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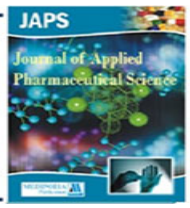

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Non-Cytotoxic Property and DNA Protective Activity against H_2O_2 and UVC of Thai GAC Fruit Extracts in Human TK6 Cells

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ABSTRACT

Momordica cochinchinensis (Lour.) fruit of Cucurbitaceae family is indigenous vegetable and fruit to Southeast Asia. It has been generally known as “GAC” whereas in Thailand it is called “Fhuck-khow”. Regarding its richness in anti-oxidants particularly lycopene and beta-carotene, GAC is named "fruit from heaven" and believed to promote longevity, health and vitality. In present study, GAC extracts were prepared from pulp (PU), skin (SK) and seed membrane (SM) by 50%, 95% ethanol and water (W). MTT assay was done in TK6 cells to determine their cytotoxicity property. The non-cytotoxic extracts were further evaluated for the DNA protective activity against H_2O_2 and UVC using high alkaline (pH >13) comet assay. TK6 cells ($1x10^5$ cells/ml) were pre-incubated with GAC extracts (25, 50 and 100µg/ml) for 24 h prior to an exposure to 50 µM H_2O_2 and a 254-nm UVC germicidal lamp for DNA damage induction. We found that H_2O_2-induced DNA damage was suppressed by 30-60 % when cells were pre-treated with PU, SK and SM extracts at all concentrations used. Regarding test condition used, UVC clearly induced DNA damage in TK6 cells greater that that by H_2O_2. The protective effect of GAC extracts against UVC was found by 20-30%. Our present results suggest that GAC possesses DNA protective ability against H_2O_2 and UVC. The degree of anti-oxidative damage activity in TK6 cells of GAC extracts is SK95>SMW>SK50.

Keywords: *Momordica cochinchinensis,* comet assay, TK6, H_2O_2, UVC.

INTRODUCTION

Momordica cochinchinensis (Lour.) Spreng belongs to Cucurbitaceae family, is an indigenous vegetable in Southeast Asia. It is generally known as the name “GAC”. It is sometimes called "fruit from heaven" and is believed to promote longevity, health and vitality. Traditionally, GAC has been used as both food and medicine in the regions in which it grows. Other than the use of its fruit for special Vietnamese culinary dishes called ‘‘xoi gac’’ (red glutinous rice), which is served at ceremonial or festive occasions such as new years and weddings (Vuong *et al.*, 2003).

Gac is also used for its medicinal and nutritional properties. In Vietnam, the seed membranes are used to aid in the relief of dry eyes, as well as to promote healthy vision. Similarly, in traditional Chinese medicine the seeds of gac, known in Mandarin Chinese as "mùbiēzǐ" (Burke *et al.,* 2006) are employed for a variety of internal and external purposes. In Thailand, *M. cochinchinensis* (GAC) is known as "Fhuck khow" and has been consumed as an indigenous vegetable. The tips and young fruits have been used for traditional food but not a ripe fruit. The seed membrane of GAC has been shown to be especially high in lycopene content (Failla *et al*., 2008). Relative to mass, it contains up to 70 times the amount of lycopene found in tomatoes. Additionally, the carotenoids present in gac are bound to long-chain fatty acids (Ishida B. *et al.,* 2004), resulting in what is claimed to be a more bioavailable form. There has also been recent research that suggests that gac contains a protein that may inhibit the proliferation of cancer cells (Tien *et al*., 2005). Over the past few decades, a number of publications reported on the chemical composition and biological activities of extracts from GAC. However, no work has been performed on the biological activities of ethanolic GAC extracts, especially on their reactive oxygen (ROS) or free radical scavenging capacity. Among the member of ROS, hydrogen peroxide (H_2O_2) radical is one of the most highly reactive species since it is relatively stable and a byproduct of normal aerobic metabolism (Fang, 2002). Generation of H_2O_2 in biological system results in immediate damage to DNA molecules and the consequent DNA degradation may lead further to apoptotic reaction and carcinogenesis in living cells. Therefore, this study was performed to investigate biological activity on anti-oxidative DNA damage activity against H_2O_2 and UVC radiation of various parts of *M. cochinchinensis* fruits in human TK6 cells by single cell gel electrophoresis (SCGE) or comet assay.

MATERIALS AND METHODS

Plant sample

The *M. cochinchinensis* (GAC) ripe fruits used in this study were purchased from private farm in Nakhonpathom province of Thailand.

Preparation of GAC ethanolic extracts

The GAC ripe fruits were cleaned and separated into three parts including skin (SK), pulp (PU) and seed membrane (SM). Each part was dried in a hot air oven at 50ºC h for 1-2 days. The dried samples were ground into powder and 100 g of each powdered sample were extracted with 50% and 95% ethanol by maceration method. The extracts were then filtered using Whatman filter paper. The filtrates were concentrated by a rotary evaporator at 45ºC. Six obtained extracts namely SK50, PU50, SM50, SK95, PU95 and SM95 representing extraction of skin, pulp and seed membrane extracted with 50% and 95% ethanol, respectively. All extracts were stored in darkness at 4ºC until used.

Preparation of GAC aqueous extracts

The GAC water (W) extracts were prepared from skin, pulp and seed membrane of GAC ripe fruits. Briefly, 500 g of each of fresh GAC part were dissolved and blended in distilled water (500 ml) in the ratio of 1:1. The resulting juices were roughly filtrated twice through a layer of muslin and followed by Whatman filter paper to remove any debris. The filtrates were dried using a lyophilization technique and stored at -20ºC until required. By this method, three aqueous extracts including SKW, PUW and SMW were obtained.

Thin layer chromatography (TLC) profile and DPPH screening

Nine samples of aqueous and ethanolic (50% and 95%) extracts of skin, pulp and seed membrane of GAC were prepared by dissolving in absolute ethanol at final concentration of 100 mg/ml. Thirty microliter of each extract solution was spotted onto TLC plates coated with silica gel (pre-coated, F_{254}; Alufolien, Merck) and separated with acetone - hexane (20:80 v/v) as mobile phase to 8 cm beyond the base. The plates were dried and sprayed with 1% of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) in absolute ethanol. The aim of DPPH spraying was to rapidly determine radical scavenging activity of GAC aqueous and ethanolic extracts on gel. The R_f values were recorded and compared with standard antioxidants including β-carotene, lycopene and α-tocopherol.

Culturing of TK6 cells

The stock TK6 human lymphoblast cells line (ATCC CRL-8015) was purchased from the American Type Culture Collection (ATCC) in Maryland, USA. The cells grow as suspension and were maintained as exponentially growth phase in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum (HS) and 1% penicillin-streptomycin (GIBCO®) in tissue culture flask (Corning®) and incubated at 37ºC in humidified atmosphere containing 5% CO_2.The cells were maintained by addition of fresh medium or replacement of fresh medium. The doubling time of TK6 cells is 12-14 h.

Determination of cytotoxicity by MTT assay

Prior or to the test, overnight culture of TK6 cells grown in 75cm^2 tissue culture flask was routinely examined under inverted microscope. The medium was removed by centrifugation at 1,500 rpm for 5 min and cells were washed with 1x of Hank's balanced salt solution (HBSS, GIBCO®). The cells ($4x10^5$cells/ml) at 0.5 ml were seeded into 24 well-plate and then treated with 0.5 ml of GAC extracts dissolved in RPMI (supplemented with 10% HS) to reach final concentrations of 500, 700, 800, 900 and 1,000µg/ml for 4 hr at 37ºC in humidified atmosphere containing 5% CO_2. After treatment, the GAC samples were removed by centrifugation (3,000 rpm for 3 min) and the TK6 cells were washed twice with 1x HBSS. The cells were re-suspended in 1 ml of 0.625 mg/ml of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in 1.5 ml eppendorf tube and kept at 37ºC in 5% CO_2 incubator for 3 hr. After this incubation period, the crystals fomazan were washed with HBSS and dissolved in 200 µl of dimethyl sulfoxide (DMSO). The amount of formazan was evaluated by measuring absorbance at 540 nm by

micro-plate reader system. The toxicity of GAC extracts was indicated by 50% inhibitory concentration (IC_{50}).

Anti-oxidative damage activity evaluation of GAC aqueous and ethanolic extracts by comet assay in TK6 cells

Pre-treatment of TK6 cells with aqueous and ethanolic extracts

TK6 human lymphoblast cell line (ATCC CRL-8015) was cultured as cell suspension and maintained as exponentially growth phase in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum (HS) and 1% penicillin-streptomycin and incubated at 37ºC in humidified atmosphere containing 5% CO_2. The cells were maintained by addition of fresh medium or replacement of fresh medium every 2-3 days. Cells at density of $2x10^5$ cell/ml were seeded onto 12 well-plate and separately exposed to medium containing various concentrations (25, 50 and 100µg/ml) of GAC aqueous and ethanolic extracts. The plates were incubated for 24 h at 37°C in 5% CO_2 incubator.

DNA damage induction by H_2O_2 treatment

By the end of pre-treatment time, GAC extracts were removed by centrifugation (3,000 rpm for 3 min) and cells were washed twice with phosphate buffered saline (PBS) and then resuspended in 1 ml of fresh RPMI containing 50 µM H_2O_2. Treatment was done at 4°C for 5 min to allow DNA damage (intracellular oxidative stress and DNA strand breaks). At the end of incubation period, H_2O_2 was removed by centrifugation at 3,500 rpm for 3 min and cells were washed twice with cold phosphate buffered saline (PBS). The comet assay was performed to evaluate DNA damage at individual cell.

DNA damage induction by UVC treatment

After GAC extracts pre-treatment, the medium was removed by centrifugation. Cells were washed twice with cold PBS and collected by centrifugation at 3,500 rpm for 3 min and resuspended in 2 ml PBS. Cells were seeded onto 12 well-plate and then exposed to UVC irradiation (0.342 mw/cm^2) using a germicidal lamp (Toshiba, Japan) for 5 min. After irradiation, cells were immediately collected by centrifugation at 3,500 rpm for 3 min. Cells were resuspended in cold RPMI and subjected to comet assay. Trolox was used as positive antioxidant compound.

Comet assay

Comet assay or single cell gel electrophoresis (SCGE) was performed according to method described by Tice *et al.,* (2000) to evaluate anti-oxidative activity of GAC aqueous and ethanolic extracts against DNA damage induced either by H_2O_2 and UVC. Following DNA damage induction, cells were washed twice with ice-cold PBS and resuspended in 200 µl PBS. A mixture of 20 µL cell suspension and 75 µl of 0.5% low melting point (LMP) agarose were immediately layered onto a glass microscope slide pre-coated with 0.75% normal melting point (NMP) agarose. The slides were allowed to solidify and 80 µl of 0.5% LMP agarose was spread on glass slides. Slides were immersed in pre-chilled lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO, 1% Triton X-100, pH 10) and kept at 4°C for 2 h. Slides were incubated in fresh electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH > 13) for 20 min at 4°C to allow unwinding of DNA and then transferred into an electrophoresis unit with the same buffer and subjected to an electrophoretic field at 300 mA and 25 V at 4°C for 20 min in dark condition. After electrophoresis, slides were fixed in neutralizing buffer (0.4M Tris, pH 7.5) for 10 min and stained with 30µl of 0.2% ethidium bromide. Cells were analyzed using a fluorescence microscope (Olympus BX51, Tokyo, Japan). Images of one hundred randomly selected cells per experimental point were scored and analyzed of damage using Comet III analysis software (Perceptive Instruments, Halstead, UK). Two major parameters including tail length (TL= distance of DNA migration) and tail moment (TM= (Distance between the centre of gravity of the head to the centre of gravity of the tail) X (Tail DNA Intensity / Total Comet DNA intensity) were taken for result analysis.

Statistical analysis

The mean values of 100 comet cells of all experiments were analyzed by one-way ANOVA (one-way analysis of variance). The significant difference between means of TL and TM values of treated groups at each GAC extract were compared with positive control (untreated group) by Tukey multiple comparisons. The significant difference between means at level of 0.05 (*p*- value <0.05) was considered as significant.

RESULTS AND DISCUSSION

TLC analysis and DPPH radical scavenging screening

Results of TLC-DPPH assay demonstrated a good DPPH radical scavenging activity of 95% ethanolic extracts including PU95, SM95 and SK95. The high level of α-tocopherol (R_f 0.41) was clearly detected in these three extracts. The other samples of GAC extracted with water (PUW, SMW and SKW) and 50% ethanol (PU50, SM50 and SK50) exhibited a trace amount of β-carotene, α-tocopherol and lycopene. This may be due to a loss during extraction steps. Previous study reported that both lycopene and β-carotene found at high level in fresh GAC fruits, with those of lycopene was up to 308 µg/g in the seed membrane of fresh material (Aoki *et al.,* 2002; Vuong *et al.,* 2003; Vuong *et al.,* 2006) However their level was decreased by heat and light in extraction process (Shi, 2002).With the aim to obtain high level of phytochemicals in GAC, the above finding suggests and supports the consumption of fresh GAC fruits instead of processed GAC.

Cytotoxicity of GAC extracts in TK6 cells

Regarding results obtained from TLC and DPPH screening methods, only three GAC extracts i.e. SK95, SK50 and SMW were selected and further evaluated for their cytotoxicity property by MTT tetrazolium assay. The cytotoxicity effect of the three GAC extracts performed at concentrations of 500, 700, 800, 900 and 1,000 µg/ml in TK6 human lymphoblast cells. Following 4 hr exposure, it was found that there was no remarkable cytotoxicity and cell proliferation inhibition effect in any

concentration tested of SK50 and SMW. Their IC_{50} values were greater than 1,000 µg/ml and % cell viability > 80% at all concentrations tested. In contrast, SK95 exhibited a slight reduction in cell viability indicated by its IC_{50} at 789µg/ml. According to classification of the cytotoxicity for natural ingredients (Ballantyne *et al.*, 1999), SK50 and SMW were classified as non-toxic whereas SK95 was potentially harmful.

Anti-oxidative DNA damage activity against H_2O_2

In the present study, we used H_2O_2 as the ROS-inducing agent *in vitro*. It was demonstrated that treatment of 50µM H_2O_2 for 5 min produced extensive oxidative DNA damage in TK6 cells (Fig.1 B) at about 10-fold greater than control or untreated cells (Fig.1 A). This was indicated by highly increased comet tail length (TL) and tail moment (TM) values in treated cells.

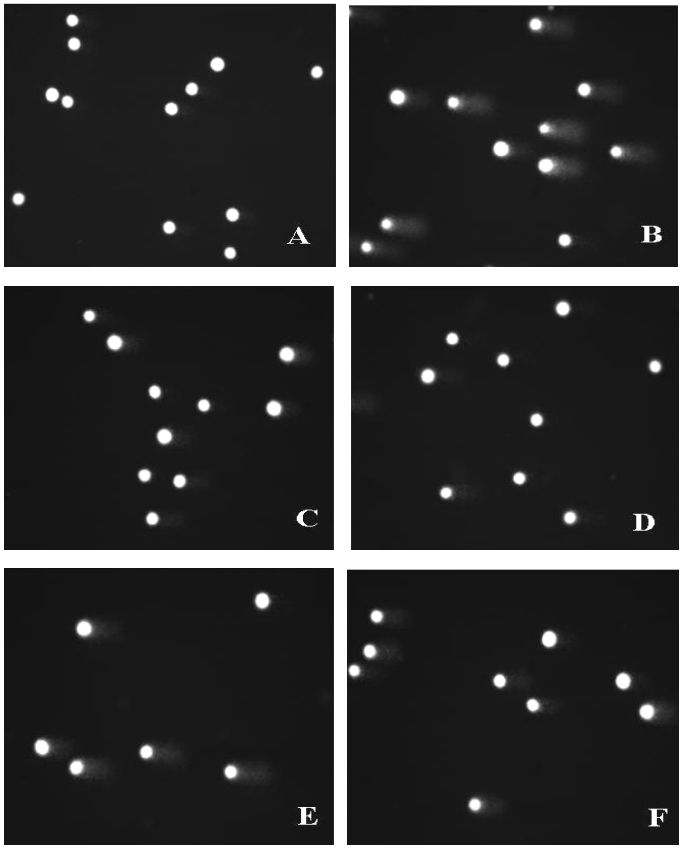

Fig.1: Fluorescent photomicrograph showing oxidative DNA damage in TK6 cells by H_2O_2 induction in comet assay: **(A)** control group (no damage), **(B)** 50µM H_2O_2 treatment**, (C)** H_2O_2+100µg/ml Trolox, **(D)** H_2O_2+100µg/ml SK95, **(E)** H_2O_2+100µg/ml SK50 and **(F)** H_2O_2+100µg/ml SMW**.**

Prior to anti-oxidative damage experimentation, we found out that treatment of TK6 cells with SK95, SK50 and SMW for 24 hr did not display inhibitory effect on cell growth rates. The viability rates of pre-treated TK6 cells were greater than 70% (data not shown). Anti-oxidative DNA damage of GAC extracts was investigated following pre-treatment TK6 cells with SK95, SK50 and SMW at 25, 50 and 100µg/ml for 24 hr followed by H_2O_2 induction. The protective effect was indicated by a reduction in TL and TM damage parameters in comparison to TK6 cells received H_2O_2 alone (Fig.1). The highest DNA protective effect was found in SK95 of all concentrations tested (54.533 ± 8.597%, 63.278 ± 15.775%, and 54.934 ±18.390 % for 25, 50 and 100µg/ml, respectively). For SK50, the highest protective activity was observed at 50.131 ± 3.524% when tested at 25µg/ml. It could be observed that in contrast to SK50 and SK95, SMW exhibited a dose-dependent increase in DNA protective effect (37.999 ± 9.689%, 36.902 ± 17.354%, and 55.245 ± 5.288% for 25, 50 and 100µg/ml, respectively) (Fig.2).

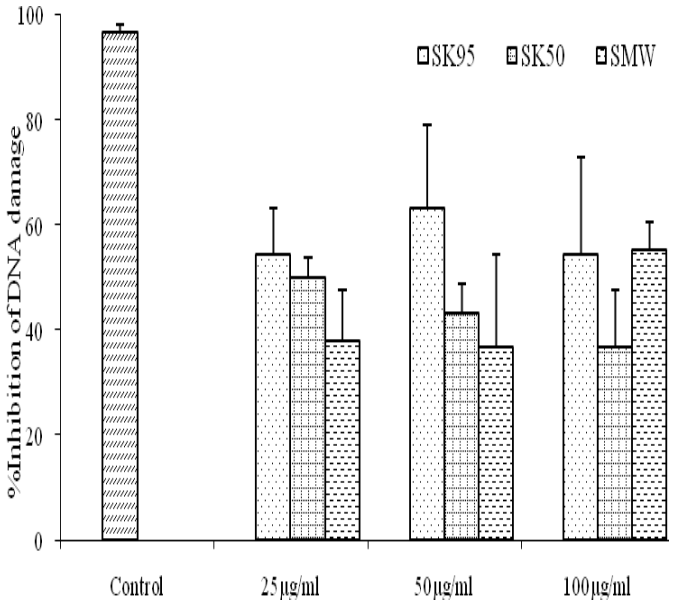

Fig.2: Inhibitory effect of GAC extracts on H_2O_2 - induced DNA damage in TK6 cells. Results are expressed as means ± SD (n=3). *Significant difference was detected from H_2O_2 treatment groups at $p \leq 0.05$ (ANOVA).

Anti-oxidative DNA damage activity against UVC

Unlike H_2O_2, the ultraviolet (UV) light produces DNA damage in different ways. Most of them are potentially hazardous to living organisms, particularly UVC (200–280 nm) has the shortest wavelengths and the highest energy. The biological effects of UV light are due to photochemical reactions, because of large quanta involved in its emission and absorption (Coohill *et al.*, 1989). Therefore, highly energetic UV photons are able to destroy chemical bonds of biomacromolecules in cells, leading to an array of cell injuries (Stapleton *et al.*, 1992).The most important cellular target site of exposure to UV radiation is DNA, resulting in DNA photoproducts and mutations (Hollósy *et al.*, 2002). In this study, we distinguished the cytotoxicity from genotoxicity effect of UVC in TK6 cells by investigating cell viability following UVC exposure. It shown that irradiation of cells for 5 min with 0.342 mw/cm^2 of standard germicidal UVC exhibited less effect on cell viability analyzed by trypan blue exclusion method. Therefore, the UVC at this dose was employed for anti-oxidative DNA damage experimentation. It was shown in this study that UVC treatment greatly enhanced DNA migration in TK6 cells (Fig.3 B) approximately by 4-fold increase in TL (58.16 µm) and a 10-fold in TM (10.36%) values over the untreated cells (TL=16.23 µm, TM=0.55%) (Fig.3 A).

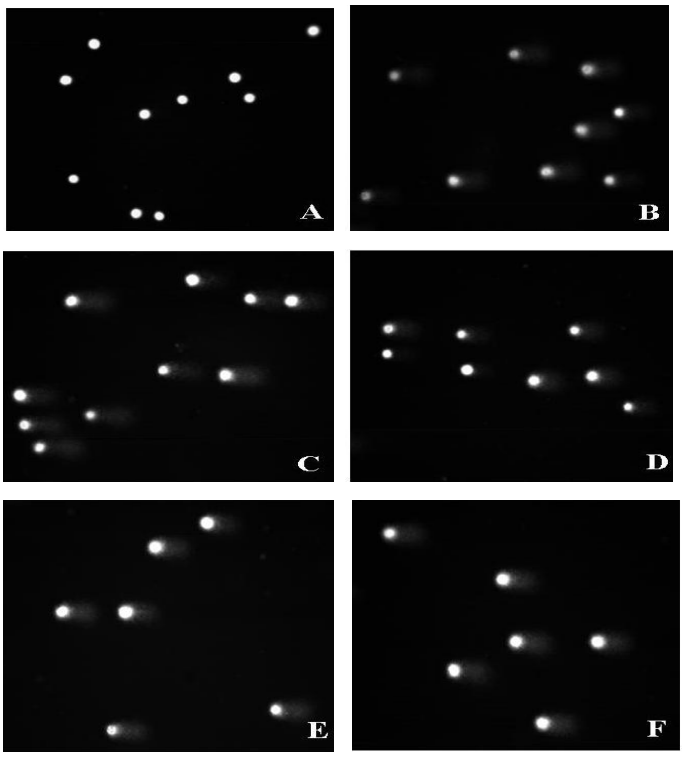

Fig. 3: Fluorescent photomicrograph showing oxidative DNA damage in TK6 cells by UVC induction in comet assay: **(A)** control group (no damage), **(B)** UVC treatment, **(C)** UVC+100µg/ml Trolox, **(D)** UVC +100µg/ml SK95, **(E)** UVC +100µg/ml SK50 and **(F)** UVC +100µg/ml SMW.

The UVC preventive effect of GAC extracts was demonstrated by a reduction in TL and TM damage parameters of pre-treated TK6 cells for 24 hr. Our results (Fig.4) suggest that pretreatment with GAC extracts (SK95, SK50 and SMW) (Fig. 3D, 3E and 3F) led to a significant decrease in DNA damage induced by UVC. The UVC preventive effect of SK95 (19.66±1.36%) and SK50 (12.97±1.93 %) was greater than that found in Trolox (10.68±6.56%) (Fig. 4).

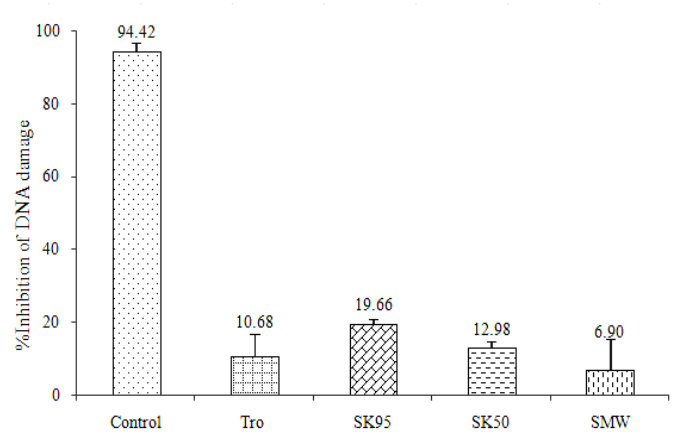

Fig. 4: Inhibitory effect of GAC extracts on UVC - induced DNA damage in TK6 cells. Results are expressed as means ± SD (n=3). *Significant difference was detected from UVC treatment groups at $p \leq 0.05$ (ANOVA).

CONCLUSIONS

Consumption of GAC could benefit public health by countering oxidative stress factors and reducing the risk of free radical-related diseases and aging. Regarding results of TLC-DPPH and comet assay, high level of α-tocopherol and DNA-protective activity in TK6 cells against H_2O_2 and UVC were found, especially SK95 (95% ethanolic extract of skin). Though, it has been reported that lycopene and β-carotene found at high level in fresh GAC fruits, by TLC detection we found a loss of these two important antioxidants after extraction with ethanol and water. We therefore conclude that α-tocopherol in GAC may play a crucial role in anti-oxidative DNA damage activity.

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