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EXTRACTION OF URSOLIC ACID FROM *OCIMUM SANCTUM:* MODULATORY EFFECT OF URSOLIC ACID ON HYDROGEN PEROXIDE INDUCED DNA DAMAGE IN HUMAN LYMPHOCYTES

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Keywords:

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ABSTRACT: The objective of the present study was to i) Extract ursolic acid , a pentacyclic triterpenoid from *Ocimum sanctum*; ii) develop a HPTLC based method for its quantification; and iii) investigate ursolic acid dependent adaptive response to cytotoxic effect of hydrogen peroxide. To elucidate the possible underlying mechanism(s), lymphocytes were co-incubated with hydrogen peroxide (0, 10, 25 and 100 μM) and ursolic acid (20 and 40 μg/ml incubation medium). After 2 h of incubation we investigated the profile of DNA strand breaks, intracellular reactive oxygen species (ROS), apurinic/apyrimidinic sites (AP sites) and 8-hydroxy-2'-deoxyguanosine (8-OH-dG) residues in DNA. Results showed that ursolic acid inhibited hydrogen peroxide induced DNA strand breaks. Ursolic also showed significant free radical scavenging as indicated by reduced ROS and decreased AP sites. The formation of 8-hydroxy-2'-deoxyguanosine (8-OH-dG) in lymphocyte DNA was also inhibited. These findings clearly showed mechanistic basis for protective effect of ursolic acid against intracellular ROS and consequent generation of AP sites in genomic DNA. In conclusion, our studies clearly suggest that ursolic acid might be a possible chemo-preventive phytochemical against oxidative stress.

INTRODUCTION: Exposure to environmental xenobiotics is often unavoidable and creates a great risk to human health [1]. Intake of sufficient amounts of antimutagens is believed to confer a preventive effect against environmental mutagens [2, 3]. Over the last two decades, an expanding body of evidence has demonstrated that edible plants as a whole, or their identified phytochemical ingredients, have substantial protective effects on onset of diseases such as carcinogenesis [4].

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Studies have demonstrated that phytochemicals taken in isolation show adequate protective effects against mutagenesis [5]. Phytochemicals are secondary metabolic products produced by plants in response to environmental stresses [6]. Many protective effects of phytochemicals have been ascribed to their potential to destroy reactive oxidative species (ROS) that initiate carcinogenesis through oxidative damage of DNA [7].

In this context, plant triterpenoids are also well recognized for their antioxidant activities [8]. These compounds scavenge free radicals and disrupt the free-radical chain reaction of lipid peroxidation, protect cell constituents against oxidative damage and therefore, limit the risk of various diseases associated with the oxidative stress [9]. Ursolic acid

(3ß hydroxy urs-12 en – 28 oic acid) is one such a triterpenoid found in plants of *Ocimum sanctum*. It has been reported to possess a wide range of pharmacological properties. Many protective effects of *O. sanctum* are determined by strong antioxidative properties of its constituent ursolic acid [10]. Since so far it is not known whether antioxidant effect of ursolic acid could protects the genomic DNA of cells, the present study was planned to unravel the effect of ursolic acid on hydrogen peroxide (H_2O_2) induced DNA damage in lymphocytes. In particular, we focused on its effects upon purine/pyrimidine free sites (AP sites) on DNA and oxidative damage to guanine base as biomarker.

Lymphocytes are known to be highly sensitive to oxidative stress [11]. The protective effect of ursolic acid on mitigation of intracellular ROS level and reduction of AP sites might be determined to ascertain potential protective mechanisms. It has been well established that biological effects of H_2O_2 induce formation of ROS, which are known to cause oxidative stress to DNA, cause 8-hydroxydeoxyguanosine (8-OH-dG) formation and DNA strand breaks [12]. H_2O_2 induced free radicals react with DNA and inflict damage to purine and pyrimidine bases [13].

Various H_2O_2 induced biochemical changes occur in cells long before the loss of integrity [14]. In this study, we focused on 8-hydroxy-2′-deoxyguanosine (8-oxo-dG), a DNA metabolite, as a marker for oxidative sensitivity. Guanine is the highly susceptible target for oxidative reactions because of its low redox potential [15-17]. Moreover, 8-oxo-dG is potentially mutagenic because of its ability to form base pairs with both cytosine and adenine [18]. Thus, cellular 8-oxo-dG levels are one of the most mutagenic lesions, and the most abundant source of AP site in genomic DNA.

Delayed repair of AP sites can also result in replication induced double strand breaks (DSB). Therefore reducing AP sites in DNA may be an approach to decrease the adverse effects of oxidative stressors such as H_2O_2.

One of the objectives of present work was to isolate and quantify ursolic acid from *O. sanctum.* As most plant extracts are composed of complex phytochemical constituents, proper method is particularly desired for extraction of desired bioactive constituent from the extract of the plant. Our other objective was to evaluate anti-genotoxic effect of ursolic acid using human lymphocytes as model system. Existing evidence suggests that antioxidants of plant origin may reduce oxidative damage to DNA. We therefore asked whether ursolic acid has the potential to mitigate H_2O_2 induced cellular ROS, AP sites and 8-oxo-dG residues in DNA. Development of new therapeutic strategies using ursolic acid as antioxidant agent rests heavily upon the elucidation of its effect on DNA repair reactions.

MATERIAL AND METHODS:

Materials and Reagents: *O. Sanctum* whole plant powder was purchased from "Total Herb Solutions Pvt. Ltd., Mumbai". The OS powder size used was 0.50–1.00 mm. Dulbacco's modified Eagle's medium (DMEM) for culturing human lymphocytes, dimethyl sulfoxide (DMSO), β-nicotinamide adenine di-nucleotide phosphate and hydrogen peroxide were purchased from Sigma (St. Louis, MO, USA). Penicillin, streptomycin, and phytohemagglutinin M were purchased from GIBCO, Invitrogen Corporation (UK). Heat-inactivated fetal bovine serum was purchased from Life Technology (UK). All solvents were purchased from Hi Media Ltd., Mumbai, India. Standard UA was purchased from Sigma Aldrich Chemical Company, USA. All the other reagents were purchased from reputed manufacturers and were of reagent grade.

Extraction and quantification of UA: The methanolic crude extract obtained was suspended in $CHCl_3$: MeOH (9:1) and subjected to gradient column chromatography by hexane, ethyl acetate and methanol in increasing polarity. The fractions were collected and monitored on TLC and tested for the presence of UA. And those fractions, which consisted of UA, were combined together and the solvent was removed under vacuum. Evaporation of the combined fractions containing UA led to a white solid. The isolated UA was well characterized by melting point, U.V. and I.R. spectra as well as by comparing with an authentic sample in TLC and HPTLC. Amount of UA in the

extract was calculated by TLC using optical densitometry, with known concentrations of the samples and of commercial UA. Different parameters affecting the extraction such as extraction time, solute to solvent ratio, extraction temperature were optimized and the final extraction experiment at the optimized conditions. The UA was quantified using Camag HPTLC apparatus with spotting device-linomat V Automatic Sample Spotter (Camag, Muttenz, Switzerland); Syringe-100µL (Hamilton), HPTLC chamber.-Glass twin trough chamber for 20× 10cm plates (Camag); Densitometer.-TLC Scanner with wincats software (Camag); HPTLC plates-20 × 10cm, pre-coated with silica gel 60, 0.2mm layer thickness (Merck, Darmstadt, Germany).

A stock solution of ursolic acid (90% pure, 72µg/mL) was prepared by dissolving 2mg of accurately weighed ursolic acid in methanol and diluting to 25mL with methanol. Aliquots (1 to 8mL) of stock solution were transferred to 10mL volumetric flasks and diluted with methanol to obtain standard solutions containing 7.2, 14.4, 21.6, 28.8, 36, 43.2, 50.4, and 57.6µg/mL ursolic acid, respectively. 10µL each of the standard solutions of ursolic acid (72 to 576 ng/spot) were applied and HPTLC was performed. After development, the plates were dried at room temperature in air, derivatized with liebermann burchard reagent and scanned at 530nm in absorbance mode using the tungsten lamp.

The peak areas were recorded. Calibration curve of ursolic acid was obtained by plotting peak areas vs applied concentrations of ursolic acid. 20µL of suitably diluted sample was applied in triplicate on an HPTLC plate. The plate was developed and scanned as described above. The peak areas and absorption spectra were recorded, and the amount of ursolic acid was calculated using its calibration curve.

Spectroscopic methods: ^{1}H-NMR spectra were obtained with a JEOL JNM-AL400 NMR spectrometer. Chemical shifts were referenced to the solvent peak as an internal standard.

Isolation of lymphocytes and experimental design of co-incubation experiments: Blood samples were obtained from five donors (Three male and two female, healthy, non-smokers, 20–30 years old). Around 0.5 ml of human venous blood was added to 3.5 ml of DMEM (human lymphocyte culture media) supplemented with 20% of fetal bovine serum to which phytohemagglutanin (PHA) (50µg/ml), antibiotics (Penicillin 100 IU/ml and streptomycin 50µg/Ml) and heparin sodium salt, 5000 IU (0.4 Ml/100 ml) were added and incubated at 37^0 C for up to 72 h.

Lymphocytes yield was usually 10^6 per ml of blood with viability above 96% (approx). After 24 hours of culture initiation, lymphocytes were treated with ursolic acid at different doses (20 and 40 µM) and hydrogen peroxide (10, 50 and 100 µM) to study their effects on DNA damage. For protective effect, studies were carried out as follows:

Group 1; Sham control Lymphocytes incubated with 0.05 % DMSO
Group 2; UA + H_2O_2 Lymphocytes exposed to UA (20 µg/ml media) and 10 µM H_2O_2
Group 3; UA + H_2O_2 Lymphocytes exposed to UA (20 µg/ml media) and 25 µM H_2O_2
Group 4; UA + H_2O_2 Lymphocytes exposed to UA (20 µg/ml media) and 50 µM H_2O_2
Group 5; UA + H_2O_2 Lymphocytes exposed to UA (20 µg/ml media) and 100 µM H_2O_2
Group 6; UA + H_2O_2 Lymphocytes exposed to UA (40 µg/ml media) and 10 µM H_2O_2
Group 7; UA + H_2O_2 Lymphocytes exposed to UA (40 µg/ml media) and 25 µM H_2O_2
Group 8; UA + H_2O_2 Lymphocytes exposed to UA (40 µg/ml media) and 50 µM H_2O_2
Group 9; UA + H_2O_2 Lymphocytes exposed to UA (40 µg/ml media) and 100 µM H_2O_2

For all experiments lymphocytes were exposed for 2 h. Ursolic acid was dissolved in DMSO at a concentration of 1000µM with subsequent dilutions in culture medium. For sham control DMSO was diluted in medium to a concentration corresponding to the 0.05 % DMSO in H_2O_2-treated groups.

Reduced glutathione (GSH) Estimation: Level of reduced glutathione was estimated as total non-protein sulphydryl group in the cell homogenates after precipitating the proteins by 5% tricholoroacetic acid (TCA). The supernatant was

mixed with 0.2 M phosphate buffer (pH 8) and 0.6 M 5, 5′-dithio-bis (2-nitrobenzoic acid) and allowed to stand for 8-10 min at room temperature. The absorbance was recorded at 412 nm using a spectrophotometer (Thermo Scientific, UV 10), and level was calculated as nMole of -SH content/mg protein from standard curve made with reduced glutathione (GSH) and finally expressed as percentage change of GSH level [19].

Protein Estimation:
The amounts of protein present in the sample were estimated using bovine serum albumin (BSA) as standard by using Folin reagent [20].

Fluorimetric Analysis of DNA Unwinding (FADU) to quantify strand breaks: The modified procedure for FADU was followed with minor modifications. Human lymphocytes at the concentration of 5×10^6 cells/ml were treated with 0.05 % DMSO control or different combinations of ursolic acid and hydrogen peroxide in a final volume of 1 ml for 2 hour. To study the ameliorative effect of UA on DNA strand breaks induced by H_2O_2, the lymphocytes were treated with H_2O_2 plus UA for 1 hour in 5% CO2 incubator at 37°C. The treatment was terminated by the addition of 5 ml ice-cold saline (0.9 % NaCl).

The treated and control cells were centrifuged at 1000 rpm for 10 minutes at 4°C, pellet was obtained and re-suspended in solution B and the volume was made up to 2.0 ml. The suspended lymphocytes were processed for FADU assay. In brief, measurement of DNA strand breaks by FADU is based on partial denaturation (unwinding) of double-stranded DNA under controlled alkaline and temperature conditions. DNA strand breaks are sites where unwinding of DNA can start. Briefly, after infliction of DNA damage, cell lysis was performed. Adding a neutralising solution terminated unwinding. To quantify the amount of DNA remaining double stranded, a commercially available fluorescence dye (SybrGreen) was used as a marker for double-stranded DNA.

Measurement of intracellular reactive oxygen species (ROS): Lymphocytes were loaded with 5 lM 20, 70-dichlorofluorescin diacetate (DCFH-DA) for 1 h. Following washing with DPBS, ROS levels were determined by measuring the fluorescent intensity at excitation wavelength 485 nm and emission wavelength 530 nm using Fluorescence microplate reader.

Quantification of purine/pyrimidine free sites (AP sites) in genomic DNA: Genomic DNA was isolated from lymphocytes using geneispin mammalian genomic DNA prep kit (Bangalore genei Pvt. Ltd. Peenya, Bangalore). AP sites in DNA were quantified using BioVision DNA damage quantification kit (Biovision Research Products, California, USA).

8-oxo-dG Quantification:
H_2O_2 induced oxidative stress was evaluated by competitive enzyme immunoassay kit (Cayman Chemical Company, Ann Arbor, MI, USA) that measured 8-oxo-dG, a marker of oxidative DNA damage, in the liver. Briefly, genomic DNA was extracted (Bangalore Genei Pvt. Ltd. Peenya, Bangalore, India) and suspended in 135 µl of 20 mM sodium acetate (pH 4.8). DNA was digested to nucleotide with nuclease P1 (40 U/ml) at 37 °C for 1 h. Then, 15 µl of 1 M Tris/HCl (pH 7.4) was added to the samples and they were then treated with alkaline phosphatase (25 U/ml) at 37 °C for 1 h.

The 8-oxo-dG assay in the digested DNA solution was based on the competition between 8-oxo-dG and 8-oxo-dG acetylcholinesterase (AChE) conjugate (8-oxo-dG tracer) for a limited amount of 8-oxo-dG monoclonal antibody. The 8-oxo-dG levels were expressed as 8-oxo dG (ng/ml)/DNA (µg/µl).

Statistical analysis:
All values were expressed as mean ± standard error of means of samples with n and p indicated in the Results section. Statistical analyses were performed using Student's t-test (GraphPad QuickCalcs, San Diego, California, USA). Values with $P < 0.05$ were considered statistically significant.

RESULTS:
Extraction and quantification of ursolic acid: Preliminary experiments showed that ursolic acid was available in free form. Consequently, ursolic acid was quantified from the methanolic extract.

The content of ursolic acid in methanolic extract of *O. sanctum* was quantified at 0.926 % w/w (Rf: 0.51). 20 and 40 µg per ml media concentrations of purified ursolic acid were used for their anti-oxidative effect against 10, 25, 50 and 100 µM concentrations of hydrogen peroxide in medium containing lymphocytes as shown in **Figure 1**.

The ^{1}H NMR spectrum (DMSO-d6) of ursolic acid contained the signals for seven skeletal methyl groups of which five were singlets at δ 0.69 (3H, s, H-24), 0.73 (3H, s, H-26), 0.87 (3H, s, H-25), 0.89 (3H, s, H-23), 1.07 (3H, s, H-27) and two were doublets at δ 0.78 (3H, d, J = 5.9 Hz, H-29) and 0.96 (3H, d, J = 6.8 Hz, H-30). The signal for H-5 and H-9 appeared as singlet at δ 0.65 and 1.55 respectively.

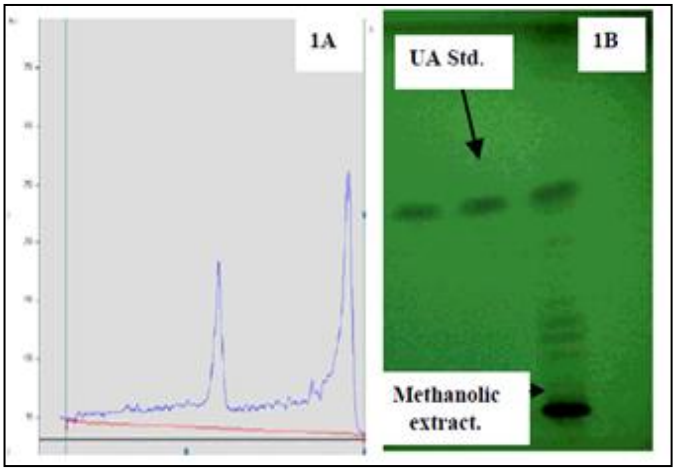

FIG.1: EXISTENCE OF URSOLIC ACID IN *O. SANCTUM* SPECIES AS INDICATED BY HIGH PERFORMANCE-TLC (HPTLC) ANALYSIS OF METHANOLIC EXTRACT. 1A) DENSITOGRAM OF URSOLIC ACID IN METHANOLIC EXTRACT WHEREIN URSOLIC ACID FROM METHANOLIC EXTRACT SHOWED RF VALUE OF 0.51; 1B) URSOLIC ACID IN METHANOLIC EXTRACT ALONG WITH PURE UA STANDARD. THE CONTENT OF URSOLIC ACID IN METHANOLIC EXTRACT OF *O. SANCTUM* WAS QUANTIFIED AT 0.926 % W/W.

The signal for olefin proton and proton at position 3 appeared as doublet of doublets at δ 5.11 (1H, dd, *J* = 13.7; 3.5 Hz; H-12) and 2.98 (1H, dd, *J* = 5.2; 9.5 Hz, H-3). Multiplet signals were observed for each two protons at δ 1.55 (2H, m, H-16), 1.57 (2H, m, H-1), 1.56 (2H, m, H-22), 1.45 (2H, m, H-2), 1.25 (2H, m, H-21), 1.23 (2H, m, H-7) and 1.02 (2H, m, H-15). Doublet of doublets was also observed for two protons at position 11 with different coupling constants at δ 2.09 (2H, dd, *J* = 13.7; 3.5 Hz, H-11). Further signals at δ 2.48 (1H, d; *J* = 11.1 Hz, H-18), 1.51 (1H, m, H-20), 1.49 (1H, m, H-6a), 1.28 (1H, m, H-19), 1.26 (1H, m, H-6b) were observed in the spectrum.

Ursolic acid mitigated oxidative strand breaks in DNA: Fig. 2 shows the protective effect of ursolic acid against oxidative stress induced by H_2O_2 in lymphocytes. When the cells were treated with ursolic acid at the doses of 20µg/ml, they showed approximately 17, 43, 61 and 79 % of DNA strand breaks after exposure to 10, 25, 50 and 100µM of H_2O_2, respectively as shown in **Fig. 2A**. These were very significant when compared to the control experiments (14%). However, exposure to 40 µg/ml of ursolic acid reduced strand breaks to 15, 17, 36 and 46 % after exposure to 10, 25, 50 and 100 µM of H_2O_2 as shown in **Fig.2B**. The maximum protection by ursolic acid was observed at 40 µg/ml showing 17 % strand breaks after exposure to 25 µM of H_2O_2 as shown in **Fig.2B**.

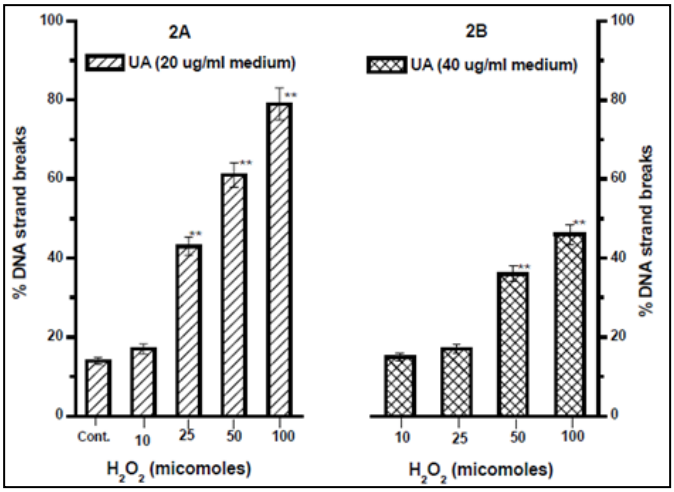

FIG. 2: URSOLIC ACID INHIBITED HYDROGEN PEROXIDE INDUCED STRAND BREAKS IN DNA OF HUMAN LYMPHOCYTES 2A) EFFECT OF URSOLIC ACID (20µG/ML) AGAINST DIFFERENT CONCENTRATIONS OF HYDROGEN PEROXIDE; 2B) EFFECT OF URSOLIC ACID (40µG/ML) AGAINST DIFFERENT CONCENTRATIONS OF HYDROGEN PEROXIDE.

All values represent mean ± standard error of samples (in triplicate) from 3 experiments in each group. (**P* < 0.05, ***P* < 0.01: indicates significance of difference between control and ursolic acid treatments).

Ursolic acid alleviated H_2O_2 induced intracellular ROS level and mitigated decrease in glutathione levels: To evaluate the activated oxygen metabolism of lymphocytes, the intracellular production of ROS in lymphocytes

was analyzed as shown in **Fig.3** after oxidative stress either with ursolic acid or with DMSO as control. Dose (H_2O_2) dependent increase in intracellular ROS was observed.

Compared to control levels, ROS levels rose up ($P < 0.01$) more than 2-fold (approx. 277%) after co-incubation with 100 µM of H_2O_2 and 20 µg/ml of ursolic acid as shown in **Fig. 3A**. However, compared to 40µg/ml ursolic acid treatment, ROS levels were observed to decline when 40 µg/ml of ursolic acid was used against 100 µM of H_2O_2 as shown in **Fig.3B**.

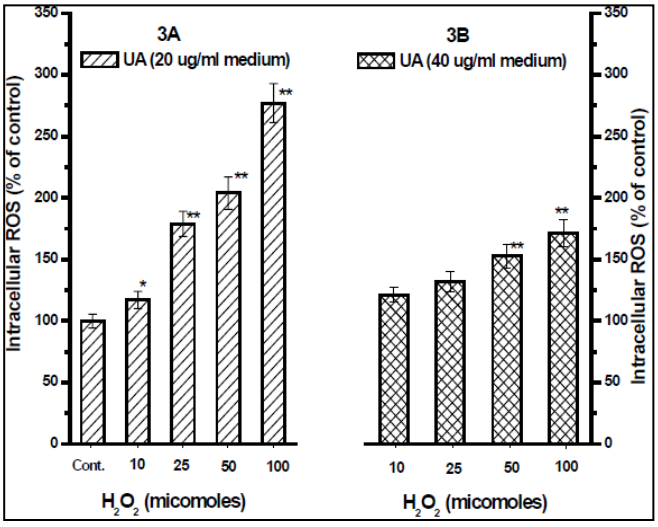

FIG.3: EFFECT OF URSOLIC ACID TREATMENT ON HYDROGEN PEROXIDE INDUCED INTRACELLULAR REACTIVE OXYGEN SPECIES (ROS) OF HUMAN LYMPHOCYTES 3A) EFFECT OF URSOLIC ACID (20 µg/ml) AGAINST DIFFERENT CONCENTRATIONS OF HYDROGEN PEROXIDE; 3B) EFFECT OF URSOLIC ACID (40µg/ml) AGAINST DIFFERENT CONCENTRATIONS OF HYDROGEN PEROXIDE.

All values represent mean ± standard error of samples (in triplicate) from 3 experiments in each group. (*$P < 0.05$, **$P < 0.01$: indicates significance of difference between control and ursolic acid treatments).

In the present study, the level of glutathione showed significant decline (56.6% with 100µM of H_2O_2) when lymphocytes were treated with ursolic acid (20µg/ml) for 2 h as shown in **Fig.4A**. However, when ursolic acid was supplemented in media at higher concentration (40µg/ml) only 28.4 % decrease ($P < 0.01$) in reduced glutathione was observed in comparison to untreated lymphocytes as shown in **Fig. 4 B**.

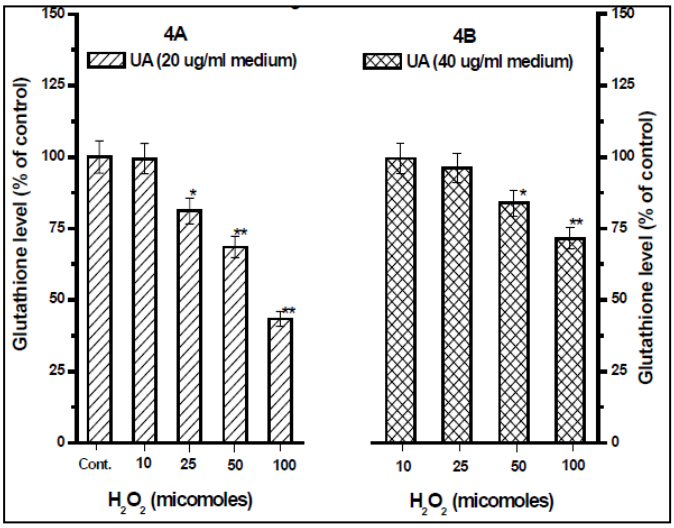

FIG. 4: EFFECT OF URSOLIC ACID TREATMENT ON GLUTATHIONE PROFILE (NMOL/MG PROTEIN) AFTER EXPOSURE OF LYMPHOCYTES TO VARIOUS DOSES OF HYDROGEN PEROXIDE 3A) EFFECT OF URSOLIC ACID (20µg/ml) AGAINST DIFFERENT CONCENTRATIONS OF HYDROGEN PEROXIDE; 3B) EFFECT OF URSOLIC ACID (40µg/ml) AGAINST DIFFERENT CONCENTRATIONS OF HYDROGEN PEROXIDE.

All values represent mean ± standard error of samples (in triplicate) from 3 experiments in each group. (*$P < 0.05$, **$P < 0.01$: indicates significance of difference between control and ursolic acid treatments).

Ursolic acid treatment conferred remarkable protection to genomic DNA by mitigating number of AP sites and 8-oxo-dG residues:

In order to explore the extent of DNA damage under various experimental conditions, AP sites were evaluated in control and H_2O_2 exposed lymphocytes in presence of 20 and 40 µg/ml of ursolic acid as shown in **Fig. 5**. The data were represented as number of AP sites per 100,000 base pairs of genomic DNA. There was no significant difference in AP sites levels between the control and 10 µM exposed lymphocytes ($P > 0.05$) on co-incubation with 20µg/ml of ursolic acid. The average frequencies of endogenous AP sites in genomic DNA in control ranged from 2.3 to 2.6 AP sites/10^5 base pairs.

As shown in **Fig.5A**, lymphocyte DNA revealed significantly elevated levels of purine/pyrimidine free sites after increase in H_2O_2 dose from 10 µM to 100µM in presence of 20µg/ml of ursolic acid. On co-incubation with 20µg/ml of ursolic acid, lymphocytes showed 15.7 ± 0.79 AP sites ($P < 0.01$; compared to control group) at 50 µM of H_2O_2

and 23.6 ± 1.2 AP sites ($P < 0.01$; compared to control group) at 100μM of H_2O_2 as shown in **Fig.5A**. AP sites in genomic DNA were significantly decreased in frequency after co-incubation with 40μg/ml of ursolic acid as shown in **Fig.5B**. Co-incubation with higher concentration of ursolic acid prevented progressive increase of AP sites. After incubation with 100μM of H_2O_2, the abundance of AP sites was 12.7 ± 0.87 as shown in **Fig.5B**.

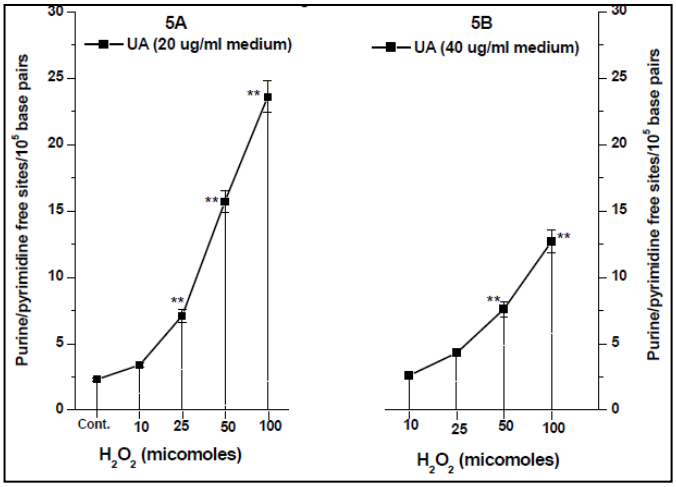

FIG.5: EFFECT OF URSOLIC ACID TREATMENT ON HYDROGEN PEROXIDE INDUCED PURINE/PYRIMIDINE FREE SITES (AP SITES) IN GENOMIC DNA OF HUMAN LYMPHOCYTES 3A) EFFECT OF URSOLIC ACID (20μg/ml) AGAINST DIFFERENT CONCENTRATIONS OF HYDROGEN PEROXIDE; 3B) EFFECT OF URSOLIC ACID (40 μg/ml) AGAINST DIFFERENT CONCENTRATIONS OF HYDROGEN PEROXIDE.

All values represent mean ± standard error of samples (in triplicate) from 3 experiments in each group. (*$P < 0.05$, **$P < 0.01$: indicates significance of difference between control and ursolic acid treatments).

Data on lymphocyte 8-oxo-dG residues profile is presented in **Fig. 6A and 6B**. Lymphocyte 8-oxo-dG level was 19.7 ± 1.3 (ng/ml)/ DNA (μg/μl) in the control. Measurement of 8-oxo-dG residues in H_2O_2 exposed lymphocytes, in general showed significant dose dependent change. However at 10μM of H_2O_2 exposure, the differences were statistically insignificant indicating sufficient protection conferred by 20 μg/ml of ursolic acid. After 25μM of H_2O_2, less pronounced change in 8-oxo-dG levels was observed in lymphocytes co-incubated with 20 μg/ml of ursolic acid as shown in **Fig. 6A**. Increase in concentration of ursolic acid to 40μg/ml significantly reduced 8-oxo-dG levels in DNA as shown in **Fig. 6B**. Maximum increase in lymphocyte 8-oxo-dG levels after 100 μM of H_2O_2 exposure was 71.5 ± 5.3 on co-incubation with 40 μg/ml of ursolic acid ($P < 0.01$).

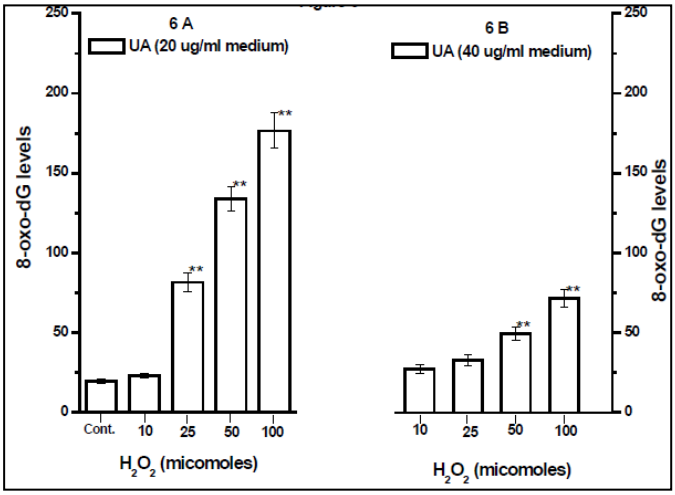

FIG. 6: EFFECT OF URSOLIC ACID TREATMENT ON 8-OXO-DG RESIDUES IN GENOMIC DNA OF HUMAN LYMPHOCYTES 5A) EFFECT OF URSOLIC ACID (20 μg/ml) AGAINST DIFFERENT CONCENTRATIONS OF HYDROGEN PEROXIDE; 5B) EFFECT OF URSOLIC ACID (40 μg/ml) AGAINST DIFFERENT CONCENTRATIONS OF HYDROGEN PEROXIDE.

All values represent mean ± standard error of samples (in triplicate) from 3 experiments in each group. (*$P < 0.05$, **$P < 0.01$: indicates significance of difference between control and ursolic acid treatments).

DISCUSSION: Till recently, the use of phytochemicals in mitigating genotoxic stress has focused on enhancing recovery using crude extracts. However, limited numbers of studies have purified bioactive constituents that confer genomic protection. In present study, HPTLC-densitometry methods were successfully applied for the quantification of ursolic acid in *O. sanctum*. The developed method is simple, precise, specific, sensitive, and accurate, and can be used for standardization and quality control purposes. Ursolic acid is an important bioactive moiety available in plants of *O. sanctum*, which is involved in protecting genomic DNA.

Studies on role of ursolic acid in protecting DNA against genotoxic agents might provide a surge of interest in the therapeutic possibilities where *O. sanctum* could be explored to alleviate oxidative injury. To explore the role of ursolic acid in protecting genomic DNA from oxidative stressors such as hydrogen peroxide, we quantified effect of

H_2O_2 on DNA strand breaks, intracellular ROS, glutathione levels, purine/pyrimidine free sites and 8-oxo-dG residues. DNA constitutes the primary vital target for cellular inactivation of a living system by oxidative stress; measurement of DNA damage is an important quantitative parameter that is used to detect the harmful effects of environmental oxidants. Generation of reactive species leads to DNA damage that causes activation of many apoptotic pathways. Therefore, we have investigated the effect of ursolic acid on intracellular ROS level and status of DNA to elucidate potential protective mechanisms.

To explore the role of ursolic acid in protecting genomic DNA from oxidative stressors such as hydrogen peroxide, we quantified effect of H_2O_2 on DNA strand breaks, intracellular ROS, glutathione levels, purine/pyrimidine free sites and 8-oxo-dG residues. Mutagenesis by oxygen free radicals has been demonstrated in mammalian cell systems. The mutations produced are usually the direct result of modifications of DNA by oxygen free radicals. The hypothesis directly linking oxygen damage to mutations is in accord with the plethora of oxygen free radical induced modifications of DNA [21, 22].

In present studies co-incubation with the ursolic acid for 2 h was found to be highly effective at scavenging hydrogen peroxide-induced intracellular ROS generated in lymphocytes. Unregulated intracellular production of ROS is highly toxic to the cell and is countered by various antioxidant defense systems, which can be categorized as enzymatic or non-enzymatic. Direct damage to DNA bases caused by ROS contributes to the development of cancer and the agents that reduce such damage decrease the risk of cancer development. Increased level of cellular antioxidants is known to provide protection against oxidative stress.

Here, in our study, pre-treatment of lymphocytes with the ursolic acid significantly restored the depleted GSH level in the H_2O_2 -treated lymphocytes. Cellular glutathione content is responsible for the redox status of cell. The rise in the levels of glutathione might be due to ROS scavenging by ursolic acid. H_2O_2 is one of the mutagenic molecules that induce many human diseases including cancer, that is why we explored possibility of ursolic acid protecting against H_2O_2 induced DNA damage in human blood lymphocytes.

The high antigenotoxic activity of ursolic acid at higher concentration indicated dose dependent protection from oxidative stress. Data presented here shows that co-incubation with ursolic acid conferred remarkable protection to lymphocytes via mitigation of AP sites in genomic DNA. Levels of AP sites in DNA are the consequence of a balance between lesion inductions from H_2O_2 induced radical processes and repair.

Here, we show that co-incubation of lymphocytes with ursolic acid plus hydrogen peroxide prevented progressive increase of AP sites. Our studies have clearly shown that ursolic acid mediated ROS scavenging might have protected DNA. Results of present studies suggested that exposure to hydrogen peroxide in absence of ursolic acid might lead to drastic increase in 8-oxo-dG levels in genomic DNA of lymphocytes.

Ursolic acid supplementation, which resulted in scavenging of intracellular ROS, lowered 8-oxo-dG level in lymphocytes. Similar to other purine/pyrimidine based lesions 8-oxo-dG is converted to AP site by action of a specific glycosylase. 8-oxo-dG levels are well-established marker for damage to purine and pyrimidine bases in DNA. Our data showed that H_2O_2 induced increase in 8-oxo-dG levels in lymphocytes was significantly abolished by protective effect of higher doses of ursolic acid.

Recent work has shown that dietary triterpenes supplementation protects by reducing oxidative stress. Therefore, mitigation of H_2O_2 induced abasic sites in genomic DNA by ursolic acid might be partly attributed to anti-oxidant potential of this bioactive constituent derived from *O. sanctum*. DNA strand breaks are the most critical lesions induced by oxidative stress, which can lead to genomic instability and cell death when misrepaired or unrepaired. The results of the present study demonstrate that ursolic acid is highly effective at protecting lymphocytes from the

hazardous effects of hydrogen peroxide, as made evident by the decrease in the ROS and AP sites.

Based on these experiments we hypothesize that stringent ROS scavenging is performed by ursolic acid so that DNA repair is not impaired under oxidative stress conditions. Taken together, our work suggests that opportunities exist for the development of phytochemicals based protective agents that mitigate ROS and reduce DNA damage. For new therapies to emerge continued progress will be needed to understand the complex regulatory mechanisms that govern ursolic acid mediated DNA protection. The results of the present study indicate that ursolic acid from *O. sanctum* possess a remarkable capacity to protect cells from oxidative stress and DNA damage.

CONCLUSIONS: Our results indicated that ursolic acid can be utilized as possible chemo-preventive phytochemical agent against oxidative stress inducing agents. Another important finding showed that anti-oxidant effect of ursolic acid is exhibited in dose-dependent manner. Pre-dominant mechanism governing antioxidant effect of ursolic acid on genomic DNA involved: reduction in intracellular 8-oxo-dG levels, mitigation of oxidative strand breaks and alleviation of purine/pyrimidine free sites. Moreover, higher level of natural antioxidant glutathione was maintained in response to ursolic acid treatment

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