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Immunomodulatory Effect of Carica Papaya and Actinidia Delicosa on Methotrexate Induced Immunosuppression in Male Rats

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Abstract This study was carried out to explore the effects of alcoholic extract of Carica papaya fruit (*C. papya*) and Actinidia delicosa (Kiwi) fruit on some immunological, proinflammatory and anti-inflammatory biomarkers in methotrexate (MTX) treated rats. Forty adult male rats were weighted (130-160gm) and equally allocated into four groups and treated daily for 8weeks with orally as follow: Group G1: (control group) received tap water, G2: rats in this group were injected (20mg/kg /B.W) of MTX in 5thweek, G3: were orally received daily (400 mg/kg /B.W) of Carica papaya fruit alcoholic extract and injected (20mg/kg /B.W) of MTX in 5thweek , G4: were orally received daily (400mg/kg /B.W) of Actinidia delicosa fruit alcoholic extract and were injected (20mg/kg /B.W) of MTX in 5thweek. At the end of experiment, all animals were sacrificed and blood was drawn from median canthus of eye for measuring Serum immunoglobulin M, Serum immunoglobulin G, TNFα, interleukin-10 and C – reactive protein concentrations. Liver tissue collected in liquid nitrogen for Real Time PCR of IL1β and IL6 mRNA expression levels. Spleen tissue collected for histopathological examination and immunohistochemistry of TNFα expression levels. The results of the current study revealed A significant increase in serum serum IgM, IgG and IL10 as well as significant decrease in serum CRP concentrations and TNFα concentrations , mRNA expression levels of IL1β IL6 and immunohistochemical expression of TNFα were return to normal following extract administration comparing to the immune-suppression effect of MTX. Histopathological examination of spleen proved the protective effect of *C.papaya* and Kiwi fruit extract against necrosis and degenerative changes caused by MTX injection. Immunohistochemically, the expression level of TNFα in spleen tissue was decreased in Carica papaya fruit and Actinidia delicosa fruit groups when compared to MTX treated group. Conclusion, alcoholic extract of Carica papaya fruit and Actinidia delicosa fruit compete the side effects of MTX correlated with immune suppressive and pro-inflammatory effect.

Keywords: *methotrexate, tumor necrotic factor alpha, c-reactive protein*

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1. Introduction

Carica papaya (*C. papaya*) belongs to caricaceae family. *C. papaya* is used in ayurvedic medicines from very long time. It is used as anti-inflammatory, antioxidant, diuretic, antibacterial, abortifacient, vermifuge, hypoglycemic, antifungal activity, antihelmenthic and immunomodulatory**.** The ethanolic extract of *C.papaya* fruit inhibiting the isopentenyl pyrophosphate (IPP) induced TNFα production in Lipopolysaccharides LPS induced dendritic cells and protecting DNA damage in lymphocytes [1]. TNFα secreted by monocytes/macrophages has an important role in the pathophysiology of inflammation by initiating other pro-inflammatory cytokines (such as IL-1β, IL-6 and IFNγ). Agents as *C.papaya* fruit extract blocked TNFα action during acute inflammatory conditions [2].

Actinidia deliciosa (Kiwi fruit) is one of the most public fruits worldwide, and it has different biological properties, inclusive antioxidant, anti-allergic, cardiovascular protective effects and immunomodulatory activity [3]. Kiwi fruit increase immune function as it is a good source of protective polyphenols along with high amount of vitamin C [4]. Kiwi fruit claims on maintaining normal immune function in a population group considered to be at risk of immunosuppression (e.g., older adults, individuals exposing to stress, heavy physical exercise, after exposure to ultraviolet radiation and immunosuppressive drugs [5].

Methotrexate (MTX) ($C_{20}H_{22}N_80_5$) is a derivative of aminopterin, an analogue and antimetabolite of folic acid [6]. As known, chemotherapy is widely used for the

treatment of cancer and chronic inflammatory diseases but it causes significant unwanted toxicity and suppression due to oxidative stress incidence [7]. MTX has been clinically applied in a wide range of diseases including systemic lupus erythematosus SLE, rheumatoid arthritis RA, psoriasis, and neoplastic diseases. The therapeutic applications of MTX is usually limited by its immunosuppressive and severe hepatotoxicity [8].

Depending on the available information reveled that natural antioxidant supplementation protected the body against immunosuppressive and inflammatory caused by methotrexate injection causing several histopathological and immunohistochemical. The present study was designed to investigate the protective effect of alcoholic extract of Carica papaya fuit and Actinidia delicosa fruit against different aspects related to liver and spleen damage induced by MTX.

2. Materials and Methods

Preparation of Ethanolic Extract of Carica papaya fruit and Actinidia delicosa fruit according to [9]:

Fresh and ripe fruits were purchased from local market in Ismailia. Samples were washed by fresh water to remove contaminants the samples were shade dried, powdered and extracted with ethanol 99.9 % in cold for a period of 5 days with occasional shaking. The extract was filtered then concentrated by drying in vacuum the resulting concentrated crude extract was used for the experimentation [9].

2.1. Animals and Experimental Design

The duration of experiment was 8weeks from beginning of (papaya and kiwi) administration. Rats were randomly divided into 4 groups (10 each) according to [10]*:* Group (1): Normal control group: they kept on a standard balanced diet all over the experimental period. Group (2): Methotrexate (MTX) injected group: They kept on a standard diet and injected I/P with (20 mg/kg b.wt.MTX) twice weekly from 5th week of experiment till the end of experimental period [11,12]. Group (3): Carica papaya group: They kept on a standard diet and given daily the ethanolic extract of CP fruit at dose 400 mg/kg/day orally for 8weeks [13,14] and injected I/P with (20 mg/kg b.wt.MTX) twice weekly from 5th week of experiment. till the end of experimental period. Group (4): Actinidia delicosa group: they kept on a standard diet and given daily the ethanolic extract of Actinidia delicosa (kiwi) fruit at dose 400 mg/kg/day orally for 8weeks [15] and injected I/P with (20 mg/kg b.wt.MTX) twice weekly from 5th week of experiment till the end of experimental period.

Blood samples were collected at end of experiment; blood was drawn median canthus of eye technique from anesthetized rats. Then serum samples were separated and frozen at 20°C until analysis of; Serum immunoglobulin M according to the methods of [16], Serum immunoglobulin G according to the methods of [17]. Using RAT IgG ELISA Kits Catalog No. MBS2513365, TNFα (TNF-α ELISA Kit Catalog No. MBS2507393 according to [18]. interleukin-10 (IL-10) ELISA Kit (Catalog no. MBS175998). according to [19]. and C – reactive protein (CRP) Rat C-Reactive Protein ELISA Catalog Number: 557825 for rat CRP according to [20] concentrations were also recorded. Liver tissue collected in liquid nitrogen for Real Time PCR of IL1β and IL6 expression as in Table 1 according to [21]. Spleen tissue collected for histopathological examination and immunohistochemistry of TNFα expression and photomicrographic images for histopathological and immunohistochemical evaluations were taken using Olympus BX41 research optical photomicroscope fitted with an Olympus DP25 digital camera.

2.2. Statistical Analysis

Analysis was done using Statistical Package for Social Sciences version 22.0 (SPSS, IBM Corp., Armonk, NY, USA) and one-way analysis of variance (ANOVA), followed by Tukey's Honestly Significant Difference (Tukey's HSD) test as post hoc test was used.

Table 1. Primers sequences used for real time PCR

Gene	Primers	*3' to 5'* Primer sequence	Reference	size
IL1β	forward	GGATGGTGGAGCAAGGG	Cao et al., 2010	190
	Reverse	GCACTGCTTCCCAGGCTT		
IL6	forward	TGTATGAACAGCGATGATG	Shirpoor et al., 2013	186
	Reverse	AGAAGACCAGAGCAGATT		
β-actin	forward	CGTTGACATCCGTAAAGACC	El-Messaoudi *et al*., 2010	176
	Reverse	TAGAGCCACCAATCCACACA		

Table 2. Effect of ethanolic extract of *Carica papaya fruit and Actinidia delicosa fruit* on serum TNFα, IL10, IgM, IgG and CRP levels against high dose of methotrexate

Parameter	Group			
	G	G2	G3	G4
TNF (pg/ml)	25.12±0.54 ^{c}	54.65±0.61 ^{a}	29.19±0.39 ^{b}	24.49±0.39 ^{c}
IL10 (pg/ml)	83.37±0.89 ^{a}	70.24±3.18 ^{b}	82.98±1.01 ^{a}	89.98±3.88 ^{a}
IgM (mg/ml)	4.51±0.11 ^{a}	2.58±0.17 ^{b}	4.4±0.18 ^{a}	4.14±0.34 ^{a}
IgG (mg/ml)	4.19±0.21 ^{a}	2.32±0.17 ^{b}	4.09±0.05 ^{a}	4.01±0.16 ^{a}
CRP (ng/ml)	4.17±0.34 ^{bc}	6.94±0.19 ^{a}	4.5±0.19 ^{b}	3.66±0.23 ^{c}

Values (mean ± SE; n = 10) of different variables within the same raw having different superscripts are significantly different (P < 0.05). *vs*.. **G1:** Control; **G2:** *MTX* (20mg/kg B.W); **G3:** papaya +MTX (400mg/kg B.W) + 20mg/kg B.W); **G4: kiwi** + MTX (400mg/kg B.W) + 20mg/kg B.W.

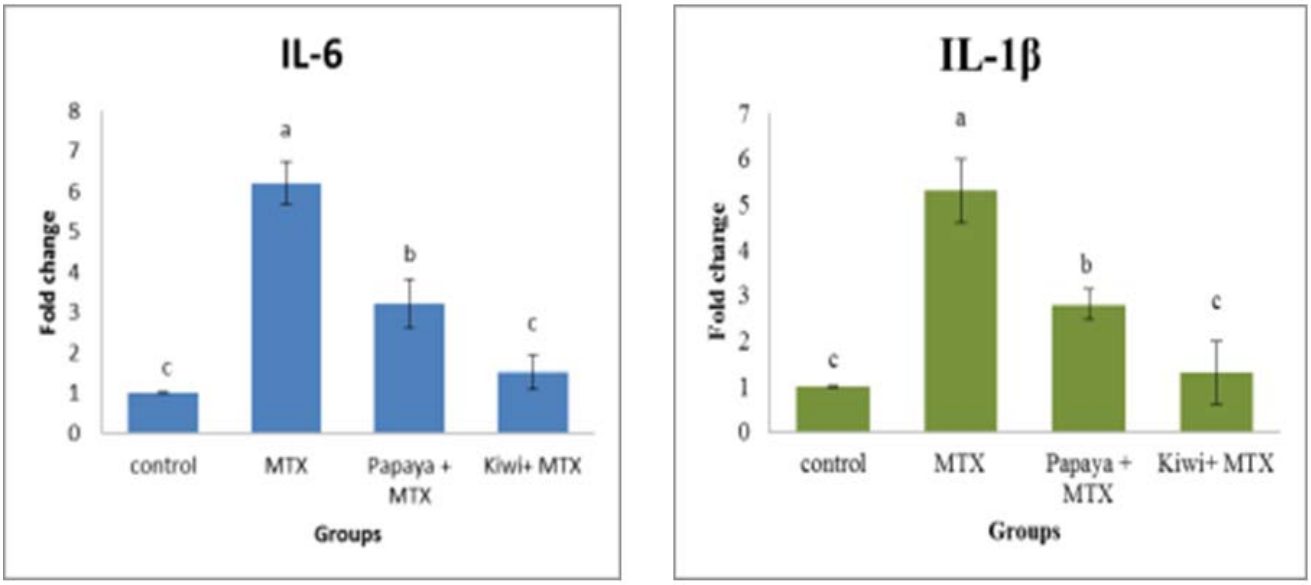

Figure 1. Effect of ethanolic extract of *Carica papaya fruit and Actinidia delicosa fruit* on expression of IL6 and IL1β in liver tissue of rats in different groups

3. Results

The results in Table 2 showed that oral administration of Ethanolic Extract of carica papaya fruit and actinidia delicosa fruit (G3)(G4) with MTX injection caused significant elevation (P≤ 0.05) in serum IgM and IgG, IL10 and significant decrease (P<0.05) in serum TNF concentration comparing P<0.05to the value in MTX (group G2) treated groups. A statistical analysis indicated that the mean values in (Table 2). The results in Figure 1 showed that oral administration of ethanolic extract of Carica papaya fruit and Actinidia delicosa fruit (G3) (G4) with MTX injection caused decrease in IL6 and IL1β expression.

4. Discussion

4.1. Effect of Ethanolic Extract of Carica Papaya Fruit and Actinidia Delicosa Fruit on Serum Concentration of TNF-α

In this study it was indicated that treatment with alcoholic extract of Carica papaya fruit and Actinidia delicosa fruit daily before MTX injection caused a significant decrease in TNFα in G3 and G4 and increased TNFα in G2. Such results proved the protective effect of Carica papaya fruit and Actinidia delicosa and thus supported the usage of these fruits for preventing MTX deleterious effect [22]. As constituents with their antioxidant properties overcame the increase in TNFα concentration by preventing oxidative stress [23]. An ethanolic papaya extract displayed significant inhibition of isopentenyl pyrophosphate (IPP) induced TNFα production in LPS (induced dendritic cells. In addition, the same extract also imparted an antioxidant effect by protecting DNA damage in lymphocytes [24]. Quercetin in Kiwi fruit extract exerted protective effect against inflammation in skin through increasing cytokine secretion [25].

4.2. Effect of Ethanolic Extract of Carica Papaya Fruit and Actinidia Delicosa Fruit on Serum Concentration of IgM and IgG

Methotrexate injected rats (group 2) showed a significant decrease (p<0.05) in IgM and IgG levels if compared with control rats (group 1). We explained our results as MTX caused depletion of tetrahydrofolate causes cell death by suppressing DNA and RNA production [26]. Also, reduction in the level of both purine and pyridine pools in primary T cells and reduced levels of ATP and GTP while increasing levels of UTP inducing reduction in T cell proliferation and increase apoptosis and inhibition of lymphocyte multiplication so make immune depression. These results agreed with [27] MTX-injected rats had bone marrow suppression, leukopenia, and so immunosuppression. The immunosuppressive effect activity of MTX through their action on spleen and lymphocytic inactivation. Our results were also, in agreement with [28] who proved that IgM and IgGconcentrations in children during chemotherapy with MTX cause potentiation of pathological lesions in the spleen tissue that cause decrease in transitional B cells and significantly lower serum immunoglobulin levels.

Our results showed that *C.papaya* and Kiwi fruit extract could increase level of IgG and IgM in group 3 and 4. The ripe transgenic papaya fruit significantly enhanced humoral immunity by increasing serum total IgM level (2062 vs. 1583 µg/ml in control group [29]. The Papaya fruit extract also significantly enhanced immunoglobulin IgG and IgM levels (from 0.120 → 0.132 and 0.892 → 0.108 mg/ml, respectively [24]. Kiwi fruit contains high level of vitamin C and strong antioxidant compounds such as carotenoids, lutein, phenolics, flavonoids and chlorophyll. It is commonly reported to be also a rich source of vitamin E fructose, galactose, minerals and polysaccharides. Recent studies have shown that kiwi fruit has antioxidant activity in vivo and vitro with immune stimulatory activity [30].

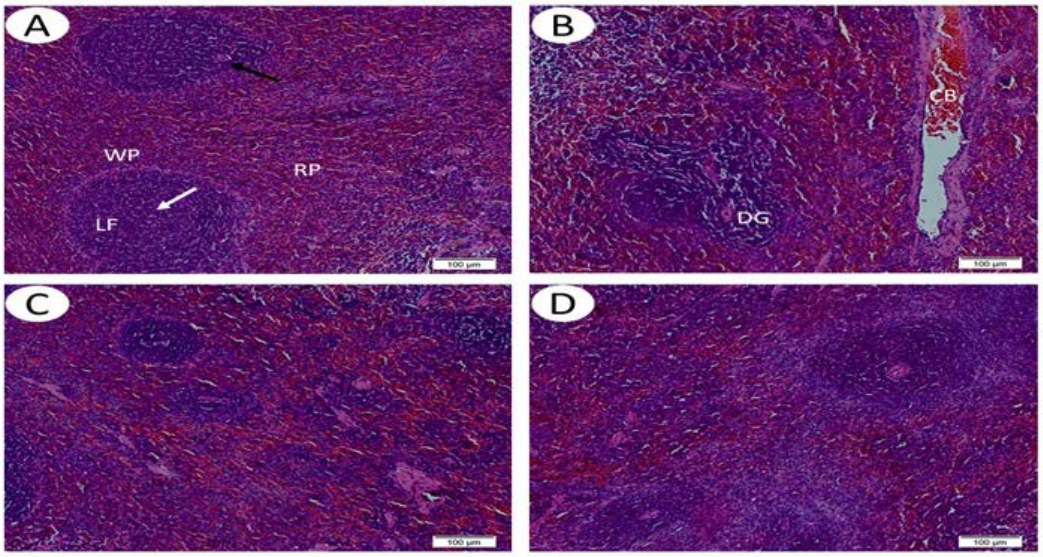

Figure 2. Histopathological examination of the spleen tissue in different groups. A representative photomicrograph of splenic sections. Control group (A): Methotrexate-treated group (B): Papaya group (C):. kiwi group (D): White pulp (WP), lymphatic follicle (LF), red pulp (RP), germinal center (white arrow), nodular artery (black arrow), degenerated germinal center (DG) and congested blood vessels (CB). H&E

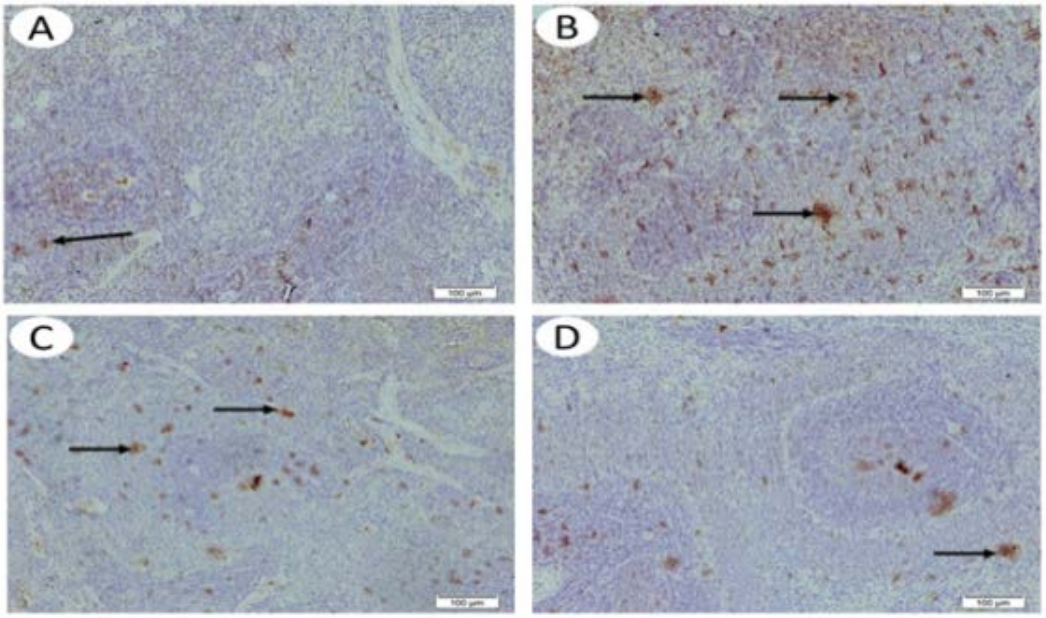

Figure 3. Immunohistochemistery (IHC) for Evaluation of TNF-α expression on spleen tissue. Immunohistochemical analysis of TNF-α expressions in spleens of untreated control group (A), methotrexate-treated group (B), papaya-treated group (C), kiwifruit-treated group (D). High expression of TNF-α was seen in the methotrexate-treated group (strong staining, 111). Meanwhile, the lowest expression of TNF-α was found in group treated with methotrexate and kiwifruit (weak staining, 1). TNF-α expression was indicated with black arrows. Stained by DAB -chromogen (Brown color) immunostaining.

4.3. Effect of Ethanolic Extract of *C. papaya* Fruit and Actinidia Delicosa fruit on Serum Concentration of IL10 and CRP

Our results showed an increase in CRP level and decrease in IL10 level in MTX- treated groups while in *C.papaya* and kiwi treated groups there was decrease in CRP and increasein IL10 level

[31] found that MTX-induced toxicity was associated with the activation of the systemic inflammatory response and proinflammatory cytokines. The acute inflammation markers TNF-α and CRP also significantly increased in MTX administrated group.

MTX generates oxidative stress by increasing ROS production causing tissue injury, which may be the main cause of its drawbacks [32,33]

4.4. Effect of Ethanolic Extract of *C. papaya* Fruit and Actinidia Delicosa Fruit on Immunohistochemistery of TNF α and Histopathological Changes in Spleen Tissue

In our Immunohistochemical examination revealed that increased expressions of spleen tissue tumor necrosis factor alpha (TNF-a) in group 2 and this evidence

suggested that oxidative stress caused by MTX can induce immunosuppression and spleen toxicity. And this in agreement with [11] who proved that Immunohistochemical examination revealed that increased expressions of TNF-α suggested the oxidative stress caused by MTX.

The harmful effect of MTX treatment was reflected by the increase in TNF-α level, which is a pro-inflammatory cytokine. The increase in TNF-α secretion is due excessive ROS formation, which leads to neutrophil infiltration and the release of pro-inflammatory cytokine triggering apoptosis, cell damage [34].

Our histopathological results of spleen tissue found that White pulp consisting of lymphatic follicles while the red pulp consisting of blood sinusoids and blood elements. Sever symptoms of congestion of the red pulp, hypocellularity and splenic parenchymal degeneration were seen in the spleens of the methotrexate-treated animals. Lymphocytic depletion, causing a complete loss of demarcation between red and white pulps as well as leading to a reduction in the density of white pulp follicles was also recorded. All these abnormal changes in spleen decreased in *C.papaya* and Kiwi treated groups as they contain high amounts of phenolics, flavonoids and vit. C which had antioxidant and immune protection to spleenocytes. These results agreed with [35] who said that kiwi fruit extract activate spleen to modulate both innate and acquired immunity in a beneficial manner.

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