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Anticancer and antimutagenic activity of *Silybum marianum* L. and *Eucalyptus camaldulensis* Dehnh. against skin cancer induced by DMBA: *In vitro* and *in vivo* models

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Abstract: The current study investigated the prospective effect of *Silybum marianum* L. and *Eucalyptus camaldulensis* Dehnh extracts against skin cancer. Skin cancer was induced by 7,12-dimethylbenz(*a*) anthracene (DMBA) in young Balb/c mice. Plant extracts were administered to animals orally, once/day (100mg/kg, 5 days/week) for the 20 weeks. Anticancer activity was examined via tumor progression, where antimutagenic activity was measured using 8-OHdG and sister chromatid exchange (SCE) levels. *Eucalyptus camaldulensis* Dehnh. leaves extract and *Silybum marianum* L. leaves extract significantly reduced 8-OHdG in cultured human lymphocytes in a dose-response manner ($P<0.05$). Similarly, the leave extracts of both plants significantly *reduced* chromosomal damage as measured by SCE levels ($P<0.05$). In the skin painting assay, the leave extracts of both plants significantly delayed the onset of tumors compared to DMBA treated group ($P<0.05$). The *Silybum marianum* leaves extract significantly reduced tumor incidence ($P<0.01$) and papilloma frequency ($P<0.01$) induced by DMBA. The *Eucalyptus camaldulensis* leaves extract significantly reduced the number of tumors per animal ($P<0.05$) and incidence of tumors ($P<0.001$). The *in vitro* and *in vivo* findings showed that leaves of *Silybum marianum* L. and *Eucalyptus camaldulensis* Dehnh. extracts might be a promising source for anticancer and antimutagenic agents against human cancer.

Keywords: Skin cancer, anticancer, antimutagenic, *Silybum marianum* L., *Eucalyptus camaldulensis* Dehnh., DMBA.

INTRODUCTION

Skin cancer (SC) is very common as it accounts for almost 40% of cancer cases worldwide (Cameron *et al.*, 2019; Bolick and Geller, 2021). The SC has three types: basal-cell SC, squamous-cell SC, and melanoma (Gruber and Zito, 2021). Nonmelanoma types are the most commonly occurred, which affects around 2–3 million people yearly (Schrom, Kim and Baron, 2020).

Silybum marianum (milk thistle) is a well-known herb used in curing liver and biliary disorders (Wang *et al.*, 2020). *Silybum marianum* was also used as cancer therapy for prostate, skin, breast, cervix cancers and hepatocellular carcinoma; though, different studies came out with varied and inconsistent findings (Greenlee *et al.*, 2007; Marmouzi *et al.*, 2021). *Silybum marianum* extracts consist of about 65-80% silymarin (a mixture of flavonoid complexes) and about 30% fatty acids (Kroll *et al.,* 2007; Abenavoli *et al.*, 2018). Silymarin, is the active ingredient that plays a protective role against drug toxicity, including chemotherapy, also is considered a strong antioxidant agent (Abenavoli and Milic, 2017; Fanoudi *et al.*, 2020).

Studies have reported an anti-inflammatory activity of silymarin by regulating different types of inflammatory mediators including TNF- ALPHA, NO, interferon-gamma, IL-4 and IL-10 (Wilasrusmee *et al.*, 2002).

Eucalyptus camaldulensis is an indigenous tree found in different world regions. The *Eucalyptus camaldulensis* leaves are commonly used for wound healing and fungal infections (Aleksic Sabo and Knezevic, 2019). The *Eucalyptus* plant extracts are frequently used as a therapy for respiratory diseases, including the common cold, influenza and sinus congestion (Kheder *et al.*, 2020). Curative properties, such as anti-inflammatory and antimicrobial properties of *Eucalyptus* spp have been reported (Aleksic Sabo and Knezevic, 2019)

Leaves have been studied extensively (Salem *et al.*, 2020; Zein *et al.*, 2020) but further investigations are needed to verify their therapeutic efficiency especially against cancers.

Different approaches were implemented to study skin cancer, either *in vitro* and/or *in vivo* systems, in addition to using carcinogens and possible anticancer agents to explore their efficiency in fighting cancer. Skin painting assay is one of the successful approaches that was used to

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verify the carcinogen ability of different chemicals or to induce a cancer model by using carcinogenic substances such as 7,12-dimethylbenz (*a*) anthracene (DMBA) and 2,4-dinitrochlorobenzene (DNCB). Commonly, a shaved mouse or hairless mouse (specially bred) is used in this technique by painting the substance onto a standard patch of skin on a frequent basis. The comparison is always made between those who have been painted with the control (without painting). The two groups need to be under the same conditions (eating, cleaning routine, day-to-day handling, etc.). This approach was used effectively in different cancer research studies by showing interesting results (Lee *et al.*, 2020). The 7,12-dimethylbenz (a) anthracene (DMBA), a polycyclic aromatic hydrocarbon, is a strong cariogenic agent that initiates tumor by suppressing the immune system and damaging DNA (Buque *et al.*, 2021). The DMBA is one of the environmental pollutants that is commonly used in many cancer studies by inducing cancer in animal models (Li and Brakebusch, 2021). It generally stimulates inflammation and genomic instability by dislocates the linkage between DNA and proteins (Wang *et al.,* 2021).

Investigating skin carcinogenesis through implementing *in vitro and in vivo* systems may help in detecting initial changes in the skin and further exposing tumor mechanisms, which may assist in developing a new and effective treatment for skin cancer. By using *in vitro* and *in-vivo* models, this study aimed to investigate the possible anticancer and antimutagenic effect of *Silybum marianum* and *Eucalyptus camaldulensis* against skin cancer induced by DMBA.

MATERIALS AND METHODS

Mouse strain and housing

Young (6-9 weeks) Balb/c mice were used in the experiments. Mice were purchased from the Animal Care Facility at Jordan University of Science and Technology. Balb/c strain has proven to be useful and reliable for the assessment of the protective effect of plant extracts against cancer development. Animals (4-6) / cage were subjected to two weeks' acclimation prior to the start of the experiments. Animals were maintained under standard conditions: 24±1°C, humidity 45±5% and light/dark cycle (12h/12h) and housed in standard cages and provided food and water *ad libitum*. Three days before the beginning of the experiment, hair on the interscapular region of the animals was clipped. The study was approved by the Animal Care and Use Committee at Jordan University of Science and Technology (approval number: 267-2013).

Plant extractions

Collection, identification, and preparation of plant material

Plant material was collected from different locations in Jordan. The taxonomic identity of plants was confirmed by Professor Jameel Lahaam, Department of Biological Sciences, Faculty of Science, Yarmouk University, Irbid, Jordan. A voucher specimen from both plants was deposited at the Medicinal Plants Museum, Department of Medicinal Chemistry and Pharmacognosy, Faculty of Pharmacy, Jordan University of Science and Technology, Irbid, Jordan. The plant material was shade-dried, then, 100gm was grounded in a Wiley grinder with a 2 mm diameter mesh. The ground material was percolated in ethanol (95%). The combined ethanolic extracts were, then, concentrated in a vacuum to give a gummy material. The yield of *S. marianum* L. was 4.51gm/100gm and for *E. camaldulensis* Dehnh. was 4.25gm/100gm. Dried plant extracts were stored in dark at desiccator.

Experimental design

For both plants tested, 3 groups (15 animals each) of mice were used and randomly distributed according to the weight of the animals using Xybion PATH/TOX software (Xybion Medical Systems; Cedar Knolls, NJ); (Meckley *et al.*, 2004). Skin cancer was induced by applying a single application of 7,12-dimethylbenz(a) anthracene (DMBA, Sigma-Aldrich, Saint Louis, MO, USA), 50 µg; (Sancheti and Goyal, 2006) dissolved in acetone (Fisher Scientific, USA) to the animal's back. The groups include: Group "I" that received vehicles used for dissolving DMBA (control group), Group "II" that received the DMBA (100µg/ 100µl of acetone) over the shaven area of the skin of the mice, and Group "III" was treated as group "II" plus administered plant extract orally, once a day (100mg/kg, 5 days/week) for the 20-week study period.

On each application day, new aliquots of plant extract were thawed and used. Following common practice (Li and Brakebusch, 2021). For DMBA, the solution was applied to the skin by micropipettes with disposable tips. For plant extract, doses were given by oral gavage.

Tumor examination

Each animal's general health was examined daily. In addition, the back of each animal was examined for the appearance of papilloma one time/week and only tumors with a diameter of 2mm or greater were counted (Roemer *et al.*, 2010). All changes were recorded and, in each group, tumor incidence (% of tumor-bearing mice), multiplicity (% of tumors/mouse), onset and malignancies were measured (Sancheti and Goyal, 2006). Also, all skin with tumors was sampled at study termination (Meckley *et al.*, 2004; Roemer *et al.*, 2010) and stored for later histopathological examinations. Tissues collected from all mice for this purpose were placed in 10% buffered formalin for fixation before processing, paraffin embedding and sectioning. Multiple sections were stained with hematoxylin and eosin for microscopic examination and histopathological evaluation. All precancerous and cancerous changes and their extent in the skin of the animals were noted.

For termination, mice were euthanized after completion of the skin painting studies by overdose with carbon dioxide as per standard methodology approved by the panel on euthanasia of the American Veterinary Medical Association (Leary *et al.*, 2020).

The 8-OH-dG assay

Human lymphocytes were cultured as previously described (Laham *et al.*, 2019). After 72 hrs. of culture initiation, cells were collected using centrifugation at 3000 xg. The collected cells were resuspended culture media without serum. Resuspended cells were then exposed to different doses of plant extracts (10, 100, 500, and 1000 in ug/ml, n=8/each treatment) for six hours (Al-Smadi *et al.*, 2019). After treatment, cells were pelleted using centrifugation at 3000 xg and an 8-OHdG marker was measured in the supernatant using commercially available assay according to the instruction provided with the kit (8-OH-dG EIA kit; Abcam, Cambridge, UK). Changes in absorbance at 405 nm were recorded using the ELx800 Universal plate reader (BIO-TEK, USA).

The sister chromatid exchanges (SCEs) assay

The setting of human lymphocyte cultures was as previously described (Khabour *et al.*, 2016). 5-bromodeoxyuridine (25µg/mL final concentration) was added to cultures immediately after setting. Cultures were exposed to plant extracts in the last 24 hours of the culturing period (72 hours). Then cultures were exposed to Colcemid (0.1µg/mL) and were added for 2 hours to arrest dividing cells in the metaphase stage. Cultured cells were then fixed and metaphase spreads were prepared as previously described (Al-Eitan *et al.*, 2020). Chromosomal spreads were differentially stained using a florescence-plus-Giemsa technique and SCEs were scored in 25 metaphases per plant extract dose. Finally, the mitotic index (MI) and proliferative index were calculated as previously described (Alzoubi *et al.*, 2018).

STATISTICAL ANALYSIS

Graphpad Prism Software (version 5, La Jolle, CA, USA) was used for statistical analysis. Multiple group comparisons were performed using one-way ANOVA followed by Tukey's post hoc test. Differences were regarded significant at a $p<0.05$.

RESULTS

The antimutagenic and anticancer effects of *Silybum marianum* (leaves) and *Eucalyptus camaldulensis* (leaves) plant extracts were examined using cultured human lymphocytes and skin-painting assay, respectively.

Fig. 1 shows the antimutagenic effect of examined extracts using an 8-OHdG assay in cultured human lymphocytes. The ethanolic extract of *Silybum marianum* (leaves) decreased oxidative DNA damage at most examined concentrations (100, 500 and 1000µg/ml) in a dose-response manner (fig. 1A, $P<0.05$). Concerning the

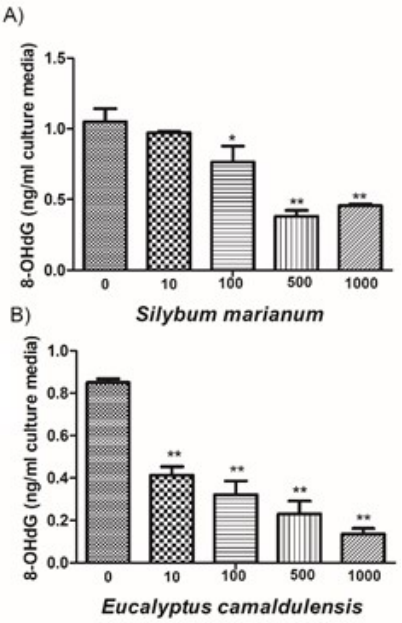

Fig. 1: The antimutagenic effect of ethanolic extracts using 8-OHdG assay in control, 10, 100, 500 and 1000 groups in A) Silybum marianum, and B) Eucalyptus camaldulensis. P ($P<0.01$) using one-way ANOVA Test. * Indicates significant difference from control.

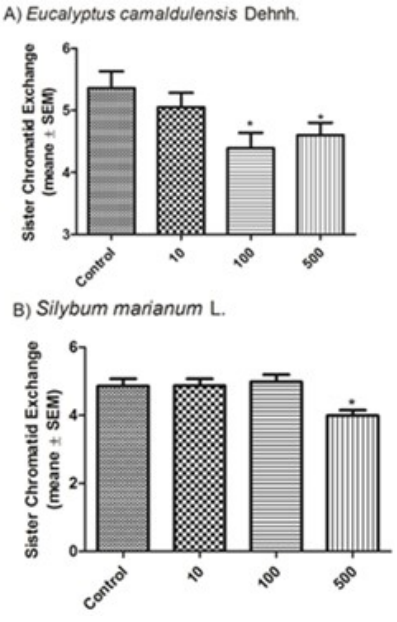

Fig. 2: The average of SCEs/cell in control, 10, 100, and 500 groups in A) Eucalyptus camaldulensis and B) Silybum marianum L. ethanolic extracts. P ($P<0.01$) using one-way ANOVA Test. *Indicates significant difference from control.

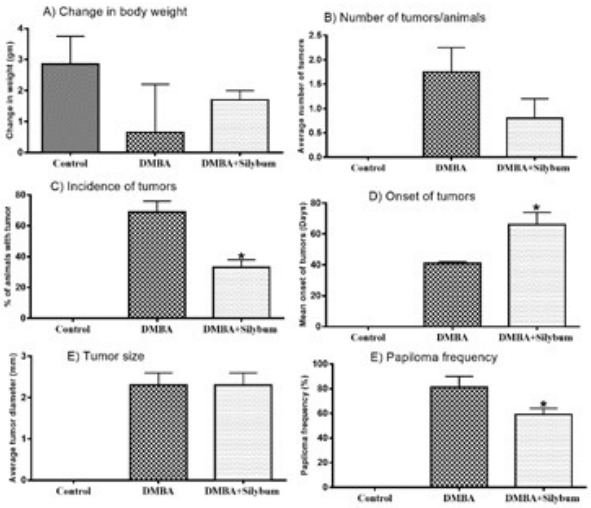

Fig. 3: The effect of the ethanolic extract of *Silybum marianum* (leaves) extract on treated mice with DMBA compared to untreated mice (control) in term of the followings A) change in body weight B) Number of tumors/animals C) Incidence of tumors D) Onset of tumors E) Tumor size E) Papilloma frequency. * Indicates significant difference from control.

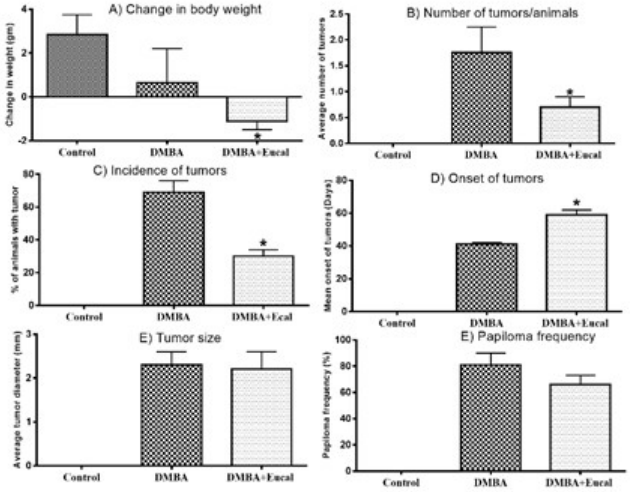

Fig. 4: The effect of the ethanolic extract of *Eucalyptus camaldulensis* (leaves) extract on treated mice with DMBA compared to untreated mice (control) in term of the followings A) change in body weight B) Number of tumors/animals C) Incidence of tumors D) Onset of tumors E) Tumor size E) Papilloma frequency. * Indicates significant difference from control.

ethanolic extract of *Eucalyptus camaldulensis*, significant decreases in 8-OHdG were observed at all examined concentrations (10, 100, 500, and 1000µg/mL) in a dose-response manner (fig. 1B, $P<0.001$).

The antimutagenic effect of examined extracts using sister-chromatid exchange (SCE) assay in cultured human lymphocytes is shown in fig. 2. The ethanolic extract of *Silybum marianum* (leaves) significantly decreased SCE levels at 100µg/ml and 500µg/ml doses (fig. 2A, $P<0.01$). The 10µg/ml dose induced a slight decrease in SCE levels, but it was not statistically significant. With respect to the ethanolic extract of *Eucalyptus camaldulensis*, significant decreases in SCE were observed only at 500 µg/ml dose (fig. 2B, $P<0.01$). Thus, *Silybum marianum* seems to be more potent in lowering SCE levels than *Eucalyptus camaldulensis*.

The impact of examined plant extract on cell kinetics parameters was examined using cultured human lymphocytes. None of the examined concentrations affected induced significant changes in proliferative or mitotic indices ($P>0.01$, data not shown).

The anticarcinogenic potential of the ethanolic extracts of *Silybum marianum* (leaves) and *Eucalyptus camaldulensis* was examined at a 100 mg/kg dose.

The results of the *Silybum marianum* (leaves) extract are shown in fig. 3. *Silybum marianum* extract did not affect the body weight of experimental animals (fig. 3A).

Treatment of mice with DMBA induced skin cancer as shown by the elevation of all examined tumor parameters (fig. 3). The *Silybum marianum* extract significantly reduced tumor incidence (fig. 3C, $P<0.01$) and papilloma frequency (fig. 3F, $P<0.01$) induced by DMBA. In addition, it significantly delayed the onset of tumors (fig. 3D, $P<0.01$) compared to DMBA treated group. A substantial decrease in the number of tumors per animal was observed but it did not reach the significant level (fig. 3B, $P=0.09$). However, *Silybum marianum* extract did not affect tumor size in animals (fig. 3E).

The overall data of *Eucalyptus camaldulensis* leaves is shown in fig. 4. The leaf extract of *Eucalyptus camaldulensis* significantly reduced the body weight of animals (fig. 4A, $P>0.05$). While treatment of animals with *Eucalyptus camaldulensis* leaves extract significantly reduced the number of tumors per animals (fig. 4B, $P<0.05$) and incidence of tumors (fig. 4C, $P<0.001$) induced by DMBA. The plant extract also delayed the onset of tumors as shown in (fig. 4D, $P<0.01$). However, the plant extract did not affect the size of tumors and papilloma frequency (fig. 4E and 4F respectively, $P>0.05$) induced by DMBA.

DISCUSSION

Herein, we examined the antimutagenic and anticancer effects of *Silybum marianum* (leaves) and *Eucalyptus camaldulensis* (leaves) extracts using *in-vitro* and *in-vivo* models. When the oxidative stress damage level of examined extracts was assessed, *Eucalyptus camaldulensis* (Leaves) resulted in a decrease in oxidative DNA damage at all examined concentrations, whereas *Silybum marianum* (leaves) decreased oxidative DNA damage at most examined concentrations in a dose-dependent manner. On the other hand, *Silybum marianum* (leaves) showed more potency in reducing SCEs levels, at higher concentrations, than *Eucalyptus camaldulensis*.

The leaves extract of *Silybum marianum* did not show any effect on either the body weight of experimental animals or the tumor size. It, though, significantly delayed the onset of tumors compared to DMBA treated group. The tumor incidence and papilloma frequency, induced by DMBA, were significantly reduced by the *Silybum marianum* extract. Additionally, a considerable decrease in the number of tumors per animal was recorded but without reaching a significant level by the *Silybum marianum* extract. It has been found that silymarin, a flavonoid compound and main component of the *Silybum marianum* extract, has a potential anti-cancer effect when studied on a mouse skin model as it reduces tumor growth rate and tumor volume (Moushumi Lahiri-Chatterjee *et al.*, 1999). Using naturally occurring medicinal herbs as an anti-cancer agent seems to be a conventional worldwide approach. For instance, silymarin was reported to show a preventive effect against different types of carcinogenic agents in various animal tumor models (de Gruijl and Forbes, 1995; Forbes, 1996; Katiyar *et al.*, 1997). Relevant treatment of silymarin inhibited 7,12-dimethylbenz (a) anthracene-initiated and several tumor promoters, induced skin carcinogenesis in mouse models (M Lahiri-Chatterjee *et al.*, 1999). Several *in vivo* studies revealed the antioxidant and anti-inflammatory capabilities of Silymarin that preclude skin cancer in *in vivo* animal models (Katiyar, 2005). It has been shown that silibinin, a main component of silymarin, inhibits the formation of UVB-induced thymine dimer and stimulates DNA repair, and/or initiates apoptosis in damaged cells (Saller *et al.*, 2007; Prasad *et al.*, 2020). This finding proposed that siblinin can be used as a potential treatment, against non-melanoma skin cancer (NMSCs) in humans (Saller *et al.*, 2007; Prasad *et al.*, 2020). Silibinin has been used as a treatment for different kinds of liver diseases and has reported possessing a significant inhibitory effect against different types of tumors including breast (Amiri *et al.*, 2016), oral (Won *et al.*, 2018), colon (Yang *et al.*, 2003), prostate (Ting *et al.*, 2013) and lung cancer (Zhu and Sun, 2018). Stimulating apoptosis, boosting tumor suppressor and cell cycle inhibitors, are the mechanisms used by silymarin to inhibit growth factors and weakening proliferative mediators in fighting cancer cells (Ravishankar *et al.*, 2013).

Eucalyptus extracts have useful therapeutic properties. For example, extracts of *E. camaldulensis* leaves were found to be highly cytotoxic to cultured carcinoma cells (Meshkani and Ranjbar, 2014; Islam *et al.*, 2015). Moreover, previous studies showed an influential cytotoxic effect of *Eucalyptus spp.* against tumor cells, which show a promising curing efficacy as a cancer treatment. For instance, leaves of *Eucalyptus*. *grandis* were found to contain A phlorogrucinol-monoterpene derivative, euglobal-G1, which showed a good anti-cancer activity using mouse skin tumor assay (Takasaki *et al.*, 2000). Additionally, leaves of *E. cypellocarpa* were found to contain several compounds that prevent the mouse from skin cancer induced by 12-O-tetradecanoyl-phorbol-13-acetate (Ito *et al.*, 2000). The exact mechanism of action of *E. camaldulensis* is not known. However, it is proposed to be related to the strong antioxidant and anti-inflammatory activity of the extract (Silva *et al.*, 2003; Huang *et al.*, 2015; Nwabor *et al.*, 2019). All these studies are in accordance with the results of the current study that showed the leaves extract of Eucalyptus camaldulensis significantly lowered the number of tumors per animal and delayed the onset of tumors.

The current investigation was conducted with the *in vivo* skin painting assay. Confirmation of the present findings using specific and standardized cancer cell lines is recommended in future investigations.

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CONCLUSION

In conclusion, the results of the current study showed that *Silybum marianum* and *Eucalyptus camaldulensis* extracts might be promising sources of compounds to be used as anticancer and antimutagenic agents against human cancer.

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