To Study the Anticancer and Apoptotic Activity of *Mentha arvensis.* Linn in ROS Induced Buccal cells.

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ABSTRACT

The anticancer activity tested has shown notable activity against the cell lines. The present study confirms that the plant will be a potential source of medicine in future and extensive study is required for the discovery of new drugs on the active biological molecules. The methanolic leaf extract *Mentha arvensis* has shown significant cytoprotective activity too. The cell treated with H2O2 has a reduced viability than the methonolic leaf extract treated cells. In H_2O_2 treated cells the cytotoxicity was more than 70% i.e. only 34.76 % is viable and the cells treated with the methanolic leaf extract of *M. arvensis* has shown viability up to 54.23 %. It can be observed that addition of hydrogen peroxide produced approximately 79.3 % increase in DNA fragmentation which can be attributed to the DNA lesions formed by breaking phosphodiester bond in intact chromosomes by OH radicals. The plant extracts produced 63.7% decrease in DNA fragmentation indices suggesting potent cytoprotective effect against deleterious effects of hydrogen peroxide.

Key word: *Mentha arvensis, anticancer,* H_2O_2, DNA fragments, Heat Shock Protein 70

I. INTRODUCTION

Plants which have one or more of its organs containing substances that can be used for the therapeutic purpose, are called medicinal plants (1). They are rich source of bioactive compounds and thus serve as important raw materials for drug production. India has 45,000 plant species and among them 8000 medicinal plants and 1200-2500 aromatic plants are present (2) (3).

Cancer is the second leading cause of death worldwide. Eleven million new cases of cancer are diagnosed every year. It is estimated that there are approximately 2 - 2.5 million cases of cancer in India at any given point of time with and more than 7 million new cases being detected every year Oral cancer is a life style related cancer. Head and neck squamous cell carcinoma occurs in 50,000 new cases annually in the USA, results in more than 13,000 deaths every year. India has the highest incidence of oral squamous cell carcinoma in the world. About 50-70% of cancer-related deaths in India are due to oral cancer. Age- standardized incidence rates of oral cancer per 100,000 populations in India were estimated to be 12.8 in men and 7.5 in women. The data from World Health Organization (WHO).

The burst of reactive oxygen species and reactive nitrogen species, has been implicated in the development of cancer. Hydrogen peroxide (H_2O_2) is considered as a mediator of apoptotic cell death. Cells undergoing apoptosis from exposure to H_2O_2 display a significant decrease in intracellular Concentration of superoxide (O^-_2) which is associated with a reduction of the intracellular milieu. Decrease in intracellular O^-_2 concentration triggers apoptosis thereby inducing a reductive as opposed to an oxidative stress. (4)

Heat shock protein, particularly HSP 90, HSP 70, are well known regulators of apoptopsis by interfering with key apoptoptic proteins. Apotopsis and cell differenciation are two physiological processes that share common features like chromatin condensation and the need of the proteases and capsases. Little is known about the role of HSP's in the differentiation process. HSP 70, during terminal blood red cells differenciation and at the onset of capsese activation, translocates into the nucleus where co-localizes and interacts with GATA-1, a transcription factor essential for red cells progenitors (erythroblasts) differenciation. In vitro and in vivo assays demonstrate that HSP 70 inhibits capsase- 3 mediated proteolysis of GATA-1, allowing the differenciation of the erythroblast. If the amount of nuclear HSP 70 is reduced, GATA-1 is cleaved and the cells die by apoptopsis (5).

The *Mentha arvensis* .Linn is a medicinally important plant species that belongs to the family Lamiaceae. It is an aromatic herb usually known as Pudina or corn mint or menthanol mint or Japanese mint. It is a culinary herb as well as possesses healing properties. (6). Mint plants contain over 40 distinct chemical compounds (7). Mint and its oil are used in China for treating indigestion, nausea, sore throat, diarrhea, colds, and (8) (9). It is also reported as a radioprotectant (10).

II. MATERIALS AND METHODS

A. Collection of Plant Material

Fresh plants were collected from a place named near Coimbatore, Tamilnadu, India. The leaves were separated, washed under running tap water and shade dried at room temperature. The dried leaves were ground to fine powder using a blender. The powder was preserved in an air tight bottle for further use.

B. Preparation of plant extract

10g of air dried powder were taken in 100mL of methanol. Plugged with cotton wool and then kept on a rotary shaker at 190-220rpm for 24hours. After 24hours the supernatant were collected and the solvent were evaporated to make the final volume one-fourth of the original volume and stored at 40c in air tight container.

C. Isolation and Culturing of Buccal Cells

Left over normal oral tissues following to teeth extraction process was kindly given by Dental Hospital in Trivandrum, Kerala and it was aseptically transferred to laboratory with Stemline Keratinocyte media (Sigma).

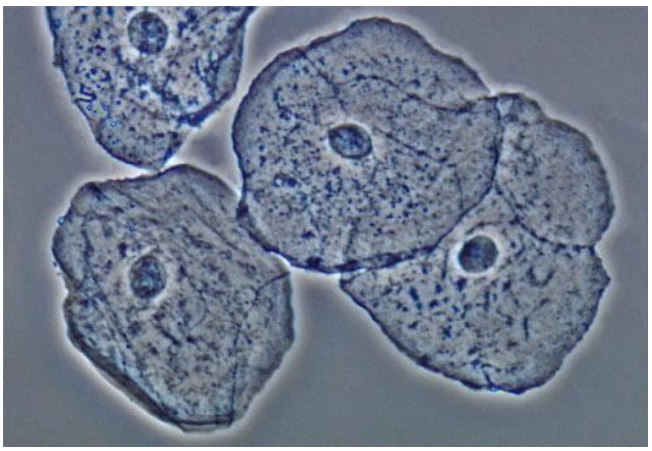

Fig. 1: Phase Contrast Microscopic view of Buccal Cells

The collected tissue was washed with PBS and stored in a T-flask along with Stemline Keratinocyte media and again washed with PBS. Tissues were finely chopped in to small pieces, transferred into a new T-flask with Stemline Keratinocyte media with growth supplement factor (Sigma Aldrich) and allowed to incubate for 72 hours in CO2 incubator at 37º C, 5% CO2. Subculturing was done after the cells were found growing attached by washing by PBS followed by addition of 500µl trypsin for detaching the cells and added fresh media. All these subculturing process were done under highly sterilized condition within the laminar air flow.

D. ROS Induction and Sample Preparation

Primary cultures of Buccal Epitheial cells were maintained in Stemline Keratinocyte Growth media (Sigma Aldrich) supplemented with 100 µg/ml of Growth supplement (Keratinocyte Growth supplement). Buccal cells were exposed to hydrogen peroxide at a final concentration of 0.5mM to one flask for 24 hours.One flask was treated with 500µg of extracts after addition of 0.5mM of Hydrogen peroxide for 24 hours and an untreated flask with buccal cells was maintained as control**.**

*E. MTT ASSAY (*Arung *et al*., 2000) (11)

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 º C for 3 hours. Removed all MTT by washing with 1x PBS and added 300µl 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 2minutes to precipitate cell debris. OD was read at 540 nm using DMSO as blank.

F. Real Time Polymerase Chain Reaction

1) RNA Isolation: Total RNA was isolated using the total RNA isolation kit according to the manufactures instruction (Chromous Biotech). Buccal epithelial cells after exposure for 24 hours with hydrogen peroxide and hydrogen peroxide with extracts was scrapped out and pelleted out at 5000 rpm in a cooling centrifuge. An untreated plate was maintained as control. 300µl RNA sol was added and mixed thoroughly. To this 200µl chloroform: isoamyl alcohol mixture (24:1) was added. Mixed well and centrifuged at 10000 rpm 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.

2) Agarose Gel Electrophoresis: 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.

3) RT-PCR analysis of HsP 70 gene: Mix 5µl of RNA template (~1µg) with 400ng of reverse primer (Hsp 70 forward primer 1.75 µl) and added DEPC water to make the volume of the reaction mix to 20µl. Incubated the above mix at 65º C for 10 min and immediately chilled on ice. 25µl reaction mix was added to fresh PCR tubes followed by 20µl RNA-primer mix, 300ng 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 housekeeping genes was also maintained (GAPDH)

Human Hsp 70
Forward: CCATGGTGCTGACCAAGATGAAG
Reverse: CACCAGCGTCAATGGAGAGAACC

GAPDH
Forward: GAGACAGCCAGGAGAAATCA
Reverse: GAAGATGGTGATGGGATTTC

Cycle Condition: The following cycles were selected as per previous references and Tm of primers. The tube were place in Eppendorf Master Cycler and programmed for the following 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.

G. DNA Fragmentation by DPA Method

After 24 hours of incubation the cells, were scraped off and centrifuged at 6000rpm at 4ºC for 10mins. The supernatants were transferred carefully in new tubes and labeled 'S' (supernatant). The pellet and 1ml of TTE (Triton Tris EDTA solution) was labeled as 'B' and vortexed vigorously. To separate the fragmented DNA from intact chromatin 'B' was centrifuged at 14,000rmp for 10mins at 4ºC. The supernatants were transferred carefully in new tubes labeled as 'T'. 1ml of TTE solution was added to the pellet in the tube 'B'. 1ml of 25% TCA was added to tubes T, B and S and vortex vigorously. Precipitation was allowed to proceed by overnight cooling at 4ºC. After the incubation, precipitated DNA was recovered by pelleting for 10 mins at 4000 rpm at 4ºC. Supernatants were discarded by aspiration. DNA was hydrolysed by adding 160µl of 5% of TCA and it was heated for 50minutes at 90ºC in a heating block. A blank was prepared with 160 µl 55% TCA alone. To each tube 320µl of freshly prepared DPA solution was added then vortexed, and colour was allowed to develop for about 4hours at 37ºC overnight at room temperature. 200ml aliquot of coloured solution (ignoring blank particles) from each tube was transferred to a well of a 96 well microtitre plate. The density was read aseptically at 600nm with a multi well spectrophotometer reader setting blank to 0. The excitation wavelength of 600nm is the optical one but wavelength from 560-620nm could be used as well. The percentage of fragmented DNA was calculated using the formula:

$$\% \text{ fragmented DNA} = \frac{S + T \times 100}{S+T+B}$$

Where S, T, B are the O.D at 600nm of fragmented DNA in the S, T and B respectively. The fragmented DNA released by all undergoing apoptosis and lysis during experiment was recovered in fraction 'S' and should therefore be taken in consideration in particular circumstances present in fraction (serum protein components of the tissue culture medium, etc) could be heavily interfere with the O.D measurement is could be more convenient to disregard the 'S' tubes (which most of the time contain only negligible amounts of DNA) and to apply the following formula.

$$\% \text{ of fragmented DNA} = \frac{T \times 10}{T+B}$$

H. Lactate Dehydrogenase Assay (12)

LDH release assay was performed with cell free supernatant collected from tissue culture plates exposed to a concentration (500 µg) of extracts. To this added 2.7ml potassium phosphate buffer, 0.1ml 6mM NADH solution, 0.1ml sodium pyruvate solution, into a cuvette and mix well. The decrease of OD was recorded at 340nm in a spectrophotometer, thermo stated at 25^0 C. (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,

$$\text{Volume of activity (U/ml)} = \frac{[(\text{Abs} - \text{Ab0}) \times 1 \text{ (ml)} \times \text{df}]}{[6.2 \times 0.1\text{(ml)}]}$$

I. Neutral Red Assay (13)

Buccal epithelial cells treated as per standard procedure 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.

$$\text{Viability} = [(C\text{-}T) \div C] \times 100$$

Where C= Absorbance of blank and T= Absorbance of testy (U/ml) = [(Abs – Ab0) x 1 (ml) x df] ÷ [6.2 x 0.1 (ml)].

III. RESULTS

A. *MTT Assay*

The methanolic mint leaves extract has shown significant cytoprotective activity. The cell treated with H2O2 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 70% i.e. only 34.76 %(Table.1) is viable and the cells treated with the methanolic leaf extract of *M. arvensis* have shown viability up to 54.23 %. It is graphically represented in Figure 2.

TABLE 1: THE CYTOTOXIC EFFECT OF METHANOLIC LEAF EXTRACT OF *MENTHA ARVENSIS* BY MTT ASSAY.

Sample Concentration	% viability
Control	**100**
H_2O_2 (500 µL)	**34.76**
EXTRACT + H_2O_2	**54.25**

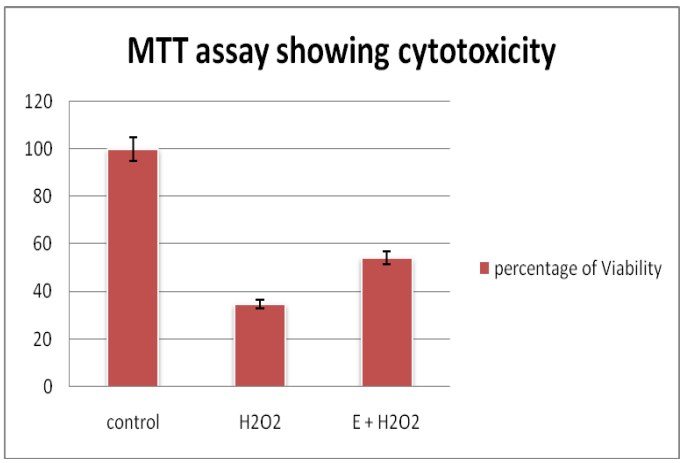

Fig. 2: Graphical representation of MTT Assay showing Cytotoxicity

B. *Real Time PCR*

Total RNA isolated was subjected to PCR analysis and the following results were obtained. From the (Figure 3) 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 *Mentha arvensis*. The potential role of oxidative stress conditions in the induction of heat shock proteins is previously described by workers like Jornot *et al.*, 1991.

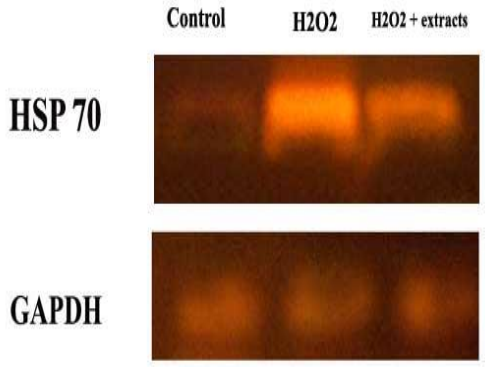

Fig. 3: RT-PCR analysis of HsP 70 gene in oxidative stress induced buccal epithelial cells treated with methanolic leaf extract of *Mentha arvensis*

C. *DNA Fragmentation*

The Figure 3 shows that the addition of hydrogen peroxide produced approximately 79.3 % increase in DNA fragments which can be attributed to the DNA lesions formed by breaking phosphodiester bond in intact chromosomes by OH radicals (Table.2) (Figure.4). The plant extracts produced 63.7 % decrease in DNA fragmentation indices suggesting potent cytoprotective effect against deleterious effects of hydrogen peroxide.

TABLE 2: DNA FRAGMENTATION ANALYSIS OF BUCCAL CELLS TREATED WITH METHANOLIC LEAF EXTRACT OF *MENTHA ARVENSIS* USING DPA METHOD

Sample	% DNA fragmentation
Control	12%
H_2O_2 (500 µL)	58%
H_2O_2 + Extract	21%

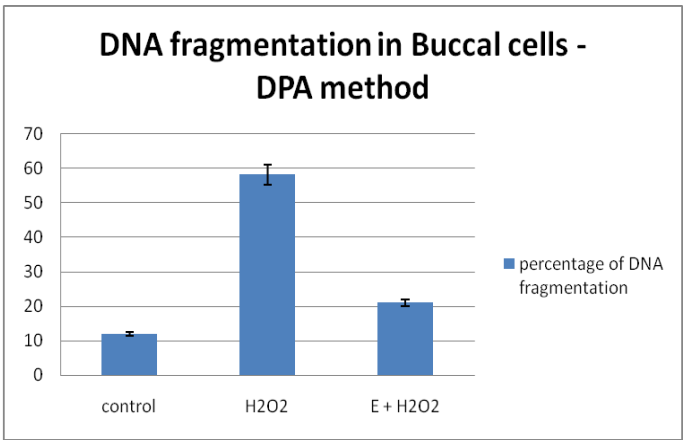

Fig.4: Graphical representation of DNA fragmentation

D. Neutral Red Assay

Neutral Red Assay is performed to study the cell viability. The methanolic leaf extract has shown significant cytoprotective activity (Figure 5). 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 *M. arvensis* has shown viability up to 62 % (Table. 3). This shows the significant cytoprotective activity of the plant extract.

TABLE 3: NEUTRAL RED ASSAY PERFORMED IN BUCCAL CELLS TREATED WITH METHANOLIC LEAF EXTRACT OF *MENTHA ARVENSIS*

sample	% viability
Control	**100**
H_2O_2	**40.12**
EXTRACT +H_2O_2	**62.0**

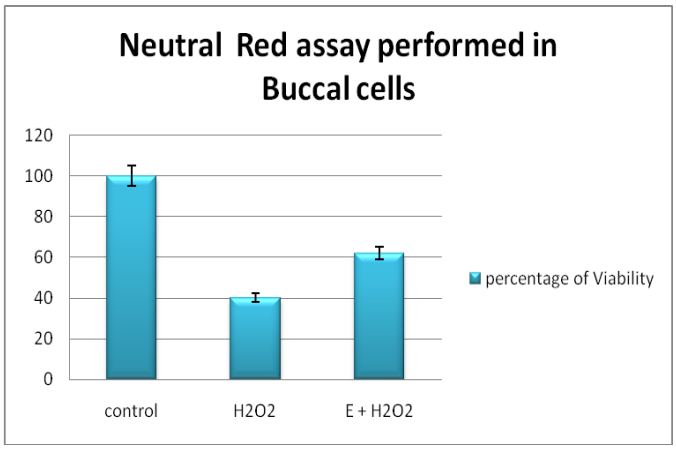

Fig 5: Graphical Representation of Neutral Red Assay Performed in Buccal Cell

E. Lactate Dehydrogenase Assay

Lactate dehydrogenase assay was performed to study the cell viability of Buccal cells (Figure. 6). The plant extract treated cells has shown significant cell protective activity towards H_2O_2 treated cells. The cell treated with H_2O_2 has a reduced viability as the LDH release was higher than the cells treated with the methanolic leaf extract of *M. arvensis* (Table. 4). This shows the significant cytoprotective activity of the plant extract.

TABLE 4: LDH ASSAY IN BUCCAL CELLS TREATED WITH METHANOLIC LEAF EXTRACT OF *MENTHA ARVENSIS*.

sample	V(U/ml) $X10^{-1}$
Control	**0.86**
H_2O_2	**2.415**
EXTRACT + H_2O_2	**1.152**

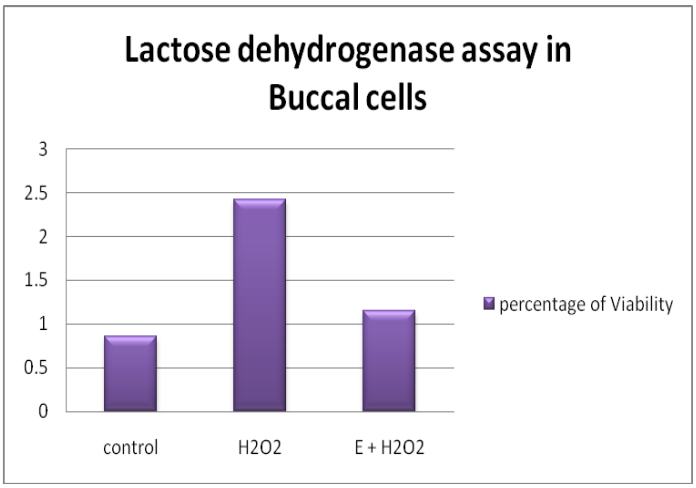

Figure 6: Graphical Representation of Lactose dehydrogenase Assay performed in Buccal Cell

IV. CONCLUSION

The objective of cancer chemotherapy is to kill cancer cells with as little damage as possible to normal cells (14). It is therefore urgent to look for novel natural or synthetic apoptosis-inducing compounds as candidate antitumor agents. Along this line, plant-derived compounds have great potential to be developed into anticancer drugs because of their multiple mechanisms and low side effects (15) (16) (17) (18) (19). Therefore, any discovery of anticancer agents must be related to novel molecular targets; *i.e.* they should be effective against specific types of cancer cells but less toxic to normal cells, or have a unique mechanism of action for specific types of cancer (15). Based on the current study, it can be concluded that the methanolic leaf extract of *Mentha arvensis* is apoptotic as well as retains cell viability in normal cells by reducing the activity of heat shock proteins. Hence further studies can reveal the more specific biomarkers that can be novel medicines for Buccal cavity cancer. The studies discussed in this paper are mainly in vitro and for more reasonable and conclusive results, it is required to do in vivo and finally human and clinical tests.

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