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# Inhibition of precancerous lesions development in kidneys by chrysin via regulating hyperproliferation, inflammation and apoptosis at pre clinical stage

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ABSTRACT

Chrysin (CH) is natural, biologically active compound, belongs to flavoniod family and possesses diverse pharmacological activities as anti-inflammatory, anti-oxidant and anti-cancer. It is found in many plants, honey and propolis. In the present study, we investigated the chemopreventive efficacy of CH against *N*-nitrosodiethylamine (DEN) initiated and Fe-NTA induced precancerous lesions and its role in regulating oxidative injury, hyperproliferation, tumor incidences, histopathological alterations, inflammation, and apoptosis in the kidneys of Wistar rats. Renal cancer was initiated by single intraperitoneal (i.p.) injection of DEN (200 mg/kg bw) and promoted by twice weekly injection of ferric nitrilotriacetate (Fe-NTA) 9 mg Fe/kg bw for 16 weeks. CH attenuated Fe-NTA enhanced renal lipid peroxidation, serum toxicity markers and restored renal anti oxidant armory significantly. CH supplementation suppressed the development of precancerous lesions via down regulation of cell proliferation marker like PCNA; inflammatory mediators like TNF-α, IL-6, NFkB, COX-2, iNOS; tumor incidences. CH up regulated intrinsic apoptotic pathway proteins like bax, caspase-9 and caspase-3 along with down regulation of Bcl-2 triggering apoptosis. Histopathological and ultra structural alterations further confirmed biochemical and immunohistochemical results. These results provide powerful evidence for the chemopreventive efficacy of CH against chemically induced renal carcinogenesis possibly by modulation of multiple molecular pathways.



## 1. Introduction

Renal cell carcinoma (RCC) is the most common malignancy of adult kidney [1]. It has been reported to be the most therapy resistant cancer and responds either very less or not at all to the conventional therapies [2]. Many novel chemotherapeutic agents have been developed over the past decade, besides an increase in deciphering molecular mechanisms implicated in the development of RCC, yet it is not curable and a fatal disease [3]. Oxidative stress is one of the risk factors for human RCC and plays an essential role in Fe-NTA induced carcinogenesis as well [4]. Diethylnitrosamine (DEN) is an effective carcinogenic *N*-nitroso compound broadly recognized for the induction of preneoplastic lesions in experimental animals [5]. Nitrilotriacetate (NTA) is chemically amino tricarboxylic acid and a potent nephrotoxic agent. Fe-NTA, ferric nitrilotriacetate is an iron chelate formed by combination of Fe and NTA, which on repeated administration produces acute and sub acute renal proximal tubular necrosis that

*Abbreviations:* CAT, Catalase; CH, Chrysin; DEN, Di ethyl nitrososamine; Fe-NTA, Ferric Nitriloacetic acid; BSA, bovine serum albumin; CDNB, 1-chloro 2, 4-dinitrobenzene; DTNB, 5, 5′-dithio bis-[2-nitrobenzoic acid]; EDTA, ethylene diamine tetra acetic acid; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; NADPH, reduced nicotinamide adenine dinucleotide phosphate; ROS, reactive oxygen species; TBA, thiobarbituric acid; LDH, lactate dehydrogenase; BUN, Blood urea nitrogen; MDA, malondialdehyde.

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ultimately results in high prevalence of renal adenocarcinoma in rats and mice. DEN and Fe-NTA stands as an outstanding model for inducing renal carcinogenesis in vivo via free radical induced damage coupled with widespread peroxidation of membrane lipids which is further illustrated by high frequency of tumor associated mortality, prevalence of pulmonary metastasis and peritoneal invasion [6,7]. Presently much research is being carried on evaluation of safe and efficient plant based products with diversified pharmacological properties chiefly against cancer because of their positive results in animal models, clinical trials as well as poor alternatives the modern system of medicine recommends for the treatment.

Flavonoids are plant polyphenolic compounds with variable phenolic structures and are extensively distributed in medicinal plants, fruits, health beverages and teas. Epidemiological studies further prove importance of flavoniods via mentioning high fruit and vegetable consumption is associated with a decreased risk of cardiovascular disease and several types of cancers including breast, colon, lung, pancreas, oral and prostate both in vitro and in vivo. This has increased the public's interest in the use of flavonoids for their potential health benefits and a growing attention in the revelation of the biological roles of flavonoids, the major components of some traditional medicinal plants by both consumers and food manufacturers. Chrysin (5,7-dihydroxyflavone) is a natural flavonoid present in many plant extracts, honey and propolis. It possesses numerous biological and pharmacological properties including antioxidant, apoptotic, anticancer, anti-inflammatory etc. Earlier studies have confirmed the importance of distribution and quantity of the hydroxl groups are related to antioxidant property of flavonoids particularly depending on their hydroxylation of ring B [8]. Recent studies have revealed that chrysin regulates key molecules involved in inflammation, cancer and aging [9]. It has also been reported that chrysin inhibits proliferation and induces apoptosis in cancer cells, making it a possible candidate as anticancer agent [10]. In vitro studies reveal that chrysin inhibits the growth of Hela cells by down regulating the expression of proliferating cell nuclear antigen (PCNA), induces apoptosis via caspase activation and Akt inactivation in various cancer cell lines. In vivo findings showed that dietary administration of chrysin significantly inhibited the development of AOM-induced colonic ACF in rats [11]. It inhibited tumor angiogenesis in vivo, which is a key step in cancer cell metastasis. It also significantly sensitizes TNF-$\alpha$ induced apoptosis in a number of human cancer cells as well as inhibited COX-2 expression and IL-6 signaling which suggests its anti-inflammatory property [12].

Therefore the current study was planned to investigate its potential against Fe-NTA induced nephrotoxicity and its preclinical chemo preventive efficacy against two stage renal carcinogenesis induced by DEN initiation and Fe-NTA promotion for 16 weeks in Wistar rats by studying its possible potential molecular targets. To this effect, we studied oxidative stress markers, anti oxidant armory profile, histopathological alterations, ultra structural changes, expressions of inflammatory marker proteins, instrinsic apoptotic pathway proteins and hyper proliferation marker PCNA known to be deregulated in cancer cells and hence might be one of the novel targets of the chemopreventive activity of CH.

## 2. Materials and methods

### 2.1. Chemicals

DEN, Fe-NTA, BSA, BUN, CDNB, DTNB, EDTA, GPx, GR, GSH, GSSG, LDH, NADPH, TBA, CH etc were obtained from Sigma-Aldrich, USA. All other chemicals and reagents were of the highest purity grade and commercially available.

### 2.2. Ethical statement

All procedures for using experimental animals were checked and permitted by the "Institutional Animal Ethical Committee (IAEC)"that is fully accredited by the Committee for Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The animals were provided by the Central Animal House Facility, Jamia Hamdard, whose registration number and date of renewal are (IAEC No: 173/Go/Re/S/2000/CPSCEA) and 30$^{th}$ march, 2015. Approval ID/project number for performing this study by permission of IAEC is 1059.

### 2.3. Animals

Male Wistar rats (150–200 g), 6–8 weeks old, were obtained from the Central Animal House of Hamdard University, New Delhi, India. Rats were housed in polypropylene cages in groups of four rats per cage and were kept in a room maintained at 25 ± 2 °C with a 12 h light/dark cycle. They were allowed to acclimatize for one week before the experiments and were given free access to standard laboratory animal diet and water ad libitum.

### 2.4. Preparation of Fe-NTA

The Fe-NTA solution was prepared by the method of Awai et al. briefly, ferric nitrate (0.16 mM/5.0 ml) solution was mixed with a fourfold molar excess or disodium salt of NTA (0.64 mM/5.0 ml) and the pH was adjusted to 7.4 with sodium bicarbonate. The solution was freshly prepared immediately before use and was injected on the basis of 10 ml/kg b wt [13].

### 2.5. Experimental design

The treatment regimen for CH and the proposal of verifying its chemopreventive efficacy against renal carcinogenesis was based on the preliminary dose dependent pilot study which was carried out in our laboratory. To study the protective effects of CH on biochemical and serological changes induced by toxicity of Fe-NTA in rats, 24 male Wistar rats were randomly divided into four equal groups.

| Groups (n = 6) | Treatment from 1st to 13th day | Treatment on 13th day |
|---|---|---|
| Group I (control) | Distilled water | Normal saline only (0.9% i.p.) |
| Group II (only Fe-NTA) | Distilled water | Fe-NTA 9 mg/kg b.wt.i.p (13th day) |
| Group III (FeNTA + CHD1) | CH 50 mg/kg b.wt. | Fe-NTA 9 mg/kg b.wt.i.p (13th day) |
| Group IV (FeNTA + CHD2) | CH 100 mg/kg b.wt. | Fe-NTA 9 mg/kg b.wt.i.p (13th day) |

All animals were sacrificed within 1 h exactly 12 h after Fe-NTA administration. Kidney tissues were processed for biochemical estimations. Blood was collected and serum separated out and