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RESEARCH ARTICLE

OXIDATIVE STRESS REDUCING CAPABILITIES OF MORACIN, THE NOVEL COMPOUND FROM THE FRUITS OF MULBERRY, *MORUS ALBA* (L) IN HYDROGEN PEROXIDE INDUCED STRESS IN SKIN FIBROBLAST CELL LINE CULTURE (AH927)

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ABSTRACT

The potential regarding antioxidant activity of Moracin has been assessed through the use of hydrogen peroxide induced stress in skin fibroblast cell line culture (AH927). The results of the attempt showed that the Moracin offers protection against oxidative stress. And cell viability was found restored to that of control on pre-incubation with the Moracin. The fibroblasts pre-incubated with Moracin had significantly lower levels of catalase; lactate dehydrogenase and malondialdehyde activity when compared to untreated ones. This attempt indicates that, the Moracin is serving as a valuable antioxidant source and therefore, it may be used to treat the cancer cells. The present attempt suggests that acetone-soluble (and water- soluble too) Moracin, from a natural source the fruits of mulberry, Morus alba (L), serve as ideal molecule to prevent oxidative stress.

INTRODUCTION

Moracin M is a phosphodiesterase-4 inhibitor isolated from *Morus alba* (L). Mulberry (*Morus alba* L., Moraceae) has been used in traditional Chinese medicine as an anti-headache, anti-hypertensive, anti-diabetic, and diuretic agent (Lee *et al.,* 1981). In particular, mulberry twigs have been widely used for the treatment of aching and numbness of joints in oriental medicine (Zhu, 1998). Several prenylflavonoids, flavonoids, coumarins and stilbenes have been isolated and identified from mulberry twigs (Ko *et al.,* 1997; Oh *et al.,* 2002; Hu *et al.,* 2011; and Chang *et al.,* 2011). Among them, prenylflavonoids and flavonoids have been reported as major principles for anti-obesity, antioxidant, anti-aging, and hepatoprotective activities of mulberry twigs (Ko *et al.,* 1997; Oh *et al.,* 2002 and Hu *et al.,* 2011). In addition, some coumarins and resveratrol derivatives in mulberry twigs were found to have strong radical scavenging and anti-inflammatory activities (Oh *et al.,* 2002 and Chung, *et al.*, 2003). Thus, mulberry twigs are receiving much interest as promising sources of functional foods with health benefits. Mulberry twigs are widely used as a promising source of well-being healthy teas, together with mulberry fruits and leaves. In addition, mulberry soups and wines made with mulberry twigs were known to have potential health benefits in folk medicine against diabetes, stroke, cough, and beriberi, etc. (Lee *et al.,* 1981).

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Therefore, study on analysis of functional constituents for standardization and quality control of mulberry twig teas, soups, and wines is required. The biochemical constituents of leaves of mulberry, *Morus alba*(L) serve a lot to orchestrate the progression of life cycle of lepidepteran insects like silkworm, *Bombyx mori*(L). Mulberry leaves are also used for food for livestock (Cattle, goat *etc.*) in the areas where dry seasons restrict the availability of ground vegetation. The traditional Chinese medicine recommend the mulberry fruits to treat the prematurely grey hair, to tonify the blood and to treat constipation and human diabetes. Zhang *et al*. (2009) reported the Moracin –M, Steppogenin–4'– O–beta-D–glucoside and mulberroside- the novel compounds of mulberry, *Morus alba* (L) for hypoglycemic effects. Naowaboot *et al.,* (2009) studied the effect of Ethanolic extract of leaves of mulberry, *Morus alba* (L) on chronic diabetic rats and observed antihyperglycemic, antioxidant and antiglycationactivity. Cancer induction is distinguished by involvement of oxidative stress in the cells. The cancer induction and its subsequent development, and associated molecular mechanism is becoming increasingly clear (Lahiri. *et al*., 1999 and Ames *et al*., 1995). The enzyme Catalase is a found in nearly all living organisms exposed to oxygen (such as bacteria, plants, and animals). This catalase enzyme help for the decomposition of hydrogen peroxide to water and oxygen (Chelikani, *et al.*, 2004). And therefore, catalase is a very important enzyme in protecting the cell fromoxidative damage by reactive oxygen species (ROS). Likewise, enzyme catalase has one of the highest turnover numbers of all enzymes; one catalase

molecule can convert millions of hydrogen peroxide molecules to water and oxygen each second. With reference to chemical structure, catalase enzyme is a tetramer of four polypeptide chains, each over 500 amino acids long. It contains four groups of porphyrinheme (iron), which allow the enzyme to react with the hydrogen peroxide. The optimum pH for human catalase enzyme is approximately 7 (Maehly, and Chance, 1954) and has a fairly broad maximum (the rate of reaction does not change appreciably at pHs between 6.8 and 7.5) (Aebi, 1984).The pH optimum for other catalases varies between 4 and 11 depending on the species. The optimum temperature also varies from species to species (Toner, *et al*, 2000). The human skin is directly and frequently exposed to sun light. Further, the skin is always in contact with oxygen, which resulting in the production of reactive oxygen species (ROS). This is implying that, the skin is always in a state of being attacked by ROS (Garmyn and Degreef, 1997). The chemical structure of Moracin is recommending a possibility of suppression of skin cancer promotion through it's antioxidant activity. A well known method on TPA– induced tumor promotion in DMBA initiated in mouse skin is availing excellent for screening the possibilities of cancer suppression through phytocompounds (Dragsted, 1998). TPA - Application triggers excessive R O S production by leucocytes in mouse skin ultimately leading tumor promotion (Nakamura *et al.,* 1998). This method had been undertaken to address whether the given compound suppress TPA – induced oxidative stress in mouse skin. TNF – alpha one of the inflammatory cytokines, acts as endogenous tumor promoter, and induces similar biochemical and biological responses as known tumor promoters (Suganuma *et al.,* 1999 and Fuji *et al.*, 1997). The increased levels of reactive oxygen species (ROS) is responsible for damage of various cellular processes. The hydrogen peroxide (H2O2) is responsible for release of hydroxyl radical, which in its turn reacting with different transition metals. The hydroxyl radical is distinguished by its harmful effects through reactive oxygen species (ROS). The harmful effects of the ROS include damage the DNA, lipid peroxidation, oxidation of proteins and deactivation of enzymes through oxidation of co-factors, leading to mutagenesis and carcinogenesis (Datta *et al.,* 2002). There is ample research with reference to discover new antioxidant compounds from plant and animal origin to prevent free radical damage (Brooker, 2011). Farrer's scallop, a marine bivalve (also known as the Chinese scallop), *Chlamys farreri* (L) release a polypeptide with antioxidant property (Han,*et al*, 2004 and Ghosh *et al*., 2006).

The insects of Lepidoptera, of both the families Bombycidae and Saturniidae are well known to produce commercially important silks. The domesticated mulberry silkworm, *Bombyx mori* (L) represents family Bombycidae and wild non-mulberry silkworms, *Antheraea mylitta*e (L) presents family Saturniidae. The silk fiber of cocoons of silkworms of boththe families, Bombycidae and Saturniidae consist of two major proteins, fibroin and sericin. Fibrion is a central core of silk fiber. And sericin forms envelop of silk fiber. Both, fibroin and sericin, of mulberry silkworm, *Bombyx mori*(L) deserve appreciable physico-chemical properties and therefore, they are now recognized as excellent biomaterials in the field of tissue engineering, biotechnology and therapy. Fibroin is the insoluble in water and sericin is water soluble. The fibroin of silk fibre of mulberry silkworm, has been recognized as a substrate for growth and adherence of cells in culture (Wang, *et al.*, 2006). Moracin treatment was found significantly suppressing the elevation in 4 - HNE level and elevated expression of c-fos, c-myc and cycloxygenase-2 (COX-2) in normal epidermis induced by double application of TPA (Vitthalrao Khyade *et al.,* 2013). The moracin was found protective influence in tumor promotion. The Moracin is a herbal compound. It may have had better effects in comparison with Sericin. It may exert it's influence in through its best way in stem cells. On this much background the attempt was planned to study the oxidative stress reducing capabilities of Moracin, the novel compound of Mulberry, *Morus alba* (L) through the use of hydrogen Peroxide Induced Stress In Skin Fibroblast Cell Line Culture (AH927).

MATERIALS AND METHODS

The attempt was carried out through the steps, which include: Cell culture of Skin Derived Fibroblast; Procurement of Moracin; Analysis of cell viability; The Phase contrast and fluorescence microscopy; Influence of pre treatment with Moracin for 24 hrs; Cell homogenate Preparation; Analysis of lactate dehydrogenase (LDH); Catalase Analysis; Lipid Peroxidation Analysis through thiobarbituric acid reactive substances (TBARS) and malondialdehyde (MDA) and Statistical Analysis of the Data.

(I). Cell culture of Skin Derived Fibroblast: The cell line of skin fibroblast (feline) (AH927) was procured from National Centre for Cell Science, Pune, India. This skin fibroblast (feline) cell line was used for further processing. The Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and streptomycin-penicillin (10 µg/ ml) were used as culture medium. Cells in the attempt were incubated at 37 degree Celsius. The five percent of carbon dioxide was maintained in the cabinet of culture. Cells were sub cultured through the method described by Zhaorigetu, (2003) at intervals of 3 days and those between passages 4-8 were used for further experimentations.

(II). Procurement of Moracin: Moracin were procured from Sigma Chemicals. Stock solution of Moracin of 1.00 ppm strength was prepared through the use of acetone as solvent. This stock solution was used for further dilution. Various concentrations of Moracin include: 00 ng/ml; 25ng/ml; 50 ng/ml; 75ng/ml; 100 ng/ml; 125ng/ml; 150ng/ml; 175 ng/ml and200 ng/ml.

(III). Analysis of cell viability: As suggested by Mossman (1983), the thiazolyl tetrazolium compound (MTT) was utilized for the purpose to analyse the cell viability, the extent of reduction. The cells of cell line of skin fibroblast (feline) (AH927) were seeded in 96-well plates (1×104 cells/well) in DMEM medium containing 10% fetal bovine serum. They were allowed for the overnight culture. Then, they were treated with different concentrations (0, 0.1, 0.2, 0.5 and 1.0 mM) of hydrogen peroxide (H2O2). The wells with cells, Dulbecco's modified Eagle's medium (DMEM) and hydrogen peroxide (H2O2) were kept in the incubator for24 hrs. Freshly prepared hydrogen peroxide (H2O2) from 30 % stock solution (Sigma) was used for treatment. Untreated cells were considered as control group. Soon after the incubation, the media in wells were removed and replaced with200 µl of fresh media containing 20 µl MTT solution (5 mg/ml) and incubated at 37 degree Celsius for 4 hrs. After the 4 hours incubation, the

media-containing MTT were removed. The addition of 200 µl of DMSO was made. This addition of DMSO was for the purpose to dissolve the for mazan crystals formed in the viable cells. The content was processed for reading the optical density through the method described by Tesco *et al.,* 1992 and recorded in Biorad 550 microplate reader at 595nm. The percent viability as in control cells, was considered as the result, extent of H2O2 mediated cell death. Each experimental attempt was repeated for four times for consistency in the results. The data collected was subjected for statistical analysis.

(IV). The Phase contrast and fluorescence microscopy: The Phase contrast and fluorescence microscopy were used for the purpose of microscopic observations. 106 cells were seeded on square coverslip (22×22 mm) and processed for microscopy. The coverslip was placed inside a petri plate and allowed to grow. The entire overnight was utilized for the growth of cells. Thereafter, the cells were treated with 0.5 mM Hydrogen Peroxide (H2O2). This Hydrogen Peroxide (H2O2) was carried for 24 hrs. The growth of the cells was observed under phase contrast and then, cells were washed with the ice-cold sterile phosphate buffered saline (PBS). The cells were then fixed in fixative consisting of methanol : acetone (1 : 1). The fixation was carried out for an hour. The cells were rinsed in PBS and incubated with Tween-20 (0.05 %). For the membrane permeabilisation, incubation was carried out for 30 minutes. This was followed by RNase (10 mg/ml) treatment for half an hour. After rinsing in PBS, cells were exposed to propidium iodide (1 mg/ml) for half an hour. The cell nuclei were visualized under fluorescence microscope Leica DMR-HC(with camera MP-60). The emission wavelength was consisting of 615 nm (Takasu *et al.,* 2002 and Dash *et al.,* 2006).

(V). Influence of pre treatment with Moracin for 24 hrs: As mentioned earlier for assessment of cell viability, the cells were subjected to pretreatment with Moracin individually at different concentrations ranging from 5 to 150ng/ml or gelatin (150 ng/ml). Preliminary experiments showed that pretreatment with sericin for 24 hours, before subjecting to oxidative stress due to 0.5 mM H2O2, gave maximum protection in comparison to 6, 12 and18 hours. The viability was checked by MTT assay after 24 hrs of exposure to hydrogenperoxide (Vitthalrao Khyade, 2016).

(VI). Cell homogenate Preparation: The cells (1×104 cells/ well) were seeded in petri plates (35 mm)and allowed to adhere for 24 hrs before treatment with Moracin. The strength of Moracin was100 ng/ml was found exhibiting the appreciable protection (from cell viability assays). Therefore, only Mracin was used for further biochemical experimentations. The cells were pre-incubated with 25 and 100 ng/ml Moracin and 150 ng/ml gelatin (these concentrations represent the concentrations where minimum and maximum protective effect was observed for Moracin and gelatin) for 24 hrs followed by 24 hours oxidative stress induction (hydrogen peroxide treatment). The cells were washed for two times with ice-cold PBS and they were harvested through the use of 0.025 %Trypsin-0.02 % EDTA solution. The cells were re-suspended in ice-cold phosphate buffered saline (PBS) and were then homogenized. The cell homogenates were then processed for centrifugation for 5 min at 5,000 × *g* . Supernatant of each sample was used for further biochemical tests (Mossman, 1983).

(VII). Analysis of lactate dehydrogenase (LDH): The quantitative assessment of fibroblast damage caused by hydrogen peroxide was carried out through the ratio of the enzyme activity released from damaged cells to the activity in undamaged / intact cells, monitored for 24 hours after induction of the stress. The medium collected after stress induction was centrifuged (for 5 min at 5,000 × *g)* and supernatant used for the assay of enzyme released from damaged cells.

% release of LDH= (released into the medium÷ Activity in sonicated cells) x100

Reduction of pyruvate by the enzyme on addition of NADH was monitored as the change in optical density of the reaction mixture containing 20 mM phosphate buffer (pH 7.4), 3.3mM sodium pyruvate and 2 mM NADH. The LDH activity was expressed as change in absorbance at 340 nm perminute as explained by Bergmeyer and Bernt, 1963).

(VIII). Catalase Analysis: Bioassay of enzyme catalase (CAT) was carried out through the method described by Maehly and Chance (1954). The catalase (CAT) was assayed by noting the decrease in optical density of NADH at 240 nm following the decomposition of hydrogen peroxide. The reaction mixture consisted of 10mM phosphate buffer (pH 7.0), 30 mM hydrogen peroxide and the enzyme source. The decrease in optical density of the reaction mixture was monitored at 240 nm for 5 min. The activity of enzyme LDH was expressed as change in optical density per minute. (Extinction coefficient =0.021). The activity of enzyme released into the medium and those in intact cells were determined as described for LDH analysis and expressed in the unit of percentage.

(IX). Lipid Peroxidation Analysis through thiobarbituric acid reactive substances (TBARS) and malondialdehyde (MDA): The concentration of thiobarbituric acid reactive substances (TBARS) in the medium after induction of oxidative stress was measured according to the protocol by Niehaus and Samuelson (1968). 0.5 ml supernatant of each sample was mixed with 0.5 ml thiobarbituric acid reagent (1 : 1, v: v, mixture of0.67% thiobarbituric acid and acetic acid). The reaction mixture was heated at 95 degree Celsius for an hour. After cooling, centrifugation was carried out at 1,000×*g* . The optical density readings of the supernatant were taken at 535 nm.

(X). Statistical Analysis of the Data: For the purpose to get consistency in the results, each experimental attempt was repeated for four times. The data was collected and subjected for statistical analysis explained by Norman and Baily (1955).The data were presented as mean ±S.E.M. and compared using one-way ANOVA and Tukeys test. $P < 0.05$ or less was considered to be statistically significant.

RESULTS AND DISCUSSION

The results pertaining the antioxidant potential of Moracin against hydrogen

Table 1. Cell viability (extent of reduction of the thiazolyltetrazolium compound (MTT) of feline fibroblast cells (AH927) in presence of Hydrogen Peroxide

Concentration of Hydrogen Peroxide (millimol)	Percent Viable Cells
0.00	100.000 (±2. 381)
0.10	073.806 (± 7.793)
0.20	051.193 (± 5.95)
0.50	027.383 (± 4.786)
1.00	008. 321 (± 2.492)

Each value is the mean of three replications.
Figures in parenthesis with(±) signs are the standard deviations.

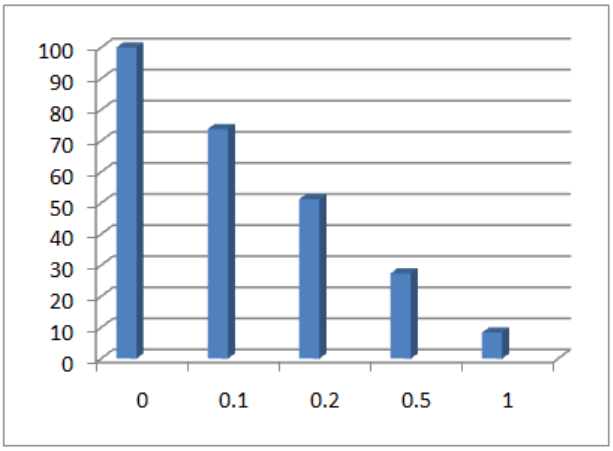


Figure 1. Cell viability (extent of reduction of the thiazolyltetrazolium compound(MTT) of feline fibroblast cells (AH927) in presence of Hydrogen Peroxide

Peroxide - induced oxidative stress in skin fibroblasts are summarized in Table – 1 to 3 ; Fig. 1 to 3 and explained through the parameters, which include: sensitivity of Fibroblast cells to hydrogen peroxide; morphological changes of the cells on exposure to hydrogen peroxide; effect of Sericin treated before the induction of oxidative stress,, LDH activity, Catalase activity and lipid peroxidation through the levels of thiobarbituric acid reactive substances (TBARS) and Malondialdehyde (MDA).

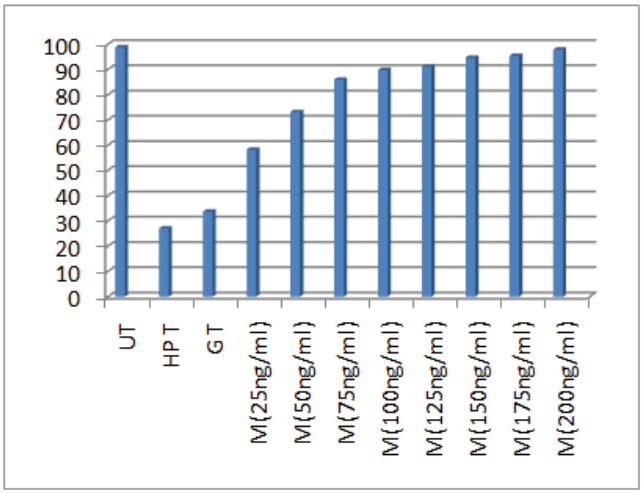
Sensitivity of Fibroblast Cells to Hydrogen Peroxide: The viability of fibroblast cells strikingly found decreased in a concentration-dependant manner on treatment with various concentrations of hydrogen peroxide. The LC50 value for 24 hr exposure to hydrogen peroxide was found measured 0.2 mM. This result is indicating that, AH927 cells were sensitive to cell damage induced through hydrogen peroxide treatment. Various concentrations of Hydrogen peroxide (0.1, 0.2,0.3, 0.4, 0.5 and 1.00 milimole) were found affecting on the viability of the cells used in the study. The concentration of 0.5mMhydrogen peroxide was found reducing the cell viability to the percentage 27.383 (± 4.786) and with that of 1.0 mM hydrogen peroxide decreased the viability to the percentage 8.321 (± 2.49) (Fig. 1). Increase in the concentration of hydrogen peroxide was found significantly affecting on the percent viability of the fibroblast cells. Reduction in percent viability of fibroblast cells treated with hydrogen is responsible for induction of stress.

Morphological changes in Fibroblast cells on hydrogen peroxide exposure: Preliminary MTT assay in the present attempt revealed in that, viability of fibroblast cells was significantly less at 0.5 mM hydrogen peroxide concentrations. The propidium iodide staining was used for the study on morphological changes in cells at concentration of 0.5 mM Hydrogen peroxide. This method helped further to assess the damage caused by oxidative stress. The observations under phase contrast microscope, the morphology of the fibroblast cells exposed to 0.5 mM Hydrogen peroxide were revealed shrunken and rounded nature of the fibroblast cells. This is in comparison to normal cells. Fluorescence staining of cells revealed that cells exposed to 0.5 mM H2O2 exhibited nuclear condensation.

Table 2. Impact of Moracin on the Cell viability (extent of reduction of the thiazolyltetrazolium compound (MTT) of feline fibroblast cells (AH927) treated with Hydrogen Peroxide

Serial No.	Group	Percent Viability of the Cells
1.	Untreated Control	98.786 (± 6.253)
2.	Hydrogen Peroxide Treated	27.084 (± 3.318)
3.	Gelatin Treated	33.719 (± 2.623)
4.	Moracin (25 ng/ml) Treated	58.215 (± 4.431)
5.	Moracin (50ng/ml) Treated	73.123 (± 4.409)
6.	Moracin (75 ng/ml) Treated	86.011 (± 5.287)
7.	Moracin(100ng/ml) Treated	89.808 (± 4.964)
8.	Moracin(125 ng/ml) Treated	91.143 (± 7.242)
9.	Moracin(150ng/ml) Treated	94.651 (± 7.521)
10.	Moracin(175 ng/ml) Treated	95.398 (± 9.463)
11.	Moracin(200ng/ml) Treated	97.853 (± 13.706)

Each value is the mean of three replications.
Figures in parenthesis with(±) signs are the standard deviations.



UT: Untreated; HPT: Hydrogen Peroxide Treated; GT: Gelatin Treated; M: Moracin

Figure 2. Impact of Moracin on the Cell viability (extent of reduction of the thiazolyltetrazolium compound (MTT) of feline fibroblast cells (AH927) treated with Hydrogen Peroxide

Effect of Moracin treated before the induction of oxidative stress: The effect of pre-treatment of cells with Moracin for 24 hrs is summerised in Table - 2. The pretreatment of gelatin with the cells for 24 hrs did not show significant protection against Hydrogen peroxide induced oxidative stress. The cells treated with 00 ng/ml; 25ng/ml; 50 ng/ml; 75 ng/ml; 100 ng/ml; 125 ng/ml; 150 ng/ml; 175 ng/ml and 200 ng/ml were found measured 98.786 % ;58.215 % ; 73.123 % ; 86.011 % ; 89.808 % ; 91.143 % ; 94.651 % ; 95.398 % and 97.853 % respectively. The one-way analysis of variance revealed that, there was an overall significant difference in cell viability between controls, H2O2-treated and Moracin treated fibroblasts ($F=44.47$, $P < 0.001$). Subsequent multiple comparisons by Tukeys test indicated that, cell viability was significantly lower ($P < 0.01$) in hydrogen peroxide-treated

when compared with control and Moracin treated fibroblasts. The concentrations of 25, 50, 75 and 100 ng/ml Moracin were found significantly (P <0.05) increasing the cell viability. Cells treated with Moracin at 125ng/ml ; 150ng/ml ; 175ng/ml and 200 ng/ml and exhibited cell viability comparable to that of control group (P >0.05). This result indicate that, pre-incubation with 150 ng/ml Moracin restore the cell viability to normal.

LDH activity: The table – 3 depicts the percentage of LDH activity released into the medium in normal fibroblast cells, fibroblast cells treated with 0.5 mM hydrogen - peroxide for 24 hrs, and fibroblast cells pre-incubated with 35 ng/ml and100 ng/ml of Moracin. The oxidative stress through hydrogen peroxide was found responsible for significant increase (P <0.01) in the release of enzyme (LDH) in comparison with untreated control and cellspre-incubated with 100 ng/ml of Moracin before hydrogen peroxide treatment. The oxidative stress through hydrogen peroxide is responsible for the loss of membrane integrity of the cells. The sericin treatment definitely serving to restore the original membrane integrity of the cells.

Catalase activity: Rate of catalase activity in various treated cells and control are presented in Table - 3. Catalase activity was significantly high (P <0.01) in medium of cells treated with hydrogen peroxide (80.608 %) compared to control(18.434%). The fibroblasts pre-incubated with Moracin at 100 ng/ml had significantly decreased (P <0.01) catalase activity. Oxidative stress through treating the cells with hydrogen peroxide is responsible for increase in the catalase activity and Moracin treatment have had protective influence on oxidative stress.

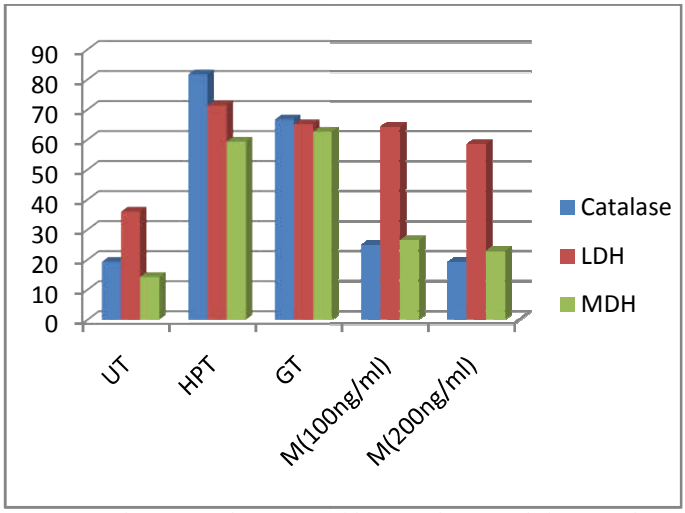
Lipid peroxidation through the levels of thiobarbituric acid reactive substances (TBARS) and Malondialdehyde (MDA)

The % of TBARS/ MDA in the media expressed as nmol /ml of the medium is shown in Table - 3. The products of peroxidation were significantly high (P <0.01) in the media of cells treated with hydrogen peroxide. On the other hand the cells pre-incubated with Moracinat 100 ng/mlhad significantly lower levels of TBARS (P <0.05). The hydrogen peroxide is model oxidant because its cellular actions and its fate has been well studied. In the present attempt, the protective effects of Moraicin induced oxidative damage in feline skin fibroblasts were observed. The mechanism of damage by hydrogenperoxide in fibroblast cultures involves the production of reactive oxygen species (ROS) (Vitthalrao B. Khyade, 2013). Level of the ROS in cultures pre-incubated with Moracin was significantly decreased as indicated by cell viability tests (MTT assay). Among the silk proteins tested, Moracin showed protective effect at a minimum concentration of 35 ng/ml and restored the viability and normal biochemical profile at 150 ng/ml which was not observed in case of sericin of *B. mori*. To study further, the effect of Moracinon hydrogen peroxide induced oxidative stress, the activities of LDH and catalase were examined. Acceleration of anaerobic metabolic pathway to cope with oxidative stress is reflected from an increase in LDH activity. The LDH activity of cells subjected to oxidative stress increased significantly in comparison to normal cells.

Table 3. Impact of Moracin on the activity of Catalase, Lactate Dehydrogenase (LDH) and Malondialdehyde (MDA) homogenate of feline fibroblast cells (AH927) treated with Hydrogen Peroxide

Group	Catalase Activity	LDH Activity	MDA Activity
Untreated Control	19.387 (± 1.853)	36.088 (± 5.411)	14.314 (± 00.893)
Hydrogen Peroxide Treated	81.789 (± 26.685)	71.413 (± 14.349)	59.378 (± 19.627)
Gelatin Treated	66.786 (± 19.372)	65.167 (± 22.417)	62.692± 18.969)
Moracin(100 ng/ml)	24.869 (± 3.903)	64.342 (± 6.673)	26.413 (± 4.516)
Moracin (200 ng/ml)	19.117 (± 4.672)	58.486 (± 6.786)	22.926 (± 3.739)

Each value is the mean of three replications.
Figures in parenthesis with (±) signs are the standard deviations.



UT: Untreated; HPT: Hydrogen Peroxide Treated; GT: Gelatin Treated; M: Moracin

Figure 3. Impact of Moracin on the activity of Catalase, Lactate Dehydrogenase (LDH) and Malondialdehyde (MDA) homogenate of feline fibroblast cells (AH927) treated with Hydrogen Peroxide

This clearly indicate the loss of membrane integrity through oxidative stress (Ujwala D. Lonkar and Vitthalrao B. Khyade, 2013). The enzyme catalase is involved in the decomposition of hydrogen peroxide to water and oxygen. Therefore, the enzyme catalase is important in protecting cells against oxidative stress (Babita Sakdeo and Vitthalrao B. Khyade, 2013). Significant increase in catalase activity in hydrogen peroxide stressed cells when compared to normal cells. A similar trend was also detected in case of the intracellular levels of products of the lipid peroxidation. Malondialdehyde (MDA) is expert to alter the structure and function of the cellular membrane and blocks cellular metabolism leading to cytotoxicity (Vitthalrao B. Khyade *et al.,* 2014). The amounts of LDH, catalase and TBARS were also significantly low in medium of Moracin-treated cells when compared to control as well as hydrogenperoxide-treated cells. This attempt indicates that Moracin might be providing protective effect on fibroblast by acting as antioxidant as well as by promoting endogenous antioxidant. Indeed, Moracin has previously been reported to possess photo - protective effect against the UVB-induced acute damage and colon carcinogenesis (Vitthalrao Khyade *et al.,* 2015). The present attempt report the cutaneous cell lines like AH927 fibroblasts are effectively protected against oxidative stress through Moracin.

Similar observations had been reported in earlier studies. The Moracinisthebeta-D-Glucopyranside and it exhibited strong anti-tumor promoting effect in the mouse skin two-stage-tumorigenesis model. The Moracin could be useful as skin - cancer preventing agent. Double application s of TPA to the mouse skin lead to excessive ROS production (Nakamura, *et al.,* 1998). The available data suggest that each concentration of Moracinapplication induces two distinguishable biochemical events, namely priming and activation. The first event, primingis characterized by infiltration of inflammatory leukocytes. The second event, the activation is characterized by ROS production from accumulated leukocytes (Murakami *et al.,* 2000 and Khyade *et al.,* 2014). Induction of inflammatory response, as seen by dermal recruitment of inflammatory cells, is thus, integral part of response of mouse skin to TPA (Skarin *et al.,* 1999). It has been also revealed that, the second TPA application significantly increases leukocyte infiltration in mouse skin (Nakamura *et al.,* 1998). The present attempt demonstrated the significant induction of leukocyte infiltration in response to application of double dose of acetone and TPA on the mouse skin. The study, further demonstrated significantly reduced TPA- induced leukocyte infiltration in the cutis in response to double application of Moracin. This clearly implying that, there is suppressing inflammatory responses due to Moracin treatment. For the purpose to observe whether the protective effect of Moracin is due to its viscous nature, gelatin (a viscous polymer) was taken as control. Surprisingly enough, it is observed that, viscous compounds like gelatin did not show any protection against oxidative stress through hydrogen peroxide. The protective influence of Moracin may be due to its unique antioxidant potential. The skin already possesses extensive and most effective network of the system of antioxidant. Practically, many more of the free radicals produced by various agents can escape this surveillance, inducing substantial damage to cutaneous constituents, especially when skin defense mechanisms are overwhelmed. The use of antioxidants has been observed to improve cutaneous wound healing significantly. Consequently, exogenous antioxidants (like Moracin) that scavenge reactive oxygen species (ROS) and restore normal redox state are supposed to be beneficial. Conclusively enough, the present attempt suggests that acetone-soluble (and water- soluble too) Moracin, from a natural source the fruits of mulberry, *Morus alba* (L), serve as ideal molecule to prevent oxidative stress. Use of Moracin may also be used in therapy with other conventional non-enzymatic antioxidants. This may open a new avenue for most suitable method for treating the cancer through the reduction of oxdative stress.

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