ORIGINAL RESEARCH ARTICLE

Inhibitory effect of *Solanum xanthocarpum* on the growth of KB human oral cancer cell line *in vitro* through ROS-induced mitochondrial pathway

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Abstract

The aim of the present study was to investigate the effect of *Solanum xanthocarpum* on KB human oral cancer cells by analyzing its anti-proliferative and apoptotic properties as well as its inhibitory effect on cell adhesion. In this study, the leaves extract of *S. xanthocarpum* was prepared using the maceration method. Cytotoxic effect of different doses of the *S. xanthocarpum* extract was assessed using MTT assay. Measurements of reactive oxygen species (ROS), lipid peroxidation and antioxidant enzymes were also performed. In addition, we also studied the impacts of *S. xanthocarpum* on the apoptosis and mitochondrial membrane potential of KB cells. Determination of antioxidant enzymes and lipid peroxidation was performed using biochemical methods. The *S. xanthocarpum* showed cytotoxic activity against KB cells with IC50 (200 µg/mL). Besides, DCFH-DA staining and acridine orange/ethidium bromide staining results demonstrated that *S. xanthocarpum* induced the generation of ROS and apoptosis in KB cells, respectively. Based on the Rh-123 staining results, *S. xanthocarpum* decreased mitochondrial depolarization in KB cells. Furthermore, the *S. xanthocarpum* treatment contributed to increased lipid peroxidation, accompanied by reduced activities of superoxide dismutase and catalase, as well as decreased glutathione content. Taken together, these findings indicate that *S. xanthocarpum* extract might comprise bioactive compounds of therapeutic significance, which can inhibit the growth of KB cells.

Keywords: Oral cancer; *Solanum xanthocarpum*; MTT; Reactive oxygen species; Mitochondrial membrane potential; Apoptosis

1. Introduction

Oral squamous cellular carcinoma (OSCC) accounts for approximately 90% of all neoplasms of the oral cavity[3]. OSCC is the fifth most common leading cause of cancer globally and emerged as the leading cause of mortality in India[4]. Overexposure of normal cells to carcinogenic metabolites from cigarette smoke and other environmental factors leads to cancerization. Oral premalignant lesions, that is, leukoplakia and erythroplakia,
are most frequent oral mucosal cancer type and the rate of malignant transformation is high. Two-third of all oral cavity cancer cases are diagnosed only when they are in advanced stage\(^3\). At present, the principal remedies for most cancers are chemotherapy, radiotherapy, and surgery. Chemotherapy is used in most cancer treatment. However, chemotherapeutic remedies are not devoid of their very own intrinsic issues\(^4\).

Herbal medicine derived from medicinal vegetation has been used as remedy for many cancers\(^5\). Plant-derived medicine have a vital supply of clinically useful anticancer compounds. India is known to for its practical knowledge of natural medication\(^6\). Despite more than 1500 anticancer medications in the pipeline with over 500 under investigations in clinical trials, there is a pressing need to develop effective powerful and less toxic medications. Studies recommend that herbs have anticancer properties against numerous cancer cell strains, such as KB human oral cancer cells\(^7\).

*Solanum xanthocarpum* (solanaceae) has a plentiful supply of steroidal glycoalkaloids. Its flowers, in particular, contain steroidal alkaloids. Solanine, isolated from *Solanum nigrum*, can induce apoptosis in HepG2 cells, with the aid of suppresing the expression of Bcl-2 protein\(^8\). Some other steroidal alkaloids have been claimed to have the potential to prevent cancer\(^9\). Solamargine and solasodine are cytotoxic to Hep3b cells at 10 mm\(^10\). Nevertheless, solasodine and diosgenin, which do not comprise carbohydrate moieties, are weakly cytotoxic. The cytotoxicity of these compounds is related to the induction of apoptosis. As compared to the cisplatin, the apoptotic effect of diosgenin on HCT-116 cells is weak\(^11\).

Therefore, in the present study, we aimed to investigate the anticancer activity of *S. xanthocarpum* extract against KB human oral cancer cells. We found that *S. xanthocarpum* extract was able to suppress the growth of KB cells and regulating several parameters, such as reactive oxygen species (ROS) and lipid peroxidation, as well as control mitochondrial depolarization.

### 2. Materials and methods

#### 2.1. Chemicals

Fetal bovine serum (FBS) was purchased from Sigma (USA). Dulbecco's modified eagle's medium (DMEM), streptomycin, penicillin-G, phosphate-buffered saline (PBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 2′,7′-dichloro fluorescein (DCFH-DA), rhodamine-123 (Rh-123), trypsin/EDTA, acridine orange (AO), ethidium bromide (EB), and dimethyl sulfoxide (DMSO) were purchased from HiMedia (USA).

#### 2.2. Preparation of *S. xanthocarpum* extract

The leaves of *S. xanthocarpum* (Figure 1) were accrued from the habitats at Thiruvarur, Tamil Nadu, India. The leaves were washed under running tap water and dried at room temperature (27 ± 2°C). An electrical blender was used to powderize the dried leaves of *S. xanthocarpum*; 300 g of the powder was used for extraction in 1 L of ethanol using the Soxhlet extractor under a boiling temperature of 68°C for 8 h. The extracts were filtered using a Büchner funnel and Whatman filter paper. The crude plant extracts were then evaporated to dryness in a rotary vacuum evaporator.

#### 2.3. Cell culture

The KB cell line was obtained from National Center For Cell Science (NCCS; Pune, India). The cells were cultured in DMEM supplemented with FBS (10%), penicillin-G (100 U/ml), and streptomycin (100 µg/ml). The cells were maintained in CO\(_2\) (5%) incubator at 37°C and the subsequent experiments were carried out after proliferation stage was attained. The DMEM medium was replaced every 2 days and the maintenance was strictly followed in accordance with the controlled conditions.

#### 2.4. Treatment of the KB cells

KB cells were grown in a 75-mm tissue subculture dish containing 10 ml of DMEM at 37°C. Following trypsinization, the cells were serially passaged using trypsin/EDTA. After attaining 80% confluence achievement, the cells were starved for 24 h in DMEM medium prior to *S. xanthocarpum* treatment.

#### 2.5. Cytotoxicity assay

The effect of *S. xanthocarpum* on cell proliferation of KB cells was measured using the protocol of Mosmann, 1983\(^12\). KB cells were seeded in the 96-well culture plates. After

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**Figure 1. Solanum xanthocarpum leaves.**
administering S. xanthocarpum extract in different doses (50 – 350 µg/ml), the cells were then incubated overnight in CO₂ incubator. Afterward, MTT dye was added to each well in a series of doses (50, 100, 150, 200, 300, and 350 µg/ml) and the cells were incubated for an additional 4 h at 37°C. After the brief incubation, the DMEM medium in each well was discarded and DMSO was added to dissolve the formazan. The absorbance was measured at 490 nm in a microplate reader (Bio-Rad). The absorbance data were expressed in percentage with respect to the control. The half maximal inhibitory concentration (IC₅₀) values were calculated and the optimum doses were analyzed at different time period.

### 2.6. Measurement of ROS in KB cells

DCF oxidized by radicals were visualized at excitation wavelength of 535 nm and emission wavelength of 485 nm. DCF is not oxidized by hydrogen peroxide or superoxide anion radical. Overnight grown cells were treated in 24-well plates for 24 h. After exposure, KB cells were subjected to centrifugation and loaded with DCFH-DA (20 µM/ml) in growth medium, and then, the cells were incubated for 30 min at 37°C. Next, S. xanthocarpum -treated cells were washed with PBS and fluorescence was measured every 5 min in over 30 min using a spectrofluorometer at 37°C.

### 2.7. Measurement of mitochondrial membrane potential (MMP) in KB cells

The effect of S. xanthocarpum on the MMP was evaluated using the lipophilic cationic fluorescent probe Rh-123 for mitochondria. The cells were incubated after they had been exposed to S. xanthocarpum at exclusive doses for 24 h. Following the addition of Rh-123 (10 µg/ml), the KB cells were incubated for 30 min at 37°C. Then, the cells were observed under a fluorescence microscope using blue filter.

### 2.8. Measurement of apoptosis in KB cells

To examine whether the IC₅₀ dose of S. xanthocarpum could lower cell proliferation by means of apoptosis, the KB cells were analyzed by AO/EB staining, 5 µl of dyes for double staining (in 1:1 ratio) was added to live cells at 37°C in the dark, followed by examination under a fluorescence microscope. The microscopic observation of apoptosis was carried out in accordance with the technique proposed by Liu et al.\cite{13}

### 2.9. Determination of lipid peroxidation and antioxidant enzymes

KB cells were suspended in 130 mm potassium chloride and 50 mm PBS containing 0.1 ml of 0.1 M dithiothreitol and then centrifuged at 10000 rpm for 15 minutes at 4°C. The supernatant was taken for biochemical determination of lipid peroxidation and antioxidant enzymes. Superoxide dismutase (SOD) and catalase (CAT) activities were measured using the methods by Beyer and Fridovich\cite{14} and Aebi\cite{15}, respectively. The measurement of glutathione (GSH) content was carried out using the method by Griffith\cite{16} and 5,5-dithiobis-2- nitrobenzoic acid, DTNB (Sigma chemical Co., USA). Measurement of MAD formation was accomplished through its response with thiobarbituric acid reactive substances (TBARS) using the method by Ohkawa et al.\cite{17}(1979).

### 2.10. Statistical analysis

The data are expressed as mean ± standard deviation (SD) and statistical comparisons were carried out by one-way analysis of variance and Duncan's multiple range test using SPSS version 17.0. The results were considered statistically significant if P < 0.05.

### 3. Results

#### 3.1. Effect of S. xanthocarpum on the proliferation of KB cells

Leaves extract of S. xanthocarpum lowered the proliferation of KB oral cancer cells after a 24-h treatment, showing notably decreased cell proliferation compared to the control cells (P < 0.05; Figure 2A). The increase in concentration resulted in much lower cell growth. The least growth rate was manifested after a 12-h treatment with 350 µg/ml of S. xanthocarpum extract. Furthermore, more than 50% of the cells died after the incubation with 200 µg/ml of S. xanthocarpum extract for 24 h (Figure 2B).

![Figure 2](https://example.com/image2.png)

Figure 2. Antiproliferative effects of Solanum xanthocarpum on KB cells. (A) The cells were treated with an increasing concentration of S. xanthocarpum (50 – 350 µg/mL) for 24 h and the results are expressed as a percentage of the control value. (B) Cellular morphological changes such as detachments, distorted shape, and dead cells were observed in KB cells treated with 150 – 200 µg/ml S. xanthocarpum for 24 h. Data are expressed as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 compared to control.
3.2. ROS generation induced by S. xanthocarpum

*S. xanthocarpum* induced ROS generation in KB cells. Excessive generation of ROS may lead to oxidative stress, loss of cell functions, and ultimately, apoptosis. To depict the role of ROS in *S. xanthocarpum*-induced cell death, intracellular ROS production was detected using an oxidant sensitive fluorescent probe, DCFH-DA. As shown in Figure 3A, the DCF signal was detected in KB cells after being treated with 150 and 200 µg/ml *S. xanthocarpum* for 24 h and in control cells. An increase of intracellular fluorescence intensity has been depicted to occur within 20 min after cell incubation with *S. xanthocarpum* at 24 h. When the concentration of *S. xanthocarpum* was increased, the oxidized form of cells was also increased (Figure 3B).

3.3. Effect of *S. xanthocarpum* on MMP of KB cells

The present study also evaluated the effect of *S. xanthocarpum* on MMP in KB cells. Rh-123 dye was used to detect the accumulation and formation of red fluorescent aggregates in mitochondria that have elevated MMP (Figure 4A). In contrast, the Rh-123 exists as a monomer that generates green fluorescence in the cytoplasm and mitochondria that have decreased MMP. When KB cells were incubated with *S. xanthocarpum* (150 and 200 µg/ml) for 24 h, *S. xanthocarpum* induced a markedly lower red fluorescence intensity compared to the control cells (Figure 4B). The present results showed that an apparent decrease in MMP occurred in cells treated with 200 µg/ml *S. xanthocarpum* for 24 h.

3.4. Effect of *S. xanthocarpum* on the apoptosis of KB cells

To verify the type of cell death caused by the *S. xanthocarpum* (150 and 200 µg/ml for 12 h) in KB cells, the AO/EB staining was carried out. The increased penetration of AO into residing cells caused an increased in the intensity of green fluorescence (Figure 5A). The second dye, EB, and emitted crimson fluorescence inside the cells with altered mobile membrane integrity. Changes in the distribution, morphology and the form of cells, as found out by fluorescence microscopic analysis, were located mostly after *S. xanthocarpum* treatment (150 and 200 µg/ml) for 24 h. The highest apoptosis rate was detected when the treatment at the IC$_{50}$ dose of 200 µg/ml *S. xanthocarpum* was used for 24 h, which was notably higher as compared to the control cells, as shown in Figure 5B.

3.5. Effect of *S. xanthocarpum* on lipid peroxidation in KB cells

The levels of TBARS decreased drastically (*P* < 0.05) in the control KB cells (Figure 6). *S. xanthocarpum* treatment (150 and 200 µg/ml) significantly increased TBARS levels in KB cells as compared to the control cancer cells.

3.6. Effect of *S. xanthocarpum* on antioxidant activity in KB cells

Figure 6 shows the activities of SOD and CAT as well as GSH content in normal and *S. xanthocarpum*-treated KB cells. Treatment with *S. xanthocarpum* (150 and 200 µg/ml) significantly decreased the activities of antioxidant enzymes such as SOD and CAT and the GSH content in KB cells (*P* < 0.05).

4. Discussion

Several phytomedicines have been acknowledged to possess strong antitumor properties, which may be recognized as a potential therapeutic compound for the
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The phytochemicals and bioactive compounds contained in different parts of the plants and vegetables can be extracted and used on numerous types of human cells to reduce the threat of proliferation of most cancers. *S. xanthocarpum* is claimed to possess high antioxidant activities. However, the anticancer effect of this herb remains largely unexplored. In this study, we demonstrated the *in vitro* effect of *S. xanthocarpum* on the growth of KB human oral cancer cell line. There is mounting evidence showing that uninhibited cell proliferation is an essential element in cancer initiation, metastasis, and progression in various organs and tissues. Furthermore, the uncontrolled tumor cell proliferation is regarded as the primary characteristic of malignant neoplasm.

MTT assay is typically utilized to evaluate the cytotoxicity of test compound and the proliferation of cancer cells. Metabolically active cells can convert MTT into a pink product, which can be determined colorimetrically. In the present study, *S. xanthocarpum* was tested for its anticancer effect on oral cancer cells. *S. xanthocarpum* reduced the proliferation of KB cells at the concentrations of 50, 100, 150, 200, 300, and 350 µg/ml. Zhang *et al.* showed that the treatment of nasopharyngeal carcinoma cells with 10 µg/ml *S. xanthocarpum*-gold nanoparticles could suppress the cell viability up to 53%.

ROS is a deleterious species that can lead to apoptosis. Overproduction of ROS could result in oxidative stress, and lipid peroxidation could result in double-stranded DNA damage, genotoxicity, and apoptosis. Kumar *et al.* reported the potential efficacy of *S. xanthocarpum* root extracts in deterring free radical damage. Intracellular ROS status can be determined through DCFH-DA staining. Microscopic examination showed that *S. xanthocarpum*...
treated KB cells emitted green fluorescence, which is an evidence of the extra accumulation of ROS. This suggests that *S. xanthocarpum* promoted apoptosis of KB cells, which was mediated by ROS. Various studies have demonstrated that plant extracts that induce ROS generation could elicit oxidative stress and DNA damage in most human cancers cells.

Mitochondria play an essential role in activating apoptosis in a mechanism that involves fluctuations in the electron transport chain, loss of mitochondrial integrity, and activation of caspase activators[26]. MMP decrease is a hallmark of apoptosis. Cancer cells show comparatively lower resting membrane potentials compared to normally multiplying cells. MMP changes can be detected through the uptake of the Rh-123 dye in KB cells. Treatment with *S. xanthocarpum* resulted in the augmented depolarization of the MMP in KB cells as evident by the elevated fluorescence intensity of Rh-123 compared to control cells. In this study, we confirmed that *S. xanthocarpum* triggered a reduction of MMP via ROS generation, which activated apoptosis subsequently.

Apoptosis is a form of cell death characterized by morphological changes and DNA damage[27]. Hence, to examine inhibitory effects of *S. xanthocarpum* on the induction of apoptosis, the morphological changes of KB cells were examined using AO/EB staining, which helped verify cell death. AO-deposited cells released green fluorescence and EB was taken up by mostly the non-viable cells which emitted purple fluorescence.

Intake of antioxidants seems to inhibit the volatile outcomes of the continuous oxidative stresses. Lipid peroxidation is one of the crucial processes of oxidative stress. The cell damages caused by oxidative stress, as well as apoptosis accompanied by the occurrence of lipid peroxidation and alterations of biomolecules, have been extensively studied[28]. In this study, we detected an elevated levels of TBARS in KB cells treated with *S. xanthocarpum* compared to the untreated control cells. The association between the increased levels of TBARS and the retarded cell proliferation confirms that apoptosis is the most essential feature of the membrane damage via lipid peroxidation[29]. Various studies have provided the evidence that antioxidants play a key role to defend against the tumor-promoting mediators.[30] Apparently, chances of metastasis or malignancy become generally higher when the activities or contents of antioxidants, such as SOD, CAT and GSH, become lower. In this study, we demonstrated that the activities or contents of SOD, CAT, and GSH were decreased on the treatment with *S. xanthocarpum* in KB cells.

*S. xanthocarpum* extract is also known to have powerful cytotoxic effect on leukemia cell line THP-1 but weaker effect on human lung cancer cell line HOP-62. This can be attributed to the distinct differences in the sensitivity to cytotoxicity of various cell lines. The results showed a strong correlation between flavonoid content and inhibition of THP-1 cell growth[31]. Similar results regarding the association between flavonoids and cytotoxic activities have also been reported in other studies. Therefore, flavonoids act as antioxidants to inhibit carcinogenesis[32].

5. Conclusion

Taken together, this study confirmed the anticancer properties of the *S. xanthocarpum* leaf extract, which inhibit the growth and promote the apoptosis of KB cells *in vitro* through an ROS-induced mitochondrial pathway. A more in-depth investigation of the therapeutic effect of *S. xanthocarpum* plant extract is needed.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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