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EVALUATION OF ANTIOXIDANT AND RADIOPROTECTIVE PROPERTIES OF HYDROXYECTOINE

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ABSTRACT

PURPOSE: The prime purpose of the present study was to evaluate the antioxidant and radioprotective efficacy of a novel osmoregulant hydroxyectoine produced by extremophilic bacteria.

METHODS: To evaluate the antioxidant and radioprotective activities of hydroxyectoine, nitric oxide radicals neutralizing, hydroxyl radicals scavenging, superoxide radicals scavenging, DPPH reducing assay, and protein radioprotection assays with native PAGE analysis was carried out.

RESULTS: Results of the study were demonstrated that hydroxyectoine exhibited nitric oxide radicals scavenging activity (12.23± 3.215488%). OH^- radicals scavenging efficacy of hydroxyectoine was estimated in terms of % inhibition in deoxy D-ribose degradation by non-site-specific assay. The maximum (16.2 ± 2.07869855%) inhibition of deoxy D-ribose degradation was observed in non-site-specific manner, at 5.0µg/ml concentration of hydroxyectoine. DPPH assay was carried out to evaluate the electron donation potential of hydroxyectoine. Maximum ~31.17% reduction in DPPH was observed at 500 µg/ml concentration of hydroxyectoine. Similarly, maximum (14.98%) hydroxyectoine mediated inhibition in superoxide radicals concentration was observed at 400µg/ml concentration. Significantly high degree of hydroxyectoine mediated protection to BSA was observed against ionizing radiation (1000-2000Gy).

CONCLUSION: In view of the observations, it can be concluded that hydroxyectoine possesses strong antioxidative and radioprotective activities and may be a potential candidate for radioprotection to cellular proteins in future.

Key words: Hydroxyectoine, Antioxidant, Radioprotection, Protein protection.

INTRODUCTION

Several synthetic and natural products have been evaluated for their radioprotective effecacy *in vitro* and *in vivo* models. Some of them are under different stages of clinical trials (1-2). However, despite concentrated efforts, not a single radioprotective drug has approved by FDA for human use. Deleterious effects of radiation on biological systems develop in a temporal sequence across the various levels of organisation, starting from the induction of primary lesions in the biomolecular structures, eliciting repair processes, leading to the cell death or transformation responsible for morbidity, genetic disorder and cancer (3).

Ionising radiation [particularly, the low linear energy transfer (LET) radiation] exerts its effect through the generation of free radicals that destroy the vital macromolecule (i.e., proteins, lipids, RNA and DNA) and structures (such as lipid membranes of the target cell, protein secondary and tertiary structures etc.). Oxygen-derived free radicals species, particularly superoxide (O_2^-), hydroxyl (OH^-), hydroperoxyl (HOO^-), peroxyl (ROO^-), alkoxyl

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(RO^-), nitric oxide (NO^-), peroxynitrite anion ($ONOO^-$) radicals, etc., are known to react with various biomolecules like functional proteins, enzymes, membrane lipids and DNA by extracting an electron from them to become stable (4-5).

Upon irradiation cellular aqueous environment produces hydroxyl radicals *(OH*$^-$), hydrated electrons, hydrogen radicals, hydroperoxy radicals (*H0*$^{2-}$*)*, etc. Among these, *OH* and solvated electrons are produced in the highest quantity and considered to be the most damaging agents (6-7). Cellular targets i.e. macromolecules, suspended in biological aqueous environment react with high energy free radicals and become ionised or convert into the free radicals (8). These kind of chemical transformations disrupt molecular organization lead to structural and functional impairment, altered cell metabolism and cell death (9-10). Radiation-induced damages in DNA in cellular milieu including damaged purine and pyrimidine bases, single-and double-strand breaks, removal of bases and cross-linking of DNA with adjacent protein molecules (11). Lipid peroxidation in all biological membranes in general and in mitochondrial membrane in particularly is the most sever effect of irradiation. As the result of lipid peroxidation short chain fatty acyl derivatives are formed which interfered in lipid-lipid as well as lipid-protein cross-linkages formation (9-10).These kind of interferences induced severe distortion in the biological functions. Similarly, oxidation of accessible amino acids, protein denaturation and scission of disulphide bonds in proteins also lead to oxidative stress in the cellular milieu. Functionally, all these changes can be expressed as altered membrane fluidity and permeability, which could trigger the release of potent physiological mediators of apoptosis (3). In view of above, several therapeutics agents, i) which are able to able to reduce the oxidative stress induced by free radicals, ii) acts as oxidative damage repairing agents and recovery enhancers along with iii) modifiers of immune system (biological-response modifiers) has been investigated to rescue the organism from radiation injury (3, 12-17). However, despite serious scientific efforts to develop efficacious and non toxic radiation counter measure nothing conclusive has come out so far. Despite serious scientific efforts to develop efficacious and non toxic radiation counter measure agents nothing conclusive has come out so far. Extremophiles produced several extremlytes to protect them self from harsh physical or chemical environment (40, 18), hydroxyectoine is one of them. It is a derivative of ectoine which belong to the family of compatible solutes (19). Ectoine and hydroxyectoine referred as stress protectectant, synthesized by halophilic bacteria such as *Halomonas elongata*. The natural environment of these organisms is characterized by high UV-radiation, dryness, extreme temperature, and high salinity. The bacteria protect themselves against damages from these hostile conditions by synthesizing ections and its other derivatives (20, 21). Ectoine and hydroxyectoine acts as proteins, membrane lipids and genetic material DNA and RNA stabilizing agent in cellular milieu in the bacterial cells. (22-25) and thus help them to survive in their inhospitable environments. Ectoins are amphoteric, organic molecules that are able to bind with water and form a large hydrated sheath that allows them to protect the biomolecules in against a variety of aggressors such as UV, osmotic stress, heat, dryness, or frost (21, 23, 26). However, its radioprotective potential against ionizing radiation has not been explored till date.

In the present study, antioxidative and radioprotective efficacy of hydroxyectoine *in vitro* models was evaluated. Detailed antioxidant activities of HE in terms of hydroxyl radicals scavenging, nitroxyl radicals neutralizing activities and radioprotective potential of hydroxyectoine was studied. Significant results of the study obtained was analysed and discussed in view of its possible applications as prominent antioxidant and radioprotector in future.

MATERIAL AND METHOD

Chemicals

All chemicals and reagents used for the present study were of high grade. HPLC grade water (Qualigens fine chemical, batch no 8234 6702-3), Bovine albumin fraction–V (Merck,RM105-5G, CAS No: 9048-46-8), λ Phase DNA (Fermentas , SD0011, Lot no. 00041790), Hind III (fermentas,ER00503,lot No 2913), Agarose (Genei, Cat AG2, lot 20123) for non-denaturing gel 30% acrylamide (Merk,1.10784.0100,lot no.K35240384), Ethidium bromide(Merck, Batch no.K38316615), Ammonium per sulphate (SRL, 014763 cat no.T829027), Tris base (Calbiochem, cat 648310, lot no. D00041401),

Glycine (Calbiochem, cat# 357002, lot D00046269) and HCl (E. Merk, no.1/17625). 2-deoxy- D-ribose, 2,2-diphenyl-1-picrylhydrazyl (DPPH) was purchased from HiMedia, India. Thiobarbituric acid (TBA), sodium nitroprusside, naphthylethylenediamine and sulfanilamide were purchased from Sigma Chemicals (St. Louis, MO, USA). Potassium ferricyanide, EDTA, trichloroacetic acid, and ferric chloride were procured from Ranbaxy, India.

Antioxidant Activities Evaluation

Superoxide Radicals Scavenging Assay

The superoxide ion quenching ability of hydroxyectoine was determined using the nitroblue-tetrazolium reduction assay (27). Different concentrations of hydroxyectoine were taken and mixed with sodium pyrophosphate buffer (0.05 M, pH 8.3) and phenazine methanosulfate (186µM). Nitroblue tetrazolium (300µM) was added to above solution and final volume adjusted to 3ml.The reaction was initiated by adding NADH (780µM) and the solution was incubated at 37°C for 3 minutes. The reaction as terminated by adding glacial acetic acid to the resultant mixture, following 4 ml of *n*-butanol, which was added and mixed vigorously. The reaction mixture was allowed to stand for 10 minutes at room temperature, the *n*-butanol layer was separated by centrifugation (1000xg) and intensity of chromogen (in the *n*-butanol layer) was measured at 560 nm.

Nitric Oxide Scavenging Activity Assay

Nitric oxide radical scavenging activity of hydroxyectoine was determined according to the method reported by Garrat (28). Sodium nitroprusside in aqueous solution at physiological pH (7.4) spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ion, which can be determined by the use of Griess Illosvoy reaction. Varied concentrations of HE was mixed with sodium nitroprusside (5 mM), and the volume made up to 1.0 ml using phosphate buffer saline (pH 7.4). The reaction mixture was incubated at 25°C for 150 min followed by addition of Griess reagent (6% sulphanilamide in 3M HCl + 0.3% napthylethylene diamine dihydrochloride + 7.5% orthophosphoric acid in 1:1:1 ratio). Pink colored chromospheres were formed. The nitric oxide scavenging activity was analyzed as percent decreased in the absorbance of the complex formed by diazotization of nitrite with sulfanilamide and subsequent coupling with naphthylethylenediamine at 546 nm. Decrease in optical density is an indicative of higher nitric oxide potential of the Hydroxyectoine.

Hydroxyl Radical Scavenging Activity Estimation

The generation and detection of non-site-specific hydroxyl radical scavenging were carried out according to a Fenton method described by Halliwell and Gutteridge (29). Different concentrations of hydroxyectoine were mixed with 0.1 mM ferric chloride solution, 0.104 mM EDTA-Na, and 0.1 mM L-ascorbic acid along with 1 mM H_2O_2, 3.6 mM 2-deoxy-D-ribose in potassium phosphate buffer (pH 7.4) in a total assay volume of 1 ml, followed by incubation at 37^0C temperature for 1 h and then treated with equal volumes of 10% TCA and 0.5% thiobarbituric acid (in 0.025M NaOH). The samples were incubated (55°C) for 15 min. The pink reactive chromogen of TBA and the malondialdehyde from deoxyribose degraded by OH• was measured at 532 nm. The decrease in absorbance at a particular concentration indicates higher hydroxyl ion scavenging potential with respect to control. The percentage inhibition of degradation of deoxyribose or hydroxyl ion scavenging potential will be calculated as follows:

$\%Inhibition=(Abs_{control} - Abs_{test})/Abs_{control} x\ 100$

2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Radical Neutralizing Activity Determination

Determination of radical scavenging capacity was based on the decay of DPPH (30) after addition of hydroxyectoine to the stock methanol solution of DPPH. Briefly, 1.0 ml of DPPH (100µM) was added to 500µl of different concentrations of hydroxyectoine The mixture was then incubated for 10 min in the dark. After incubation, absorbance was taken at 517 nm. Percent scavenged DPPH radical by hydroxyectoine was calculated according to the equation:

$\%Inhibitio =(Abs_{control} - Abs_{test})/Abs_{control} x\ 100$

***In Vitro* Evaluation of Radioprototective Activities**

Analysis of Radioprotection offered by Hydroxyectoine to BSA

Observation of structural damage in BSA followed by irradiation of different doses (1000-2000Gy) and its protection by hydroxyectoine (25µg/ml) treatment, native-PAGE analysis was performed. 10%

polyacrylamide gels of 0.75 mm thickness were prepared. Protein mixture was added to PAGE sample buffer [0.0625M Tris/HCl, pH 6.8, 5% (v/v) glycerol, 2% (v/v) 3-mercaptoethanol and 0.01% (w/v) Bromophenol Blue]. Equal amounts of protein samples (20µg) were loaded in each well. Electrophoresis was carried out at a constant voltage (90V). After electrophoresis, the gels were stained with gentle shaking in 0.1% Coomassie Brilliant Blue R-250 in methanol/acetic acid/water (4:2:4, by vol.) at room temperature and destained in a washing solution [methanol/acetic acid/water (1:0.7:8.3)] to obtain distinct bands over a clear background.

RESULTS

Nitric Oxide Radical Scavenging Activity

Oxidative stress induced by gamma radiation generates nitric oxide radicals, which play a critical role in the initiation and progression of oxidative damage in the biological system. Inhibition in nitric oxide radicals by hydroxyectoine was studied. Results of the present study revealed significant inhibition in the nitric oxide radicals concentration where hydroxyectoine was used as free radical scavenger. The maximum nitric oxide inhibitory activity of hydroxyectoine (12.23± 3.215488%), was observed at maximum tested concentration (i.e., 20µg/ml) **(Figure 1)**

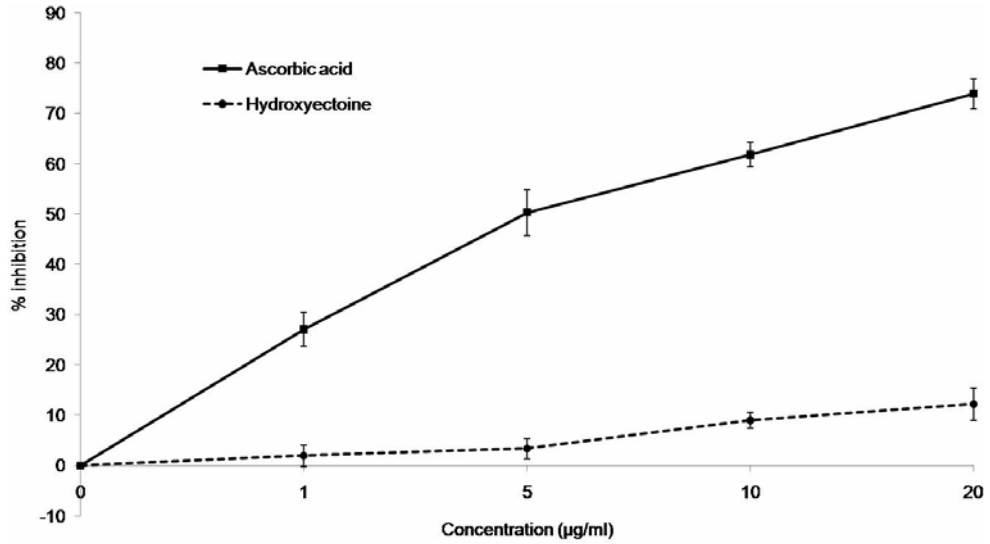

Fig. 1. Estimation of Nitric oxide radical scavenging capacity of hydroxyectoine. Ascorbic acid was used as positive control. The data was represented the percentage (%) nitric oxide inhibition.

Hydroxyl Radical Scavenging Potential

The hydroxyl radical scavenging potential hydroxyectoinewas evaluated in terms of % inhibition of deoxy D-ribose degradation. Results of the study demonstrated significant inhibition in the deoxy D-ribose degradation by hydroxyectoine in dose dependent manner. However, maximum % inhibition in deoxy D-ribose degradation was observed to be (16.23± 2.07869855%) at 5µg/ml concentration of hydroxyectoine **(Figure 2).**

Superoxide Radicals Scavenging Potential

Superoxide free radicals scavenging ability of hydroxyectoine was evaluated. The results of the study were demonstrated a dose dependent increase (100-400µg/ml) in the superoxide scavenging activities of hydroxyectoine. The superoxide scavenging activities of hydroxyectoine was found comparable to the same activity of quercetin used as positive control. At the highest tested concentration (i.e. 400µg/ml), hydroxyectoine exhibited (14.98 ± 1.547433 %) inhibition in the superoxide radicals generation and thus corresponding inhibition in the formazan crystals formation **(Figure 3).**

DPPH Reduction Potential

DPPH is a stable radicals in solution (methanolic solution) and appears as violet colour solution. Any electron donating agents (antioxidant) can reduced DPPH radicals resulted disappearance of the characteristic violet colour and thus decreased in the absorbance at 517nm. Hydroxyectoine exhibited concentration dependent decrease in absorbance of DPPH solution, indicated its reduction by hydroxyectoine. Maximum hydroxyectoine mediated DPPH reduction (31.17±1.787685%) was observed at 500µg/ml concentration of hydroxyectoine which was found highly comparable to the ascorbic acid used as positive control in the study **(Figure 4)**.

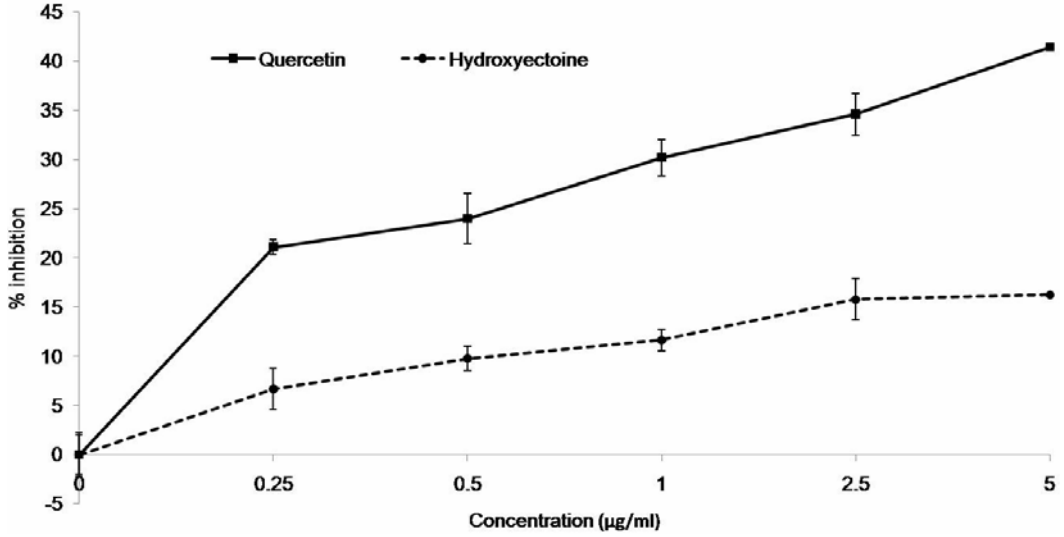

Fig. 2. Hydroxyl radical scavenging activities of the hydroxyectoine.
The results are mean ± SD. Hydroxyectoine exhibited significant percentage inhibition of deoxy-D-ribose degradation in non-site specific assay. Quercetin was used as positive control.

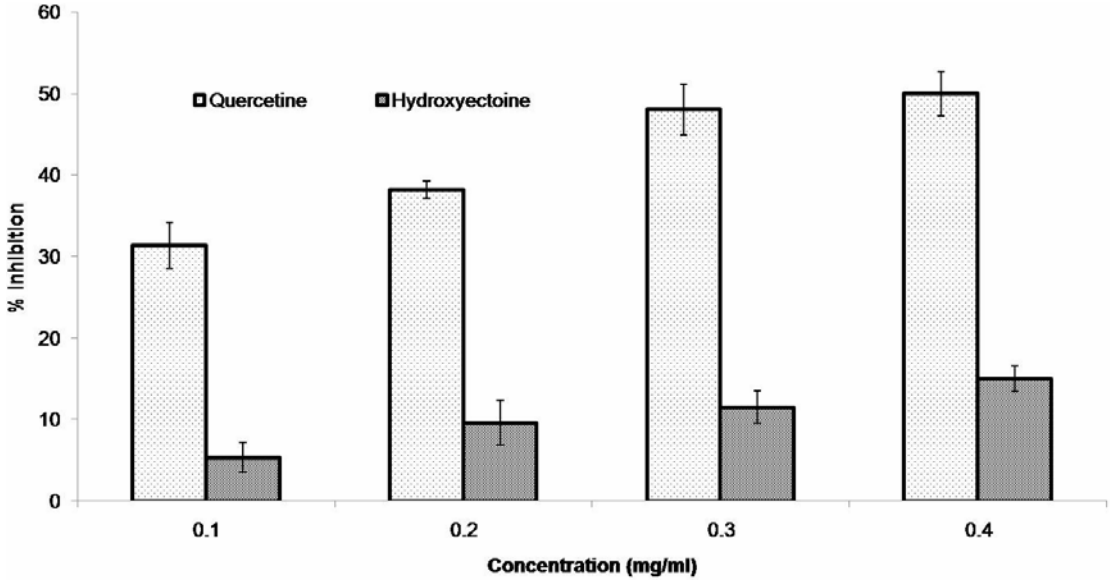

Fig. 3. Determination of Superoxide radicals scavenging activities of by hydroxyectoine.
Hydroxyectoine exhibited significant % inhibition of formation of formazan crystals vs control. The results are mean ± SD.

Protein protective efficacy of hydroxyectoine against radiation induced damage

To observe the radioprotective potential of hydroxyectoine in terms of bovine serum albumin (BSA) protection against oxidative stress induced by gamma radiation SDS-PAGE electrophoresis study was carried out. Aqueous solution of BSA was irradiated with different doses of gamma radiation in presence or absence of hydroxyectoine and protein degradation was evaluated on SDS-PAGE analysis. Gamma radiation dose (1000-2000 Gy) dependent degradation in BSA was observed as intense smeared bands appeared on SDS-PAGE as compared to untreated controls. However, intense smearing of BSA bands was found to disappeared upon irradiation (1000-2000Gy) in presence of hydroxyectoine. Therefore, results of the present study were clearly demonstrated significantly high radioprotective efficacy of hydroxyectoine towards BSA against supra lethal ionizing radiation induced damage **(Figure 5).**

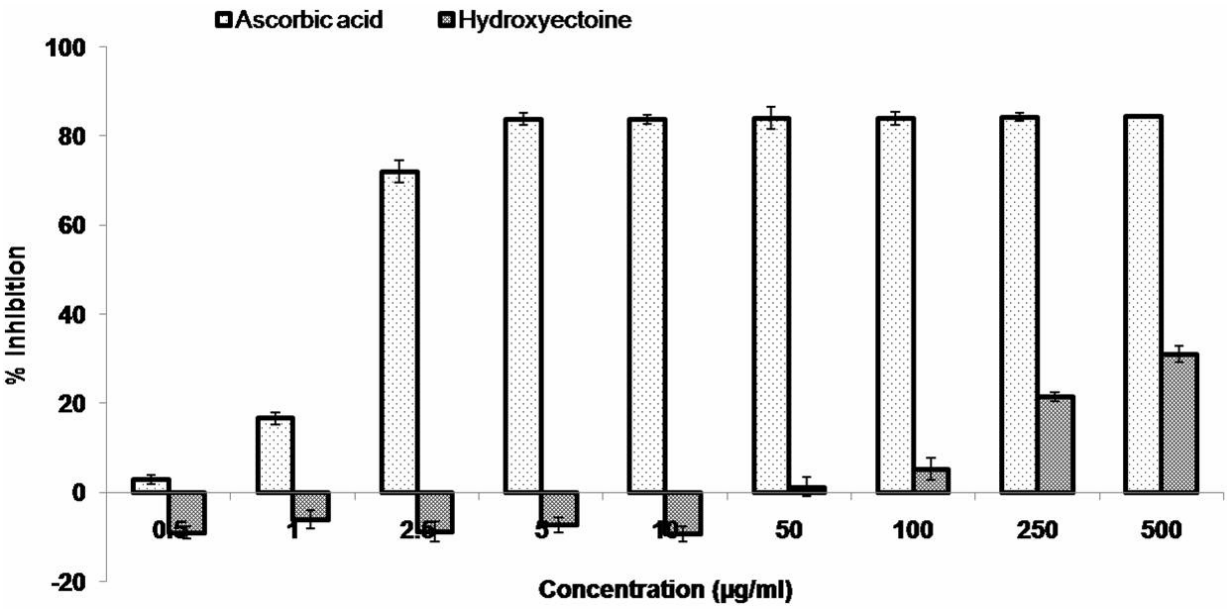

Fig. 4. DPPH radical neutralizing efficacy of hydroxyectoine
Alcoholic suspension of DPPH (100µM) was treated with different concentrations (0.5-500µg/ml) of hydroxyectoine and incubated for 10 min in the dark at room temperature. The reduction of the DPPH radicals was estimated as the function of decrease absorbance as monitored at 517 nm along with increasing hydroxyectoine concentration.

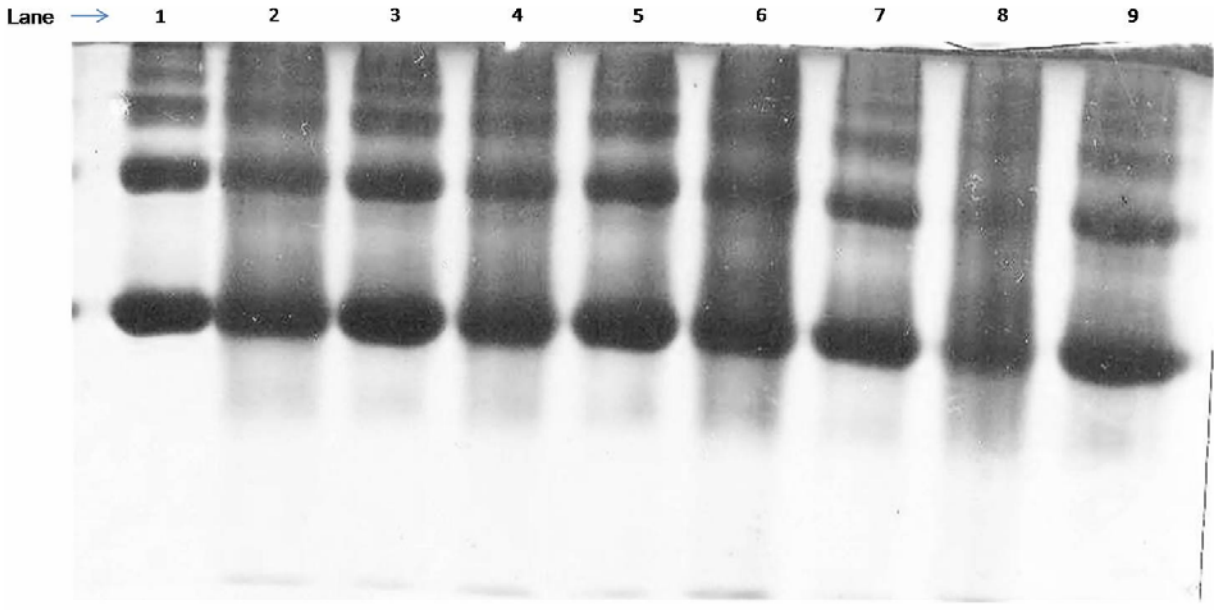

Fig. 5. Analysis of protein protection offered by hydroxyectoine on Native PAGE
Observance of protein protection (BSA) offered by hydroxyectoine (25µg/ml) at different radiation doses (1000-2000Gy). Lane1: control BSA; Lane2: only 1000Gy; Lane3: HE + 1000Gy; Lane4: only 1200Gy; Lane5: HE + 1200Gy; Lane6: only 1500Gy; Lane7: HE +1500Gy; Lane8: only 2000Gy; Lane9: HE +2000Gy

DISCUSSION

Radiation-induced oxidative damage is a multi-facet phenomenon that impaired regulation of vital cellular processes such as proliferation, repair, and recovery etc (31). Ionizing radiation exposure induces reactive oxygen/nitrogen species (ROS/RNS) such as superoxide radicals (O_2^-), hydroxyl radicals (OH^-), hydrogen peroxide (H_2O_2), and singlet oxygen in cellular milieu. Further radiation

induced water radiolysis also induced ROS/RNS formation, lead to membranes blebing, DNA fragmentation and lesions, oxidation and denaturation of functional proteins and other precious biomolecules resulted cell death (32). In the present study, hydroxyectoine was evaluated for its antioxidant and radioprotective activities. Several biochemical methods such as hydroxyl ion scavenging assay (as measured by inhibition of hydroxyl ion) and free radical scavenging properties using DPPH assay (41) were performed.

Nitric oxide (NO), a diffusible free radical, plays an important role in various biological functions including neuronal messenger, vasodilation, antimicrobial and antitumor activities (33). However, over-production of nitric oxide and superoxide radicals contributes to the pathogenesis of some inflammatory diseases (34). Moreover, in the pathological conditions, nitric oxide reacts with superoxide anion and form peroxynitrite which lead to serious toxic reactions with biomolecules, like protein, lipids, and nucleic acids (35-36). Hydroxyectoine showed significant inhibition (12.23%) in the nitric oxide radical generation in *in vitro* system (Figure 1). Thus taking into account, hydroxyectoine may be contemplated with its similar antioxidant action under radiation-induced pathological situations. Hydroxyectoine -induced inhibition of nitric oxide radicals may be one of the mechanisms for its radioprotective effect.

Hydroxyl radical is the most reactive oxygen centred radical, formed during the reaction of various peroxides with transition metal ions. It bears the shortest half-life compared to other ROS. OH-radicals are known to attack on proteins, DNA, polyunsaturated fatty acid in membranes and other biological molecules in the surrounding cellular milieu (37). OH radicals capable enough to abstracts hydrogen atoms from membrane lipids (38). The hydroxyl ion scavenging activity of hydroxyectoine was evaluated using deoxy-D-ribose assay. It was used to study the non-site specific [Fe^{2+} + H_2O_2 + EDTA] hydroxyl ion scavenging activity in aqueous system. In Non-site-specific assay, the presence of EDTA makes Fe^{2+} non-available for attacking deoxyribose directly (39). The quenching ability of hydroxyectoine to hydroxyl radicals seems to be directly related to the prevention of propagation of the process of lipid peroxidation and seems to be a good scavenger of active oxygen species. This study indicated significantly higher non-site-specific hydroxyl ion scavenging potential of hydroxyectoine **(Figure 2)**.

The most efficient tool to evaluate antioxidant and free radical properties of any compound is DPPH assay. 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) is one of the most widely used stable free radicals react with antioxidants, extract electron from it and gets reduced (Refer reaction mention below).

$DPPH^{\bullet}$ + AH = DPPH-H + $A^{\bullet}$
Or $DPPH^{\bullet}$ + $R^{\bullet}$ = DPPH-R

We have found that hydroxyectoine expressed a good DPPH radical scavenging capacity in a concentration-dependent manner (Figure 4). These findings were in accordance with all above presented observations and characterized hydroxyectoine as an efficient antioxidant.

As antioxidant potential has been recognised as the contributory factor to radioprotection offered by any compound, hydroxyectoine was evaluated for its radioprotective efficacy *in vitro* models. To evaluate hydroxyectoine mediated protection to the Bovine Serum Albumin (BSA) against lethal ionizing radiation, native-PAGE analysis was performed. Results of the study indicated clear intense smearing in the BSA bands pattern with increasing radiation doses (Figure 5), suggested intense oxidative damage in the secondary structure of BSA. However, no such smearing was observed in the BSA bands when it was irradiated in the presence of hydroxyectoine (Figure 5), provided a credible evidence of hydroxyectoine mediated protection to the BSA against supralethal gamma irradiation.

Based on the current study, it can be concluded that hydroxyectoine carried strong antioxidant properties, and thus can neutralize radiation induced free radicals efficiently in *vitro.* Though, there is no such *in vivo* study on radioprotection was done so far with hydroxyectoine. However, based on the preliminary experimental evidences, it can be worth to conclud that hydroxyectoine can be used clinically as potential radioprotector / mitigator agent to overcome the radiation toxicuty in the cancer patient undergoing radiotherapy regime. However, further studies

to evaluate *in vivo* radioprotective efficacy of hydroxyectoine will certainly explore its future application in planned (radiotherapy of cancer patients) and unplanned (accidently or deliberately) radiation exposure eventuality.

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REFERENCES

1. Weiss, JF., and Landauer, MR., Protection against ionizing radiation by antioxidant antrients and phytochemicals. *Toxicology,* 189:1-20, 2003.
2. Arora, R., Gupta, D., Chawla, R., Sagar, R., Sharma, AK., Prashad, J., Singh, S., Kumar, R., Sharma, RK.,Radioprotection by plant products: present status and future prospects. *Phytother Research* 19:1-22, 2005.
3. Upadhyay,SN., Dwarakanath, BS., and T,Ravindranath.,Chemical Radioprotectors *Defence Science Journal,* 55(4):403-425,2005.
4. Cao, G., Prior, RL., Measurement of oxygen radical absorbance capacity in biological samples. *Methods in Enzymology*., Academic Press, pp 50–62,1999.
5. Valko, M., Rhodes, CJ., Moncol, J., Izakovic, M., Mazur, M., Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chem Biol Interact,* 160:1–40,2006.
6. Alper,T., *Cellular radiobiology*. Cambridge University Press, London,1979 .
7. Carr, J., Huff, JE., Fisher, KD., Huber, TE., Protective agents modifying biological effects of radiation. *Archives Environ Health,* 20:88-98,1970.
8. Chapman, JD., Reuvers, AP., The time scale of radioprotection in mammalian cells. *Experentia,* 27: 9-18, 1977.
9. Edwards, JC., Chapman, D., Cramp, WA., Yatvin, MV., The effects of ionising radiation on biomembrane structure and function. *Prog. Biophys. Mol. Biol,* 43:7 l-93, 1984.
10. Ferle Vidovic, A., Petrovic, D., Vidic, Z., Osmak, M., Kadizak, K., Absence of 2-aminoethyl isothiouronium bromide hydrobromide protection against fast neutrons: Cellular effects. *Radiat. Environ. Biophys*,19: 197-204,1981.
11. Téoule, R., Radiation-induced DNA Damage and Its Repair.*International Journal of Radiation Biology* ,51(4):573-589,1987.
12. Ghose, A., Ganguly, SK., Kaur, J., Protection with combination of hydroxytryptophan and some thiol compounds against whole body gamma radiation. *Int. J. Radiat. Biol,* 44:175-181, 1983.
13. Goel, HC., Ganguly, SK., Prasad, J., Jain, V., Radioprotective effect of diltiazem on cytogenetic damage and survival in gamma ray-exposed mice. *Ind. J. Exp. Biol*, 34:1194-2000, 1996.
14. Yuhas, JM., Proctor, JO., Smith, LH., Some pharmacologic effects of WR-2721: Their role in toxicity and radioprotection. *Radiation Research,* 54: 222-33, 1973.
15. (15)Yuhas, JM., Biological factors affecting the radioprotective efficiency of S-2-(3- aminopropylamino) ethylphosphorothioic acid (WR- 2721): LD (30) doses. *Radiation Research,* 44: 621-628, 1970.
16. Satyamitra, M., Devi, Uma P., Murase, H., Kagiya, VT., In vivo post-irradiation protection by a vitamin analogue, alpha-TMG. *Radiation Research*, 160 (6):655-661,2003.
17. Nair, CKK., Salvi, V., Kagiya, VT., Rajagopalan, R., Relevance of radioprotectors in radiotherapy: Studies with tocopherol monoglucoside. *J. Env. Pathol. Toxicology Oncol*, 23:153-160,2004.
18. Reuter, K., Pittelkow, M., Bursy, J., Heine, A., Craan, T., Synthesis of 5-Hydroxyectoine from Ectoine: Crystal Structure of the Non-Heme Iron(II) and 2-Oxoglutarate-Dependent Dioxygenase EctD. *PLoS ONE,* 5(5): e10647, 2010.
19. Hideki, Nakayama., Kazuya, Yoshida., Hisayo, Ono., Yoshikatsu, Murooka., and Atsuhiko, Shinmyo., *Plant Physiology*,122, pp. 1239–1247,2000.
20. Galinski, EA., Pfeiffer, HP., Truper, HG., 1,4,5,6-Tetra-hydro-2-methyl-4-pyr-imidinecaroxylic acid.A novel cyclic amino acid from halophilic phototrophic bacterial of the genus Ectothiorhodospira. *Eur J Biochem*, 149, 135-139,1985.

21. Galinski, EA., Truper, HG., Microbial behaviour in salt-stressed ecosystems. *FEMS Microbial Reviews*, 15,1994.
22. Galinski, EA., Compatible solutes of halophilic eubacteria: molecular principles, water-solute interaction, stress protection. *Experientia,* 49, 487-496,1993.
23. Lippert, K., Galinski, EA., Enzyme stabilization by ectoin-type compatible solutes: protection against heating, freezing and drying. *App Microbiol Biotech-nol*, 37,61-65,1992.
24. Inbar, L., Lapidot, A., Metabolic Regulation in Streptomyces parvulus during Actionomycin D Synthesis, *Journal Bacterio*, 170, 4055-4064,1988.
25. Inbar, L., Lapidot, A., The structure and biosynthesis of new tetrahydropyrimidine derivatives in actionomycin D producer streptomyces parvulus, *J Biological Chemistry*, 263, 16014-16022.1988.
26. Louis, P., Truper, HG., Galinski, EA., Survival of Escherichia coli during drying and storage in the presence of compatible solutes. *Appl Microbiol Biotechnology*, 41,684-688,1994.
27. Kakkar, P., Das, B., Viswanathan, PN., A modified spectrometric assay of superoxide dismutase. *Ind J Biophys Biochem*, 21:110-133,1984.
28. Garrat, DC., The quantitative analysis of drug. Chapman and Hall, Hirakawachi,1964.
29. Halliwell, B., Gutteridge, JM., Aruoma, OI., The deoxyribose method: a simple test tube assay for determination of the rate constants for reactions of hydroxyl radicals. *Anal Biochem,* 165:215–219,1987.
30. Brand-Williams, W., Cuvelier, ME., Berset, C., Use of free radical method to evaluate antioxidant activity. *Lebensmittel Wissenschaft und Technologie,* 28:25–30,1995.
31. Giambarresi, L., Jacobs, AJ., Radioprotectant. In: Conklin JJ, Walker RI (eds) *Military radiobiology.* Academic Press, London, pp 265–301,1987.
32. Sies, H., Oxidative stress. Academic press, New York,1985.
33. Miller, MJ., Sadowska-Krowicka, H., Chotinaruemol, S., Kakkis, JL., Clark, DA., Amelioration of chronic ileitis by nitric oxide synthase inhibition. *J Pharmacol Exp Ther,* 264:11–16,1993.
34. Guo ,X., Wang, WP., Ko, JK., Cho, CH., Involvement of neutrophils and free radicals in the potentiating effects of passive cigarette smoking on inflammatory bowel disease in rats. *Gasteroenterol,* 117:884–890,1999.
35. Moncada, S., Palmer, RM., Higgs, EA., Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev*, 43:109–142,1991.
36. Yermilov, V., Rubio, J., Becchi, M., Friesen, MD., Pig-Natelli, B., Ohshima, H., Formation of 8-nitroguanine by the reaction of guanine with peroxynitrite in vitro. *Carcinogenesis,* 16:2045–2050,1995.
37. Aruoma, OI., Free radicals, antioxidants and international nutrition. *Asia Pacific J Clin Nutr,* 8:53–561,1999.
38. Yen, G., Duh, P., Scavenging effect of methanolic extracts of peanut hulls on free-radical and active-oxygen species. *J Agric Food Chem*, 42:629–632,1994.
39. Kitts, DD., Wijewickreme, AN., Hu, C., Antioxidant properties of a North American ginseng extract. *Mol Cell Biochem,* 203:1–10,2000.
40. Kumar, R., Patel, DD., Bansal, DD., Mishra, S., Mohammed, A., Arora, R., Sharma, A., Sharma, RK., and Tripathi, RP., Extremophiles: Sustainable Resource of Natural Compounds-Extremolytes, *Sustainable Biotechnology*, 279-294,2010.
41. Kumar, R., Bansal, DD., Patel DD., Mishra, S., Karamalakova, Y., Zheleva, A., Gadjeva .,V., Sharma,RK., Antioxidative and radioprotective activities of semiquinone glucoside derivative (SQGD) isolated from Bacillus sp. INM-1, *Mol Cell Biochem,*349:57–67,2011.