Chapter-IV

Chrysin protects against cisplatin-induced colon toxicity via amelioration of oxidative stress and apoptosis: probable role of p38MAPK and p53
1. Background

Cisplatin [cis-diamminedichloroplatinum (II) (CDDP) or cisplatinum] (fig.1) is a platinum (Pt) containing antineoplastic drug, widely used as a foremost therapy against numerous forms of cancer including testicular cancer, ovarian germ cell tumors, epithelial ovarian cancer, head and neck cancer, advanced cervical cancer, colon cancer, bladder cancer, mesothelioma, endometrial cancer, non-small cell lung cancer, malignant melanoma, carcinoids, penile cancer and adrenocorticol carcinoma (Adenis et al. 2005; Van Basten et al. 1997; Thigpen et al. 1994; Wang et al. 2004; Saad et al. 2004; Lebwohl & Canetta 1998). It is used as an adjuvant therapy following surgery or radiation and also used in combination with other anticancer drugs (Basu & Krishnamurthy 2010). The therapeutic efficacy of CDDP is enhanced by dose augmentation but its therapeutics intervention is due to its damaging effects on normal cells consequently cause pronounced adverse effects viz., nephrotoxicity, ototoxicity, neurotoxicity, hepatotoxicity, nausea, emesis and 67% of patients experienced diarrhoea (Koc et al. 2005; Kris et al. 1988; Langerak & Dreisbach 2001; Zicca et al. 2004; Bearcroft et al. 1999). The precise mechanism of CDDP toxicity is not fully understood but the plausible mechanism may be through the DNA adduct formation and the generation of panoply of reactive oxygen species (ROS) e.g., superoxide anion ($\text{O}_2^-$), hydrogen peroxide ($\text{H}_2\text{O}_2$), hydroxyl radical ($^\bullet\text{OH}$) etc. which may interact with DNA, lipids and proteins (Sun, 1990). CDDP can act on the sulfhydryl (-SH) groups of cellular proteins (Basu & Krishnamurthy 2010) but DNA is the main cellular target of CDDP that may lead to DNA damage induced by ROS and Pt-DNA adduct formation, thus hampers the cell division or DNA synthesis and its repair mechanism which leads to apoptotic cell death (Sherman et al. 1985; Eastman 1990).

Several lines of evidence exhibited that this chemotherapeutic drug is not specific in action against tumors but also cytotoxic to rapidly dividing normal cells viz., intestinal epithelial cells,
Chrysin protects…

through the production of ROS which provides a nidus for the development of oxidative stress (Wadler et al. 1998; Vijayalakshmi et al. 2006). In this perspective, there is unequivocal evidence that natural compounds with antioxidant properties subsides CDDP toxicity (Atessahin et al. 2007; Chang et al. 2002; Guerrero-Beltrán et al. 2010; Kim et al. 2005; Longo et al. 2011). Therefore, there is a need to explore the natural compound that can effectively diminish the CDDP-induced toxicity to improve its chemotherapeutic efficacy. Now-a-days, dietary supplements containing natural products have many pharmacological properties and have potential to fight against numerous human diseases (Khan and Sultana 2011). Flavonoids are the natural polyphenols ubiquitously present in many plants (Wang and Morris 2007). Several epidemiological studies suggest that dietary supplements rich in flavonoids prevent various diseases viz., cancers (Clere et al. 2011; Hoensch et al. 2010; Pierini et al. 2008), cardiovascular diseases (Garcia-Lafuente et al. 2009), diabetes (Fu et al. 2011), and neurodegenerative diseases (Mandel et al. 2008; Rezai-Zadeh et al. 2005). Chrysin (5, 7-dihydroxyflavone) (fig. 2) belongs to this category which is found in bee propolis, honey and various plants (Barbaric et al. 2011; Pichichero et al. 2010). It has several important biological properties viz., antioxidant (Lapidot et al., 2002), anti-inflammatory (Cho et al., 2004) and anti-cancer properties (Gonçalves et al. 2011; Cardenas et al. 2006; Wang et al. 2004; Miyamoto et al. 2006). Chrysin also reported to enhance the level of testosterone by inhibiting the aromatase enzyme which converts testosterone into estradiol and is already available in market as a dietary supplement in the form of capsule (500mg per capsule) (iHerb Inc., Monrovia, CA; VitaDigest, Walnut, CA) and 6 capsules per day as the highest suggested dose (Wang and Morris 2007).

Previously, it has been reported that chrysin induces cell death in human colorectal cancer cell line i.e., HCT-116 by sensitizing these cells to TNFα-mediated apoptosis (Li et al. 2010) and it has anti-proliferative effects via G2/M cell-cycle arrest in human colon cancer cell lines.
(Goncalves et al. 2011; Wang et al. 2004). It is also reported to modulate NF-κB pathway in human colon cancer cells i.e., Caco-2 cells via diminishing IκBα level, inhibiting NF-κB activation and lowering the IL-8 secretion (Romier et al. 2008).

Recently, it has been shown that chrysin induce cancer cell death synergistically with doxorubicin by chemosensitizing these cells to chemotherapy via GSH depletion within the cancer cells (Brechbuhl et al. 2011). These insights of chrysin help to envisage for reducing the CDDP toxicity which may lead to improve the chemotherapeutic efficacy of CDDP.

In the light of above facts, we hypothesized that prophylactic treatment of chrysin may have protective effects against CDDP-induced colon toxicity by interfering with apoptotic pathway and oxidative processes. In the present study, we investigated the protective role of chrysin against CDDP induced oxidative stress, apoptotic responses and colonic damage in Wistar rats.

2. Treatment schedule

To study the effect of prophylactic treatment with chrysin against CDDP-induced colon toxicity, 30 male Wistar rats were randomly allocated to 5 groups of 6 rats each. The rats of group I (control group) received corn oil through oral gavage at the dose of 5ml/kg body weight once daily for 14 days, which was used as a vehicle for chrysin. Group III received chrysin through oral gavage at the dose of 25 mg/kg body weight once daily for 14 consecutive days. Group IV and V received chrysin through oral gavage at the dose of 50 mg/kg body weight once daily for 14 days. Group II, III and IV rats were given a single intraperitoneal injection of cisplatin at the dose of 7.5 mg/kg body weight on day 14 after 1 hour of the last treatment of chrysin. All the rats were anaesthetized with mild anaesthesia and sacrificed by cervical dislocation after 24 hour of the cisplatin injection. Selection of cisplatin dose was based on the previous published studies (Guerrero-Beltrán et al. 2010; Chirino et al. 2004; Boogaard et al. 1991).
2.1 Schematic representation of the experimental design

- **Corn oil (5ml/kg b. wt.)**
- **Cisplatin (7.5mg/kg b.wt. i.p. once at day 14)** arrow indicates cisplatin injection
- **Chrysin (25mg/kg b. wt. orally everyday for 14 days) + Cisplatin (7.5mg/kg b.wt. i.p. once at day 14)** arrow indicates cisplatin injection
- **Chrysin (50mg/kg b.wt. orally everyday for 14 days) + Cisplatin (7.5mg/kg b.wt. i.p. once at day 14)** arrow indicates cisplatin injection
- **Chrysin only (50mg/kg body weight, orally everyday for 14 days)**

*Sacrifice on day 15*
3. Results

3.1 Effect of prophylactic treatment of chrysin against CDDP-induced lipid peroxidation

The level of MDA was significantly enhanced (p<0.001) in Group II as compared to Group I. Chrysin pretreatment significantly decreased the level of MDA in Group III (p<0.001) and Group IV (p<0.001) respectively as compared to Group II. No significant difference was found in the level of MDA between Group I and Group V. (Fig.3)

3.2 Effect of chrysin pretreatment and CDDP on XO activity in colonic tissue

The activity of XO was significantly increased (p<0.001) in Group II as compared to Group I. Chrysin pretreatment significantly decreased the activity of XO in Group III (p<0.001) and Group IV (p<0.001) as compared to Group II. Group V exhibited no significant change in the activity of XO as compared to Group I. (Fig.4)

3.3 Effect of prophylactic treatment of chrysin against CDDP-induced GSH depletion

The level of GSH was depleted significantly (p<0.01) in CDDP treated group (Group II) as compared to control group (Group I). Chrysin pretreatment showed a significant increase in the level of GSH in Group III (p<0.05) and Group IV (p<0.05) when compared with group II. No significant difference was found in the level of GSH between Group I and Group V. (Fig.5)

3.4 Effect of chrysin supplementation and CDDP on the activities of glutathione dependent enzymes in colonic tissue

CDDP treatment caused a significant decrease in the activities of GPx (p<0.001), GST (p<0.05), GR (p<0.001) and G6PD (p<0.001) in Group II as compared to Group I. Chrysin supplementation at the dose of 25 mg/kg b. wt. significantly increased the activity of GST only
(p<0.05) but not other enzymes in Group III as compared to Group II. But the higher dose of chrysin (50 mg/kg b. wt.) significantly attenuated the activities of GPx (p<0.05), GST (p<0.05), GR (p<0.01) and G6PD (p<0.05) in Group IV as compared to Group II. However, the activities of these enzymes in Group V did not change significantly as compared to Group I. (Table.1)

3.5 Effect of chrysin supplementation and CDDP on the activities of colonic antioxidant enzymes

The activities of CAT and QR were decreased significantly at the level of (p<0.01) and (p<0.001) respectively in Group II as compared to Group I while the activity of SOD was increased significantly (p<0.001) in Group II as compared to Group I. Chrysin pretreatment at the dose of 25 mg/kg b.wt. significantly augmented the activities of CAT (p<0.05) and QR (p<0.05) in Group III as compared to Group II while the activity of SOD was significantly decreased (p<0.001) in Group III as compared to Group II. The higher dose of chrysin (50 mg/kg b. wt.) also showed significant increase in the activities of CAT (p<0.05) and QR (p<0.01) in Group IV as compared to Group II while the activity of SOD was significantly decreased (p<0.001) in Group IV as compared to Group II. However, the activities of these enzymes in Group V did not change significantly as compared to Group I. (Table.2)

3.6 Effect of chrysin pretreatment and CDDP on the expression of phospho-p38, p53, Bak and cleaved caspase-3 in colonic tissue

The colonic sections of CDDP treated group (Group II) have more phospho-p38, p53, Bak and cleaved caspase-3 immunopositive staining (arrows) as indicated by brown colour as compared to control group (Group I) while pretreatment of chrysin in Group III and IV reduced phospho-
p38, p53, Bak and cleaved caspase-3 immunostaining as compared to Group II. However, there were no significant differences in the immunostaining of all proteins in Group V as compared to Group I. For immunohistochemical analyses, brown colour indicates specific immunostaining of phospho-p38, p53, Bak and cleaved caspase-3, and light blue colour indicates haematoxylin staining. Original magnification: 40x. (Fig. 6-9)

3.7 Effect of chrysin pretreatment against CDDP-induced goblet cell disintegration

The colonic sections of CDDP treated group (Group II) showed extensive disintegration of goblet cells whereas there is no goblet cell disintegration in control group (Group I). In Group III and IV, chrysin supplementation at both the doses (25 and 50 mg/kg b.wt.) showed protection against CDDP-induced goblet cells disintegration as compared to Group II. (Fig. 10)

3.8 Effects of chrysin pretreatment and CDDP on colon histology

The H&E stained sections exhibited normal histoarchitecture with mild inflammatory cells infiltration in control group (Group I) while CDDP-treated groups showed distorted mucosal glandular architecture, crypt ablation with intense inflammatory cells infiltration in mucosal and submucosal layers as well as crypt abscess formation. In Group III and IV, chrysin significantly attenuated the CDDP-induced histopathological changes at both the doses (25 and 50 mg/kg b.wt.). There is no significant difference in the histological changes in Group V as compared to Group I. (Fig.11)

4. Discussion

In this study, we have examined the protective effects of chrysin against CDDP-induced colon toxicity in Wistar rats. The CDDP-induced intestinal toxicity is well documented as it causes
emesis and diarrhoea (Bearcroft et al., 1999). Although, the exact mechanism underlying CDDP-induced intestinal toxicity is still unclear but it may be due to ROS generated by CDDP which leads to the condition of oxidative stress. Therefore, the natural compounds with antioxidant properties are gaining much attention of several investigators. In the present study, the protective effects of chrysin observed may be associated with amelioration of oxidative stress and apoptotic damage in the colon of CDDP treated rats. CDDP generates superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (•OH) which may play a part in the initiation of lipid peroxidation (Sun, 1990). Lipid peroxidation is a marker of oxidative stress and several studies have reported that remarkable elevation in the level of malondialdehyde (MDA), a lipid peroxidation product, was observed after CDDP treatment (Koc et al. 2005; Chang et al. 2002). Our results corroborated with the above mentioned previous findings which showed that there is remarkable increase in the level of MDA in rats treated with CDDP and pretreatment with chrysin significantly reduced the level of MDA.

Apart from lipid peroxidation, it was observed that CDDP-induced GSH depletion and enhanced xanthine oxidase (XO) activity corroborating the CDDP-induced oxidative damage in the colon of Wistar rats. Chrysin supplementation significantly attenuated the GSH depletion and XO activity. XO is an enzyme that reduces O$_2$ to superoxide anion radical (O$_2^-$) and consequently produce oxidative stress (Heunks and Dekhuijzen 2000) while GSH is a low molecular weight tripeptide cellular antioxidant. It protects the peroxidation of lipid membrane by conjugating with the electrophile such as 4-Hydroxy-3-nonenal (HNE), formed during lipid peroxidation and thus gets depleted in this conjugation reaction (Kawanishi and Yamamoto 1991). This conjugation of GSH via sulphahydryl (-SH) group to electrophile is catalysed by an
antioxidant enzyme i.e., glutathione-s-transferase (GST), and thus the GST activity decreased in this process (Forman et al. 2009; Douglas 1987).

Additionally, it was observed that the activities of antioxidant enzymes viz., CAT, GPx, GR and G6PD and phase-II detoxifying enzymes viz., GST and QR were diminished whereas the activity of SOD was increased in rats treated with CDDP. Pretreatment with chrysin significantly attenuated the activities of these antioxidant and phase-II detoxifying enzymes. In this study, the increase in SOD activity in CDDP treated group is in agreement with the previous finding which exhibit that CDDP treatment leads to the over-expression of SOD to lessen the CDDP toxicity (Vijayalakshmi et al. 2006). The GST enzyme detoxifies a number of ROS via catalyzing the conjugation with GSH (Douglas 1987; Manar et al. 2004). QR is a phase-II enzyme involve in xenobiotic metabolism that catalyses the two-electron reduction and thus protects cells against free radicals and ROS generated by the one-electron reductions catalyzed by cytochromes P450 and other enzymes (Benson et al. 1980; Dinkova-Kostova and Talalay 2000). The diminished activities of antioxidant and phase-II detoxifying enzymes in CDDP-treated group supporting the involvement of oxidative stress in the pathophysiology of CDDP-induced colon toxicity.

It has already been reported that CDDP-induced toxicity was closely associated with ROS generated by CDDP (Kim et al. 2010). CDDP damages cellular DNA by forming Pt-DNA adducts and additionally CDDP-generated ROS also fostering the DNA damage within the cell thus leading to the activation of p38 mitogen-activated protein kinase (MAPK) (Reinhardt et al. 2007; Nishida et al. 2005). CDDP-induced DNA damage leads to the activation of DNA damage sensor kinases viz., Ataxia-Telangiectasia mutated (ATM) and Ataxia-Telangiectasia and Rad-3 related (ATR). These sensor kinases phosphorylate and activate thousand and one (Tao) kinases and the later further phosphorylate and activate MAPK kinase 3/4/6 (MKK3/4/6) which
ultimately leads to the phosphorylation and activation of p38MAPK (Ashwell 2006; Raman et al. 2007; Thornton and Rincon 2009). In our study, it was observed that CDDP-treated group have more phospho-p38 immunopositive staining as compared to control group while pretreatment with chrysin significantly attenuated the phospho-p38 immunopositive staining. These results further supported the involvement of oxidative stress in CDDP-induced toxicity. (Fig.12)

The p53 is a tumor suppressor protein and also acts as a transcription factor that regulates the transcription of genes involved in cell cycle, DNA repair and apoptosis (Riley et al. 2008). Mdm-2 is a co-repressor of p53 and it maintains the low level of p53 via ubiquitin-mediated proteosomal degradation (Haupt et al. 1997). In response to DNA damage, phosphorylated and activated p38MAPK directs the phosphorylation and activation of p53 and thus the cellular level of p53 protein increases (Bulavin et al. 1999; She et al. 2001, She et al. 2000; Huang et al. 1999; Maya et al. 2001). In response to irrepaired or irrepairable DNA damage, p53 translocates to the mitochondria and induces apoptosis via interacting and activating the mitochondrial membrane protein Bak (Bcl-2–antagonist/killer). Bak, instead of Bax, plays a critical role in mitochondrial fragmentation during apoptosis and p53-Bak interaction causes homo-oligomerization of Bak within the outer mitochondrial membrane ensuing mitochondrial outer membrane permeabilization (MOMP) they cause, unleashes the pro-apoptotic proteins viz., cytochrome c into the cytosol (Green and Kroemer 2009; Leu et al. 2004; Brooks et al. 2007). Released cytochrome c ultimately results in activation of caspase-3 which is the main executioner caspase because it can be activated through both intrinsic and extrinsic pathway (Liu et al. 1996; Narula et al. 1999). Activated caspase-3 leads to DNA fragmentation and cleavage of specific cellular proteins like poly(ADP-ribose) polymerase (PARP), actin, fodrin and lamin during apoptosis (Sakahira et al. 1998).
The present study has demonstrated that CDDP-treated group have more p53, Bak and cleaved caspase-3 immunopositive staining as compared to control group while prophylactic treatment with chrysin significantly attenuated the p53, Bak and cleaved caspase-3 immunopositive staining. These results further supported the involvement of apoptotic colonic damage and oxidative stress in CDDP-induced toxicity. (Fig.12)

Goblet cells, the specialized exocrine cells of colonic crypts, synthesize and secrete mucins. Mucins are high molecular weight, highly glycosylated proteins which forms a protective layer in the form of gel in intestinal lumen (Specian and Oliver 1991; Robbe et al. 2004). It was observed in our study that chrysin significantly attenuated the CDDP-induced disintegration of the goblet cells in colonic crypt. These results exhibited the protective effects of chrysin against CDDP-induced toxicity.

The above mentioned findings corroborated with the histological data which exhibited the protective effects of chrysin against CDDP-induced distorted mucosal glandular architecture, crypt ablation with orchestration of intense inflammatory cells infiltration in mucosal and submucosal layers as well as crypt abscess formation.

The precise mechanism of protective action of chrysin against CDDP is still unknown but it can be concluded from the findings of the present study that chrysin exhibit the protective effect against CDDP-induced colon toxicity probably through the attenuation of CDDP-induced oxidative stress and apoptotic tissue damage. It could be used as a combinational therapy with CDDP but before that further studies are still needed to elucidate the exact protective mechanism of chrysin.
Figure 1. Cisplatin

![Cisplatin structure](image)

Figure 2. Chrysin

![Chrysin structure](image)
Figure 3. Effects of chrysin and cisplatin on the colonic MDA level

Figure 3: Effect of prophylactic treatment of chrysin against CDDP-induced lipid peroxidation (MDA level) in colon of Wistar rats. Data were expressed as mean ± S.D. (n=6) and measured as nmol MDA formed/g tissue. MDA level was significantly increased (***p<0.001) in CDDP treated group (Group II) as compared to Group I. Pretreatment with chrysin significantly attenuated the level of MDA in Group III (###p<0.001) and Group IV (###p<0.001) as compared to Group II.

GP1- Vehicle Treated Control Group (Corn oil – 5ml/kg b. wt.)

GP2- Cisplatin Treated Group (7.5mg/kg b.wt.)

GP3- Dose 1 of Chrysin (25 mg/kg b.wt.) + Cisplatin (7.5mg/kg b.wt.)

GP4- Dose 2 of Chrysin (50 mg/kg b.wt.) + Cisplatin (7.5mg/kg b.wt.)

GP5- Only Dose 2 of Chrysin (50 mg/kg b.wt.)
Figure 4. Effects of chrysin and cisplatin on the colonic XO activity

**Figure 4** Effect of chrysin pretreatment and CDDP on XO activity. Data were expressed as mean ± S.D. (n=6) and measured as µg uric acid formed/min/mg protein. XO activity was significantly increased (**p<0.001**) in CDDP treated group (Group II) as compared to Group I. Pretreatment with chrysin significantly attenuated the activity of XO in Group III (###p<0.001) and Group IV (####p<0.001) as compared to Group II.

**GP1** - Vehicle Treated Control Group (Corn oil – 5ml/kg b. wt.)

**GP2** - Cisplatin Treated Group (7.5mg/kg b.wt.)

**GP3** - Dose 1 of Chrysin (25 mg/kg b.wt.) + Cisplatin (7.5mg/kg b.wt.)

**GP4** - Dose 2 of Chrysin (50 mg/kg b.wt.) + Cisplatin (7.5mg/kg b.wt.)

**GP5** - Only Dose 2 of Chrysin (50 mg/kg b.wt.)
Figure 5. Effects of chrysin and cisplatin on the colonic GSH level

![Graph showing effects of chrysin and cisplatin on colonic GSH level]

Figure 5 Effect of prophylactic treatment of chrysin against CDDP-induced depletion of colonic GSH content. Data were expressed as mean ± S.D. (n=6) and measured as μmol DTNB conjugate formed/g tissue. GSH content was significantly decreased (**p<0.01) in CDDP treated group (Group II) as compared to Group I. Pretreatment with chrysin significantly prevented the depletion of colonic GSH level in Group III (#p<0.05) and Group IV (#p<0.05) as compared to Group II.

GP1- Vehicle Treated Control Group (Corn oil – 5ml/kg b. wt.)

GP2- Cisplatin Treated Group (7.5mg/kg b.wt.)

GP3- Dose 1 of Chrysin (25 mg/kg b.wt.) + Cisplatin (7.5mg/kg b.wt.)

GP4- Dose 2 of Chrysin (50 mg/kg b.wt.) + Cisplatin (7.5mg/kg b.wt.)

GP5- Only Dose 2 of Chrysin (50 mg/kg b.wt.)
Figure 6. Immunohistochemical staining of phospho-p38

Photomicrographs of colonic sections depicting (A) Vehicle treated control group (Group I), (B) CDDP treated group (7.5mg/kg b. wt.) (Group II), (C) Dose 1 of Chrysin (25 mg/kg b.wt.) + CDDP (Group III), (D) Dose 1 of Chrysin (50 mg/kg b.wt.) + CDDP (Group IV), (E) Only Dose 2 of Chrysin (50 mg/kg b.wt.) (Group V). For immunohistochemical analyses, brown colour indicates specific immunostaining of phospho-p38 and light blue colour indicates nuclear haematoxylin staining. The colonic section of CDDP treated group (Group II) has more phospho-p38 immunopositive staining (arrows) as indicated by brown colour as compared to control group (Group I) while pretreatment of chrysin in Group III and IV reduced phospho-p38 immunostaining as compared to Group II. However there was no significant difference in the phospho-p38 immunostaining in Group V as compared to Group I. Original magnification: 40x.
Figure 7. Immunohistochemical staining of p53

Figure 7 Effect of chrysin pretreatment on CDDP-induced p53 expression. Photomicrographs of colonic sections depicting (A) Vehicle treated control group (Group I), (B) CDDP treated group (7.5mg/kg b.wt.) (Group II), (C) Dose 1 of Chrysin (25 mg/kg b.wt.) + CDDP (Group III), (D) Dose 1 of Chrysin (50 mg/kg b.wt.) + CDDP (Group IV), (E) Only Dose 2 of Chrysin (50 mg/kg b.wt.) (Group V). For immunohistochemical analyses, brown colour indicates specific immunostaining of p53 and light blue colour indicates nuclear haematoxylin staining. The colonic section of CDDP treated group (Group II) has more p53 immunopositive staining (arrows) as indicated by brown colour as compared to control group (Group I) while pretreatment of chrysin in Group III and IV reduced p53 immunostaining as compared to Group II. However there was no significant difference in the p53 immunostaining in Group V as compared to Group I. Original magnification: 40x.
Figure 8. Immunohistochemical staining of Bak

(A) Vehicle treated control group (Group I), (B) CDDP treated group (7.5mg/kg b. wt.) (Group II), (C) Dose 1 of Chrysin (25 mg/kg b.wt.) + CDDP (Group III), (D) Dose 1 of Chrysin (50 mg/kg b.wt.) + CDDP (Group IV), (E) Only Dose 2 of Chrysin (50 mg/kg b.wt.) (Group V). For immunohistochemical analyses, brown colour indicates specific immunostaining of Bak and light blue colour indicates nuclear haematoxylin staining. The colonic section of CDDP treated group (Group II) has more Bak immunopositive staining (arrows) as indicated by brown colour as compared to control group (Group I) while pretreatment of chrysin in Group III and IV reduced Bak immunostaining as compared to Group II. However there was no significant difference in the Bak immunostaining in Group V as compared to Group I. Original magnification: 40x.
Figure 9: Immunohistochemical staining of cleaved caspase-3

Photomicrographs of colonic sections depicting (A) Vehicle treated control group (Group I), (B) CDDP treated group (7.5 mg/kg b. wt.) (Group II), (C) Dose 1 of Chrysin (25 mg/kg b.wt.) + CDDP (Group III), (D) Dose 1 of Chrysin (50 mg/kg b.wt.) + CDDP (Group IV), (E) Only Dose 2 of Chrysin (50 mg/kg b.wt.) (Group V). For immunohistochemical analyses, brown colour indicates specific immunostaining of cleaved caspase-3 and light blue colour indicates nuclear haematoxylin staining. The colonic section of CDDP treated group (Group II) has more caspase-3 immunopositive staining (arrows) as indicated by brown colour as compared to control group (Group I) while pretreatment of chrysin in Group III and IV reduced cleaved caspase-3 immunostaining as compared to Group II. However there was no significant difference in the cleaved caspase-3 immunostaining in Group V as compared to Group I.

Original magnification: 40x.
Figure 10. Effects of chrysin against cisplatin-induced goblet cells disintegration

Photomicrographs of alcian blue staining of histological sections of colon depicting (A) Vehicle treated control group (Group I), (B) CDDP treated group (7.5mg/kg b. wt.) (Group II), (C) Dose 1 of Chrysin (25 mg/kg b.wt.) + CDDP (7.5mg/kg b. wt.) (Group III), (D) Dose 1 of Chrysin (50 mg/kg b.wt.) + CDDP (7.5mg/kg b. wt.) (Group IV), (E) Only Dose 2 of Chrysin (50 mg/kg b.wt.) (Group V). (A) Goblet cells stained blue due to acidic mucin secreted by these cells and exhibited the normal integrated goblet cells along the colonic sections. (B) Extensive disintegration of goblet cells was observed (bold arrows) in CDDP-treated group. (C) & (D) Chrysin pretreatment showed protection against CDDP-induced goblet cells disintegration. Both the doses of chrysin (25 and 50 mg/kg b.wt.) maintained the integrity of goblet cells. (E) Normal integrated goblet cells were observed as in control group. Original magnification: 40x.
Figure 11. Effects of chrysin and cisplatin on the colonic histoarchitecture

(A) Normal histology of the rat colon with mild inflammatory cells infiltration (arrows) (normal appearance of the mucosal crypts of Lieberkuhn, submucosa, and muscular layers). (B) Distorted mucosal glandular architecture (bold arrows), crypt ablation (arrowheads) with intense inflammatory cells infiltration in mucosal and submucosal layers (arrows) as well as crypt abscess formation (stars) was observed in CDDP treated group. (C) & (D) Chrysin supplementation prevented the mucosal damage and ameliorated inflammatory cells infiltration as well as crypt ablation and crypt abscess formation. (E) Normal histology was observed as in Group I. Original magnification: 40x.
Figure 12. Proposed intracellular targets of chrysin against cisplatin induced damages in the colon of Wistar rats.

Cisplatin causes toxicity via DNA damages and ROS generation. DNA damage leads to activation of p38MAPK and the later results in phosphorylation and activation of p53 that allows the cells to repair the DNA by blocking the cell cycle. If DNA remains unrepaired, it leads to apoptosis via Bak and consequent caspases activation. Chrysin pre-treatment shows reduction in XO activity (1) leading to reduction in ROS formation. Further attenuation in antioxidants like SOD (2), CAT (3) activities and GSH content and related redox cycle enzymes (GR, GPx, and...
G6PD) (4) potentiate its role against oxidants induced damages. Moreover chrysin pretreatment also increased phase II metabolising enzyme (GST and QR) activities (5 a & 5 b). These effects are evident by reduction in LPO of cellular membranes (6). Chrysin shows the promising role against cisplatin induced apoptotic injuries in colons by reducing the levels of phospho-p38, p53, Bak and cleaved Casp-3 (7, 8, 9 & 10 respectively). Cleaved Casp-3 = Cleaved Caspase-3; CAT = Catalase; GPx = Glutathione peroxidise; GR = Glutathione reductase; GSH = Reduced glutathione; GSSG = Oxidised glutathione; GST = Glutathione S transferase; G-6-P = Glucose-6-phosphate; G-6-PD = Glucose-6-phosphate dehydrogenase; 6-PG = 6-phosphogluconate; NADPH = Nicotinamide adenine dinucleotide phosphate reduced; NADP+ = Nicotinamide adenine dinucleotide phosphate (Oxidised); O2− = Superoxide radical; QR = Quinone reductase; R = Xenobiotic; ROS = Reactive Oxygen Species; R-SH = thiol conjugated xenobiotics; SOD = Superoxide dismutase; XO = Xanthine oxidase; Bak = Bcl-2–antagonist/killer. Arrows indicates the activation.