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EXTRACTION OF URSOLIC ACID FROM *OCIMUM SANCTUM* AND SYNTHESIS OF ITS DERIVATIVES: COMPARATIVE EVALUATION OF ANTI-OXIDANT ACTIVITIES

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ABSTRACT: The objective of this study was to extract ursolic acid (UA) from *Ocimum sanctum*, to synthesize its derivatives and to examine the improvement of antioxidative activity. Present work involved structural modification of UA at the C-3 and C-28 positions. UA derivatives namely UA-4 and UA-7 with distinct property were synthesized. Comparative evaluation of anti-oxidant activity of UA, UA-4 and UA-7 using *in vitro* assays and lymphocyte model system involved: (i) inhibition of liposomal lipid peroxidation by UA and its derivatives (ii) assessment of trolox equivalent antioxidative capacity (TEAC) (iii) ABTS and DPPH radicals scavenging ability and (iv) cell death (MTT assay) under hydrogen peroxide (H₂O₂) induced oxidative stress. Moderate increase in antioxidant activity of compound UA-4 compared to UA was observed. In contrast, significant improvement in antioxidant activity of UA-7 was observed. To evaluate antioxidant effects on lymphocytes, we exposed cells with UA and its derivatives along with hydrogen peroxide. This was followed by measurement of cell viability by trypan blue exclusion assay and cell death using MTT assay. UA-7 significantly protected lymphocytes against H₂O₂ induced oxidative stress. In conclusion, we propose that UA might be chemically modified to increase its antioxidant potential. Our studies also clearly showed that UA derivatives significantly decreased hydrogen peroxide induced cell death due to mitigation of oxidative injury.

INTRODUCTION: The mechanisms by which natural compounds protect against different stressors are complex and varied¹. Individual phytochemicals have been implicated as modulators of many metabolic and regulatory processes².

Triterpenoids are a class of naturally occurring compounds that are found in a variety of plants^{3, 4, 5}.

Ursolic acid (UA) (3β hydroxy urs-12 en-28 oic acid) is one such a triterpenoid found in plants like *Ocimum sanctum*^{6, 7}. It has been reported to possess a wide range of pharmacological properties⁸. Many protective effects of *O. sanctum* are determined by strong antioxidative properties of its constituent UA⁹. Earlier studies have shown that UA inhibits radiation-induced oxidative damage to human keratinocytes¹⁰. However, UA derivatives have not been thoroughly explored for

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their anti-oxidant activity. In order to find biologically more active derivatives of this naturally occurring triterpene, we selected methanolic extract of *O. sanctum* as the source of UA.

Oxidative stress occurs in the cells due to imbalance between the prooxidant/antioxidant systems¹¹. It results in injury to bio-molecules such as nucleic acids, proteins, structural carbohydrates, and lipids¹². Assessments of the effectiveness of UA and its derivatives as antioxidants can be taken, asking the following questions. Does the compound act as an antioxidant *in vitro*? And, in lymphocyte model system does it render lymphocytes more resistant to challenges with oxidizing agents *in vitro*? Earlier investigators have studied lymphocytes as a model system as these cells contain variety of redox and free radical scavenging systems¹³. Hence, studies on antioxidant effect of UA and its derivatives in blood lymphocytes could be of immense significance.

In brief, the objective of present work was to synthesize novel derivatives of UA with high antioxidant potential. To this aim, this study synthesized derivatives of UA by retaining ester functionality at C-3 and hydrogen donor group at either C-3 position and/or C-28 position of UA. Our present studies show that derivatives of UA are important candidate as antioxidants. This information is currently unavailable and is essential to develop potential strategies to manage systemic dysfunction induced by oxidative stressors.

MATERIAL AND METHODS:

Materials and Reagents: OS whole plant powder was purchased from Total herb solutions Pvt. Ltd., Mumbai. Plant material was identified at Department of Botany, G. N. Khalsa College, Mumbai against voucher specimen number 311013. 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 methanol crude extract obtained was suspended in

CHCl₃: 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 for the presence of ursolic acid. All those fractions, which consisted of ursolic acid, were combined together and solvent was removed under vacuum. Evaporation of the combined fractions containing ursolic acid led to a white solid. The isolated ursolic acid 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 as shown in **Figure 1**.

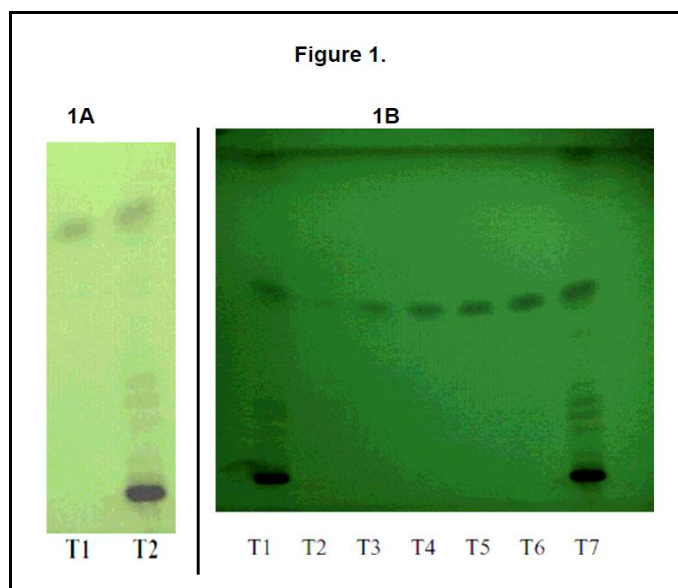


FIGURE 1. EXISTENCE OF URSOLIC ACID IN *O. SANCTUM* SPECIES AS INDICATED BY HPTLC ANALYSIS OF METHANOLIC EXTRACT.

1A) T1; URSOLIC ACID IN METHANOLIC EXTRACT T2; URSOLIC ACID STANDARD. 1B) T1: EXISTENCE OF URSOLIC ACID IN METHANOLIC EXTRACT; T2-T7- URSOLIC ACID STANDARDS OF VARYING CONCENTRATION.

The ursolic acid was quantified using CAMAG HPTLC apparatus. Calibration curve of ursolic acid was obtained by plotting peak area versus applied concentrations of ursolic acid. 10 µl of each standard solution of ursolic acid (72-576 ng/spot) were applied and HPTLC were performed. After development, the plates were dried at room temperature in air, derivatized with Liebermann Burchard reagent and scanned at 530 nm in absorbance mode using tungsten lamp. The peak areas were recorded. 20µl of suitably diluted

sample was applied in triplicate on 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.

Synthesis of UA derivatives: Ursolic acid (3 β -hydroxy-urs-12-en-28-oic acid) was used as the lead compound and the structure modification was done as shown in **Figure 2**. Synthesis of UA derivatives UA-4 and UA-7 and their structure confirmation by mp, IR ^1H NMR spectra and elemental analysis was done following procedure standardized in our laboratory ¹⁴.

In brief, to prepare compound UA-4; UA was reacted with acetic anhydride in Tetrahydrofuran (THF) in the presence of 4-dimethyl amino pyridine (DMAP), then reacted with chromium trioxide in acetic acid solution containing 5% acetic

anhydride to obtain intermediate compound (3 β -acetoxy-urs-11-oxo-12-en-28-oic acid). This intermediate compound was reacted with chromium trioxide in acetic acid solution containing 5% acetic anhydride to obtain an intermediate, which was treated with oxalyl chloride to yield a crude intermediate (3 β -acetoxy-urs-11-oxo-12-en-28-acyl chloride), which was further condensed with aniline in the presence of triethylamine to give UA-4 (N- [3 β -acetoxy-urs-11-oxo-12-en-28- acyl] aniline). Compound UA-7 (Methyl N- [3 β -butyryloxy-urs-12-en-28-oyl]-2-amine acetate) was prepared by UA reacted with butyric anhydride in the presence of DMAP in THF. The intermediate UA-6 was obtained from UA-5 by treating with oxalyl chloride, which was then reacted with methyl glycinate to yield compound UA-7 (Methyl N-[3 β -butyryloxy-urs-12-en-28-oyl]-2-amine acetate).

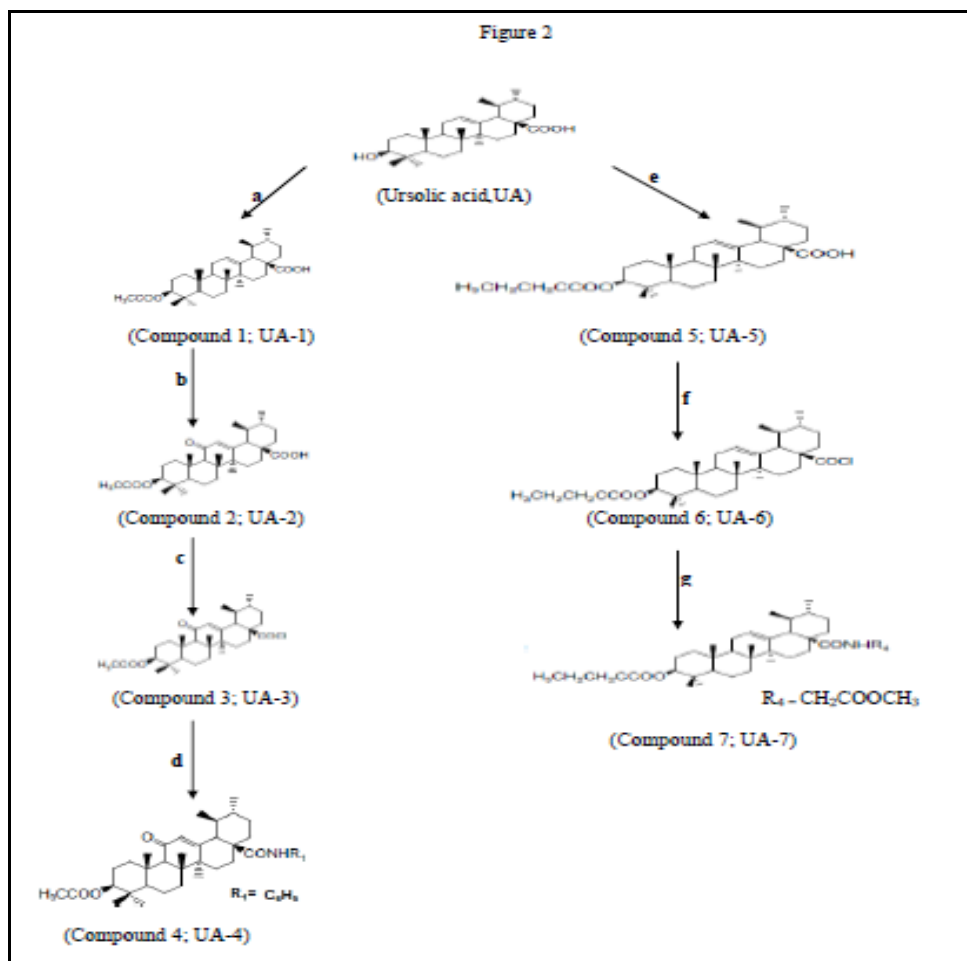


FIGURE 2. SYNTHESIS OF NOVEL DERIVATIVES UA-4 AND UA-7 FROM URSOLIC ACID. REAGENTS AND CONDITIONS:

(COMPOUND UA-1) AC_2O , DMAP, THF, R.T.; (COMPOUND UA-2) CRO_3 , AC_2O , ACOH , R.T.; (COMPOUND UA-3) CH_2CL_2 , $(\text{COCL})_2$, R.T.; (COMPOUND UA-4) ET_3N , NH_2R_1 , R.T.; (COMPOUND UA-5) $(\text{CH}_3\text{CH}_2\text{CH}_2)_2\text{O}$, DMAP, THF, R.T.; (COMPOUND UA-6) CH_2CL_2 , $(\text{COCL})_2$, R.T.; (COMPOUND UA-7) ET_3N , NH_2R_4 , R.T.

Evaluation of antioxidant activity of UA and derivatives using various *in vitro* assays

Inhibition of lipid peroxidation in liposomes:

The lecithin was dried under vacuum onto the wall of a round-bottom flask. The resulting lipid film was hydrated in 50 mm of Tris-HCl buffer at pH 7.4, shaken for 15 min and sonicated (samples thermostated at 0-2°C). Before the end of sonication a fluorescent probe, 3-[*p*-(6-phenyl)-1, 3, 5-hexatrienyl]-phenylpropionic acid was added to the liposome suspension. The assay was conducted by procedure described earlier¹⁵.

Trolox equivalent antioxidative capacity (TEAC) for UA, UA-4 and UA-7:

The assay was performed as previously reported with slight modifications¹⁶. Samples (40 µg/ml concentrations) were mixed with 200 µl of ABTS+ radical cation solution in 96-well plates and absorbance was read (at 750 nm) in a microplate reader. Trolox equivalents (in µM) were derived from the standard curve at 5 min of incubation.

Determination of IC50-value of UA, UA-4 and UA-7 for ABTS and DPPH radical scavenging:

The radical scavenging activity of UA, UA-4 and UA-7 against DPPH radical was determined spectrophotometrically^{17, 18}. Assay was based on DPPH radical reaction with an antioxidant compound that can donate hydrogen and gets reduced. IC50-value is the concentration of compound required to inhibit 50% of radical production.

Isolation of peripheral blood lymphocytes:

Blood samples were obtained from five donors (all males, healthy, non-smokers, 20–30 years old). Fresh whole blood (100 ml) was suspended in 1 ml RPMI-1640 containing 10% FCS in an Eppendorf 1.5 ml tube, then left on ice for 30 min. This was followed by addition of 200 ml lymphoprep to samples and centrifugation at 1200 rpm for 8 min at 4°C. Lymphocytes were retrieved above the boundary between RPMI and lymphoprep, washed with PBS (pH 7.4), centrifuged at 1000 × g and re-suspended in 30 ml PBS. The yield of lymphocytes was usually 10⁶ per ml of blood with viability above 96%.

Co-Incubation of lymphocytes with purified UA or UA-4 or UA-7 and hydrogen peroxide for MTT assay:

After 24 hours of culture initiation in DMEM supplemented with 20 % fetal bovine serum, lymphocytes (at concentration of 1×10⁶ cells/mL) were treated with UA & derivatives (40 µg/ml) and hydrogen peroxide (25 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 alone Lymphocytes exposed to 40 µg/ml UA

Group 3; UA-4 alone Lymphocytes exposed to 40 µg/ml of UA-4

Group 4; UA-7 alone Lymphocytes exposed to 40 µg/ml of UA-7

Group 5; H₂O₂ alone Lymphocytes exposed to 25 µM H₂O₂

Group 6; UA + H₂O₂ Lymphocytes exposed to 40 µg/ml UA and 25 µM H₂O₂

Group 7; UA-4+ H₂O₂ Lymphocytes exposed to 40 µg/ml UA-4 & 25 µM H₂O₂

Group 8; UA-7 + H₂O₂ Lymphocytes exposed to 40 µg/ml UA-7 & 25 µM H₂O₂

For all experiments lymphocytes were exposed for 2 h. UA or UA-4 or UA-7 were dissolved in DMSO at a concentration of 1000 µM with subsequent dilutions in culture medium. For sham control DMSO was diluted in medium to obtain 0.05 % concentration.

MTT assay: The MTT assay involved co-incubation of lymphocytes with UA or UA-4 or UA-7 as per experimental design. This was followed by addition of 0.5mg/mL of MTT to the incubated cells and cells were kept for another 4 h at room temperature. After incubation tubes were centrifuged for 10min, medium with MTT was removed and 200 µL of DMSO was added. Absorbance was measured to at 570 nm.

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 AND DISCUSSION:

Protective Effect of UA derivatives on lipid peroxidation:

UA, UA-4 and UA-7 (all at 10 μM concentrations) were evaluated for the ability (compared to synthetic antioxidant TBHQ at 10 μM) to inhibit lipid peroxidation induced by Fe (II) ions in a liposomal model as seen in **Figure 3A**. In the lipid peroxidation assay, the relative decrease in fluorescence showed that UA-4 was the moderately stronger antioxidant compared to UA ($p < 0.01$) as appeared in **Figure 3B**. The derivatization of C-28 position with $R_1-C_6H_5$ groups attached to the UA skeleton elevated their ability to inhibit the rate of

Fe (II)-induced lipid peroxidation. Compound UA-7 (Methyl N-[3 β -butyryloxy]-urs-12-en-28-oyl]-2-amine acetate) with modification at C-3 as well as C-28 position was the most active under these conditions and its antioxidant capacity was comparable to commercial synthetic antioxidants TBHQ.

Lipid peroxidation has been shown to perturb the bilayer structure and modify membrane fluidity¹⁹. UA-7 showed the most potent effect against lipid peroxidation. There is a causal relationship between increased production of ROS and lipid peroxidation²⁰. Our results show that UA derivative UA-7 rendered significant protection against $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ -induced lipid peroxidation.

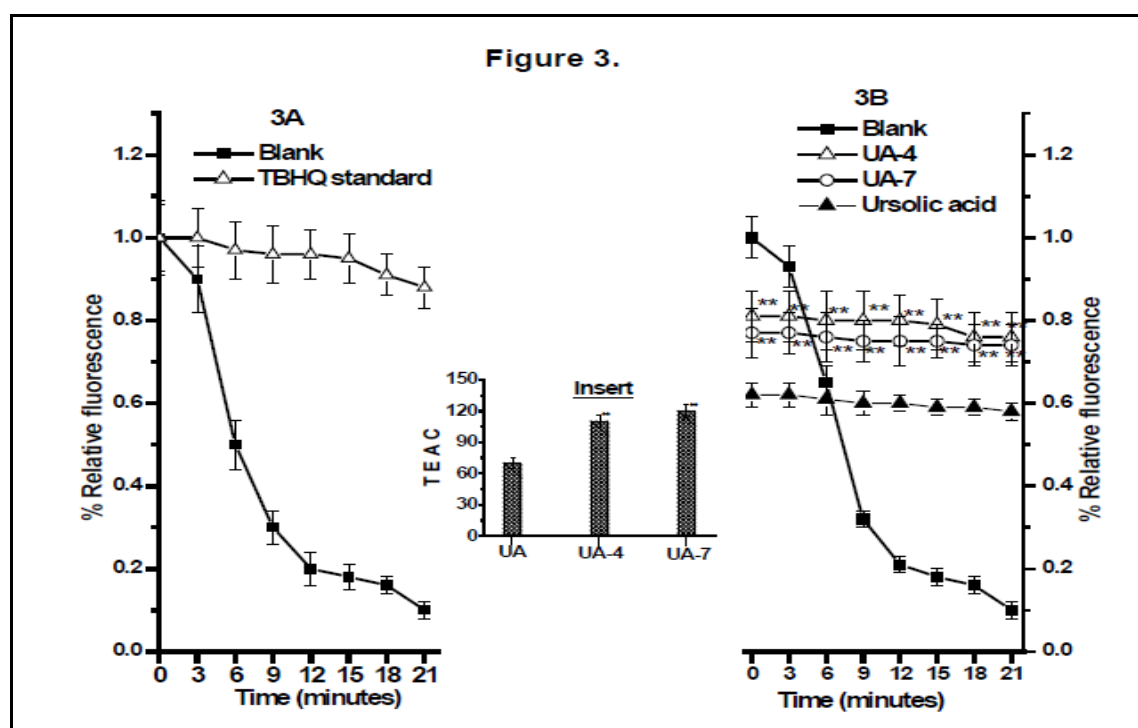


FIGURE 3. INHIBITION OF LIPID PEROXIDATION INDUCED BY FE (II) OF

(A) COMMERCIAL SYNTHETIC ANTIOXIDANTS TBHQ (40 $\mu\text{G}/\text{ML}$);

(B) URSOLIC ACID (UA) AND ITS NOVEL DERIVATIVES UA-4 AND UA-7 AT CONCENTRATION OF 40 $\mu\text{G}/\text{ML}$. TROLOX EQUIVALENT ANTIOXIDANT CAPACITY (TEAC) OF UA AND DERIVATIVES (CONC. 40 $\mu\text{G}/\text{ML}$) WAS ALSO STUDIED (INSERT).

All values are mean \pm SE of samples (in triplicate) from 3 experiments in each group. (* $P < 0.05$, ** $P < 0.01$).

Trolox equivalent antioxidant capacity (TEAC) of UA, UA-4 and UA-7:

In the TEAC assay, UA, UA-4 and UA-7 had values of 70, 110 and 120 μM trolox equivalents, respectively. TEAC is the concentration of Trolox required to give the same antioxidant capacity as 1

mM test substance. The order of antioxidative potency of the UA and derivatives showed that UA-7 is more effective antioxidant than UA-4. Moreover, both compounds were more potent antioxidant compared to parent compound UA as appeared in **Figure 3-Insert**. TEAC assay further

confirmed higher antioxidant capacity of UA derivatives compared to parent compound.

Free radical scavenging ability of UA and its derivatives (UA-4 and UA-7):

Figure 4A, 4B and 4C shows the scavenging ability of UA on DPPH and ABTS radicals. The IC₅₀-value of UA in DPPH and ABTS radicals were 8.4 ± 0.7 , 9.3 ± 0.9 $\mu\text{g}/\text{mL}$ required to inhibit 50% of radical production as appeared in **Figure**

4A. The IC₅₀-value of UA-4 in DPPH and ABTS radicals were 7.6 ± 0.6 , 8.1 ± 0.8 $\mu\text{g}/\text{mL}$ required to inhibit 50% of radical production **shown in Figure 4B**. The IC₅₀-value of UA-7 in DPPH and ABTS radicals were 4.5 ± 0.3 , 5.9 ± 0.5 $\mu\text{g}/\text{mL}$ required to inhibit 50% of radical production as seen in **Figure 4C**. These results clearly suggested that UA-7 exhibited more pronounced ABTS and DPPH radical scavenging activity.

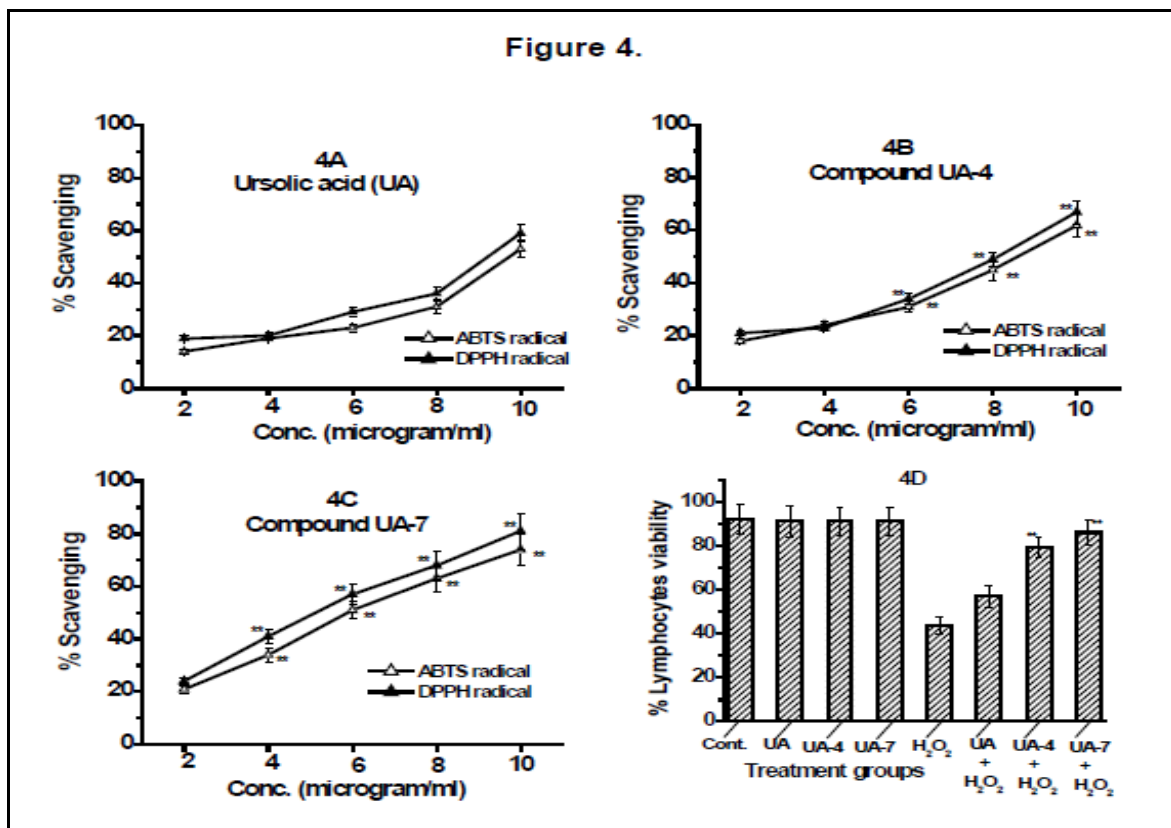


FIGURE 4. DETERMINATION OF IC₅₀-VALUE OF URSOLIC ACID (UA)

(FIGURE 4A) AND ITS NOVEL DERIVATIVES UA-4

(FIGURE 4B) AND UA-7

(FIGURE 4C) FOR ABTS AND DPPH RADICAL SCAVENGING. MTT ASSAY SHOWING ANTI-OXIDANT POTENTIAL OF UA, UA-4 AND UA-7 (CONC. 40 $\mu\text{g}/\text{mL}$) AGAINST HYDROGEN PEROXIDE (25 MM) INDUCED OXIDATIVE STRESS IN LYMPHOCYTES WAS ALSO STUDIED (FIGURE 4D).

All values are mean \pm SE of samples (in triplicate) from 3 experiments in each group. (* $P < 0.05$, ** $P < 0.01$).

Protective effect of UA and its derivatives against oxidative stress (hydrogen peroxide induced) induced cell death in lymphocytes:

Our results showed that cell death was greatly increased in hydrogen peroxide exposed lymphocytes (43.6 ± 3.9). Simultaneous treatment with UA and its derivatives significantly improved cell survival (Approx. 57% with UA; 79% with UA-4 and 86% with UA-7) when hydrogen

peroxide exposure was combined with UA or its derivatives. UA-7 showed most significant protective effect on lymphocytes (Figure 4D). Earlier studies have shown that grape seed, rich in UA prevents UV-induced keratinocyte death.

CONCLUSIONS: In this study, UA derivatives possessing two hydrogen-bond forming groups (an H-donor and a

carbonyl group) at positions 3 and 28 exhibited significantly improved antioxidant activity. It is evident from the present study that UA-7 offers a remarkable protection against lipid peroxidation and free radical stress induced cell death. We report that reduction potential of UA is influenced by the presence of electron-donating or electron-withdrawing groups. The altered redox potential could affect the ability of the UA to scavenge deleterious oxy radicals. Further development of new therapeutic strategies using UA derivatives as antioxidant agent rests heavily upon the elucidation of its effect on biological reactions.

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DECLARATION OF INTEREST: The authors report no conflict of interest.

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