

Full Length Research Paper

Effect of *LAWSONIA INERMIS* treatment on mice with sarcoma

Mehmet Emin Zumrutdal¹, Mehmet Ozaslan^{2*}, Mehmet Tuzcu³, Mehmet Emin Kalender⁵, Kenan Daglıoğlu⁴, Atilla Akova¹, Isık Didem Karagöz², İbrahim Halil Kilic², Omer Colak⁵ and Fatih Köksal⁷

¹Department of General Surgery, Numune Training Hospital, Adana/Turkey.

²Department of Biology, University of Gaziantep, 27310 Gaziantep/Turkey.

³Veterinary Research Institute, 01330 Adana/Turkey.

⁴Animal Research and Implementation Center, 01330 Adana/Turkey.

⁵Department of Oncology, Faculty of Medicine, Gaziantep/Turkey.

⁶Department of Biology, University of Cukurova, 01330 Adana/Turkey.

⁷Department of Microbiology, University of Cukurova, 01330 Adana/Turkey.

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LAWSONIA INERMIS commonly is used as cosmetic material. However many studies had shown that *L. INERMIS* had antitumoural, antimicrobial and anti-tuberculostatic effects. The aim of this study was to investigate whether *L. INERMIS* can destroy cancer cells by induction of apoptosis due to decreasing of intracellular H⁺ ion level or increasing intracellular free radicals and H₂O₂ levels in cancer cells as a result of oxidative effect or not. We used 70 female Swiss albino mice and divided them into four groups. Group 1 was given only tap water. Group 2 was given only *L. INERMIS*. Group 3 was given Ehrlich ascites tumour (EAT) cells + tap water and Group 4 was given EAT + *L. INERMIS*. At the result of this study the thickness of subcutaneous lipid tissue, diameters of gluteal mass, the pH levels of gluteal mass, the GSH levels at the liver tissue samples and the MDA levels of the liver tissue samples of these groups were measured. This study showed that, *L. INERMIS* can be used as a supplementary agent for cancer treatment.

Key words: *L. inermis*, Ehrlich ascites tumour, Ehrlich ascites carcinoma, antitumoural.

INTRODUCTION

Many reports about medical and cosmetic usage of *Lawsonia inermis* (*L. inermis*) has been published in literature. Antitumoural, antimicrobial and antituberculostatic effects of *L. inermis* are prominent in these reports (Malekzadeh, 1968; Curreli et al., 2001; Dasgupta et al., 2003; Habbal et al., 2005; Singh and Pandey, 1989; Sharma, 1990; Kok et al., 2005). Also the serious oxidant effect of *L. inermis* has been shown (Curreli et al., 2001; Dasgupta et al., 2003; Soker et al., 2000). In addition,

L. inermis can induce glucose-6-phosphate dehydrogenase (G6PD) enzyme deficiency in children (Raupp et al., 2001; Soker et al., 2000; Devocioğlu et al., 2001; Zinkham and Oski, 1996).

It has been thought that *L. inermis* can show decreasing effect on glutathione (GSH) level due to oxidative stress and increasing effect on malonal di aldehyde (MDA) (Dasgupta et al., 2003). It has been reported that measuring of GSH level is significant to follow up of breast and over cancer progression. Pentose phosphate pathway and G6PD enzyme are important for GSH synthesis.

The aim of this study was to investigate whether *L. inermis* can destroy cancer cells by induction of apoptosis due to decreasing of intracellular H⁺ ion level or increas-

*Corresponding author. E-mail: ozaslanmd@gantep.edu.tr, ozaslanmd@yahoo.com. Tel: (+90)-342-317-1945. Fax: (+90)-342-360-1200-1032.

Table 1. Experimental groups of mice.

Experimental group	1	2	3	4
Treatment	Only tap water	Only <i>L. inermis</i>	EAT + tap water	EAT + <i>L. inermis</i>

EAT: Ehrlich ascites tumor.

ing intracellular free radicals and H₂O₂ levels in cancer cells as a result of oxidative effect or not. Antitumoral effect of *L. inermis* was evaluated to measure of pH, GSH and MDA levels, tumor volume, subcutaneous lipid tissue thickness and calculate mean survival time in experimental groups consisting of mice with sarcoma formed by Ehrlich ascites tumour (EAT) cells in gluteal muscle tissue.

MATERIALS AND METHODS

Animals

We have used 70 female Swiss albino mice which were 25 - 30 g in weight and 10 weeks old. Mice were from Medical Experimental Research Laboratory of Cukurova University and were kept 12 h under light and 12 h under dark cycles at 25°C for 5 days before study. Mice were fed with standard pellet diet and tap water *ad libitum*. Experimental groups were designed as seen at Table 1.

The formation of sarcoma in Swiss albino mice

Peritonitis carcinomatosa was formed with EAT at sterile conditions and ascites fluid were taken from mouse with paracentesis (Cria et al., 2004, Ozaslan et al., 2006). Tumour cells were stained with Tryphan blue and counted in hemocytometer. The tumour ascites liquid which contained 6×10^6 cells (0.2 ml) was inoculated intramurally to posterior region of left gluteal muscle. By the same way 0.2 ml of sterile 0.9% NaCl solution was injected to Group 1 and Group 2 mice which were not inoculated with EAT.

L. INERMIS solution

L. inermis powder purchased from herbal market. 0.3% *L. inermis* solution was prepared as powder and resolved in distilled water. This solution was boiled and then filtered. *L. inermis* solution was given to mice in two equal doses daily for 12 days. The tap water was given to Group 1 and 3 at same doses and manner.

Thickness of subcutaneous lipid tissue

Thickness of subcutaneous lipid tissue in posterior cervical area was measured in the first and 12th days after tumor inoculation by using vernier callipers.

Measurement of gluteal mass

The diameter of sarcoma developed in median line between hip joint and knee joint on the femur bone was measured on the first and 12th days after tumor inoculation using vernier callipers.

The evaluation of pH in tissue with sarcoma

At the 12th day of the inoculation of EAT, mice were sacrificed by cervical dislocation. Then approximately 1 g tissue sample was taken by incision from sarcoma in gluteal area and placed in tubes with 0.02 M EDTA (1.5 ml) in shortest time. Tissue samples were homogenized in ice (Ultra Turrax T25) and centrifugated at +4°C and then pH of samples were measured with a pH meter (InoLab).

The preparation of liver tissue samples

After cervical decapitation of mice, whose abdomen was dissected by median incision, approximately 200 mg liver tissue to measure GSH and 1 g to measure MDA was sampled and washed with 0.9% NaCl solution. Tissues were taken in tubes with 0.02 M EDTA (8 ml) for GSH and in tubes with 0.15 N KCl (5 ml) for MDA immediately and they were stored at -20°C.

The measurement of GSH

Liver tissue (200 mg) was homogenized in 0.02 M EDTA (8 ml) and stored in ice bath until use. Homogenate (5 ml) was prepared from liver tissue in tubes with EDTA, then it was mixed with 4 ml distilled water and 1 ml 50% trichloroacetic acid (TCA). Then it was centrifuged at 3000 rpm (15 min) and 2 ml of liquid supernatant was mixed with 0.4 M Tris buffer (1 ml) (pH 8.9) and 0.1 Ellman reagent [5,5'-dithiobis-(2-nitrobenzoic acid)] (DTNB) (Sigma). The absorbance at 412 nm in spectrophotometer was recorded. The result values were signified as $\mu\text{mol GSH/g tissue}$ (Sedlak and Lindsay, 1968).

The measurement of MDA

10 ml homogenate was prepared with 1 g liver tissue in 0.15 N KCl. 0.2 ml sample was taken from homogenate and was mixed with 0.8 ml distilled water, 1.5 ml 2-thiobarbituric acid (TBA), 1.5 ml acetic acid and 0.2 ml sodium dodecyl sulphate. For standardization of homogenate, 0.2 ml tetraethoxylpropane was used instead of homogenate. Blank was prepared with distilled water. Tubes were vortexed and boiled in water bath at 95°C for 1 h. Tubes were centrifugated at 4000 rpm (10 min.) after cooling. Supernatant was collected and the absorbance of supernatant was recorded in spectrophotometer at 532 nm. The results were recorded as nmol/ml.

Survival analysis

Mean Survival Time (MST) and Average Survival Time (AST) were calculated according to these formulas:

$$\text{MST} = (\text{the first death day} + \text{the last death day}) / 2$$

$$\text{AST} = \text{the total death days} / \text{total mice number}$$

The percentage of Increasing Mean Standard Life (IMSL) and The

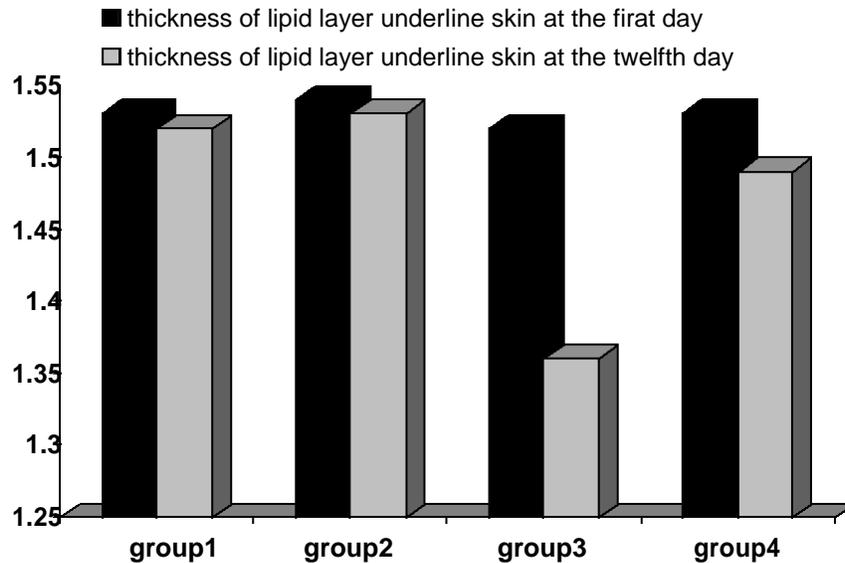


Figure 1. Thickness of subcutaneous lipid tissue (mm) of mice with sarcoma treated with *Lawsonia inermis* (See Table 1 for the definition of the various groups).

Percentage of Increasing Average Standard Life (IASL) were calculated with these formulas (Jagethia and Rao, 2006; Jagethia et al., 2005; Jagethia and Baliga, 2003).

$$\text{IMSL (\%)} = \frac{(\text{experimental group MST} - \text{control group MST}) \times 100}{\text{Control group MST}}$$

$$\text{IASL (\%)} = \frac{(\text{experimental group AST} - \text{control group AST}) \times 100}{\text{control group AST}}$$

Statistical analysis

The statistical analysis of data was performed with SPSS 12.0 package program. Student t-test was used for normal dispersion data and Mann-Whitney U test for abnormal dispersion data. The limit of statistical significance was set at $p < 0.05$.

RESULTS

Thickness of subcutaneous lipid tissue at the first and 12th days is shown in Figure 1. In group 1 mice there was no difference between measurement values, but in group 2 mice measurement at the first day was 0.02 mm thicker than the twelfth day measurement. In group 3 mice, thickness in first day was 1.51 mm and the thickness in twelfth day was decreased to 1.31 mm. The thickness was 1.54 mm in first day and it was decreased to 1.47 mm in 12th day in group 4 mice.

Diameters of gluteal mass are shown in Figure 2. In group 1 and 2 mice, there was no difference between diameters measured at first and 12th days. Although in group 3 and 4 mice, diameters at first day were same, but the diameters at the twelfth day increased. In addition, it was detected that the increasing diameter of gluteal mass in group 3 was higher than the others.

The pH levels of gluteal mass is given in Figure 3. The pH levels in group 1 and 2 were 6.23. Whereas, the measured pH level of group 3 and 4 were 6.24 and 6.27, respectively.

The GSH levels at the liver tissue samples is shown in Figure 4. Liver tissue GSH levels were 4.5 mol/g in groups 1 and 2 and there was no significant difference between these two groups ($p < 0.05$). The GSH levels were 2.9 mol/g in group 3 and 3.3 mol/g in group 4.

The MDA levels at the liver tissue samples is shown in Figure 5. Although there was no significant difference between groups 1 and 2, the highest MDA level was detected in group 3.

Accordingly, the first death date was 30th day and the last death date was 52th inoculation day of EAT in group 3. The survival results of this group were MST: 41 day and AST: 40.1 day. In group 4 the first death date was 35th day and the last death date was 56th day. The survival results were MST: 45.5, IMSL: 11%, AST: 42.1 and IASL: 5% (Table 2).

DISCUSSION

Damage due to oxidative stress and free radicals is one of the important factor in carcinogenesis. Electrophilic compounds occur after metabolism of carcinogens and these compounds must be detoxified in order to avoid DNA damage and lipid peroxidation. GSH is a very important factor in detoxification procedure and MDA is a sign of lipid peroxidation.

Cancer cells can occur in different tissues and proliferation and metastasis can occur by different mechanisms. Oxidative stress due to metabolism of cancer cells in-

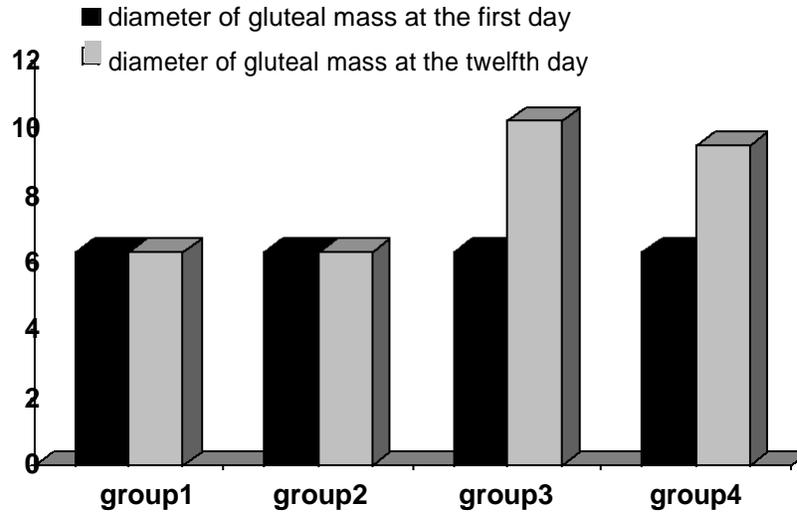


Figure 2. Diameter of gluteal mass (mm) of mice with sarcoma treated with *Lawsonia inermis* (See Table 1 for the definition of the various groups).

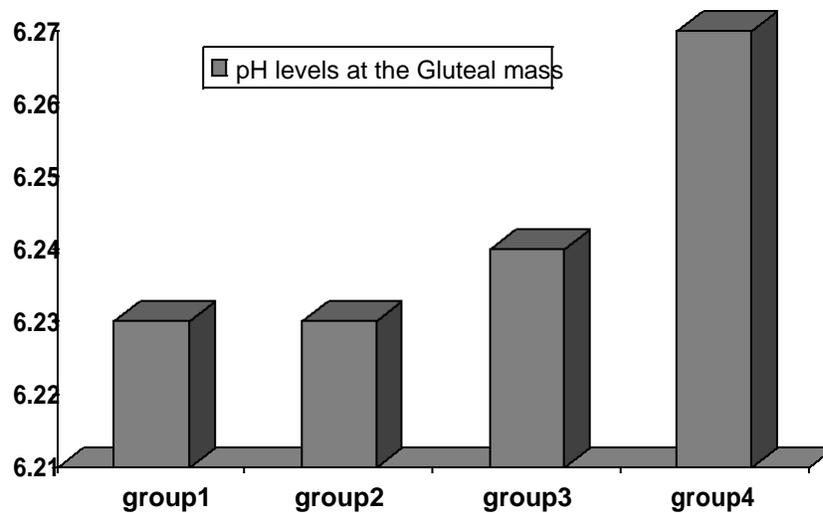


Figure 3. pH levels of the gluteal mass of mice with sarcoma treated with *Lawsonia inermis* (See Table 1 for the definition of the various groups).

creases the cell damage.

Dasgupta et al. (2003) showed the inhibitor effect of *L. inermis* on gastric and skin papillomatous tumors, and GSH and SOD levels were higher, while MDA level was lower with *L. inermis* treated group. Sauriasari et al. (2007) observed that *L. inermis* increase the H₂O₂ level. It is known that the SOD and superoxide anion together form H₂O₂. It has been shown that apoptosis is induced by increase in H₂O₂ (Kim et al., 2003).

L. inermis induce hemolysis in children with G6PD deficiency (Raupp et al., 2001). The G6PD is an H⁺ source for cells through the NADPH + H⁺. McMillan et al.

(2004) showed the oxidative role of *L. inermis* on stressed erythrocytes with hemolytic anemia. These findings indicated that oxidative effect due to *L. inermis* can induce serious alteration in intracellular H⁺ balance.

Cancer cells need more H⁺ and intracellular H⁺ concentration of cancer cells can be decreased by oxidative effect of *L. inermis*. Cancer cells can be destroyed with induction of apoptosis via oxidative stress, by increased intracellular free radicals and by blocking H₂O₂ detoxification with *L. inermis* usage.

Austin and Wray (1993) showed that, for toleration of tissue pH changes, changes at per unit of extracellular

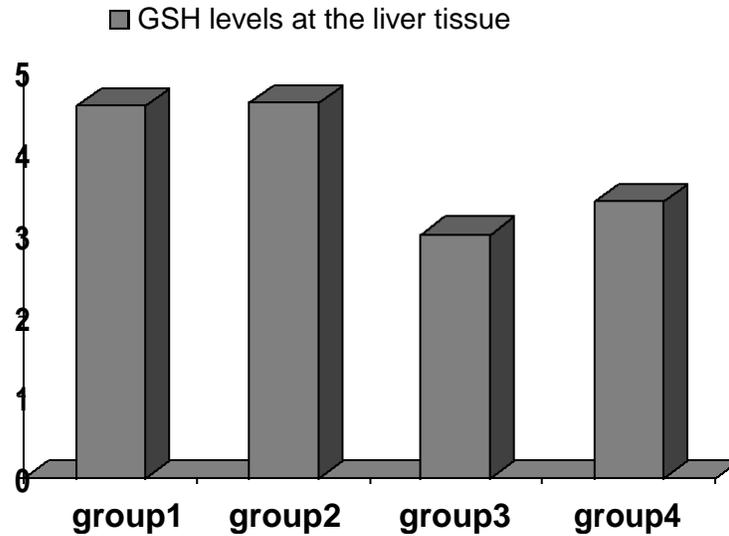


Figure 4. GSH levels at the liver tissue samples ($\mu\text{mol/g}$) of mice with sarcoma treated with *Lawsonia inermis* (See Table 1 for the definition of the various groups).

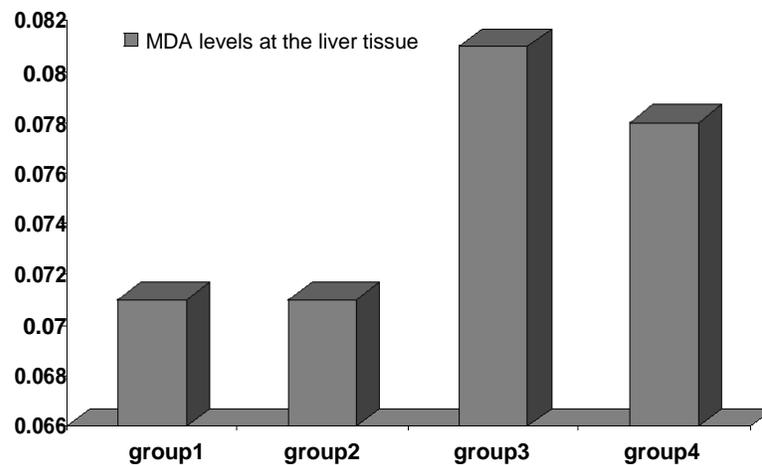


Figure 5. MDA levels at the liver tissue samples (nmol/ml) of mice with sarcoma treated with *Lawsonia inermis* (See Table 1 for the definition of the various groups).

Table 2. The survival of Group 3 and 4 mice with sarcoma treated with *Lawsonia inermis* (See Table 1 for the definition of the various groups).

Parameter	Group 3	Group 4
	Sarcoma + tap water	Sarcoma + <i>L. INERMIS</i>
MST	41	45.5
AST	40.1	42.1
IMSL (%)	-	11
IASL (%)	-	5

pH induce 0.73 fold intracellular pH change. Our aim was

to determine intracellular pH of sarcoma cells with pH of sarcoma tissue. Sarcoma tissue pH of *L. inermis* treated group 4 mice was meaningful more alkaline than water treated group 3 mice ($p < 0.05$) and this finding support our hypothesis. The more alkaline pH in sarcoma tissue can be due to either increased H^+ consumption by ribonucleotide reductase activation or decreased H^+ level by oxidative effect of *L. inermis*. With this condition and usage of *L. inermis*, cancer cells can be induced to apoptosis by constrained intracellular H_2O_2 detoxification due to increased SOD activity and decreased detoxification of intracellular free radicals.

The growth of sarcoma tissue at *L. inermis* treated mice

was meaningfully less ($p < 0.05$) and this finding indicates that the tumor doubling time of sarcoma tissue is longer with *L. inermis* treated mice. Also lesser loss of thickness of subcutaneous lipid tissue ($p < 0.001$), higher level of GSH at liver tissue ($p < 0.05$), lower level of MDA at liver (not significant), longer MST and AST were observed in *L. inermis* treated mice (group 4) than group 3 mice and results of IMSL and IASL were 11 and 5%, respectively. These findings support our hypothesis.

In conclusion *L. inermis* showed tumour suppressor effect and prolonged MST and AST time in mice with gluteal sarcoma formed with EAT cells. These findings indicate that *L. inermis* can be used as a supplementary agent for cancer treatment. Further research is needed to fully explain the ribonucleotide reductase activity of *L. inermis*, free radical formation, evaluation of pH and oxidative potentials, relation between apoptosis and these parameters, and importance of *L. inermis* in cancer treatment.

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