Abstract

Objective: To evaluate the effect of selected flavonoids diosmin, morin and chrysin on chang cell (normal human liver cells) line by using cell viability assay.

Methods: The cell viability assay on chang cell was determined using MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay. Diosmin, morin and chrysin were subjected in the concentration of 1.625 µM, 3.125 µM, 6.25 µM, 12.5 µM, 25 µM, 50 µM, 100 µM and 500 µM respectively.

Results: The cytoprotective activity by MTT method showed that the IC 50 value of diosmin, morin and chrysin was 101.91 µM, 14.62 µM and 70.00 µM respectively.

Conclusion: Out of the three flavonoids, diosmin and chrysin were proven to have very good cytoprotective activity against Chang cell line. The order of activity was found to be Disomin > Chrysin > Morin.

Key words: Flavonoid, Chang cell line, Diosmin, Morin, Chrysin

Introduction

Plant produces different types of secondary metabolites and one such group is flavonoid. They are polyphenolic in nature and present in different part of the plant like leaves, flowers, fruits, vegetable etc. Flavonoid possesses various pharmacological actions like antioxidant, anti inflammatory, antiulcer, anticancer and hepatoprotective actions. Inspite of various biological flavonoids, diosmin, chrysin and morin were found to be some of the bioflavonoid having various pharmacological actions and available in market for various therapeutic purpose. My present study is with reference to hepatoprotective activity. All the selected flavonoids are proven to be hepatoprotective in various animal models.
Diosmin was proven to be hepatoprotective in ethanol induced toxicity and high carbohydrate induced hepatic damage.

Chrysin was proven to be hepatoprotective in ethanol induced toxicity and galactosamine induced hepatic damage.

Similarly morin was also proven to be hepatoprotective in ethanol induced liver toxicity. But so far no attempts have been taken to study the effect these hepatoprotective flavonoid on normal human liver cell line (chang cell line). There is no proven statement for effect of these flavonoids on normal human liver cell.

Disomin is a flavonoid glycoside introduced in the year 1969 as a therapeutic agent. It was first isolated from Scrophularia nodosa. It has been used for more than 30 years for its phlebotonic properties. It is a polyphenolic compound present in various plants, Hyssopus officinalis, Hyssopus decumbens, Vicia trunctula, Evodia rutaecarpa, Chrysanthemum morifolium, and Buddleja asiatica [1,2]. Mechanisms of action of diosmin include improvement of venous tone, increased lymphatic drainage, protection of capillary blood microcirculation, inhibition of inflammatory reactions, and reduced capillary permeability.

![Structure and Chemistry of diosmin](image)

| Synonym: | 3',5,7-trihydroxy-4'methoxyflavone-7-rutinoside |
| Empirical formula: | C_{28}H_{32}O_{15} |
| Molecular weight: | 608.54 |

**Table 1: Structure and Chemistry of diosmin.**

An increasing number of research showed that diosmin have multiple pharmacological actions. Antioxidant and anti inflammatory actions of diosmin lead to its other pharmacological actions.

**Table 2: Reported pharmacological actions of diosmin.**

<table>
<thead>
<tr>
<th>Pharmacological action</th>
<th>Invitro/invivo studied model</th>
<th>References</th>
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<tbody>
<tr>
<td>Antioxidant</td>
<td>Rat</td>
<td>[3]</td>
</tr>
<tr>
<td>Antinflammatory</td>
<td>Rat</td>
<td>[4]</td>
</tr>
<tr>
<td>Anti-apoptotic effect</td>
<td>Mice</td>
<td>[5]</td>
</tr>
<tr>
<td>Chemopreventive effect</td>
<td>Rat</td>
<td>[6]</td>
</tr>
<tr>
<td></td>
<td>Mice</td>
<td>[7]</td>
</tr>
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<td>Antihypertensive</td>
<td>Rat</td>
<td>[8]</td>
</tr>
<tr>
<td>Antiulcer Rat</td>
<td>[9]</td>
<td></td>
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<td>----------------</td>
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<td></td>
</tr>
<tr>
<td>Diabetic Rat</td>
<td>[10]</td>
<td></td>
</tr>
<tr>
<td>Diabetic neuropathy Rat</td>
<td>[11]</td>
<td></td>
</tr>
<tr>
<td>Anti-hyperlipidemic activity Rat</td>
<td>[12, 13]</td>
<td></td>
</tr>
<tr>
<td>Nephroprotectant Rat</td>
<td>[14]</td>
<td></td>
</tr>
<tr>
<td>Clinical studies Human</td>
<td>[15]</td>
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**Diosmin and hepatoprotective action**

Tahir et al 2012 [16] studied the effect of diosmin on ethanol-induced hepatotoxicity in rats. An attempt was made to find the mechanism of action by taking into account the certain factors like generation of free radicals, oxidative damage to membrane lipids, activation of transcription factors, imbalance to oxidant and antioxidant status, ethanol metabolizing enzymes and elevation in inflammatory markers involved in ethanol- induced hepatic damage in female wistar rats.

Diosmin administrated in three different doses of 10, 20 mg/kg BW. In ethanol-treated group, ethanol metabolizing enzymes CYP 450 2E1 and alcohol dehydrogenase(ADH) have significantly increased in liver tissue by 77.82% and 32.32% respectively as compared with control group and this enhancement is significantly normalized with diosmin administration (20 mg/kg). Similarly the oxidative stress markers lipid peroxidation(LPO), reduced glutathione (GSH), glutathione peroxidase( GPx), and xanthine oxidase have significantly reduced by diosmin. The study further showed that diosmin alleviated ethanol-induced NF-kB activation, enhanced expression of TNF-α, COX-2 and iNOS.

The study also concluded that diosmin alleviates alcoholic liver injury via modulating ethanol metabolizing pathway, inhibition of oxidative stress markers and suppression of inflammatory markers. This may represent a novel protective strategy against ethanol-induced liver diseases. T. Devaki et al 2013 studied the effect of diosmin in regulating triglyceride accumulation during high-carbohydrate diet (HCD)-induced hepatic steatosis[17]. Male albino wistar rats were fed with high carbohydrate diet for 28 days. Abnormal increase in liver enzymes like alanine transaminase and aspartate transaminase, protein, bilirubin levels in HCD rats were attenuated by diosmin treatment. Elevated levels of serum total cholesterol (TC), triglycerides (TG), and very low density lipoproteins (VLDL), liver Malondialdehyde (MDA) were attenuated by disomin, while reduced levels of enzymic antioxidants like superoxide
dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione-S-transferase (GST), Glutathione reductase (GR) and non-enzymic antioxidants like glutathione (GSH), vitamin C and vitamin E in HCD administered rats were restored by disomin treatment.

The study concludes that diosmin has effect in regulating the triglyceride accumulation during high-carbohydrate diet (HCD)-induced hepatic steatosis.

Morin

Morin is one of the naturally occurring bioflavonoids, originally isolated from members of the Moraceae family. It is found in herbs and fruits including onion, see weeds, mill, fig, almond, red wine and osage orange, *Psidium guajava* [18, 19].

![Structure and chemistry of morin](image)

Table 3: Structure and chemistry of morin.

<table>
<thead>
<tr>
<th>Synonym: 2',3',3,4',5,7– pentahydroxyflavone</th>
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<tbody>
<tr>
<td>Emprical formula : C_{15}H_{10}O_{4}</td>
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<td>Molecular weight : 254.24</td>
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Morin exhibited several pharmacological actions

Table 4: Reported pharmacological actions of morin.

<table>
<thead>
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<tr>
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</tr>
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<td>[23,24]</td>
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<td></td>
<td>leukemia HL-60 cells</td>
<td>[25]</td>
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<td>[27], [28]</td>
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<td>[29]</td>
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<td>[30]</td>
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<tr>
<td>Diabetic osteopenia</td>
<td>Rat</td>
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Morin and hepatoprotective action

Mohammed Talat Abbas et al 2012 studied the effect of morin on Isoniazid and Rifampicin Induced hepatotoxicity in Rats [35]. Study was designed on concurrent administration of morin (30 mg/kg /day) and INF-RIF (100mg/kg/day) for 21 days. There is an increase in the activity of alanine aminotransferase, aspartate aminotransferase and malondialdehyde level. there is a significant decrease in the activity of superoxide dismutase, glutathione peroxidase, glutathione s-transferase and catalase in animal groups treated with INH and RIF as compared to control groups. morin significantly decreased alanine aminotransferase, aspartate aminotransferase activity and malondialdehyde level and significant increase in superoxide dismutase, glutathione peroxidase, glutathione s-transferase and catalase. so it is concluded that morin ameliorates the antituberculosis drugs adverse effects in liver.

Saravan et al 2013 studied the effect of morin on ethanol-induced dyslipidemia and oxidative stress in liver mitochondria of rats [36]. Morin (15, 30, 60 and 120 mg/kg BW) was administered to the ethanol-fed rats after 30 days of the experimental period and the treatment was continued up to 60 days. Ethanol administered rats showed a significant elevation in hepatic markers. Decreased activities/levels of superoxide dismutase, catalase, glutathione peroxidase and reduced glutathione were observed in ethanol. Oral administration of morin (60 mg/kg BW) showed its high potentiality in hepatoprotective and antioxidant effects.

Chrysin

Chrysin (5, 7-dihydroxyflavone) is a flavone present at high levels in honey, propolis, blue passion flower *Passiflora caerulea*, *Passiflora incarnata* and in *Oroxylum indicum*. It is also found in *Pleurotus ostreatus* (mushroom) [37]

<table>
<thead>
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**Table 5: Structure and chemistry of chrysin.**
Table 6: Reported pharmacological actions of chrysin.

<table>
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<td>Antioxidant</td>
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<td>Antinflammatory</td>
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<td>Antilipidemic</td>
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<tr>
<td>Anxiolytic</td>
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<td>Rat</td>
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Chrysin and hepatoprotective action

Jayanthi sathiavelu et al 2010 studied the effect of chrysin on ethanol induced hepatic damage [53]. Chrysin had significantly lowered enzymic and non-enzymic antioxidant activity of superoxide dismutase, catalase and glutathione-related enzymes such as glutathione peroxidase, glutathione reductase, glutathione-S-transferase, reduced glutathione, vitamin C and vitamin E.

The results also showed significantly elevated levels of tissue and circulatory thiobarbituric acid reactive substances, conjugated dienes and lipid hydroperoxides in ethanol-treated rats compared with the control. The study concluded that
chrysin produces significant protection against free radical-mediated oxidative stress in rats with ethanol-induced liver injury.

Summaya rahid et al 2013 studied the Hepatoprotective effect chrysin against doxorubicin (DXR) induced oxidative stress [54]. Rats were subjected to concomitant oral administration of chrysin at the dose of 40 and 80 mg/kg BW. Treatment with chrysin significantly decreased the level of serum toxicity markers and elevated the antioxidant defence enzyme levels.

Pushpavalli et al 2010 investigated the effect of chrysin on d-galactosamine-induced hepatic toxicity in rats [55]. Treatment with chrysin (25, 50 and 100mg/kg body weight) decreased hepatic marker enzyme activities and lipid peroxidation products and also increased the activities of free-radical scavenging enzymes superoxide dismutase, catalase and glutathione peroxidase. The levels of non-enzymatic antioxidants reduced glutathione, vitamin C and vitamin E. The study has concluded that chrysin acts as a hepatoprotective and antioxidant agent against d-galactosamine-induced hepatotoxicity. From the literature review, it was concluded that all the selected flavonoids diosmin, chrysin and morin possess various pharmacological actions, particlulary all have hepatoprotective actions against different toxic agent in animal model. So far no attempt has been taken to check the effect of these flavonoids on normal liver cell lines. Hence the current study was aimed to evaluate the cytoprotective effect of diosmin, morin and chrysin on chang cell (normal human liver cells) line by using cell viability assay.

Material and methods

Chemical and reagents

Morin, chrysin and diosmin were purchased from sigma Aldrich. DMEM (Dulbecco’s Modified essential medium) purchased from In vitrogen Gibco BRL , Dimethyl sulfoxide (DMSO), penicillin, streptomycin, Tryspin-EDTA, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), all other chemicals were procured from Sigma Chemical Co. (St. Louis, MO, USA).

Cell culture and drug preparations

Changs liver cell lines were purchased from National centre for cell sciences (NCCS) Pune. Cell lines was grown as monolayer cultures maintained in Dulbecco’s modified Eagle’s medium supplemented with heat inactivated 10% Fetal bovine serum and 2 mM L-glutamine 100 units/ml penicillin and 100 μg/ml streptomycin and maintained at 37°C in a
atmosphere of 5% CO₂ incubator at 95% air humidified. The concentrations used for the study was freshly prepared for each experiment with a final DMSO concentration of 0.1%. All the experiments were performed as three biological replicates with minimum of three independent experiments for compound, and concentration.

**Principle**

Measurement of cell viability is the basis for numerous in vitro assays. Microculture tetrazolium (MTT) assay is widely used to assess cytotoxicity and cell viability. The yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2), 5-diphenyltetrazolium bromide) is reduced by metabolically active cells.

The live cells produce dehydrogenase enzymes. It is based on the capacity of Mitochondria succinate dehydrogenase enzymes in living cells to reduce the yellow water soluble MTT into an insoluble, coloured formazan product which is measured spectrophotometrically.

The level of activity is a measure of the viability of the cells.

Yellow tetrazolium dye $\rightarrow$ succinate dehydrogenase enzymes $\rightarrow$ Purple formazan product

**Procedure:**

Approximately $(5 \times 10^3$ cells/well) chang cells were plated in each well of 96 well plates and incubated for 24 h for attachment. After incubation, supernatant media was replaced with an equal amount of fresh media containing different concentrations of each flavonoid dissolved in DMSO.

After incubation for indicated times, MTT solution was added to the plate at a final concentration of 5 mg/mL and incubated for 4 hr in dark at 37°C.

The resulting MTT-products was dissolved by DMSO. Cell Viability was calculated by measuring optical density at 650 nm using ELISA reader.

Cell survival was calculated by the following formula:

$$\% \text{ Viability} = \frac{\text{Test OD}}{\text{Control OD}} \times 100$$

$$\% \text{ Cytotoxicity} = 100 - \text{Viability} \%$$

Each flavonoids were made into 8 different concentration like 1.625 µM, 3.125 µM, 6.25 µM, 12.5 µM, 25 µM, 50 µM, 100 µM and 500 µM
Result and discussion

To evaluate the cytotoxic activity of three different flavonoids Diosmin, Morin and Chrysin on human normal liver cells (chang cell line) the cell lines were incubated with different doses flavonoids with concentrations s1.625 µM, 3.125 µM, 6.25 µM, 12.5 µM, 25 µM, 50 µM , 100 µM and 500 µM.

After 24 hours of incubation, cell viability was determined by the MTT assay. The results of cytotoxicity assay are presented in (Table .7). Diosmin showed maximum percentage of cell viability of 99 % at the concentration of 3.125 µM and minimum percentage cell viability of 47% at 500 µM. Morin showed maximum percentage of cell viability of  79.45 % at 1.625 µM concentration and minimum cell viability of 24.4% at 500 µM. Chrysin showed maximum percentage of cell viability of  96.81 % at 1.625 µM concentration and minimum cell viability of 44.43 % at 500 µM.

![Figure 1: Percentage viability of chang liver cell at different concentration of diosmin, morin and chrysin. Data expressed as mean ±SD, n= 3. To compare mean between the groups one way ANOVA followed with posthoc test (Tukey HSD) used.](image)

All the three flavonoids induced cell cytotoxicity in a concentration dependent manner. The MTT assays for each flavonoid were carried out in triplicate on 3 different days. The IC50 values were calculated using GraphPad Prism version 5.02-2008 software (GraphPad Software, Inc., La Jolla, CA, USA). The images of diosmin and chrysin given in figure : 2 & 3

![Figure 2: Mtt assay image of chrysin in chang cell line at different concentrations.](image)
To the best of our knowledge this is the first study to report the effect of diosmin, morin and chrysin on chang cell line. The effect of flavonoids on chang cell line was studied using MTT assay. IC 50 value of diosmin is 101.91 µM, IC 50 value of Morin is 14.62 µM, Ic 50 value of chrysin 70.00 µM. Diosmin and chrysin showed good cell protective action against chang cell line. Out of the three flavonoids, diosmin has very good cytoprotective activity against Chang cell line. The order of activity was found to be disomin > chrysin > morin.

The present study was intended to explore whether the selected flavonoids could have protective effect on human liver cells which may contribute to the development of new formulation for the treatment of liver disease.

Acknowledgement:

We are thankful to the authorities of Sri Ramachandra University for providing necessary facilities to carry out this work.

References


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