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# EFFECT OF HESPERTIN ISOLATED FROM ORANGE PEELS ON CISPLATIN-INDUCED NEPHROTOXICITY

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## ABSTRACT

The present study is to determine the effect of hespertin isolated from orange peels on cisplatin-induced toxicity and Isolation of hespertin from the hesperdin by acid degradation to determine its effect on cisplatin – induced nephrotoxicity.In the present study, the hespertin (200 mg & 400 mg/ kg bd.wt) was examined for its protective effect against cisplatin induced renal injury in rats. Thirty healthy male and female albino rats (150- 200 g weight) were choosen and divided into five groups. Vehicle, cisplatin and hespertin were given according to the experimental design. After 8 days of treatment, urinary functional parameters were analysed. Histopathological characters were examined after scarifying and dissecting the rats. Cisplatin (6 mg/kg) alone significantly elevated serum markers level, increased urinary protein excretion, reduced urine to serum creatinine ratio and creatinine clearance. In curative regimen, the extract significantly reduced the elevated serum creatinine and urea levels. Renal antioxidant defense systems, such as superoxide dismutase, catalase, glutathione peroxides activities and reduced glutathione level, depleted by cisplatin therapy were restored to normal by treatment with the extract.The present investigation suggests the hespertin protective effect against cisplatin induced nephrotoxicity.

**KEYWORDS:** Cisplatin; hespertin; nephrotoxicity; Creatinine; hesperdin.

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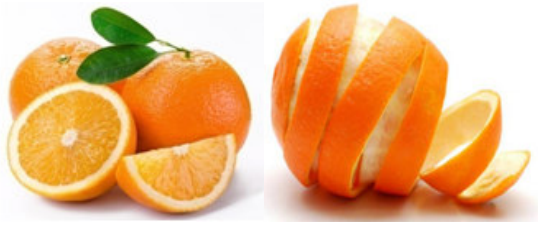
# INTRODUCTION

### *Plant*

The orange is both a literal and symbolic embodiment of the sun, from whose light it is formed. As a whole food it irradiates us with a spectrum of healing properties, the most prominent of which some call "vitamin C activity," but which is not reducible to the chemical skeleton known as 'ascorbic acid.' Science now confirms the orange has a broad range of medicinal properties, which is why the ancients knew it both as a food and a medicine [1]. Oranges is an important medicinal plant of the family Rutaceae. It is cultivated mainly for its alkaloids, which are having anticancer activities and the antibacterial potential in crude extracts of different parts (viz., leaves, stem, root and flower)[2] .The limonene and flavonoids found in orange peel seem to have anti-carcinogenic properties by acting as a blocking agent. Studies have shown that limonin and limonene, both found in high concentrations in the peel, can induce the enzyme activity of glutathione S-transferase, which is an important detoxifying enzyme. The citric acid found in orange peels also helps starve cancer cells by cutting off their energy supply. A number of studies have shown decreased risk of several cancers, most notably skin, breast and colon cancer, linked with the increased consumption of orange peel. In addition, orange peels and orange peel extract can provide an extra benefit to diabetics and those looking to reduce overeating. This is due to the fact that orange peels are a natural source of pectin, a natural fiber that helps decrease the rise in blood sugar after a meal. It may also be helpful in lowering cholesterol. D-limonene, another biochemical found in orange peels, is helpful in dissolving kidney stones. Orange peel has antiseptic, bactericidal, and fungicidal properties, which are extremely beneficial in eliminating any kidney or urinary tract infections. Orange peels may also help with heartburn. According to a 2007 “Alternative Medicine Review” article by Jidong Sun, Ph.D., Sun, d-limonene possesses the ability to neutralize gastric acid and support normal peristalsis [3].

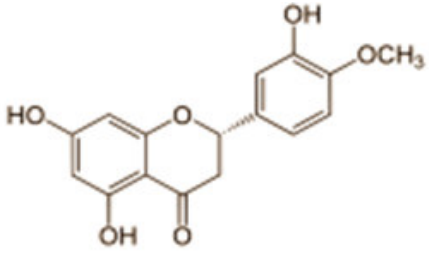


### *Flavonoids*

Phytochemicals are defined as the substances found in edible fruits and vegetables that exhibit a potential for modulating human metabolism in a manner beneficial for the prevention of chronic and degenerative diseases [4]. The most important natural pigments are carotenoids which are tetrapyrrole derivatives of naturally occurring phenolic compounds ubiquitously distributed in plant kingdom [5]. Phenolics are defined as a class of polyphenols which are important secondary metabolites present in plants[6] and are also responsible for their antioxidant action and various beneficial effects in a multitude of diseases[7,8]. Among these compounds, flavonoids constitute one of the most ubiquitous groups of all plant phenolics. So far, over 8,000 varieties of flavonoids have been identified [5]. Flavonoids are low molecular weight[9,10] bioactive polyphenols[11] which play a vital role in photosynthesising cells[10] .Flavonoids are secondary metabolites characterised by flavan nucleus[13] and C6-C8-C6 carbon-skeleton[14,15]. These are group of structurally related compounds with a chromane-type skelton having phenyl substituent in C2-C3 position[16].They are ubiquitously found in fruits, vegetables, tea, and wine, and are usually subdivided into six classes including flavonols(e.g., quercetin, kaempferol), flavones (e.g., apigenin, luteolin), flavanones(e.g., hesperidin, naringenin), flavan-3-ols (e.g., catechin, theaflavin, and gallic esters of catechin and theaflavins), anthocyanidins (e.g., pelargonidin,cyanidin), and isoflavones (e.g., genistein, daidzein). Secondary metabolism of flavonoids in plants generates glycosides through linkage to a sugar moiety,which enhances absorption in humans[17].Flavonoids occur as aglycones, glycosides and methylated derivatives[18] flavonoids aglycones (i.e., flavonoids without attached sugar) occur in a variety of structural forms. All contain fifteen carbon atoms in their basic nucleus: two six-membered rings linked with a three carbon unit which may or may not be a part of a third ring[19].

### *Hesperetin*

It is a biofavonoid and, to be more specific, a flavanone A trihydroxyflavanone having the three hydroxy gropus located at the 3'-, 5- and 7-positions and an additional methoxy substituent at the 4'-position.




***Molecular Formula: $C_{16}H_{14}O_6$***

***IUPAC Name: (2S)-5,7-Dihydroxy-2-(3-hydroxy-4-methoxyphenyl)-2,3-dihydro-4H-chromen-4-one***

***The average intake of hesperetin estimate is 28.3 mg/d.***

Hesperetin (and orange juice) has been shown to inhibit chemically induced mammary [20], urinary bladder [21], and colon [22,23] carcinogenesis in laboratory animals. Hesperetin[24]as well as the other major citrus flavanone naringenin[25,26], also possess some antioxidant activities, although this activity is poorer compared with many other polyphenols. Other possible effects of hesperetin and naringenin are on lipid metabolism. They have been reported to regulate apolipoprotein B secretion by HepG2 cells, possibly through inhibition of cholesterol ester synthesis[27], and to inhibit 3-hydroxy-3-methylglutaryl-coenzyme A reductase and acyl coenzyme A: cholesterol O-acyltransferase in rats[28,29]. Furthermore, a decrease in plasma low-density lipoprotein levels and hepatic cholesterol levels in rabbits fed a high-cholesterol diet has been observed [30].

***Nephrotoxicity***

Nephrotoxicity is an intrinsic adverse effect of certain anticancer drugs. Anticancer drugs have a narrow therapeutic index and therapeutic dose of such drugs usually produces significant nephrotoxicity. The dosage used in clinical trials, mostly, the maximum tolerated dose determined during phase I drug evaluation. But even greater toxicity is expected during curative therapy than during palliative therapy. But cancer patients often exhibit excretory reduced organ function. Modulation of pharmacokinetics and pharmacodynamics of these drugs in cancer patients is therefore necessary in order to improve tolerance [31]. Cancer patients are particularly liable for the development of renal abnormalities. Interestingly, patients with renal abnormalities who have undergone kidney transplantation are at high risk for malignancy. Clinical symptoms of renal involvement are diverse and harmful. Inspite of the recent advances in understanding the mechanism of anticancer drug and nephrotoxicity, prevention still relies on drug dosage decrease. Hence an active screening of drugs for renal abnormalities for patients treated with anticancer drugs is still a major field of research [32].

## METHODS AND MATERIALS

***Chemicals***

All chemicals used were of analytical grade. Cisplatin, 5-5[1] – Dithiobis 2-nitrobenzoic acid (DTNB) purchased from sigma, St. Louis, M.O. USA. n-Butanol, xylene, silicagel, petroleum ether, ethanol, thiobarbituric acid (TBA), Trichloro acetic acid and (TCA), disodium hydrogen phosphate, Sodium dihydrogen phosphate were obtained from S.d fine chemicals, India. Sodium nitropruesside Sulphanilamide, O-phosphoric acid, napthylethylene diamedihydro chloride (EDTA), Pyridine, haematoxylene purchased from Merck (INDIA). Methanol, Sulphuric acid ($H_2SO_4$), Isopropanol, Celite, Acetic acid, Petroleum ether (40-60$^0$), Charcoal, formamide purchased from S.d fine chemicals (INDIA).

***Instruments***

Purity of the compounds was checked by thin layer chromatography (TLC) using glass plates coated with silica gel G, and spots were detected by iodine vapours. Melting points were determined in open capillaries on a Tempo apparatus, and are Uncorrected. Samples were send to IICT, Hyderabad to obtain IR, NMR and MASS IR spectra.

***Plant material***

The orange peels were collected from local juice centers of tirupathi, hespertin is isolated from orange peels in Tirupathi.

***Isolation of hespertin***

Hesperitin (HTN) was isolated by acid degradation of hesperdin (HDN).1 gram hesperdin and 20 ml ethylene glycol containing 1 ml sulfuric acid were heated on a steam bath for 40 min. The clear yellow solution is poured into 50 ml water. The precipitated hespertin was filtered on a Buchner funnel and washed with water. Crystallized from ethanol gives crystals (yield: 350 mg).

***Selection of animals***

All animal experiments were performed in accordance with our Institutional Animal Ethics Committee and by the animal regulatory body of the government (Regd.No.1629/PO/A/12/CPCSEA). Albino rats of either sex obtained from Ghosh Enterprises, Kolkata were used in the study. Under controlled environment the animals were placed six per cage at a temperature of 22 ± 2˚C with 12h light / dark cycle. Rats were fed with standard pellet diet ( Ratan Brothers, Hyderabad ), and water ad libitum. Animals were kept for twelve days in laboratory for habituation.

**Table 1**
***Preliminary phytochemical screening***

| S.no | Name of the constituent | Chemical test | Result |
|---|---|---|---|
| 1 | Carbohydrates | Molisch's test | -ve |
| 2 | Proteins and free amino acids | Biuret test | -ve |
| 3 | Flavonoids | Shinoda's test | +ve |
| 4 | Alkaloids | Dragand roff's test | -ve |
| 5 | Saponins | Foam test | -ve |

## EXPERIMENTAL PROCEDURE

***The Effect of Hespertin on cisplatin induced nephrotoxicity***

The animals were divided in to five groups, each group consists of six animals (n=6)

- The Animals of Group – $I_b$ received 1% Carboxy Methyl Cellulose (CMC) in distilled water for 10 days.The animals of group – $II_b$ received Cisplatin (6mg/kg body weight i.p) on 5th day.
- The animals of Group-$III_b$ received the low dose of Hesperitin (20 mg/kg body) suspended in the vehicle for 10 days.

- The animals of group – $IV_b$ received the high dose of Hesperitin (40 mg/kg bd wt) suspended in the vehicle for 10 days.
- The animal of group –$V_b$ received only hespertin suspended in the vehicle for 10 days. In addition to this the animals of groups $III_b$, $IV_b$ were co-administered with cisplatin (6mg/kg body weight i.p) on day 5. Group – $I_b$ received normal saline instead of cisplatin.

***Assessment of nephro-protector activity***

On the day 9, urine was collected with the help of metabolic cages and the urine samples were subjected for estimation of urinary functional parameters. The animals were sacrificed by cervical decapitation and blood samples were collected by cardiac puncture and were used for estimation of Blood urine nitrogen (BUN, Di acetylmonooxime method), Serum creatinine (SC, Alkaline Picrate method). In kidney tissue Malondialdehyde levels were estimated.

**Table 2**
***Effect of Hespertin on normal rats***

| S NO | PARAMETER | GROUP I | GROUP IV |
|---|---|---|---|
| | Treatment (mg/kg) | 1% carboxy methyl cellulose throught the treatment | Hespertin given throught the period |
| 1 | BUN(mg/dl) | 24.56±0.15 | 26.75±1.5 |
| 2 | SC(mg/dl) | 0.69 ± 0.16 | 0.71±0.01 |
| 3 | $U_{TP}$(mg/24hrs) | 6.20±0.33 | 7.25±0.12 |
| 4 | $Cl_{cr}$ (ml/hr/100g bd.wt) | 18.90±1.3 | 18.98±1.9 |
| 5 | LPO(n moles/gm) | 73.56±2.16 | 72.06±2.6 |

**Table 3**
***Effect of isolated compound of hespertin on cisplatin induced nephrotoxicity***

| Group | Treatment (mg/kg) | BUN (mg/dl) | SC (mg/dl) | UTP(mg/24hrs) | Clcr (ml/hr/100g bd.wt) | LPO (n moles/g) |
|---|---|---|---|---|---|---|
| I | Normal (1%carboxy methyl cellulose) | 26.76±1.5 | 0.72±0.2 | 7.8±0.33 | 19.80±1.6 | 11.7±2.6 |
| II | Cisplatin (6mg/kg) | 62.2±2.3 | 2.3±1.6 | 19.9±1.06 | 5.13±2.6 | 14.7±0.3 |
| III | Low dose (200mg/kg) | 28.02±2.90 | 1.6±0.9 | 6.8±0.6 | 11.7±1.4 | 14.1±0.5 |
| IV | High dose (400mg/kg) | 29.02±2.9 | 1.4±0.1 | 6.3±0.33 | 10.6±1.56 | 12.1±0.9 |

***Statistical analysis***

The results are expressed as mean±SEM and the data analysed using one way analysis of variance followed by post hoc Student-Keuls test using SPSS computer software for *in vivo* studies. Statistical significance was set at P≤ 0.05.

## RESULTS

***HESPERTIN***
***(S)-2,3-dihydro-5,7-dihydroxy-2-(3-hydroxy-4-methoxyphenyl)-2-methylchromen-4-one***

sIt was obtained as white needles from petroleum ether and methanol fraction. M.P ranges between 226 to 228$^0$C. The molecular formula $C_{16}H_{14}O_6$ was established by Mass spectral data and elemental analysis.

***IR data (cm$^{-1}$)***

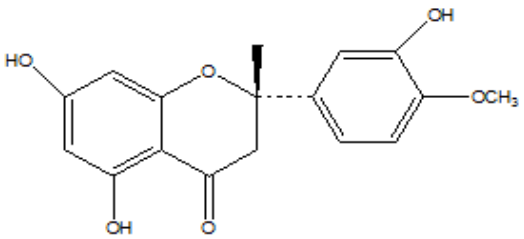
In IR spectra the characteristic bands were observed. In IR spectra the characteristic bands were observed at 3472.92 cm$^{-1}$ (phenolic OH), a band at 2917.20 cm$^{-1}$ for C-H present in $OCH_3$, C-O-C band was observed at 1203 cm$^{-1}$, a band at 1647.76 cm-1 (C=0).

***H$^1$NMR***

In H$^1$NMR spectra signals at δ 4.2 (d, 1H) and δ 5.3 (d, 1H) for phenolic hydroxyl are present at 5 and 3$^1$ positions respectively. Signal at δ 3.7 (m, 3 H) for $OCH_3$ group present at 4$^1$ position. A signal at δ 3.12 (m, 3H) for proton is present at 3 position. Signals at δ 5.8, 5.34, 5.98 (m, 3H) protons present at 5, 2, 8 positions respectively.

***Mass spectra***

Mass spectra showed characteristic fragmentation peaks 302 ($M^+$), 179 (M-133), 150 (M-152), 137 (M-165), 124 (M-188).Based on above physical and spectral data the isolated compound was confirmed as Hespertin.




***3.1 Effect of hespertin on normal rat kidney***

***Effect on Serum markers***

With the administration of hespertin to group $II_b$ animals there was no significant changes were observed in BUN and SC levels (Group II 26.75±1.5 and 0.71±0.01) when compared with normal group $I_b$ animals (24.56±0.15 and 0.69 ± 0.16).

***Effect on Urinary parameters***

Urinary total proteins ($U_{TP}$) were expressed in mg/24hrs. Animals which received hespertin showed almost similar values (7.25±0.12) to that of normal control group $I_b$ animals (6.20±0.33). There were no significant changes in $Cl_{cr}$ levels hespertin treated animals (18.98±1.9) when compared with normal group $I_b$ animals (18.90±1.3).

***Effect on LPO activity***

The animals which received only hespertin on oral administration there was no significant changes observed in LPO (72.06±2.6) when compared with normal Group-$I_b$ animals (73.56±2.16)

***Effect on antioxidant enzymes***

With the oral administration of only hespertin there was no significant changes observed in GSH, CAT,SOD values (22.01±0.10, 1.66±0.15 and 104.5±5.07 of GSH, CAT and SOD respectively) when compared with normal group $I_b$ animals (22.14±0.13, 1.65±0.12, 105.8±6.48).

***3.2 Effect of Hespertin on cisplatin-induced nephrotoxicity***

***Effect on Serum markers.***

Animals which received cisplatin (6mg/kg) alone showed significant elevated levels of BUN (62.2±2.3) when compared to group Ib animals (26.76±1.5). Animals which belong to Gr $III_b$ (20 mg/kg) and Gr $IV_b$ (40 mg/kg) has exhibited dose dependent protection (i.e, Gr $III_b$ 28.02±2.90; Gr $IV_b$ 29.02±2.9). The SC levels were increased in Gr IIb which received cisplatin (2.3±1.6) when compared with normal control animals (0.72±0.2). Animals treated with hespertin of 20 mg/kg Bd.wt showed moderate protection (1.6±0.9) and animals treated with 40mg/kg Bd.wt showed significant protection (1.4±0.1).

***Effect on Urinary parameters***

Animals administered with cisplatin excreted high amount of $U_{TP}$ (19.9±1.06) when compared with normal group $I_b$ animals (7.8±0.33) where as animals belonging to Gr $III_b$ (20mg/kg) and Gr $IV_b$ (40 mg/kg) reversed the effect caused by cisplatin (i.e, 6.8±0.6, 6.3±0.33).The animals received cisplatin alone exhibited decreased levels of $Cl_{cr}$ (5.13±2.6) when compared with normal animals (19.80±1.6). On oral administration of hespertin fraction showed significant increase in $Cl_{cr}$ and the values are Gr IIIb animals 20mg/kg bd wt (11.7±1.4) and Gr $IV_b$ animals 40 mg/kg bd wt (10.6±1.56) respectively.

***3.3 Effect on LPO activity:***

The animals received cisplatin alone increased levels of LPO (14.7±0.3) when compared with normal Group-$I_b$ animals (11.7±2.6). On oral administration of hespertin to the groups of $III_b$ (20mg/bd wt), $IV_b$ (40mg/kg Bd wt) showed decreased levels of LPO (14.1±0.5), (12.1±0.9)

***3.4 Effect on antioxidant enzymes***

Animals which received cisplatin alone increased levels of decreased levels of GSH (76.5±2.3), CAT (7.10±0.57), SOD (23.1±1.15) when compared with normal group $I_b$ animals (143.14±1.6, 1.25±0.12 and 105.8±6.48, of GSH, CAT and SOD respectively). On oral administration of hespertin fraction to animals belonging to Gr $III_b$ (20 mg/kg Bd wt) and Gr IVb (40 mg/kg Bd wt) showed increased levels of GSH, CAT, SOD indicating moderate protection at 20 mg/kg bd wt (85.42±3.7, 12.5±1.50 and 43.1±3.2) and significant protection at 40 mg/kg bd wt (89.13±1.9, 14.0±1.0 and 68.52±9.0

# DISCUSSION

Cisplatin is an antitumor drug; it has been successful in the bladder, lung, head, neck, cervical especially testicular and ovarian cancers. The precise mechanism of cisplatin-induced nephrotoxicity has not been elucidated, but it has been suggested that the oxygen free radicals play an important role. Cisplatin-induced nephrotoxocity is related to increase in lipid peroxide levels in kidney. Reports also suggest that there is an involvement of nitric oxide which induces the nephrotoxicity by cisplatin. Tsulsumishitha and coworkers reported the involvement of $H_2O_2$ in ciplatin-induced nephrotoxicity in outer medullary cortical tubule (OCMT) cells. Number of studies has shown that reduction of the glomerular filtration rate is common in patients receiving cisplatin treatment. Moreover, cisplatin-induced nephrotoxicity in humans is secondary to the accumulation of this drug in the tubular epithelial cells from segment of the proximal kidney tubules. However the generation of oxygen free radicals in tubular cells has been proposed as an important pathogenic process. Various data indicate that cisplatin induces oxidative stress, lipid peroxide and DNA damage. Flavonoids are the polyphenolic compounds are found in fruits and vegetables, nuts and seeds as well as most types of tea and red wine. Flavonoids show antioxidant and anti inflammatory activity. In present study, the effect of cisplatin is similar to those previously described i.e., elevated serum urea, serum creatinine levels, reduced creatinine clearance, increased urinary protein excretion. Previous reports also evidenced that cisplatin exerted its nephrotoxic effects through LPO. In the present study, cisplatin induced accumulation of lipid peroxides and depletion of glutathione and its related antioxidant enzymes in kidney may play the critical role of oxidative stress in cisplatin nephrotoxicity. A Cisplatin-induced alteration in lipid peroxides is markedly improved by HDN and HTN. The mechanism of cisplatin nephrotoxicity is related to depletion of the antioxidant defence system. In present study, GSH depletion by cisplatin is in agreement with other reports pertaining to cisplatin-induced renal GSH depletion. The results of the present study showed CAT, SOD activities were significantly decreased in the cisplatin treated animals. hesperitin treated animals significantly improved the levels of GSH, CAT and SOD respectively compared to cisplatin treated group The HDN, HTN showed the protective activity against the cisplatin induced nephrotoxicity. The HDN showed more

prominent action when compared with HTN. HDN is a glycoside and HTN is the aglycon of HDN. Presence of sugar moiety in hesperidin may play a critical role in transportation and fast on- set of action. The inhibition of antioxidant enzyme activity in cisplatin nephrotoxicity and prevention of this altered in rats treated with hesperidin and hesperitin support the rationale for the use of antioxidants to ameliorate cisplatin nephrotoxicity In conclusion, the results provide HDN, HTN are attenuates the nephrotoxicity of cisplatin in rats. The results provide further insight into the mechanisms of cisplatin-induced nephrotoxicity and confirm the antioxidant potential of hesperitin.

## CONCLUSION

Hespertin is a bioflavonoid, to be more specific a flavones. It contains Aglycon moiety and good anti-oxidant properties. It also enhances lipid metabolism and regulate apolipoprotein B secretion by HepG2 cells, possibly through inhibition of cholesterol ester synthesis. Due to its high anti-oxidant properties there is chance of this compound in treating diseases like Diabetes.

## CONFLICT OF INTEREST

Conflict of interest declared none.

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