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Essential Oils of Spice Plants Prevent DNA Damage

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Abstract

Essential oils of six spice plants namely *Piper nigrum*, *Nigella sativa, Cinnamomum zeylanicum, Syzygium aromaticum, Cuminum cyminum* and *Ptychotis ajowan* were evaluated for their protective properties against chemical mutagen (hydrogen peroxide). The experiments were performed on fresh human cheek cells, *in vitro*, using single cell gel electrophoresis/comet assay technique. Hydrogen peroxide (H_2O_2) was used in concentrations of 25, 50, 100, 150, 200, 250, 300, 350 and 900 µM respectively to get decipherable damage in DNA of cheek cells. Conspicuous DNA damage (comparatively larger tail lengths) was observed in human cheek cells on application of H_2O_2 (350 µM). The effectiveness of essential oil against H_2O_2 (350 µM) was tested by evaluating the inhibitory effect of essential oils against chemical mutagen on human cheek cells . All the six essential oils were found evenly effective in shielding the DNA damage caused by H_2O_2 (350 µM). Furthermore the comet assay technique was found to be sternly potent contrivance to appraise the DNA damage of individual cells.

Keywords

Single Cell Gel Electrophoresis/Comet Assay, Anti mutagenic Effect, DNA Damage, Cheek Cells, Essential Oils, Hydrogen Peroxide.

Introduction

Oxidant species generated during metabolism can impair bio molecules (Javaid, S 2015) but in response a lot of natural antioxidant molecules present in the body scavenge these oxidant species and nullify their harmful effect. Disparity between levels of bio molecules oxidant and antioxidant characters leads to many biochemical changes which can cause severe disorders. This disproportion puts on oxidative stress that probably lead to geno-toxic effect and might cause damage to DNA and bio molecules like lipids, proteins etc. (Olinski, 2002). DNA damage induces mutagenesis in individuals (Olinski, 2002) so the estimation and measurement of DNA damage could be used to examine the possible beneficial and damaging effects of environmental and dietary agents (Szeto *et al.,* 2005).

DNA mutagens could be inhibited by exploiting antioxidants like plant extracts that possess natural anti-oxidants properties against DNA mutagens. The use of natural nutritive (Jahangir, T.) and non-nutritive antioxidants against DNA mutagens had become interesting and major area of research (Mimica *et al*., 2010). Their protective properties against mutagenicity and cyto-toxicity had been attributed to the phenolic substances such as tocopherols (vitamin E & related compounds) and various classes of flavonoids, phenolic acid, tannins and lignans etc. (Larson, 1997). Numerous plants have been studied for their anti-oxidant properties and phenolic compounds, and their phenolic contents have been reported as superior antioxidant (Carlsen *et al*., 2010 and Pellegrini *et al*., 2006).

The present study aimed to investigate the antioxidant potential of spice plants extracts against oxidative damage.

Materials and Methods

Extraction of Essential Oils

Hydro distillation method by Shahwar *et al.,* (2012) was used to extract essential oils of six selected spices (Table-1) . The plant species were obtained from Department of Chemistry, Government College University Lahore, Pakistan and were chopped and grinded to powder after drying. The powdered material was weighed and placed in the round bottom flask of 1 liter along with some water. Steam was generated in a steam generator and circulated throughout the plant material. The essential oils were obtained from oily layer above the water. The oily layer was separated from water by solvent extraction method. Contents were poured into a separating funnel of 1 liter and extracted with diethyl ether. Anhydrous Na_2SO_4 was used to remove moisture and kept in air-tight sample vial at 4°C. All samples were prepared separately to avoid contamination.

Spice	**Local name**	**Botanical Name**		**Family**
		Genus	**Species**	
Black pepper	Kali Mirch	*Piper*	*Nigrum*	Piperaceae
Cinnamon	Dar Chini	*Cinnamomum*	*Zeylanicum*	Lauraceae
Black seed /seasam	Kalonji	*Nigella*	*Sativa*	Ranunculaceae
Clove	Long	*Syzygium*	*aromaticum*	Myrtaceae
Carom Seeds	Ajwain	*Ptychotis*	*ajowan*	Umbelliferae
Cumin	Zeera	*Cuminum*	*cyminum*	Apiaceae

Table-1 Nomenclature of the plants tested for anti-mutagenic potential

Cheek cell collection:

The cheek cells were collected from randomly selected normal, healthy, non-smoker male and female students of age between 18-25 years belonging to Department of Industrial Biotechnology, Government College University, Lahore, Pakistan during their internship program at CAMB. The cheek cells were collected from saliva of volunteers, with their full consent, in separate sterilized (50ml) beakers. The saliva samples were pipetted into micro tubes (1.5 ml) and cells were pelleted at 5000 rpm at room temperature for 15 minutes. The cell pellet was washed with autoclaved double distilled water at room temperature at 5000 rpm for 15 minutes and re-suspended in 100 µL of autoclaved double distilled water. The cell suspension so obtained was then further diluted to required.

Treatments with Hydrogen Peroxide

To evaluate the extent of DNA damage induced by Hydrogen peroxide, the cheek cells were treated with nine different concentrations of hydrogen peroxide (25, 50, 100, 150, 200, 250, 300, 350 and 900 µM H_2O_2). The washed cell pellet were re-suspended in 100 µL of respective stock of Hydrogen peroxide while one cell pellet was re-suspended in 100 µl of H_2O to be used as negative control.

Treatments with Essential Oils

The protective efficiency of the essential oils was studied against mutagenic effect of hydrogen peroxide. The method of cell treatment by Jayakumar (2012) was followed with modifications as they have used cell lines and not the fresh human cheek cells. The pellet of cleaned cheek cells was suspending in 30 µl of essential oil and 70 µl of 350 µM H_2O_2 with continuous mixing. This emulsion was shaked several times during the treatment period (10 minutes) to keep the oil in contact with cells.

Cleaning of cells after treatments

After 10 minutes 1.0 ml of autoclaved double distilled water was added to stop the reaction. Every time this practice was started from tube which was treated first and so on. At the end the tubes were centrifuged at 5000 rpm for 15 minutes at room temperature to pellet out cells. Supernatant was discarded and the cells were re-suspended in 0.75% low melt Agarose prepared in Phosphate Buffered Saline (PBS) for comet assay.

This procedure was enough to clean the H_2O_2 treatments but when treated with essential oils, extra cleaning was required as essential oils made lubricant layer at the surface of supernatant. For this purpose no organic solvents were used for washing as it could damage the cells and the precision of experiment. Therefore, oil was removed from tube manually as described in Figure-1 and required concentrations of cell suspension were prepared by using Neubauer Improved MARIENFELD Heamocyto meter (attached to OLYMPUS CKX41 microscope) for cell counting.

Single Cell Gel Electrophoresis:

Comet Assay

Single cell gel electrophoresis technique commonly known as Comet Assay was performed according to the ITRC: The scge/comet assay protocol (http://www.cometassayindia.org/Protocol%20for%20Comet%20Assay.pdf) with some modifications. The cell pellet was re-suspended in 0.75% low melt gel and diluted up to 1×10^5 cells then 75 µl of this suspension was poured onto the center of a frosted slide by keeping it in tilted position afterwards all successive steps were performed in diffused light or in dark to avoid any harm to the DNA. Suspension quantified at 75µl of 0.75% high melt agarose was dropped over the layer of gel having cells; the slides were kept at 4 °C and then immersed in lysing buffer for two hours; and put again at 4°C in dark. Then the slides were transferred s to chilled alkaline electrophoresis buffer and were kept at 4 °C in dark to let the damaged DNA to completely unwind. After 20 minutes the slides were transferred to electrophoresis chamber having chilled alkaline electrophoresis buffer and a paper box over it to provide darkness. The power was supplied at 1Volt/cm distance between two electrodes and the current was set at 300 m A. After 30 minutes the slides were removed out on a tissue towel and neutralizing buffer was applied gently over the slides (drop wise). The neutralizing buffer was applied again after 5 minutes and excessive moisture was sucked gently with the tissue and 80 µl of staining solution was dropped over the slides. Staining solution was let to dry and studied the slides under fluorescent microscope (Olympus BX61 DP Controller).

Results

Mutagenic effect of Hydrogen Peroxide

A steadily increased DNA damage was observed with increase in concentration of H_2O_2 in ratio of 25, 50, 100, 150, 200, 250, 300, 350, and 900 µM (Figure-2 B to J) except in control cells with no treatment of H_2O_2 which clearly exhibited round nuclei (Figure-2 A). Slight DNA migration was observed in slides prepared from cells treated with 25, 50, and 100 µM H_2O_2 (Figure-2 B, C, and D respectively**)**. No nuclei could be observed in 900 µM H_2O_2 because in this treatment only a very few nuclei with very light fluorescence were observed in only one replicate. (Figure-2 J). A modest quantity of DNA was left in the head of comet and rest of the DNA was migrated away showing that entire DNA was badly damaged on application of 900 µM H_2O_2. The DNA deterioration on application of 350 µM H_2O_2 was conspicuous in all the replicates with elongated tails in treated cells (Figure-2 J). Hence, this can be confidently stated that H_2O_2 passes from membranes to cells and can cause damage to the DNA and the spoilage fosters with enhancement in the concentration of H_2O_2.

Cheek cells selection as a model to study the damage in human DNA proved to be effective as the sampling was easiest, cheapest and . But after thorough review a strong idea was developed that buccal cells were not a good selection material for DNA damage. One of the reasons might be due to the fact that lysing the cheek cells was not so easy as in earlier experiments breakdown of cell membrane was difficult. Furthermore, comet assay was found to be a highly effective tool for DNA damage studies in individuals.

Evaluation of Potential of Spices' Essential Oil to Inhibit DNA Damage

Essential oils of six spice plants namely Black pepper, Cinnamon, Black seasam, Clove, and Carom Seeds locally known as Kali Mirch, Dal Chini, Kalonji, Long, Ajwain, and Zeera respectively, were tested for the anti-mutagenic activity. For this purpose the cheek cells were pretreated with essential oils and then with a standard mutagen concentration as described in methodology. Three hundred fifty µM H_2O_2 was used as standard mutagen, as it caused maximum damage to the DNA and the cheek cells were treated with all the six essential oils discretely before exposure to mutagen (oil was present in cell suspension when exposed to H_2O_2). Nearly complete defense was observed by all the tested oil samples as circular DNA were seen glowing with Ethidium Bromide under fluorescence microscope. No substantial DNA damage was observed in any slide of a treatment when observed under microscope (Figure-3 A to F). The Figure E and F showed enhance image of intact nuclei of experiment with Clove and Black seed respectively.

Discussion

Mutagenic effect of Hydrogen Peroxide

In the presented study buccal cells were used as model cells for studying different aspects of DNA damage through comet assay as previously studied by Szeto *et al.,* (2005). A comparison of DNA damage extent with and without oxidant pretreatment with declaration of comet assay as the preeminent option among all other methods to study the DNA damage was also reported in this study which coincides with the results reported by Szeto *et al.,* (2005). In current studies various concentrations of H_2O_2 were used to evaluate the extent of DNA damage as reported by Miranda-Vilela

et al., (2010) who reported positive correlation between the H_2O_2 concentration and DNA deterioration levels while no damage was found in control. Zaika *et al.,* (2011) premeditated the role of p73 protein in DNA damage by employing single cell gel electrophoresis/comet assay technique and figured out that deficiency of p73 protein fosters the DNA damage. Jayakumar and Kanthimathi (2012) performed a dose-activity assay with hydrogen peroxide to select the mutagenic concentration of Hydrogen peroxide and anti mutagenic activity of cumin seed, black peppers and cloves extracts through single cell gel electrophoresis technique. The concentrations of Hydrogen peroxide used in their studies were 25, 50 and 100 µM and maximum tail length in DNA was observed at 100 µM H_2O_2 for application of spice plants extracts to evaluate their protective properties against DNA damage. Present study explored maximum DNA damage in 900 µM H_2O_2 treatment while identifiable DNA damage was observed on application of H_2O_2 in concentration of 350 µM. The 350 µM concentration of H_2O_2 was selected for the present study to maintain the precision of the successful experiments because in most of the cases whole of the DNA vanished when subjected to higher concentration of H_2O_2 i.e. 900 µM.

Evaluation of Potential of Spices' Essential Oil to Inhibit DNA Damage

The potential of spice plants to protect DNA damage was evaluated by number of researchers. The anti-mutagenic activity of cumin, black pepper and clove extracts were evaluated by Jayakumar and Kanthimathi (2012) through single cell gel electrophoresis technique as an evaluation tool. They used different concentrations of Hydrogen peroxide to select the effective dose for induction of mutagenesis and to evaluate the anti-mutagenic potential of spices namely Cumin, black pepper and clove extracts, among six other spices against H_2O_2. Clove and pepper were found significantly effective against DNA damage at low concentrations but other spices showed anti-mutagenic effect only at higher concentrations of their water extracts. The results obtained in this study as shown in Figure-3 (E & A) respectively are in harmony with the results reported by Jayakumar and Kanthimathi (2012).

Correspondingly antioxidant properties of spices were also carried out by Parveen and Shadab (2012) through probing *in vitro* anti-genotoxic cause of *Nigella sativa* seed extract to control the number of chromosome aberrations induced by Chlorambucil (CLB) which caused genetic mutation through generating free radicals that can damage DNA. *Nigella sativa's* possess anti-carcinogenic activity that could inhibit DNA damage but it cannot completely protect cells from damage. On the basis of these evidences and reports *Nigella sativa* was used in present studies for evaluation of its protective properties against DNA damage caused through H_2O_2 application and results clearly revealed protective role of *Nigella sativa* as shown in **Figure-3 (F)**.

Cinnamon extract was also found to possess protective properties against DNA damage caused by H_2O_2 application in the present study as shown in **Figure-3 (B)** as reported by Jayaprakasha *et al.,* (2007) while studying antioxidant property of cinnamon extract from fruit in different solvents i-e water, methanol, acetone, and ethyl acetate. They found maximum antimutagenic activities of cinnamon fruit when extracted in water. Black pepper was found to possess anti-mutagenic properties in present study as shown in **Figure-3 (A)**. The results of present study coincides with results of Hamssa *et al.,* (2003) who reported anti-mutagenic effect of bell pepper and black peppers in *Drosophila melanogaster*. Alkylating agent were used by them, e.g. the methyl methane sulfonate in combination with bell pepper and black peppers extract with the result that anti-mutagenic and anti-carcinogenic effect of food components is based on their capability to inhibit carcinogen activation and improving the detoxification of carcinogens, scavenging the reactive agents that damage DNA, by modifying some properties of the cancer cell and hindering the early pre neoplastic lesions from abnormal proliferation. Previous reports and present results have suggested that black pepper show anti mutagenic effect due to inhibition of certain mutagen formation and their direct interaction with electrophilic species.

Conclusion

From DNA damage of human cheek cells through hydrogen per oxide during these studies it can be suggested that the essential oils of spice plants effectively restrain DNA damage caused by hydrogen peroxide. This points towards another fact that natural plant extracts (essential oils) of clove, cinnamon, black pepper, black seed, carom seeds and cumin are incredibly good source of anti mutagenic materials. Therefore, it can be suggested that essential oils of spice plants can be added to the formula of hydrogen per oxide containing products to avoid or to minimize the risk of DNA damage. It would also minimize the risk of diseases generated due to mutagenic effect of such products. Furthermore, Single Cell Gel Electrophoresis is a highly dependable technique that could be used for studies on DNA damage. This technique was

very versatile and can be easily modified according to research project. However more studies to understand the mechanism of essential oils inhibition against hydrogen peroxide are inevitable.

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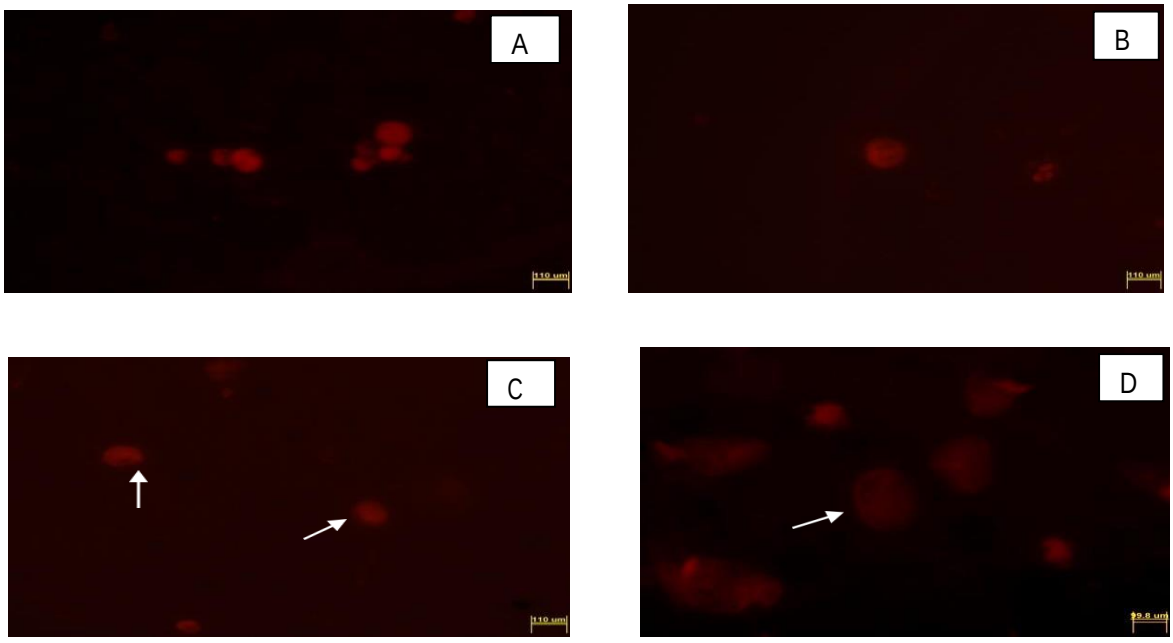
Figure-1 Cleaning of cells after treatment with essential oils

A. Tissue towel was wrapped over the pointed tip of a small spatula and a piece of tape was wrapped to fix the tissue at the back end of covering **B.** Carefully cleaned the oil from inner surface of tube, without touching the cell pellet; and sucked the remaining supernatant with layer of oil at top **C.** After cleaning, carefully removed the spatula head without touching the walls of tube and discarded in disinfectant.



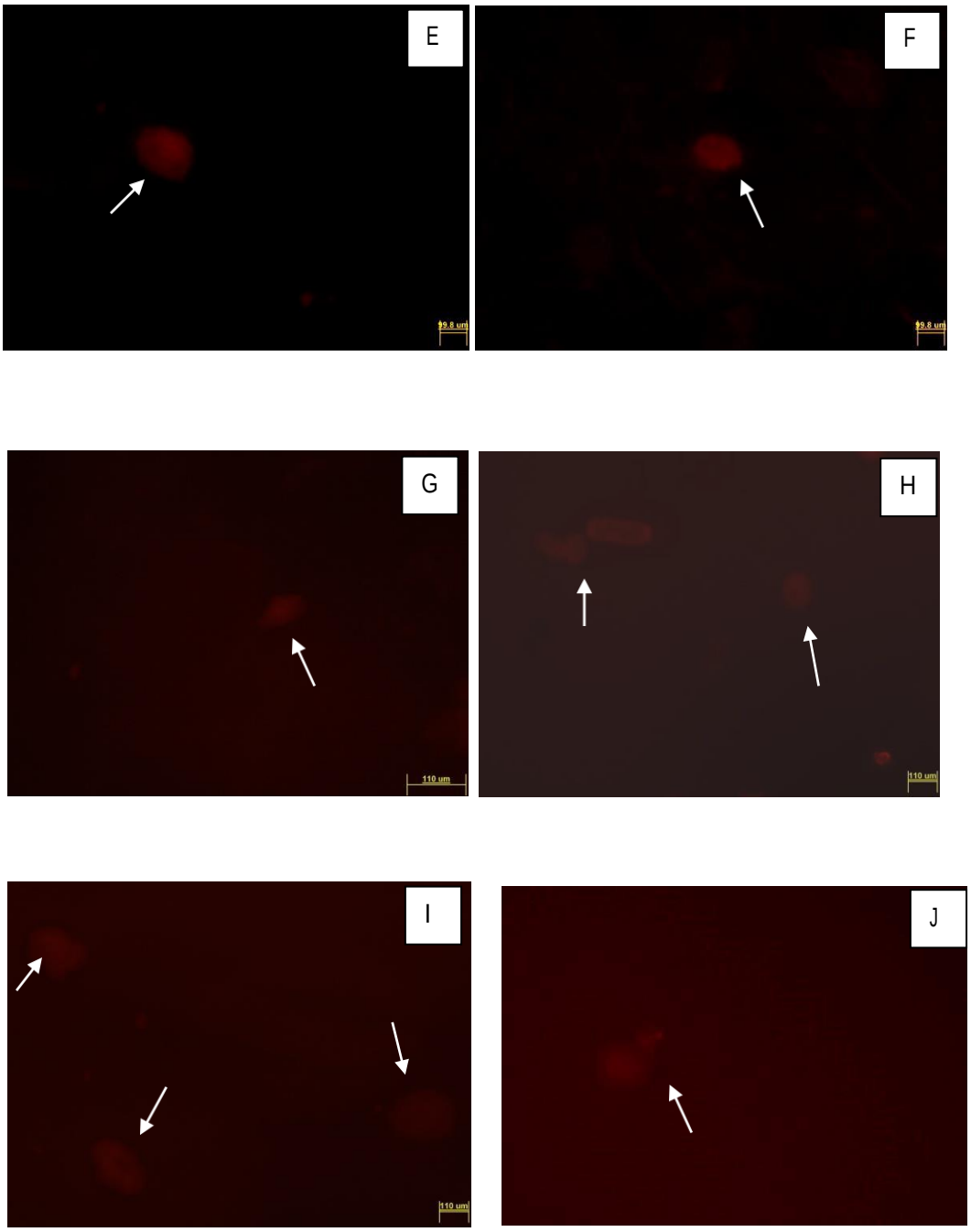


Figure-2 Comet Assay: Effect of different concentrations of H2O2 on cheek cells. (A) control cells without treatment with H2O2), **(B to J)** DNA damage is shown against 25, 50, 100, 150, 200, 250, 300, 350, 900 µM H2O2 respectively (Ethidium Bromide, RFP filter, 20x magnification of Olympus BX61 DP Controller, D- 40x magnification).

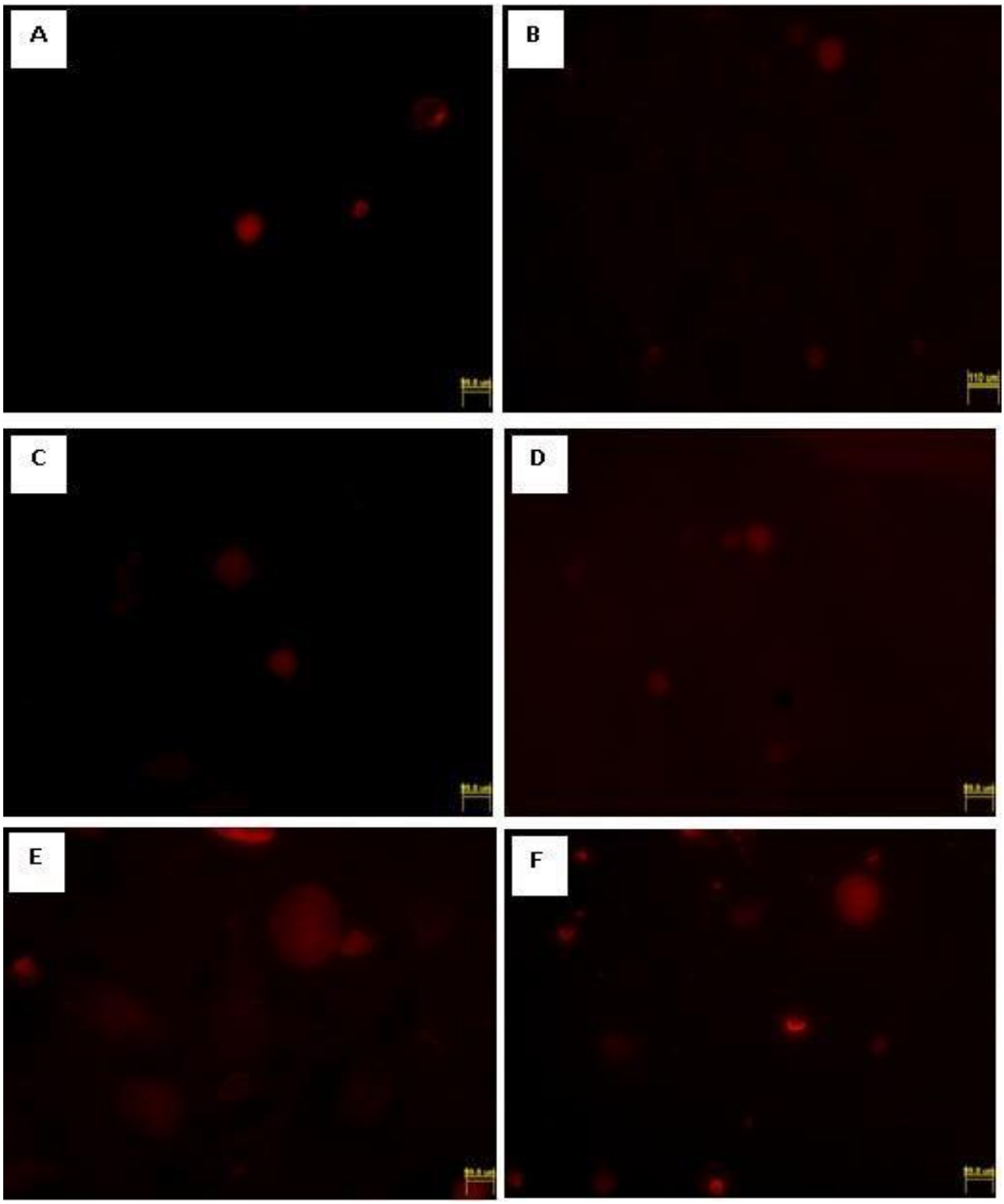


Figure-3 Comet Assay: Capability of Essential oils to inhibit DNA damage by H_2O_2 (A. Black pepper B**.** Cinnamon **C.** Carom Seeds **D.** Cumin (20x magnification of Olympus BX61 DP Controller) and **E.** Clove **F.** Black seasam. (40x magnification of Olympus BX61 DP Controller), Ethidium Bromide, RFP filter

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