Anti-inflammatory Effects of *Hypericum triquetrifolium* Turra Methanol Extract in Albino Male Mice

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**Abstract**

**Background:** Herbal medicines containing *H. triquetrifolium* which have been used in traditional Arab herbal medicine to treat various diseases; however, only few studies have been conducted to evaluate the effects of plant on inflammation, but the probable anti-inflammatory effect of *H. triquetrifolium* in a rat model of carrageenan induced inflammation was explored.

**Methods:** Total and absolute counts of leukocytes was determined in albino males mice after interactions between the plant extract and cyclophosphamide, in addition to assessment of anti-inflammatory activity in a mouse model of carrageenan induced inflammation.

**Results:** Compared to vehicle groups, the extract was able to normalize total and absolute counts of leukocytes and had strong anti-inflammatory activity. Conclusion: The plant extract can be considered as an immune modulator agent with anti-inflammatory activity.

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1. Introduction

Herbal medicine is the oldest form of health care known to mankind, and medicinal plants had been used by all cultures throughout the history. It represents an integral part of modern civilization development (Fuemmeler et al., 2009). Scientific investigations confirmed these medicinal potentials, and presented *in vitro* and *in vivo* evidences that medicinal plants or their secondary metabolites have shown different biological effects with a wide range of pharmacological properties; for instance, immune stimulator, anti-bacterial, anti-viral, anti-inflammatory, anti-oxidant, anti-mutagenic, anti-cancer, hepatoprotective, and many other properties (Butler and Newman, 2008; Mike et al., 2010). Modulation of immune response by using medicinal plant products has become a subject of scientific investigations, and medicinal plants are commonly used for the treatment of various ailments and are considered by many, to have advantages over the conventionally used medicines, which are known to have harmful side effects. The immune system plays a vital role in the protection against various infections, and its integrity and efficiency is important during the treatment of many diseases (Arokiyaraj et al., 2009).

Among these plants are species of the genus *Hypericum*, which belongs to the family Clusiaceae and includes about 450 species of trees, shrubs and herbs (Robson, 2006). Many species of the *Hypericum* genus have a long traditional value as medicinal plants. *Hypericum triquetrifolium* Turra is one of these species, which has received a considerable scientific interest in recent years, because it is a rich source of a variety of bioactive compounds that lend *H. triquetrifolium* to be a medicinal plant with a wide range of medicinal applications (Aleisa, 2008; Rouis et al., 2013). The
plant is distributed as native in Europe (Albania, Cyprus, France, Greece, Italy and Former Yugoslavia), Asia (Cypress, Egypt, Iran, Iraq, Palestine, Jordan, Lebanon, Syria and Turkey), and Africa (Algeria, Libya). It is also naturalized in Australia. The plant has been traditionally used for its sedative, anti-emetic, anti-inflammatory and anti-septic effects (Rousi et al., 2013). In addition, several studies have reported the potential use of its crude extracts as therapeutic substances; for instance, treatment of burns and gastroenteritis, as well as its anti-inflammatory and anti-oxidant potentials have been suggested (Saad et al., 2011; Özkan and Mat, 2013). Accordingly, the present investigation aimed to assess the immunological effects of the plant methanol extract in albino male mice through total (TLC) and absolute counts of leukocytes and anti-inflammatory activity.

2. Materials

2.1 Preparation of plant extract and doses:
The aerial parts of *H. triquetrifolium* (leaves) were supplied as powdered dried material. It was collected from mountain regions in Tasloga (Sulaymaniyah); a city 330 Km north the capital Baghdad. Methanol extract of *H. triquetrifolium* was prepared according to Fua et al., (2010). Briefly, 50 grams of the plant powder were extracted with 80% methanol (250 ml) at 65°C for 3 hours using the soxhlet apparatus. The extract solution was concentrated to dryness under reduced pressure in a rotary evaporator to yield dried crude extract, which was frozen at -20°C until use to prepare the required doses. Three doses of the extract were tested (50, 100 or 200 mg/kg) in albino male mice as suggested by Kumar et al. (2010).

2.2 Experimental Design

To assess the immunological effects (total and absolute counts of Leukocytes) of these three doses, the mice were distributed into six groups, each of four animals (total: 24 mice). In Groups I and II, mice were administrated with physiological saline (Blank and Vehicle controls). Group III mice were administrated with vitamin C at a dose of 120 mg/kg (Positive controls).

Groups IV, V and VI Mice were administrated with the first, second and third doses (50, 100 and 200 mg/kg, respectively) of *H. triquetrifolium* methanol extract. The tested materials were injected intraperitoneally (IP) as a single dose (0.1 ml) per a day and for 10 days. Each mouse of groups II - VI was IP injected with 0.1 ml of CYP (80 mg/kg) on days 4 - 6. At day 11, the mice were sacrificed for laboratory assessments (Wang et al., 2012).

2.3 Total Count of Leukocytes

Blood samples were collected by heart puncture using a disposable insulin syringe (1 ml). The method of Sood (1986) was followed, in which the conventional procedure of total and absolute counts of leukocytes were determined.

2.4 Anti-inflammatory Activity

Five different groups of further male mice (total number = 20 animals) were investigated in assessing the anti-inflammatory activity of *H. triquetrifolium* methanol extract, but with similar doses (50, 100 and 200 mg/kg). The first three groups (each of four mice) were IP injected with 0.1 of the doses 50, 100 and 200 mg/kg, respectively, while the fourth group was similarly injected but with physiological saline (vehicle control).

A fifth group was IP injected with 0.1 ml of indomethacin (10 mg/kg; a non-steroidal anti-inflammatory drug), and considered as a positive control. Sixty minutes later, the paw edema was induced by injecting 0.1 ml of 1% carrageenan into the subplantar tissues of the right hind paw of each mouse. The paw edema was measured (millimeter; mm) with vernier before and 1, 2, 4 and 6 hours after carrageenan injection, and difference between before and after was recorded (Tunalier et al., 2007).

2.5 Statistical Analysis

Data were given as mean ± SD (standard deviation), and significant differences between means were assessed by ANOVA (analysis of variance) followed by Duncan test. The analyses were carried out by using the statistical package for social sciences (SPSS) version 13.

3. Results

3.1 Total Count of Leukocytes

Total leukocyte count in blank control mouse was 7550 ± 665 cells/ cu. mm. blood, but such count was significantly decreased to 2650 ± 550 cells/ cu. mm. blood after CYP treatment (vehicle controls), and the reduction was 64.9%. Treating mice with vitamin C or the first dose of methanol extract (50 mg/kg) was associated with a significant recovery of TLC, which reached 6950 ± 929 and 6800 ± 909 cells/cu.mm.blood, respectively, with approximated percentages of reduction 7.9 and 9.9%, respectively. A better TLC recovery was observed at the dose 100 mg/kg of methanol extract, and it was almost approximated the blank control count (7450 ± 718 vs. 7550 ± 665 cells/cu.mm.blood). However, the dose 200 mg/kg was able to increase the count to 8900 ± 621 cells/cu. mm. blood, which exceeded the blank control count by 17.9%. (Table 1).
3.2 Absolute Count of Lymphocytes
Mice treated with CYP (vehicle controls) showed a significant decreased absolute count of lymphocytes compared to blank controls (566 ± 174 vs. 4641 ± 469 cells/cu.mm.blood), and the reduction was 87.8%. Vitamin C and the first two doses of *H. triquestrifolium* methanol extract (50 and 100 mg/kg) exceeded the blank control count by 6.1, 15.2 and 34.3%, respectively, but without a significant difference. Whereas the dose 200 mg/kg of extract increased the absolute lymphocyte count significantly to 7027 ± 480 cells/cu.mm.blood, and represented 51.4% increase of blank control count. (Table 2)

3.3. Absolute Count of Neutrophils
The absolute count of neutrophils in blank control mice was 2435 ± 273 cells/cu.mm.blood, but when mice treated with CYP, a 66.0% reduction was observed, which was significant. Vitamin C was able to minimize the reduction to 38.9%, but a less reduction was observed at the 50 and 100 mg/kg doses of methanol extract (58.1 and 46.8%, respectively). However, the highest reduction (17.0%) was reached at the dose 200 mg/kg of extract (2003 ± 355 cells/cu.mm. blood), but it was significantly lower than the blank control count (Table 3).

3.4. Absolute Count of Monocytes
Treatment with CYP caused a significant reduction (82.7%) in monocyte count, but vitamin C and the third dose (200 mg/kg) of *H. triquestrifolium* methanol extract were able to counter such effect and increased the count significantly to 513 ± 128 and 580 ± 227 cells/cu.mm.blood, respectively compared to the count in vehicle mice (82 ± 47 cells/cu.mm.blood). (Table 4)

3.5. Anti-inflammatory Activity
The result of anti-inflammatory activity showed that the plant extract has different inhibition percentages, which were dependent on dose, as well as time of edema post-induction. The best percentage of inhibition was observed four hour post-induction at the dose 200 mg/kg (50.0%), followed by the same dose but six hours post-induction (45.8%). For indomethacin, a maximum inhibition of 33.3% was observed at the first and sixth hour post-induction. (Table 5)

**Discussion**
Analysis of blood parameters is relevant to risk evaluation and the changes in the hematological system have a higher predictive value for human toxicity, when the data are translated from animal studies (John et al., 2012). The assessment of hematological parameters could be used to reveal the protective or deleterious effects of foreign compounds including plant extract on the blood cellular constituents of animals (Li et al., 2014).

The results declared the ability of *H. triquestrifolium* methanol extract to counteract the reducing effects of CYP and restoring the normal counts of leukocytes or their subsets (lymphocytes, neutrophils and monocytes). Such modulation could be attributed to the presence of phenol compounds (i.e. flavonoids), which have the ability to enhance immune system functions and increase the number of blood cells *in vivo* (John et al., 2012). The significant increase in leukocytes and their absolute counts in the tested animals suggest that the extract may have immunological properties, by stimulating the production of these cells; thereby boosting the defense mechanisms of the animals. (Konaté et al., 2012).

It is generally accepted that monocytes and granulocytes, and humoral elements, like lysozyme, agglutinin and metal ion binding proteins are the main components of the non-specific immune system (Ardo et al., 2008). There are many studies reporting that herbal medicine extracts can be used as immuno-stimulants to enhance non-specific immune system (Sahu et al., 2007),

**Table 1:** Effect of *H. triquestrifolium* methanol extract and vitamin C on total count of leukocytes in cyclophosphamide-treated albino male mice

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose (mg/kg)</th>
<th>Mean ± SD (cells/cu.mm.blood)</th>
<th>Percentage of Blank Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (Blank controls)</td>
<td>-</td>
<td>7550 ± 665&lt;sup&gt;h&lt;/sup&gt;</td>
<td>0.0</td>
</tr>
<tr>
<td>II (Vehicle controls)</td>
<td>80</td>
<td>2650 ± 550&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-64.9</td>
</tr>
<tr>
<td>III (Vitamin C)</td>
<td>120</td>
<td>6950 ± 929&lt;sup&gt;h&lt;/sup&gt;</td>
<td>-7.9</td>
</tr>
<tr>
<td><em>H. triquestrifolium</em> Methanol</td>
<td>IV 50</td>
<td>6800 ± 909&lt;sup&gt;h&lt;/sup&gt;</td>
<td>-9.9</td>
</tr>
<tr>
<td>Extract</td>
<td>V 100</td>
<td>7450 ± 718&lt;sup&gt;h&lt;/sup&gt;</td>
<td>-1.3</td>
</tr>
<tr>
<td></td>
<td>VI 200</td>
<td>8900 ± 621&lt;sup&gt;a&lt;/sup&gt;</td>
<td>+17.9</td>
</tr>
</tbody>
</table>

Different letters: Significant difference (P ≤ 0.05) between means.
Table 2: Effect of *H. triquetrifolium* methanol extract and vitamin C on absolute count of lymphocytes in cyclophosphamide-treated albino male mice.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose (mg/kg)</th>
<th>Mean ± SD (cells/cu.mm.blood)</th>
<th>Percentage of Blank Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (Blank controls)</td>
<td>-</td>
<td>4641 ± 469&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.0</td>
</tr>
<tr>
<td>II (Vehicle controls)</td>
<td>80</td>
<td>566 ± 174&lt;sup&gt;A&lt;/sup&gt;</td>
<td>-87.8</td>
</tr>
<tr>
<td>III (Vitamin C)</td>
<td>120</td>
<td>4925 ±1097&lt;sup&gt;B&lt;/sup&gt;</td>
<td>+6.1</td>
</tr>
<tr>
<td><em>H. triquetrifolium</em> Methanol IV</td>
<td>50</td>
<td>5345 ± 441&lt;sup&gt;B&lt;/sup&gt;</td>
<td>+15.2</td>
</tr>
<tr>
<td>Extract VI</td>
<td>200</td>
<td>7027 ± 480&lt;sup&gt;A&lt;/sup&gt;</td>
<td>+51.4</td>
</tr>
</tbody>
</table>

Different letters: Significant difference (P ≤ 0.05) between means.

Table 3: Effect of *H. triquetrifolium* methanol extract and vitamin C on absolute count of neutrophils in cyclophosphamide-treated albino male mice.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose (mg/kg)</th>
<th>Mean ± SD (cells/cu.mm.blood)</th>
<th>Percentage of Blank Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (Blank controls)</td>
<td>-</td>
<td>2435 ± 273&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.0</td>
</tr>
<tr>
<td>II (Vehicle controls)</td>
<td>80</td>
<td>828 ± 256&lt;sup&gt;A&lt;/sup&gt;</td>
<td>-66.0</td>
</tr>
<tr>
<td>III (Vitamin C)</td>
<td>120</td>
<td>1488 ± 251&lt;sup&gt;B&lt;/sup&gt;</td>
<td>-38.9</td>
</tr>
<tr>
<td><em>H. triquetrifolium</em> Methanol IV</td>
<td>50</td>
<td>1020 ± 324&lt;sup&gt;DE&lt;/sup&gt;</td>
<td>-58.1</td>
</tr>
<tr>
<td>Extract VI</td>
<td>200</td>
<td>2003 ± 355&lt;sup&gt;B&lt;/sup&gt;</td>
<td>-17.7</td>
</tr>
</tbody>
</table>

Different letters: Significant difference (P ≤ 0.05) between means.

Table 4: Effect of *H. triquetrifolium* methanol extract and vitamin C on absolute count of monocytes in cyclophosphamide-treated albino male mice.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose (mg/kg)</th>
<th>Mean ± SD (cells/cu.mm.blood)</th>
<th>Percentage of Blank Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (Blank controls)</td>
<td>-</td>
<td>474 ± 194&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.0</td>
</tr>
<tr>
<td>II (Vehicle controls)</td>
<td>80</td>
<td>82 ± 47&lt;sup&gt;B&lt;/sup&gt;</td>
<td>-82.7</td>
</tr>
<tr>
<td>III (Vitamin C)</td>
<td>120</td>
<td>513 ± 128&lt;sup&gt;B&lt;/sup&gt;</td>
<td>+8.2</td>
</tr>
<tr>
<td><em>H. triquetrifolium</em> Methanol IV</td>
<td>50</td>
<td>413 ± 202&lt;sup&gt;A&lt;/sup&gt;</td>
<td>-12.9</td>
</tr>
<tr>
<td>Extract VI</td>
<td>200</td>
<td>580 ± 227&lt;sup&gt;B&lt;/sup&gt;</td>
<td>+22.4</td>
</tr>
</tbody>
</table>

Different letters: Significant difference (P ≤ 0.05) between means.

Table 5: Anti-inflammatory activity of *H. triquetrifolium* methanol extract and indomethacin in albino male mice.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose (mg/kg)</th>
<th>Foot-pad Thickness (Mean ± SD; mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>One Hour</td>
<td>Two Hours</td>
</tr>
<tr>
<td><em>H. triquetrifolium</em> Methanol I</td>
<td>50</td>
<td>2.2±0.1&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>II</td>
<td>100</td>
<td>2.5±0.4&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>III</td>
<td>200</td>
<td>2.1±0.1&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>IV (Vehicle)</td>
<td>-</td>
<td>1.8±0.2&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>V (Indomethacin)</td>
<td>10</td>
<td>2.4±0.3&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Different letters: Significant difference (P ≤ 0.05) between means of columns.

and the presented results might be due to the enhancement of the non-specific immune system by the plant extract (Mohamad and Abasali, 2010). The blood level of TLC showed a significant change after administration of CYP when compared to control group, and such changes suggest that the immune system was compromised. However, *H. triquetrifolium* at all tested doses produced significantly increased blood cell counts of leukocytes, especially lymphocyte and monocyte at the dose 200mg/kg of the extract when compared to control group. This could be due to the presence of some active phytochemicals in the extract which confers the extract with immunostimulatory property as evident by significantly elevated lymphocyte count (Catoni et al., 2008). Increase in the number of lymphocytes is a measure of the strength of the immune system; thus, foreign or invading organisms can easily be identified and destroyed (Fidan et al., 2008). Chemical analysis of *H. triquetrifolium* showed that it is a source of a variety of biologically active compounds including phenolics like flavonoids (Cirak et al., 2011), and flavonoids can display a huge array of biochemical and pharmacological effects that affect the function of the immune system and inflammatory processes (Costantini and Dell’Omo 2006; Genestet et al., 2014).
Traditional use of *Hypericum* products includes wound healing and resolving infection, which could result from potential anti-inflammatory properties (Hammer and Birt, 2014). These anti-inflammatory properties of *Hypericum* are due to its compounds: hypericin, adhyperforin, amentoflavone, hyperoside, isoquercitrin, hyperforin and rutin (Sosa et al., 2007; Wolff et al., 2014).

Many plants containing flavonoids have also shown to have diuretic, laxative, antispasmodic, anti-hypertensive, and anti-inflammatory actions (Rajan et al., 2012). Flavonoids are well known for their anti-inflammatory properties due to their inhibitory effects on enzymes involved in the production of the chemical mediator of inflammation (Jothimanivannan et al., 2010; Hossain et al., 2011).

Carrageenan-induced edema has been commonly used as an experimental animal model for acute inflammation and is believed to be biphasic (Perazzo et al., 2008). The early phase (1-2 hour) of the carrageenan model is mainly mediated by histamine, serotonin, and increased synthesis of prostaglandins (PGs) in the damaged tissue surroundings. The late phase is sustained by PG release and mediated by bradykinin, leukotrienes, polymorphonuclear cells, and prostaglandins produced by tissue macrophages. The presence of PGs in the inflammatory exudates from the injected foot can be demonstrated at three hours and periods thereafter (Agrawal, 2011). Non-steroidal anti-inflammatory agents are known to inhibit cyclooxygenase (COX-2) enzymes involved in PG synthesis (Bankhle, 2001). Based on those reports it is possible that the inhibitory effect of the methanol extract of *H. triquetrifolium* on carrageenan-induced inflammation in mice could be due to inhibition of the enzyme cyclooxygenase leading to inhibition of PG synthesis. Significant inhibition of paw edema in the first hours could be attributed to the inhibition of histamine and/or serotonin release (Rahman et al., 2011; Dey et al., 2014). Since the extract significantly inhibited paw edema induced by carrageenan in the second phase, this finding suggests a possible inhibition of cyclooxygenase synthesis by the extract and this effect is similar to that produced by nonsteroidal anti-inflammatory drugs such as indomethacin, whose mechanism of action is inhibition of the cyclooxygenase enzyme (Perianayagam et al., 2006).

Another compound found in many *Hypericum* species is hyperforin, which is the major lipophilic compound that has been shown to be able to inhibit 5-lipoxygenase (5-LO) formation and suppressed products with equal potency to the well-documented 5-LO inhibitor zileuton (Koeberle et al., 2011). Hyperforin induced significant inhibition of various ion channels (such as P-type Ca²⁺ channels) via interaction with calmodulin or through calmodulin-activated pathways involving at least one second messenger and inhibit paw oedema (Krishnal et al., 2001).

The ability of flavonoids to inhibit eicosanoid biosynthesis has also been documented. Eicosanoids, such as PGs, are involved in various immunological responses and are the end products of the cyclooxygenase and lipoxygenase pathways (Rathee et al., 2009). These enzymes play an important role as inflammatory mediators. They are involved in the release of arachidonic acid, which is a starting point for a general inflammatory response. Phenolic compound were shown to inhibit the cyclooxygenase and lipoxygenase pathways (Lafuente et al., 2009). Furthermore, flavonoids are able to inhibit neutrophil degranulation and thereby decrease the release of arachidonic acid. Another anti-inflammatory fetchers is that, flavonoids inhibit both cytosolic and tyrosine kinase (Bellik et al., 2013). *H. triquetrifolium* significantly inhibited the edema induced by carrageen in mice and this may be due to the mechanisms explained above, and shared the effects of indomethacin, which has been shown to produce its anti-inflammatory effect by inhibiting the enzyme, cyclooxygenase; thus inhibiting PG synthesis (Chopade et al., 2014). It has also been shown that the nonsteroidal anti-inflammatory drugs may antagonize mediators such as serotonin, bradykinin, and capsaicin, some of which have been implicated in carrageenan-induced paw oedema (Amabeoku and Kabatende, 2012).

**Conclusion**

Based on the results, the methanol extract of *H. triquetrifolium* may have the potential of immune enhancement and anti-inflammatory activity, but further investigations are certainly required to establish such potentials.

**References**


