Current Trends in Biotechnology and Pharmacy
Vol. 15 (3) 248-255, July 2021, ISSN 0973-8916 (Print), 2230-7303 (Online)
DOI: 10.5530/ctbp.2021.3.27

Evaluation of *In-vitro* Cytoprotective, Wound Healing and Antioxidant Effects of *Ocimum sanctum* Leaf Extract

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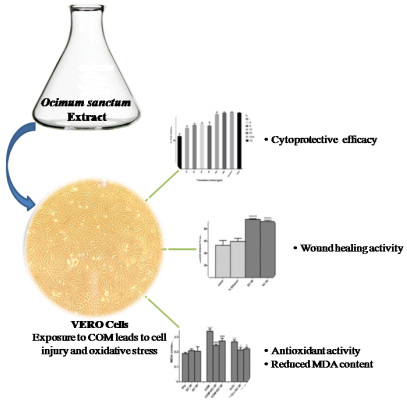
Abstract

Exposure of renal epithelial cells to Calcium oxalate monohydrate crystals leads to cellular injury and play a significant role in the formation of kidney stones. Additionally, lipid peroxidation of polyunsaturated fatty acids further accelerates the generation and progression of the disease. Availability of safe and effective treatment of the disease is the need of hour. The objective of this research work was to evaluate the impact of hydro-alcoholic extract of *Ocimum sanctum* leaves on renal epithelial cell injury, oxidative stress and wound healing. Renal epithelial cells (Vero cells) were exposed to Calcium oxalate monohydrate crystals to cause injury and then injured cells were treated with *Ocimum sanctum* extract. Cell viability of the treated group was compared against control using MTT assay. Antioxidant potential was evaluated using malondialdehyde content as biomarker. Wound healing potential was evaluated using scratch assay. A significant increase in cell viability and wound closure rate was observed in the treated group compared to that in control group was observed. We observed reduced malondialdehyde content in the treated group as compared with control. It can be concluded that hydro-alcoholic extract of leaves of *Ocimum sanctum* possesses strong cytoprotective, antioxidant and wound healing potential and can be effective in the prevention and treatment of kidney stones.



Key words Calcium oxalate monohydrate crystals, Malondialdehyde, Oxidative stress, Cytoprotective, Wound healing

Introduction

Recent studies have marked the significance of injury and reactive oxygen species in the formation of kidney stones. Excessive generation of free radical either endogenously or exogenously, leads to oxidative stress and tissue damage which eventually results in pathological conditions. Calcium oxalate monohydrate (COM) crystals elevate the production of reactive oxygen species and oxidative stress ultimately leading to cell damage in renal tissues [1,2]. Lipid peroxidation of polyunsaturated fatty acids

producing malondialdehyde (MDA) is a major consequence of oxidative stress. For this reason malondialdehyde is frequently used as an indicator of lipid peroxidation [3,4]. Additionally crystal binding sites are more exposed in case of renal epithelial wound or injury and these exposed adhesion sites facilitate attachment of COM crystals hence formation of kidney stones [5].

Therefore management of oxidative stress and renal tubular cell injury or wound healing can be an effective approach to avoid the crystal adhesion to renal epithelium cells and ultimately the formation of stone. Herbal medicines have once again emerged as safe and effective alternative of disease prevention and treatment. Phytochemicals present in the medicinal plants exhibit potential to be effective in retarding oxidative stress and tissue damage. Each part of the plants including roots, stem, leaves, fruits and seeds are known to possess bioactive components and have been used as a primary source of therapeutics. Among various medicinal plants *Ocimum sanctum* also known as *Ocimum tenuiflorum* or holy basil has been traditionally used as the mother medicine provided by nature due to its therapeutic potential in almost every part of the plant [6]. The plant has already been investigated for its radioprotective, ulcer healing, antibacterial, dyslipidemia reversing and many other therapeutic values [7,8]. Extract of *Ocimum sanctum* is reported to reduce tissue damage due to oxidative stress and minimize the pathological changes in kidney [9,10]. Aqueous extract of *Ocimum sanctum* reportedly exhibit cytoprotective effect on renal cells [11]. Though the herb has not been much investigated for its antiurolithiatic potential and needs to be explored thoroughly.

In the present study hydroalcoholic extract of *Ocimum sanctum* leaves (OLE) was evaluated for its impact on renal epithelial cell injury, oxidative stress and wound healing. For this purpose malondialdehyde content in different experimental conditions was investigated as a marker of oxidative stress. Renal epithelial cells (Vero cells) were exposed to COM crystals and assessment of renal cell viability was done using MTT cell viability assay. Wound healing scratch assay was used to evaluate the impact of extract on wound healing.

Materials and Methods

Chemical and reagents

Fetal bovine serum (FBS), Dulbecco's modified eagles's medium (DMEM) and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H tetrazolium bromide) were purchased from HiMedia Laboratories, India. Dimethyl sulfoxide (DMSO) was purchased from Sigma Aldrich.

Plant materials

Ocimum sanctum was collected from local nursery and was identified and authenticated by Department of Botany, University of Delhi. A voucher specimen of it was submitted there with accession number DUH14478.

Cell culture

Vero cells were procured from National Centre of Cell Sciences (NCCS, Pune). Cells were cultured as subconfluent monolayers in DMEM supplemented with 10% FBS and antibiotics penicillin 10 mg/ml and streptomycin 100 mg/ml in a humidified incubator at 37°C with 5% CO2.

Preparation of COM crystals

COM crystals were prepared using the method described by Semangoen et al (2008). Purity of COM crystals was confirmed by FTIR analysis. Crystals were sterilized by giving UV treatment before performing the experiment to avoid any contamination [12]

Effect of OLE on calcium oxalate crystal induced Vero cell injury

The experiment was performed by inoculating $5x10^3$ cells/well Vero cells (in triplicates) using 96 well tissue culture plates in DMEM media with 10% FBS at 37°C in a CO_2

incubator for 24 hrs. Media was replaced with COM crystals (200 µg/ml) containing serum free DMEM media to induce cell injury. After 24 hrs cells were exposed with different concentrations of OLE for 24 hrs at 37°C in the incubator.

$$\% \text{ Cell Viability} = \left[\frac{(At\text{-}Ab)}{(Ac\text{-}Ab)}\right] X\ 100$$

Effect of OLE on viability of Vero cells was assessed by performing MTT assay using the method described by Karamustafa et al [13] . The absorbance was recorded using micro plate reader at 570 nm. Cells incubated in DMEM media without any test material were used as control. Percentage cell viability was calculated using the following formula:

Where At- Absorbance of test sample; Ac- Absorbance of control; Ab- Absorbance of blank

Effective concentrations, EC 50 and EC 90 were calculated using AAT bioquest EC50 calculator (https://www.aatbio.com/tools/ec50-calculator/)

Evaluation of wound healing potential of OLE

In order to study the wound healing potential of OLE, cells were plated at a density of $2x10^5$ cells/well in a 12 well plate [14,15] and incubated in a humidified incubator at 37°C with 5% CO_2 until the formation of subconfluent monolayer. Wells containing cells were divided into control and test groups. To simulate wounding, a cell free gap was created by scratching the monolayer using a sterile pipette tip (200 µl) in the middle of each well. Cells were then washed with PBS to remove all the debris and unattached cells. The cells in the test group were treated with equal volumes of EC 50 and EC 90 values of OLE (dissolved in 70% ethanol) as test compounds while the cells in control were supplemented with DMEM without any test compound and 70% ethanol. The gaps were inspected at different time points (0, 3, 18 and 24 hrs) by taking pictures of scratched surface under an inverted microscope (Olympus Life Science) at 10x magnification. Image Pro-Plus software (Media Cybernetics) was used to compare the images at different time intervals [14] and wound closure percentage (WCP) was calculated [16].

Malondialdehyde (MDA) assay

Malondialdehyde (MDA) is a reliable marker of oxidative stress induced cell injury. To assess the COM and H_2O_2 exerted oxidative stress on Vero cells, MDA content was measured in different experimental sets using the standard procedure [17]. Cells were divided into control, injury and repair groups. Control groups included exposure of DMEM (serum free), EC 50 and EC 90 of . For the injury groups, serum free DMEM containing H_2O_2 (150 µm) and COM (200 µg/ml) crystals were added to the cells. For the repair groups, EC 50 and EC 90 of OLE were added to the cells along with H_2O_2 and COM crystals. MDA content was expressed as nmol/mg protein. .

Statistical analysis

Each experiment was performed in triplicates (n=3) and results are presented as mean ± Standard deviation. Graphpad prism 6.0 software (GraphPad Software, San Diego, CA, USA) was used for data presentation. Statistical analysis was performed using one way ANOVA. Associated probability (P) value of < 0.05 was

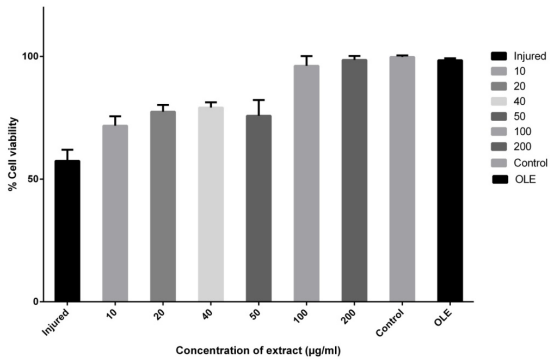


Figure 1: Cytoprotective effect of OLE on COM crystal injured Vero cells in concentration dependent manner. Values are expressed as Mean±SD

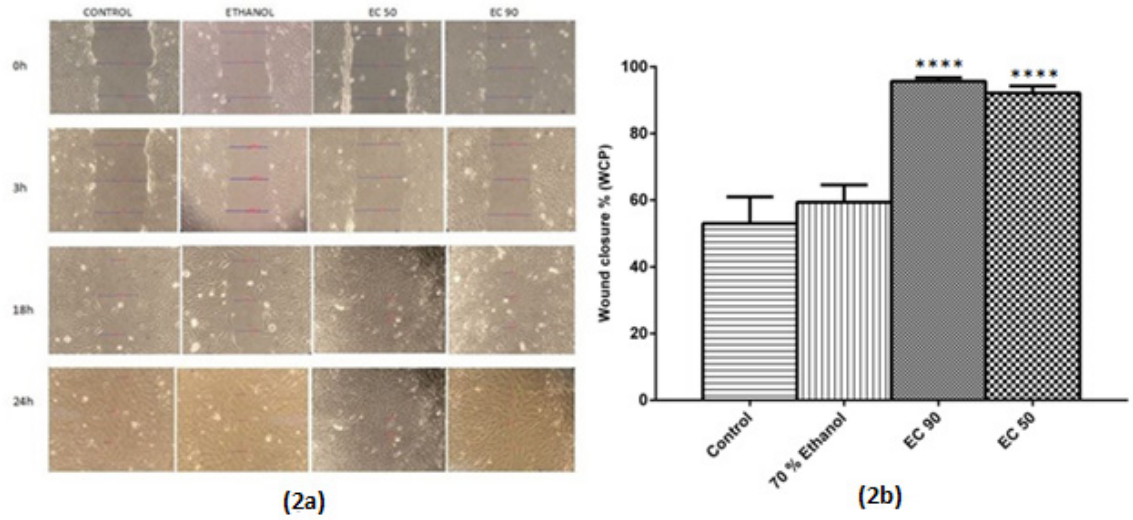


Figure 2: (a) Wound healing potential of EC 50 and EC 90 of OLE at different time intervals. (b) Bar graph expressing the percentage wound closure after 24 hrs. Values are expressed as Mean±SD. ****p < 0.0001 versus control.

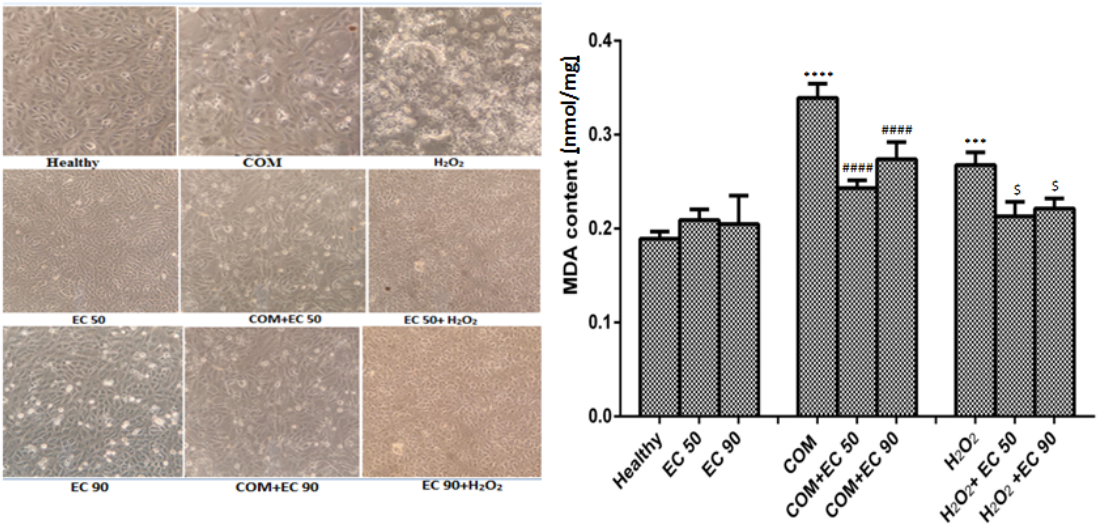


Figure 3: (a) Cells observed during different experimental conditions (b) Bar graph expressing the MDA content. Values are expressed as Mean±SD. *compared with control healthy group, #compared with the COM injured group, $compared with injured group. ###/***p< 0.0005 and ####/****p< 0.0001.

considered as statistically significant.

Results

Effect of OLE on calcium oxalate crystal induced Vero cell injury

OLE exhibited protective effect on Vero cells injured by COM crystals. Cell viability of injured cells was found to be increased in the presence of different concentration of OLE. Even the lower concentrations of OLE including 10, 20 and 40 µg/ml resulted in 71.77%, 77.50% and 79.16% cell viability respectively which

was found to be at comparable difference with calcium oxalate crystals injured group (57.45%). Interestingly, 100 and 200 µg/ml of OLE were showing maximum cell viability (96.18% and 98.62% respectively) with a slight decline in cell viability at 50 µg/ml (75.83%) (Fig.1). Effective concentrations, EC 50 and EC 90 were calculated to be 87.63 and 90.36 µg/ml respectively.

Evaluation of wound healing potential of OLE

Wound closure percentage (WCP) was calculated to evaluate the wound healing potential of OLE. Treatment with both the concentrations (EC 50 and EC 90) resulted in significant improvement in WCP of Vero cells as compared to the control groups (Fig. 2a). WCP for both the doses of OLE was found to be 92.13% (EC 50) and 95.62% (EC 90) in comparison with controls which resulted in 53.03% and 59.38% WCP in DMEM and 70% ethanol control groups respectively after 24 hrs (Fig. 2b).

MDA content estimation in injured and treated cells

In control groups, MDA levels in healthy, EC 50 and EC 90 treated cells were 0.189, 0.209 and 0.204 nmol/mg protein respectively. MDA levels in the control groups treated with OLE (EC 50 and EC 90) were not at significant difference when compared to healthy control group of cells. In injury group, oxidative injury by COM crystals and H_2O_2 resulted in remarkable increase in MDA levels, which was 0.339 and 0.261 nmol/mg protein respectively. This increase in MDA levels was at comparable difference with the control healthy group (0.189). A drop in MDA levels was observed in repair group as compared to injury group. Treatment with EC 50 and EC 90 of OLE along with COM crystals reduced the MDA content from 0.339 to 0.279 and 0.303 nmol/mg protein respectively when compared with COM crystal injured group. Moreover treatment with EC 50 and EC 90 of OLE along with H_2O_2 reduced the MDA concentration from 0.261 to 0.201 and 0.238 nmol/mg protein respectively which was at comparable difference with H_2O_2 injured group. It is clear from the above data that antioxidant activity of extract is capable of reducing the oxidizing effect of COM crystal and H_2O_2. Figure 3a explains the changes in cellular morphology during different experimental and control conditions while figure 3b depicts the estimated MDA content in different experimental sets.

Discussion

Generation of free radicals during cellular or tissue injury exert damaging effects and contribute to the pathology and occurrence of various diseases. Furthermore such diseases usually result from the imbalance between the excessive radical production and body's defense mechanism to combat the situation [18]. Exposure of calcium oxalate crystals to renal epithelial cells is injurious to cells and is considered as a major risk factor for the formation and retention of stones [19].

Such condition focuses on the necessity of free radical scavenging potential of traditionally used herbs to restore the antioxidant balance in the body. Presence of large number of biologically active phytocomponents in OLE is probably responsible for antioxidative potential and protective effect of OLE on renal cell injury caused by calcium oxalate crystals.

Due to crystal injury of cells reactive species are produced which further initiate kidney stone formation [20]. In the present study COM crystals injured Vero cells were treated with different concentrations of OLE and it was observed that OLE significantly increased the % retained cell viability as compared to control group of cells in a concentration dependent manner. The extract analysis of Ocimum leaves revealed presence of many phytocompounds that possess antioxidative properties and the protective effect of OLE against injured Vero cell might be due to the synergistic effect of the phytocompounds present in it. Presence of large number of anti oxidant phytocompounds

in OLE results into cytoprotection of crystal injured Vero cells.

In this study we examined the ability of OLE to manage oxidative stress using MDA assay. Being the end product of lipid peroxidation, increased level of MDA is considered as a biological marker for increased oxidative stress [21].Cells in injury group exhibited significantly increased MDA levels, this increase was at comparable difference for COM injured ($p< 0.0001$) as well as for H_2O_2 injured group ($p< 0.0005$) when compared to control healthy group. Furthermore addition of OLE reduced the MDA levels in repair groups. Elevated MDA levels in injured group suggest that cellular injury is primarily due to oxidative stress. Antioxidants present in OLE are supposed to counteract with oxidative stress and thus retarding the lipid peroxidation leading to decreased MDA levels. Flavonoids present in *Ocimum sanctum* have already been reported to exhibit free radical scavenging activity thus inhibiting lipid peroxidation [22]. Previous studies also suggest that antioxidants can reduce the MDA concentration and thus lipid peroxidation in kidney tissues [23].

In wound healing scratch assay, treatment with OLE markedly improved the WCP as compared with control ($p < 0.0001$). This data supports that OLE is helpful in accelerating the wound closure procedure. It is postulated that wound healing capacity of OLE is due to synergistic activity of anti oxidative phytocompounds present in it. There are various reports suggesting that the wound healing capacity of a plant extract is possible due to the presence of antioxidative phytocompounds [24]. This antioxidant activity of plant flavonoids has been linked to wound healing activity of extracts [25]. Redox properties of plant derived antioxidants can be helpful in managing the oxidative damage and thus can enhance the healing of wound [26].

Conclusion

The results of this study conclude that hydroalcoholic extract of leaves of *Ocimum sanctum* (OLE), possess significant cytoprotective and wound healing abilities. Extract was also capable of reducing MDA content in oxidatively stressed conditions. Thus it can be hypothesized that OLE can be helpful in preventing COM crystal induced renal cell injury by its radical scavenging effect and wound healing potential.

Acknowledgement

We acknowledge “Physics and Materials Science Department of Jaypee Institute of Information Technology for providing FTIR facility.

Conflict of Interest

Authors declare no conflict of interest.

Funding

None

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