Cell culture studies - Role of fucoidan on oxalate induced apoptosis
INTRODUCTION

Renal epithelial injury is regarded as a major risk factor for crystallization of crystal deposition in the kidneys (Asselman et al., 2003). Sublethal injury provides sites for crystal attachment to the injured cell membranes (Wiessner et al., 1999) whereas lethal injury results in disintegration of the cells supplying membrane vesicles for heterogeneous nucleation of the crystals, and their aggregation and retention within the renal tubules (Khan et al., 2002). Lethal injury also exposes the epithelial basement membrane for crystal attachment, thereby, securing their retention within the renal tubules (Khan, 1995) and ports show that apoptosis might be the possible pathway through which oxalate induces lethal injury. In general, oxalate exposure has been found to produce a variety of changes in renal epithelial cell morphology and function. Interaction of oxalate with renal epithelial cells elicits a programmed sequence of events that can lead to either cell proliferation or death. The mode of cell death produced by oxalate has not been defined until recently, though initial studies were consistent with necrotic renal cellular injury (Hackett et al., 1995). However, recent report how that oxalate together with calcium oxalate crystal formation/deposition may induce renal tubular cell damage and/or dysfunction which may express itself as cell apoptosis (Sarica et al., 2001).

Apoptosis, also described as programmed cell death, is a physiological phenomenon characterized by cellular morphological and biochemical alterations that cause a cell to commit suicide. By these means, delinquent cells are marked to be safely removed and any disturbances in the general “to live or not to live” decision-making process, may have calamitous consequences for the organism.
Two general pathways have been described by which cells undergo apoptosis. The extrinsic pathway is triggered via cell surface receptors, leading to activation of initiator (i.e. caspase-8) and effector (i.e. caspase-3) proteolytic enzymes. The intrinsic pathway involves the mitochondria resulting in cytochrome c release and activation of the initiator caspase, caspase-9, with subsequent activation of effector caspases (i.e. caspase-3) (Takahashi et al., 2004).
owed that exposure of oxalate to human renal tubular epithelial HK-2 cells induces apoptosis mediated by an intrinsic pathway (Jeong et al., 2005). Induction of intrinsic pathway of apoptosis by oxalate is also supported by the observation that oxalate exposure increased the abundance of membrane ramide, a biochemical marker and a possible mediator of cellular apoptosis (Lannun and Obeid, 1997).

Though the exact mechanism through which oxalate initiates apoptosis is not clear, it is possible that oxalate-induced reactive species production might play a vital role in this process (Khan, 2004). Pro-oxidant agents, such as H$_2$O$_2$, ramide, etoposide and semiquinones, induce apoptosis whereas antioxidants, such as N-acetylcysteine, suppress apoptosis by acting as scavengers for ROS, and their actions provide evidence that ROS act as signaling molecules to poptosis. Thus, the present study was instigated to examine the characteristics of oxalate-induced cell death. Cell cultures were chosen for this purpose for they have served as prefect tools to analyze the interaction of oxalate with cells. Indeed, most of the studies which explicate the changes associated with oxalate exposure and its consequences have been provided by cell culture studies. Madin Darby Canine Kidney (MDCK) epithelial cells, of distal tubular origin were used in this study to assess the features of oxalate-induced apoptosis and the effect of fucoidan.

4.2. MATERIALS AND METHODS

4.2.1. Drugs and Chemicals

Fucoidan from F. vesiculosus and BSA were obtained from Sigma Chemicals, St Louis, MO, USA. The plastic culture wares were bought from TPP, Switzerland. Dulbecco’s Modified Eagle’s Medium (DMEM), FBS and
other tissue culture reagents were obtained from Seromed\textsuperscript{a}-Biochrom AG, Germany. All other chemicals and solvents used were of highest purity and analytical grade. MDCK cells were gifted from Department of Animal Biotechnology, Tamil Nadu Animal Sciences and Veterinary University, Chennai, India.

4.2.2. Culture and drug exposure

MDCK cells were maintained in DMEM with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose, 10% FBS, penicillin, streptomycin solution (10,000 IU and 10,000 µg/ml respectively) and amphotericin B solution (250 µg/ml). The cells were maintained in growth conditions of 37 \textdegree{}C and 5% carbon dioxide. Oxalate and fucoidan were dissolved in the medium and MDCK cells were exposed to varying concentration of these compounds. Cells were exposed to 0.5 mM to 1 mM concentration of oxalate for 24 h and effective dosage causing 50\% of cell death was used to induce oxalate toxicity. Another set of cells were treated with 0 to 100 µg/ml concentrations of fucoidan along with oxalate for 24 h, among which an effective dosage was considered for the treatment of oxalate toxicity. Solvent control and drug controls were also included. After 24 h, all cells were harvested for experimental studies.

4.2.2.1. Cell viability assay

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl-tetrazolium bromide (MTT) quantitative assay capable of detecting viable cells (Mosmann, 1983). The assay is based on the ability of mitochondria from viable cells to cleave the tetrazolium rings of the pale yellow MTT resulting in the formation of dark blue formazan product. The cells were treated in triplicates with oxalate and with or without fucoidan for 24 h. At the end of each
the point, 20 μl of MTT (5 mg/ml) was added to each well and the plates were incubated for 4 h at 37 °C. The MTT formazan precipitate was subsequently dissolved in dimethyl sulfoxide (DMSO) and the absorbance was read at 562 nm (test wavelength) and at 660 nm (reference wavelength) using an ELISA microplate reader against a blank.

4.2.2.2. Estimation of ROS production

Intracellular ROS generation on oxalate exposure in MDCK cells was monitored using DCF - DA by the protocol described in the section 3.2.12.2.

4.2.2.3. Assessment of mitochondrial membrane potential with JC-1 staining by confocal microscope

MDCK cells were incubated in media containing 0.5 μM JC-1 (Sigma Chemicals) dye for 30 min at 37 °C and washed with PBS. The cells were then excited at 488 nm and the fluorescence emission recorded at 534 nm and 596 nm and images were captured using a CCD camera (Hamamatsu Digital Camera: Meta Vue software). Levels of green and red fluorescence were coded on a scale (0 – 4095) representing pixel intensity and 1280 × 1024 pixel images were collected. The stock solution of JC-1 was dissolved in 100% DMSO, but the final concentration of DMSO in the media was < 0.1%.

4.2.2.4. Analysis of the expression of Bcl-2 by western blotting

Cells collected by trypsinization were washed with PBS and subjected to lysis with 50 mM Tris HCl (pH 8.0), containing 150 mM NaCl, 1% Triton X-100, 100 μg/ml phenylmethylsulfonyl fluoride and 1 μg/ml aprotinin. This was followed by incubation for 30 min in ice and then centrifuged at 10,000 × g for 20 min at 4 °C. Bcl-2 was analyzed in the supernatant using western blotting.
as described in the section 3.2.14.2. Primary antibody of Bcl-2 obtained from Cell Signaling Technology, Danvers, MA, USA was used in this study.

4.2.2.5. Estimation of cytochrome c

For analysis of cytochrome c, the cells were washed and collected by centrifugation at 1000 $\times$ g for 10 min at 4 °C. The cell pellets were washed once with ice-cold PBS and resuspended in lysis buffer (20 mM HEPES of pH 7.5, 10 mM KCl, 1.5 mM MgCl$_2$, 1 mM sodium EDTA, 1 mM sodium P-GTA, 1 mM DTT, 0.1 mM PMSF, and 250 mM sucrose) supplemented with protease inhibitors (10 μg/ml leupeptin, 10 μg/ml aprotinin and pepstatin A). The cells were then homogenized in a glass homogenizer, and the nuclei and cell debris were removed by centrifugation at 1000 $\times$ g for 15 min at 4 °C. Mitochondrial and cytosolic fractions were separated by differential centrifugation. Cytochrome c content in both the fractions was assessed by ELISA as described in section 3.2.13.3. The primary antibody for cytochrome c was procured from Cell Signaling Technology, Danvers, MA, USA.

4.2.2.6. Immunocytochemistry

Cells grown on coverslips were fixed in 3% paraformaldehyde in PBS followed by incubation in 80% ethanol for 30 min for permeabilization. The cells were washed thrice with PBS and then incubated in 2% BSA in PBS for 60 min at room temperature. A fresh solution of 0.3% H$_2$O$_2$ in PBS was used to treat the cells for 30 min at 37 °C to inhibit endogenous peroxidase activity. After washing the cells with primary antibody for cleaved α-todrin (150 kDa) (Cell signaling Technology, Danvers, MA, USA) was added and the cells were incubated overnight at 4 °C. The cells were then rinsed with PBS and incubated with appropriate HRP conjugated secondary antibodies, for 30 min at room
temperature. After three rinses with PBS, 1 ml of DAB reagent was added. The cells were mounted with anti-fade mounting solution and examined under light microscope, Nikon Eclipse E400, model 115, Japan.

4.2.2.7. Detection of apoptotic cells by Annexin V-FITC/PI staining

The frequency of apoptotic cells was detected by flow cytometry using apoptosis detection kit (Sigma Chemicals). This procedure is based on the binding of Annexin V-FITC to phosphatidylserine in the membrane of cells which is at the start of the apoptosis and the binding of PI to the cellular DNA in cells where the cell membrane has been totally compromised (Vermes et al., 1995). Briefly, 5 µl of Annexin V-FITC (50 µg/ml in 50 mM Tris-HCl, pH 7.5 containing 100 mM NaCl) and 10 µl of PI (100 µg/ml in 10 mM potassium phosphate buffer, pH 7.4 containing 150 mM NaCl) were added to 0.5 ml of trypsinized cell suspension (1 × 10⁶ cells/ml) suspended in binding buffer (10 mM HEPES/NaOH, pH 7.5, containing 140 mM NaCl and 2.5 mM calcium chloride) and protected from light. Exactly after 10 min of incubation at room temperature, the fluorescence of the cells was determined with a BD FACS Calibur flow cytometer. Cells which are in the early apoptotic process are stained with Annexin V-FITC alone. Live cells showed no staining by either PI or Annexin V-FITC. Necrotic cells were stained by both PI and Annexin V-FITC. Cells which were damaged during the isolation procedure were stained only with PI.

4.2.3 STATISTICAL ANALYSIS

The results are expressed as mean ± S.D. for three experiments. Differences between groups were assessed by ANOVA using the SPSS (Statistical Package for Social Sciences) software package for Windows. Post
hoc testing was performed for inter-group comparisons using the least
Significance Difference (LSD) test; significance at $P$-values < 0.001, < 0.01,
< 0.05 have been given respective symbols in the figures.

4.3. RESULTS AND DISCUSSION

Apoptosis is an important mechanism of cell death in the kidney during
development and after injuries. Studies with cell cultures have shown that cells
exposed to oxalate exhibit characteristic features of apoptosis. Among which,
redistribution of the membrane phospholipid, activation of phospholipases,
generation of lipid signaling molecules like arachidonic acid, lysophosphatidyl
choline and ceramide are of vital importance due to their inexplicable role in
apoptosis. As a part of the present study, cell injury and cell death induced by
oxalate was investigated in MDCK cells, detailing the stages and mechanisms of
apoptosis along with the putative role of fucoidan in oxalate-induced apoptosis.

4.3.1. Dose dependant effect of oxalate and fucoidan on MDCK cells

Oxalate toxicity on renal cells depends on the concentration and time of
exposure of oxalate. MDCK cells were exposed to varying concentrations of
oxalate 0.02, 0.05, 0.1, 0.2, 0.5 and 1 mM for 24 h. among which 0.1 mM oxalate
were found to be the most effective dosage which induces 54.3% cell death
(Figure 4.2). To test the effectiveness of fucoidan, MDCK cells were exposed to
0.1mM oxalate and different concentrations of fucoidan (Figure 4.3). 25 µg of
fucoidan was found to be effective in preventing the damage (73.33%); and,
further increase in fucoidan had no discernable effect on cell viability. Also,
fucoidan did not have any harmful effect on viability of cells. On the basis of
these preliminary experiments, studies were designed to assess the effect of
fucoidan on oxalate-induced apoptosis.
Figure 4.2. Dose dependant effect of oxalate on MDCK cells

![Graph showing the dose-dependent effect of oxalate on MDCK cells.]

Figure 4.3. Dose dependant effect of fucoidan on oxalate-induced toxicity in MDCK cells

![Graph showing the dose-dependent effect of fucoidan on oxalate-induced toxicity in MDCK cells.]

Values are expressed as mean ± SD of 3 experiments.
Figure 4.4. Effect of fucoidan on levels of ROS generation in oxalate exposed MDCK cells

Values are expressed as mean ± S.D. for 3 experiments. Comparisons are made between: a – Group I and Groups II, III, IV; b – Group II and Group IV. The symbols represent statistical significance: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. 
The channels 1-3 represent the green, red and merged channel fields observed respectively. Oxalate exposed MDCK cells show decrease in red fluorescence characteristic of loss of mitochondrial membrane potential (b1-3). Fucoidan treatment to the cells restored the near normal red fluorescence (d1-3). Control and fucoidan treated cells show normal fluorescence (a,c 1-3 respectively).
proteins is a key regulator of the mitochondrial response to apoptotic signals in the intrinsic pathway and contains both pro- and anti-apoptotic members. Alteration/decrease in Bcl-2 protein is strongly associated with the release of cytochrome c, an important secondary mediator of apoptosis. In the present study, Bcl-2 expression and cytochrome c levels were assessed in oxalate exposed MDCK cells (Figure 4.6 and 4.7 respectively). Figure 4.6 depicts the Bcl-2 protein expression and there was a significant decrease in Bcl-2 protein expression on oxalate exposure. This correlates well with the present in vivo studies which showed a decrease in Bcl-2 mRNA. Moreover, Itoh et al. (2005) have also shown a decrease in Bcl-2 protein on oxalate exposure. Further support also comes from the study of Miller et al. (2000), who showed that genetic manipulations which enhance the expression of Bcl-2 decreases apoptosis during oxalate exposure. Fucoidan was able to restore the Bcl-2 level to near normalcy and thereby modulate apoptosis.

In the present study, increased cytosolic cytochrome c in MDCK cells on oxalate exposure was synchronous with decreased Bcl-2 expression. Cytochrome c, a component of the mitochondrial electron transfer chain, initiates caspase activation when released from mitochondria during apoptosis (Iiu et al., 1996b). In the present study, an increase in cytochrome c in the cytosolic fractions along with a decrease in its content in the mitochondrial fractions was observed in oxalate exposed MDCK cells (Figure 4.7). Cytochrome c released from mitochondria is known to have an important role in apoptosis. It binds to Apaf-1, and forms apoptosome, which subsequently recruits multiple procaspase-9 molecules to the complex and facilitate their autoactivation. Only, the caspase-9 bound to the apoptosome is able to efficiently cleave and activate downstream executioner caspases, such as caspase-3 (Rodriquez and Lazebnik, 1999). The present observation is strengthened by the finding of Meimaridou et al. (2006).
Figure 4.5. Effect of fucoidan on mitochondrial membrane potential oxalate exposed MDCK cells (100x)

The channels 1-3 represent the green, red and merged channel fields observed respectively. Oxalate exposed MDCK cells show decrease in red fluorescence characteristic of loss of mitochondrial membrane potential (b1-3). Fucoidan treatment to the cells restored the near normal red fluorescence (c1-3). Combined fucoidan treated cells show normal fluorescence (a,c 1-3 respectively).
Figure 4.6. Effect of fucoidan on Bcl-2 expression in oxalate exposed MDCK cells

Lane 1  Lane 2  Lane 3  Lane 4  Lane 5
215 kDa
120 kDa
84 kDa
60 kDa
39.2 kDa
28 kDa
18.3 kDa

Lane 1  Protein Marker
Lane 2  Control
Lane 3  Oxalate
Lane 4  Fucoidan
Lane 5  Oxalate + Fucoidan
Figure 4.7. Effect of fucoidan on cytochrome c levels in oxalate exposed MDCK cells

Values are expressed as mean ± S.D. for 3 experiments. Comparisons are made between: a – Group I and Groups II, III, IV; b – Group II and Group IV. The symbols represent statistical significance: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. 
who showed increased levels of cytosolic cytochrome c in hyperoxaluric rats. Fucoidan was able to restore the mitochondrial integrity and thereby prevent cytochrome c release. Moreover, fucoidan was able to increase Bcl-2 protein level, which effectively inhibits cytochrome c release. Prevention of cytochrome c release by fucoidan is of relevance as drugs targeting to prevent cytochrome c release are efficient in preventing apoptosis (Matsuki et al., 2003).

The outcome of cytochrome c release is ultimately the activation of executioner caspases like caspase-3. These executioner caspases subsequently cleave many important intracellular substrates, leading to characteristic morphological changes in apoptosis such as chromatin condensation, nucleosomal DNA fragmentation, nuclear membrane breakdown, externalization of phosphatidylserine and formation of apoptotic bodies (Hengartner, 2000). Some of the substrates of the caspases are given in Table 4.1. among which α-fodrin, a membrane component is one of the first substrate to be cleaved by caspases during the initiation phase of apoptosis. α-fodrin, an abundant membrane-associated cytoskeletal protein, is rapidly and specifically cleaved during apoptosis, and this appears to be related to the membrane blebbing, as it is associated with ion pumps and channels. α-fodrin is cleaved into 145/150 kDa fragment by caspase-3 (Wang et al., 2001). Initially, it was proposed that α-fodrin is cleaved by calpain I (Martin et al., 1995), but recent studies show that the cleavage is probably due to caspase activation (Cryns et al., 1996; Vanags et al., 1996). In the present study, the presence of cleaved α-fodrin (150 kDa fragment) was assessed as an indicator of caspase activation. Immunocytochemical detection of cleaved α-fodrin is given in Figure 4.8. Oxalate exposed MDCK cells showed an increased expression of cleaved α-fodrin implying the activation of caspase-3 (Figure 4.8b). Addition of fucoidan to oxalate exposed cells decreased the expression of cleaved α-fodrin
Table 4.1. Protein substrates of caspases

<table>
<thead>
<tr>
<th>Protein substrate</th>
<th>Caspase</th>
<th>Function of substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly (ADP-ribose) polymerase</td>
<td>3.7</td>
<td>DNA repair enzyme</td>
</tr>
<tr>
<td>U1-70 kDa (70 kDa protein component of the U1 small nuclear ribonucleoprotein)</td>
<td>3</td>
<td>Splicing of RNA</td>
</tr>
<tr>
<td>DNA-PKCS (catalytic subunit of DNA-dependent protein kinase)</td>
<td>3</td>
<td>DNA double-strand-break repair</td>
</tr>
<tr>
<td>Protein kinase C δ</td>
<td>3</td>
<td>Cleaved to active form in apoptosis</td>
</tr>
<tr>
<td>D4-GDP dissociation inhibitor</td>
<td>3</td>
<td>Regulator of Rho GTPases</td>
</tr>
<tr>
<td>Heteroribonuclear proteins C1 and C2</td>
<td>3.7</td>
<td>Processing of pre-mRNA</td>
</tr>
<tr>
<td>Huntingtin</td>
<td>3</td>
<td>Huntington disease gene product</td>
</tr>
<tr>
<td>Fodrin</td>
<td>3</td>
<td>Membrane-associated cytoskeletal protein 127</td>
</tr>
</tbody>
</table>
Control (a) and fucoidan exposed (c) MDCK cells show mild staining for cleaved α-fodrin. Oxalate exposed cells (b) show strong immunostaining for α-fodrin indicating the activation of caspase. Oxalate + fucoidan exposed cells (d) show mild staining for α-fodrin.
significantly (Figure 4.8d). Control and fucoidan treated cells showed near normal appearance with negative staining for α-fodrin (Figure 4.8a and 4.8c). Increase in caspase activity in MDCK cells on oxalate exposure corroborates with the observation of Cao et al. (2004). They have also suggested that this effect of oxalate might be through activation of secondary lipid signaling molecules. A decrease in caspase activation on fucoidan administration shows the inhibitory role of fucoidan in apoptosis and supports the observation of Jhamandas et al. (2005) who showed a decrease in caspase activity on administration of fucoidan to neuronal cultures.

4.3.5. Frequency of apoptotic and necrotic cells in oxalate exposed MDCK cells

Decrease in Bcl-2, release of cytochrome c and activation of apoptotic factors are clear indicators of induction of apoptosis in oxalate exposed cells. However, the mode of cell death induced by oxalate is vague as some suggest a necrotic pathway of cell death rather than apoptosis (Miller et al., 2000). Hence, to further characterize the nature of cell death caused by oxalate, frequency of apoptotic and necrotic cells were assessed by labeling with both Annexin V-FITC and PI. During the onset of apoptosis, phosphatidylserine, which is normally restricted to the inner leaflet of the plasma membrane, is translocated to the outer surface of the membrane and this externalization of phosphatidylserine acts as a “flag” for apoptosis. A recently discovered family of proteins, the annexins, has been found to have high affinity for phosphatidylserine and is used to probe phosphatidylserine externalization (Martin et al., 1995). In the present study, fluorescently labeled Annexin V (Annexiv V-FITC) was used to detect membrane bound phosphatidylserine externalization. Additionally, PI staining indicates the membrane disruption that
Figure 4.9. Flow cytometric distribution of MDCK cells stained with a combination of annexin V-FITC and PI

Four sub populations of cells in the dot plot of annexin V-FITC is shown in the figure Green fluorescence (FL1-H) vs PI red fluorescence (FL2-H) were made on the basis of annexin V-FITC binding and PI uptake. 1 Damaged cells (annexin V-FITC negative/PI positive), 2 Late apoptotic and necrotic cells (annexin V-FITC positive/PI positive), 3 Early apoptotic cells (annexin V-FITC positive/PI negative), 4 Live cells (annexin V-FITC negative/PI negative)

Table 4.2. Effect of oxalate and fucoidan on frequency of apoptotic and necrotic cells

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I Control</th>
<th>Group II Oxalate</th>
<th>Group III Fucoidan</th>
<th>Group IV Oxalate + Fucoidan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Live cells (%)</td>
<td>94.54 ± 0.89</td>
<td>79.91 ± 0.95</td>
<td>94.28 ± 0.30</td>
<td>91.48 ± 1.22</td>
</tr>
<tr>
<td>Early apoptotic cells (%)</td>
<td>2.25 ± 0.45</td>
<td>12.39 ± 0.74</td>
<td>2.22 ± 0.70</td>
<td>3.92 ± 1.15</td>
</tr>
<tr>
<td>Late apoptotic necrotic and cells (%)</td>
<td>1.82 ± 0.78</td>
<td>6.70 ± 0.90</td>
<td>1.98 ± 0.67</td>
<td>3.33 ± 0.31</td>
</tr>
<tr>
<td>Damaged cells (%)</td>
<td>1.38 ± 0.13</td>
<td>1 ± 0.05</td>
<td>1.53 ± 0.37</td>
<td>1.27 ± 0.53</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± S.D for 3 experiments. Comparisons are made between a – Group I and Groups II, III, IV, b – Group II and Group IV. The symbols represent statistical significance * P < 0.05, ** P < 0.01, *** P < 0.001
occurs during necrosis. Frequency of apoptotic and necrotic cells in oxalate exposed cells is given in Figure 4.9. Control and drug control treated groups did not exhibit any pathological changes, whereas significant increase in apoptotic cells was seen on oxalate exposure rather than necrosis. Increase in apoptotic cells in the present study is also supported by the observation of Cao et al. (2001) who demonstrated that oxalate is capable of inducing phosphatidylserine externalization. However, fucoidan administration to oxalate exposed MDCK cells was able to decrease the frequency of apoptotic and necrotic cells. Inhibition of apoptosis in MDCK cells by heparin strengthens the anti-apoptotic role of fucoidan (Ishikawa and Kitamura, 1999).

From the results of the present study, it can be concluded that oxalate provokes cell death which follows mitochondrial mediated intrinsic pathway. Characteristic features of apoptosis like loss of mitochondrial transmembrane potential, decrease in Bcl-2, release of cytochrome c and activation of caspase-3 were observed in oxalate goaded MDCK cells. Fucoidan administration was able to effectively circumvent these changes. Decrease in apoptosis on fucoidan administration is very crucial because apoptosis apart from exposing the basal lamina for crystal attachment also increases matrix components essential for stone formation. Hence, dwindling in apoptotic events on fucoidan administration proves that fucoidan can serve as a potential candidate in circumventing lithogenesis.