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Prevention of DMBA-induced Mammary Tumors from Pulmonary Metastases by *Saussurea costus* (Falc.) Lipsc Root Ethanolic Extract in Sprague Dawley Rats

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ABSTRACT

Background: Breast cancer is an important problem in human females and therapies used for treatment often found to produce side effects. The present study was conducted to evaluate the effect of *Saussurea costus* root extract (SC) against metastatic breast cancer to the lungs.

Methods: The anti-neoplastic activity of SC extract was assessed by calculating the % cytotoxicity on Leydig cell testicular tumour cells-540 (LC-540). The anti-metastatic effect of SC extract at 3 different doses (100, 250 and 500 mg/kg BW) for a period of 18 weeks against 12-dimethylbenz (a) anthracene (DMBA) induced mammary tumours in Sprague Dawley (SD) female rats was studied through radiography, gross lesion scoring, histopathology and immunohistochemistry (MMP-9).

Result: SC extract exhibited the highest anti-neoplastic activity in a time and concentration-dependent manner. After 72 h of duration SC extract exhibited highest anti-neoplastic activity at 200 µg of concentration. On radiological and gross pathological examination, the inhibition of metastases was maximal in the rats treated with highest dose of the extract as compared with only DMBA treated group. The histopathological changes and immunohistochemical expression of metastases marker MMP-9 in lung tissue were more severe in the rats treated with DMBA only and were reduced in the groups treated with SC extract in a dose-dependent manner. SC extract has potential anti-neoplastic activity and prevents the rate of metastases from mammary tumours to lungs. Further research in exploring the possible therapeutic usage of SC extract against various neoplastic conditions is warranted.

Key words: Anti-neoplastic, Breast cancer, Lungs, Metastases, *Saussurea costus*.

Abbreviations: DMBA- 12-dimethylbenz (a) anthracene, DMSO- Dimethyl sulfoxide, HandE- Haematoxylin and Eosin, IHC- Immunohistochemistry, LC-540- Leydig cell testicular tumour cells, MMP-9- Matrix-metalloproteinase- 9, SC- *Saussurea costus* root extract, SD rats- Sprague dawley rats, SRB- Sulforhodamine B colorimetric.

INTRODUCTION

Cancer is the world's second largest cause of mortality (Akhouri *et al*., 2020). In 2019, the World Health Organisation (WHO) supplemented statistics showing that cancer deaths rank first or second in 112 nations before the age of 70 and third or fourth in 23 countries (Bray *et al*., 2018). Female breast cancer has the greatest prevalence data (11.7%) among the most commonly reported tumours, followed by lung (11.4%), colorectal (10.0%), prostate (7.3%) and stomach cancers (5.6%). However, lung cancers continue to be the leading cause of death in humans (18.0%), followed by colorectal (9.4%), hepatic (8.3%), gastric (7.7%) and female mammary tumours (6.9%) (Goldschmidt *et al*., 2016). Metastasis is one of the leading causes of death in breast cancer and there is presently no standardized treatment for metastatic breast cancer. Metastasis is a complex process that consists of several distinct steps, including epithelial-mesenchymal transition (EMT), in which cancer cells lose cell-to-cell contact; local tissue invasion, which is caused by the breakdown of extracellular matrix (ECM); extravasation, in which cancer cells pierce the wall of a blood vessel and enter the circulation and homing, in which cancer cells must persist in the bloodstream (Liu *et al*., 2015). Death in breast cancer in human females is augmented metastases to different

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organs especially lungs (Largillier *et al*., 2008; Lu and Kang, 2007).

More than 3000 plant-derived anti-neoplastic agents have been incorporated in recent years to treat tumours and curiously, they have considerably fewer side effects than commonly used chemo-preventative medications (Kumari *et al*., 2022). Extensive scientific study has led to the development of numerous therapeutic techniques, including hormonal therapy, chemotherapy and surgical interventions, in order to reduce the significant increase in the death toll. Anti-neoplastic drugs or therapies used to treat tumours improve longevity but degrade quality of life due to severe adverse effects (Samant *et al*., 2007).

Saussurea costus (Falc.) Lipsch is one of these indigenous plant species that may be found at altitudes ranging from 2000 to 3500 meters above sea level in the North Western Himalayan region. This plant grows in cold desert environments from April to September. The bioactive constituents present in this plant are frequently used to treat malignancies such as breast, ovarian, prostate, colon, urinary bladder, liver cancer and leukaemia (Semenza, 2012). The genus Saussurea has around 420 bioactive compounds, the most important of which are sesquiterpenes, triterpenes, lignans and flavonoids amongst others (Yang *et al*., 2017). A study conducted by Hsu *et al*. (2009) reflected the anti-cancerous effects of *Saussurea costus* derived dehydrocostus lactone *in vivo* and *in vitro* on hepatocellular carcinoma.

Saussurea costus is an excellent medicinal herb, therefore the current study aimed to evaluate the anti-metastatic potential of *Saussurea costus* against pulmonary metastases from neoplastic cells from DMBA-induced breast tumours in female Sprague Dawley rats.

MATERIALS AND METHODS

Sampling and preparation of SC extract

The *Saussurea costus* plant material was collected from Lahul and Spiti, Himachal Pradesh, India and authenticated at CSIR-Institute of Himalayan Bioresource and Technology (CSIR-IHBT), Palampur, Himachal Pradesh, India. The roots of the plant were shade dried and 70% aqua-ethanolic extract was prepared and concentrated over rotatory evaporator (Buchi Rotavapor R-210) at 40°C and 175 mbr vacuum pressure. The slurry obtained was subjected to lyophilisation.

Sulforhodamine B (SRB) colorimetric assay

Rat Leydig cell testicular tumour LC-540 cells were procured from National Centre for Cell Science (NCCS), Pune, India. Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) (Invitrogen Biosciences, India) and sodium bicarbonate (2 g/L) was supplemented with Penicillin (10,000 units/100 ml) and Streptomycin (10 mg/100 ml) (Invitrogen Biosciences, India) and was used for routine maintenance of LC-540 cell line. LC-540 cells were maintained at 37°C in 5% CO_2 environment at pH 7.4. Dimethyl sulfoxide (DMSO) was used as a solvent for the SC extract and Vinblastine (10 ìM) served as a positive, control. LC-540 cells were seeded in 96 well plates and treated with 4 different concentrations of SC extract *i.e*., 20 µg/ml, 50 µg/ml, 100 µg/ml and 200 µg/ml. After every time line LC-540 cells were treated with SRB dye and the optical density was measured at 540 nm through microplate reader (BioTeK Synergy H1 Hybrid Reader) after adding 50 µl tris base.

$$\text{Per cent \% cytotoxicity} = \frac{\text{Optical density of control - Optical density of test}}{\text{Optical density of control}} \times 100$$

Experimental study design

The experimental work was conducted as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and study on adult female Sprague Dawley (SD) rats was approved by institutional Animal Ethics Committee (IAEC) with issued IAEC No. QSD.IAEC/VetPath/CSK HPKV/2019/246-50. A total of 40 adult female rats weighing around 160±20g and 6-8 weeks old were procured from National Institute of Pharmaceutical Education and Research (NIPER), Mohali, Punjab, India and were maintained in the Animal House of College of Veterinary and Animal Sciences, Himachal Pradesh Agricultural University, Palampur, Himachal Pradesh, India. The rats were maintained at standard laboratory house conditions (temperature: 22±3°C; humidity: 50±10%; light: 12:12 h light/dark cycle) and provided with *ad libitum* autoclaved feed and water.

Rats were randomized to five groups with 8 rats in each group. Group I (CX) served as a control group; group II (DX) received breast cancer inducing carcinogen DMBA only (25 mg/kg BW, single subcutaneous dose near mammary gland), groups III (SC1), IV (SC2) and V (SC3) were provided with SC extract at 100 mg/kg BW, 250 mg/kg BW and 500 mg/kg BW, respectively through oral route after the induction of DMBA. Animals were examined for any clinical symptoms and mortality for 18 weeks. After the end of the experiment eats were sacrificed using diethyl ether.

Radiology

Radiological evaluation of mammary tumours was done to assess the level of metastases to lungs in rats in the Department of Veterinary Surgery and Radiology, DGCN COVAS, Palampur. For radiographic exposure, the X-ray machine (Allengers HF MARS 80' of Allengers Medical System) with a moving horizontal bucky table or stationary grid with grid ratio of 6:1 and 8:1 was used. However, for the scanning of exposed X-ray plates, a computed radiography machine (Regius 110, KONIKA Minolta Healthcare Pvt Ltd.) was used.

Gross pathology

The lungs were examined for gross lesions, scored and recorded. The gross examination of lungs was performed

by counting the number of foci evident, the % area of lungs involved by metastatic foci and size of the metastatic foci in millimeters.

Histopathology

The lung sections approximately 0.5 cm in thickness were collected in 10% neutral buffered formalin (NBF) for histopathological and immunohistochemical evaluation of MMP-9 expression. The lung sections were dehydrated with ascending grades of alcohol, cleared with benzene, embedded in paraffin and sectioned with microtome. Tissues after sectioning were stained with Haematoxylin and Eosin (H and E) stain as per the standard procedure (Bancroft and Gamble, 2008). The histopathological lesions were examined and photographed under light microscope (OLYMPUS BX40).

Immunohistochemical analysis

Immunohistochemistry for MMP-9 expression was done as per the standard protocol (Chhimwal *et al.*, 2020). The lung sections were collected on poly-L-lysine coated slides, deparaffinized with xylene and dehydrated with alcohol. The sections were treated with 3 cycles of sodium citrate buffer (pH-6.0) in microwave for antigen retrieval. Expression of metastasis marker MMP-9 at 1:150 dilution was done as per the protocol mentioned in ImmPRESS Excel staining kit (Vector Labs, Burlingame, United States). The expression of MMP-9 was analyzed by using DAB reagent and counter staining with Mayer's haematoxylin.

Statistical analysis

The results of this study are presented by Mean ± Standard Error and the statistical analysis was done by using one-way variance analysis (ANOVA) by using SPSS software at $P \leq 0.05$.

RESULTS AND DISCUSSION

Effect of SC extract on Rat Leydig cell testicular tumour LC-540 cells

The screening of *Saussurea costus* (roots) for the % anti-proliferative activity on LC-540 cell line revealed highest activity at 200 µg/ml concentration after 72h of incubation (87.01±4.21). The anti-neoplastic activity of SC extract was found to be increased in a dose and time-dependent manner. The LC-540 cell line treated with DMSO did not exhibit marked changes in the morphology at three different time points. Vinblastine used as a standard drug to evaluate the anti-proliferative activity on LC-540 cell line showed drastic morphological changes. The 70% aqua ethanolic extract of *Saussurea costus* (roots) elicited the prominent morphological changes including irregular nuclei, rounding of cells, fragmentation of cells and membrane blebbing on LC-540 cell line at 200 µg/ml concentration (Fig 1 and Fig 2).

Radiology

A qualitative radiological examination was done to assess the metastases of mammary tumours to lungs. The rats kept in group DX showed multifocal, 1-2 sized round foci on the lung parenchyma as compared with the groups treated with SC root extract. The rats in group SC3 revealed the presence of few foci on the lung surface, whereas animals in group SC1 were observed to show numerous whitish foci involving maximum surface of lung parenchyma corresponding to the lesions in the DMBA-only treated group (Fig 3).

Effect of SC extract on gross lesions in DMBA-induced pulmonary metastatic lesions

On gross examination of lungs number of metastatic foci (Fig 4A) and % area (Fig 4B) involved by metastatic foci were found to be significantly ($P \leq 0.05$) more in group II treated with DMBA only as compared with the group treated

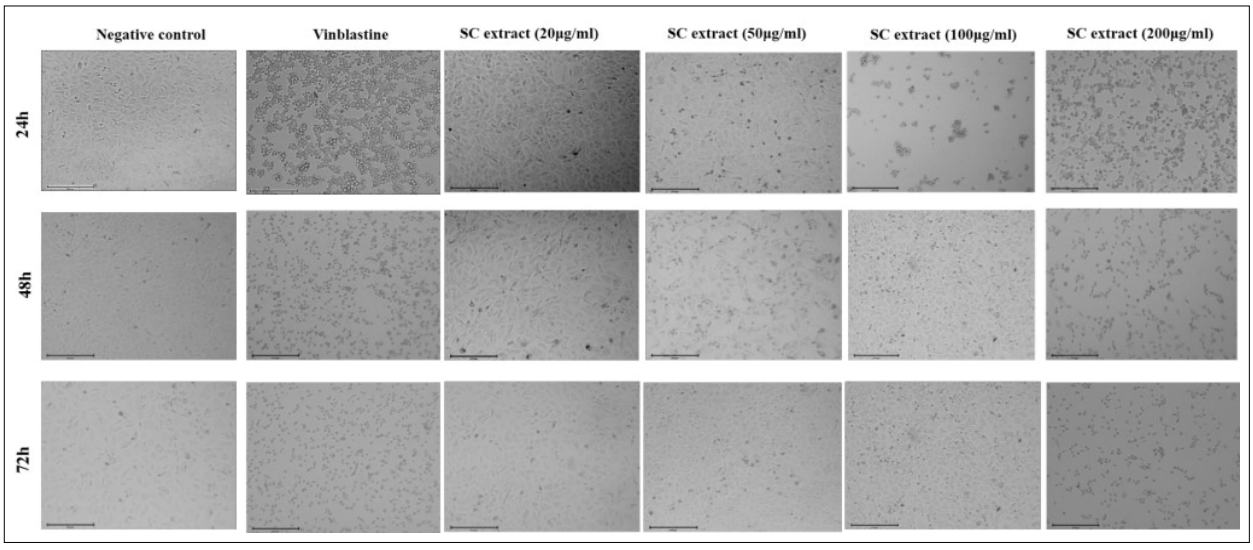


Fig 1: Phase contrast micrographs of LC 540 cells treated with *Saussurea costus* root extract.

with the highest dose of the extract *i.e.* 500 mg/Kg bw. The size of the metastatic foci in treatment groups was reduced in a dose-dependent manner as compared with group II. However, no level of significance was achieved (Fig 5A and Table 1).

Effect of SC extract on histopathological findings in DMBA-induced pulmonary metastatic lesions

A total of 7 rats in group I showed lung tumours, with 5 showing papillary carcinomas and 2 showing solid carcinomas on the lungs. Six rats in groups III and IV each had lung tumours, with 3 papillary carcinomas and 3 solid carcinomas. Five of the 8 rats in group V were found to have lung tumours with 2 having papillary carcinomas and 3 having solid carcinomas. In this study, the incidences of papillary carcinomas and solid carcinomas were 54.2% and 45.8%, respectively. The incidence of lung papillary carcinomas was decreased in the groups treated with SC extract in a dose-dependent manner as compared with group II treated with DMBA only. In papillary carcinomas involving epithelial cells in the bronchioles and alveoli displayed atypical proliferative changes, as well as marked pleomorphism, hyperchromasia and increased mucin secretions as a result of increased goblet cell activity. These proliferative changes were maximal in the group treated with DMBA only in comparison to the treatment groups provided with SC extract. Sheets of neoplastic cells with altered cellular morphology, hyperchromatic nuclei, prominent nucleoli and mitotic activity were more prominent in solid lung carcinomas (Fig 5B).

Effect of SC extract on MMP-9 expression in DMBA-induced pulmonary metastatic lesions

Immunohistochemical expression of MMP-9 was reduced in the groups treated with SC extract in a dose-dependent manner as compared with group II, however the significant ($P \leq 0.05$) decline was only seen in the group treated with the highest dose of the extract *i.e.* 500 mg/Kg bw. MMP-9 expression was reduced in the groups III and IV as compared with only DMBA-treated group, but no level of significance ($P \leq 0.05$) was achieved (Fig 5C).

Breast cancer is the most common cancer diagnosed in women and the leading cause of death (Ferlay *et al*., 2019). Metastatic breast cancer is the primary cause of

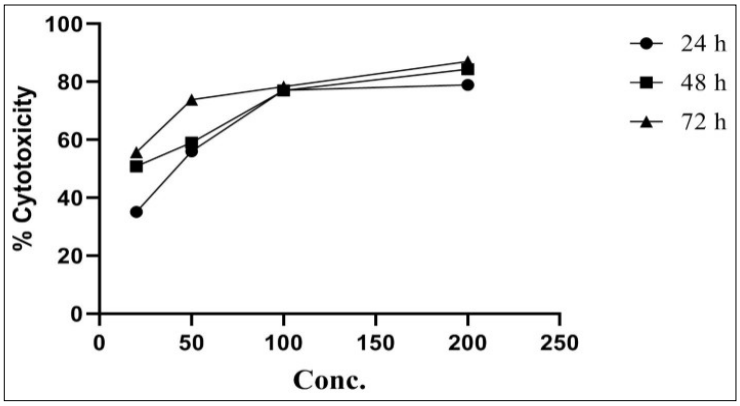


Fig 2: Graphical presentation depicting % cytotoxicity of *Saussurea costus* root extract on LC 540 cells after 24, 48 and 72 h of treatment.

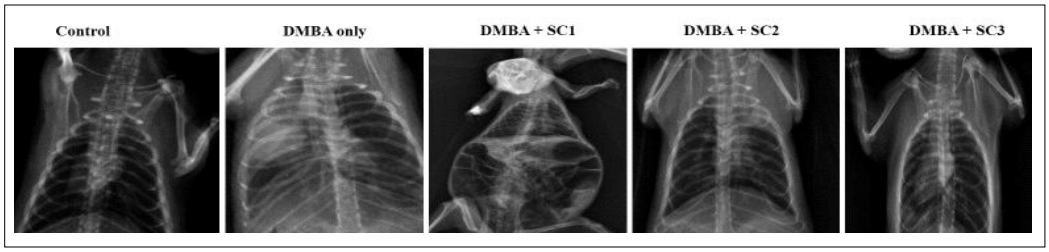


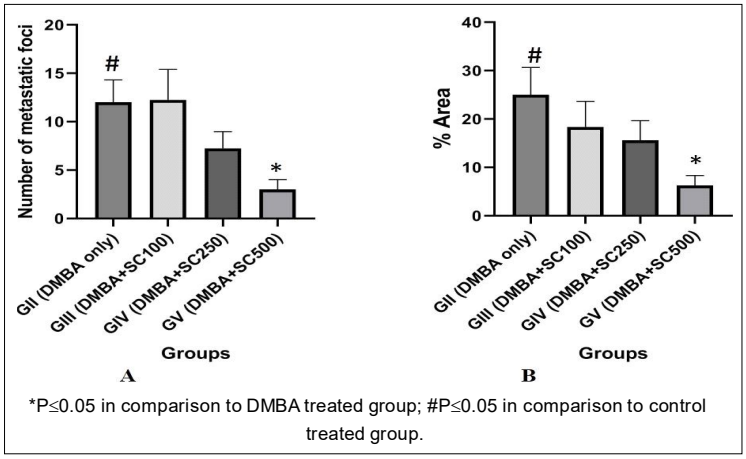
Fig 3: The pictures demonstrating the effect of SC extract on the presence of neoplastic foci on lungs in various treatment groups in comparison to DMBA only treated group.

Table 1: Incidence of lung papillary carcinomas and mixed solid carcinomas in different treatment groups.

Type of tumour	GI (Control)	GII (DMBA only)	GIII (DMBA+ SC100)	GIV (DMBA+ SC250)	GV (DMBA+ SC500)	Incidence (%)
Lung papillary carcinoma (13)	0	5	3	3	2	54.2
Mixed solid carcinoma (11)	0	2	3	3	3	45.8
Total =24	0	7	6	6	5	100

breast cancer-related deaths, globally. The anti-neoplastic medications or therapies that are used to treat cancers do increase life expectancy but the quality of life is drastically decreased by their severe adverse side effects. For more than 2500 years, indigenous people have used *Saussurea costus* in traditional and ethnopharmacological therapies for ailments such as gastroenteritis, ulcers, piles, throat infections, allergies, liver problems, typhoid, arthritis and impaired states of vata, pitta and kapla or trichosha (Nadda *et al*., 2020). The active constituents present in *Saussurea costus* are already documented to exhibit anti-cancerous potential against leukemia, breast cancer, prostate cancer, hepatocellular carcinomas, colon cancer, ovarian cancer *etc*. (Rathore *et al*., 2021).

In our study, *Saussurea costus* root extract exhibited maximum anti-proliferative activity at 200 µg/ml concentration after 72 h of incubation. The anti-neoplastic activity revealed by SC extract was in a time and dose-dependent manner. In a previous study, conducted by Okubo *et al*. (Okubo *et al*., 2021) have documented the reduced HepG2 cells viability treated with methanolic extract of *S. costus* roots. In AGS (a human gastric adenocarcinoma cell-line), *S. costus* was reported to upregulate the expression of p53 and p21 (Ko *et al*., 2004). Dehydrocostus lactone, a bioactive molecule of *S. costus* root extract has also been shown to promote endoplasmic reticulum stress-mediated apoptosis and inhibit the PI3K/Akt/Bad pathway a dose-dependent manner in Hep-2 and TU212



*P≤0.05 in comparison to DMBA treated group; #P≤0.05 in comparison to control treated group.

Fig 4: A: Graphical presentations showing the effect of SC extract on presence of number of neoplastic foci on lungs in various treatment groups; B: Graphical presentations showing the effect of SC extract on the % area on lungs involved by neoplastic foci in various treatment groups.

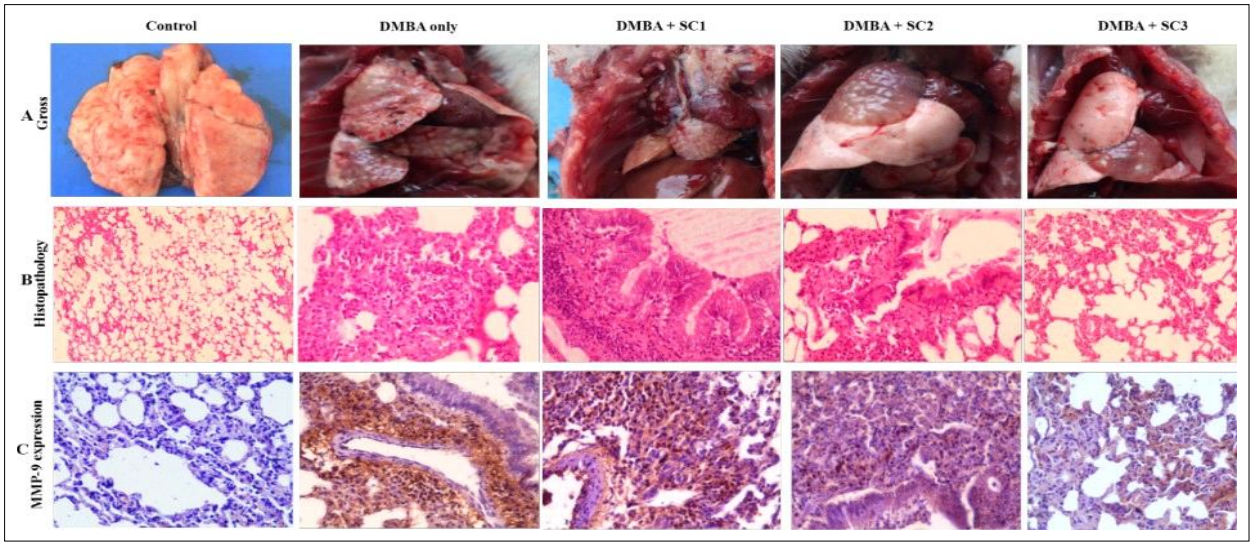


Fig 5: A: Effect of SC extract on neoplastic foci on lungs in various treatment groups; B: Effect of SC extract on various groups treated with SC extract in comparison to DMBA only treated group; C: Effect of SC extract on immunological expression of MMP-9 protein on various groups treated with SC extract in comparison to DMBA only treated group.

cells, but little cytotoxicity was seen in human normal larynx epithelial HBE cells (Zhang *et al*., 2020).According to Ahmed *et al*. (2022), *S. costus* oil had antineoplastic effects on MCF-7 (Breast carcinoma cells), HCT (colon carcinoma cells) and HepG-2 (liver carcinoma cells) cells. *S. costus* extract triggered apoptosis in man hepatocellular carcinoma (HepG2), colorectal adenocarcinoma (HCT116) and breast adenocarcinoma (MCF-7) cells by increasing the expression of Bax and by diminishing Bcl-2 and caspase-3 expressions (Shati *et al*., 2020).

X-ray examination is one of the first line of investigation in the lung cancers and metastasis (Bradley *et al*., 2019). Thoracic radiographs are comparatively cheap, accessible and have low radiation dose (Hamilton, 2010). In the present study, the metastatic foci were more in number and size as compared with SC treatment groups in a dose-dependent manner. X-ray findings in our study were in concordance with the observations of Fouriez Lablée *et al*. (2017), which were also validated on post-mortem examination. One of the investigation concluded by Currier *et al*. (2005), reported that DMBA can produce metastatic lesions in tissues other than mammary gland, including lungs, skin and lymphoid tissues. In the present experimental trial, gross examination of lungs in groups treated with DMBA alone exhibited substantially more metastatic foci in terms of both number and percent area affected as compared with groups treated with SC extract. Interestingly, the incidence of lung tumours was reduced in the groups treated with SC extract and a significant difference was observed in the group treated with the highest dose of the extract. Mice with DMBA induced tumor has shown high incidence of lung metastases in rat and mice models (Arroyo-Acevedo *et al*., 2015; Plante *et al*., 2011). In our study, the incidence of lung tumours was reduced in the groups treated with SC extract and a significant difference was observed in the group treated with the highest dose of the extract *i.e*. 500 mg/Kg and the group treated with DMBA only. On parallel lines, one of the studies has demonstrated the 100% incidence of pulmonary metastases in DMBA-induced mammary tumors in Hotlzman rats, which were significantly reduced in the group treated with ethanolic extract of *Piper aduncum* (Arroyo-Acevedo *et al*., 2015). In the present experimental study, lung papillary carcinomas occurrence was observed to be lower in the groups treated with SC extract, where the more pronounced solid carcinomas were present. Lung papillary carcinomas were more pronounced in the group II exclusively receiving DMBA treatment. Another study in DMBA-induced mammary tumors in BALB/c mice through oral route has documented 14.58% and 8.33% incidences of papillary lung tumor and mixed solid carcinomas, respectively (Duro De Oliveira *et al*., 2015). As per one of the reports by Wang *et al*. (2014) have shown the inhibition of pulmonary metastases in spontaneous and experimental study in Balb/c mice by *Panax notoginseng* Saponins against 4T1 breast cancer cells.

Matrix metalloproteinases (MMPs) are the enzymes that breakdown collagen IV and V in extracellular matrix (ECM), which plays a crucial role in growth and metastases of tumours (Stamenkovic, 2000). In the present study, a dosage-dependent reduction in the immunological expression of MMP-9 was observed in the groups treated with SC extract as compared to group II, although the significant fall was observed in group V given the highest dose of the extract. According to Doyle and Miller (2008) and Manshadi *et al*. (2018), increased immuno-expression of MMP-9 in breast cancer in humans is linked to advanced cancer stage, increased degree of histological undifferentiation, increased risk of metastases and death. Based on a study by Aresu *et al*. (2011), MMP-9 expression is reported to be higher in females with more advanced and metastatic breast cancers and is in consonance with this study.

CONCLUSION

The present study has demonstrated the inhibition of pulmonary metastases from DMBA-induced breast cancers in female SD rats supplemented with SC extract in a dose-dependent manner. *In vitro* studies, X-ray examination, gross and histopathological studies and immuno histochemical findings of MMP-9 expression all together support the protective effect of SC root extract by inhibiting the pulmonary metastases from mammary tumours in rats. Therefore, the present experimental study warrants the need to use bioactive constituents of *S. costus* root extract as a therapeutic regimen against breast cancer-related metastases to lungs and other organs.

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Ethics approval

The experimental work was approved by the Institutional Animal Ethics Committee (IAEC) with IAEC No. QSD.IAEC/Vet Path/CSK HPKV/2019/246-50.

Declaration of competing interest

The authors declare no competing interest.

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