IN VIVO EFFICACY OF MYRTUS COMMUNIS AQUEOUS
LEAVES EXTRACT AGAINST METACESTODE OF
ECHINOCOCCUS GRANULOSUS

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ABSTRACT

Hydatidosis is an endemic disease of human and animals which is difficult to treat. Experimental studies indicated that Myrtus communis leaves had an efficiency against some bacterial and parasitic infections. The study aimed to evaluate the in vivo efficiency of M. communis aqueous leaves extract against hydatidosis. A total of 40 white albino mice were divided into 4 equal groups. Each mouse in the first, second, and third groups were injected intraperitoneally (IP) with 2000 protoscolices, whereas the forth group was left as non-infected control. Four months post infection, mice in the second and third group treated with 6 and 12mg of M. communis aqueous leaves extract respectively in every other day for one week. Mice in the first group were untreated and considered as infected control group. Morphological and histopathological changes in cysts and infected organs were studied. Enzyme activities of adenosine deaminase (ADA) and alkaline phosphatase (ALP) were recorded in each mouse. Mice from treated groups with either concentration had lower number and diameter of cysts and higher cyst reduction percentage than infected control group. Histological sections of the liver from mice treated with either concentration showed hepatocytosis, aggregation of inflammatory cells, intensive hyalinization which surrounds the hepatocytes and degeneration of some hepatocytes. An elevation of ADA and dropping in ALP activities were recorded in treated mice especially with 12 mg as compared with non-infected control group.

Key words: Hydatid cyst, Echinococcus, protoscolices, Myrtus communis leaves

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كفاءة المستخلص المائي لأوراق نبات آلوس في علاج Myrtus communis

الإصابة بالأكياس المائية: دراسة في الجسم الحي

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الخلاصة

بدأ الأكياس المائية من الأمراض المتواترة الصعبة العلاج والتي تسبب الإنسان والحيوان على حد سواء. أشارت العديد من الدراسات التجريبية إلى كفاءة أوراق نبات آلوس في قتل وتشتيت أنواع مختلفة من البكتيريا والطفيليات. أستهدفت الدراسة تقييم كفاءة المستخلص المائي لأوراق نبات آلوس ضد الإصابة التجريبية بالأكياس المائية. أستعمل 40 فأراً تم تقسيمها إلى أربع مجاميع متساوية. حققت الفئران في المجاميع الأولى والثانية والثالثة بـ 2000 رويس أولى/ل.أ. في المجموعة الرابعة كمجموعة سيطرة غير مخمجة، بعد أربعة أشهر من الخمج عولجت الفئران في المجموعة الثانية وثالثة بـ 6 مليترزغم و 12 مليترزغم من المستخلص المائي لأوراق نبات آلوس على التوالي بين يومًا وآخر لمدة أسبوع فيما ترتكز المجموعة الأولى كمجموعة سيطرة مخمجة. درست التغيرات المظاهرية والنسجية للأكياس المائية وأعضاء المحمجة بها. كما قاس الفاعلية الإزيمية لإزيم Adenosine deaminase (ADA) و Alkaline phosphatase (ALP) في الفئران المعالجة والمستخلص بأي من التركيزين أعلاهما. ومعدل أقمار للأكياس المائية أقل من فئران مجموعة السيطرة المخمجة، فيما أظهرت المقاطع النسيجية للكبد للفئران المعالجة زيادة في أعداد الخلايا الكبدية وجمعيات من الخلايا الالتهابية مع ترخيج شديد حول الخلايا الكبدية وتتكيس بعضها منها. سجل إرتفاع في النشاط الأزيمي لإزيم ADA وانخفاض في النشاط الإزيمي لإزيم ALP في الفئران المعالجة لاسيما تلك التي عولجت بتكرير 12 مليترزغم/مليتر مقارنة مع مجموعة السيطرة غير المخمجة.
INTRODUCTION

Echinococcosis is a cosmopolitan zoonosis caused by adult stages of cestodes belonging to the genus *Echinococcus* (family Taeniidae). Larval infection (hydatid disease, hydatidosis) is characterized by long-term growth of metacestode (hydatid) cysts in the intermediate host. Current treatment of hydatidosis is mainly surgery, and to less extend percutaneous aspiration and medicinal treatment using benzimidazole compounds(1). In some cases, surgery may not be possible because of the patient’s condition, location of the cyst(s), or when there are multiple cystic loci. Even when surgical operation is done, some risk factors may associated such as recurrence (2) massive peritoneal dissemination, long-stay hospitalization and biliary fistula(3), aside from the high economical cost of the operation (4). Percutaneous drainage is relatively a new treatment method for hydatidosis and it has provided a useful alternative to surgery. Rupture of cyst is considered as one of the most important risk factor of this alternative (3). The effect of benzimidazole derivatives is due to their metabolites which reach a definite serum concentration and passes to the hydatid fluid. However, some of these metabolites are potentially toxic or cause transient abnormality of the liver function test (4,5), aside from resumption of cystic growth following cessation of treatment (6). Some reports pointed out serious adverse reactions due to albendazole usage such as encephalitis syndrome, influenza-like syndrome, allergic purpura, and rash (7,8). A large number of medicinal plants and their purified constituents have been used in treatment of different diseases as an alternative medicine. *Myrtus communis* leaves extract (an evergreen shrub belonging to the family of Mirtaceae) have been employed as a traditional medicine for the treatment of variety of ailments. Oil extract of the leaves of this plant are gaining remarkable interest for the potential multipurpose use as antioxidant, antibacterial, antifungal, and antiseptic agent(9,10,11,12). More recently, this extract was found to be effective against *Trichomonas vaginalis* under certain conditions (13). This study aimed to evaluate the efficiency of aqueous solution of *M. communis* leaves extract in treatment of hydatidosis in vivo.
MATERIALS AND METHODS

Parasite materials

Eight fresh *Echinococcus granulosus* hydatid cysts were obtained from patients who carried hepatic and pulmonary cysts after surgical extripation in Al-Kadhimiya, Ibn-El-Nafees, and Al-Kindy hospitals. The cysts were wrapped carefully in clean plastic bags to the Medical Research Unit/College of Medicine/Al-Nahrain University. The outer surfaces of the cysts were sterilized with 70% ethanol before being dissected. Protoscolices were extracted according to Smyth(14).

Extracted protoscolices were preserved in a sterile medium made of a mixture of Kerb’s Ringer solution (KRS) and hydatid cyst fluid (4:1). The viability of protoscolices was confirmed prior to experiments. It was determined by body movement under light microscope and vital staining with 0.1% methylene blue (15). Crystalline penicillin G and streptomycin sulfate were added to the mixture to keep it free from bacterial contamination.

Plant collection and extraction

Leaves of *M. communis* were collected from the garden of the College of Science, Al-Mustansiryia university. The leaves were washed with water several times and dried in shed at 25°C for two weeks with continuous turning over. Leaves extract was prepared according to Al-Zohyri (16). Stock solution was prepared by dissolving 600 mg of dried powder in 10 mL of distilled water. From this solution, two concentrations (6 and 12 mg/mL) were prepared for the experiment.

Animals

A total of 40(4-6 week old) white albino mice (17 males and 23 females) were divided into 4 groups each with 10 mice. Mice in the first, second, and third groups were injected (IP) with 2000 protoscolices/mouse (equivalent to 1 mL), whereas each mouse in the fourth group was injected IP with 1mL of normal saline and represented as non-infected control group. After 4 months of infection, mice in the second and third group were respectively injected IP with 6 and 12mg (equivalent to 1mL) of aqueous leaves extract of *M. communis* leaves in every other day for 7 days (a total of 4 doses). Mice in the fourth group were not treated and were considered as a infected control group.

Biochemical tests

Blood samples were taken only once from mice in non-infected and infected control groups four months post infection, and from treated mice (second and third group) 60 days post treatment. The samples were drawn directly from the heart after the animals were anesthetized.
Serum was separated from each sample and stored at -20°C to be used later for the estimation of activity of adenosine deaminase (ADA) where Giusti method (17) was used, and Alkaline phosphatase (ALP) where alkaline phosphatase ready kit was (sera and vaccines institute) was used. All animals were weighted and sacrificed 60 days post treatment and the internal organs of which were examined for infection with hydatid cysts. Number and diameter of secondary hydatid cysts were determined in each animal. Reduction percentage of hydatid cysts was determined as following:

\[
\text{Reduction percentage of hydatid cysts} = \frac{\text{average number of hydatid cysts in infected control group} - \text{average number of hydatid cysts in treated group}}{\text{average number of hydatid cysts in infected control group}} \times 100
\]

**Liver and spleen hypertrophy indicator**

Liver and spleen were removed from each mouse and weighted. An organ hypertrophy indicator for these organs was calculated as follow:

\[
\text{Organ Hypertrophy Indicator} = \frac{\text{organ weight}}{\text{animal weight}} \times 1000
\]

**Histopathological study**

Sections from liver, spleen and kidney from infected mice were prepared and examined histologically (3).

**Statistical analysis**

The data were expressed as mean value ± standard deviation and tested with one way ANOVA followed by least significant difference for multiple comparisons. Statistical probability of 0.05 was considered significant.

**RESULTS AND DISCUSSION**

**Number, diameter, and reduction percentage of cysts**

Dissecting of mice in infected control group revealed presence of secondary hydatid cysts in liver, peritoneal membrane, diaphragm, and spleen. In the liver (which was especially infected), cysts either partially embedded in the parenchyma or attached to the surface Figure (1). Protoscolices viability test from these cysts revealed 100% viability. Cysts in treated mice group (2-3) were found in the visceral organs especially the liver, however, viability test revealed no viable protoscolices. Dissecting of mice in negative control group showed no hydatid cysts.
Table (1) shows number, diameter, and reduction percentage of secondary hydatid cysts in the studied groups. Mice treated with 6 and 12 mg have less average number of cysts (2.25±1.28 and 1.28±0.4 respectively) than infected control mice (4.12±1.8) with significant difference, while there were no significant differences in average diameter of cyst between these groups despite the lower diameter in treated mice with either concentration than infected control mice.

While cyst reduction percentage in infected control mice was zero, it was 43.44 and 68.93 in mice treated with 6 mg and 12 mg respectively with significant difference between the two groups.

**Liver and spleen hypertrophy indicators**

Mice treated with 6 and 12 mg had lower liver hypertrophy indicator (51.05±7.34 and 48.98±8.87 respectively) than infected control group (61.79±3.11) with significant difference. Mice treated with 12 mg had the least spleen hypertrophy indicator (56±0.99) and differed significantly from mice in infected control group (7.21±0.6) and un-significantly from mice treated with 6mg (6.32±1.42).

**Histopathological changes**

Histopathological section of liver from infected control mice group (4) showed infiltration with neutrophil, lymphocyte, and macrophage in between hepatocytes with portal area was infiltrated with lymphocyte and monocyte. Some hepatocyte underwent balloon degeneration Figure (2). Sections of spleen from these mice revealed undistinguished white and red pulp, epitheloid histiocyte infiltration, and extramedullary hemopiesis Figure (3). Histological sections of the liver from treated mice with either concentration show hyperplasia, infiltration of inflammatory cells, extensive hyalinization around the hepatocytes, and degeneration of some hepatic cells simultaneously with formation of new cells Figure (4). Sections of spleen show mild extramedullary hematopoiesis and infiltration of some inflammatory cells Figure (5).

**Biochemical changes**

Table (3) shows enzyme activity of ADA and ALP in the studied groups. After 4 months of infection, enzyme activity of ADA in infected control mice group (1) was 1.89±0.03 U/L compared with 0.71±0.12 U/L in non-infected control mice with significant difference. Sixty days post treatment, mice treated with 12 have higher ADA activity (1.74±0.04 U/L) than either mice treated with 6mg (0.80±0.14 U/L) or infected control group (0.71±0.12 U/L) with significant difference. Two months after infection, enzyme activity of ALP in non-infected and infected control groups were 21.33 ± 1.12 U/L and 63.83 ± 3.75 U/L respectively.
Sixty days post treatment, the activity of this enzyme was 53.10 ± 4.47U/L and 53.10 ± 4.47U/L in mice treated with 6 and 12 mg respectively with significant differences among the four groups.

Plants constitute a rich source of bioactive compounds. *Myrtus communis* leave extract has been used for treatment of variety of ailments. The two concentrations of *M. communis* leaves extract were determined according to previous work (unpublished data). Screening test of this extract revealed many constituents among which are flavonoids, saponins, sterols, tannins and glycosides (20).

The bioactivity-guided separation resulted in the isolation of an important chromatographically pure compound which is flavonoid to which many pharmacological activities including anticancer, antimicrobial, antiviral, anti-inflammatory, antioxidant, and hepatoprotective effects can be attributed (21). These effects are assumed to be resulted mainly from two properties: modulation of certain enzymes (hexokinase, aldose reductase, phospholipase C, protein kinase C, cytoxygenase, lipoxygenase, myeloperoxidase, NADPH oxidase, and xanthine oxidase) and antioxidant activity (22). As the liver is the main target of hydatid cysts, the less destructive effect of these cysts in the livers of treated mice especially with 12mg group (3) (as it is indicated by liver hypertrophy indicator and histopathological changes) can be referred to the effect of flavonoids and to less extend to the other constituents of *M. communis* leaves extract. The hepatoprotective role of flavonoids was well established; silymarin (a flavonoid derivative) has been widely used in Europe in the treatment of alcoholic liver diseases associated with increased vascular permeability and capillary fragility (23). They also prevented glutathione depletion and lipid peroxidation induced by acute intoxication with ethanol in rats (24). In Swiss mice, flavonoids pretreatment prevented phaloidin induced acute hemorrhagic necrosis of the liver, and significantly reduced the leakage of liver enzymes into the blood stream (25). As a space occupying lesion, hydatid cysts main effects is ischemia. Flavonoids derivatives were shown to reduce ischemia in the liver (26). The induction of hepatic ischemia is accompanied by elevation of hepatocellular enzymes, which were significantly reduced by pretreatment with flavonoids derivatives. The study revealed significant reduction in number and increased reduction percentage of hydatid cysts in mice treated with either concentration (group 2 and 3). These effect can’t be referred to the action of flavonoids because it has been proved that these compounds can inhibit the releasing of histamine from basophils and reduce inflammatory reaction (27). Rather, these effects may be attributed to saponins which have the ability to modulate the cell mediated immune system, enhance antibody production, induce strong cytotoxic CD8+ lymphocyte responses and amplified phagocytosis of macrophage. The mechanisms of immune-stimulating action of saponins have not been clearly understood, but Saponins reportedly induce production of cytokines such as interleukins and interferon that might mediate their immunostimulant effects (28).
Adenosine deaminase (ADA) is an enzyme involved in the catabolism of purine base. Its plasma activity is found to be elevated in disease eliciting a cell-mediated immune response (29). Earlier studies showed increase in ADA activity in liver diseases and is proposed to reflect the amplified phagocytic activity of macrophage (30). Increased ADA activity in treated mice especially with higher concentration (12 mg) confirmed this proposal as some active ingredients of *M. communis* leaves enhance the activity of macrophage and consequently increase serum activity of this enzyme.

Alkaline phosphatase (ALP) is an orthophosphoric-monoester phosphohydrolase catalyzes the alkaline hydrolysis of large variety of naturally occurring substrates. ALP activity is present in most organs of the animal bodies but it is especially associated with liver and convoluted tubules of kidney. The activity of ALP increases in liver diseases that principally affect parenchymal cells (31). Decreased ALP activity in treated mice especially with 12 mg may be referred to effect of flavonoids which have protective property and reduced the leakage of liver enzymes into the blood stream (25).

*Figure (1): Secondary hydatid cysts in mouse from infected control group*
Table (1): The effect of aqueous *M. Communis* leaves extract on number, diameter and size reduction rate of hydatid cyst in mice.

<table>
<thead>
<tr>
<th>Groups (number)</th>
<th>No. of mice</th>
<th>No. of death</th>
<th>Total No. of cysts</th>
<th>Average No. of cyst / mouse ± S.D</th>
<th>Average diameter of cysts ± S.D</th>
<th>Reduction rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected control (1)</td>
<td>10</td>
<td>2</td>
<td>33</td>
<td>4.12±1.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.87±2.41&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td>Treated with 6 mg (2)</td>
<td>10</td>
<td>2</td>
<td>18</td>
<td>2.25±1.28&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.12±1.55&lt;sup&gt;a&lt;/sup&gt;</td>
<td>45.38&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Treated with 12 mg (3)</td>
<td>10</td>
<td>3</td>
<td>9</td>
<td>1.28±0.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.14±1.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>68.93&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Non-infected control (4)</td>
<td>10</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Note: Different letters indicate significant difference

Figure (2): Histological section of liver from mouse in infected control (group 1) (H and E stain) (A) degenerated and swelled hepatocytes. (B) infiltrated inflammatory cells (100X).
Figure (3): Histological section of spleen from infected control mouse (group 1) showing un-distinct white and red pulp (H and E stain, 500X).

Figure (4): Histological section of liver from mouse treated with 12mg/mL of *M. communis* leaves extract. A: degenerated hepatocytes, B: new formed hepatocytes (H and E stain, 1000x)
Figure (5): Histological section of spleen from mouse treated with 12mg/mL of *M. communis* leaves extract Shows extramadullary hematopoiesis (arrows) (H and E stain, 500x)

Table (2): The effect of aqueous *M. communis* leave extract on liver and spleen hypertrophy in mice infected with hydatid cyst.

<table>
<thead>
<tr>
<th>Groups (number)</th>
<th>No. of mice</th>
<th>Average body wt(g)± S.D</th>
<th>Average spleen wt(g)±S.D</th>
<th>Average liver wt(g)±S.D</th>
<th>Spleen hypertrophy indicator±S.D</th>
<th>Liver hypertrophy indicator±S.D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected control (1)</td>
<td>8</td>
<td>30.96±3.6</td>
<td>0.22±0.02</td>
<td>1.91±0.27</td>
<td>7.21±0.6</td>
<td>61.79±3.11</td>
</tr>
<tr>
<td>Treated with 6 mg (2)</td>
<td>8</td>
<td>32.20±4.96</td>
<td>0.20±0.02</td>
<td>1.65±0.39</td>
<td>6.32±1.42</td>
<td>51.05±7.34</td>
</tr>
<tr>
<td>Treated with 12mg (3)</td>
<td>7</td>
<td>30.75±2.46</td>
<td>0.17±0.03</td>
<td>1.51±0.32</td>
<td>5.56±0.99</td>
<td>48.98±8.87</td>
</tr>
<tr>
<td>Non-infected control (4)</td>
<td>9</td>
<td>30.24±2.82</td>
<td>0.15±0.01</td>
<td>1.23±0.35</td>
<td>4.91±0.62</td>
<td>40.29±8.72</td>
</tr>
</tbody>
</table>

Note: Different letters indicate significant differences
Table (3): Enzyme activity of ADA and ALP in treated and control mice.

<table>
<thead>
<tr>
<th>Groups (number)</th>
<th>Enzyme activity of ADA U/L ±S.D</th>
<th>Enzyme activity of ALP U/L ± S.D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected control (1)</td>
<td>0.71 ± 0.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>63.83 ± 3.75&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Treated with 6 mg (2)</td>
<td>0.90 ± 0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>55.66 ± 4.96&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Treated with 12 mg (3)</td>
<td>1.74 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>45.16 ± 3.38&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Non-infected control (4)</td>
<td>1.89±0.03</td>
<td>21.33±1.12</td>
</tr>
</tbody>
</table>

Note: Different letters indicate significant differences

REFERENCES


