

# Cystone – An ayurvedic polyherbal formulation inhibits adherence of uropathogenic *E. coli* and modulates H<sub>2</sub>O<sub>2</sub>-induced toxicity in NRK-52E cells

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**Abstract:** Gentamicin is a widely used antibiotic for the treatment of adverse urinary tract infections (UTI), which in turn causes nephrotoxicity to uroepithelial cells and hence an alternative safe herbal remedy is much desired to compensate these toxic effects. The bacterial adhesion to the uroepithelial cells is the primary step in UTI and it induces various immunogenic reactions leading to the generation of reactive oxygen species (ROS), which are detrimental to the cells survival. Inhibition of bacterial adherence to urinary tract epithelial cells has been assumed to account for the beneficial action ascribed to cystone (an ayurvedic polyherbal formulation) in the prevention of UTI. In this study, we have examined the effect of cystone on the adherence of pathogenic [2-<sup>14</sup>C]-acetate labeled *Escherichia coli* (MTCC-729) to rat proximal renal tubular cells (NRK-52E cells). Further, the antioxidant property of cystone was studied using hydrogen peroxide (400 μM) as a pro-oxidant in NRK-52E cells. The results showed that cystone inhibited the adherence of *E. coli* to NRK-52E cells significantly. Additionally cystone effectively combats the toxicity induced by H<sub>2</sub>O<sub>2</sub> in NRK-52E cells. The cytoprotective effect of cystone is brought about by inhibiting lipid peroxidation by 36% in cells treated with cystone compared to H<sub>2</sub>O<sub>2</sub>- treated cells without cystone. The antioxidant enzymes catalase, glutathione were increased by 53% and 68% respectively and superoxide dismutase activity was increased 3-fold. The glutathione content was significantly increased by 2.4-fold in NRK-52E cells treated with cystone compared to H<sub>2</sub>O<sub>2</sub> control group. These results suggest that cystone effectively inhibits bacterial adherence to NRK-52E cells and attenuates H<sub>2</sub>O<sub>2</sub>-induced toxicity in NRK-52E cells by inhibiting lipid peroxidation and increasing the antioxidant defense mechanism.

**Keywords:** bacterial adhesion, lipid peroxidation, antioxidant enzymes, hydrogen peroxide, urinary tract infection

## Introduction

The ability of bacteria to adhere to epithelial cells is considered an important factor in infective virulence at mucosal surfaces throughout the body, including the gastrointestinal, respiratory and genitourinary tracts.<sup>1-4</sup> Urinary tract infection (UTI) is one of the most common infectious diseases in humans, with a prevalence strongly influenced by gender and age. UTI is common in young girls, and about half of adult women report at least one episode of UTI<sup>4</sup>, and 20%–50% of women have recurrent episodes of UTI.<sup>5</sup> The frequency of UTI increases with age in males due to bladder outflow obstructions, predominantly caused by prostatic diseases.<sup>4</sup>

The normal urinary tract is sterile. Uropathogenic bacteria often originate from the fecal flora around the perineal area.<sup>6</sup> If microorganisms overcome the bladder defense mechanisms they may colonize the lower urinary tract. Bacterial virulence factors

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and the susceptibility of the host decide the subsequent scenario.<sup>7</sup>

*Escherichia coli* is the predominant pathogen in UTI. Uropathogenic *E. Coli* are a subset of clones, with varying virulence in the urinary tract. Bacterial adherence to mucosal surfaces is thought to be a critical factor in virulence of this infection. Fecal isolates, cystitis and pyelonephritis strains differ in their adherence capacity to uroepithelial and vaginal cells.<sup>8</sup> Identification of the underlying mechanisms have shown that *E. coli* express different types of adhesion (Dr and Afa, the S and P fimbriae, and the type-1 fimbriae), that mediate binding to receptors or receptor epitopes present on the uroepithelium.<sup>9</sup> The fimbriae are thin hair-like structures, up to 2 µm long and with between 100–400 fimbriae per bacteria. Some *E. coli* strains may produce three to four different fimbriae while others produce only one. In some strains no fimbriae are produced.<sup>10</sup> Adherence factors other than Type I and the P fimbriae, are well studied, and their potential role as virulence determinants has been convincingly shown. On the other hand, the host pathogen interaction results in various immunogenic responses and generates reactive oxygen species (ROS).<sup>11</sup> The ROS is implicated in the etiology of various age related and chronic diseases such as cardiovascular, cancer, diabetes, neurological and in renal failure. It mediates a wide range of renal impairments during diabetic nephropathy, rhabdomyolysis, obstructive nephropathy, hyperlipidemia, urolithiasis and UTI.<sup>12–15</sup> The abundance of polyunsaturated fatty acids makes the kidney an organ particularly vulnerable to ROS attack. In addition renal cells are susceptible to many toxicants since the kidneys are also involved in the excretion of various toxicants.<sup>12</sup> A variety of oxidation products are found in urine, which reflects both local and systemic oxidative stress.<sup>16</sup> Urinary malondialdehyde (MDA) is increased during alpha thalassemia, renal failure and pancreatic disease.<sup>17–19</sup> However there are few studies pertaining to the direct correlation between UTI and oxidative stress.

Gentamicin an aminoglycoside along with other classes of antibiotics such as fluoroquinone, β-lactam antibiotics, and the combination of two or more of these antibiotics, are widely used for the treatment of bacterial infection. However, such treatment has been shown to cause nephrotoxicity when used long in the term<sup>20</sup> and nephrotoxicity is the predominant side effect that seriously limits their use. Gentamicin treatment causes acute renal failure with acute tubular necrosis in about 20% of patients.<sup>20</sup> In this context, an alternative, safe, traditional herbal remedy for urinary tract infections and the attenuation of host pathogen

induced ROS toxicity has a scope in the treatment of renal disorders. Many herbal-based therapies are used to treat urological disease amongst them; Chinese herbs, green tea extracts, saw palmetto, bee pollen and cranberry juice are prominent.<sup>21–23</sup>

In this context, cystone, a polyherbal formulation based on ancient ayurvedic system of medicine has been used for many years (>70 years) to treat urinary calculi and UTI. It has previously been shown that cystone is very effective in preventing the supersaturation of lithogenic substances and additionally it possesses antioxidant activity.<sup>24,25</sup> Since adherence of *E. coli* to uroepithelial cells is the first step in the pathogenesis of UTI, we hypothesized that cystone might play a significant role in the alleviating the UTI by inhibiting the bacterial adhesion to uroepithelial cells and the generation of ROS *in vitro*.

In this context, we investigated the effect of cystone on bacterial adherence to NRK-52E cells. The nephroprotective effect of cystone against H<sub>2</sub>O<sub>2</sub>-induced toxicity was studied using NRK-52E cells to ascertain the role of cystone during UTI-associated complication.

## Material and methods

### Reagents

Bradford reagent, cytochrome-C, Dulbecco's modified Eagle medium (DMEM), ferric chloride (anhydrous), fetal bovine serum (FBS), glutathione, hydrogen peroxide, MTT, NADPH, thiobarbituric acid, xanthine and xanthine oxidase were purchased from Sigma-Aldrich (St. Louis, MO, USA). All reagents were of analytical grade.

### Composition of cystone

Cystone is an approved ayurvedic proprietary medicine by the drug regulatory authority, Department of AYUSH, Ministry of Health and Family Welfare, Government of India. Five milliliters of cystone liquid concentrate contains extracts of the following medicinal plants in definite proportions: Gokshura (*Tribulus terrestris*) 91 mg; punarnava (*Boerhaavia diffusa*) 67 mg; Pashanabheda (*Saxifraga ligulata*) 53 mg; Mustaka (*Cyperus rotundus*) 42 mg; Satavari (*Asparagus racemosus*) 21 mg; Kulattha (*Dolichos biflorus*) 21 mg; Ushira (*Vetiveria zizanioides*) 21 mg and Karchura (*Curcuma zedoaria*) 14 mg. Good agricultural practices (GAP) were employed for plants used in the formulation. The plants were identified and certified by a botanist and a voucher specimen of each constituent plant has been archived in the herbarium of the Research and Development Department, The Himalaya Drug Company, Bangalore, India.

## Cell culture

NRK-52E cells (rat proximal renal tubular cell line), obtained from the National Center for Cell Science (NCCS) Pune, India, were maintained in culture in 25 cm<sup>2</sup> polystyrene flasks (Tarsons) with DMEM containing 10% FBS, 1% antibiotic-antimycotic solution, and 3.7 g/L sodium bicarbonate under an atmosphere of 5% CO<sub>2</sub> at 37 °C with 95% humidity.

## Cytotoxicity

NRK-52E cells were plated in 96-multiwell culture plates at  $1 \times 10^5$  cells per well. To study H<sub>2</sub>O<sub>2</sub>, cystone and gentamicin cytotoxicity, twenty-four hours after plating, the medium was discarded and fresh medium containing 200, 400, 600, 800 and 1000 μM H<sub>2</sub>O<sub>2</sub>, 1%, 2%, 3%, 4%, 5% cystone and 15.5, 31.2, 62.5, 125, 250 and 500 μg/mL gentamicin were added. At different timepoints, cellular viability was determined by the MTT assay.<sup>26</sup>

## Antibacterial activity assay

Antibacterial activity of test formulations/antibiotics were determined by the Kirby–Bauer disk diffusion method with limited modifications. Briefly, the test inoculum was prepared by adjusting the bacterial culture, (24 hours old) to a minimum of  $1 \times 10^5$  cfu/mL in Müller–Hinton broth solution using a McFarland nephelometer. Sterile Müller–Hinton Agar No. 2 (20 ml) was poured into petri dishes and allowed to cool in aseptic conditions. 100 μl of the test inoculum was uniformly spread onto the plates and 5 mm diameter cups were made in the plates using a sterile borer. Different concentrations of cystone and gentamicin were prepared in sterile distilled water and 50 μl of each dilution was introduced in triplicate wells, along with the test drug dilutions, and solvent controls. All petri dishes were refrigerated at 4 °C for 60 minutes for drug diffusion to occur before being incubated at 37 °C for 24 hours. Further, the minimum inhibitory concentration (MIC) of the above test compounds was determined by the liquid broth dilution method described earlier and the least concentration of both cystone and gentamicin, which inhibited the microbial growth, was taken as the MIC.<sup>27</sup>

## Bacterial culture and radioactive isotope labeling

Uropathogenic *E. coli* (MTCC 729) characterized for F7 fimbriae and uroepithelial cell adhesiveness were obtained from the microbial type culture collection (MTCC), Institute of Microbial Technology, Chandigarh, India were used in this experiment. The bacteria were adapted to

grow in Müller–Hinton broth (as described earlier) before experimentation. All viable colony counts were performed using Müller–Hinton agar. A 1.0% (v/v) inoculum was inoculated into Müller–Hinton broth and incubated overnight at 37 °C. In the morning, a 2.5% (v/v) inoculum was made into 10 mL of fresh broth containing 10 μCi of [2-<sup>14</sup>C]-labeled sodium acetate/mL (BARC, Mumbai, India). Culture growth was monitored using a McFarland nephelometer. The culture was then placed in a centrifuge and spun at 3,000 × g for 10 minutes to sediment the bacteria. The bacterial pellet was then washed thoroughly and resuspended in phosphate-buffered saline (PBS) with a pH 6.4 and the radioactivity measured to confirm the bacterial [2-<sup>14</sup>C]-acetate incorporation using a Packard scintillation counter. The labeled bacterial suspension was then suspended in PBS and added to NRK-52E cells.<sup>28</sup>

## Interaction of NRK-52E cells and bacteria

$1 \times 10^5$ /mL of NRK-52E cells were incubated with or without non-cytotoxic concentration of cystone and gentamicin with same number of [2-<sup>14</sup>C]-acetate labeled *E. coli* in duplicates for 30 minutes at 37 °C. After the incubation period the supernatant was decanted and the cell monolayer washed with PBS. The cells were then lysed using 2 mM NaOH and the radioactive counts recorded using a Packard scintillation counter.<sup>29</sup>

## Effect of cystone on lipid peroxidation, glutathione levels and antioxidant enzymes in NRK-52E cells

NRK-52E cells were plated in 60 mm culture plates at  $7.5 \times 10^5$  cells per well. Twenty-four hours after plating, the medium was discarded and fresh medium containing 400 μM H<sub>2</sub>O<sub>2</sub> and 1% cystone was added. Twenty-four hours later, the cell culture medium and cell scrapings were harvested and kept at –80 °C for the quantification of several parameters. Cell scrapings were harvested in lysis buffer (25 mM KH<sub>2</sub>PO<sub>4</sub>, 2 mM MgCl<sub>2</sub>, 5 mM KCL, 1 mM EDTA, 1 mM ethylene glycol tetra-acetic acid (EGTA), 100 μM phenylmethanesulphonyl fluoride (PMSF; pH 7.5) after rinsing the cells with PBS (pH 7.4).

## Biochemical analysis

### Lipid peroxidation

The extent of lipid peroxidation was estimated by measuring the malondialdehyde levels as thiobarbituric acid reactive substances, (TBARS) at 535 nm.<sup>30</sup> The results are expressed

as nmol/mg of protein using a molar extinction coefficient of  $1.56 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$ .

## Measurement of nonenzymic antioxidants

Cells were homogenized in trichloroacetic acid (5% w/v), and deproteinized supernatant was used for total glutathione (GSH) assay. The glutathione levels from the cell homogenates was determined by the 5,5'-dithio-bis (2-nitrobenzoic acid)-glutathione disulfide (DTNB-GSSG) reductase recycling assay as previously described<sup>31</sup> with some modifications. The results are expressed as nmol GSH/mg of protein.

## Measurement of enzymic antioxidants

The activity of antioxidant enzymes, namely superoxide dismutase (SOD), catalase and glutathione peroxidase, (GPx) were assayed in  $1000 \times g$  supernatants of cell homogenates. Total SOD activity was determined by monitoring the inhibition of the reduction of ferricytochrome C at

550 nm, using the xanthine-xanthine oxidase system as the source of superoxide. One unit of the SOD is defined as the amount of the enzyme required to inhibit 50% of the rate of cytochrome C reduction.<sup>32</sup> Catalase activity was measured by following the rate of  $\text{H}_2\text{O}_2$  consumption spectrophotometrically at 240 nm and expressed as  $\mu\text{mol H}_2\text{O}_2$  oxidized/min/mg protein.<sup>33</sup> Glutathione peroxidase activity was determined by following the enzymatic NADPH oxidation at 340 nm.<sup>34</sup>

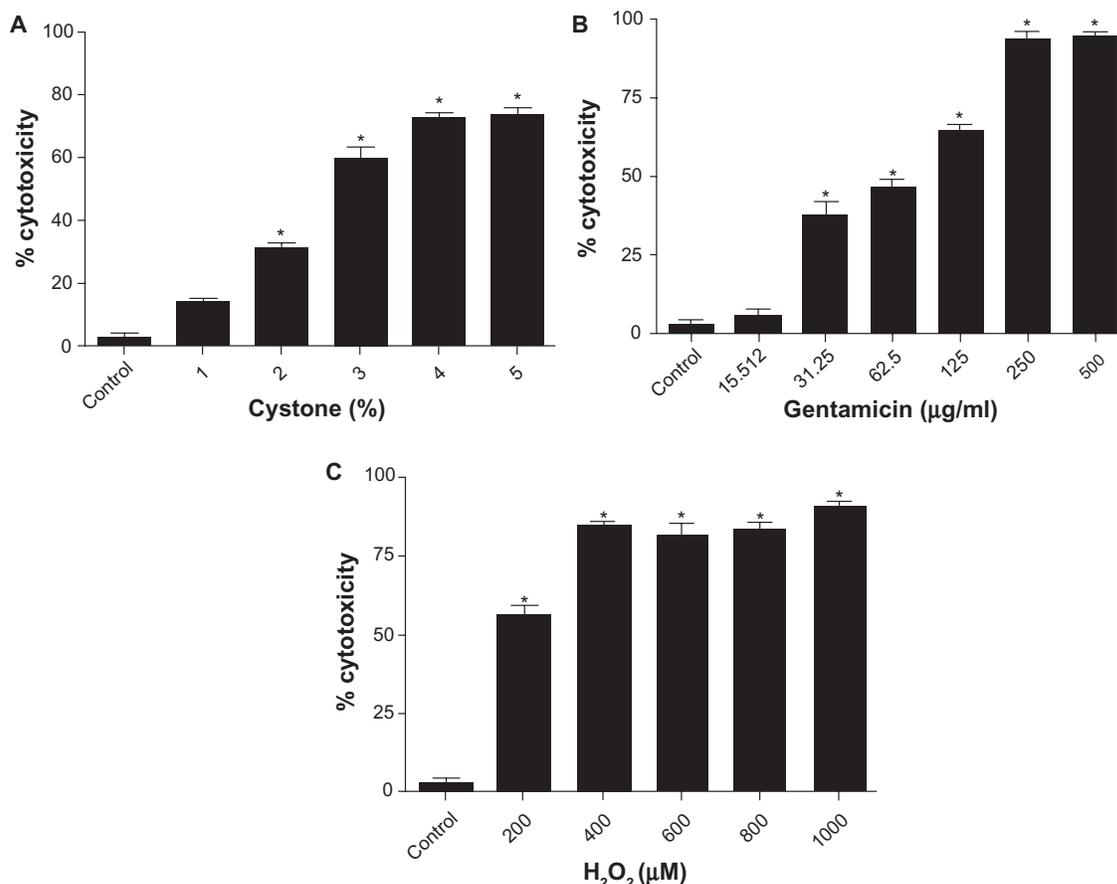
## Statistical analysis

Results were expressed as Mean  $\pm$  SEM. Statistical significances were determined using GraphPad Prism 4 (Graphpad Software Inc., Latolla, CA, USA). Results are considered to be significant at  $P < 0.01$ .

## Results

### Cytotoxicity

The results pertaining to cytotoxicity of different test compounds used in the present study are given in Figure 1. When



**Figure 1** Dose-dependent effect of (A) Cystone, (B) Gentamicin and (C)  $\text{H}_2\text{O}_2$  on NRK-52E cell viability. NRK-52E cells were treated for 24 h with or without test substances at different concentrations and the cell viability was determined by MTT.

**Notes:** Values are Mean  $\pm$  SEM of 18 samples taken from three independent experiments.

\*Significant difference at  $P < 0.01$  compared to control.

cystone was tested for its cytotoxic effect in NRK-52E cells, it was observed that exposure to >1% of cystone markedly increased the cytotoxicity Figure 1A. Hence 1% of cystone was used in all the experiments. The gentamicin induced 50% cytotoxicity at 125 µg/mL. Hence the sub-lethal concentration of gentamicin was used in the experiments Figure 1B. The H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity in NRK-52E cells and exposure to H<sub>2</sub>O<sub>2</sub> markedly increased the cytotoxicity along with the increased concentrations (Figure 1C).

## Determination of antibacterial activity

The antibacterial effect of cystone and gentamicin were determined by agar diffusion method. The wide range of concentration of gentamicin and cystone were tested for their antimicrobial activity. The results showed that cystone moderately inhibited the *E. coli* growth compared to gentamicin at the concentrations tested. On the other hand gentamicin inhibited the *E. coli* growth at all concentrations tested. The antibacterial results are given in Table 1. The MIC of gentamicin was found to be 3.12 µg/mL.

## [2-<sup>14</sup>C]-acetate labeling to *E. coli* and effect of cystone on bacterial adherence

Incubation of bacteria with [2-<sup>14</sup>C]-acetate resulted in the incorporation of the radioactive isotope labeled substrate to the bacteria. The results (Figure 2A) showed that, the amount of radioactive isotope labeled acetate incorporation

into bacteria increased rapidly with time up to ≈30 minutes incubation. Thereafter, the incorporation was slow and at ≈8 hours the maximal incorporation was recorded.

The adherence of *E. coli* to NRK-52E cells was determined by the measuring the radioactive isotope labeled *E. coli* adhering to NRK-52E cells upon co-incubation for 30 minutes. In a control test carried out before the antibiotic challenge, the isotope labeled bacterial were found to be adhering to the NRK-52E cells. Upon incubation with MIC (as determined by agar diffusion) of gentamicin the bacterial adhesion was completely inhibited. On the other hand, when incubated with 1% cystone the bacterial adhesiveness decreased significantly compared to control (Figure 2B). Upon exposure to a sub-inhibitory concentration of gentamicin the bacterial adherence was decreased significantly as compared to the control (Figure 2C). Similarly when the cystone was incubated at <1% the bacterial adhesiveness decreased but the results were not significant when compared to control (Figure 2D).

## Cytoprotection, lipid peroxidation and glutathione content

The H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity in NRK-52E cells at 400 µM concentration (Figure 3A). Upon co-incubation with 1% cystone, the cytotoxicity was significantly reduced by 73.5% showing the cytoprotective effect of cystone in NRK-52E cells. A similar effect was also observed with cells co-incubated with vitamin C. On the other hand 1% cystone could not rescue gentamicin induced toxicity in cells.

The H<sub>2</sub>O<sub>2</sub> addition resulted in the 4.15 fold increase in lipid peroxidation in NRK-52E cells (Figure 3B). Cystone inhibited the lipid peroxidation by 36% when compared to toxicant group. The GSH level was significantly depleted by 63% upon addition of H<sub>2</sub>O<sub>2</sub>. Cystone effectively inhibited the depletion of reduced glutathione levels in the cells and increased its concentration by 136% when compared to H<sub>2</sub>O<sub>2</sub>-treated cells (Figure 3C). Vitamin C was used as a control in both the experiments and results show that cystone exerts a strong antioxidant activity in NRK-52E cells.

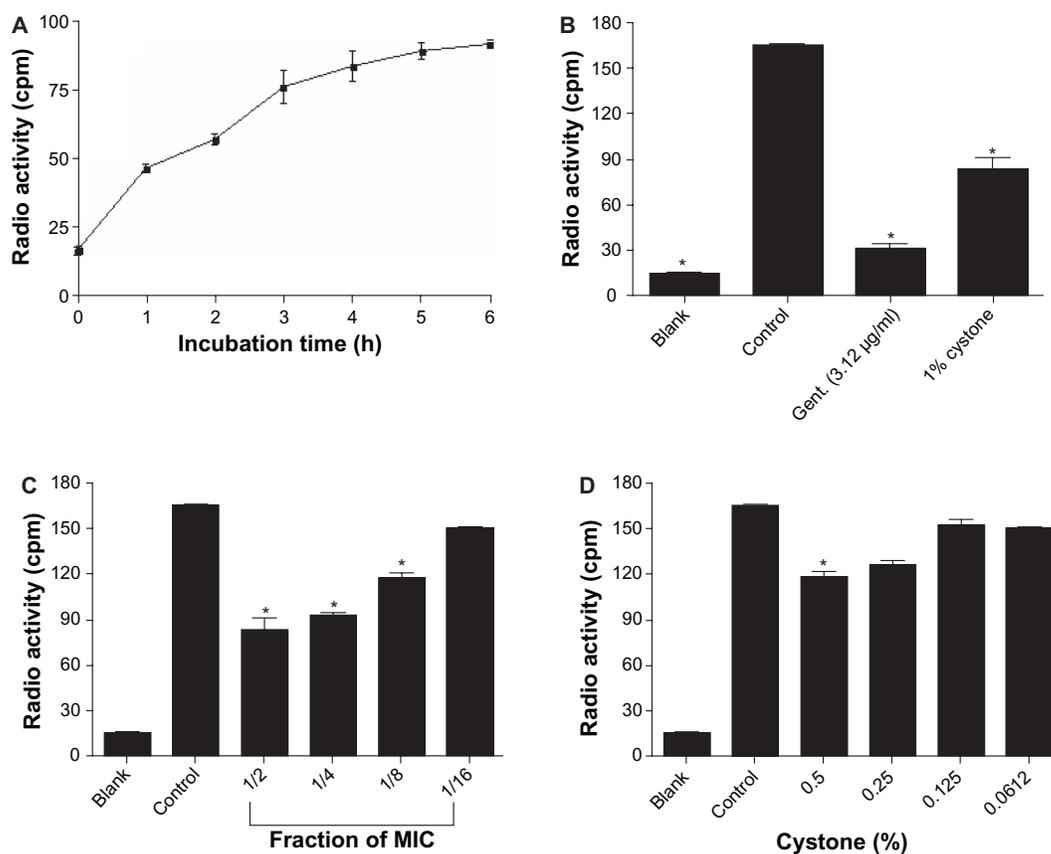
## Antioxidant enzymes

The effect of cystone in H<sub>2</sub>O<sub>2</sub>-treated cells on antioxidant enzymes was evaluated and the results are given in Table 2. In H<sub>2</sub>O<sub>2</sub>-treated cells catalase, glutathione peroxidase and superoxide dismutase activity increased significantly by 22%, 46%, and 29% respectively. On the other hand addition of cystone increased the activity of catalase and glutathione peroxidase by 53% and 68%, respectively, as compared to

**Table 1** Antibacterial activity of gentamicin and cystone on uropathogenic bacteria *E. coli* (MTCC 729)

Sample	Concentration	Zone of inhibition (mm)
Gentamicin (µg/mL)	500	45 ± 3.20
	200	43 ± 2.40
	100	40 ± 2.20
	50	39 ± 1.50
	25	37 ± 1.20
	12.5	35 ± 1.40
	6.25	34 ± 1.50
	3.12	22.13 ± 2.31
	1.56	10.23 ± 3.21
Cystone (%)	1	5.63 ± 0.96
	2	6.35 ± 0.26
	3	6.89 ± 0.85
	4	7.12 ± 0.52
	5	6.59 ± 0.69

**Notes:** Values are mean ± SEM of 18 samples taken from three independent experiments. Zone of inhibition was 0.00 in solvent control against *E. coli*.



**Figure 2** Effect of gentamicin and cystone on the adherence of *E. coli* to NRK-52E cells. **(A)** Time course of incorporation of [2-<sup>14</sup>C]-acetate to *E. coli*. **(B)** Inhibition of bacterial adhesion to NRK-52E cells by gentamicin and cystone. **(C)** Effect of sub-inhibitory concentration of gentamicin and **(D)** cystone. The labeled *E. coli* cells ( $1 \times 10^5$  *E. coli* cells/mL) were incubated with  $1 \times 10^5$  NRK-52E cells for 30 min with or without gentamicin/cystone and the results are given as described in materials and methods.

**Notes:** Values are mean  $\pm$  SEM of 18 samples taken from three independent experiments.

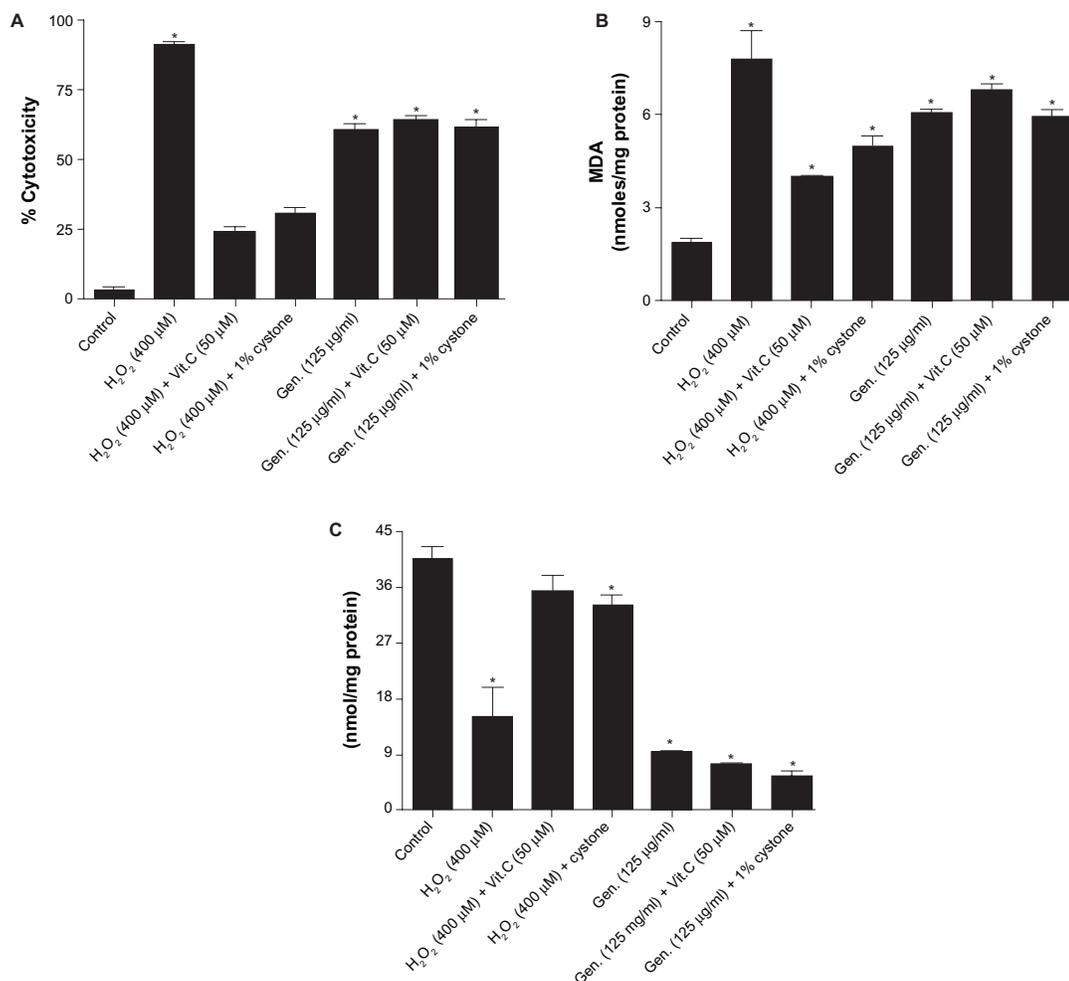
\*Significant difference at  $P < 0.01$  compared to control.

H<sub>2</sub>O<sub>2</sub>-treated cells. Similarly, cystone significantly increased the superoxide dismutase enzyme activity by 3-fold, when compared to H<sub>2</sub>O<sub>2</sub>-treated cells-respectively. Vitamin C was effective against H<sub>2</sub>O<sub>2</sub>-induced toxicity and increased the antioxidant enzyme activity at similar levels to cystone. Gentamicin treatment resulted in the depletion of antioxidant enzyme activity and neither cystone or vitamin C could replenish the enzyme activity.

## Discussion

The diagnosis and treatment of recurrent UTI is very complicated in view of the increasing prevalence of antibiotic resistant strains of *E. coli*. Hence, an alternative therapy directed at diminishing bacterial susceptibility and enhancing host defense against UTI rather than using antibacterials is of importance using alternative approaches. During UTI many immunogenic and cellular responses determine the bacterial pathogenicity and other secondary manifestations. Consequently the virulence of bacteria infecting mucosal surfaces is thought to depend on the ability of the microorganism to adhere to epithelial tissue in the body.<sup>1-4</sup>

In the present study it was clearly demonstrated that the labeled *E. coli* adhered to the renal tubular epithelial cells upon co-incubation. On the other hand it is observed that in the presence of sub-lethal concentrations of gentamicin to NRK-52E cells, the labeled *E. coli* adherence was significantly decreased suggesting that the gentamicin effectively inhibited the bacterial adherence to the renal tubular epithelial cells. When the NRK-52E cells were co-incubated with cystone the adhesion of labeled bacteria to NRK-52E cells was significantly decreased, by 50%, suggesting that cystone is effective in inhibiting the bacterial adherence to the NRK-52E cells. The results of the present study showed that both gentamicin and cystone are toxic to NRK-52E cells at higher concentrations. Hence the nontoxic concentrations of the gentamicin and cystone were used to study the bacterial adhesion using NRK-52E cells. Previously it has been reported that the colonization by uropathogens in the urinary tract is dependent on the bacterial adherence to epithelial cells and that there is increased adherence of bacteria to cells of cystitis-prone females in an *in vitro* experiment.<sup>35</sup> In another study, it was shown that adherence of *E. coli* type O4



**Figure 3** The cytoprotective and antioxidant effect of cystone in NRK-52E cells. **(A)** The NRK-52E cells were treated with or without 1% cystone along with 400 μM H<sub>2</sub>O<sub>2</sub> and Gentamicin (125 μg/mL) and the cell viability was determined by MTT assay. In another set of experiments **(B)** Lipid peroxidation and **(C)** reduced glutathione (GSH) content were measured in NRK-52E cells.

**Notes:** Values are mean ± SEM of 18 samples taken from three independent experiments.

\*Significant difference at  $P < 0.01$  compared to control.

to vaginal epithelial cells did not vary between a control and cystitis-prone women at either pH 6.4 or pH 4.0.<sup>29</sup> The advantage of using the radioactive isotope labeled method, over conventional microscopic observation techniques, in studying bacterial adhesion has been previously demonstrated.<sup>29</sup> In similar lines in the present study the *E. coli* was labeled with [2-<sup>14</sup>C]-acetate and bacterial adherence was studied. The results showed that bacterial radioactive isotope labeling was time dependent and increased with time.

Cystone showed moderate bactericidal activity unlike gentamicin. Gentamicin at all the concentrations tested showed significant bactericidal activity. But gentamicin at 3.12 μg/mL and cystone at 1% were effective in inhibiting the adherence of radioactive isotope labeled *E. coli* to NRK-52E cells. It was also observed that, at lower concentrations cystone (<1%) was effective in inhibiting the bacterial adhesion. Similarly the sub-MIC, concentrations of gentamicin was also

shown to inhibit bacterial adhesion. The results pertaining to inhibition of bacterial adherence shows the paradoxical effect of cystone in inhibiting the uropathogenic bacterial adhesion to NRK-52E cells. Along similar lines the antiadhesive effects *in vitro* of cystone may be attributed to various mechanisms: (a) Changes in bacterial shape interfering with the ability of bacteria to approach receptors on human cell surfaces; (b) the induced release of adhesins from the surface of bacterial cells; (c) the formation of nonfunctional aberrant adhesins; and (d) the inhibition of the synthesis or expression of adhesive bacterial cell surfaces, (as it was earlier demonstrated with sub-MIC's of gemifloxacin).<sup>36</sup> On the other hand it is speculated that the beneficial action of cystone is by its action on the host cell by modulating the cellular and immunological responses, which in turn may inhibit the bacterial adhesion. However, the underlying mechanisms are out of scope in the present study.

**Table 2** Effect of cystone on activity of antioxidant enzymes in H<sub>2</sub>O<sub>2</sub> and gentamicin-induced toxicity in NRK-52E cells

Groups	Catalase <sup>1</sup>	Gpx <sup>2</sup>	SOD <sup>3</sup>
Control	0.36 ± 0.11	11.54 ± 0.74	7.32 ± 0.60
1% cystone	0.44 ± 0.03 <sup>a</sup>	14.37 ± 1.14	11.0 ± 1.54 <sup>a</sup>
H <sub>2</sub> O <sub>2</sub>	0.60 ± 0.23 <sup>a</sup>	13.18 ± 1.10	32.44 ± 9.29 <sup>a</sup>
H <sub>2</sub> O <sub>2</sub> (400 μM) + Vit.C (50 μM)	0.48 ± 0.06 <sup>b</sup>	15.37 ± 1.33	19.35 ± 2.54 <sup>b</sup>
H <sub>2</sub> O <sub>2</sub> (400 μM) + 1% cystone	0.55 ± 0.13	19.44 ± 1.64 <sup>b</sup>	22.16 ± 2.82 <sup>b</sup>
Gen. (125 μg/mL)	0.24 ± 0.03 <sup>a</sup>	4.37 ± 1.14 <sup>a</sup>	11.0 ± 1.54 <sup>a</sup>
Gen. (125 μg/mL) + Vit.C (50 μM)	0.30 ± 0.23	5.63 ± 1.10	6.44 ± 9.29 <sup>c</sup>
Gen. (125 μg/mL) + 1% cystone	0.18 ± 0.06 <sup>c</sup>	2.37 ± 1.33	4.52 ± 2.54 <sup>c</sup>

**Notes:** NRK-52E cells were incubated with 400 μM H<sub>2</sub>O<sub>2</sub> and gentamicin (125 μg/mL) with or without 1% cystone and the antioxidant enzyme activity was determined as described in materials and methods. <sup>1</sup>μmoles of H<sub>2</sub>O<sub>2</sub>-decomposed/min/mg protein, <sup>2</sup>μmoles of NADPH oxidized/min/mg protein, <sup>3</sup>Units/mg protein. Values are mean ± SEM of 18 samples taken from three independent experiments. <sup>a</sup>Significant difference at  $P < 0.01$  compared to control; <sup>b</sup>Significant difference at  $P < 0.01$  compared to H<sub>2</sub>O<sub>2</sub>; <sup>c</sup>Significant difference at  $P < 0.01$  compared to gentamicin.

**Abbreviations:** UTI, urinary tract infection; MTT-thiazolyl blue tetrazolium bromide; MDA, malondialdehyde; TBARS, thiobarbituric acid reactive substances; SOD, superoxide dismutase; ROS, reactive oxygen species.

It is known that prevention of UTI by *E. coli* in mice and primates has been achieved by blocking bacterial adherence with inhibitory sugars.<sup>37,38</sup> Thus it is reasonable to assume that consumption of foods containing inhibitors of bacterial adherence might affect the infectious process. It has also been shown that although cranberry juice failed to exhibit bactericidal activity it efficiently inhibited bacterial adherence.<sup>39</sup> Along similar lines to the present study, cystone with moderate bactericidal activity may prevent adhesion of *E. coli* to NRK-52E cells. In another study it has been shown that cranberry juice has a bearing on the expression of Tamm–Horsfall glycoprotein found in urine, which is known to interfere in the bacterial adherence to mucosal cells.<sup>40</sup>

UTI is widely treated with broad-spectrum antibiotics such as gentamicin and other classes of antibiotics which may cause considerable damage (primarily to proximal renal tubular cells in both humans and animals), and induces nephrotoxicity.<sup>20</sup> In the present study it is observed that the cytotoxic concentration of gentamicin is  $>1000 \times$  higher than MIC to *E. coli*. Hence it is inferred that gentamicin induced toxicity to renal tubular epithelial cells is due to systemic administration of long term, large dosages of antibiotics in the absence of effective xenobiotic metabolism. It has been previously shown that gentamicin induces apoptosis by the generation of ROS.<sup>20</sup> Also during the host

pathogen interaction various immunogenic reactions take place to generate ROS, which in turn are responsible for the generation of various inflammatory responses.<sup>11</sup> Cystone is rich in polyphenols and known to be a very good source of antioxidants.<sup>24–25</sup> Hydrogen peroxide (400 μM) was used to study the cytoprotective and antioxidant effects of 1% cystone in NRK-52E cells. The results indicated that cystone effectively restored cell viability against H<sub>2</sub>O<sub>2</sub> toxicity in NRK-52E cells. Along similar lines, it has been previously reported that cystone is effective against cisplatin induced nephrotoxicity in rodents.<sup>24–25</sup>

It is well documented that UTI causes oxidative stress and increases lipid peroxide levels leading to the insufficiency of antioxidant enzymes.<sup>11</sup> In the present study when NRK-52E cells were challenged with H<sub>2</sub>O<sub>2</sub>, the lipid peroxides were increased by 4.15-fold and the antioxidant enzyme activity was decreased. These biochemical perturbations were effectively countered by cystone which effectively inhibited the lipid peroxidation induced by H<sub>2</sub>O<sub>2</sub> in NRK-52E cells. Similar observations have been reported previously with other phenolic compounds in various oxidative models.<sup>41</sup> Apart from reduced lipid peroxidation cystone was very effective in attenuating the toxicity caused by H<sub>2</sub>O<sub>2</sub> by increasing antioxidant enzyme activity. The superoxide dismutase activity was increased 3-fold compared to H<sub>2</sub>O<sub>2</sub> control group. Cystone also increased both glutathione peroxidase and catalase activity by 68% and 53%, respectively. Several other studies have also shown that phenolic compounds can remove oxygen free radicals, by increasing the activities of SOD, catalase and glutathione peroxidase.<sup>42</sup> In addition glutathione is the major antioxidant molecule, which determines the cell viability. From this study, it is clearly evident that cystone significantly restored depleted glutathione levels in cells treated with H<sub>2</sub>O<sub>2</sub>.

In conclusion cystone is effective in inhibiting the bacterial adherence to NRK-52E cells *in vitro* and effectively negated the toxic effects caused by H<sub>2</sub>O<sub>2</sub> in NRK-52E cells by inhibiting the lipids peroxides and increasing antioxidant defense mechanisms. Hence cystone may be beneficial while treating UTI patients with traditional antibacterial treatments.

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## Disclosures

The authors report no conflict of interest in this work.

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