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Research Article

ANTIPROLIFERATIVE POTENTIAL OF *PERGULARIA DAEMIA* (FORSK.) ON HUMAN ORAL EPIDERMOID CARCINOMA (KB) CELLS BY INDUCING APOPTOSIS AND MODIFYING OXIDANT ANTIOXIDANT STATUS

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ABSTRACT

Objective: Management of cancer without any side-effects is still a challenge for the medicinal system. This leads to an increasing search for improved anticancer drugs. Few of plant products have been used in traditional medicine for thousands of years and have been drawing of great deal of attention to suppress cancer. The main objective of this study is to evaluate the anti-proliferative effect of Pergularia daemia (Forsk) against KB cells.

Methods: Different concentrations of areal part of P. daemia methanolic extract (PDME) (10, 20, 40, 80, 160, 320 µg/mL) were subjected to cytotoxic study. The antiproliferative effect of PDME was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, analysis of reactive oxygen species (ROS) generation, mitochondrial membrane potential, cell cycle arrest, and antioxidant status.

Result: Increased level of intracellular ROS, lipid peroxidation marker (Thiobarbituric acid reactive substances), DNA damage (comet assay), apoptotic death and cell cycle arrest in PDME treated cells. Whereas decreased activity of antioxidants and altered mitochondrial membrane potential were observed in PDME treated cells.

Conclusion: The current investigation suggested that the phyto constituents of P. daemia responsible for anticancer activity. Thus, the long-term consumption of P. daemia could be considered and promoted as an adjuvant therapy for various malignancy.

Keywords: Pergularia daemia, KB cells, Mitochondrial membrane potential, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Reactive oxygen species, Apoptosis, Comet assay, Cell cycle arrest.

INTRODUCTION

Cancers are the most life-threatening health problems in the World [1]. Oral squamous cell carcinoma (OSCC), the fifth most common neoplasm worldwide and accounting for over 500,000 cases annually [2]. In the past, squamous cell carcinoma of the oral cavity (OSCC) was primarily found in elderly men with the risk factors being tobacco and excessive alcohol use. However, some studies have shown an increased incidence of OSCC among young patients under 40 years of age [3]. Development of OSCC is a multi-step process involving genes related to cell cycle, growth control, apoptosis, DNA damage response and other cellular regulators [4]. Chemotherapy, being a major treatment modality used for the control of advanced stages of malignancies and as a prophylactic against possible metastasis [5]. Several chemotherapeutic, cytotoxic and immunomodulating agents are available in Western medicine to treat cancer. Besides being enormously expensive, these drugs are associated with serious side-effects and morbidity. Still, the search continues for an ideal treatment that has minimal side-effects and is cost-effective. Today, in Western medicine, only a limited number of plant products are being used to treat cancer.

Medicinal plants are used by 80% of the World population as the only available medicines, especially in developing countries. Plants have an almost unlimited capacity to produce substances that attract researchers in the quest for new and novel chemotherapeutics [6]. Ayurveda, the traditional Indian system of medicine, has been successful from ancient times in using natural drugs, mostly herbal preparations, in preventing or suppressing various tumors using several lines of treatment. Thousands of herbal and traditional compounds are being screened worldwide to validate their use as anti-cancerous drugs [7].

Pergularia daemia (Asclepiadaceae) species is a perennial twining herb and is widely distributed in the tropical and subtropical regions of Asia and South Africa and have multiple applications in different folk medicines. The phytochemical screening on quantitative analysis shows that the leaves of the P. daemia are rich in popular phytochemical constituents such as flavonoids, terpenoids, carbohydrates, steroids and alkaloids. In Indian Ayurvedic system, the various parts of this plant are reported to possess numerous pharmacological properties such as [8], wound healing [9], anti-diabetic [10], hepatoprotective [11], cardioprotective effect [12] and antibacterial activity [13]. However, there is little scientific investigation has been carried out on anticancer activity of the plant and there is a wide scope for investigation. Hence, we have made an attempt to congregate the anti-proliferative potential of P. daemia methanolic extract (PDME) against KB cells.

METHODS

Chemicals

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2-7-diacetyl dichlorofluorescein, rhodamine 123 (Rh-123), heat-inactivated fetal bovine serum (FBS), minimum essential medium (MEM), glutamine, penicillin and streptomycin, EDTA, trypsin, thiobarbituric acid (TBA), phenazinemethosulphate, nitrobluetetrazolium and 5,5-dithiobis (2-nitrobenzoic acid) were purchased from Sigma Chemicals Co., St. Louis, USA. All other chemicals, solvents and other analytical grades were obtained from S.D Fine Chemical, Mumbai and Fisher Inorganic and Aromatic Limited, Chennai.

Plant material

The fully matured aerial parts of P. daemia were collected from Pudukkottai District, Tamil Nadu, India. The plant was identified by Dr. V. Venkatesalu, Professor, Department of Botany, Annamalai University. A voucher specimen (ACC: 196) was deposited in the herbarium of Department of Botany, Annamalai University.

Preparation of extracts

The flowering aerial parts of plants were dried in the shade and powdered, so that all the material could be passed through a mesh not larger than 0.5 mm. Powdered materials of plant (1000 g) was soaked in 3 L of methanol (Merck Co., Germany) for 1 day, and the steps were repeated twice, and then Soxhlet apparatus by using methanol for 72 hr. At the end of extraction, it was passed through Whatman No.1 filter paper (Whatman Ltd., England). The PDME (9%) was concentrated to dryness under vaccum on rotary evaporator at 40°C then reconstituted in minimum amount of DMSO and stored at 4°C for further use. 5 mg of PDME was dissolved in 0.05% of DMSO and final volume of 5 mL sample made up to MEM medium.

Cell culture

The human oral cancer cell line KB (NCCS, Pune, India) was grown in MEM supplemented with 10% FBS, 1% penicillin and streptomycin (Sigma, USA). Cells were maintained in humidified air with 5% CO_2 at 37°C. During subculture, cells were detached by trypsinization when they reached 80% confluency. Cells were used for various anticancer experiments.

Drug sensitivity assay

The proliferation activity of cell populations under different treatment conditions were determined by the MTT assay based on the detection of mitochondrial dehydrogenase activity in living cells. MTT assay was first proposed by Mosmann et al., (1983) [14]. Ten microliters of MTT solution (5 mg/mL in phosphate-buffered saline [PBS]) was added to each culture well after 24 hr of incubation with PDME treatment. The color was allowed to develop for additional 4 hr incubation. An equal volume of DMSO was added to stop the reaction and to solubilize the blue crystals. The absorbance was taken using UV-visible spectrophotometer (Elico SL159, India) at a wavelength of 570 nm.

Cell treatments

Cells were treated with different concentration of PDME (10, 20, 40, 80, 160, 320 µg/mL) incubated at 24 hr and the cytotoxicity was observed by MTT assay. IC_{50} values were calculated and the optimum dose was used for further study.

Group I: Control (Untreated KB cancer cells)
Group II: KB cells + Positive control (kaempferol 50 µg/mL)
Group III: KB cells + PDME (80 µg/mL)
Group IV: KB cells + PDME (160 µg/mL)

Clonogenic cell survival assays

Cell survival colonies were assessed for clonogenic cell survival study was early described by Bradbury et al. [15]. Briefly, KB cells were seeded at densities of 2 × 10^5 cells/100-mm tissue culture dish and allowed to grow in a 37°C incubator until they reached 75% confluence. PDME was dissolved in water and added directly to the growth media for the indicated times, after which the plates were exposed, cells were trypsinized, diluted, counted, and seeded into 60 mm cloning dishes at the densities of 200-20,000 cells/dish. Colonies were allowed to grow in a humidified 5% CO_2, 37°C environment for 7-10 days, after which they were fixed with methanol, stained (crystal violet) and counted. Individual assays were performed with multiple dilutions with a total of six cloning dishes per data point, repeated at least thrice. Dose response survival curves were plotted on a log-linear scale.

Determination of intracellular reactive oxygen species (ROS) generation

ROS was measured by using a non-fluorescent probe, 2,7-dichloro dihydro fluorescein diacetate (H2-DCFDA) that can penetrate into the intracellular matrix of cells, where it is oxidized by ROS to fluorescent dichloro fluorescein (DCF). Cells were incubated with 1 µl of H2-DCFDA (13 mM) fluorescence probe for 15 minutes [16]. The intensity of excitation and emission at 490 and 520 nm respectively was then measured using a fluorescence spectrophotometer. Fluorescence microscopy images were taken at 450-490 nm using a Nikon TS 500 fluorescence microscope.

Alterations in mitochondrial membrane potential

The study of mitochondria and changes in the mitochondrial membrane potential has become a focus of apoptotic analysis. The changes in ΔΨm were observed microscopically and determined fluorimetrically using fluorescent dye Rh-123 (Shimadzu RF-5301 PC spectroflurometer). After treatment with PDME for 24 hr, fluorescent dye Rh-123 (10 µg/mL) was added to the cells and kept at incubation for 30 minutes [17]. Then, the cells were washed with PBS and viewed under fluorescence microscope using blue filter (Nikon TS500).

Apoptotic morphological changes

Acridine orange (AO) and ethidium bromide (EBr) staining with DNA to allowed visualization of the condensed chromatin of dead apoptotic cells [18]. The control, PDME treated cells were seeded in 6-well plate (3 × 10^4/well) and incubated in CO_2 incubator for 24 hr. The cells were fixed in methanol:glacial acetic acid (3:1) for 30 minutes at room temperature. The cells were washed in PBS, and stained with 1:1 ratio of AO/EBr. Stained cells were immediately washed again with PBS and viewed under a fluorescence microscope (Nikon, Eclipse TS100, Japan) with a magnification of ×40. The number of cells showing features of apoptosis was counted as a function of the total number of cells present in the field.

Alkaline single-cell gel electrophoresis (Comet assay)

DNA damage was estimated by alkaline single-cell gel electrophoresis (comet assay) according to the method of Singh et al. 1988 [19]. A layer of 1% NMPA was prepared on microscope slides. The control and PDME treated cells (50 µL) were mixed with 200 µL of 0.5% LMPA. The suspension was pipette onto the precoated slides. Slides were immersed in cold lysis solution at pH 10 (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris pH 10, 1% Triton X-100, 10% DMSO) and kept at 4°C for 60 minutes. To allow denaturation of DNA, the slides were placed in alkaline electrophoresis buffer at pH 13 and left for 25 minutes. Subsequently, slides were transferred to an electrophoresis tank with fresh alkaline electrophoresis buffer. Electrophoresis was performed at field strength of 1.33 V/cm for 25 minutes at 4°C. Slides were neutralized in 0.4 M Tris (pH 7.5) for 5 minutes and stained with 20 µg/mL EBr. For the visualization of DNA damage, observations were made using a ×40 objective in an epifluorescent microscope equipped with an excitation filter of 510-560 nm and a barrier filter of 590 nm. One to two hundred comets on duplicated slides were analyzed. Images were captured with a digital camera with networking capability and analyzed by image analysis software, CASP [20]. DNA damage was quantified by tail moment (TM), tail length, olive tail moment (OTM). OTM is the product of the distance (in × direction) between the center of gravity of the head and the center of gravity of the tail and the percent tail DNA [21].

Biochemical estimations

The cells were harvested by trypsinization. The cell pellet obtained was suspended in PBS. The suspension was taken for biochemical estimations. The concentration of TBA reactive substances (TBARS) was estimated by the method of Niehaus and Samuelsson [22]. In this method, malondialdehyde and TBARS were measured by their reaction with TBA in an acidic condition to generate a pink colored chromophore, which was read at 535 nm. Superoxide dismutase (SOD) was assayed by the method of Kakkar and Viswanathan [23]. The assay is based on the inhibition of the formation of Nicotinamide adenine dinucleotide (NADH), phenazine methosulphate, nitroblue tetrazolium formazon. The reaction was initiated by the addition of NADH. The activity of catalase (CAT) was determined by the method of Sinha, [24]. Dichromate in acetic acid was converted to perchromic acid and then to chromic acetate, when heated in the presence of H_2O_2. The chromic acetate formed was measured at 620 nm. Glutathione peroxidase (GPx) was estimated by the method of Rotruck et al., [25]. A known amount

of enzyme preparation was allowed to react with H_2O_2 in the presence of glutathione (GSH) for a specified period. Then, the GSH content remaining after the reaction was measured. The total GSH content was measured based on the development of a yellow color when 5,5'-dithiobis-2-nitrobenzoic acid was added to compound containing sulfydryl groups [26].

Statistical analysis

Statistical analysis was performed using one-way analysis of variance followed by Duncan's multiple range test by using Statistical Package of Social Science version 12.0 for windows (SPSS, Inc., Chicago). The values are mean ± standard deviation for six samples in each group. $p<0.05$ were considered as level of significance.

RESULTS

Effect of PDME on cell proliferation

The effect of PDME on cancer cell proliferation was determined by MTT assay (Fig. 1). Various concentration of PDME (10, 20, 40, 80 and 160 µg/mL) were treated with KB cancer cells for 24 h. The results from MTT assay revealed that PDME at lower concentration did not inhibit the cell growth (10, 20 and 40 µg/mL). However, the higher concentrations (80, 120, and 160 µg/mL) of PDME treatment significantly showed increased cytotoxic effect on KB cells. Moreover, the IC_{50} value of PDME was found to be 80 µg/mL and 160 µg/mL could greatly inhibit the cell growth. Hence, we have selected the 80 and 160 µg/mL concentration of PDME for further experiments. In this assay, we compared the anticancer potential of PDME with the positive control kaempferol (50 µg/mL).

Clonogenic survival assay

The results of clonogenic survival assays were represented in Fig. 2. In this assay, we found that the treatment with PDME at doses of 80 and 160 µg/mL significantly reduced the surviving fraction of KB cells colonies when compared with that of the standard kaempferol (50 µg/mL).

PDME generates intracellular ROS

Fig. 3 shows the effect of PDME on ROS generation at 24 hrs incubation with presence or absence of PDME in oral KB cells. Relative DCF fluorescence intensity was increased in PDME treated cells. PDME (160 µg/mL) showed maximum generation of ROS in KB cells when compared with untreated control cells. Furthermore, fluorescence images were also observed under fluorescence microscope based on green DCF fluorescence. Among the two concentrations tested (80 and 160 µg/mL), 160 µg/mL of PDME showed the maximum generation of ROS (95%) in KB cells. We observed similar result with positive control, kaempferol (50 µg/mL).

PDME modulates mitochondrial membrane potential

Mitochondrial membrane potential changes were measured by Rh-123 fluorescence dye in control, and PDME treated cells. Fluorescence microscopic images (Fig. 4) showed that the more accumulation of Rh-123 dye in the untreated control group and little accumulation was observed in PDME (160 µg/mL) treated cells as revealed by decreased membrane potential. Spectro fluorimetric analysis showed that the mitochondrial membrane potential has been found to be reduced in the PDME treated cells. We observed similar result with positive control, kaempferol (50 µg/mL).

Effect of PDME on apoptotic morphology

Apoptotic features with condensed or fragmented chromatin, indicative of apoptosis, were observed in control and PDME treated KB cells. PDME at doses of 80 and 160 µg/mL treated KB cells showed 56% and 85% of apoptotic cells, respectively (Fig. 5). Control cells showed evenly distributed AO stain (green fluorescence), which indicates cells not undergoing apoptosis. PDME (160 µg/mL) treated KB cells showed apoptotic morphological features indicates formation of orange fluorescence. We observed similar result with positive control, kaempferol (50 µg/mL).

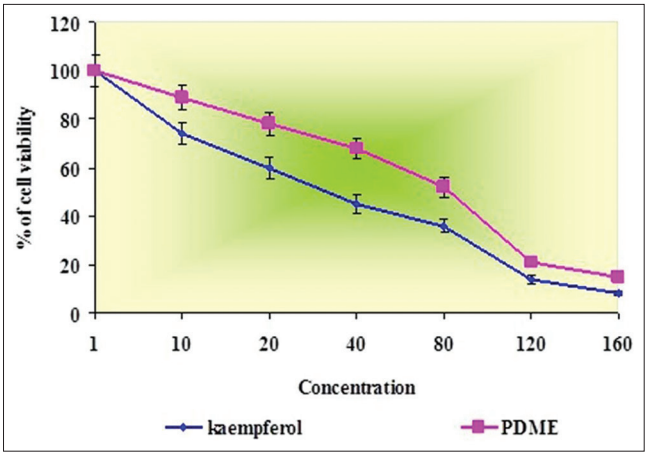

Fig. 1: Cell viability of *Pergularia daemia* methanolic extract (PDME) on KB cells (dose fixation study). PDME was incubated with cancer cells for 24 hr and % cell viability was observed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Cell viability was observed in a concentration (10-160 µg/mL) dependent manner in KB cell lines. The values are given as mean ± standard deviation of six experiments in each group (analysis of variance followed Duncan's multiple range test)

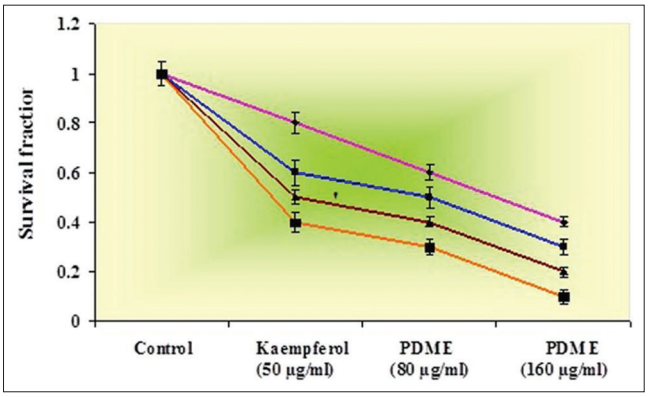

Fig. 2: Clonogenic cell survival in control and of *Pergularia daemia* methanolic extract (PDME) treated KB cells. KB cells were seeded in 100 mm tissue culture plates and subjected to PDME treatment. After 8 days incubation survival colonies were counted and surviving fractions were calculated. Values are given as means ± standard deviation. of six experiments in each group. Values not sharing a common marking (a, b, c etc.,) differ significantly at $p<0.05$ (Duncan's multiple range test)

PDME induced DNA damage

Fig. 6 shows the photomicrographs of DNA damage (comet assay) in different treatment groups. The control cells show largely non-fragmented DNA. We observed that the fragmented DNA in PDME treated cells which appear as a comet during single cell gel electrophoresis. The comet lengths were analyzed by CASP software. There was no change observed in levels of DNA damage in the untreated control cells. PDME (80 and 160 µg/mL) treatment significantly increased tail length, percentage of DNA, OTM, and TM in KB cells. Among the two doses tested, 160 µg/mL of PDME showed maximum tail length (55%), DNA tail (60%), OTM (26%), and TM (22%). We observed similar result with positive control, kaempferol (50 µg/mL).

PDME enhances TBARS in KB cancer cell line

We observed the levels of lipid peroxidation (TBARS) in control and PDME-treated cells (Fig. 7). PDME treatment showed increased levels of lipid peroxidation in KB cells. Among the two doses (80 and 160 µg/mL) tested, 160 µg/mL of PDME showed maximum levels of TBARS and compared with kaempferol (50 µg/mL).

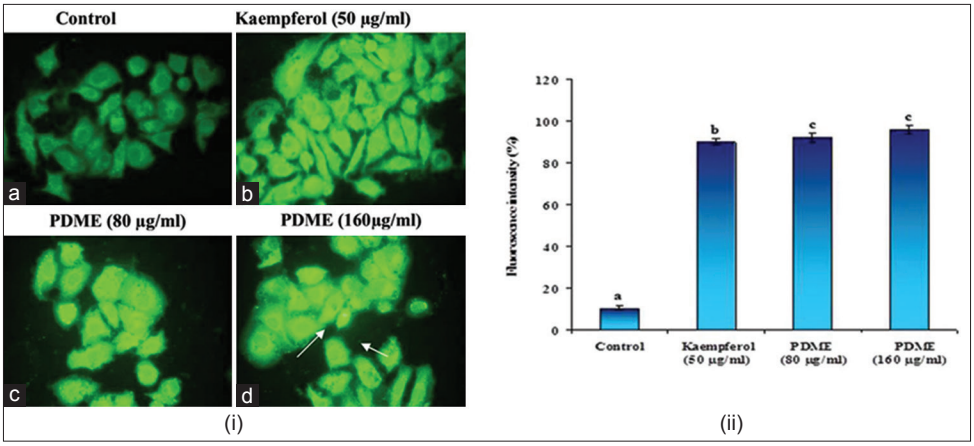

Fig. 3. (i) Fluorescence microscopic images of intracellular reactive oxygen species (ROS) measurement by 2-7-diacetyl dichlorofluorescein staining. Arrow mark (→) represents high dichloro fluorescein fluorescence in 160 µg/mL PDME treatment. (ii) Intracellular ROS measurement by spectrofluorometer. The values are given as mean ± standard deviation of six experiments in each group (analysis of variance followed Duncan's multiple range test). Bars not sharing the common superscripts differ significantly at p<0.05

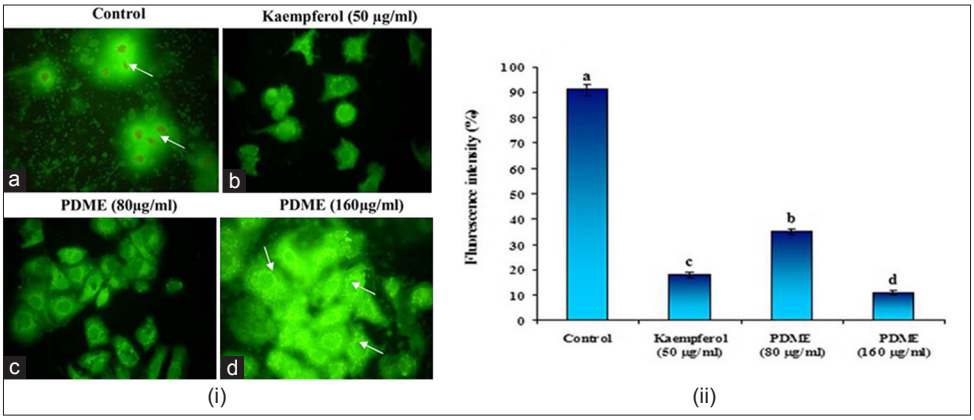

Fig. 4: Fluorescence microscopic images of mitochondrial membrane potential by rhodamine 123 (Rh-123) staining. Arrow marks (→) represents dye accumulation in control group (a). Arrow marks (→) represents no dye accumulation in 160 µg/mL PDME treatment group (d). There is little accumulation of Rh-123 in b and c. The values are given as mean ± standard deviation of six experiments in each group (analysis of variance followed Duncan's multiple range test). Bars not sharing the common superscripts differ significantly at p<0.05

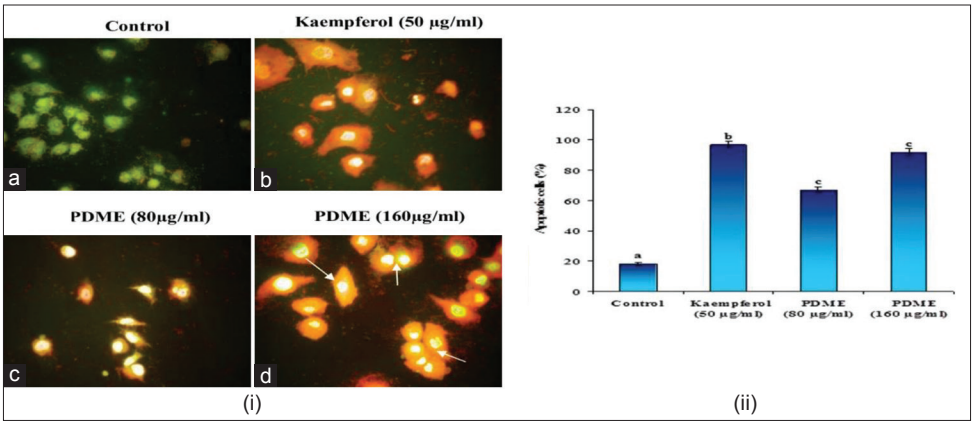

Fig. 5: (i) Fluorescence microscopic images of apoptotic morphology by dual staining. Arrow mark (→) represents orange-colored cells which are late apoptotic cells. (ii) Percentage of apoptosis. The values are given as mean ± standard deviation of six experiments in each group (analysis of variance followed Duncan's multiple range test). Bars not sharing the common superscripts differ significantly at p<0.05

Changes in the activities of antioxidant enzymes

Fig. 8 shows the effect of PDME on the status of enzymatic antioxidants such as SOD, CAT and GPx on KB cells. PDME (80 and 160 µg/mL) treatment significantly decreased the activities of SOD, CAT, and GPx in KB cells, when compared with untreated control cells. Among the two doses tested, 160 µg/mL of PDME significantly decreased enzymatic antioxidants in KB cells.

Changes in the levels of reduced GSH

Reduced GSH is an important cellular antioxidant to donating thiol groups. The effect of reduced GSH levels in PDME treated cells were significantly decreased in KB cells. Levels of GSH in control and PDME treated cells were depicted in Fig. 9. The treatment of PDME (160 µg/mL) maximum decreased GSH levels in KB cells when compared control cells. Among all the doses tested, 160 µg/mL of PDME significantly decreased GSH levels.

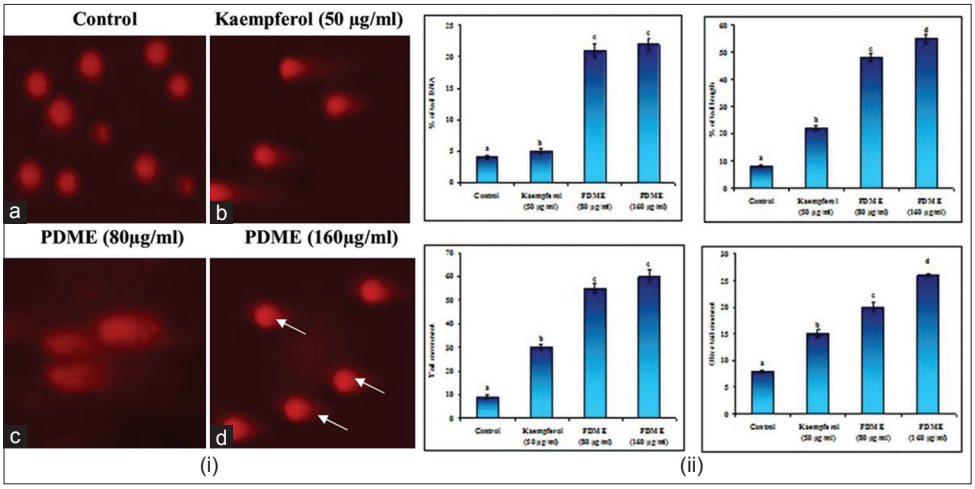

Fig. 6. (i) Fluorescence microscopic images of oxidative DNA damage (comet assay). (ii) Comet parameters (% tail DNA, % tail length, tail moment, and olive tail moment). The values are given as mean ± standard deviation of six experiments in each group (analysis of variance followed Duncan's multiple range test). Bars not sharing the common superscripts differ significantly at p<0.05

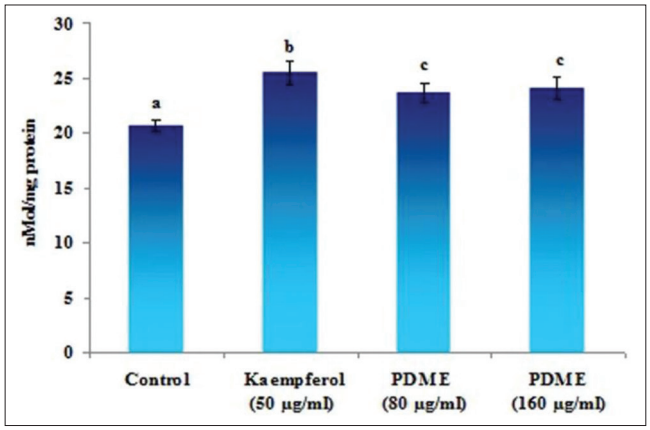

Fig. 7: Levels of lipid peroxidation markers (thiobarbituric acid reactive substances) in KB cells. The values are given as mean ± standard deviation of six experiments in each group (analysis of variance followed Duncan's multiple range test). Bars not sharing the common superscripts differ significantly at p<0.05

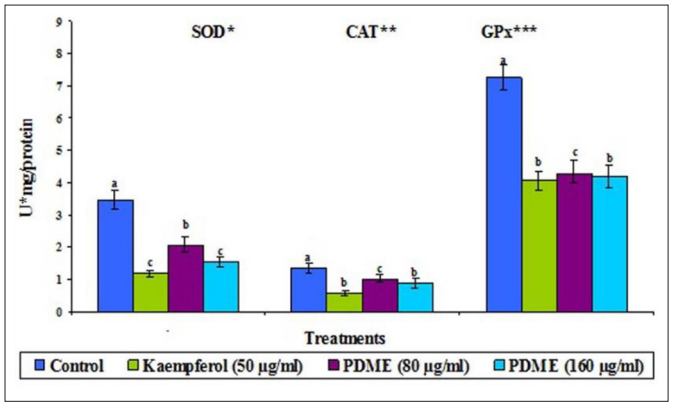

Fig. 8: Activities of enzymic antioxidants in KB cells. Enzyme concentration required for 50% inhibition of *nitroblue tetrazolium reduction in 1 minute. **µmol of hydrogen peroxide consumed per minute. ***µg of glutathione consumed per minute. The values are given as mean ± standard deviation of six experiments in each group (analysis of variance followed Duncan's multiple range test). Bars not sharing the common superscripts differ significantly at p<0.05

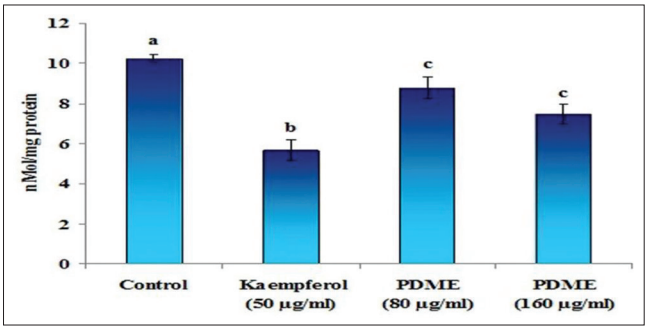

Fig. 9: Levels of reduced glutathione in KB cells. The values are given as mean ± standard deviation of six experiments in each group (analysis of variance followed Duncan's multiple range test). Bars not sharing the common superscripts differ significantly at p<0.05

DISCUSSION

Cancer is a major public health problem all over the world because of its significantly high rates in morbidity and mortality. In OSCC, approximately 50% of tumors exhibited dysfunctional p53, resulting in the loss of a check point control. Thus, cells with damaged genomes would not be able to undergo apoptosis, allowing the defective genome to persist and replicate in the offspring cells [27]. Chemotherapeutic drugs currently being used for OSCC, head and neck cancers and found to induce specific apoptotic pathways include cisplatin and 5-fluorouracil. The commercially available chemotherapeutic agents are highly toxic and poor in bioavilability. Hence, there is a need for the development of new drugs with more bioavailabilty and minimum side-effects.

Many leafy plants, either fruits or vegetables, are high in phenolic compounds (also referred to as phenols, phenolics and/or polyphenols), which have been studied extensively in recent years. The phenolic compounds form the largest category of phytochemicals and are the most widely distributed in nature [28]. Phytochemicals are one of the major sources of anticancer drugs [29]. Recently, P. daemia extract has drawn great attentions because of its beneficial effects against many diseases [30]. In this investigation, we examined the anticancer effect of PDME on oral KB cells in vitro.

The MTT assay showed the higher concentrations (80, 120, and 160 µg/mL) of PDME treatment significantly showed increased cytotoxicity in oral KB cells (Fig. 1). It was found that the IC_{50} value was found to be 80 µg/mL of PDME and 160 µg/mL could greatly inhibit

the cell growth at nearly 90% cytotoxicity. The previous studies were suggested that cytotoxicity potential of calotropisprocera extract showed 46, 67 and 73% cytotoxicity at 10, 30, and 100 µg/mL against human oral cancer cell line [31]. Supportively the anti-proliferative activity of essential oil extracted from Thai medicinal plants on KB and P388 cell lines [32]. Thus, we observed methanolic extract of PDME had the most pronounced cytotoxicity against KB cell lines. The present results also indicate that PDME treatment significantly decreased surviving fraction of KB cells (Fig. 2). Therefore, the PDME greatly inhibits cell viability and colony-forming capacity of KB cells, possibly because of increased oxidative stress.

Cancer cells often show high levels of ROS and must maintain a delicate balance between prooxidants and antioxidants [33]. As ROS may act as growth inhibitory and apoptotic signaling agents, perturbing the balance of ROS in cancer cells may offer an opportunity for prevention by phytochemicals [34]. Recent studies have suggested that the phytochemicals that would target ROS metabolism and raising the levels of ROS in cancer cells [35]. A significant increase in the ROS levels during PDME treated 160 µg/mL of KB cells was further noticed (Fig. 4). The increased ROS levels during the PDME treatment might be due to its prooxidant property. The initial oxidation of catechols generates a semiquinone that can react with oxygen to form superoxide anion $O_2^{\bullet -}$. This reaction has an autocatalytic character as $O_2^{\bullet -}$ will oxidize the phenolic compound to regenerate the semiquinone and H_2O_2. Further, peroxidases present in the cancer cells catalyze a one electron oxidation of phenols to form phenoxyl radicals which, in turn, rapidly oxidize NADH to $NAD^{\bullet}$. Then this NAD radical reduces O_2 to $O_2^{\bullet -}$. This might be the reason for the increased ROS generation during PDME treatment.

Further, we observed the changes in mitochondrial membrane potential in PDME treated and control cells. Mitochondrion is one of the most important organelles in regulating cell death, as well as a maker in apoptosis [36]. The authors observed that the mitochondrial membrane potential has been altered during PDME treatment. The release of cytochrome C is known to be a function of oxidative stress [37] and the change in mitochondrial potential caused by PDME might be an essential requirement for apoptosis. Previous studies suggested that the treatment of PC12 cells with BEL resulted in a reduction of mitochondrial membrane potential, similar to that observed in the treatment with the mitochondrial depolarizing agent FCCP [38].

The increasing apoptotic cells during PDME treatments clearly indicated that this combination would be able to induce apoptosis. We observed that the PDME (160 µg/mL) pre-treatment significantly increased apoptotic morphological changes in KB cells. The increased ROS levels and subsequent reduced matrix metalloproteinases levels might be the reason for the increased apoptotic morphological changes in the PDME treated cells. Previous results demonstrated that TSL-1 induces HOSCC cell death by either apoptosis or a combination of apoptosis and necrosis. These differing effects could be due to cell type-dependent reaction after treatment with TS leaf extract. The mechanism for the apoptotic and/or necrotic cell death observed in UM2, SCC4, and SCC9 cells, remain to be elucidated [39].

DNA is an important molecular target for tumor cell killing [40]. Fig. 5 shows significant DNA damage in PDME treated KB cells. Polyphenols are known to enhance oxidative DNA damage in cancer cells [41]. Previous studies suggested that the genotoxicity of herbicide such as glyphosate, which up to now has heterogeneous results. The comet assay was performed in Hep-2 cells. The level of DNA damage in control group (5.42±1.83 arbitrary units) for TM measurements has shown a significant increase ($p<0.01$) with glyphosate at a range concentration from 3 to 7.5 mM. As cancer cells possess centrally acidic region, the most phenolics present in PDME act as prooxidant in cancer cells. Flavonoids auto-oxidize in an aqueous medium and may form highly reactive free radicals. Polyphenols may act as substrate for peroxidases and other metalloenzymes, yielding quinone-type prooxidants [42]. This might be the reason for the increased oxidative DNA damage (% tail DNA, % tail length, TM, and OTM) observed in PDME treated cancer cells.

We observed significant increase in lipid peroxidation in PDME treated cancer cells. Prooxidant activity of hydroxycinnamic acids on DNA damage and lipid peroxidation in the presence of Cu (II) ions and the structure activity relationship have been elucidated [43]. In this study, the authors observed decreased activities of antioxidant enzymes, i.e., SOD, CAT, and GPx in PDME treated cancer cells. They have also noticed prominent decrease of GSH levels in cancer cells treated with PDME.

The results summarize that PDME initiates cancer cell death by decreasing cell proliferation, antioxidant status, increasing intracellular ROS, alteration in mitochondrial membrane potential, lipid peroxidation, DNA damage and apoptosis in KB oral cancer cell line. These observations revealed that the cytotoxic potential of PDME, which could be attributed to their pro-oxidant property on the cancer cells. It is evident from the observation made in the present study that the PDME have potential anticancer effect. However, it is worth emphasizing that the protective role of PDME can be considered as an anticancer agent to combat against oral cancer. Further studies are highly warranted to isolate the active compounds from P. daemia responsible for this potent activity against oral cancer.

CONCLUSION

The current investigation suggested that the polyphenolic constituents of P. daemia responsible for anticancer activity. Thus, the long-term consumption of P. daemia could be considered and promoted as an adjuvant therapy for various malignancies.

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