### ORIGINAL ARTICLE \_

# Sorghumol triterpene inhibits the growth of circulating renal cancer cells by promoting cell apoptosis, G2/M cell cycle arrest and downregulating m-TOR/PI3K/AKT signalling pathway

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#### Summary

**Purpose:** To investigate the growth inhibitory effect of Sorghumol on the circulating renal cancers cells and to investigate the underlying mechanisms including its effects on apoptosis, cell cycle phase distribution and m-TOR/PI3K/ AKT signalling pathway.

**Methods:** The antiproliferative effects were assessed by WST-1 and colony formation assay. Apoptosis was detected by the Hoechst and AO/EB staining using fluorescence microscopy. Cell cycle analysis was carried out by flow cytometry. Protein expression was checked by western blotting.

**Results:** The results revealed that Sorghumol inhibited the growth of the renal cancer cell (RCC) line A498 and circulating RCCs. However, more profound effects were observed on

the RCC cells. The anticancer effects were found to be due to induction of apoptosis. Moreover, Sorghumol could also caused G2/M cell cycle arrest of the RCC cells. Besides, examination of the effect of Sorghumol on m-TOR/PI3K/AKT revealed that Sorghumol inhibited the expression of p-mTOR, p-PI3K and p-AKT in a concentration-dependent manner.

**Conclusion:** Taken together, we conclude that Sorghumol inhibited the proliferation of circulating RCCs and may therefore prove to be an important lead molecule for the treatment of renal cancer.

*Key words:* apoptosis, cell cycle, circulating renal cancer cells, flow cytometry, sorghumol

#### Introduction

Plants have been used for management of human diseases since times immemorial. Over the years a diversity of chemical scaffolds have been isolated from plants and have been used as drugs for the treatment of several diseases and disorders [1]. The plant derived natural products such as taxol and cisplatin have been used for the treatment of cancers [2]. Similarly artemesnin has been used in the treatment of malaria [3]. Plants continue to be the reservoir of natural drugs and many more

drugs are expected to be isolated from plants in the coming time. Among plant secondary metabolites, triterpenes have been reported to be of immense pharmacological potential [4]. They have been reported to exhibit a vast array of bioactivities such as anticancer and antimicrobial [5]. Sorgumol is an important triterpene that has been reported to exhibit a diversity of pharmacological properties [6]. However, in this study we report for the first time the anticancer activity of shorgumol against the

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circulating RCCs. Renal cancer in adults generally arises from the renal parenchyma or renal pelvis. The pelvis cancers include around 10% of all the diagnosed renal cancer cases and the rest 90% of renal carcinomas are adenocarcinomas [7]. Renal cancer is responsible for significant mortality and its incidence is still increasing. Although there are treatments available for this type of cancer, there are frequent relapses and the current treatments have a number of side effects [8]. Therefore, there is a need to explore new drugs or identify specific therapeutic targets for renal cancer treatment. In this study we observed that Sorghumol inhibits the growth of the circulating RCCs. The anticancer effects of Sorghumol were found to be due to the induction of apoptosis. Furthermore, Sorghumol could also induce G2/M cell cycle arrest in the circulating RCCs. Furthermore, evaluation of the effects of Sorghumol on the mTOR/PI3/AKT signalling pathway revealed that Sorghumol could inhibit the expression of some of the important proteins of this pathway. Taken together, we conclude that Sorghumol may prove useful in the treatment of renal cancer.

#### Methods

#### Cell lines and culture conditions

Renal cancer cell line A498 and circulating RCCs were procured from American Type Culture Collection. This cell line was maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), antibiotics (100 units/mL penicillin and 100  $\mu$ g/mL streptomycin), and 2 mM glutamine. The cells were cultured in CO<sub>2</sub> incubator (Thermo Scientific Waltham, Massachusetts, USA) at 37°C with 98% humidity and 5% CO<sub>2</sub>.

## Determination of cell viability and colony formation potential

The cell viability of the RCCs was assessed by WST-1 colorimetric assay. Briefly, RCCs were seeded in 96well plates at a density of 2×10<sup>5</sup> cells/well. The cells were then incubated with WST-1 at 37C for 4 hrs. The absorbance at 450 nm was then taken by a microplate reader to determine the viability of RCCs. To assess the impact of Sorghumol on the colony formation potential, RCCs were collected at the exponential phase of growth and were then counted using a hemocytometer. The platting of the cells was carried out at 200 cells /well. The plates were then kept at 37°C for 48 hrs to permit the cells to adhere. This was followed by the addition of various concentrations (0, 10, 20 and 40 µM) of Sorghumol. Following treatment with Sorghumol, the cell plates were again incubated for 6 days. Following this, they were subjected to washing with PBS and fixation with methanol. The RCCs were then stained with crystal violet followed by microscopy and then counted under light microscope.

#### Detection of apoptosis

The nuclear morphology of the RCC was assessed by fluorescence microscopy after subjecting the cells to cell-permeable Hoechst 33342 dye. Ten fields with 100 cells/ field were selected randomly for estimation of the cells with condensed nuclei. AO/EB double staining was used for the determination of apoptotic RCCs as described previously [9].

#### Cell cycle analysis

To investigate the distribution of the RCCs in different phases of the cell cycle, approximately  $1 \times 10^5$  cells in each well of 6-well plates were kept at 37°C overnight to allow the cells to adhere. This was followed by treatment with various doses of Sorghumol (0, 10, 20 and 40  $\mu$ M) followed by incubation for 24 hrs at 37°C. Finally the distribution of the RCCs in various cell cycle phases was evaluated by flow cytometry.

#### Western blot analysis

RCCs were lysed using ice-cold hypotonic buffer. After estimating the protein concentrations in each of the cell extracts, the samples containing the proteins were loaded and separated on SDS–PAGE. This was followed by transfer to a nitrocellulose membrane and incubation with the primary antibodies [AKT (cat.no.sc-135829), phosphorylated (p)-AKT (cat.no.SC-7985-R), PI3K (cat. mo. SG136298), mTOR (cat.no.sc-517464) and p-mTOR (cat.no.SC-291133]; all 1:1000 for 24 hrs at 4°C, all from Santa Cruz Biotechnology Inc., Dallas, TX, USA]. Thereafter, the membrane was incubated with anti-rabbit secondary antibody (cat.no.sc-2357-CM;1:1000) for 1 hr at 24°C. The visualisation of the proteins was carried out by enhanced chemi-luminescence reagent.

#### Results

## Antiproliferative effects of Sorghumol on A498 RCC cell line

To examine the antiproliferative effects of Sorghumol (Figure 1) on A498 renal cancer cells and RCC circulating cells, the RCCs were subjected to treatment with Sorghumol at varied doses. It was observed that Sorghumol displayed significant anticancer effects against A498 RCCs with an observed IC<sub>50</sub> of 30 and 20  $\mu$ M respectively. The effect of Sorghumol on the growth of RCCs exhibited a



Figure 1. Chemical structure of sorghumol.

concentration-dependent pattern (Figure 2). In addition, it was observed that Sorghumol treatment caused a considerable reduction in the RCC colonies in a concentration-dependent manner (Figure 3).

#### Apoptosis in RCCs

To explore the reason behind the anticancer effects, Sorghumol-treated RCCs were subjected to DAPI staining. It was observed that Sorghumol caused apoptosis in RCCs dose-dependently (Figure 4). Analysis of apoptotic cell population was further studied by AO/EB staining and showed increased apoptosis with increase in the doses of Sorghumol as indicated by the cells with orange color nuclei (Figure 5).



**Figure 2.** Sorghumol inhibits the cell viability of renal cancer A498 cells and circulating cancer cells in a concentration-dependent manner. The experiments were repeated three times and presented as mean  $\pm$  SD (\*p< 0.05).

#### Sorghumol triggered cell cycle arrest

To examine the impact of Sorghumol on the distribution of the RCC in different phases of the cell cycle, the cells were treated with 0, 10, 20 and 40  $\mu$ M of Sorghumol for 24 hrs and found that the percentage of cells at G2 phase increased in a dose-dependent manner causing cell cycle arrest in the G2/M cell cycle (Figure 6).





40µM

10 µ M





**Figure 4.** Sorghumol triggers apoptosis in the RCCs as depicted by the Hoechst staining. The experiments were repeated three times.



**Figure 3.** Sorghumol inhibits the colony formation potential of circulating cancer cells in a concentration-dependent manner. The experiments were repeated three times.



**Figure 5.** Effect of Sorghumol on the induction of apoptosis as indicated by the AO/EB staining. The experiments were repeated three times.

#### Sorghumol inhibited the m-TOR/PI3K/AKT signalling **Discussion** pathway in RCC

The effect of Sorghumol was also investigated on the m-TOR/PI3K/AKT signalling pathway. The results revealed that Sorghumol inhibited the expression of some of the important proteins of this pathway. It was observed that the expression of pmTOR, p-PI3K and p-AKT was significantly downregulated. However, the expression of PI3K, AKT and mTOR remained almost unaltered (Figure 7).



Figure 6. Effect of Sorghumol on the triggering cell cycle arrest in RCC cells. Arrows show G2/M phase cells. The experiments were repeated three times.



Figure 7. Effect of Sorghumol on the mTOR/PI3K/AKT signalling pathway as indicated by western blotting. The Figure shows that the expression of p-PI3K, p-AKT and p-mTOR was downregulated upon Sorghumol treatment. The experiments were repeated three times.

Advanced renal cancer is a lethal disease and its treatments are limited and exhibit a number of side effects [10]. Hence there is urgent need to either screen new molecules for their anticancer activity or to explore novel therapeutic targets [11]. In this study we investigated the anticancer effects of Sorghumol against renal cancer and the circulating RCCs. The results showed that although Sorghumol could inhibit the proliferation of both types of cancer cels (A498 and circulating RCCs). The anticancer effects were more profound on the circulating RCCs. While in this study  $IC_{50}$  was found to be 30 µM against RCCs, Sorghumol exhibited an  $IC_{50}$  of only 20  $\mu$ M against the circulating RCCs. These results are also supported by previous investigations wherein Sorghumol has been found to inhibit the proliferation of several types of cancer cells [6]. Moreover, many of the triterpenes have been found to inhibit the proliferation of cancer cells. For instance many triterpenes from olive tree have been reported to inhibit the proliferation of breast cancer cells [12]. Moreover, our results were further validated by the results of the colony formation assays wherein it was observed that Sorghumol inhibits the formation of colonies by RCCs in a dose-dependent manner. It has been reported that a wide array of plant-derived secondary metabolites prompt apoptosis in cancer cells. Apoptosis is an important mechanism which helps, among others, remove the harmful malignant cells from the body [13]. We observed that Sorghumol induces apoptosis in the RCCs in a dose-dependent manner as revealed by the Hoechst staining. Apoptosis was further confirmed by the AE/OB staining wherein the red coloured apoptotic cells were found to increase when the concentration of Sorghumol was increased.

Apart from apoptosis, cell cycle arrest is yet another mechanism by which anticancer agents halt the growth of cancer cells [14]. In this study, we observed that Sorghumol caused arrest of RCCs in the G2/M phase of the cell cycle, further adding to the anticancer effects of this molecule. Several signalling pathways have been reported to be activated in cancer cells and currently researches are being carried out to target these pathways for the treatment of cancers [15]. In this study we examined the effects of Sorghumol on the m-TOR/PI3K/ AKT signalling pathway which is one of the highly important activated pathways in cancer cells [16]. The results showed that Sorghumol could block this pathway in the circulating RCCs suggesting the potent anticancer effects of Sorghumol on the RCCs.

### Conclusion

The results of this study indicated that Sorghumol inhibits the proliferation of the RCCs dosedependently. The anticancer effects were found to be due to the induction of apoptosis and G2/M cell cycle arrest. Moreover, Sorghumol could inhibit the mTOR/PI3K/AKT signalling pathway in cancer cells, suggesting that Sorghumol could prove to be an important lead molecule to prevent the spread of the circulating RCCs.

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#### **Conflict of interests**

The authors declare no conflict of interests.

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