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Protective Effect of Distillate and Redistillate of Cow's Urine in Human Polymorphonuclear Leukocytes Challenged With Established Genotoxic Chemicals

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Objective From the ancient period cow's urine has been used as a medicine. In Veda, cow's urine was compared to the nectar. In Susrut, several medicinal properties of cow's urine have been mentioned and are known to cause weight loss, reversal of certain cardiac and kidney problems, indigestion, stomach ache, edema, etc. However, the literature and scripture did not mention the antigenotoxic properties of cow's urine. **Methods** In the present investigation, the antigenotoxic/ antioxidant properties of cow's urine distillate and redistillate were studied *in vitro*. The antioxidant status and volatile fatty acid levels were determined. Actinomycin-D (0.1 μmol/L) and hydrogen peroxide (150 μmol/L) were used for inducing DNA strand break with 0.1% DMSO as negative control. Dose for the antigenotoxic effect of cow's urine was chosen from the dose response study carried out earlier. **Results** Both actinomycin-D and H_2O_2 caused statistically significant DNA unwinding of 80% & 75% respectively (P<0.001) as revealed by fluorimetric analysis of DNA unwinding (FADU), and the damage could be protected with the redistilled cow's urine distillate (1, 50 & 100 μL) in simultaneous treatment with genotoxic chemicals. **Conclusion** The redistillate of cow's urine was found to possess total antioxidant status of around 2.6 mmol, contributed mainly by volatile fatty acids (1500 mg/L) as revealed by the GC-MS studies. These fatty acids and other antioxidants might cause the observed protective effects.

Key words: Ammonia; Antioxidants; Cow's urine distillate; Cow's urine redistillate; Human polymorphonuclear leukocytes (PMNL's); DNA damage; Fluorimetric analysis of DNA unwinding (FADU); Gas chromatography mass spectrometer (GC-MS); Genotoxicity; Volatile fatty acids

INTRODUCTION

From the ancient period in India, cow's urine has been used as a medicine. In Veda, cow's urine was compared to the nectar (Rigveda 10.15). In Susrut several medicinal properties of cow's urine have been mentioned (45/221). Cow's urine is known to cause weight loss, and reverse certain cardiac and kidney problems, indigestion, stomach ache, edema, *etc*. The medicinal properties of cow's urine have also been mentioned in Charak

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Biographical note of the first author: Dr. K. KRISHNAMURTHI is a scientist, having an experience of more than 12 years in research and development, currently is engaged in genotoxic and carcinogenic risk assessment of hazardous waste and toxic chemicals, protective mechanism of natural products, molecular toxicology of contaminants, bioremediation of contaminated sites and biological degradation of recalcitrant chemicals and wastes.

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(sloka-100) where it is considered useful in treating renal colic, jaundice, anemia, diarrhea, gastric infection, piles and skin diseases including vitilago. It is also considered as an appetizer and is known to reverse inflammation, or a diuretic as well as a nephroprotective agent.

In Ayurveda, clinical effects of cow's urine (Gomutra) were described to counter kapha and pitta dosha. It also acts at cellular level and generates bioenergy[1]. However, the antioxidant property of cow's urine has not been reported in the literature.

Reactive oxygen species are produced in the form of superoxide radical, hydroxy radical, singlet oxygen and hydrogen peroxide as a result of biological processes and exogenous factors[2,3]. The reactive oxygen species play positive roles such as energy production, phagocytosis of intracellular signaling and synthesis of biologically important compounds. However, reactive oxygen species can also play a negative role by attacking DNA and other macromolecules thereby leading to mutation and cancer[4].

FADU is one of the sensitive techniques for measuring DNA damage induced by chemicals *in vivo* as well as *in vitro*[5] and was used in the present investigation to assess DNA damage causing potential of actinomycin-D, H_2O_2 and protection of the damage by cow's urine distillate and redistillate.

MATERIALS AND METHODS

Chemicals and Media

DMEM culture media, actinomycin-D and ethidium bromide were purchased from Sigma (St. Louis, MO, USA). Dimethyl sulphoxide (DMSO), hydrogen peroxide, trypan blue, were from Sisco Research Laboratories, India. The total antioxidant status kit was procured from Randox Laboratories UK limited. All other chemicals and solvents used in this study were of the highest analytical grade available.

Solutions for DNA Strand Break Assay

Solution A: 0.87% ammonium chloride and 10 mmol/L Tris-Hcl. Solution B: 0.25 mol/L mesoinositol, 0.01 mol/L sodium phosphate (monobasic and dibasic) pH 7.2. Solution C: 9 mol/L urea, 10 mmol/L NaOH, 2.5 mmol/L EDTA, 0.1% SDS. Solution D: 0.2 mmol/L NaOH. Solution E: 1 mol/L glucose, 14 mmol/L mercaptoethanol. Solution F: 0.6 mg of ethidium bromide dissolved in 100 mL of 10 mmol/L Tris pH 8.0.

Emergence of Ammonia in Cow's Urine Following Storage

Cow's urine after collection was stored in closed containers for unspecified time prior to distillation. If this storage time was long, it resulted in production of ammonia due to microbial activity (microbial biohydrolysis of urea present in urine).

Distillate of Cow's Urine

Cow's urine was distilled at 100℃ using a temperature-controlled distillation apparatus. A portion of the single distilled cow's urine was then acidified by lowering the pH below 2.0 with the addition of 85% orthophosphoric acid and distilled again at 100℃ using a temperature-controlled distillation apparatus to remove ammonia present in the distillate. Both single distillate and redistillate were used for the antigenotoxic study.

Antioxidant Status

Total antioxidant status of cow's urine distillate and redistillate was detected by using a kit provided by Randox Laboratories, UK. In this assay, ABTS® (2,2'-Azino-di-[3-ethylbenzthiazoline sulphonate]) was incubated with a peroxide (metmyoglobin) and H_2O_2 to produce a radical cation $ABTS^{®*+}$. This had a relatively stable blue green in colour, which was measured at 600 nm. Antioxidants in the added sample caused suppression of this colour production to a degree proportional to their concentration.

Ammonia Estimation

Ammonia in cow's urine generated at various detention time (0-19 h) was quantified using standard method 20th edition APHA, AWWA and WPCF (1998).

Volatile Fatty Acid Estimation, Extraction and Esterification

The estimation of volatile fatty acids was carried out according to the protocol given in the 20th edition of the standard method APHA, AWWA and WPCF (1998). The cow's urine was extracted and esterified for the identification of volatile fatty acids using gas chromatography and mass spectrometer[6].

Volatile Fatty Acid Analyses

The analysis of volatile fatty acid in the redistilled cow's urine was carried out by gas chromatography-mass spectrometer (GC-MS, Saturn Model Varian) with capillary column 50 Q c2 / FFAP, MS column (M/s. J &W Scientific, USA 50 m×0.22 mm id×0.25 μ) and helium gas as carrier. The injector temperature was 150℃. The oven was programmed at 40℃ for 2 min, raised to 200℃ at 10℃/min. The fatty acid and its derivatives were identified by computer search of the National Institute of Science & Technology (NIST-1998) Library of Mass Spectra on the basis of retention time and mass fragmentation pattern.

Cytotoxicity Test

Cytotoxicity of distillate and redistillate was tested using tryphan blue assay[7]. Approximately 1×10^6 human polymorphonuclear leukocytes were incubated with different concentrations such as 1, 10, 25, 50, 100, and 200 μL of distillate and redistillate for different time intervals up to six hours.

Fluorimetric Analysis of DNA Unwinding (FADU)

Human polymorphonuclear leukocytes having a density of $5.0\text{-}6.0\times10^6$ cells/mL were treated with actinomycin-D (0.1 μmol/L) and H_2O_2 (150 μmol/L) as positive controls, 0.1% DMSO (negative control) and different concentrations of distilled and redistilled cow's urine (1 μL, 50 μL, and 100 μL). For protective study the positive controls were simultaneously treated with different concentrations of distillate and redistillate (1 μL, 50 μL, and 100 μL) for 1 h at 37℃. The treatment was terminated by the addition of ice cold saline (0.9% NaCl). The treated and control cells were centrifuged at 400×g for 10 minutes at 4℃ and resuspended in solution B and the volume was made up to 2.0 mL. The suspended peripheral leukocytes were processed for FADU assay as described by Birnboim & Jevcak (1981)[8] modified by Krishnamurthi *et al*. (2003)[9].

Statistical Analysis

The results were statistically analyzed using ANOVA one way test with "Analyze it software" and expressed as *P* values, which were not considered significant when $P > 0.05$.

RESULTS

Experimental analysis revealed that ammonical nitrogen (NH_3-N) levels in cow's urine collected freshly was between 50 mg/L and 100 mg/L. However, when it was stored for 18-19 h, the concentration of NH_3-N reached 1000-2000 mg/L (Fig. 1**)**. Ammonia, being volatile, appeared in the distillate of cow's urine following microbial biohydrolysis of urea present in the fresh urine. This observation prompted the investigators to redistill the above distillate in the presence of 85% orthophosphoric acid at pH<2.0 to obtain a redistillate whose ammonical nitrogen (NH_3-N) content was around 15 mg/L.

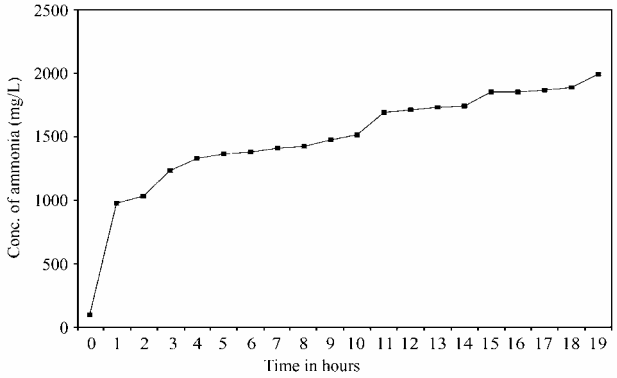


FIG. 1. The values are average of three sets of experiments.

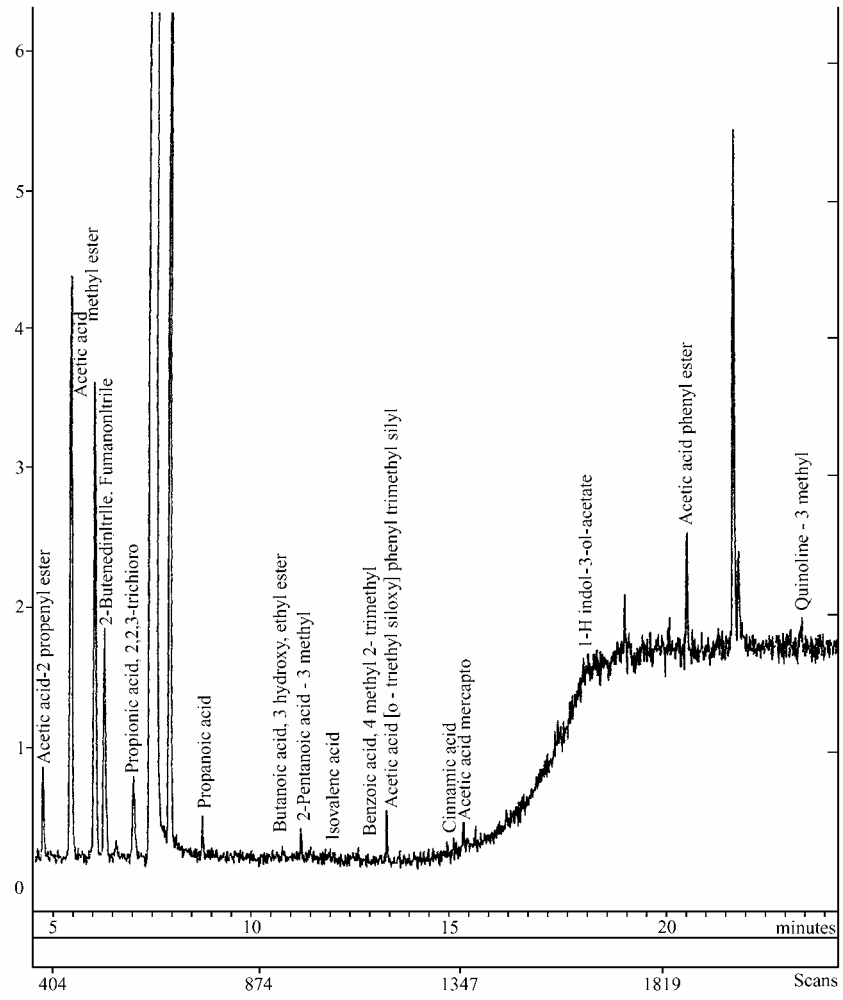
The volatile fatty acid concentration in redistillate of cow's urine was found to be around 1500 mg/L. The literature survey revealed that the volatile fatty acids were known as a potent antioxidant[10]. Further studies were carried out to determine the antioxidant status of the cow's urine distillate and redistillate, the total antioxidant status was found to be 0.8 mmol in distillate and 2.6 mmol in redistillate.

TABLE 1

Compounds Identified in Redistillate of Cow's Urine by Gas Chromatography Mass Spectrometer (GC-MS)

Sr. No.	Compound Name	RT	Scan No.
1.	Acetic Acid- 2propenyl Ester	4.739	379
2.	Acetic Acid Methyl Ester	6.032	501
3.	Propioinc Acid, 2,2,3-trichloro	6.954	588
4.	Propioinc Acid	8.843	765
5.	Butanoic Acid-3 Methyl, Propyl Ester (Valeric Acid)	10.234	898
6.	Butanoic Acid, 3 Hydroxy, Ethyl Ester	10.558	927
7.	Butanoic Acid, 3 Methyl Propyl Ester(Iso Valeric Acid)	11.828	1047
8.	Acetic Acid[o-(triethyl siloxy)] Triethyl Silyl	13.161	1173
9.	Propionic Acid, 3 Phenyl (Cinnamic Acid)	14.834	1331
10.	1-H indol-3-ol-acetate	18.073	1637
11.	Acetic Acid Phenyl Ester	20.646	1880
12.	Quinoline, 3-methyl Thio	24.879	2281

Table 1 and Fig. 2 show the reconstituted total ion chromatogram and mass spectrum of the individual compounds present in the redistillate. Figs. 3 and 4 show the cytotoxicity effect, if any, of distillate and redistillate in human polymorphonuclear leukocytes (PMNL's). The results showed that the distillate was cytotoxic at all the concentrations tested (1 µL, 10 µL, 25 µL, 50 µL doses) at the 3rd h to the 5th h following exposure, whereas the 100 µL and 200 µL doses caused significant cytotoxicity from the 2nd h onward upon exposure. However, the redistillate was not found to be cytotoxic at all the concentrations tested in the present study. Fig. 5 shows the DNA strand breaks induced by the distillate and redistillate in human polymorphonuclear leukocytes. The distillate caused significant DNA damage. However, the redistillate did not cause significant DNA strand break at the dosages tested in this study, which merited redistillate as non-genotoxic. The distillate of cow's urine at 1 µL, 50 µL, and 100 µL showed respectively 53%, 55% and 86% of DNA strand break compared to the negative control showing 24% strand break. The redistillate of cow's urine at 1 µL, 50 µL, and 100 µL showed respectively 25%, 27% and 30% of DNA strand break following one-hour exposure.



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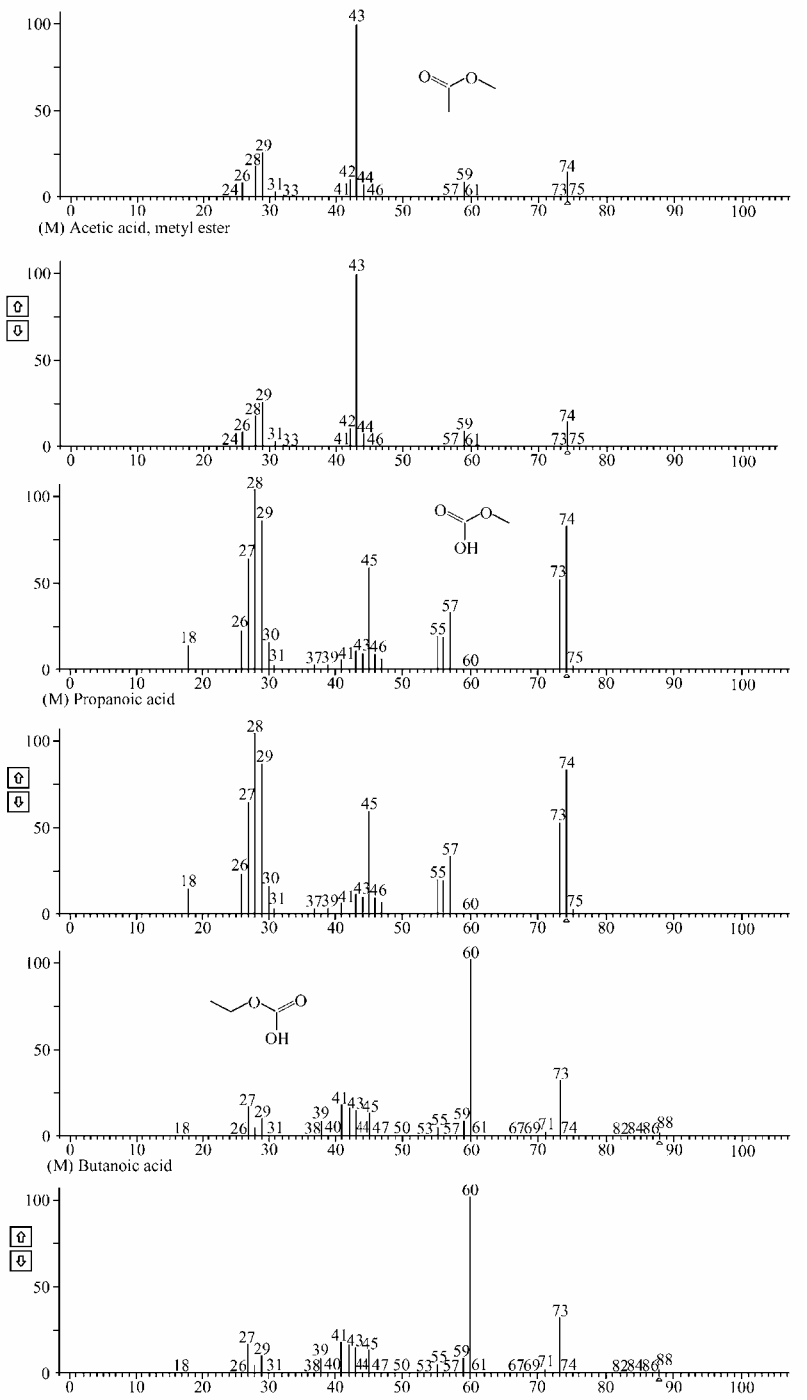

FIG. 2. Reconstituted total ion chromatograph and mass fragmentation pattern of redistillate showing volatile fatty acids.

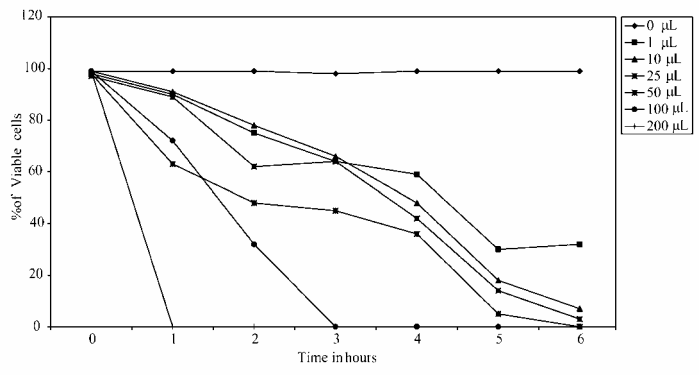

FIG. 3. The results are average of five sets of experiments.

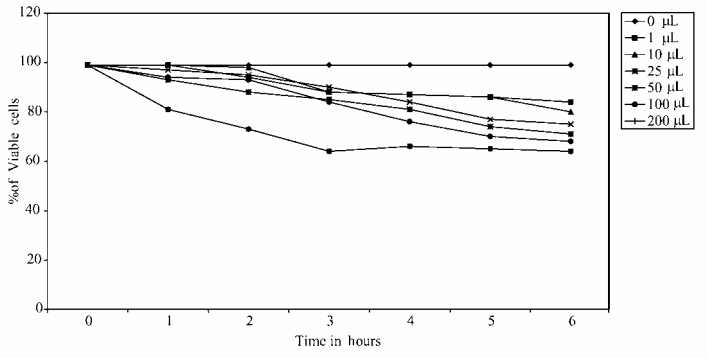

FIG. 4. The results are average of five sets of experiments.

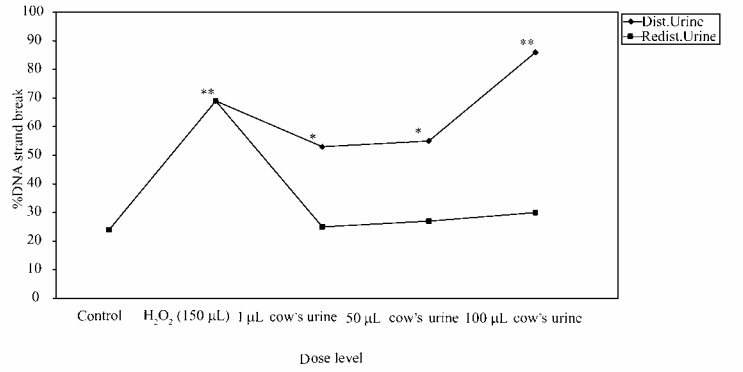

FIG. 5. The results are average of five sets of experiments, level of significant protection of DNA strand break was calculated against positive control and expressesd as P value. $^{*}P<0.01$, $^{**}P<0.001$.

The DNA damage induced by hydrogen peroxide was studied and the maximum level of DNA damage induced by hydrogen peroxide at a concentration of 150 µmol/L was 69%. Actinomycin D on DNA strand break was studied at a dose level of 0.1 µmol/L, its maximum DNA damage level was around 62%. Figs. 6 and 7 show the protective effect of the distillate and redistillate against genotoxic chemicals such as hydrogen peroxide and actinomycin-D following simultaneous treatment. The distillate had no significant protective

effect on DNA strand break caused by actinomycin-D and H_2O_2, whereas, the redistillate provided significant (P<0.001) protection against the gentoxicants induced DNA damage.

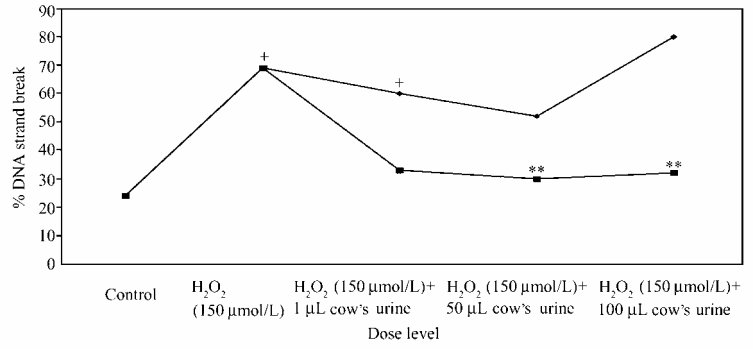

FIG. 6. The results are average of five sets of experiments, level of significant protection of DNA strand break was calculated against positive control and expressesd as P value. $^{*}P<0.01$, $^{**}P<0.001$. Significant DNA strand break was calculated against negative control $^{+}P<0.001$.

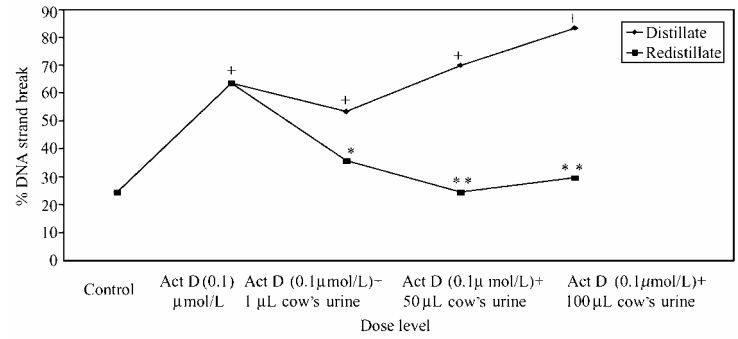

FIG. 7. The results are average of five sets of experiments, level of significant protection of DNA strand break was calculated against positive control and expressesd as P value. $^{*}P<0.01$, $^{**}P<0.001$. Significant DNA strand break was calculated against negative control $^{+}P<0.01$.

DISCUSSION

The availability of fresh urine for distillation is not always possible, as often it needs to be stored for some time prior to distillation. The stored urine has, therefore, high alkaline pH of around 9-10 because of microbial biohydrolysis of urea over a period of time. In the laboratory pH of the distillate was reduced to less than pH 2.0 by the use of 85% orthophosphoric acid.

Our results indicated that treatment of polymorphonuclear leukocytes with H_2O_2 and actinomycin-D along with the redistillate of cow's urine showed 60%-77% protective effects (P<0.001). On the other hand, the distillate had no statistically significant protective effect against the chemical treatment. This inability to cause protection by the distillate was attributed to the presence of toxic ammonia. Actinomycin-D was used to cause DNA damage, as it had been used as an anti tumor drug[11]. The rationale behind selection of H_2O_2 and actinomycin-D was that actinomycin-D was known to cause the production of reactive

oxygen species and hydrogen peroxide itself was a free radical. The phenoxasone chromophore region of actinomycin-D intercalated between bases in the DNA. As a result, the 2 amino group of the guanine formed a stable actinomycin-DNA complex[12]. This complex could prevent the unwinding of DNA to facilitate the interaction with RNA polymerase by preventing the synthesis of RNA via the DNA dependent RNA polymerase and this blockage was responsible for the cytotoxic effect in any phase of the cell cycle[13].

Literature shows that H_2O_2 could induces oxidative damage to the DNA base pairs[14] as a result of the generation of free reactive oxygen species produced by Fenton type reaction due to the interaction between metal ions like Fe (II) and H_2O_2. This type of mutation occurred because of base mispairing at sites of cytosine dimers produced by free $OH^{\cdot}$[15] resulting in the modification of DNA bases which may lead to DNA protein cross-link[16,17].

The antigenotoxic effect of cow's urine redistillate was studied at different concentrations such as 1 µL, 50 µL, and 100 µL as the doses did not show any significant cytotoxicity. Generally the oxidative damage could be prevented/protected by enzymes present in the system such as glutathione peroxidase (GPX), glutathione reductase (GR) and super oxide dismutase (SOD), etc.[18]. In our *in vitro* experimental system, however, the damage caused by H_2O_2 and actinomycin-D could not be prevented by the normal cellular mechanism. With the treatment of redistillate, however the damages caused by genotoxicants in the form of DNA strand breaks were significantly prevented.

The GC MS data also revealed that the redistillate of cow's urine contained a large number of volatile fatty acids and the total antioxidant kit test showed the presence of about 2.6 mmol of antioxidants, which might play a role in the prevention/protection of the free radicals mediated DNA strand breaks due to the established genotoxicants used in the present study.

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