Cyto-protective effect of kaempferol against H_2O_2 induced human blood mononuclear cells

Lavudi Saida* and Chepuri Kalyani
Centre for Biotechnology, Institute of Science and Technology, JNTUH, Kukatpally, Hyderabad, Telangana State 500085, INDIA
*lavudisaida@jntuh.ac.in

Abstract
Kaempferol is a natural flavonoid acting as an antioxidant and is currently under consideration as a possible anti-proliferative compound. Kaempferol possibly will help by augmenting the body's antioxidant defense which impedes the development of cancer. Of late, Kaempferol was found to control cellular signal transduction pathways linked to apoptosis, inhibiting cancer cell growth, but on the other hand, kaempferol also seems to preserve normal cell viability exerting a protective effect. The aim of this study is to assess the cytoprotective effect of phytochemical kaempferol and conventional chemotherapeutic drugs like doxorubicin and cisplatin on human blood mononuclear cells and to evaluate the extent of DNA damage by COMET assay. In the present study, hydrogen peroxide (H_2O_2) was used as an oxidant to induce oxidative damage with lymphocytes as the model system. The cytotoxic effect of H_2O_2 on lymphocytes was evaluated by considering the range of concentrations from 25 to 500µM.

Cytotoxicity was enhanced in dose dependent manner. 100µM H_2O_2 was used to evaluate the antioxidant activity of kaempferol on lymphocytes. When the lymphocytes were incubated with the kaempferol, doxorubicin and cisplatin at IC_{50} concentrations for 3 hrs before exposure to H_2O_2 (100µM), the protective effect was less apparent in leucocytes incubated with chemotherapeutic drugs. Furthermore, the percentage cell viability (MTT assay) is significant with kaempferol compared with chemotherapeutic drugs. When comet assay was performed to evaluate the protective effect of kaempferol against H_2O_2 induced DNA damage on lymphocytes, it was found that in lymphocytes treated with individual compounds, the tail lengths of kaempferol, doxorubicin and cisplatin, were found to be 12.12±1.93, 34.9±1.68 and 22.1±2.01 respectively.

Similar DNA damage indices were observed with percentage tail DNA and olive moment also. In the present study, it was found that kaempferol was found to reduce DNA damage compared to doxorubicin and cisplatin. Our findings demonstrate the kaempferol's protective role against DNA damage.

Keywords: Kaempferol, Chemotherapeutic drugs, Human blood mononuclear cells, MTT assay, Comet assay, DNA damage.

Introduction

Oxidative stress (OS) denotes the disturbance in attaining equilibrium between generation and degeneration of reactive oxygen species (ROS). ROS are named to play crucial role in many metabolic reactions in the body and are generated at the home of electron transport chain [9]. Mostly, hydroxyl radical (•OH), superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) are included under ROS family[13,15]. On the other hand, over production of ROS may have deleterious effects on typical biomolecules like lipids, proteins and DNA which ultimately lead to severe pathological changes. Severe ailments like cancer, neurodegerative disorders, inflammation, diseases related to cardiovascular, respiratory and digestive systems are some of the evidences related to pathogenicity of oxidative stress (OS)[10].

A number of dietary flavonoids are reported to act against oxidative damage[3,11].Flavonoids are abundantly available natural polyphenolic compounds and reported to be used as anticancer agents by many epidemiological and laboratory studies. Kaempferol (3,5,7-trihydroxy-2-(4-hydroxy phenyl)-4H-1-benzopyran-4-one) is one among flavonoids with many pharmacological functionalities like reducing osteoporosis, neurodegenerative diseses, anixiety and allergies etc.[1,4,7] Furthermore, consumption of kaempferol rich diet is shown to have typical role in reducing many types of cancers like lung, gastric, pancreatic and ovarian cancers[16].

Therefore, the current study was to investigate the protective effects of kaempferol against H_2O_2 treated human blood lymphocyte cells in comparison with chemotherapeutic drugs doxorubicin and cisplatin.

Material and Methods

Materials: Kaempferol was purchased from Calbiochem. Doxorubin and Cisplatin from Sigma; culture medium (RPMI 1640), antibiotics penicillin-G and streptomycin, L-glutamine, fetal bovine serum (FBS), trypsin, lymphocyte isolation medium (Histopaque) and related cell culture chemicals were procured from Himedia (India).

Lymphocyte culture: Venous blood (3ml) from healthy donor who were not on any medication, was collected and the study was approved by JNTU ethical committee. To the collected venous blood, 3ml of lymphocyte isolation medium (Histopaque 1077) was added carefully and layered

evenly on the whole blood and centrifuged at 400g for 30 min[20]. After centrifugation was complete, topmost translucent layer was discarded by removing it carefully and the middle opaque inter phase layer containing mononuclear cells was collected into a sterile centrifuge tube. To the collected mononuclear cells, 10ml of PBS was added and centrifuged immediately at 1200 rpm for 10min. Washing step was performed again to the collected pellet with PBS. The washing procedure was repeated twice with RPMI-1640 media and to the retained pellet, 5ml of fresh RPMI-1640 media was added.

MTT assay: The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was used to screen cytotoxic activity of kaempferol, doxorubicin and cisplatin on lymphocyte cells[11]. Briefly, the freshly isolated lymphocytes were treated with increasing concentrations of kaempferol, doxorubicin and cisplatin and incubated for 24 hrs under culture conditions and evaluated for cytotoxicity. Following washing with PBS, 0.1 ml of MTT (0.1% w/v in PBS) solution was added to the cells and incubated for 4 hrs at 37^0C in dark. Blue colored crystals indicate the formation of formazan salts.

To dissolve the crystals, 0.1ml of Dimethyl Sulphoxide (DMSO) was added after careful removal of MTT from the wells[8]. After 1hr, the intensity of purple color was measured using microplate reader (Wallac 1420 Multilabel counter, PerkinElmer) at a wavelength of 560 nm. The data were presented as percent post treatment recovery (% live cells) whereas the absorbance from untreated control cells was defined as 100% live cells.

Assessment of DNA damage caused by H_2O_2 on lymphocytes; MTT assay: In order to assess the DNA damage caused by H_2O_2, the freshly isolated lymphocytes were seeded into 6 well plates and exposed to H_2O_2 at varying concentrations (25 µM, 50 µM, 100 µM, 200 µM and 500 µM). The exposure was for 10 min in dark at 37^0C. Then, the lymphocytes were analyzed for viability using MTT assay protocol. The effect of H_2O_2 on lymphocytes was expressed in terms of percentage viable cells.

Cell viability analysis of H_2O_2-induced cytotoxicity on lymphocytes; Effect of chemotherapeutic drugs and kaempferol: To evaluate the effect of doxorubicin, cisplatin and kaempferol, the lymphocytes cultured in 6 well plates were incubated with doxorubicin (50 µg/ml), cisplatin (50µg/ml), kaempferol (100µg/ml) for 3 hrs at 37^0C separately. After the incubation period, the cells were exposed to 100 µM H_2O_2 for 10 min in dark. At the end, MTT assay was performed to assess the cell viability. The cells treated only with H_2O_2 and without H_2O_2 were also considered in the experiment.

Cytoprotective effect of kaempferol; Comet assay: To assess the DNA damage, comet assay was performed. The pre incubated lymphocytes with doxorubicin, cisplatin and kaempferol for 3 hrs were exposed to 100 µM H_2O_2 for 10 min in dark. Then, the cells were collected by centrifugation and comet assay was performed [14]. To compare the extent of DNA damage, cells exposed only with H_2O_2 and without H_2O_2 were also considered along with other combinations. OPEN COMET was used to score 50 comets.

Results and Discussion

Cell viability analysis: The MTT assay, a gold standard assay was used to determine the cytotoxicity of kaempferol, doxorubicin and cisplatin against lymphocytes and mononuclear cells. To evaluate the cytotoxicity, the cells were treated with increasing concentrations (5µg-100µg) of doxorubicin, cisplatin and kaempferol individually. The percentage of cell inhibition was determined after 24 hrs of incubation. DMSO treated cells acted as control. As shown in graph, doxorubicin and cisplatin treatment decreased cell viability of lymphocytes by 50% compared to the control. Interestingly, the growth of human blood lymphocytes did not affect when treated with kaempferol (Fig. 1).

Flavonoids are found to have potential antioxidant activities[6]. The present study has shown that viability of lymphocytes was higher at its high concentration of 100 µg when compared to doxorubicin and cisplant at their highest concentrations. This indicates no toxicity of kaempferol towards lymphocyte cells.

Effect of H_2O_2 on viability of lymphocytes: Hydrogen peroxide, a reactive compound at sub-lethal concentrations generates hydroxyl radicals (OH) through the fenton reaction. The OH has high affinity towards DNA and causes strand break. This overall process results in DNA instability, mutagenesis and carcinogenesis[17].

In the present study, hydrogen peroxide (H_2O_2) was used as an oxidant to induce oxidative damage with lymphocytes as the model system. The cytotoxic effect of H_2O_2 on lymphocytes was evaluated by considering the range of concentrations from 25 to 500µM. Cytotoxicity was enhanced in dose dependent manner (Fig. 2). The 50% cell survival was observed with 100µM concentration of H_2O_2. Hence, 100µM H_2O_2 was used to evaluate the antioxidant activity of kaempferol on lymphocytes.

Protective effect of kaempferol against H_2O_2-induced cytotoxicity on lymphocytes: The protective role of flavonols in opposition to hydrogen peroxide on DNA damage was very well reported. The prominant geno-protective role of flavonoids was very well documented in hydrogen peroxide stressed cells. Among various flavonoids, kaempferol had been found to be very effective in giving protection to the oxidatively DNA damaged cells caused by hydrogen peroxide[18].

In the present study, the lymphocytes were incubated with the individual compounds at the specific concentrations doxorubicin (50µg), cisplatin (50µg) and kaempferol

(100µg) for 3 hrs before exposure to H_2O_2 (100µM). MTT assay was performed to determine the percentage cell viability. The protective effect was less apparent when leucocytes were incubated with chemotherapeutic drugs. Furthermore, the percentage cell viability is significant with kaempferol alone compared with drug combination. Our findings demonstrate that kaempferol has potent antioxidant property supporting the hypothesis of a beneficial effect of kaempferol in oxidative stress in human system (Fig. 3).

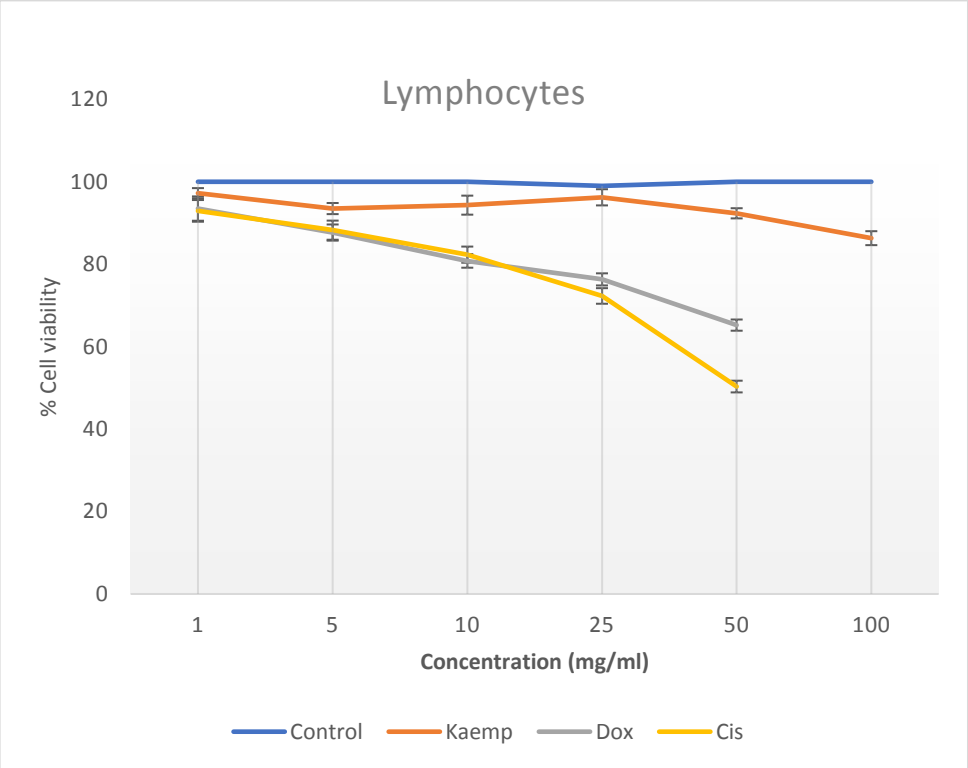


Fig. 1: Graphical illustration showing cytotoxic effect of different concentrations of kaempferol, doxorubicin and cisplatin on Lymphocytes after 24 hrs of treatment. Percentage cell viability measured by MTT assay. Data represents the mean +/- SD, n = 5.

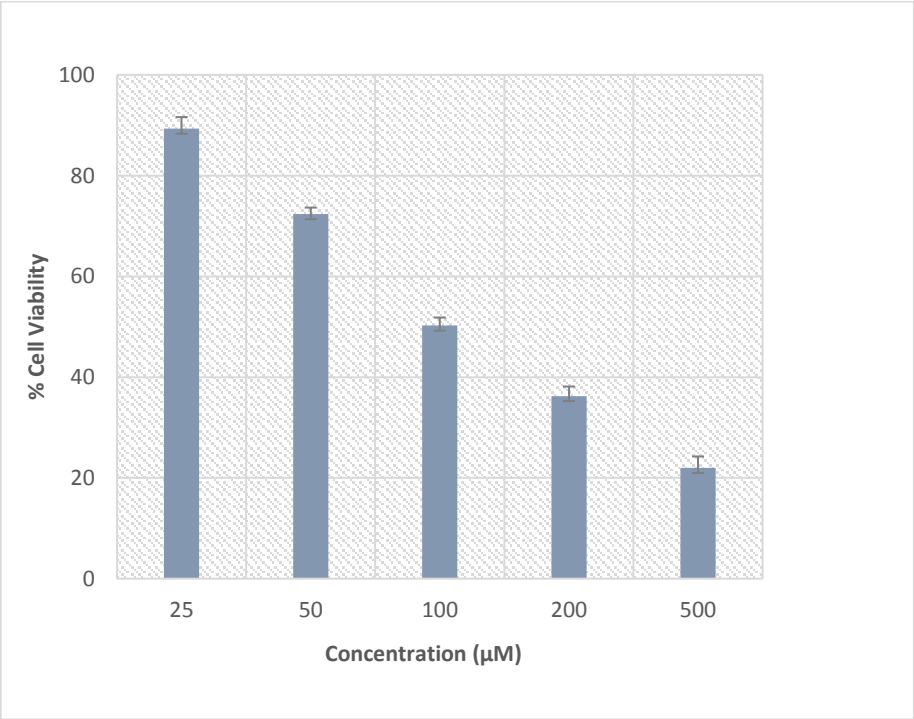


Fig. 2: Graphical representation showing the cell viability assay on lymphocytes treated with H_2O_2. Data represents the mean +/- SD, n = 3.

Protective effect of kaempferol against H_2O_2-induced DNA damage on lymphocytes using comet assay: Flavonoids are revealed to have significant geno-protective effects in hydrogen peroxide stressed cells. According to Nuutila et al,[19] luteolin followed by apigenin and kaempferol was shown to be the most effective in protecting DNA from oxidative damage induced by hydrogen peroxide. However, the investigated flavonoids also induced DNA damage which indicates their pro-oxidative capacity[2]. The balance between the protection of DNA from oxidative damage and pro-oxidative effects was strongly dependent on flavonoid concentration and the incubation period.

The oxidative damages caused by reactive oxygen species (ROS) are proved to be deleterious and associated with many of the human diseases such as cancer, atherosclerosis and rheumatoid arthritis. Among ROS, hydrogen peroxide (H_2O_2), one of the reactive oxygen intermediates has been proved to alter the immune responses of lymphocytes. At sublethal concentrations, H_2O_2 inhibits the function and proliferation of human T cells without causing cellular damage[5].

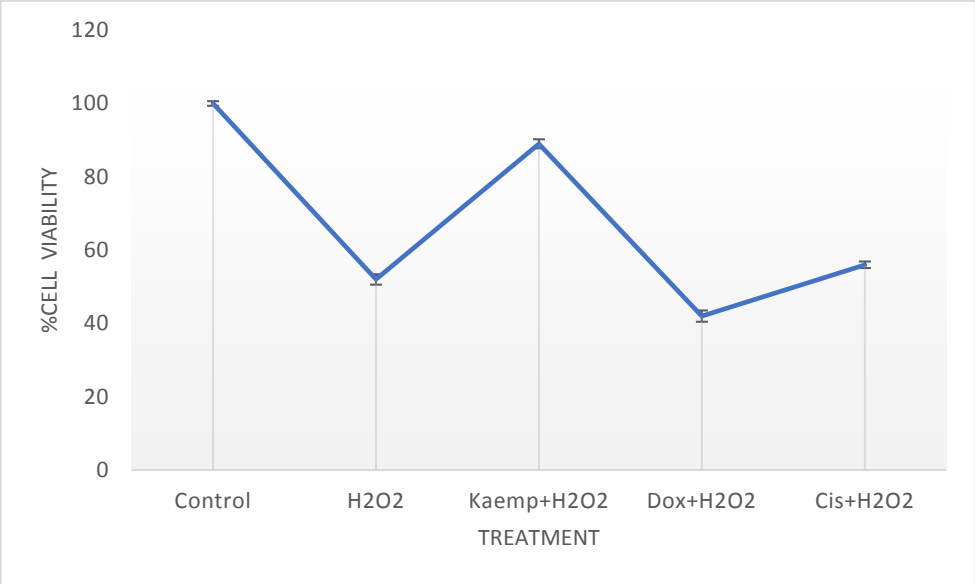


Fig. 3: The graphical representation showing the cyto-protective activity of individual compounds and kaempferol on lymphocytes and mononuclear cells. Data represents the mean +/- SD, n = 3.

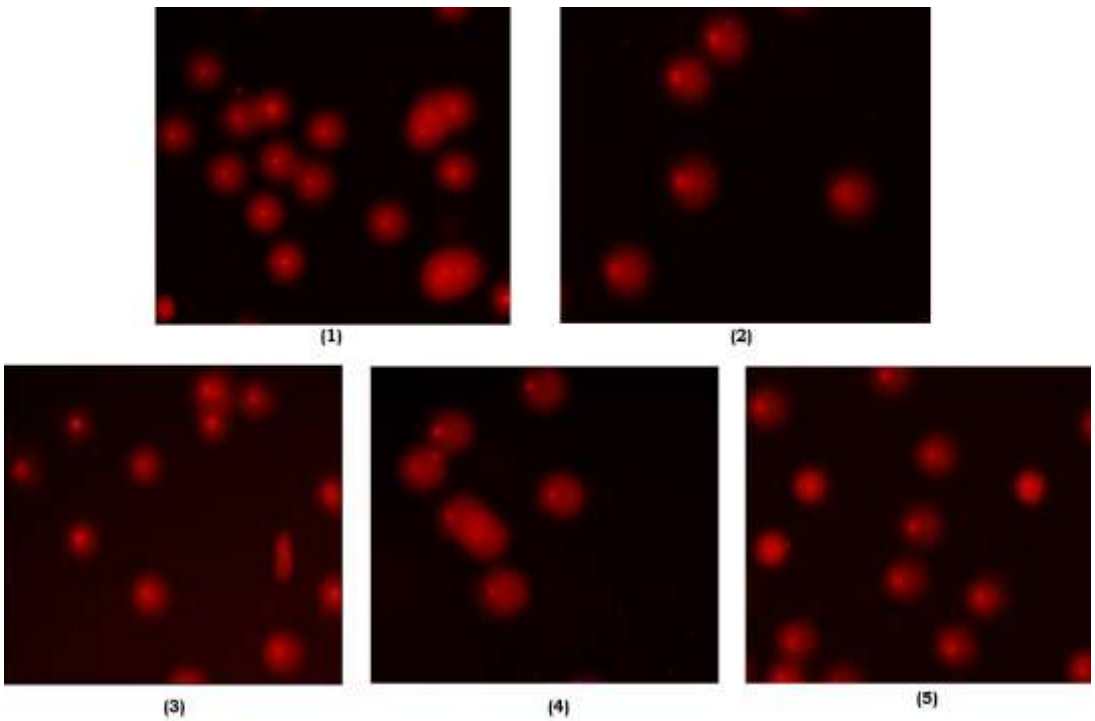
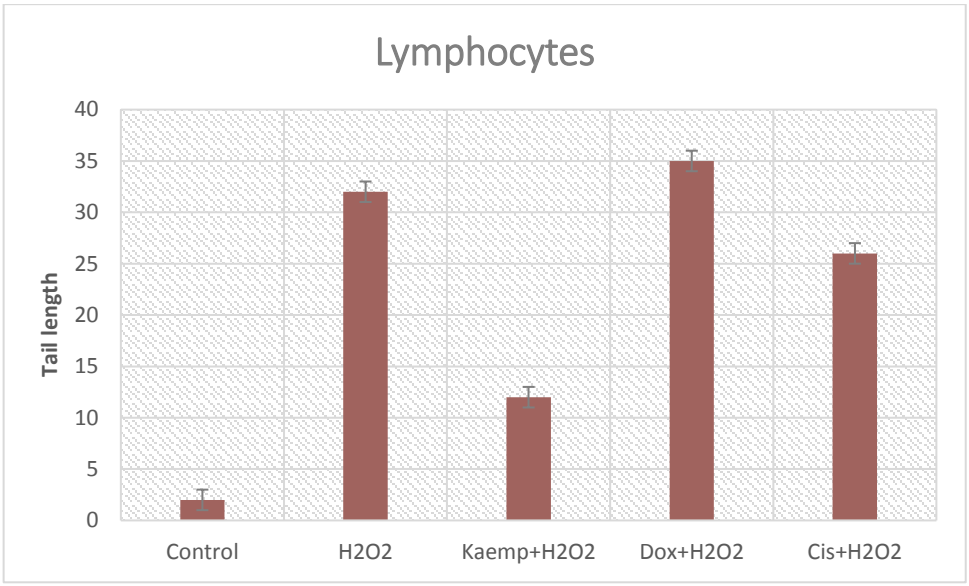
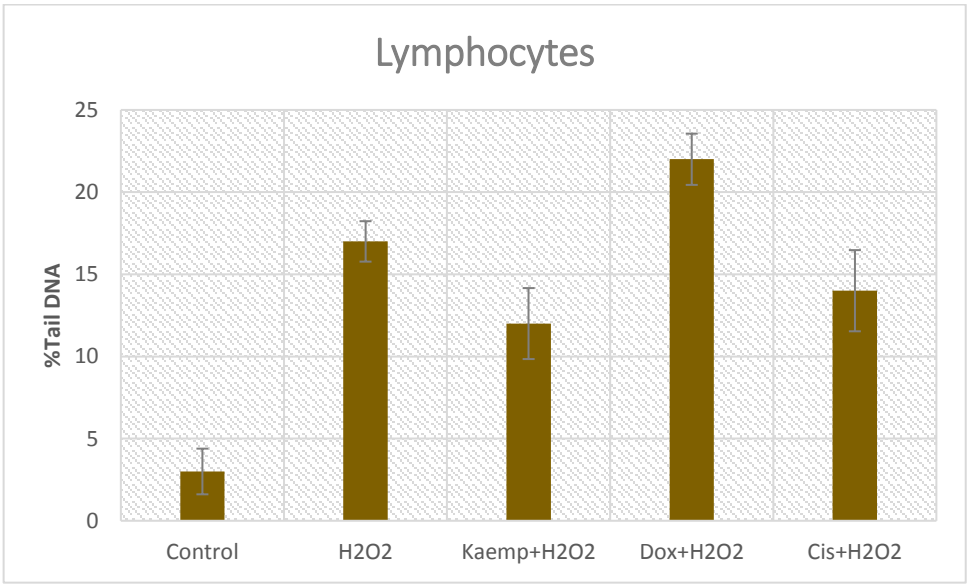


Fig. 4: Cytoprotective effect of phytochemical and drugs :1) control cells without H_2O_2; 2)control cells with H_2O_2 3) treated with doxorubicin (50ug/ml); 4) treated with cisplatin (25ug/ml); 5) treated with kaempferol (120ug/ml).





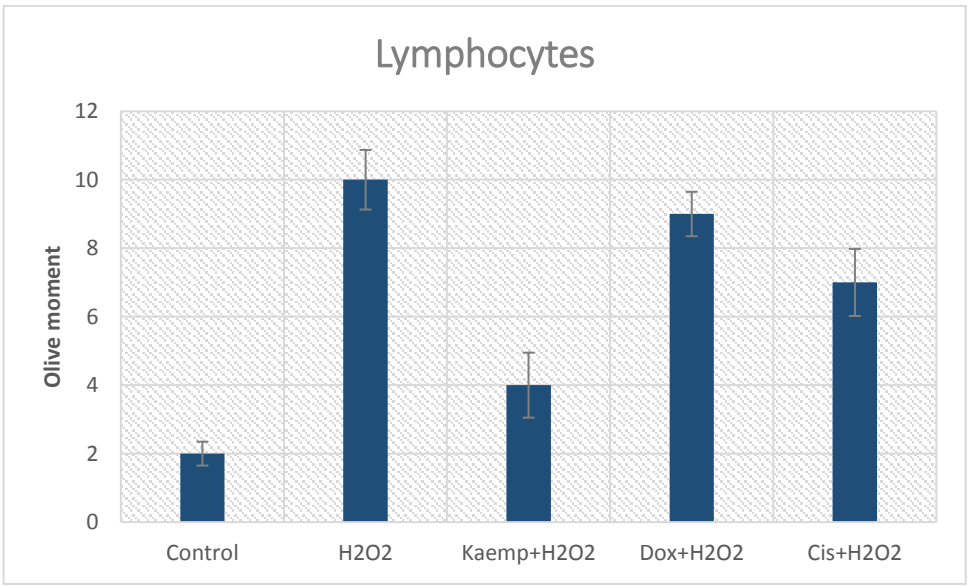


Fig. 5: Cyto-protective effect of Kaempferol and drugs on Lymphocytes induced with H_2O_2; DNA damage by COMET assay. Data represents results as mean+/-SD, n=3.

To evaluate the DNA damage by hydrogen peroxide exposure, a fast and reliable comet assay under alkaline conditions was used. The cells pre-incubated with doxorubicin, cisplatin and kaempferol prior to H_2O_2 exposure were evaluated for the protective effect. Figure 4 represents the tail length, percentage tail DNA and olive moment in lymphocyte cultures from doxorubicin, cisplatin and kaempferol after 30 minutes of H_20_2 exposure. The total DNA damage in H_2O_2 incubated lymphocyte culture is highly significant compared to control (without H_2O_2) cells. Similarly, doxorubicin and cisplatin treated cell cultures presented high DNA damage indexes.

However, kaempferol treated cells were also observed with DNA damage induced by hydrogen peroxide which represents the pro-oxidant activity of kaempferol at high concentration[12]. Earlier reports indicate that kaempferol at low concentration had exhibited antioxidant activity by its ability to protect DNA against oxidative damage to purines. The above results clearly demonstrate the protective role of kaempferol against DNA damage caused by either drugs or H_2O_2 (Fig. 5).

Conclusion

In the present study, kaempferol was found to be protective when compared with conventional chemotherapeutic drugs doxorubicin and cisplatin on human blood lymphocytes. It was evident with comet assay also. Further, the protective effect of kaempferol has to be analysed on normal breast epithelial cells.

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