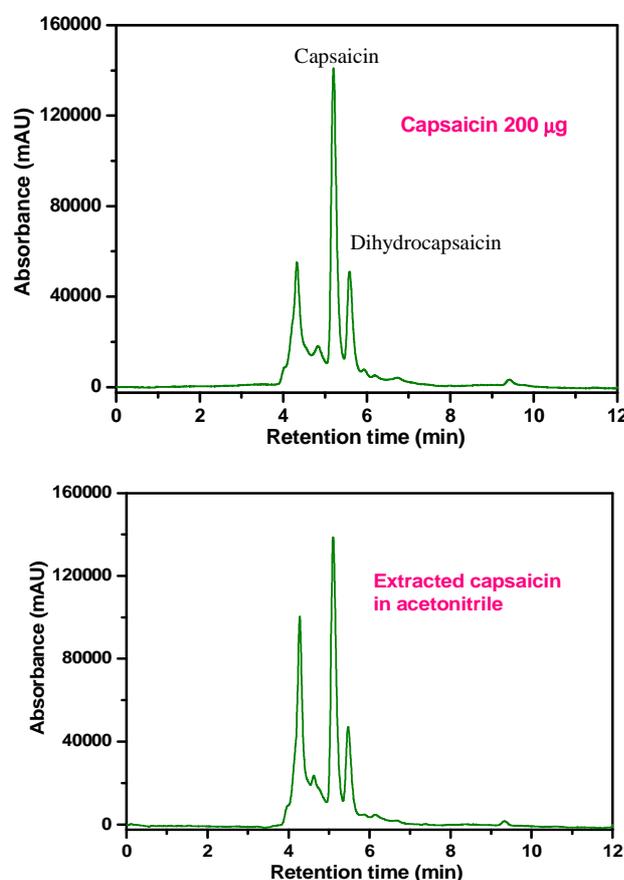


Fig. 1: shows high-performance liquid chromatography (HPLC) chromatogram of A. Standard capsaicin, B. acetonitrile extract of *C. chinense* fruit.

Excessive generation of radical oxygen scavenger (ROS) and other radicals can damage proteins, carbohydrates, polyunsaturated fatty acids and DNA and lead to oxidative stress for certain cancers [36]. Induced cytotoxic effects in a time- and dose-dependent manner and increased reactive oxygen species (ROS) in human colon cancer colo 205 cells after exposure to capsaicin was reported [37]. Nitric oxide at appropriate levels is involved in normal function of organs whereas uncontrolled release can cause destruction of target tissue during inflammation and septic shock [38]. *Capsicum annuum* showed significant NO inhibitory effect (53.5%) in macrophages [39]. LDH is a more reliable and accurate marker of cytotoxicity,

because damaged cells are fragmented completely during the course of prolonged incubation with substances [40]. Malondialdehyde, being a major breakdown product of lipid peroxides, is a useful index of lipid peroxidation [41]. Enhanced hepatic MDA, a major reactive aldehyde resulting from the peroxidation of polyunsaturated fatty acids in the cell membrane, reflects a causal role of lipid peroxidation in liver damage [42]. Several studies showed that exposure of cancer cell lines to capsaicin induce cell cycle arrest at the G1 phase [43-44]. Lee et al., determined the apoptosis mechanism by capsaicin in A172 human glioblastoma cells [16]. According to Hagan [45] capsaicin induces apoptosis through elevating the levels of intracellular ROS and Ca²⁺, promoting the levels of Bax, GADD153 and GRP78, decreasing membrane potential, Bcl-2 XIAP and CIAP1 and then increasing caspase - 3 activation in HepG2 cells. ROS play significant role in the induction of apoptosis in HepG2 cells by capsaicin. In our investigation, acetonitrile extracts of *C. chinense* fruits also induced apoptosis in HepG2 cells. Thus the study confirmed anticancer property of the capsaicinoids in AN extract, through modulating the free radicals release and also established anti-inflammatory potential.

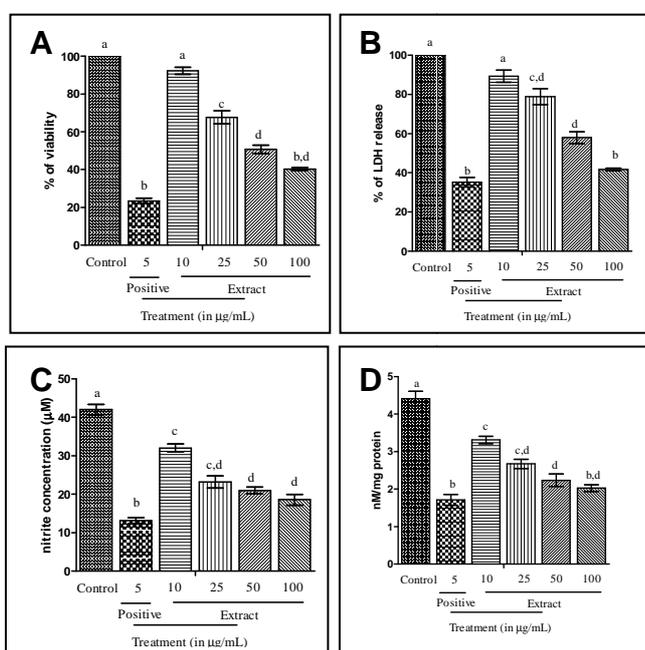


Fig. 2: Shows MTT assay for cell viability in percentage (A), LDH enzyme activity for LDH leakage test in percentage (B), NO release in nitrite µM concentration (C) and Lipoperoxidase in nitrite µM concentration (D).

Control - without treatment, Positive - treated with Cyclophosphamide (5 µg) and Acetonitrile Crude extract (10, 25, 50 & 100 µg/ml)

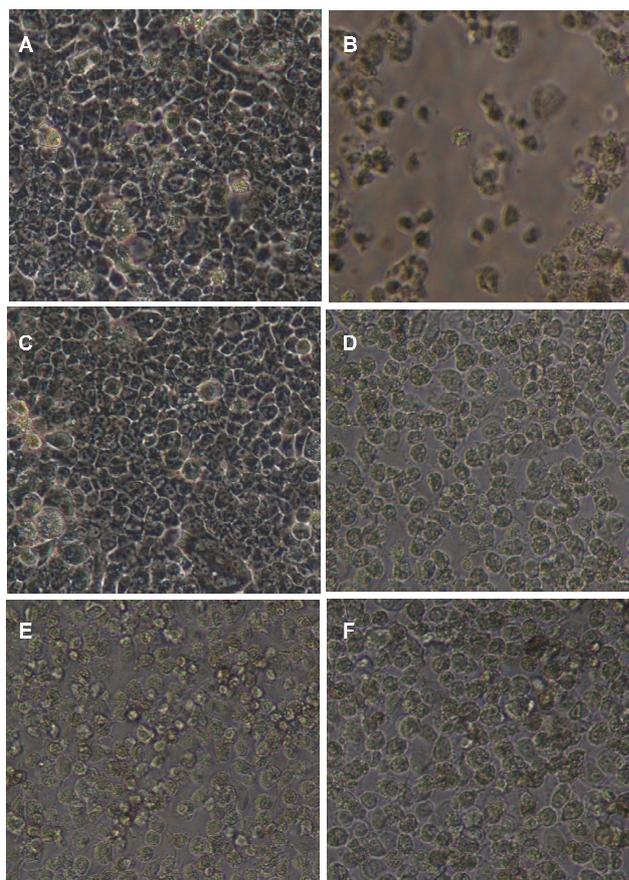


Fig. 3: Shows HepG2 cell lines after 24 h of incubation- A. Control, B. Cyclophosphamide µg/ml, C. AN extract 10 µg/ml, D. AN extract 25 µg/ml, E. AN extract 50 µg/ml, F. AN extract 100 µg/ml

CONCLUSION

Our findings clearly demonstrated that exposure of HepG2 cells to acetonitrile extract, reduces cell viability. The capsaicinoids in the extract significantly suppressed the release of LDH, LPO and NO production at dose dependant manner. In summary present investigation suggest that acetonitrile extract of capsaicinoids possess anticancer activity and *C. chinense* Bhut jolokia of spice has a potential as an anticancer agent.

Table 1: shows HPLC quantification of capsaicin and dihydrocapsaicin in acetonitrile extract from *C. chinense*

Solvent	Capsaicin (µg/g)	SHU	Dihydrocapsaicin (µg/g)	SHU	Total Capsaicinoid (µg/g)	SHU
Acetonitrile	697.5644	11,161,030	301.8868	4,830,189	999.4512	15991219

REFERENCE

- Chung SY, Sung MK, Kim NH, Jang JO, Go EJ, Lee HJ. Inhibition of P-glycoprotein by natural products in human breast cancer cells. Arch Pharm Res. 2005; 28(7):823-8.
- Bennett J, Kirby GW. Constitution and biosynthesis of capsaicin. J Chem Soc C 1968; 442-446.
- Surh YJ, Lee SS. Capsaicin, a double edged-sword: toxicity, meta-bolism, and chemopreventive potential. Life Sci 1995; 56:1845-55.
- George F. Antonious, Terry Berke, Robert L. Jarret. Pungency in Capsicum chinense: Variation among countries of origin Journal of Environmental Science and Health Part B 2009; 44:179-184
- Maria de Lourdes Reyes-Escogido, Edith G, Gonzalez-Mondragon, Erika Vazquez-Tzompantzi. Chemical and Pharmacological Aspects of Capsaicin Molecules 2011; 16: 1253-1270
- Bernal MA, Ros Barceló A. 5', 5'- dicapsaicin, 4'-O-5-dicapsaicin ether, and dehydrogenation polymers with high molecular weights are the main products of the oxidation of capsaicin byperoxidase from hot pepper. J. Agric. Food Chem.1996; 43: 352-355.
- Walpole CS, Bevan S, Bloomfield G, Breckenridge R, James IF, Ritchie T, Szallasi A, Winter J, Wrigglesworth R. Similarities and differences in the structure-activity relationships of capsaicin

- and resiniferatoxin analogues. *J. Med. Chem.* 1996; 39: 2939-2952.
8. Bhutani M, Pathak AK, Nair AS, Kunnumakkara AB, Guha S, Sethi G, et al. Capsaicin is a novel blocker of constitutive and interleukin-6-inducible STAT3 activation. *Clin Cancer Res* 2007; 13:3024-3032.
 9. Kim JD, Kim JM, Pyo JO, Kim SY, Kim BS, Yu R, Han IS. Capsaicin can alter the expression of tumor forming-related genes which might be followed by induction of apoptosis of a Korean stomach cancer cell line, SNU-1. *Cancer Lett* 1997; 120: 235-241.
 10. Zhang R, Humphreys I, Sahu RP, Shi Y, Srivastava SK. In vitro and in vivo induction of apoptosis by capsaicin in pancreatic cancer cells is mediated through ROS generation and mitochondrial death pathway. *Apoptosis* 2008; 13:1465-1478.
 11. Chou CC, Wu YC, Wang YF, Chou MJ, Kuo SJ, Chen DR. Capsaicin-induced apoptosis in human breast cancer MCF-7 cells through caspase-independent pathway. *Oncol Rep* 2009; 21:665-671.
 12. Mori A, Lehmann S, O'Kelly J, Kumagai T, Desmond JC, Pervan M, McBride WH, Kizaki M, Koeffler HP. Capsaicin, a component of red peppers, inhibits the growth of androgen-independent, p53 mutant prostate cancer cells. *Cancer Res* 2006; 66(6):3222-9.
 13. Bruix J, Sherman M. Management of Hepatocellular Carcinoma. *Hepatology* 2005; 42:1208-1236.
 14. Chia-Han Lin, Wei-Cheng Lu, Che-Wei Wang, Ya-Chi Chan and Mu-Kuan Chen Capsaicin induces cell cycle arrest and apoptosis in human KB cancer cells. *BMC Complementary and Alternative Medicine* 2013; 13:46
 15. Gong XF, Wang MW and Ikejima T. Mechanisms of capsaicin-induced apoptosis of human melanoma A375-S2 cells. *Zhonghua Zhong Liu Za Zhi* 2005; 27: 401-403.
 16. Lee YS, Nam DH, Kim JA. Induction of apoptosis by capsaicin in A172 human glioblastoma cells. *Cancer Lett* 2000; 161:121-130.
 17. Szolcsanyi J. Future perspectives of capsaicin research. In: De, A.K. (Ed.), *The Genus Capsicum*. Taylor and Francis London 2003; 248-269.
 18. Moscone EA, Scaldaferrero MA, Grabielle M, Cecchini NM. The evolution of chili peppers (*Capsicum Solanaceae*): a cytogenetic perspective. *Acta Hort* 2007; 745:137-169.
 19. Dias GB, Gomes VM, Moraes TMS, Zottich UP. Characterization of Capsicum species using anatomical and molecular Genet Mol Res 2013; 12(4):6488-6501.
 20. Margaret D, Collins1 Loide Mayer Wasmund, Paul W. Bosland. Improved Method for Quantifying Capsaicinoids in Capsicum Using Highperformance Liquid Chromatography. *Hortscience* 1995; 30(1): 137-139.
 21. Nwokem CO, Agbaji EB, Kagbu JA, Ekanem EJ. Determination of capsaicin content and pungency level of five different peppers grown in Nigeria. *NY Sci. J.* 2010; 3: 17-21.
 22. Sanatombi K, Sharma, GJ. Capsaicin content and pungency of different Capsicum spp.cultivars. *Not. Bot. Hort. Agrobot. Cluj* 2008; 36: 89-90.
 23. Wu D, Cederbaum A. Cytochrome P4502E1 sensitizes to tumor necrosis factor alpha-induced liver injury through activation of mitogen-activated protein kinases in mice. *Hepatology* 2008; 47(3):1005-17.
 24. Perucka I, Materska M. Phenylalanine ammonia-lyase and antioxidant activities of lipophilic fraction of fresh pepper fruits *Capsicum annuum L.* *Innovat. Food Sci. Emerg. Tech.* 2001; 2:189-192.
 25. Kosuge S, Furuta M. Studies on the Pungent Principle of Capsicum. Part XIV: Chemical Constitution of the Pungent Principle. *Journal of Agricultural and Biological Chemistry* 1970; 34: 248-256.
 26. Lopez-Hernandez J, Oruna-Concha MJ, Simal-Lozano J, Gonzales-Castro MJ, Vazquez-Blanco ME. Determination of capsaicin and dihydrocapsaicin in cayenne pepper and padron peppers by HPLC. *Dtsch Lebensmitt Rundsch* 1996; 92: 393-395.
 27. Zeid Abdullah Al Othman, Yacine Badjah Hadj Ahmed, Mohamed Abdelaty Habila, Ayman Abdel Ghafar. Determination of Capsaicin and Dihydrocapsaicin in Capsicum Fruit Samples using High Performance Liquid Chromatography. *Molecules* 2011; 16: 8919-8929
 28. Todd P, Bensinger M, and Biftu T. Determination of Pungency due to Capsicum by Gas-Liquid Chromatography. *Journal of Food Science* 1977; 42: 660-665.
 29. Attuquayefio VK, Buckle KA. Rapid sample preparation method for HPLC analysis of Capsicum fruits and oleoresins. *J. Agr. Food Chem* 1987; 35:777-77.
 30. Karnka R, Rayanakorn M, Watanesk S, Vaneesorn Y. Optimization of high-performance liquid chromatographic parameters for the determination of capsaicinoid compounds using the simple method. *Anal. Sci.* 2002; 18: 661-665.
 31. Saravanan S, Hairul Islam VI, Gabriel Paulraj M, Ignacimuthu S. Protective effect of *Enicostema axillare* on mutagenicity of *Salmonella typhimurium* by increasing free radical scavenging abilities. *Pharmaceutical Biology* 2011; 50(6): 698-706.
 32. Shakya AK, Sharma N, Saxena M, et al. Evaluation of the antioxidant and hepatoprotective effect of Majoon-e-Dabeed-ul-ward against carbon tetrachloride induced liver injury. *Exp Toxicol Pathol* 2012;64:767-73.
 33. Center SA. Metabolic, antioxidant, nutraceutical, probiotic, and herbal therapies relating to the management of hepatobiliary disorders. *Vet Clin Small Anim Pract* 2004; 34:67-172.
 34. Sumita Verma and Paul J. Thuluvath. Complementary and Alternative Medicine in Hepatology: Review of the Evidence of Efficacy *Stroenterology and Hepatology* 5: 4
 35. Alia M, Mateos R, Ramos S, Lecumberri E, Bravo L, Goya L. Influence of quercetin and rutin on growth and antioxidant defense system of a human hepatoma cell line (HepG2). *Eur. J. Nutr* 2006; 45(1): 19-28.
 36. Ebenharder R, Grünhage D. Free radical scavenging abilities of flavonoids as mechanism of protection against mutagenicity induced by tert-butyl hydroperoxide or cumene hydroperoxide in *Salmonella typhimurium*TA10. *Mutat Res. Genet. Toxicol. Environ. Mutagen* 2003; 540:1-18
 37. Lu HF, Chen YL, Yang JS, Yang YY, Liu JY, Hsu SC, Lai KC, Chung JG. Antitumor activity of capsaicin on human colon cancer cells in vitro and colo 205 tumor xenografts in vivo. *J Agric Food Chem.* 2010; 58(24):12999-3005.
 38. Saravanan S, Prakash Babu N, Pandikumar P, Karunai Raj M, Gabriel Paulraj M, Ignacimuthu S. Immunomodulatory potential of *Enicostema axillare* (Lam.) A. Raynal, a traditional medicinal plant. *Journal of Ethnopharmacology* 2012; 140: 239-46.
 39. Lei Chen, Young-Hwa Kang. Anti-inflammatory and antioxidant activities of red pepper (*Capsicum annuum L.*) stalk extracts: Comparison of pericarp and placenta extracts. *Journal of Functional Foods* 2013; 5(4):1724-1731
 40. Raju Asirvatham, Arockiasamy Josphin Maria Christina, and Anita Murali In Vitro Antioxidant and Anticancer Activity Studies on *Drosera Indica L.* (*Droseraceae*) *Adv Pharm Bull* 2013; 3(1): 115-120.
 41. Saravanan S, Pandikumar P, Pazhanivel N, Gabriel Paulraj M, Ignacimuthu S. Hepatoprotective role of *Abelmoschus esculentus* (Linn.) Moench on carbon tetrachloride induced liver injury. *Toxicology mechanisms and methods* 2013; PMID: 23581558.
 42. You Y, Yoo S, Yoon HG, et al. In vitro and in vivo hepatoprotective effects of the aqueous extract from *Taraxacum officinale* (dandelion) root against alcohol-induced oxidative stress. *Food Chem Toxicol* 2010; 48:1632-7.
 43. Zhang J, Nagasaki M, Tanaka Y, Morikawa S. Capsaicin inhibits growth of adult T-cell leukemia cells. *Leuk Res* 2003; 27:275-283.
 44. Hail N Jr, Lotan R. Examining the role of mitochondrial respiration in vanilloid-induced apoptosis. *J Natl Cancer Inst* 2002; 94:1281-1292.
 45. Hogan MV, Grant RE, Lee L Jr. Analgesia for total hip and knee arthroplasty: a review of lumbar plexus, femoral, and sciatic nerve blocks. *Am J Orthop (Belle Mead NJ)* 2009; 38: 129-133.