Antimetastatic, superoxide anion and nitric oxide reduction potential of *Solanum xanthocarpum* on human lung cancer cell line A549

Mital Bhatt* & Mandadi Narsimha Reddy

Department of Biosciences, Veer Narmad South Gujarat University, Surat-395 007, Gujarat, India

E-mail: mital.bhatt14@gmail.com

Received 01 September 2017, revised 19 March 2018

*Solanum xanthocarpum* Schrad. & H. Wendl is a prickly herb, extensively known for its medicinal properties across the tribal and rural parts of India. Its efficacy in lung disorders and related respiratory diseases is well established in *Ayurveda*. In present time, cancer is considered as a significant challenge and metastasis is the most devastating hallmark in cancer progression. The present study evaluates the effects of whole plant and root of *S. xanthocarpum* on highly metastatic human lung adenocarcinoma cell line A549. Extracts were prepared using ethyl acetate and chloroform in various dilutions. Cytotoxicity of the plant and root extracts was analyzed. Wound healing assay was employed to check the effects on migration of highly metastatic A549 cells. Moreover, plants effects on cellular superoxide anion \( \text{O}^\cdot \) and nitric oxide (NO) quantities were evaluated to check effects on the redox regulation of the cell. All the extracts of *Solanum xanthocarpum* are not cytotoxic. However, all the extracts efficiently inhibit migration of A549 cells. The whole plant in ethyl acetate treatment shows a three-fold decrease in wound healing capacity of the cells. Ethyl acetate extracts of the plant show potential inhibition of \( \text{NO}^\cdot \) and all the extracts show decreased levels of \( \text{O}^\cdot \) at higher concentrations.

**Keywords:** *S. xanthocarpum*, Lung cancer, Antimetastatic, A549, Wound healing

**IPC Int. Cl.**: A61K 36/00, A01D 4/04, A01D 20/47, A01D 20/48, A61K 39/395

India has an enormous biodiversity and rich knowledge of traditional systems of therapy such as *Ayurveda*, Siddha, Unani, Amchi and folk medicines. *Solanum xanthocarpum* Schrad. & H. Wendl., a synonym of *Solanum virginianum* L., is the plant found throughout India, a member of *Dashamula* and is of significant use in *Ayurvedic* formulations. Different names of *Solanum xanthocarpum* are known in various languages in India, *Kantakari* (Sanskrit), *Kateri* and *Bhatkataiya* (Hindi), *Bhornignd* (Gujarati), *Kantankattiri* (Tamil), *Kantkaricunta* (Malayalam), *Vakudu* (Telugu), *Nelagulle* (Kannada)\(^1\). *Kantakari* is used as an ingredient in many of the compound formulations including *Vyaghriharitaki avaleha*, *Chavanaprasha*, *Dasamoolarishta*, *Vyaghri tailam*, *Vyaghi ghrtam*, *Vyaghiyadi kwatha*, etc.\(^1\). Whole plant, roots and fruits are used to treat various ailments such as vitiated conditions of *Vata* and *Kapha*, helminthiasis, dental caries, inflammations, flatulence, constipation, dyspepsia, anorexia, leprosy, skin diseases, hypertension, fever, cough, bronchitis, hiccough, lumbago, haemorrhoids and epilepsy. The plant is bitter, acrid, thermogenic, anthelmintic, anti-inflammatory, digestive, carminative, appetizer, stomachic, febrifuge, expectorant, laxative, stimulant, diuretic, rejuvenating, emmenagogue and aphrodisiac\(^1\), and helpful in bronchial asthma\(^1\,2\), tympanitis, peristalsis, piles and dysuria\(^2\). Many of the reported secondary metabolites of the plant such as lupeol, apigenin, solamargine have shown apoptosis inducing activity in cancer cells. Moreover the phytochemicals – stigmasterol, carpsterol, diosgenin and lupeol found to show immunomodulatory and anti-inflammatory effects\(^2,3\). In our previous *in silico* study, lupeol interaction with proteins involved in MAPK pathway was reported\(^4\). The plant is abundant in phytoconstituents such as alkaloids, phenolics, flavonoids, sterols, saponins and their glycosides, carbohydrates, fatty acids, tannins and amino acids\(^5\). It also shows antioxidant activity\(^6\). According to tribal practices in the area of South Gujarat, *S. xanthocarpum* is an anticancer and antimetastatic herb.

Adenocarcinoma, a type of cancer occurring in non-smoker patients, is one of the most prevalent types of lung cancer, and its types are found to be challenging because of drug resistance and metastasis. An increase in Non-small cell lung carcinoma (NSCLC) proportion is observed in never smokers.
Nitrogen species synthesized by many cells, such as concentrations of nitric oxide (NO), a reactive endothelial, immune, and tumor cells, are found to be dramatically increased in lung cancer environments. Thus plant’s ability to inhibit migration of A549 cancer cells and effects on redox status will be a right approach in cancer treatment. In present study cell viability and redox status is analyzed in A549 lung carcinoma cell line. Moreover, antimetastatic potential of the plant on lung cancer cells is the most critical aspect to be accessed. Though metastases occur due to several interrelated, intracellular and intercellular events, the ability of cancer cells to migrate gives crucial insights of the malignancy. Solanum xanthocarpum is an efficient plant for respiratory and lung-related ailments. Thus plant’s ability to inhibit migration capacity of A549 cancer cells and effects on redox status will be a right approach in cancer treatment.

**Methodology**

**Plant extract preparation**

*S. xanthocarpum* whole plant and root was collected from VNSGU campus on an afternoon of March, which is also the flowering and fruiting season of the plant. The plant specimen was identified by Prof. Minoo Parabia and submitted to departmental herbarium at Shri Bapalal Vaidhya Botanical Research Centre & Aspee Dhanvantray Udyan (BVBRC1204). The collected samples were dried at room temperature. Dry whole plant and root materials were used for extraction. *S. xanthocarpum* plant materials were homogenized and macerated in ethyl acetate and chloroform. Extracts of the whole plant and roots abbreviated as SEW, SER respectively and chloroform extracts of whole plant and roots abbreviated as SCW, SCR respectively. After that, mixtures were filtered and residues were suspended in same amount of solvent for next 24 h. Obtained residues were allowed to dry in rotary evaporator under vacuum at 40 °C. The per cent yield of all the extracts was calculated. Extracts were then dissolved in 1 % ethanol at 1 mg/mL stock solution and stored at 4 °C for further experiments.

**Cell culture**

Human lung carcinoma A549 cells were obtained from NCCS, Pune and propagated in a humidified atmosphere with 5 % CO₂ at 37 °C and maintained in Dulbecco’s Modified Eagle's Medium (DMEM) supplemented with 10 % Fetal Bovine Serum (FBS). Cells were maintained in 25 cm² flask and grown up to 70-80 % confluence for all the further assays.

**Cell viability evaluation**

MTT assay was performed to check the cytotoxicity of the plant. 1 × 10⁵ cells/mL were seeded in a 96 well plate [200µL media with cells] and incubated for 24 h at 37 °C in 5 % CO₂. 5µL extracts of SEW, SER, SCW, SCR in different dilutions [50 µg/mL, 100 µg/mL, 200 µg/mL, 300 µg/mL, 500 µg/mL] were added to appropriate wells except in blanks. Fluorouracil (5-FU), a drug used to treat cancer was used (50 µg/mL and 100 µg/mL) for anticancer reference results. Control wells did not receive any additional treatment. The plate was incubated (37 °C, 5 % CO₂) for 36 h followed by addition of 20 µL MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) 5 mg/mL in Dulbecco’s phosphate-buffered saline (PBS) to each well and incubated. After 3 h complete content of the plate was decanted and formazan was suspended with 200µL Dimethyl sulfoxide (DMSO) in each well. Optical density readings were taken at 490 nm in epoch microplate spectrophotometer immediately after adding DMSO. Viability was expressed in % of control.

**Quantification of nitrite production**

The amount of nitrites was determined by performing Griess assay. The Griess coloured reaction represents the spectrophotometric determination of nitrites –NO₂ (an indicator of the nitric oxide-NO level). Cells were seeded in 96 well plates and incubated till 80 % confluence. After treatment with plant extracts cells were again incubated for 24 h.

Across Asia. Approximately 80-85 % of the lung cancer patients diagnosed with an advanced stage of NSCLC that has limited therapeutic options due to metastasis. Lethality of lung cancer often attributed to late diagnosis and metastasis. Lung cancers frequently metastasize to bone, brain and liver causing shorter survival. Metastasis is the primary cause of deaths occurring due to cancer. Metastases represent the end products of a multistep cellular-biological process. Superoxide anion and nitrite production is one of the important aspects of carcinogenesis. The malignant phenotype of cancer cells can be reversed just by reducing the cellular levels of O₂⁻. Overexpression of the O₂⁻ detoxifying enzymes superoxide dismutases can reduce tumor cell growth, metastasis and other malignant features of cancer cells from different origin. Similarly, the concentrations of nitric oxide (NO), a reactive nitrogen species synthesized by many cells, such as endothelial, immune, and tumor cells, are found to be dramatically increased in lung cancer environments. Impact of plant extracts on cancer cells superoxide and nitrite production is one of the important aspects to understand. In present study cell viability and redox status is analyzed in A549 lung carcinoma cell line. Moreover, antimetastatic potential of the plant on lung cancer cells is the most critical aspect to be accessed. Though metastases occur due to several interrelated, intracellular and intercellular events, the ability of cancer cells to migrate gives crucial insights of the malignancy. Solanum xanthocarpum is an efficient plant for respiratory and lung-related ailments. Thus plant’s ability to inhibit migration capacity of A549 cancer cells and effects on redox status will be a right approach in cancer treatment.
Then Griess reaction mixture, 50 µL 0.1 % N-(1-naphthyl) ethylenediamine and 50µL of 1 % sulphanilamide (solution in 5 % phosphoric acid), was added and absorbance was measured in epoch microplate spectrophotometer following incubation of 10 min. The results were obtained in µM from a standard curve established in each test.

**Determination of superoxide anion radicals**

The reduction rate of nitroblue tetrazolium (NBT) was estimated to check the presence of $O_2^-$. The A549 cells were seeded in appropriate wells in 96 well plate with 200 µL media and incubated for 24 h. Later, cells were treated with different dilutions of extracts of whole plant and roots. After incubation of 24 h, the assay was performed by adding 10µL NBT (5mg/mL in PBS) to each well. One hour later the formazan products were quantified by adding 100µL of DMSO and colour reaction was measured at 550 nm in epoch microplate Spectrophotometer.

**Wound healing scratch assay**

In *vitro* wound healing scratch assay is one of the convenient methods for primary analysis of the capability of cells to migrate. $1 \times 10^5$ cells were seeded in six well plates and allowed to grow up to 70 % confluence in serumless conditions. Later a uniform scratch was made using 100 µL tip in each well, and arbitrary places were marked at the bottom, cells washed with PBS. 10 µM 5-FU was used as a standard for inhibition of wound healing reference. Media with relevant extracts were added without FBS. After incubation of 24 h, media was replaced again with fresh DMEM with 10 % FBS and analyzed on the next day by fixing with 3.7 % Formaldehide and staining with crystal violate. The measurement and analyses of images were done using Image J ver. 1.46r (NIH, USA). Wound closure was expressed as the average of ± SEM of the difference the measurements at time 0 h and 24 h period.

**Statistical analysis**

Results expressed as MEAN ± SD of the number of experiments performed. A student’s t-test for paired or unpaired values was performed and a p-value of < 0.05 was considered statistically significant.

**Results**

**The total yield of crude extract**

The percentage yield of crude extracts from *S. xanthocarpum* whole plant and roots by using ethyl acetate was 1.41 & 0.23 and by using chloroform 1.25 & 0.44, respectively. Whole plant extracts appeared dark green while roots extracts were yellowish green.

**A549 cell viability**

A549 cell viability evaluation by MTT assay indicated that different dilutions of SCR SCW and SER did not show observable toxicity. However, 500 µg/mL concentrations of SEW show a decrease in cell viability as shown in Fig. 1. According to US National Cancer Institute, a crude extract is considered to possess *in vitro* cytotoxic activity if its IC50 value is lesser than 20 µg/mL. Therefore none of the extracts can be considered as cytotoxic *in vitro*.

**NO$_2^-$ production in A549 cells**

Griess assay estimated Nitrite (NO$_2^-$) which is found to promote tumor growth, metastasis and angiogenesis in some cancer cells. Table 1 indicates that different extracts of the plant show decrease in NO$_2^-$. Chloroform extracts do not show significant effects on NO$_2^-$. Only SCR 500 µg/mL treatment shows a significant decrease in NO$_2^-$ whereas SCW treatment with all concentrations does not show a significant change in NO$_2^-$. Ethyl acetate extracts of the plant show significant decrease in NO production the cells.

**Superoxide anion determination**

Among all reactive oxygen species, superoxide anion radical is one of the most important radical involved in many processes in human cells, because of its content and high reactivity. NBT assay results show the effects of plant extracts on superoxide anion.
content in A549. SCR, SER and SEW are efficiently decreasing $O_2^-$ at 200, 300 and 500 µg/mL concentrations as shown in Fig. 2.

Wound healing capability analysis

Initial wound size is shown in Fig. 3(A). Control – untreated A549 cells show almost complete wound healing after 24 h, as shown in Fig. 3(B). Cells treated with 5-FU shows inhibition of migration. Fig. 3(C). The cells treated with plant extracts show significant inhibition on wound closure migration of cells after 24 h, Fig. 3(D). Here the result images shown are for cells treated with 300 µg/ml dilutions extracts. Fig. 4 shows the quantitative reduction in the area covered with cells after 24 h. Significant inhibitory effects of the plant extracts are observed on A549 cell migration.

Discussion

Viability of A549 cells after treating it with SCR, SCW, and SER at 500 µg/mL concentration is not affected significantly. It has been reported that this plant is non cytotoxic and has IC50 value at 625 µg/mL on other cell lines. On A549 cells, SEW at 500 µg/mL, shows observable cytotoxicity, but all of the treated cells are found to be viable at 100, 200, 300 µg/mL dilution. At these same non cytotoxic concentrations, NO$^-$, which indicates level of NO, $O_2^-$ and cell migration is significantly decreased.

Metastasis is the cause of higher rates of deaths due to cancer. Migration of cancer cells from the primary site to other is one of the crucial stages of metastases. Wound healing scratch assay gives the preliminary accuracy about the migratory ability of the cells. A549 is a highly metastatic cell line and all the extracts of S. xanthocarpum used in present study show decrease in migration of cells. Additionally, redox reactions have an essential role in cell survival, apoptosis and regulation of various signaling pathways. Changes in ROS/RNS production could modify signal pathways in the cell by direct modification of bio molecules and proteins.

<table>
<thead>
<tr>
<th>Concentrations (µg/mL)</th>
<th>SCR Nitrites (µM)</th>
<th>SCW Nitrites (µM)</th>
<th>SER Nitrites (µM)</th>
<th>SEW Nitrites (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (500 µg/mL)</td>
<td>55±0.101</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5FU treated (100 µg/mL)</td>
<td>38.19±0.015</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>50</td>
<td>49.51±0.014</td>
<td>52.15±0.008</td>
<td>47.77±0.014</td>
<td>45.48±0.005*</td>
</tr>
<tr>
<td>100</td>
<td>49.027±0.010</td>
<td>50.62±0.012</td>
<td>47.353±0.009*</td>
<td>44.23±0.001***</td>
</tr>
<tr>
<td>200</td>
<td>49.30±0.018</td>
<td>50.17±0.012</td>
<td>46.80±0.009*</td>
<td>43.54±0.005***</td>
</tr>
<tr>
<td>300</td>
<td>48.68±0.008</td>
<td>49.58±0.014</td>
<td>44.65±0.008*</td>
<td>43.36±0.008***</td>
</tr>
<tr>
<td>500</td>
<td>47.63±0.02*</td>
<td>48.40±0.02</td>
<td>43.54±0.02**</td>
<td>43.47±0.007**</td>
</tr>
</tbody>
</table>

Data represents MEAN±SD of independent set of experiment carried out in triplicate. (*p<0.05, **p<0.005)
indicates oxidative stress. Here results show that given the treatment, superoxide anion reduced significantly, which suggests modification in pathways of A549 cells. NO\textsuperscript{2−} has been reported to exert dichotomous effects within the multistage model of cancer. Reduced NO is suggested to be correlated in antimetastatic effects in MDA-MB-231 breast cancer cells\textsuperscript{16}. It modulates different cancer-related events including angiogenesis, apoptosis, cell cycle, invasion, and metastasis. However, NO\textsuperscript{2−} has found to show dual effects. In contrast to tumor promoting effects, NO\textsuperscript{2−} has also been reported to have tumoricidal effects\textsuperscript{19}. In this study, NO\textsuperscript{2−} is found to be significantly decreasing in ethyl acetate extracts of \textit{S. xanthocarpum} treated groups of cells.

This is the primary study to express antimetastatic potential of \textit{S. xanthocarpum} extracts. Wherein further studies can be performed to find the accurate mechanism responsible for modification in cell migration ability, NO\textsuperscript{2−} and O\textsuperscript{2−} level regulation in \textit{S. xanthocarpum} treated cells. Moreover SER and SEW shows better results in almost all experiments on A549 in comparison to SCR and SCW. Ethyl acetate and chloroform extracts are reported to have different phytochemical contents in the plant\textsuperscript{4,20}. Reports suggest that chloroform extract of the plant contains tannins, phenolic compounds, sterols, terpenoids and flavonoids whereas ethyl acetate extract is rich in alkaloids\textsuperscript{4}. It is worth noting that biological and botanical - semi-synthetic or synthetic derivatives of plants are proven to be effective therapeutics but have inherent limitations if focused only on few targets in polygenic diseases like cancer\textsuperscript{21,22}. Thus secondary metabolites, a combination of phytochemicals or compounds in their pure state can be analyzed against various candidate genes and pathways involved in cancer cells to provide better directions for finding putative therapeutic molecules.

**Conclusion**

\textit{S. xanthocarpum} is a non cytotoxic plant. Even though its anticancer efficacy is not very well established, the inhibitory action of \textit{S. xanthocarpum} on lung cancer cell line A549 migration is promising and needs further exploration. The plant also shows regulation of superoxide anion and nitric oxide, which suggest the metabolic impact of the plant on the cells. Mechanism through which \textit{S. xanthocarpum}, a \textit{Dashamula} member, regulates cellular motility and redox metabolism should be identified.

**Acknowledgment**

We thank Department of Biosciences, VNSGU and Minoo Parabia Endowment Fund for providing all needed help in the work.

**References**

8. Lee Sau Har, Indu Bala Jaganath, Rishya Manikam & Shamala Devi Sekaran, Inhibition of Raf-MEK-ERK and Hypoxia pathways by \textit{Phyllanthus} prevents metastasis in...