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In Vitro Anticancer Activity of Ethanolic Extracts of *Syzygium Aromaticum* in HEP2 Cell Line

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Abstract: Cloves are the aromatic flower buds of a tree in the family Myrtaceae, *Syzygium aromaticum*. They show various medicinal properties, conventionally used as respiratory and digestive ailments. Its medicinal properties are antioxidant, anti-septic, anti-helminthic, anesthetic, anti-inflammatory, rubefacient and anti-flatulent. It shows anti-cancerous property on various cancerous cell line such as HeLa cell, lungs cancer, prostate cancer, breast cancer, esophageal cancer. Ethanolic extract of clove has been taken to show anti-cancerous effect on HEP2 cell line. Different concentration of clove extract has been taken to show effect on HEP2 cell line, 1000 µg/ml, 100 µg/ml, 10 µg/ml and 1 µg/ml shows cytotoxicity on HEP2 cell line. Least cytotoxic effect was observed in 1 µg/ml and highest in 1000 µg/ml. thus cytotoxic effect was observed in all concentrations but the effect is varying different doses. This cytotoxicity was determined by performing MTT Assay, NRU Assay and DNA Fragmentation Assay. All shows cytotoxic effect on 1000 µg/ml but there is varying result in MTT and NRU assay but the effect properly observed in DNA fragmentation assay. DNA Fragmentation assay indicated the apoptotic cell death at all doses. Thus it can be concluded that clove have cytotoxic effect on HEP2 cell line.

Keywords: *Syzygium aromaticum*, HEP2 cell lines, MTT assay, NRU assay, DNA Fragmentation assay

I. INTRODUCTION

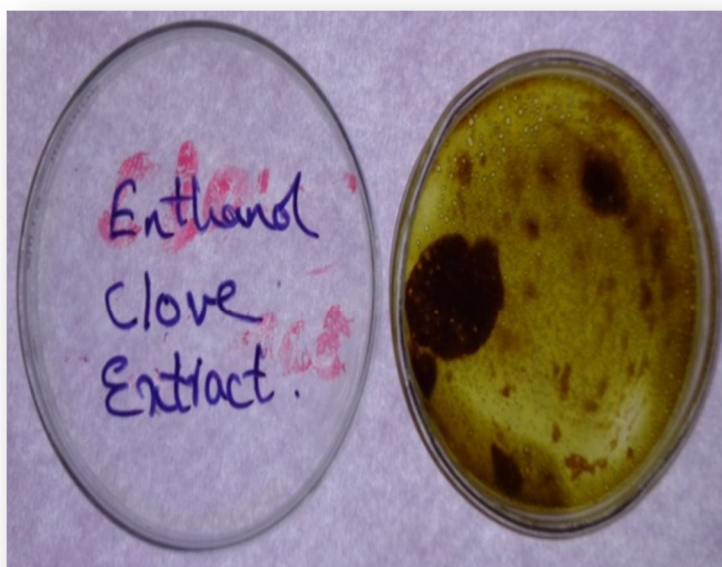
Clove, the sun-dried unopened flower bud from the plant *Syzygium aromaticum* is a commonly used spice and food flavour. Clove oils play important roles in prevention and treatment of various cancers. The objective of the present study is to compare the in vitro anticancer activities of ethanol extracts of Clove (*Syzygium aromaticum*), against Hep-2 cancer cell line. The main constituents in clove are polypropenoids thymol, carvacol, cinnamaldyhyde, eugenol, eugenol acetate, caryophyllene, and sesquiterpenes. Eugenol, which accounts for the majority of the essential oil of the plant, has been well proven to possess anti-cancerous effect. The volatile clove oil containing up to 85% eugenol and acetyl eugenol, methyl salicylate, pinene and vanillin. It contains gum, tannins, flavonoids and sterols. Eugenol comprises 72-90% of the essential oil extracted from cloves, and is the compound most responsible for the cloves' aroma. This study was under taken to investigate anti-cancer effect of ethanol extracts from the buds of *S. aromaticum*. Cancer is a class of diseases characterized by out-of-control cell growth. There are over 100 different types of cancer, and each is classified by the type of cell that is initially affected. Cancer harms the body when damaged cells divide uncontrollably to form lumps or masses of tissue called tumours (except in the case of leukaemia where cancer prohibits normal blood function by abnormal cell division in the blood stream)[1]. Tumours can grow and interfere with the digestive, nervous, and circulatory systems and they can release hormones that alter body function. HEP2 cell lines are abbreviated for Human epithelial type 2. These cells allow recognition of over 30 different nuclear and cytoplasmic patterns that are given by upwards of 50 different autoantibodies with various autoimmune conditions. These cell lines are thought to be derived from epidermoid carcinoma of the larynx. Cells of these cell lines are found to contain HeLa marker chromosomes, and were derived of via HeLa contamination. Cells contain keratin and contain papilloma virus (AE Moore et al., 1995). Various studies have indicated the anticancer effect of ethanolic extract of clove but its activity against Hep2 cell line has not been explored yet. Hence Ethanolic extract of Clove was taken to study the anti-cancerous effect on HEP2 cell line. Different concentration of clove extract has been taken to study its effect on HEP2 cell line. This cytotoxicity was determined by performing MTT assay, NRU assay and apoptosis was analysed with DNA Fragmentation assay. Clove extract shows anti-proliferative effects on various cancerous cell lines like HeLa cells, breast cancer, prostate cancer, esophageal cancer. EAEC shows its inhibitory effect on lungs cancer by reduction in proliferation and enhances apoptosis (Dwivedi and Shrivastava et al., 2009). Earlier some work has been done on cytotoxicity of certain compounds on HEP2 cell lines. Silver nano particles exhibited excellent cytotoxic effect on HEP2 cell lines (Narayanan et al., 2012). Several radiotherapy and chemotherapy are given which are often leading to toxicity. So we move towards herbs, clove extract provide a better option. Clove extract with low dose of gemcitabine that was highly toxic for cancerous cells. Clove extract increases the efficiency of gemcitabine and it was found less toxic to normal cells (Hussain A., Sasidharan S., Ahmed T., Ahmed M., 2009). Different solvents (Hexane, Diethyl-ether, Ethyl

acetate, Ethanol, Acetone and aqueous) extracts were prepared based on varying polarity index. Each of these extracts had shown anticancer activity, but most pronounced effect was observed in hexane. The Hexane extract of clove showed an activity against cancer cells at a minimum concentration of 125 μ g/ml, and anti-cancer activity at a concentration 350 μ g/ml showed 50% inhibition (IC50) against MCF-7 cells (Sharma Pankaj et al., 2014).

II. METHODOLOGY

A. Extract Preparation

Clove extracts is prepared from ethanol solvent. Buds of clove were bought from market, India. They were milled to fine powder with the aid of a clean electric blender. 6 g milled clove powder was soaked in 200 ml of ethanol to prepare the ethanolic extract. After removal of water powered clove 6g was loaded in the inner tube of Soxhlet apparatus and then filter into a round bottomed flask containing in 200 ml of 100% ethanol to prepare the ethanolic extract. The extracts of clove were boiled gently over a water bath using adjustable rheostat. The extraction was continued for 8h and the solvent was removed at the reduced pressure with the help of vacuum pump distillation unit. Left residual volume is taken in small petri-plate and preserved for several days to let ethanol evaporation from clove extract. After that clove extract is taken for dose preparation.



B. Cell Line Screening

HEp-2 cell lines were derived from epidermoid carcinoma of the larynx. Cells of these cell lines are found to contain HeLa marker chromosomes, and were derived from HeLa contamination. Cells contain keratin and papilloma virus [7].

C. Chemicals and Reagents

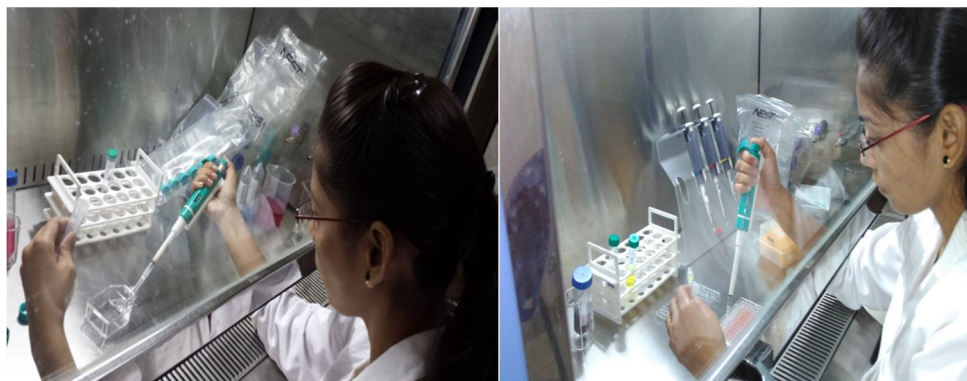
EMEM, Trypsin-EDTA, MTT reagent, NRU reagent, Ethanol, PBS(Phosphate Buffer Saline), TAE Buffer, TE Buffer, Lysis Buffer, Fetal Bovine Serum (FBS).

D. Cell Culture

EMEM (Earles minimal essential medium) is used for the culture of HEp2 cells. Once Hep-2 cells reached approximately 80% confluence on plates, decant media from flask. Add 1.5 ml of 0.25% trypsin-EDTA solution to cells to detach cell layer. Do not agitate the cells during dispersal, either by hitting or shaking the flask.

This may cause clumping of the detached cells). Once Hep-2 cell layers is detached, deactivate trypsin by adding 2 ml of incomplete media in above T-25 flask. Aspirate cells by gently pipetting.

Transfer the cells into the 25 ml centrifuge tube. Centrifuge cells in 25 ml eppendroff tube for 5 minutes at 300 RCF. Discard the supernatant and add 6 ml complete media and mix it well with micropipette. Differentiate the cells into three T-25 flasks and make their volume 6ml each with complete media. Store the T-25 Flask at 37.7 °C in CO2 incubator with 5% CO2 supply for 48 h.



E. MTT Assay

MTT assay is used for observing anti-proliferative activity. Measurement of cell Viability and proliferation forms the basis for numerous in vitro assays of a cells populations response to external factors. The reduction of tetrazolium salts is now widely accepted as a reliable way to examine cell proliferation. The yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl)-2, 5-diphenyltetrazolium bromide) is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intra-cellular purple formazan can be solubilized and quantified by spectrophotometric means. The MTT Cell Proliferation Assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability. The number of assay steps has been minimized as much as possible to expedite sample processing. The MTT Reagent yields low background absorbance values in the absence of cells. For each cell type the linear relationship between cell number and signal produced is established, thus allowing an accurate quantification of changes in the rate of cell proliferation (Mosmann et al., 1983).

F. Dose Preparation

Doses were prepared with 100% ethanol extract of clove and weighed 50mg/ml and diluted to 20 mg/ml (T_1) concentration and they are further serial diluted in T_2 , T_3 and T_4 eppendroff tubes. Doses prepared for treatment are of concentration of 1000 μ g/ml, 100 μ g/ml, 10 μ g/ml and 1 μ g/ml. They are 5 μ l transferred to each well of 96 well plate as per different column decided for treatment T_1 , T_2 , T_3 and T_4 . 100 μ l of 20 mg/ml is taken and added with 900 μ l-distilled water and further diluted.

G. NRU Assay

Harvest suspension cells by centrifugation. Adherent cells should be released from their substrate by trypsinization or scraping. Resuspend cells at 1×10^6 per mL. Prepare serial dilutions of cells in culture medium from 1×10^6 to 1×10^5 cells per ml. Plate out, in triplicate, 100 μ l of the dilutions into wells of a micro-titerplate. Include one control well of medium alone to provide the blanks for absorbance readings. Incubate the cells under conditions appropriate or the cell line for 6 to 48h (to recover from handling). The time required will vary but 12h to overnight is sufficient for most cell types. Suck out media from well plate and add 100 μ L of the NR. The plates were incubated at 37°C with 5% CO_2 for further 2h. After incubation, plates were taken out, NRU is completely poured out and 100 μ L of NR desorb added per well. Plates were protected from light and well mixed with pipette. Plates were taken to 96-well plate reader and their absorbance is measured in each well at 540nm, 550nm and 650nm.

H. DNA Fragmentation Assay

DNA Fragmentation Assay is a tool to study apoptosis in treated cells. This assay was performed to determine the effect of ethanolic extract of clove in the DNA of the HEp2 cell lines. This assay will highlight the DNA damaging potential of clove extract. In this assay cells were treated with different doses of clove extract. Then treated cells DNA will be isolated from cell and their damaging will be seen in Gel Electrophoresis. The entire damaged DNA will be fragmented into several fragments and produces smear in agarose gel whereas intact DNA forms a clear band. High amount of dead cells will be there with increasing concentration of doses and thus the greater number of fragments in the culture which can be estimated in Gel Electrophoresis. All the cells which are live will have intact DNA so there will no fragmentation. Thus as in experiment with the doses of different concentration different type of effects will be shown. As cells with highly concentrated doses will have high amount of cell death and it will decrease with the decreasing concentration of extract. Thus DNA Fragmentation assay will clearly prove the cytotoxicity of the ethanolic extract of clove.

III. RESULTS AND DISCUSSION

A. Cell Culture

HEp2 Cell growth is shown in figure 1.

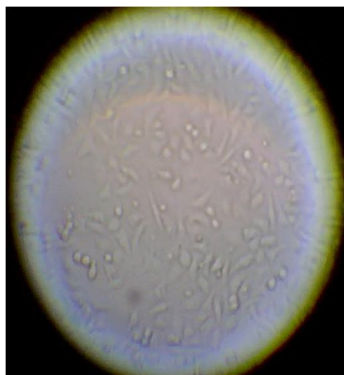


Figure 1:HEp2 cell line

MTT treatment on 96 Well plates is shown in fig. 2.

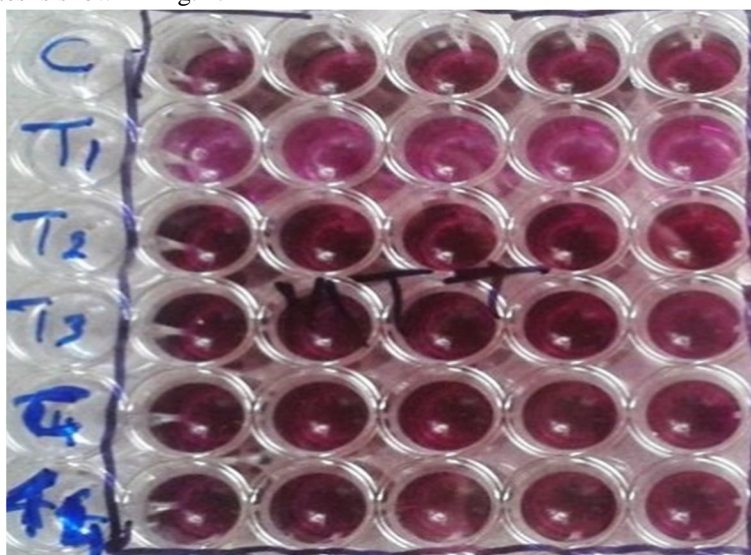


Figure 2: MTT Assay 96-well

Graph (fig.3) of MTT Assay compares the result of each treatment with the control. This shows that 1000 $\mu\text{g/ml}$ of dose had drastic effect on the cancerous cell lines. Other does not show any effect on the cancerous cells. However, high amount of decrement in the cell line shows that about 300 $\mu\text{g/ml}$ and above will show cytotoxicity in the HEp2 cell lines. Here 3 stars show 99.9% chances of same result in the case of experiment done in the same way.

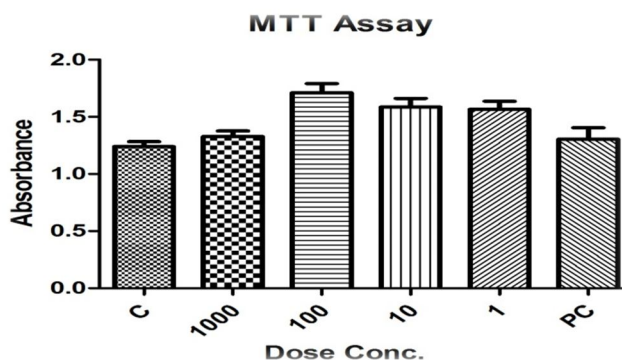


Figure 3: MTT Assay for clove

NRU treatment on 96 Well plates is shown in fig. 4.



Figure 4: NRU Assay 96-well

Graph (fig.5) of NRU Assay shows apoptosis in the 1000 $\mu\text{g/ml}$ of dose, other dose does not show any proper effect on the cancerous cell line. This confirms that the clove have cytotoxicity in the HEp2 cell lines.

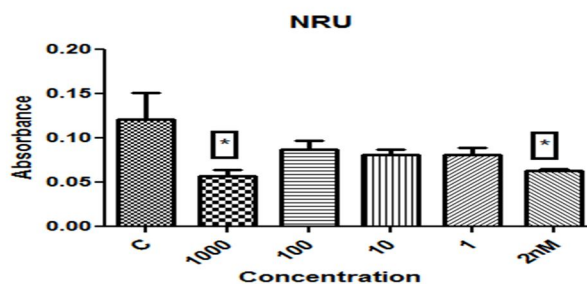


Figure 5: NRU Assay for Clove

DNA Fragmentation Assay on 96 Well plates is shown in fig. 6.

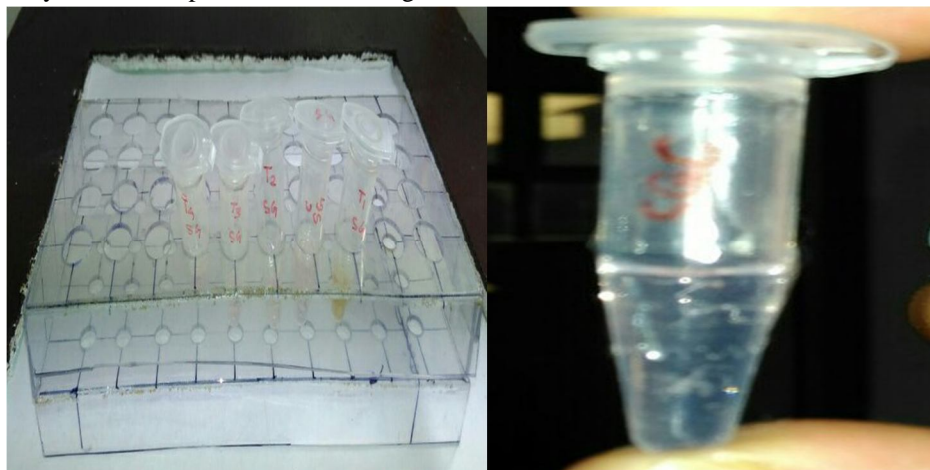


Figure 6: DNA Fragmentation Assay

DNA Fragmentation Assay perfectly determines the cytotoxicity of the Clove on the HEp2 cell line. Decreasing the concentration of the Clove extract in every well of the cell plate has some cytotoxic effect on HEp2 cell line. DNA got fragmented and separates in electrophoresis gel. Long lines in the gel represent the high concentration of fragmented DNA. Control has very less DNA fragments and others have DNA fragments. T_1 have more DNA fragments than T_2 , T_3 and T_4 . In the following way, there is the increase in the order of DNA fragments: $C < T_4 < T_3 < T_2 < T_1$. Ethanolic extract had been used in various Anti-cancerous experiments.

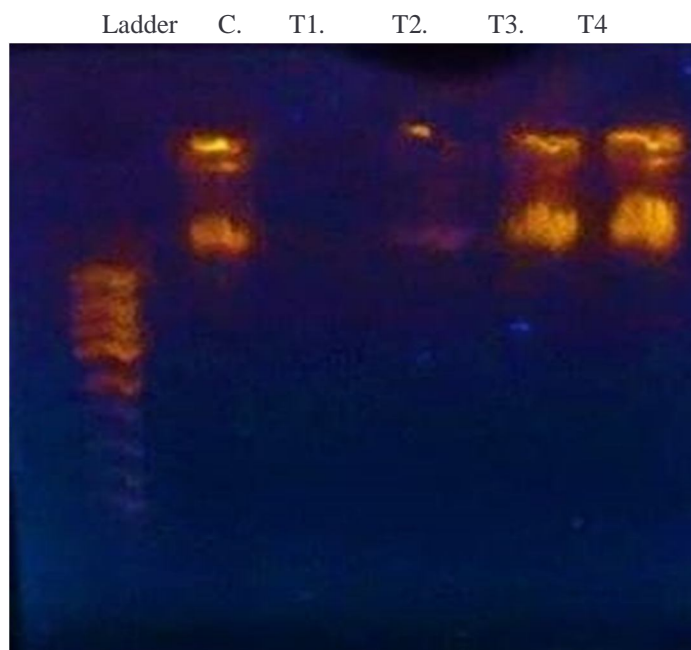


Figure 7: DNA Fragmented Assay of clove

Water, ethanol and oil extract were taken from clove, which show different activity on different cell line. Oil extract shows maximal cytotoxicity. Morphological analysis and DAPI staining showed cytotoxicity to be a result of cell disruption with subsequent membrane rupture. Maximum cell death and apoptosis occur in TE cell within 24 h whereas minimal death is in DU-145 cells [11]. Clove ethyl acetate extract (EAEC) is also beneficial for anti-cancerous activity. Mainly molecular changes are associated with it, Oleanolic acid (OA), a component of EAEC is responsible for anti-cancerous activity, EAEC is superior to OA. EAEC promoted G0/G1 cell cycle arrests and induces apoptosis in dose wise manner. Thus, in the same way, we took ethanolic extract of Clove and it induces the apoptosis in the cell in a dose wise manner high concentration shows high amount of apoptosis. Ethanolic extract also had same effect as like the EAEC induces apoptosis on cell line. As like the lung cancer and other cancer has been inhibited by Clove extracts, the ethanolic extract shows inhibitory effect on the HEp2 line.

IV. CONCLUSION

MTT, NRU and DNA fragmentation assays were proved the cytotoxicity of ethanolic extract of Clove on HEp2 cell line. MTT Assay at 1000 $\mu\text{g/ml}$ shows drastic effect on the cell line but others low concentration do not show that effect on HEp2 cell line. NRU assay also shows cytotoxic effects of ethanolic extract of Clove on the HEp2 cell line at 1000 $\mu\text{g/ml}$ concentration but other concentration do not show any effect on cell line. The more proper way of determining is DNA Fragmentation assay, which is indicator of apoptosis, since apoptosis can be seen with different concentration of ethanolic extract of Clove thus we can conclude the cytotoxic effect of ethanolic extract on HEp2 cell line. Here by DNA Fragmentation assay, we observe the effect of different concentration on cancerous cell line at 1000 $\mu\text{g/ml}$, 100 $\mu\text{g/ml}$, 10 $\mu\text{g/ml}$ and 1 $\mu\text{g/ml}$ induces decreased effect on HEp2 cell line. Highest effect is of 1000 $\mu\text{g/ml}$ and least is of 1 $\mu\text{g/ml}$ ethanolic extract of Clove. .

V. ACKNOWLEDGEMENT

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VI. CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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