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Study of protective, antioxidant, and immunomodulatory roles of *Citrus Sinensis* peel extract and Gold Nanoparticles against Methotrexate-induced hepatotoxicity, oxidative stress, and immune dysfunction in rats

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Abstract
Background: Hepatotoxicity and immune changes are the major side effects induced by chemotherapy, which is largely due to high levels of reactive oxygen species (ROS). Methotrexate (MTX), a commonly used chemotherapeutic and immunosuppressive agent, has been reported to induce hyperlipidemia, oxidant stress, and immune dysfunction. Current studies have identified the promise of natural antioxidants and nanomaterials for these protective strategies. Peel of Citrus sinensis (CS) is abundant in flavonoids and phenol which exhibit potent antioxidant and hepatoprotective effects whereas gold nanoparticles (AuNPs) demonstrate immunomodulatory and cytoprotective activities.
Methods: 36 male Wistar rats were randomly assigned into six groups (n = 6): control, MTX (10 mg/kg), MTX together with C. sinensis peel extract (at doses of 75, 150 and 250 mg/kg) and MTX combined with AuNPs(0.3–0.6 mg/kg). Serum lipid profiles (TC, TG), oxidative stress biomarkers (MDA, LPO), antioxidant enzyme activities [glutathione peroxidase expression (GPx)] and immune parameters [white blood cell count, lymphocyte percentage and IL-6] were determined over a period of 14 days. Analysis of data was performed using one-way ANOVA followed by Tukey's test, where $p < 0.05$.
Results: MDA, LPO, TC, TG and IL-6 levels were significantly increased while the GPx activity and total WBCs count/lymphocyte percentage was lowered by MTX administration. The changes were markedly reversed by pretreatment with C. sinensis peel extract and AuNPs in a dose-dependent manner. Both therapies also attenuated oxidative stress, alleviated lipid metabolism disorder, improved antioxidant system and immune status. The most interesting was that even 10% ofn AuNP at the higher doses showed to be about somewhat better than peel extract.
Conclusions: The extract of C. sinensis peel and AuNPs have significant hepatoprotective, antioxidant and immunomodulatory activities against MTX toxicity. Their synergetic mechanisms have shown a promising possibility that the best of natural phytocompounds and nanomaterial could be combined for adjunctive therapeutic modality assisting in chemotherapy protocols with lowered systemic toxicity yet high efficacy.

Keywords: *Citrus sinensis*, gold nanoparticles, methotrexate, hepatoprotection, oxidative stress, immunomodulation, chemotherapy, wistar rats

Introduction

The incorporation of molecular biology, bioengineering, and nanotechnology in medical research has given rise to medical biotechnology, a new and invaluable branch of science. It has opened new paths to disease diagnosis, therapy and drug administration. In this medical biotechnology framework, gold nanoparticles (AuNPs) research has become popular because of some unique and needed properties, like a high surface area, biocompatibility, and chemical stability, as well as adjustable sizes (10 to 50 nm) [1, 4]. Gold nanoparticles are useful for penetrating biological membranes, increasing cell uptake, and serving as targeted drug carriers. In addition, their selective functionalization with biomolecules enhances their selective modulators of oxidative and inflammatory responses to low-toxic targets [5, 7]. Before, however, there has been much research on drug-induced organ toxicity and it has been determined that these gold nanoparticles possess potent antioxidant properties and therefore, can alleviate organ toxicity [6, 8].

At the same time that there are new advances in nanotechnology, phytotherapy has become a supplemental and preventative practice throughout the globe, with almost 80% of the world population dependent on plant-derived medicines [9]. Citrus fruits such as *Citrus sinensis* (sweet orange) are a plentiful source of phytochemicals of flavonoids, carotenoids and polyphenolic compounds. Their peels, often discarded as agricultural waste, are especially abundant in secondary metabolites such as nobiletin, D-limonene, and polymethoxylated flavones [10, 13]. These bioactive molecules possess well-documented antioxidant, anti-inflammatory, hepatoprotective, and immunomodulatory properties, enabling them to scavenge reactive oxygen species (ROS), inhibit lipid peroxidation, and stabilize cellular structures [14, 15]. Recent *in vivo* studies have demonstrated that citrus peel extracts significantly improve lipid metabolism, enhance enzymatic antioxidant defense (e.g., catalase, superoxide dismutase, and glutathione peroxidase), and reduce oxidative stress biomarkers in animal models [16, 17].

Methotrexate (MTX), a folic acid antagonist used commonly in cancer chemotherapy, is still an essential therapeutic tool in autoimmune diseases, such as rheumatoid arthritis and psoriasis. Nevertheless, the clinical applications of CsA are severely compromised because of its dose-related side effects such as hepatic damage, oxidative stress hyperlipidemia and immune suppression [18, 21]. MTX is thought to be toxic through the increase in

ROS, depletion of intracellular glutathione, mitochondrial dysfunction and the induction of proinflammatory cytokines such as TNF-α and IL-6 [22, 23]. These abnormalities impede hepatic processes, disturb whole body antioxidant status and immune activity. Thus, the need for adjunctive treatments that minimize MTX-induced toxicity and retain therapeutic efficacy is pressing.

This combination of nanotechnology and phytotherapy may offer a better solution for this problem. It has been reported that AuNPs and phytocompounds from the peels of citrus can independently alleviate oxidative stress by lowering MDA levels, suppressing the lipid peroxidation process and augmenting activities of antioxidant enzymes [10, 12, 24]. Furthermore, they both demonstrate immunomodulatory effects through the regulation of cytokine production and immune homeostasis [7, 13, 25]. The combined use of these could have an accumulative protective action against the oxidation and immunological changes caused by MTX, which would enhance the systemic defense meanwhile decrease organ damage.

For this purpose, the present study was planned to investigate both individual and combined role of peel extract of Citrus sinensis (CsPE) and gold nanoparticles (AuNPs) against toxicity induced by methotrexate in male albino rats during protective, antioxidant and immunomodulatory activities. This review attempts to unveil the new trends in combining endogenous antioxidants and nanostructured materials for the enhancement of chemotherapy outcome and amelioration of its side effects. The holistic view of possible synergistic interactions will be provided by the evaluation of biochemical, antioxidant and immunological parameters.

Material and method

Plant Material and Extraction: Local markets provided peels of *Citrus sinensis* L which were then washed three times with tap water followed by distilled water and then air dried. The objective of the washing was to remove dust, pesticides, and the other contaminants. The peels were later ground into a fine powder and the produced powder was stored in polypropylene tubes which were cooled to 4 degrees Celsius until the time of extraction.

The extraction was done using Soxhlet apparatus. To prepare the Soxhlet apparatus, we needed to heat 100 gr of powdered peel with 100 ml of 80% for 24 hours. The ethyl alcohol was then removed with the rotary evaporator which was set at a reduced pressure. The concentrate had to be kept cooled at 4°C until the moment of use. The method provided enough bioactive compounds including but not limited to flavonoids, D-limonene, and D-nobiletin.

Gold Nanoparticles (AuNPs): C Gold nanoparticles (33–40 nm) were purchased from an approved supplier and kept according to the manufacturer´s instructions. The nanosize and dispersions of the particle was verified by DLS and transmission electron microscopy (TEM) to make them uniform and reproducible [5, 6]. Stock solutions were made with sterile distilled water and sonicated before the addition to the samples in order to avoid aggregations.

Animals and Experimental Design: Adult male Wistar rats (n = 60, weight: 190–330 g; age 9 to 11 weeks) were purchased from the central animal facility. Rats were kept under standard laboratory conditions: 25°C,12 h light/dark cycle and relative humidity 50–60% with the access to chow and tap water ad libitum. The animals were acclimatized 7 days prior to beginning the experiment**.** Rats were randomly divided into six groups (n=10 per group) as follows

T1: Saline 0.85% (control)
T2: MTX 10 mg/kg + *C. sinensis* 75 mg/kg
T3: MTX 10 mg/kg + *C. sinensis* 150 mg/kg
T4: MTX 10 mg/kg + *C. sinensis* 250 mg/kg
T5: MTX 10 mg/kg + AuNPs 0.3 mg/kg
T6: MTX 10 mg/kg + AuNPs 0.6 mg/kg

All treatments were administered orally once daily for 14 consecutive days. After 30 days of administration, blood samples were obtained through cardiac puncture under light anesthesia. Serum was obtained by centrifugation at 3000 rpm for 10 min and preserved in –40°C for further investigations [22, 23].

Biochemical Assays: Serum total cholesterol and triacylglycerides levels were determined with the particular routine biochemical kits (Biomaghreb Company) according to manufacturer guideline [22]. All experiments were conducted in triplicate to ensure reproducibility.

Oxidative Stress and Antioxidant Assays: Level of LPO and MDA (as indicators of oxidative stress) were measured with the help of commercial kits (Chin Bioassay Technology).GPx activity was established as an index of the enzymatic antioxidant potential. The assays were performed according to the respective kit instructions and results were presented as a ratio to serum protein [23].

Immunology Assay: The white blood cell (WBC) count and percent of lymphocyte were also detected with hematology analyzer.Concentration of IL-6 was quantified by ELISA (Chin Bioassay Technology) according to the manufacturer's protocol.Duplicate measurements were carried out for each sample to guarantee the accuracy [25].

Statistical Analysis: The data were expressed as mean± SD. One-way ANOVA was performed and pair wise comparison of means was tested using Tukey's post hoc test for multiple comparisons. A P value of ≤ 0.05 was considered to be statistically significant. Least significant difference (L.S.D) values were further computed mentioning minimum detectable difference between treatment means for the respective characters. L.S.D gives a good approximation of the differences among groups and helps understand the meaning of pairwise comparisons at molecular, oxidant/antioxidant and immunological level [26]. All statistical analyses were conducted using SPSS software version 26 (SPSS Inc., Chicago, Illinois).

Results

Treatment with methotrexate (MTX) led to marked changes in biochemical, oxidative and immunological parameters in male Wistar rats. MTX treatment (T2) resulted in significantly hyperlipidemia, oxidative stress status and an immune response as well as increased serum total cholesterol, triacylglycerides, malondialdehyde (MDA), lipid peroxidation (LPO), interleukin-6 (IL-6) concomitant with lower glutathione peroxidase (GPx) activity, total WBC and lymphocyte percentages. Combining the C.

sinensis peel extract and gold nanoparticles (AuNPs) together resulted in a dose-dependent alleviation of these harmful impacts, which is indicative of their hepatoprotective and immunomodulatory effects.

Biochemical Parameters: The treatment with MTX (T2) produced a significantly increase in serum triacylglycerides (99.56 mg/dL) and total cholesterol (142.22 mg/dL) than control group (T1: 67.34 mg/dL and 103.34, respectively). Co-treatment with C. sinensis peel extract or AuNPs reduced lipid levels in a dose dependant manner (T3-T6) and nearly brought the values to normal in the highest treatment groups.

Table 1: Lipid Profile in MTX-Treated Rats with *C. sinensis* Peel Extract and AuNPs (Mean ± SD)

Group	Triacylglycerides (mg/dL)	Total Cholesterol (mg/dL)
T1	67.34 ± 2.41	103.34 ± 3.12
T2	99.56 ± 3.28	142.22 ± 4.15
T3	87.65 ± 2.98	134.34 ± 3.87
T4	77.85 ± 2.54	123.55 ± 3.24
T5	66.86 ± 2.11	98.45 ± 2.89
T6	49.65 ± 1.88	79.76 ± 2.33

L.S.D. Values: Triacylglycerides (2.87 mg/dL); Total Cholesterol (3.91 mg/dL)
Inter-presentation: L.S.D. separation between group means larger than the values is significant at ($P \leq 0.05$). This indicates that MTX significantly increased lipids contents, whereas C. sinensis extract and AuNPs adversely altered LP dose dependently.

Oxidative Stress Markers and Activity of Antioxidant Enzymes: MTX profoundly increased MDA (0.854 µmol/L) and LPO (8.45 µmol/L) levels as well as decreased GPx activity (40.85 µmol/L) levels reflecting heightened oxidative stress and decreased antioxidant defence mechanism. Coadministration with C. sinensis or AuNPs drastically increased GPx activity and decreased the MDA and LPO levels in a dose-dependent manner

Table 2: Oxidative Stress Markers and Antioxidant Enzyme Activity (Mean ± SD)

Group	MDA (µmol/L)	LPO (µmol/L)	GPx (µmol/L)
T1	0.634 ± 0.03	6.43 ± 0.21	65.11 ± 2.44
T2	0.854 ± 0.04	8.45 ± 0.27	40.85 ± 1.97
T3	0.754 ± 0.03	7.54 ± 0.25	45.45 ± 2.11
T4	0.694 ± 0.02	6.76 ± 0.22	49.76 ± 2.18
T5	0.534 ± 0.02	4.87 ± 0.19	56.93 ± 2.09
T6	0.501 ± 0.02	3.43 ± 0.16	61.39 ± 1.87

L.S.D. Values: MDA (0.035 µmol/L); LPO (0.27 µmol/L); GPx (2.15 µmol/L)
Interpretation*:* The much higher values of the group means are significantly different from each other as shown were than L.S.D. MTX-induced oxidative stress could be significantly alleviated by C. sinensis or AuNPs, suggesting potent antioxidant and cytoprotective activities.

Immunological Markers: MTX resulted in significant decrease of total WBC counts (5.67 ×104/µL) and lymphocyte (%) (42.8) and increase levels of IL-6 (35.6 pg/mL), indicating the immune depressed and pro-inflammatory status respectively. WBC count, lymphocyte percentage and IL-6 were increased dose-dependently with the decrement of Co-administration of C. sinensis peel extract or AuNPs restored gradually WBC count, increased lymphocyte percentage and reduced IL-6 in a dose-dependent manner; it is immunoprotective action.

Table 3: Immunological Markers in MTX-Treated Rats (Mean ± SD)

Group	WBC (×10³/µL)	Lymphocytes (%)	IL-6 (pg/mL)
T1	9.15 ± 0.42	60.2 ± 2.11	12.3 ± 0.87
T2	5.67 ± 0.33	42.8 ± 1.98	35.6 ± 1.21
T3	6.85 ± 0.38	48.7 ± 2.05	28.4 ± 1.12
T4	7.45 ± 0.41	52.1 ± 2.14	22.7 ± 1.05
T5	8.21 ± 0.39	57.0 ± 2.07	18.3 ± 0.97
T6	8.89 ± 0.42	59.5 ± 2.12	14.6 ± 0.89

L.S.D. Values: WBC (0.44 ×10³/µL); Lymphocytes (2.19%); IL-6 (1.18 pg/mL)
Interpretation: Differences more than the L.S.D are considered as a significant change for the hematological and inflammatory markers. Phytochemicals and nano-materials both played an important role in alleviated the immune suppression and inflammation caused by MTX.

Summary of Results: Hyperlipidemia, increased oxidative stress and immune dysregulation were demonstrated after MTX exposure in rats. Simultaneous supplementation of C. sinensis peel extract and AuNPs strongly modulated biochemical, oxidative, and immunological relationships in a dose dependent manner suggesting a powerful hepatoprotective, antioxidant and immune stimulatory capabilities. Results Analysis Comparisons L.S.D analysis verified the difference between groups as statistically significant.

Discussion

The current study explored the protective and immunomodulatory potential of Citrus sinensis peel extract and gold nanoparticles (AuNPs) against methotrexate (MTX)-induced biochemical, oxidative, and immunological changes in male Wistar rats. 30, 31 MTX, a very popular folate antagonist for chemotherapy and treatment of autoimmune diseases but with well-documented hepatotoxicity, hyperlipidemia, oxidative stress and immune dysfunction. These present findings consistently argue toward these toxic effects and also understandable mechanism by which there is protection of phytochemicals and nanomaterials.

MTX-Induced Toxicity: MTX treatment (T2) led to marked increase ($p<0.05$) in the serum total cholesterol 142.22, mg/dL and triacylglycerides 99.56, mg/dL when compared with control (103.34, mg/dL and 67.34mg/Dl, respectively; Table T1). These results verify that MTX induced lipid disorder is supported by previous reports on the association of MTX treatment with increased hepatic synthesis of lipids and abnormal metabolism of lipids [17, 21]. The perturbation of lipid homeostasis can be through the biochemical disturbance caused by MTX in accordance with known oxidative stress and mitochondria damage breast hepatocyte injury by MTX.
The markers of oxidative stress namely malondialdehyde (MDA) and lipid peroxidation (LPO) were significantly

higher in MTX-administered rats as compared to the controls with a concomitant decrease in glutathione peroxidase (GPx) activity (Table 2). Our observations are consistent with previous reports showing that MTX overproduces ROS, and cause lipid peroxidation, protein oxidation, mitochondrial injury, and apoptosis in hepatocytes [13, 15, 23, 24]. Moreover, the surplus of used ROS directly destructs subcellular structures as well as enhances pro-inflammatory signaling cascades to amplify systemic toxicity [25, 26]. Immunologically, MTX decreased total WBC and lymphocyte%, and increased IL-6 (Table 3) was observed, which implied immunosuppression and excessive inflammatory response [24, 25].

Protective Effects of *Citrus sinensis* Peel Extract: Co-treatment with C. sinensis peel extract (T3–T4) produced dose-dependent reduction of MTX-induced biochemical and oxidative alterations. The highest dose (250 mg/kg) significantly reinstated the activity of GPx and brought down levels of both MDA and LPO to near normal values (Table 2). The protective effects are related to the presence of a high proportion of bioactive flavonoids, such as nobiletin, polymethoxyflavones, carotenoids (particularly β-carotene), vitamin C and citric acid in orange peel [30, 36]. All of these components do scavenge ROS well and protect cellular membranes and balance redox.

Apart from antioxidative activity, C. sinensis flavonoids exhibit hypolipidemic effects represented by a decrease in the concentrations of total cholesterol and triacylglyceride (T3–T4, Table 1). Flavonoids affect lipid metabolism by inhibiting HMG-CoA reductase, one of the rate limiting steps in cholesterol synthesis and stimulating LDL receptor activity [37, 39]. The above results support the dual antioxidant defense and lipid homeostasis functions of citrus phytochemicals.

Immune: According to the immunological analysis, WBC counts and lymphocyte percentage were increased while IL-6 levels gradually decreased in a dose-dependent manner with C. sinensis extract treatment (Table 3), coinciding with possession of anti-inflammatory and immune modulatory activities. Inhibition of pro-inflammatory cytokine signaling by flavonoids may play a role in the re-establishment of immune homeostasis.

Protective Effects of Gold Nanoparticles (AuNPs): AuNPs (0.3–0.6 mg/kg) exhibited dose-dependent antioxidant, hepatoprotective and immunomodulatory activities (T5–T6). Treatment with AuNPs strongly decreased oxidative stress indexes and the activity of GPx (Table 2). The small size and large surface-to-volume ratio of the nanoparticles increase cellular uptake and enzyme interaction, leading to stabilization of cellular membranes, ROS scavenging and protection against lipid peroxidation [1, 6, 40, 41].

AuNPs also prevented the alteration of lipid profiles by decreasing levels of total cholesterol and triacylglycerides (Table 1), indicating potential amelioration on MTX-induced hepatotoxicity. Immunologically, AuNPs enhanced WBC counts and lymphocyte percentages and suppression of IL-6 (Table 3), indicating the potential for modifying inflammatory response and immune recovery.

Synergy mechanism of C. sinensis and AuNPs: The above phenomena demonstrated that the protective effects of phytochemicals and nanomaterial were synergistic. Citrus flavonoids owe multi-target antioxidant and hypolipidemic effects, and AuNPs increase cellular resistance, enzymic functioning, and immunomodulation. All biochemical, oxidative, and immunological parameters were dose-dependent effects that indicated the possible therapeutic benefit from combining the 2 drugs. Maximal doses resulted in an almost complete normalization of oxidative stress, lipid metabolism and immune endpoints showing remarkable hepatoprotective and immunoprotective synergy.

Clinical Implications: The results provide evidence for the possibility of using C. sinensis peel extract and AuNPs as adjuvants in decreasing chemotherapy-induced systemic toxicity.Through decreasing oxidative stress, reprogramming lipid metabolism, and optimizing immunity, these interventions are likely to mitigate MTX-induced toxicities by improving the safety profile of MTX. Further studies on combination phytochemical/nanomaterial formulations, pharmacokinetics and long-term safety profiles are required.

Summary: In conclusion, MTX-induced hyperlipidemia was remarkably severe in association with strong oxidative stress and immunosuppressive status in rats. C peel extract and AuNPs, which were responsible in providing notable relief against these disorders dose-dependently by their antioxidative, hypolipidemic and immunomodulatory pathways. The biochemical, oxidative and immunological studies provide a glimpse at their protective and synergistic abilities; thereby reflecting their translational utility in adjuvant cancer management.

Conclusion

The current study evaluates the protective and immunomodulatory role of Citrus sinensis (CS) peel extract and gold nanoparticles (AuNPs) on methotrexate (MTX)-induced toxicity in male Wistar albino rats. MTX hepato-toxicity (increased serum cholesterol, triglycerides, oxidative stress indices and immune parameters). In combination therapy of C. sinensis extract and AuNPs, the alterations which have been found were significantly reduced in a call dose manner including lipid profile, oxidative stress indices and immune function. These protective properties are thought to be because of the bioactive molecules in the extract and/or tolerance provided by AuNPs. This combination might serve as an adjuvant for diminishing MTX side effects in chemotherapy.

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