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RESEARCH ARTICLE

Nano - Structured Hesperidin: Evaluation the Antioxidant Activities *In Vitro*

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Abstract

Hesperidin from citrus peels is known to have anti-inflammatory, vaso-protective, hypo-lipidemic, anti-allergic, anti-carcinogenic and antioxidant actions. Current study was designed to evaluate the antioxidant capacity of hesperidin and hesperidin nanoparticles by using simple *in vitro* free radical scavenging system including, DPPH free radical-scavenging, hydrogen peroxide scavenging and Resazurin dye scavenging activities. Hesperidin nanoparticles showed strong scavenging effects against DPPH, hydrogen peroxide and Resazurin dye, and this effect was more potent than pure hesperidin. The scavenging activity of hesperidin nanoparticles was concentrations dependent, in the range of 100-400 µg mL^{-1}showing a maximum activity of 80% at 400 µg mL^{-1}in similar way to Vit.C which was used as positive control. In conclusion, this work displayed that hesperidin exhibited antioxidant activity and could be considered as a source of antioxidants from nature.

Keywords: *Hesperidin nanoparticles, Antioxidants, Resazurin, DPPH, Hydroxyl.*

Introduction

Antioxidants are necessary substances that maintain the body in normal state and protect from damage caused by free radicals. These free radicals can be come from internal sources such as metabolic pathways that take place within the body tissues and can also be come from external sources like food, UV radiation, drugs and environmental pollution [1]. A large number of diseases caused by these free radicals including cardiovascular diseases, neural disorders, ulcerative colitis, aging, Alzheimer's disease, alcohol induced liver disease and hepatotoxicity, atherosclerosis and different types of cancers [2].

Antioxidants show to have a vital role in the protective effect of plant foods, where evidence suggests that high consumption of vegetables and fruits participate in improving human health. Various reasons can cause depletion of antioxidants such as, limited absorption of these compounds (e.g. Crohn disease), decrease dietary intake of antioxidants, smoke, environmental pollution, renal dialysis, traumas and other reasons [3]. Importantly, there are synthetic antioxidants are widely used in the pharmaceutical and food industry such as trolox, butylatede hydroxy anisole (BHA) and butylated hydroxy toluene (BHT), but these substances appeared to have toxic and/or mutagenic effects. And because of their toxicity, there was need to develop and isolate of natural antioxidants from plant, especially edible plants, such as polyphenols, flavonoids and silymarin [4, 5].

Flavonoids are a large group of phenolic components that are vastly found in plants. They are the constituents of efficient conventional therapies [6]. The flavonoid hesperidin is a flavanone (a class of flavonoids) and consists of hesperitin and the disaccharide rutinose. (Figure1). Hesperidin is the dominant flavonoid in oranges and lemons. The highest hesperidin concentrations were found in the peels and membranous parts of these fruits. So, orange juice containing pulp is richer in hesperidin than that without pulp [7, 8]. Hesperidin is a white to yellow crystalline powder with low aqueous solubility. Thus, this limits its dissolution rate in water, and finally results

in low in vivo bioavailability [9]. Recently, an interesting method to overcome the solubility and dissolution problem of the poorly soluble compounds were improved by reduction the particle size to nanometer range. The nanoparticles improve the *in vivo* action of low soluble drugs consequently, leading to an increased the dissolution velocity and surface area [8].

Hesperidin is known to have anti-inflammatory, vaso-protective, hypo-lipidemic, anti-allergic, anti-carcinogenic and antioxidant actions [10, 11]. *In vitro* studies have elucidated that the antioxidant properties of flavonoids are related with their capacity for scavenging free radicals, inhibiting the activity of oxidases and chelating metals [12]. The aim of current study is to estimate the antioxidant activity of hesperidin and hesperidin nanoparticles by accomplished diverse *in vitro* assays.

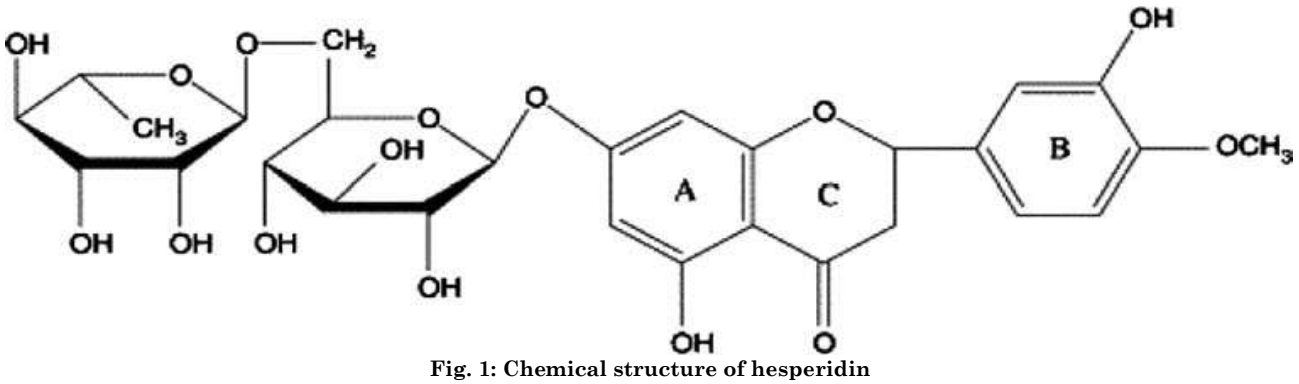

Fig. 1: Chemical structure of hesperidin

Material and Methods

Dimethyl sulfoxide (DMSO) (BDH, UK),D, L-lactide and glycolide (PLGA), Poloxamer 407 and hesperidin were purchased by Sigma-Aldrich (St. Louis, MO, USA), ascorbic acid, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), were purchased from Sigma Chemical Co. (St. Louis, MO, USA), Resazurin dye was obtained from HIMEDIA- India, Hydrogen peroxide was obtained from BDH- England and Sodium salicylate from Fluka-Switzerland.

Characterization of Prepared Hesperidin Nanoparticles

Different analysis techniques were used to characterize hesperidin nanoparticles such as SEM, XRD, FTIR and particle size analysis (PSA), as shown in our previously work [13].

Determination of Free Radical Scavenging Activity

Resazurin Dye Scavenging Activity

About 0.1 g of Resazurin dye was dissolved in 100 mL of distilled water to prepare Resazurin dye solution and a vortex mixer was used to obtain a homogenous solution. The ability of hesperidin and hesperidin nanoparticles to scavenge free radicals was determined in accordance with Yadav, *et.al* [14] with some modification. 20 µL of hesperidin and nano-hesperidin from three different concentration (100, 200 and 400 µg mL^{-1}) were added to 20 µL of Resazurin and by using distilled water, the volume was completed to 1 mL.

After 15 min the absorbance of Resazurin at 600 nm was measured against a blank solution contain distilled water. And ascorbic acid (Vit. C) was used as positive control. After that the percentage of Resazurin scavenging was calculated by the equation

$$\text{Resazurin radical activity}(\%) = (AC - AS / AC) \times 100$$

Where Ac and as are the intensity of peak at 517 nm for control (DPPH) and sample, respectively.

Diphenyl-1-Picrylhydrazy (DPPH) Free Radical Scavenging Activity

This assay was described in accordance with Sulaiman, *et.al* [15]. DPPH solution was prepared by dissolving 2.366 mg of 2, 2-diphenyl 1-picrylhydrazyl in 100 mL of absolute ethanol to obtain 60 µM DPPH. As the following procedure, three different concentrations of hesperidin and nanohesperidin (100, 200 and 400 µg mL^{-1}) were used in three different tubes and complete the volume to 500 µL by using

ethanol for each sample. And then 500 µL of DPPH solution was added to all samples (Vit. C 5 µg $mL^{-1}$was used as positive control and DPPH with ethanol only was used as negative control) and incubate in the dark for 30 min. after that time the absorbance of DPPH at 517 nm was measured for all samples by using UV-VIS spectrophotometer. The percentage of DPPH scavenging activity of both compounds was calculated using the following equation:

$$DPPH\,Scavenging\;\;Activity(\%) = (AC - AS / AC) \times 100$$

Where AC is the absorbance of DPPH and AS is the absorbance of hesperidin or hesperidin nanoparticles sample solvent.

Hydroxyl Radical Scavenging Assay

Hydroxyl ($OH^{\cdot}$) radical scavenging activity was determined in accordance with Smirnoff, *et.al* [16]. With minor modification 500 µL of $FeSO_4$ (3 mM) was added to 500 µL of different concentration of hesperidin and hesperidin nanoparticles, and the reaction was started by addition 500 µL of H_2O_2 (3 mM). After incubation for 10 min, 500 µL of salicylic acid (6 mM) was added to each sample and incubated for 15 min. after that time all the samples were centrifuged at 8000 rpm for 5 min. and the absorbance of the supernatant was measured at 510 nm. Hydroxyl radical scavenging activity was calculated using the following equation:

$$Hydroxyl radical\;\;activity(\%) = \;(AC - AS / AC) \times 100$$

Where Ac and as are the intensity of peak at 510 nm for control (Hydroxyl) and sample, respectively.

Statistical analysis

The grouped data were statistically evaluated using ANOVA with SPSS program (SPSS/14.0; SPSS Inc., Chicago, IL, USA). Values were presented as the mean± SD of the three replicates of each experiment.

Results

Figure 2 represent the reducing power of Resazurine dye by hesperidin and hesperidin nanoparticles. The results show that the reducing power increased with increasing concentration, and the effect of hesperidin nanoparticles was more potent than pure hesperidin. This figure is also shows the percentage inhibition of Resazurine, the inhibition power of hesperidin at a concentration of 400 µg mL^{-1} was 55.4%, while hesperidin nanoparticles was 80.9 and similar to that of Vit. C inhibition power.

This indicates that hesperidin and hesperidin nanoparticles were electron donors. Figure 3 shows the radical-scavenging activity of hesperidin and hesperidin nanoparticles and the results were expressed as percentage inhibition of DPPH. The results showed that hesperidin significantly reduced the level of the DPPH. While, hesperidin nanoparticles led to boost the antioxidant activity and this could be attributed to the enhanced dissolution rate and solubility. The freshly prepared DPPH solution appeared as a deep purple color with a maximum absorbance at 517 nm, the disappearance of purple color might is due to presence of hesperidin and nano-hesperidin as antioxidant.

Free radical scavenging activity of hesperidin nanoparticles on DPPH• radical was found to increase with increase in concentration, showing a maximum inhibition of 80% at 400 µg mL^{-1}andin a similar way to the antioxidant Vit. C, showing a maximum inhibition of 91 %. DPPH, is amolecule containing a stable free radical, and converts into a stable compound by reacting with hesperidin as an antioxidant which can donate an electron to DPPH. While, Figure 4 represents the hydroxyl radical scavenging effect of hesperidin and hesperidin nanoparticles, the results were expressed as percentage inhibition of hydrogen peroxide (H_2O_2) and this effect was concentration dependent. Hesperidin nanoparticles effect was better than pure hesperidin; hesperidin nanoparticles exhibited strong hydroxyl

radical scavenging activity and showed higher percentage inhibition at 400 µg mL^{-1}.

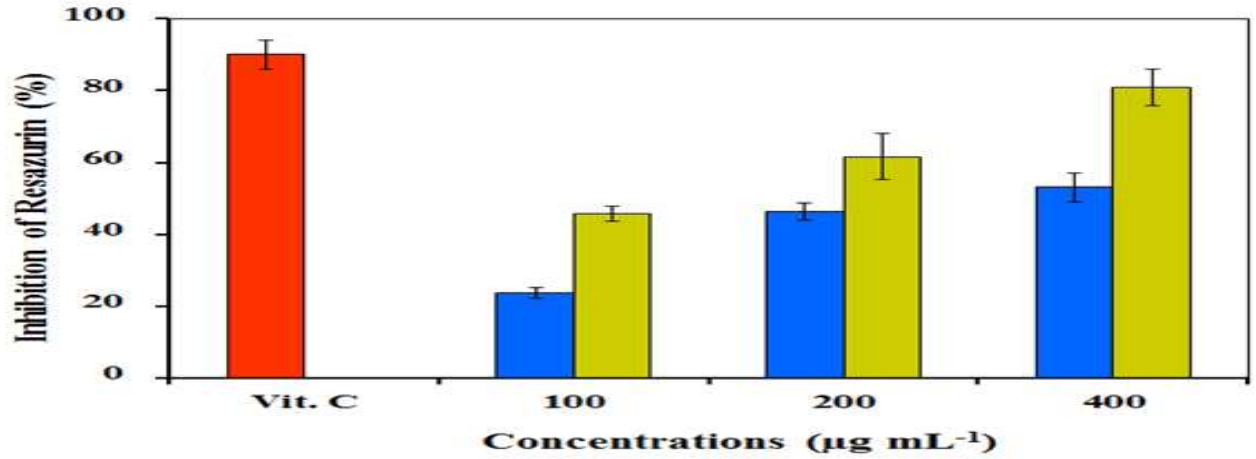

Fig. 2: Reducing power of different concentrations of pure hesperidin (Blue color) and hesperidin nanoparticles (Yellow color) along with Vit. C.

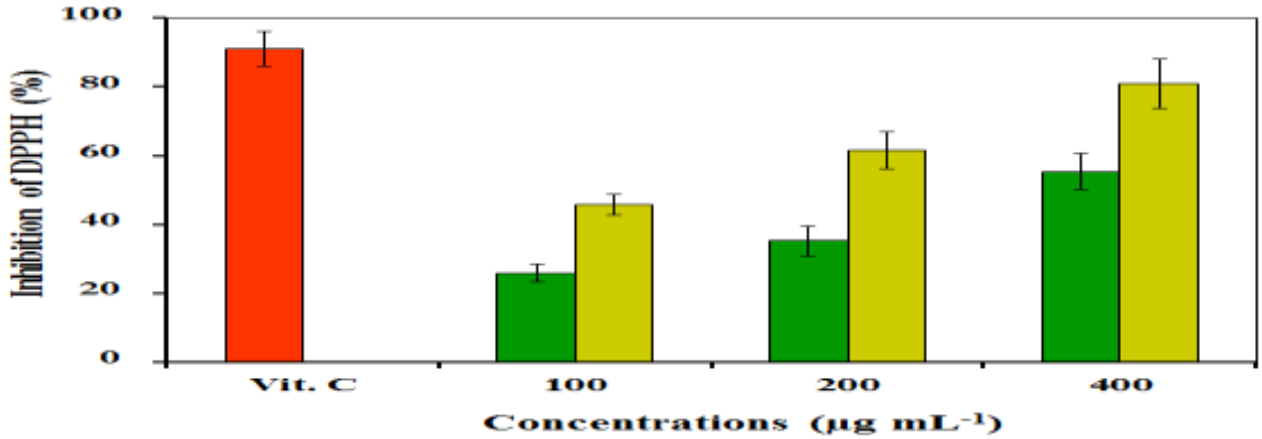

Fig. 3: DPPH free radical scavenging assay of hesperidin (Green color) and nanohesperidin(Yellow color)

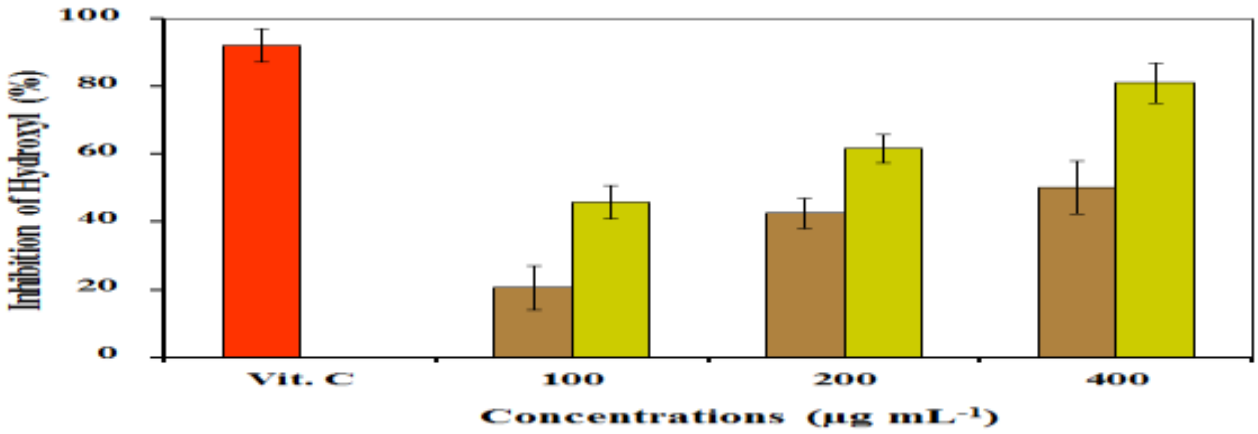

Fig. 4: Hydroxyl radical scavenging activities of hesperidin (Brown color) and hesperidin nanoparticles (Yellow color) with Vit.C as positive control

Discussion

The main bioactive nutraceutical compounds in plants are flavonoids as phenolic compounds that have strong antioxidants and metal chelating potentials. Other properties have also been reported such as anti-allergic, anti-inflammatory, hepato-protective, anti-carcinogenic antithrombotic and antiviral activities [17]. Free radicals are deleterious secondary products generated during normal cellular metabolism, and could initiate oxidative damage inside the body. The antioxidant activity of phenolic compounds is mainly attributed to their redox-potentials, which play a significant role in neutralizing free radicals, quenching singlet and triplet oxygen [18].Hesperidin has been widely examined by a number of researchers to evaluate the antioxidant activity and radical scavenging potentials by using different assay systems [19]. In the reducing power assay, Resazurin (blue and

non-fluorescent) is an oxidation-reduction indicator and the principle of this assay depending on the converting of the blue color of Resazurinein to pink and highly fluorescent when reduced to resorufin. Resorufin is further reduced to hydro-resorufin (uncolored and non-fluorescent). In addition to the antioxidant compounds which are able to transfer the hydrogen toresazurin quench the color and produce a de-coloration of the solution and this reaction is rapid and stable [14].

For DPPH free radical scavenging assay and this is the simplest and most widely used method for investigating antioxidant activity in foods and many plant drugs. The purple chromogen radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH•) is reduced by antioxidant compounds to the corresponding pale yellow hydrazine [20]. Hydrogen peroxide oneself is not very reactive, however it can give rise to hydroxyl radical in the cells for this reason it can sometimes be poisonous to cells. Therefore, the removal of H_2O_2 is very important for preservation the cell from oxidants effects. H_2O_2 can penetrate the membranes and may oxidize a number of compounds inside the cell [18].

The antioxidant activity of flavonoids is directly related to their structure. In the case of hesperidin, the ability of hesperidin to scavenge the hydroxyl radicals produced from hydrogen peroxide may be attributed to the presence of a hydroxyl group at position 3′of ring B [21]. Importantly, the overall antioxidant properties of hesperidin rely upon the arrangement and number of the hydroxyl groups and the range of structure conjugation [7]. There were other investigators previously studied the antioxidant potentials of flavonoids by using 2, 2′-azinobis (3-ethylbenzothiazoline)-6-sulfonate radicalcation ($ABTS^{\bullet+}$), superoxide anion ($O_2^{-\cdot}$), and 2hydroxyl radical ($OH^{\bullet}$) produced photolytically or byFe^{2+}-H_2O_2 [22]. Flavonoids practice their anti-inflammatory ad anti-oxidative effects through the free radical scavenging properties. Thus, they play significant roles in the prevention of metabolic abnormalities such as inflammation, oxidative stress, dyslipidemia, hypertension, insulin resistance, and glucose intolerance.

In addition, anticancer effects of flavonoids rely on their capacity to enhance DNA repair processes and inactivate carcinogens. Also, flavonoids exhibit their cardio-protective actions by providing antiplatelet effects, regulating blood pressure and protecting against oxidized low-density lipoprotein induced damage. There is evidence that diets rich in flavonoids are advantageous for improved health and prevention of non-communicable diseases (NCDs) or chronic diseases [23].

Conclusion

In the current study we examined the antioxidant potential of hesperidin and hesperidin nanoparticles. The results illustrated that it exhibited highly antioxidant activity against free radical $DPPH^{\cdot}$, hydroxyl radical generated from hydrogen peroxide (H_2O_2) and reducing power of Resazurin. In addition, hesperidin nanoparticles have the stronger effect than pure hesperidin and this effect was concentration dependent. So, hesperidin could be considered as a source of antioxidants from nature. Hence, clinical studies are needed to determine the importance of this natural compound in the prevention or treatment of human diseases.

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