Journal of Scientific and Innovative Research 2015; 4(1): 43-48
Available online at: www.jsirjournal.com

Research Article

ISSN 2320-4818
JSIR 2015; 4(1): 43-48

Received: 19-01-2015
Accepted: 15-02-2015

Bindu Jacob
Department of Biotechnology, Kongunadu Arts and Science College, Coimbatore- 641029, Tamilnadu, India

Suresh Natarajan
Department of Biotechnology, Sree Narayana Guru College, K.G. Chavadi, Coimbatore- 641029, Tamilnadu, India

Sagadevan.Pattiyappan
Department of Biotechnology, Kongunadu Arts and Science College, Coimbatore- 641029, Tamilnadu, India

Rathish Kumar Sivarman
Department of Biotechnology, Kongunadu Arts and Science College, Coimbatore- 641029, Tamilnadu, India

Syama. M. Suresh
Department of Biotechnology, Kongunadu Arts and Science College, Coimbatore- 641029, Tamilnadu, India

Correspondence:
Suresh Natarajan
Department of Biotechnology, Sree Narayana Guru College, K.G. Chavadi, Coimbatore- 641029, Tamilnadu, India

Study on anticarcinogenic and apotoptic properties of methanolic stem extract of *Cardiospremum halicacabum* in MCF 7 cell line

Bindu Jacob, Suresh Natarajan, Sagadevan.Pattiyappan, Rathish Kumar sivarman, Syama. M. Suresh*

Abstract

The present study reveals the medicinal properties of the present study reveals the medicinal properties of the *Cardiospermum halicacabum*. The Cytotoxic activity of methanolic stem extract of *Cardiospermum halicacabum* was tested against breast cancer cell line (MCF-7) using MTT assay. The Cytotoxic activity of methanolic stem extract of *Cardiospermum halicacabum* showed significant activity against tested cell line. The different concentrations (100, 500, 1000) was tested at an optical density of 540nm was observed to calculate the percentage of viability. Hundred percent viability was seen against control. The maximum inhibitory activity was seen in lower concentration (100%) at a optical density of 6.753 was observed. The minimum percentage of viability (50%) was observed at an optical density of 0.392 was observed in 1000 µg/ml). From the results it can be observed that the added compounds produced significant decrease in cell viability which confirms antiproliferative activity of the studied extracts. Acridine orange fluorescent staining, Neutral Red staining and Lactose dehydrogenase assays were carried out in MCF-7 breast cancer cell lines to study apoptopsis. The plant extract has shown significant apoptoptic activity against cancer cell lines.

Keywords: *Cardiospermum halicacabum*, Anticancer, (MCF-7), Antiproliferative activity, Apoptotic activity.

Introduction

The medicinal properties of plants are the most precious gift of Mother Nature to mankind. It has been used to treat various human ailments in the traditional system of medicine from centuries, in India. The number of modern medicine which is used today has a record of being used from the ancient times. An impressive number of modern drugs have been isolated or derived from natural sources, based on their use in traditional medicine.[1] It has been estimated by the World Health Organization (WHO) that approximately 80% of world's population rely on traditional medicinal plants for their health care.

The emergence of multiple drug resistance bacteria (MDR) has been a major cause of failure of treatment of infectious diseases.[2, 3] Even though pharmacological industries have produced a new antibiotic in the last three decades, resistance to these drugs by micro-organisms have increased. The screening of plant extracts and their products for antimicrobial activity has shown that higher plants represent a potential source of novel antibiotic prototypes.[4] Plant based antimicrobials represent a vast untapped source of medicines even after their enormous therapeutic potential and effectiveness in the treatment of infectious disease hence, further exploration of plant antimicrobials need to occur.[5]

Plants posses various phytochemicals and active bimolecular, which play a major role in the treatment of cancer. Many plants have been examined to identify new and effective anticancer compounds, as well as to elucidate the mechanism of cancer prevention and apoptosis.[6]

Compounds like vinblastine, vincristine, etoposide, teniposide, taxol, navelbine, taxotere, topotecn, and irinotecan have been approved for the use of anticancerous drugs. There are more than 2, 70,000 higher plants existing on this planet. But only a small portion has been explored phytochemically. So it is anticipated that plants can provide potential bioactive compounds for the development of new leads to combat cancer disease.

Cardiospermum halicacabum Linn. Belongs to family *Sapindaceae*, commonly known as Balloon vine or Love in a puff. *Cardiospermum* is the combination of the Latin words cardio, meaning heart, and sperma, meaning seed and refers to the white heart-shaped pattern on the seed. *Halicacabum* is derived from the Latin word halicacabus, a plant with inflated fruits.[7] It is an annual or sometimes perennaial climber, widely distributed in tropical and subtropical Africa and Asia. Often found as a weed along roads and rivers, it has been examined for antidiarrhoeal as well as homoeopathic medicinal properties. *C. halicacabum* has been used in the treatment of rheumatism, nervous diseases, stiffness of the limbs and snakebite. Young leaves can be cooked as vegetables. The leaf juice has been used as a treatment for earache as well. It has been used for centuries to treat many ailments yet it is best known by modern society as a weed. Although, numerous weeds have been shown to have a medicinal properties very little attention has been paid to them .[8]

Cancer is one of the second leading causes of death in worldwide killing more people globally. It is estimated that there are nearly 2.5 million cases in India with nearly 400,000 deaths occurring due to cancer. In India, around 5, 55,000 people died of cancer in 2010; according to estimates publish in The Lancet Today.[9] The most frequently reported cancer sites in males are lung, oesophagus, stomach, and larxynx. In females, cancers of cervix, breast, ovary and oesophagus are the most commonly encountred, in India. Cancers of female reproductive tract and breast has a high incidence amongst Indian women. The incidence of breast cancer in India is rapidly becoming the number one cancer in females. It is reported that one in twenty-two women in India and one in eight women in America is likely to be victim of breast cancer. Men are also at risk for breast cancer, but the death rate is quite low, at 0.22% or two-tenth of a percent.

BRCA1 and BRCA2 are human genes that belong to a class of genes known as tumor suppressors. Mutation of these genes has been linked to hereditary breast and ovarian cancer. A woman's risk of developing breast and/or ovarian cancer is greatly increased if she inherits a deleterious (harmful) BRCA2 or BRCA2 mutation. Men with these mutations also have an increased risk of breast cancer. Both men and women who have harmful BRCA1 or BRCA2 mutations may be at increased risk of other cancers. Genetic test are available to check for BRCA1 and BRCA2 mutations. A blood sample is required for these tests, and genetic for, and genetic counseling is recommended before and after the tests. Many research studies are being conducted to find newer and better ways of detecting, treating, and preventing cancer in BRCA1 and BRCA2 mutation carriers. Latest findings reveal that all conventional medical treatment for cancer is not helpful. As per the new studies it is found that survival in breast cancer is four times longer without conventional treatment. It is also found that people who refused treatment lived for an average of twelve and half years. Those who accepted other kinds of treatment lived on an average of only 3 years. Hence the significance of novel herbal remedies can be clearly understood.

Materials and Methods

Anticancer studies and apoptotic study

Culturing of MCF-7 Breast Cancer Cell Line

MCF-7 purchased from NCCS Pune, was maintained in Dulbecco's modified eagles media and grown to confluency at 37oc and 5 % CO2 in a humidified atomosphere in a CO2 incubator. The cells were trypsinized (500µl of 0.025% Trypsin in PBS/ EDTA solution) for 2 minutes and passaged to T flasks in complete aseptic conditions. Extracts were added to grown cells at a concentration of 100 µg, 500 µg and 1000 µg from a stock of 100 mg/ml and incubated for 24 hours. The antiproliferative effect of extracts were determined by MTT, Neutral red and LDH leakage assay

MTT Assay

MTT is a colorimetric assay that measures the reduction of yellow 3-(4, 5dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells are then solubilised with an organic solvent (eg. isopropanol) and the released, solubilised formazan reagent. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells.

The cell culture suspension was washed with 1x PBS and then added with 200µl MTT solution to the culture (MTT - 5mg/volume dissolved in PBS) and incubated at 37 ºC for 3 hours. Removed all MTT by washing with 1x PBS and added 300 µl DMSO to each culture. Incubated at room temperature for 30 minutes until all the cells get lysed and a purple colour is obtained. The solution was transferred to centrifuge tubes and centrifuged at top speed for 2 minutes to precipitate cell debris. OD was read at 540 nm using DMSO as blank.

Real Time PCR

RNA Isolation

- 5 ml or 2 ml micro centrifuge tubes
- Chloroform: Isoamylalcohol
- Isopropanol
- DEPC extracted 3M sodium acetate

- DEPC extracted 70% ethanol
- DEPC extracted TE 10mm Tris HCl pH 8.0 1mm EDTA

Total RNA was isolated using the total RNA isolation kit according to the manufactures instruction (Chromous Biotech). Addition of RNA sol solution causes the disruption of cells and the release of RNA. Chloroform extraction following centrifugation, and separates the mixture into a lower red phenol-chloroform phase, inter phase and the colourless upper aqueous phase. RNA remains exclusively in the aqueous phase where as proteins are in the interphase and organic phase. On mixing with isopropanol RNA gets precipitated as a white pellet on the side and bottom of the tube.

MCF-7 cell line after 24 hr exposure with extracts was scrapped out and pelleted out at 5000 rpm in a cooling centrifuge. An untreated plate was maintained as control. 300 µl RNA sol was added and mixed thoroughly. To this 200 µl chloroform: isoamyl alcohol mixture (24:1) was added. Mixed well and centrifuged at 10000rpm for 5 min at 4ºc. The clear upper aqueous layer was transferred into another vial, added $1/10^{th}$ the sample volume of 3M sodium acetate (p^H 5.2) and 1:1 isopropanol. The contents were thoroughly mixed and centrifuged at 1000rpm for 10 min at 4ºC. The RNA pellets were collected and washed with 70% ethanol and centrifuged at 1000rpm for 5 min at 4ºC. The pellet obtained were dried at 37ºC and then suspended in 5µl TE buffer.

Agarosegel Electrophoresis

Reagents

- 1% Agarose
- 1X TE buffer
- EDTA
- TBE buffer

Procedure

1% Agarose gel was prepared in 1xTE buffer. This was melted in hot water bath at 90ºc , and the melted agarose was cooled down to 45ºc, 8µl of 0.5 mg/ml of ethidium bromide was added and poured into gel casting apparatus with gel comb. After setting, the comb was removed from the gel. The platform with gel was placed into an electrophoretic tank with sufficient electrophoretic buffer (TBE) to immerse the gel. RNA sample was prepared with an appropriate amount of loading buffer and was loaded into well. The gel was allowed to run. The stained gel was then observed under UV transilluminator.

RT-PCR analysis of BRCA1 in MCF-7 Breast Cancer cell line.

Mix 5µl of RNA template (~1µg) with 400 ng of reverse primer (BRACA1) forward primer (1.75 µl) and added DEPC water to make the volume of the reaction mix to 20µl. Incubated the above mix at 65ºC for 10 min and immediately chilled on ice. 25µl reaction mix was added to fresh PCR tubes followed by 20µl RNA-primer mix, 300 ng of forward primer (2 µl) and finally added 2µl enzyme mix and the total reaction mix was made up to 50 µl with DEPC treated water. A set of control reaction and house keeping genes was also maintained (GAPDH).

Primers

BRCA1

Forward: aaaagatatagatgtatgttttgctaatgtgt
reverse: TCCCAAATTAATACACTCTTGTGCTGA

GAPDH

Forward: GAGACAGCCAGGAGAAATCA
Reverse: GAAGATGGTGATGGGATTTC

Cycle Condition

The following cycles were selected as per previous references and Tm of primers. The tubes were place in Eppendorf Master Cycler and programmed for the following cycle.

42ºc	**94ºc**	**94ºc**	**55ºc**	**72ºc**	**72ºc**	**4ºc**
30min	**15min**	**30sec**	**30sec**	**30sec**	**2min**	∞
		←30 cycles→				
42ºc	**94ºc**	**94ºc**	**55ºc**	**72ºc**	**72ºc**	**4ºc**
30min	**15min**	**30sec**	**30sec**	**30sec**	**2min**	∞
		←30 cycles→				

The amplification products were run on 1% agarose gel and view on UV transilluminator (UV tek UK) and compared with control using Image J gel analysis software.

Apoptotic Studies (Acridine orange fluorescent staining)

The induction of apoptosis in MCF-7 by plant extracts was determined using Epifluroscent microscopy. Acridine orange is intercalating nucleic acid specific fluorochromes which emit a green fluorescence. Acridine orange can cross the plasma membrane of viable and early apoptotic cells. Viewed by fluorescence microscopy, viable cells appear to have a bright green nucleus with intact structure while apoptotic cells exhibit a bright green nucleus showing condensation of chromatin as dense green areas. Cells treated with extracts were washed with PBS to remove interfering plant material and added 1ml of DMEM medium. 10 µl of Acridine orange (Sigma Aldrich) at a final concentration of 1mg/ml was added to culture flasks and incubated for 10mts. The excess acridine orange was drained off, washed with PBS and observed under Blue filter of Olympus epifluorescent Microscope (CKX41) attached with Olympus CMOS camera.

Lactate Dehydrogenase Assay

Lactate dehydrogenase is used as a quantitative marker enzyme for the intact cell, its activity providing information on cellular glycolytic capacity. Measurement of LDH release (leakage) is an important and frequently applied test for severe irreversible cell damage. Enzymatic reaction catalyzed by lactate dehydrogenase. Pyruvate + NADH + H^+ → L- lactate + NAD^+ LDH release assay was performed with cell free supernatant collected from tissue culture plates exposed to different concentration of extracts (100 µg, 500 µg and 1000 µg). To this added 2.7 ml potassium phosphate buffer, 0.1 ml 6mM NADH solution, 0.1 ml sodium pyruvate solution, into a cuvette and mix well. The decrease of OD was recorded at 340nm in a spectrophotometer, thermo stated at 25^0C. (Elico SL 177) The blank solution was prepared by adding enzyme dilution buffer instead of sample. Activity of lactate dehydrogenase was be calculated by using the formula,

Volume of activity (U/ml) = [(Abs – Ab0) x 1 (ml) x df] ÷ [6.2 x 0.1 (ml)]

Neutral Red Assay

The neutral red cytotoxicity test was based on the ability of living cells to uptake and bind neutral red (NR). NR was a positively charged dye that easily diffuses through the cellular membrane of the cells, accumulates in the cellular cytoplasma and was stored in the acidic environment of lysosomes. The principle of the test consists in the fact that NR are able to absorb and bind only live cells while this ability declines in damaged or dead cells. The amount of accumulated NR was thus directly proportional to the amount of live cells in the cell culture. MCF-7 was treated as per standard procedure described above, were incubated for 3 hours with neutral red dye. The p^H of the neutral red solution was adjusted in all the experiments to 6.35 with the addition of KH_2PO_4 (1M). Cells were then washed with phosphate buffer saline (PBS) and the addition of 1ml of the elution medium (ethanol/ acetic acid, 50%/ 1%) followed by gentle shaking for 10 minutes, so that complete dissolution was achieved. Aliquots of the resulting solutions were transferred to cuvettes and the absorbance at 540nm was recorded using the spectrophotometer.

Viability = [(C-T) ÷ C] ×100 Where C = Absorbance of blank T= Absorbance of test

Y (U/ml) = [(Abs – Ab0) x 1 (ml) x df] ÷ [6.2 x 0.1 (ml)]

Result

Anticarcinogenic activity and Apoptotic Activity

The cytotoxic activity of methanolic stem extract of *Cardiospermum halicacabum* was tested against breast cancer cell line (MCF-7) using MTT assay. The Cytotoxic activity of methanolic stem extract of Cardiospermum halicacabum showed significant activity against tested cell line. The different concentrations (100, 500, 1000) was tested at an optical density of 540nm was observed to calculate the percentage of viability. Hundred percent viability was seen against control. The maximum inhibitory activity was seen in lower concentration (100%) at a optical density of 6.753 was observed. The minimum percentage of viability (50%) was observed at an optical density of 0.392 was observed in 1000 µg/ml). (Table-1, Figure-1 plate-1 & 2)

Table 1: The cytotoxic effect (MTT Assay) of Methanolic Stem Extract Cardiospermum halicacabum

Sample Concentration	OD (540 nm)	% Viability
Control	0.770	100
100	0.753	97.1
500	0.448	58.1
1000	0.392	50.9

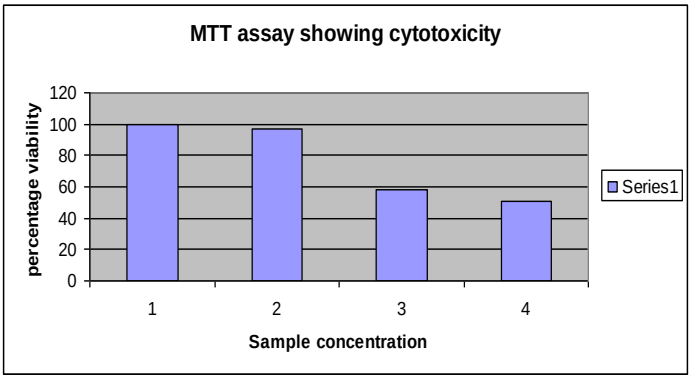

Figure 1: Graphical Representation of MTT Assay showing cell cytotoxicity in MCF-7

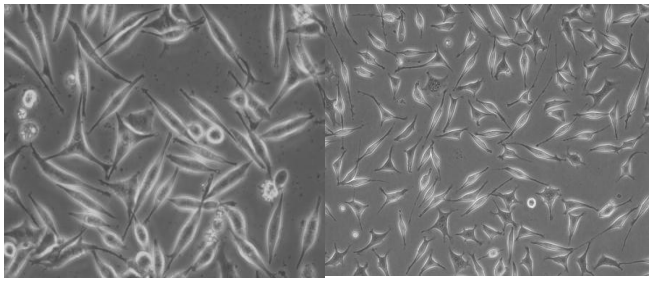

Plate 1: Phase Contrast Microscopic view of MCF 7 Cell Lines

From the results it can be observed that the added compounds produced significant decrease in cell viability which confirms antiproliferative activity of the studied extracts.Acridine orange fluorescent staining, Neutral Red staining and Lactose dehydrogenase assays (Figure-3) were carried out in MCF -7 breast cancer cell lines to study apoptopsis. The plant extract has shown significant apoptoptic activity against cancer cell lines.

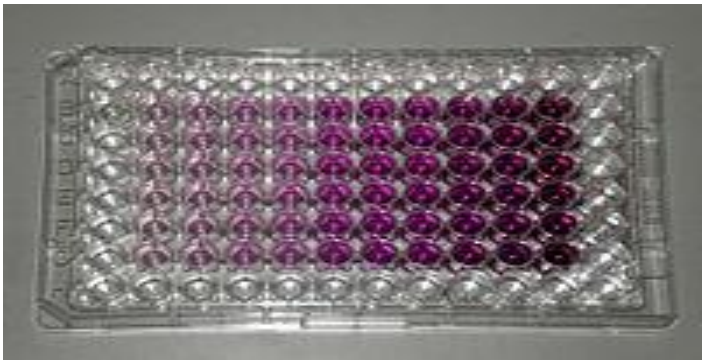

Plate 2: MTT ASSAY in 96 well microtiter plate; Acridineorange Fluorescent Staining

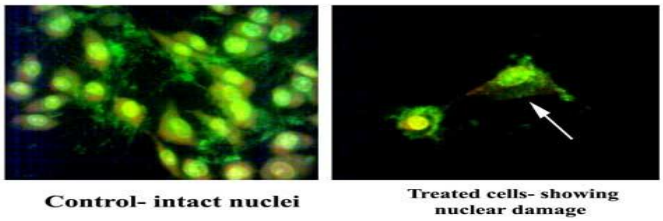

Plate 3: Acridine orange fluorescent staining of MCF-7 Cells lines

Real Time PCR

Total RNA isolated was subjected to PCR analysis and the following results were obtained (Plate 4). From the results it can be observed that hydrogen peroxide induces expression of Heat Shock Protein in buccal cells which can suppress to an extent (approximately 50%) by the methanolic leaf extract of *Cardiospermum halicacabum*. The potential role of oxidative stress conditions in the induction of heat shock proteins is previously described by workers like.

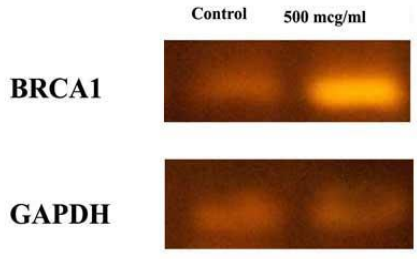

Plate 4: R T-PCR Analysis of BRACA1 in MCF-7 Cell line treated with methanolic stem extract of *Cardiospermum halicacabum*

Neutral Red Assay

Neutral Red Assay is performed to study the cell viability. The methanolic leaf extract has shown significant cytoprotective activity. (Table-2, Figure-2). The cell treated with H_2O_2 has a reduced viability than the H_2O_2 and methanolic leaf extract treated cells. In H_2O_2 treated cells the cytotoxicity was more than 60% i.e. only 40.12 % has uptaken the dye, is viable and the cells treated with the methanolic leaf extract of *Cardiospermum halicacabum* has shown viability up to 62 %. This shows the significant cytoprotective activity of the plant extract.

Table 2: Neutral Red Assay

Sample Concentration (500µg/ml)	% Viability
Control	100
MCF-7	51.03

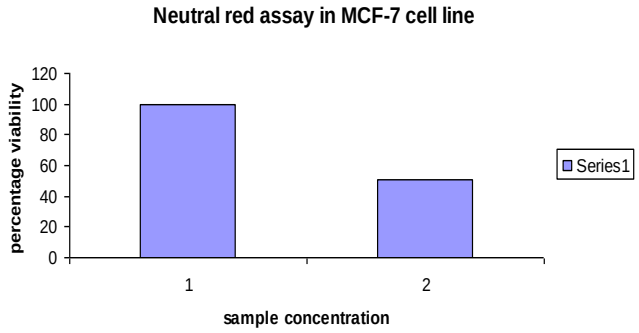

Figure 2: Graphical Representation of Neutral Red assay in MCF-7 Cell line

Lactate Dehydrogenase Assay

Lactate dehydrogenase assay was performed to study the cell viability of buccal cells (Table-3) (Figure-3). The plant extract treated cells has shown significant cell protective activity towards H_2O_2 treated cells.

Table 3: LDH Assay in MCF-7 Cell line

Sample Concentration	V(U/ml) X 10^{-1}
CONTROL	0.94
SAMPLE TREATED	2.5

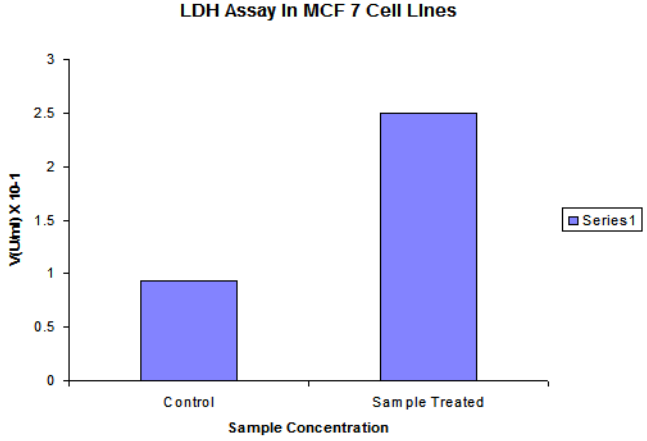

Figure 3: Graphical representation in Lactose Dehydrogenase Assay in MCF-7 Cell Line

Discussion

Historically, plants have provided a source of inspiration for novel drug compounds, as plant derived medicines have made large contributions to human health and well being. Their role is twofold in the development of new drugs: (1) they may become the base for the development of a medicine, a natural blue print for the development of new drugs or; (2) a phytomedicine to be used for the treatment of diseases.[10] Herbal-based and plant-derived products can be exploited with sustainable comparative and competitive advantage. Higher plants, as sources of medicinal compounds continue to play a dominant role in maintenance of human health since antiquities. Over 50% of all modern clinical drugs are of natural product origin[11] and natural products play an important role in drug development programs of the pharmaceutical industry. Presently, many scientists and organizations are in search of traditional remedies as alternate medicine.[12]

Another secondary metabolite compound observed in the stem bark extract of *C. halicacabum* was alkaloid. One of the most common biological properties of alkaloids is their toxicity against cells of foreign organisms. These activities have been widely studied for their potential use in the elimination and reduction of human cancer cell lines.[13] Alkaloids which are one of the largest groups of phytochemicals in plants have amazing effects on humans and this has led to the development of powerful pain killer medications[14, 15] revealed the inhibitory effect of saponins on inflamed cells. Saponin was found to be present in *C. halicacabam* extracts and has supported the usefulness of this plant in managing inflammation. Phenolic compounds are known as powerful chain breaking antioxidants[17], may contribute directly to antioxidative action.[16] These compounds are very important constituents of plants and their radical scavenging ability is due to their hydroxyl groups.[17]

Conclusion

The present study reveals the medicinal properties of the present study reveals the medicinal properties of the Cardiospermum halicacabum .The phytochemical screening of methanolic leaf extracts *Cardiospermum halicacabum* showed the presence of secondary metabolites such as Alkaloids, Terpenoids, Tannins, Saponins, Flavonoids and Steroids. There is a remarkable anticancer potential was observed against the breast cancer cell lines in methanolic leaf extract of *Cardiospermum halicacabum* There is a correlation was observed in the concentration and % of growth inhibition against the extract and breast cancer cells. Total RNA isolated was subjected to PCR analysis and the following results were obtained. From the results it can be observed that hydrogen peroxide induces expression of Heat Shock Protein in buccal cells which can suppressed to an extent (approximately 50%) by the methanolic leaf extract of *Cardiospermum halicacabum* .The potential role of oxidative stress conditions in the induction of heat shock proteins is previously described by workers like .

Reference

1. Afolayan, A. J. Extracts from the shoots of Arctotis artotoides inhibit the growth of bacteria and fungi. Pharm. Biol., 2003;41: 22-25.

2. Brown, R.W. Composition of Scientific Words. Washington, D.C., USA: Smithsonian Institution Press, 1954.

3. Cortella, A.R., Pocheltino, M.L. Plants employed for infusions in urban areas of Argentina. Pharm. Biol. 1999;37: 97-104.

4. Cragg, M.G., and Newman, D.J. Natural Product Drugs Discovery in Next Millennium, National Cancer Institute, Frederick, USA, 2001.

5. Duh, P.D., Tu, Y.Y., Yen, G.C. Antioxidant activity of water extract of harn jyur (*Chyrsanthemum morifolium* Ramat). LWT-Food Sci. Technol. 1999;32: 269–277.

6. Gibbons, S. Plants as a source of bacterial resistance modulators and anti-infective agents. Phytochemistry Rev. 2005;4: 63-78.

7. Hatano, T., Edamatsu, R., Hiramatsu, M., Mori, A., Fujita, Y., Yasuhara, D. Effects of tannins and related polyphenols on superoxide anion radical and on DPPH radical. Chem. Pharm. Bull. 1989;37: 2016-2021.

8. Iwu, M.W., Duncan, A.R., Okunji, C.O. New antimicrobials of plant origin.In janick J, ed, perspectives on new crops and New uses, Alexanderia, VA: ASHS Press: 1993, pp:457-462

9. Just, M.J., Recio, M.C., Giner, R.M., Cueller, M.J., Manez, S., Bilia, A.R., Rios, J.L. Anti-inflammatory activity of unusual lupine saponins from *Bupleurum fruticescens*. Planta Medica. 1998;64: 404-407.

10. Kam, P.C.A., Liew. Traditional Chinese herbal medicine and anaesthesia. Anaesthesia 2002;57(11): 1083-1089.

11. Mathias, A.J., R.K. Somashekar, S. Sumithraand and S. Subramanya. An Assessment of Reservoirs of Multi-resistanct Nosocomial Pathogens in a Secondary care hospital. Indian J. Microbiol., 2000;40: 183-190.

12. Nobori, T., Miurak, K., Wu, D.J., Takabayashik, L.A., Carson, D.A. Deletion of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. Nature 1994;368 (6473): 753-756

13. Parekh, J., Darshana, J. and Chanda, S. Efficacy of aqueous and methanol extracts of some medicinal plants for potential antibacterial activity. Turk. J. Biol., 2007;29:203-210.

14. Pieroni, A., Nebel, S., Quave, C., Münz, H., & Heinrich, M. Ethnopharmacology of Liakra: traditional weedy vegetables of the Arbëreshë of the Vulture area in southern Italy. Journal of Ethnopharmacology. 2002;81(2): 165-185.

15. Shahidi, F., Wanasundara, P.K.J.P.D. Phenolic antioxidants. Cr. Rev. Food Sci. Nutr. 1992;32: 67-103.

16. Stuffness, M., Douros, J. Current status of the NCI plant and animal product program. J. Nat. Prod. 1982;45: 1-14.

17. Swammy, S.M.K., and Tan, B.K.H. Cytotoxic and immunopotentiating effects of ethanolic extracts of *Nigella sativa* L. seeds. Journal of Ethnopharmacology 2000;70: 1-7.