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Original article

Protective effect of vanillic acid in hydrogen peroxide-induced oxidative stress in D.Mel-2 cell line

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ABSTRACT

The overproduction of reactive oxygen species (ROS) causes oxidative stress, such as Hydrogen peroxide (H_2O_2). Acute oxidative stress is one of the main reasons for cell death. In this study, the antioxidant properties of vanillic acid- a polyphenolic compound was evaluated. Therefore, this study aims to check the effectiveness of vanillic acid in H_2O_2-induced oxidative stress in D. Mel-2 cell line. The efficacy was determined by biochemical tests to check the ROS production. The cytotoxicity of H_2O_2 and vanillic acid was checked by MTT assay. The DNA fragmentation was visualized by gel electrophoresis. Protein biomarkers of oxidative stress were analyzed by western blotting. The results depict a promising antioxidant effect of vanillic acid. The IC_{50} value of vanillic acid and H_2O_2 was found 250 μg/ml and 125 μg/ml, respectively. The catalase activity, SOF, GPx, and PC was seen less in H_2O_2 treated group compared with the control and vanillic acid treated group. However, the TBRAS activity was hight in H_2O_2 treated group. The effect of H_2O_2 on DNA fragmentation was high as compared with vanillic acid-treated cells. The protein expression of Hsp70, IL-6 and iNOS was seen significant in a vanillic acid-treated group as compared with H_2O_2 treated group. These results reinforce that at low concentration, vanillic acid could be used as an antioxidant agent in the food and pharmaceutical industries.

1. Introduction

It is a well-known fact that the production of free radicals in aerobic respiration cause cumulative oxygen damage, which may result in cell death. Oxygen plays a vital role for the production ATP; however, it is often transformed into highly reactive forms such as reactive oxygen species (ROS), which results in cell damage or even death (Nita and Grzybowski, 2016; Hill, 1996; Lacombe et al., 1996). It has been estimated that about 2% of the oxygen reduced by the mitochondria then forms superoxide or the dismutation production of hydrogen peroxide (H2O2). The reaction of metal ions with either peroxides or superoxides further promotes the overproduction of radical generation, in particular with the generation of hydroxyl radicals. Which furthers reacts with cells components like but not limited to DNA and proteins (Goldstein, 1996), and damages these molecules (Imlay and Linn, 1988). Therefore, in such conditions, an imbalance between oxidants and defence system leads to oxidative stress. The oxidative stress normally results due to ROS production excessively, Imparied antoxoidant system, mitochondrial dysfunction, or it could be due to combination of all these three factors (Nita and Grzybowski, 2016). Thereby causes severe damage to cells which results in metabolic dysfunctions, and finally results in the pathogenesis of human diseases, like inflammation and cancer and so on.

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The P450 complex possesses detoxifying enzymes: predominantly, the Nicotinamide Adenine Dinucleotide (NADPH) oxidase complexes, and xanthine oxidase. These enzymes play a decisive role in internal oxidative stress (Sosa et al., 2013). To minimize the aftermath of ROS, several anti-ROS mechanisms are present in organisms to manage the levels of intracellular and extracellular ROS, including oxygen radicals and some nonradical derivatives of O_2 (Muller et al., 2007; Fang et al., 2009). However, most reactive radicals produced from the Fenton and Haber reactions are hydroxyl radicals —OH (Barbara, 2012). Yet the connection between oxidative stress and mitogenic signals remains obscure (Xu et al., 2017).

Vanillic acid is a naturally occurring aromatic acid belonging to phenolic compounds which is usually used as an additive agent in the food industry. Due to its phenolic nature, it insulates the biological membrane and constrains lipid peroxidation in cells. Available literature suggests that vanillic acid can scavenge and remove ROS radicals, such as hydroxyl and lipid peroxyl (Ohkawa et al., 1979). Moreover, it has antimicrobial, anti-inflammatory, antitumorigenic, and hepatoprotective effects (Anbalagan and Raju, 2017). Diets rich in vanillic acid could reduce free radical cancer promotion (Bezerra et al., 2016) and slow down mutagenesis caused by chemical and physical mutagens in different models. Therefore, this research was performed to assess the efficacy of vanillic acid in hydrogen peroxide-induced oxidative stress D. Mel-2 cell line.

2. Materials and methods

2.1. Cytotoxicity activity using MTT assay

The D.Mel-2 cell line was collected from the National Center for Cell Sciences (NCCS), Pune, India. The cells were maintained in a Schneider's medium having 10% FBS and 100 μg/ml of streptomycin and penicillin 100 U/ml in a humidified environment with 5% CO_2 at 37 °C temperature. Additionally, the MTT reduction by mitochondrial-mediation (formazan was added to calculate cell respiration) is cell viability. In brief, the cells (1×10^5 per well) were added to a 24-well plate and kept for incubation at 37 °C. Once the cells reached the confluence stage, adherent cells were treated with distinct sample contractions and were further incubated for 24 h. The media was withdrawn from the well, and the cells were given PBS (pH 7.4) wash.

Further, MTT (0.5 mg/ml) was added to the cells and further incubated for four hours at a temperature of 37 °C, supplied with 5% CO_2. Finally, the media was discarded, and DMSO was added to solubilize the formazan precipitate, later absorbance was taken at 570 nm using a microplate reader (Rafiquzzaman et al., 2015). The concentration required for a 50% inhibition (IC50) was determined. Besides, the percentage of cell viability was calculated using the following formula:

$$\% \text{ cell viability} = \text{A570 of treated } \frac{\text{cells}}{\text{A750}} \text{ of control cells} \times 100$$

2.2. Estimation of oxidative stress markers and antioxidant assays

In this study, the cells were homogenated and treated with 29% trichloroacetic acid. The mix was then centrifuged for ten mins at 5000 g. The protein carbonyl (Pc) was evaluated by adding 0.5 mg of protein into a 10 mM of 2 N HCL and dinitrophenol hydrazine. The mix was kept for one-hour incubation at Room Temperature (RT). Moreover, Lowry's method was followed to measure absorbance and the total amount of protein (Lowry et al., 1951). Similarly, Ohakawa's method was used to measure TBRAS (Sinha, 1972). CAT activity was checked by the protocol utilized by Sinha (1972). SOD activity was checked by following the protocol of Kostyuk (1989), and GSH assay was performed using the protocol of Ellman (1959).

2.3. DNA fragmentation

Briefly, the samples at their IC_{50} concentration were added to D. Mel2 cells after they attain confluency and were incubated for 24 h. Then, the cells were harvested using TPVG. About 1.5 ml of cell suspension was subjected to centrifugation at 200xg for 10 mins at 4 °C. Further, 0.5 ml of TTE solution was added and vortexed vigorously. The process allows the release of fragmented chromatin from nuclei, after cell lysis. Then centrifugation was done to separate the fragmented DNA from the intact chromatin at 20,000xg for 10 mins at 4 °C. The supernatant was removed carefully then 500 μl of TTE solution was added to the pellet. Besides, NaCl was used to remove the histones from DNA followed by addition of 700 μl of ice-cold isopropanol and vortex. The mixture was subjected to overnight precipitation at 20 °C. Then centrifugation was done to pellet down DNA at 20,000xg for 10 mins at 4 °C followed by washing with 500–700 μl of 70% ice-cold ethanol and re-centrifugation. Finally, the DNA was dissolved in 50 μl of TE solution and stored at 4 °C. The DNA sample was loaded in gel with loading buffer and dye. The electrophoresis was carried, and DNA was visualized using a UV transilluminator.

2.4. Western blot analysis

For the analysis using Western blot, *cells* were homogenized in an ice-chilled RIPA buffer. The homogenized mixture was then centrifuged at 14,000 rpm for ten minutes at a temperature of 5 °C. The samples were electrophoresed using a 10% SDS page gel. Finally, the blot-transferred proteins were placed onto a PCDF membrane and incubated in a blocking buffer for 170 min, followed by incubation with primary and secondary antibodies at a temperature of 5 °C overnight and for two hours, respectively. The membranes were assiduously washed three times with TBST roughly for 15 to 20 min. Finally, using an ELC kit (GenScript ECL Kit, USA) by following the chemiluminescence method, the protein bands were visualized, scanned, and analyzed using the Image J software program (Bethesda, USA).

2.5. Statistical analysis

The results obtained were further examined using the SPSS windows student version software for entire data sets. One-Way ANOVA and Student Newman Keul (SNK Test) were used to retrieve the statistical difference between the control and treated groups. A statistically significant difference of $p < 0.05$ was recorded.

3. Results

3.1. Cytotoxicity effect of hydrogen peroxide

The MTT was carried out to evaluate the cytotoxicity of H_2O_2. The transformation of MTT to formazan by mitochondrial dehydrogenase measures cell viability. Consequently, a decrease in activity straightly correlates with a reduction in cell (feasibility) viability. Exposing cells to H_2O_2 reduces cell viability in a concentration-dependent manner (Fig. 1 and Fig. 2). The toxicity level decreased with a low dose of hydrogen peroxide. At 62.5 μg/ml, the toxicity was less compared to the dose at 250 μg/ml. The highest toxicity

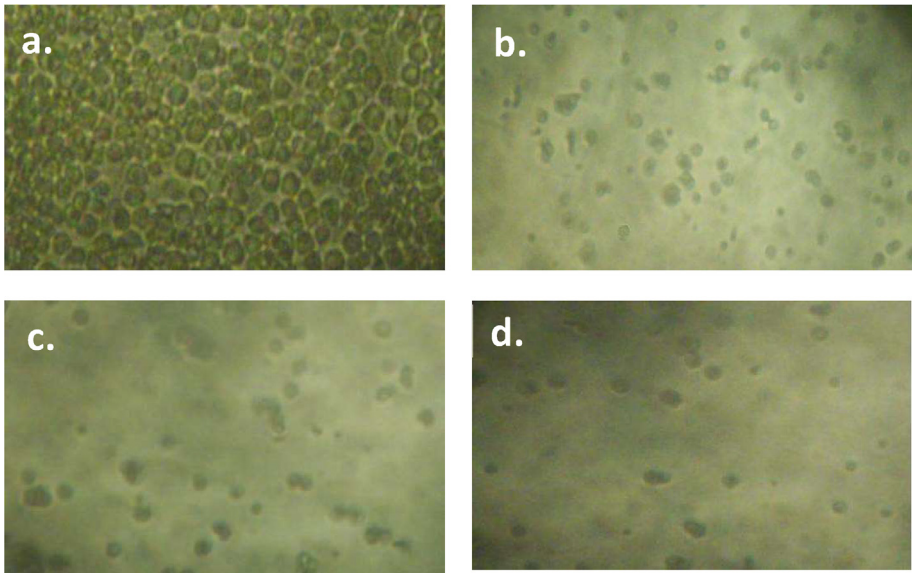

Fig. 1. Cytotoxicity of hydrogen peroxide on D.Mel-2 cell line. (a). Normal cells without any treatment. (b) Cells treated with 62.5 μg/ml H_2O_2. (c). Cells treated treated with 125 μg/ml H_2O_2. (d). Cells treated with 1000 μg/ml H_2O_2.

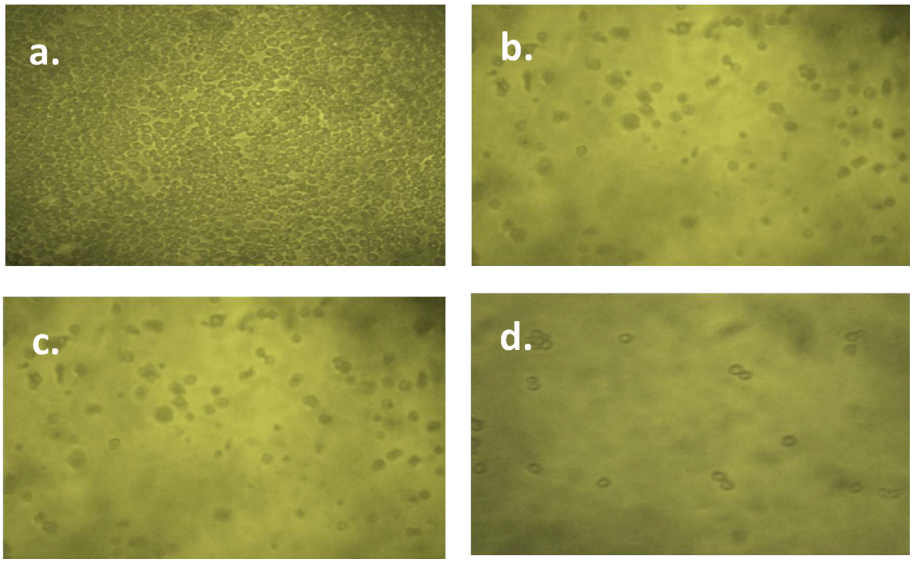

Fig. 2. Cytoxicity of vanillic acid on D.Mel-2 cells. (a). Normal cells without any treatment. (b) Cells treated with 62.5 μg/ml vannilic acid. (c). Cells treated treated with 125 μg/ml vannilic acid (d). Cells treated with 1000 μg/ml vannilic acid.

of H_2O_2 was observed at 1000 μg/mg, as shown in Fig. 1. The IC_{50} value of vanillic acid and H_2O_2, were found to be 250 μg/ml and 125 μg/ml, respectively (Fig. 3).

3.2. Biochemical analysis

Besides, catalase activity was seen at its least in the H_2O_2-treated group compared with the control group, whereas the H_2O_2 + vanillic acid-treated group showed significantly increased levels of catalase activity ($P < 0.001$). Likewise, the vanillic acid-treated group significantly showed the highest CAT activity as contrary to the control group ($P < 0.001$), as shown in Fig. 4a. The SOD activity was seen to be significantly less in the H_2O_2-treated group compared to the control used and the vanillic acid only–treated group. Nevertheless, a significant difference in activity was seen among the H_2O_2-treated group and the H_2O_2 + vanillic acid–treated group ($P < 0.001$), as shown in Fig. 4b. SOD is a vital mechanism of the intrinsic antioxidant defence system accounting for the distribution of superoxide radicals. Throughout the oxidative stress, body's defence mechanism is used to subside the elongation of lipid peroxidation *via* antioxidant enzymes such as SOD; hence, the task of this enzyme increases during the initial period of exposure. But if it progresses further, the enzyme gets exhausted and becomes inept, rendering it ineffective to combat the effect of free radicals. Therefore, with the advancement of peroxidation activity, the function of SOD diminishes. The TBARS activity of H_2O_2 increased remarkably in the H2O2-treated group as compared with the control group, whereas the activity decreased with H2O2 + vanillic acid, however, not significantly so. The lowest output of activity was shown when treated alone with vanillic acid ($P < 0.054$), as shown in Fig. 4c. Additionally, Glutathione Peroxidase (GPx) levels were relatively less in the H_2O_2-treated group as that of the control and vanillic acid + H_2O_2 groups ($P < 0.001$), as shown in Fig. 4d. Furthermore, decreased Glutathione (GSH)

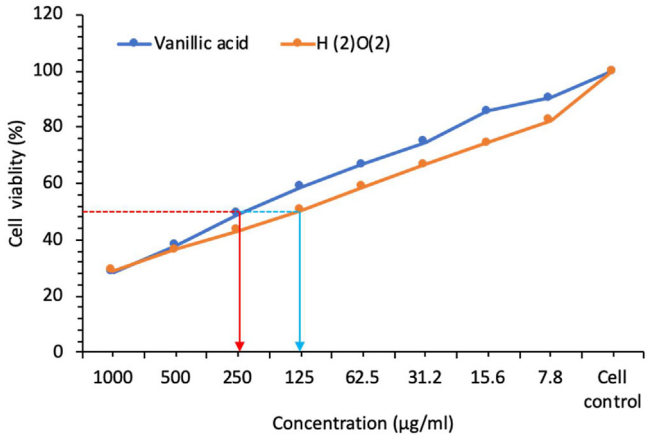

Fig. 3. Effect of vanillic acid and H_2O_2 on cell viability evaluated by MTT assay. The red and blue arrow shows the IC_{50} values of vanillic acid and H_2O_2, respectively.

levels were seen in the H_2O_2-treated group compared with the control group, whereas vanillic acid + H_2O_2 showed increased levels of GSH when compared with the H_2O_2 only–treated group (Fig. 4e).

3.3. DNA fragmentation

The DNA band analysis after electrophoresis shows that more breaks were seen in samples treated with H2O2 alone as compared to the samples treated with H2O2 + vanilic acid, as shown in Fig. 5a and 5b. The H_2O_2 and vanillic acids were added at their IC_{50} concentration, 125 µg/ml and 250 µg/ml, respectively.

3.4. Western blot

The expression of heat shock protein (Hsp70) was checked as a biomarker of oxidative stress. Expression of Hsp70 to toxic antigens is a key marker of early toxicity. In the control group, the expression of Hsp70 protein was low compared with the H2O2-treated *cells*, where the expression of Hsp70 was highly upregulated. Further, the expression of Hsp70 in *cells*, treated with H2O2 plus vanillic acid, was downregulated compared with the H_2O_2-treated group. Also, the expression of Hsp70 was low in *cells* treated with vanillic acid alone. Similarly, the expression levels of IL-6 and iNOS were also high in the H2O2-treated group compared with the H2O2 and vanillic acid–treated group. These results depict that vanillic acid could be used as a novel antioxidant; however, it is not limited to H2O2-induced oxidative stress (Fig. 6).

4. Discussion

Biological rhythms are synchronized to the environment by the natural light–dark cycle. The biological clock exhibits an ability to regulate processes endogenously, and numerous physiological and behavioural traits are maintained during constant conditions. The temporal changes in metabolism include those connected to brain functions and lead to oxidant formation (Hardeland et al., 2003). Sosa et al. (2013) reported that internal oxidative stress is mainly caused by detoxifying enzymes from the P450 complex, recurrently active in the mitochondria. The radical and nonradical components of ROS family within cell membranes instigated the oxidative stress, thereby deteriorating the percentage of antioxidants; this leads to an elevated generation of ROS, in addition to toxic aldehydes. The increased values of harmful geotaxis activity

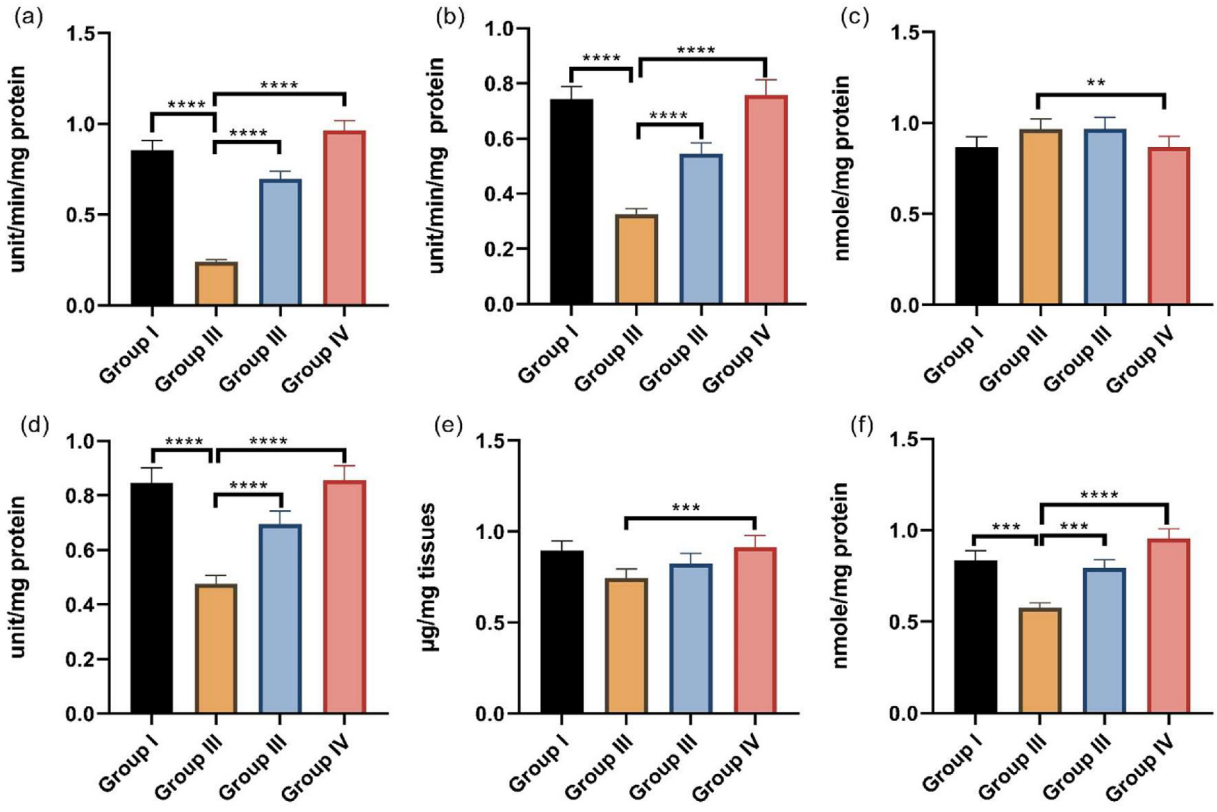

Fig. 4. Antioxidant effect of vanillic acid determined by various *in vitro* antioxidant assays. Group I (control); Group II (H_2O_2 treated); Group III (H_2O_2 + Vanillic acid treated); Group IV (Vanillic acid only treated). Hydrogen peroxide (100 nM) and vanillic acid (0.25%). (a) Catalase activity assay (b) SOD (c) TBRAS (d) GPX (e). GSH (f) PC. The data represents the mean ± SDs (Standard deviation of independent repeats.).

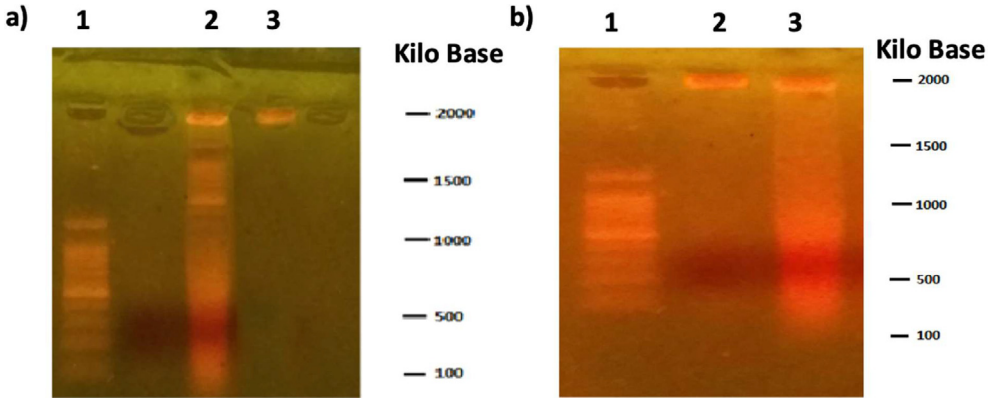

Fig. 5. DNA fragmentation in D.Mel-2 cells. a). 1-Marker, 2-H_2O_2 treated, 3. Control. b). 1- Marker, 2-Control, 3-H_2O_2 + Vanillic acid treated.

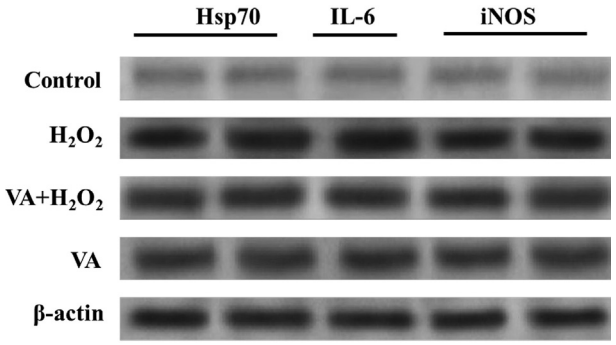

Fig. 6. Effect of vanillic acid on the levels of oxidative stress-related proteins in D. Mel-2 cell line.

in vanillic acid-treated flies could be due to an increase in total antioxidant status. Additionally, hydrogen peroxide rapidly causes DNA fragmentation. Here, hydrogen peroxide acts as a degrader of the DNA. DNA fragmentation has an advantage in cloning and apoptosis. However, complications inside a cell at times can trigger the fragmentation of red blood and sperm cells' DNA fragmentation.

SOD in control group and the variation of SOD activity in cells was maximum. The increased values of SOD in vanillic acid treated group could be due to increase in the total antioxidant status in the cells. Increased value in antioxidant enzymes superoxide dismutase, catalase, reduced glutathione and normalization of negative geotaxis activity by the administration of vanillic acid maybe due to its radical scavenging and antioxidant properties. Stress proteins have been shown to assist cells by transiently reprogramming cellular metabolic activity, acting as chaperones thereby protecting cells from further oxidative and heat damage (Mathew and Morimoto, 1998). Among different heat shock proteins, the stress inducible heat shock protein (Hsp70)70 is well characterized, the most widely studied member of stress protein family, especially in response to proteotoxicity or cytotoxicity (Krone et al., 2005). Expression of Hsp70 and other stress proteins have been studied in the context of individual environmental chemical exposure to display potential as a candidate biomarker (Ait-Aissa et al., 2000; Krone et al., 2005; Gupta et al., 2010). It has been shown to have potential as a first bioindicator of cellular damage due to its conservation through evolution, inducibility by a wide diversity of inducers and being a part of the cellular defense machinery (Krone et al., 2005). Ceriani et al. (2002) have used microarrays to assay genome-wide message levels in adult flies, and reported that Hsp22 is regulated, and that the expression of Hsp70-related gene is regulated. In our study the Hsp70 protein expression level was noticed in D. Mel-2 cells. In different organism's oxidative stress is causally implicated in the induction of Hsp70 genes during aging (Landis and Tower, 2005). The patterns of Hsp expression observed here suggest that the regulation of is associated with expression of Hsp.

Cytokines that are induced by IL-6 can also directly activate the NF-κB pathway, thus establishing a positive auto regulatory loop that can augment the inflammatory response and duration of inflammation (Beg and Baltimore, 1996). IL-6 is one of the most multifaceted cytokines known to regulate inflammation, abnormal cell growth, and immunomodulation. Intriguingly, in our study, H_2O_2 possesses anti-inflammatory property and lead to decreased IL-6 expression. The NF – κB translocates the transcriptional activators of target genes including IL-6, iNOS, which play roles in preventing oxidative stress mediated reactions and inflammation (Izawa et al., 2013; Redwine et al., 2000; Vgontzas et al.,2005).

In this study, the oxidative stress of Hsp expression observed here suggests that the regulation is associated with the expression of Hsp. The main effect of the stimulation of stress-sensitive signaling pathways is the production of gene products, for example, nitric oxide, which eventually accounts for the later problems of oxidative stress. In this condition, nitric oxide could react with ROS, like superoxide radicals, to produce peroxynitrite, a reactive oxidant species, thereby causing more vigorous oxidative as well as nitrosative stress. In the current study, hydrogen peroxide upregulated the expression of iNOS. The iNOS plays a major role among the radicals causing oxidative stress. The expression of iNOS was increased in vanillic acid–treated cells, which shows that iNOS may be enhanced by the cytokines and/or free radicals generated by vanillic acid. In contrast, the reduction of iNOS expression in vanillic acid with hydrogen peroxide–treated cells may be as a result of the anti-inflammatory property of hydrogen peroxide. The variation of protein expression in experimental groups reported a strict regulation of oxidative stress in hydrogen peroxide–treated groups. Thus, it normalizes the protein expression of iNOS.

5. Conclusion

The accumulation of oxidative free radicals is due to decreased activities of the free radical defense enzymes. Hence, a potential therapy which augments antioxidative defense system may prove beneficial under various disease conditions. Since hydrogen peroxide is a widely employed compound for inducing oxidative stress in mammalian model, we chose the cell line model to test if antioxidant effect of vanillic acid can mitigate hydrogen peroxide induced oxidative stress. The results showed, vanillic acid–treated

cells were least affected both in terms of production of ROS and DNA fragmentation. These results suggest that vanillic acid could be prove beneficial as a novel antioxidant agent in food and pharmaceuticals industries.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contributions

Conceptualization: S.T, EAB; methodology: S.T, NS; validations: EAB, NS, JSM, AQ,SR; analysis: ST, JSM, AQ, IAR; funding acquisition: IAR; writing-original draft, ST; review and editing, IAR, SR.

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