



# International Journal of Pharmacology

ISSN 1811-7775

**science**  
alert

**ansinet**  
Asian Network for Scientific Information

## ***In vivo* Antitumoral Effect of Diffractaic Acid from Lichen Metabolites on Swiss Albino Mice with Ehrlich Ascites Carcinoma: An Experimental Study**

<sup>1</sup>I.D. Karagoz, <sup>1</sup>M. Ozaslan, <sup>1</sup>I. Guler, <sup>1</sup>C. Uyar, <sup>1</sup>T. Yalim, <sup>2</sup>U. Kazanci, <sup>3</sup>A. Aslan and <sup>4</sup>A. Cakir

<sup>1</sup>Department of Biology, University of Gaziantep, Gaziantep, 27310, Turkey

<sup>2</sup>Department of Pathology, Dr. Ersin Aslan Government Hospital, Gaziantep, Turkey

<sup>3</sup>Department of Biology, Education Faculty of Kazim Karabekir,  
University of Ataturk, Erzurum, 25240, Turkey

<sup>4</sup>Department of Chemistry, University of Kilis 7 Aralik, Kilis, 79000, Turkey

**Abstract:** Lichens synthesize very special metabolites as differ from other plant species. *Usnea longissima*, which is usually spread in moist and shady areas, is a lichen species. *Usnea longissima* has many biological activities. Diffractaic acid is one of the major metabolites of *Usnea* species. The present study aimed to investigate the anticancer efficacy of a lichen metabolite, diffractaic acid, on experimentally induced *in vivo* Ehrlich ascites carcinoma. Diffractaic acid was isolated and its chemical structure was confirmed by UV, IR, <sup>1</sup>H-NMR, <sup>13</sup>C-NMR, 1D and 2D-NMR spectroscopic methods. A total of 50 Balb/C male mice were divided into 5 groups (n:10). About 1×10<sup>6</sup> carcinoma cells were inoculated intraperitoneally to the animals and after 2 days 50-200 mg kg<sup>-1</sup> diffractaic acid were given for 13 days. Haematological parameters were measured from serum samples. The EAC fluid was collected by paracentesis and the cells were counted according to trypan blue dye exclusion method. Stomach, liver, kidney and small and large intestine tissues were examined histopathologically. Diffractaic acid showed the antitumor effect on EAC cells and histopathological and haematological studies showed lower doses of diffractaic acid has protective effect as compared with its higher doses.

**Key words:** Diffractaic acid, *Usnea longissima*, ehrlich ascites carcinoma, antitumoral, lichen

### **INTRODUCTION**

Lichens synthesize very special metabolites as differ from other plant species. *Usnea longissima*, which is usually spread in moist and shady areas, is a lichen species. The investigations have shown that *U. longissima* lichen species have many biological activity such as antiulcer (Halici *et al.*, 2005), anti-tuberculosis (Yamamoto *et al.*, 1995), anti-inflammatory (Choudhary *et al.*, 2005; Engel *et al.*, 2007), inhibitory effect in melanogenesis (Kim and Cho, 2007), antimicrobial (Cansaran *et al.*, 2006), antiplatelet and antithrombotic (Lee and Kim, 2005), antioxidant (Odabasoglu *et al.*, 2004), inhibitory effect in plant growing (Nishitoba *et al.*, 1987), antitumor and cytotoxic activity (Einarsdottir *et al.*, 2010). Diffractaic acid is one of the major metabolites of *Usnea* species (Kumar and Muller, 1999). It has been documented that diffractaic acid has gastroprotective effect (Bayir *et al.*, 2006; Odabasoglu *et al.*, 2006).

This study was aimed to investigate that the anticancer efficacy of the lichen metabolite, diffractaic acid, on the experimentally induced *in vivo* Ehrlich Ascites Carcinoma (EAC) cells.

### **MATERIALS AND METHODS**

**Experimental animals:** A total of 50 Swiss albino male mice, weighing average 18-20 g (provided by Gaziantep University, Medical Faculty, Department of Physiology) were used in this study. All experiments were carried out according to the guidelines for the care and use of experimental animals and approved by the Local Animal Ethics Committee, Cumhuriyet University, Sivas, Turkey (No: B.30.2.CUM.0.01.00.00-50/31).

**Ehrlich ascites carcinoma cells:** The parent line of EAC cells was supplied by Istanbul University, Turkey. The carcinoma cell line was maintained by serial intraperitoneal (i.p.) transplantation of 1×10<sup>6</sup> EAC cells/0.1 mL in male Swiss albino mice.

**Extraction of diffractaic acid:** *Usnea longissima* lichen species were obtained from Erzurum (Turkey). At first, dichloromethane was added to the lichen samples for 24 h in the extraction apparatus to extract the diffractaic acid. Then the extracts were filtered, dichloromethane was added on the same lichen sample again and this process was repeated five times. The resulting extracts were combined and the dichloromethane was removed from the extracts. The extract was subjected to silica gel column chromatography using the appropriate solvent system and taken of each fraction was checked by thin layer chromatography. At the end of the this procedure, diffractaic acid which is an important metabolite of *U. longissima* lichen was isolated and its chemical structure was confirmed by UV, IR, <sup>1</sup>H-NMR, <sup>13</sup>C-NMR, 1D and 2D-NMR spectroscopic methods.

**Experimental design:** A total of 50 Swiss albino male mice were used for EAC modeling and the animals were divided into 5 groups including 3 experimental groups and 2 control groups as positive control and negative control (10 mice per group). Group 1 was kept as the negative control group and given only 10 mL kg<sup>-1</sup> physiological water (SF) i.p., group 2 mice were inoculated i.p. with 1 × 10<sup>6</sup> EAC cells for 13 days and kept as the positive control group. The experimental groups were treated with 50, 100 and 200 mg kg<sup>-1</sup> diffractaic acid solution which were dissolved in DMSO and diluted with distilled water and then inoculated i.p. for 13 days. The groups of mice and the applications were shown in Table 1. The bodyweight changes and water and food consumptions were followed daily and the survival analysis was carried out for each group (Ozaslan *et al.*, 2007).

**Counting of ehrlich ascites carcinoma cells:** After the applications for 13 days, all animals were anesthetized with 100 mg kg<sup>-1</sup> ketamine/5 mg kg<sup>-1</sup> diazepam and euthanized with 200 mg kg<sup>-1</sup> thiopental at the 14th day. The EAC fluid accumulated in the peritoneal cavity of the animals was taken by paracentesis and the cells were counted in the Cedex XS (Innovatis Diagnostic, USA) cell counter according to trypan blue dye exclusion method. The percentage of viable and dead cells was calculated, in addition to the calculation of total number of cells (Islam *et al.*, 2012).

**Hematological studies:** At the end of the application, blood samples were collected from all animals by heparinized injector into lithium heparinized tubes and hematological parameters were measured from separated serum. The values of Red Blood Cells (RBC), White Blood Cells (WBC), hemoglobin (HGB), platelets (PLT) and lymphocytes (LYM) were measured automatically (Garg and Goyal, 1992).

**Histopathological analysis:** Stomach, liver, kidney, small and large intestine tissues were removed from the sacrificed animals at the end of the application and all of the tissue samples were fixed in 10% buffered formaldehyde. Then, tissue specimens were processed by the paraffin slice technique and sections were stained with hematoxylin and eosin (Guldur *et al.*, 2006). Paraffin-embedded tissues were examined morphologically by a pathologist under light microscopy (Olympus BX53). The slide of tissues by hematoxylin and eosin staining were assessed according to cellular changes such as an inflammatory reaction, autolysis, necrosis and intense necrosis (Agrawal *et al.*, 2011).

**Statistical analysis:** All statistical analyses were performed by using software GraphPad Instat. Statistical analysis was carried out using ANOVA-TUKEY-HSD (*Post hoc*) test for the parametric values. Kruskal-Wallis test (Kruskal and Wallis, 1952) were used for the non-parametric values. The value p<0.05 was considered statistically significant. All values were expressed as Mean±Standard Error (SEM).

## RESULTS

The changes of weight in the control and experimental groups with different concentration of diffractaic acid were showed in Table 2. It was found that the average weight had increased in treated groups with 100 mg kg<sup>-1</sup> diffractaic acid in the experimental groups but in the other experimental groups the average weight had decreased. Body weight changes resulted by tumor burden among groups were not significant (p>0.05) (Fig. 1a).

Effect of three different concentrations of diffractaic acid on total number of EAC cells, number of viable and dead cells in mice-bearing EAC were showed in Table 3.

Table 1: Groups of mice and the applications of diffractaic acid for each experimental group

Groups	n	Experimental design	EAC cells inoculated i.p.	Concentration (mg kg <sup>-1</sup> )
1	10	Negative control	-	-
2	10	Positive control (EAC)	1×10 <sup>6</sup> EAC cells	-
3	10	EAC+Diffractaic acid	1×10 <sup>6</sup> EAC cells	50
4	10	EAC+Diffractaic acid	1×10 <sup>6</sup> EAC cells	100
5	10	EAC+Diffractaic acid	1×10 <sup>6</sup> EAC cells	200

Table 2: Body weight changes (Mean values±SEM) in control and experiment groups of swiss albino male mice with the different concentration of diffractaic acid

Groups	Experimental design	Beginning weight (g)	Weight after 13 days (g)	Body weight changes±SEM (g)
1	Negative control (Healthy)	29.21	27.78	-1.43±0.70
2	Positive control (EAC)	26.28	27.20	0.92±0.70
3	EAC+50 mg kg <sup>-1</sup> diffractaic acid	28.64	25.64	-0.53±0.59
4	EAC+100 mg kg <sup>-1</sup> diffractaic acid	22.57	23.31	0.74±0.61
5	EAC+200 mg kg <sup>-1</sup> diffractaic acid	28.36	27.31	-1.05±0.67

Table 3: Effect of diffractaic acid concentrations, total cells, viable and dead cells (Mean values±SEM) in mice-bearing EAC

Groups	Experimental design	Total cells (cells mL <sup>-1</sup> )×10 <sup>6</sup>	Viable cells	Dead cells
1	Negative control (Healthy)	-	-	-
2	Positive control (EAC)	551.76±63.07	407.71±71.02	144.06±15.12
3	EAC+50 mg kg <sup>-1</sup> diffractaic acid	12.76±6.980***	6.76±3.850***	6.00±3.130***
4	EAC+100 mg kg <sup>-1</sup> diffractaic acid	3.95±1.450***	2.29±0.870***	1.67±0.580***
5	EAC+200 mg kg <sup>-1</sup> diffractaic acid	3.71±1.300***	2.7±1.1100***	1.01±0.240***

\*\*\*Statistically different (p<0.001) from the control (healthy group)

Table 4: Effect of diffractaic acid on hematological parameters (Mean values±SEM) in mice-bearing EAC

Groups	WBC count×10 <sup>3</sup> μL <sup>-1</sup>	RBC count×10 <sup>6</sup> μL <sup>-1</sup>	PLT count×10 <sup>3</sup> μL <sup>-1</sup>	LYM count×10 <sup>3</sup> μL <sup>-1</sup>	HGB (g dL <sup>-1</sup> )
Healthy	20.87±4.28	5.15±0.82	1637.44±3.1800	19.58±4.02	20.15±0.76
EAC	19.21±5.30	11.61±1.30*	1472.40±349.95	16.88±1.59	18.38±3.37
EAC+50 mg kg <sup>-1</sup> diffractaic acid	26.99±3.20	11.94±0.74	1272.33±139.14	17.65±2.67	19.24±1.00
EAC+100 mg kg <sup>-1</sup> diffractaic acid	16.76±5.97	10.95±2.05	635.29±200.79*	12.15±2.87	14.81±1.96
EAC+200 mg kg <sup>-1</sup> diffractaic acid	13.05±4.49	9.96±1.81	577.17±131.85**	19.05±5.07	9.88±3.29

\*\*Differ significantly at p<0.05, \*\*\*Differ significantly at p<0.01

Table 3 indicated that total number of EAC cells were decreased in the experimental groups in a dose-dependent manner and this was found statistically more significant (p<0.001). In addition, there was a reduction in the number of dead cells in the animals received EAC 1×10<sup>6</sup> i.p.+ three different concentrations of the diffractaic acid for 13 days and these results were found to be very significant (p<0.001). These findings suggested that diffractaic acid had strongly reduced the EAC growth in mice.

The effect of 3 different concentrations of diffractaic acid on hematological parameters was analyzed. The values of Red Blood Cells (RBC), White Blood Cells (WBC), hemoglobin (HGB), platelets (PLT) and lymphocytes (LYM) were summarized in Table 4. The EAC group was compared with the healthy group. The EAC+diffractaic acid groups were compared with the EAC group. It was determined that WBC counts were similar among groups (Fig. 1b).

It was found that there is a statistical difference between EAC and healthy groups in terms of RBC values. The difference between RBC mean value of the healthy group and of EAC group was found statistically significant (p<0.05) in Fig. 1c. In addition to this, it was found that RBC value was decreased with increase of the concentration of diffractaic acid. However, these differences were not statistically significant (p>0.05).

Effect of diffractaic acid on the amount of platelet in the blood was shown in the Fig. 1d. The PLT values in the

experimental groups were decreased with increasing the amount of diffractaic acid as compared with EAC group. These decreases were found statistically significant in the group treated with 100 and 200 mg kg<sup>-1</sup> doses of diffractaic acid and very significant (p<0.05 and p<0.01, respectively). The LYM values among the groups were not different (p>0.05) (Fig. 1e).

Effect of diffractaic acid on the value of HGB in the blood was shown in the Fig. 1f. As can be seen from this figure, the value of HGB was decreased in the groups administrated with 100 and 200 mg kg<sup>-1</sup> doses of diffractaic acid as compared to those of the control groups, healthy and EAC. The maximum significant decrease was observed in the group treated with 200 mg kg<sup>-1</sup> dose of diffractaic acid (p<0.05).

Stomach, liver, kidney and small and large intestine tissues from healthy control group were normal intact histological structures but all tissues from EAC group had severe inflammatory reactions and also deep intense necrosis. Diffractaic acid treated animals had not any changes in stomach and large intestine architecture as compared to those of EAC group. However, the histology of kidney, small intestine and liver in the group treated with 50 mg kg<sup>-1</sup> dose of diffractaic acid showed that they possessed the little inflammatory cells. Histopathological examinations of tissues obtained from the experimental groups were shown in Fig. 2.

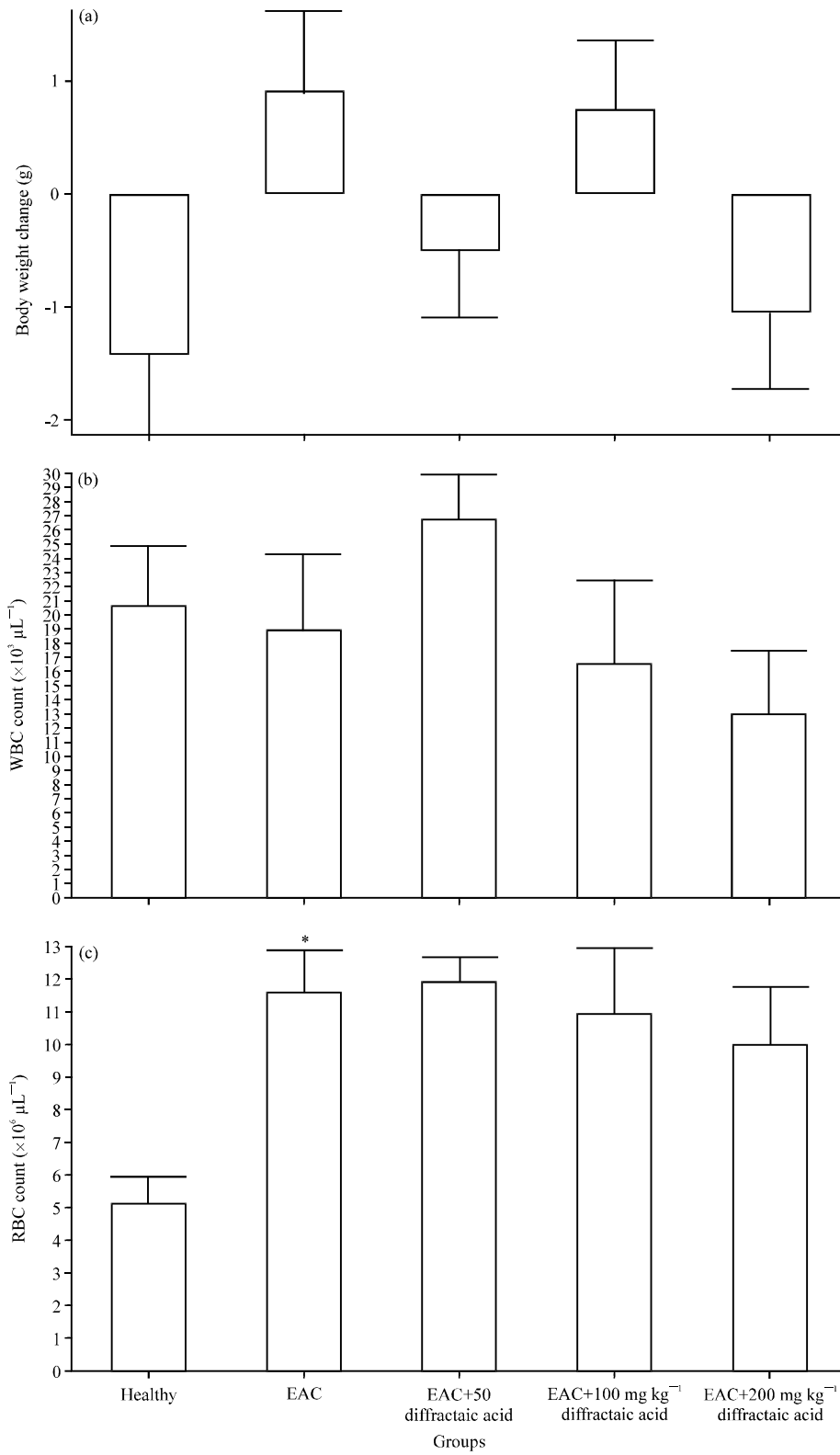


Fig. 1(a-f): Countinue

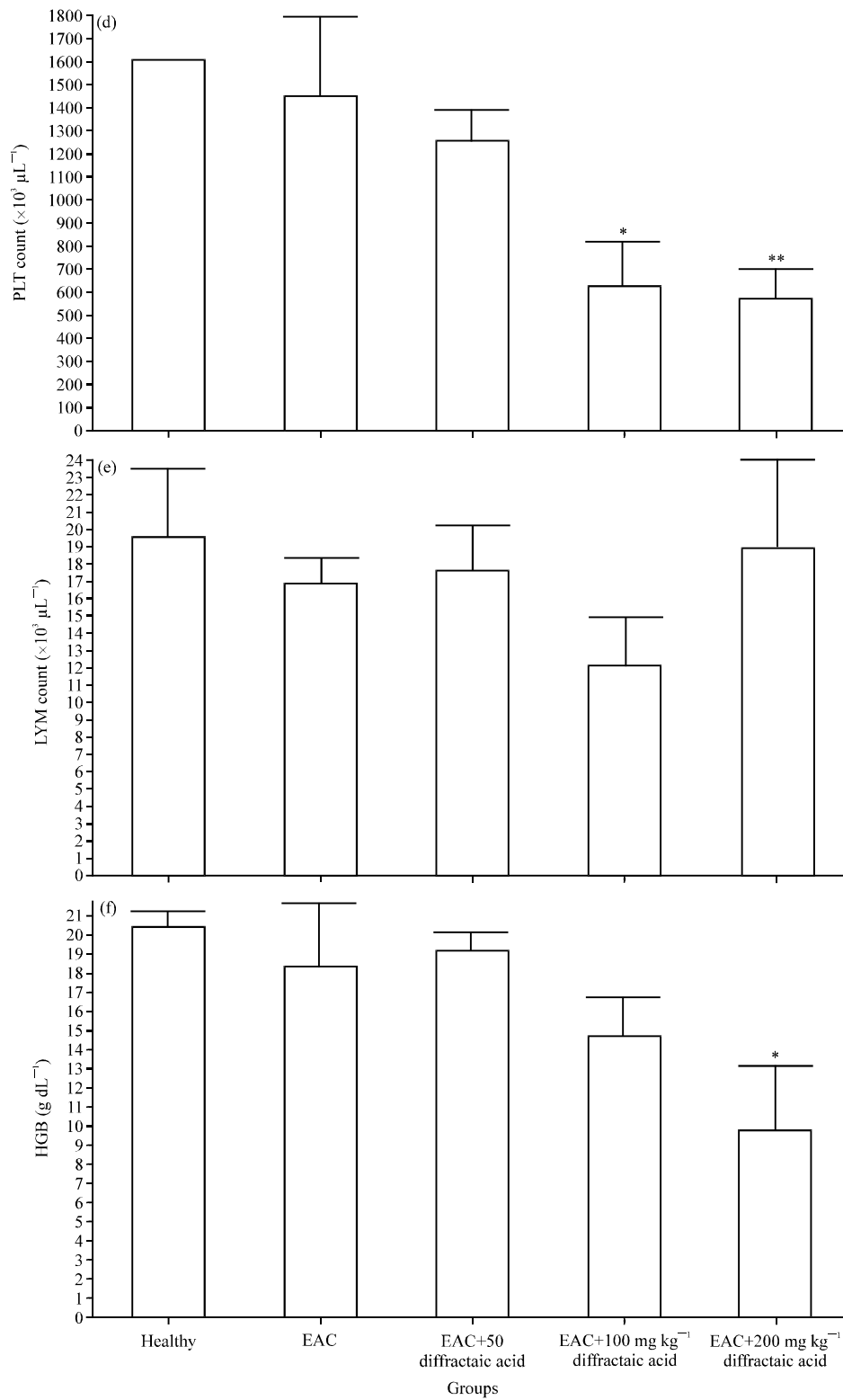


Fig. 1(a-f): (a) Body weight changes, (b) WBC, (c) RBC, (d) PLT, (e) LYM and (f) HGB values of animals. Data is Mean $\pm$ SEM. \*\*\*Significant at  $p < 0.05$  and  $p < 0.01$ , respectively

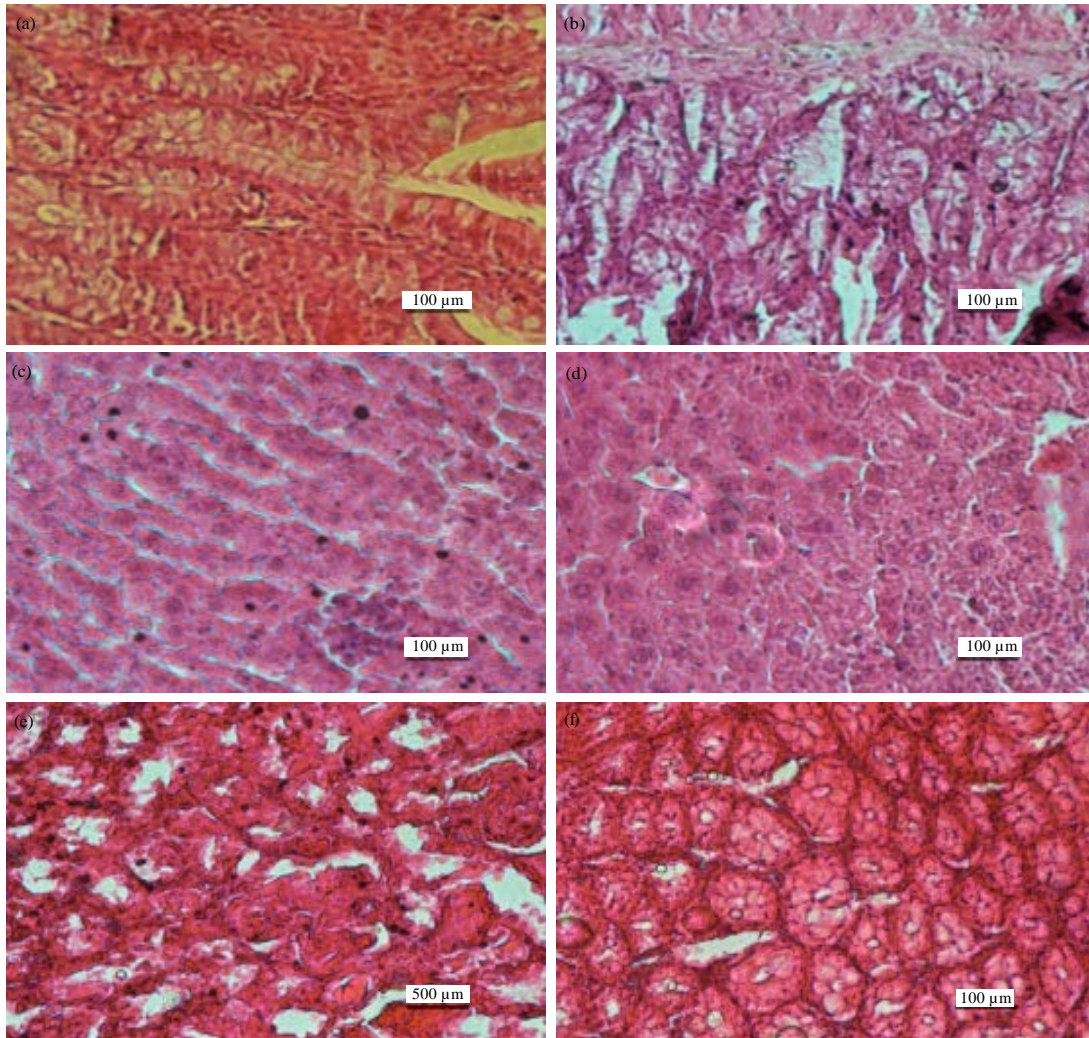


Fig. 2(a-f): Histopathological results in the small intestine (H and E, 100x) in (a) EAC group, (b) Small intestine (H and E, 100x) of the group treated with 50 mg kg<sup>-1</sup> dose of diffractaic acid, (c) Liver (H and E, 100x) of EAC group, (d) Liver of the group treated with 50 mg kg<sup>-1</sup> doses of diffractaic acid, (e) Kidney (H and E, 100x) of the EAC group and (f) Kidney (H and E, 100x) of the group treated with 50 mg kg<sup>-1</sup> doses of diffractaic acid

### DISCUSSION

In terms of weight changes compared with EAC cells inoculated group and healthy group, observed that weight gain in the EAC cells inoculated group and observed similar situation in treatment group which applied the dose of 100 mg kg<sup>-1</sup> diffractaic acid. This increase in the body weight may be due to growth of the accumulation of tumor fluid (Jaiswal *et al.*, 2012). Although decrease in the body weight was observed in other treatment groups administrated with

50 and 200 mg kg<sup>-1</sup> doses of diffractaic acid and this situation indicate that diffractaic acid prevents the growth of tumor, nevertheless this is not statistically significant (Islam *et al.*, 2012).

The application of diffractaic acid to the animals that inoculated with EAC has altered the number of cells in each group. In the present study, at the end of the experiments was determined that the decrease in the number of cells in the animals treated with only EAC compared to the animals treated with the different dose of diffractaic acid. The decrease in the number of cells may

be caused the cytotoxic effect of diffractaic acid, so it can be said that diffractaic acid has the effect of killing tumor cells (Islam *et al.*, 2012).

In this study, it was concluded that hematological parameters have changed, depending on the tumor growth. The hemoglobin content, RBC counts and PLT counts decreased, whereas the other parameters didn't decrease after the inoculation of EAC cells. When compared the hematological parameters in terms of the WBC in the healthy group and the group that only inoculated with EAC, it was observed that the values to be around each other, but the RBC values have increased significantly in the group that only inoculated with EAC. The increase of RBC values in the group that only inoculated with EAC has been observed in the groups treated with different doses of diffractaic acid. The increase in the values of RBC might be due to the hemorrhagic effect of diffractaic acid.

When compared the group that only inoculated with EAC and the group treated with different doses of diffractaic acid in terms of LYM and WBC values have not been observed a significant difference among the groups; it has been observed that the levels of HGB and PLT has decreased in the groups with treated diffractaic acid. The decrease in the values of PLT might be due to the antiplatelet activity of *U. longissima* extract (Lee and Kim, 2005). The decrease in the values of HGB may be due to the deficiency of iron in hemolytic condition (Fenninger and Mider, 1954).

Histological evaluations of all tissues in EAC inoculated group showed that an intensive damages to cell such as severe inflammation, autolysis, necrosis and severe necrosis when compared with those of normal animals. Treatment with diffractaic acid at high doses (100-200 mg kg<sup>-1</sup>) induced inflammation and caused cell dead due to its cytotoxic activity in all tissues (Islam *et al.*, 2013). Diffractaic acid at low dose (50 mg kg<sup>-1</sup>) had comparatively beter protection of kidney, liver and small intestine by reduction in inflammation and necrosis area.

## CONCLUSION

In conclusion, the diffractaic acid compound which isolated from *U. longissima* has demonstrated the antitumor effect on EAC cells in mice. Besides of this result, histopathological evaluation and hematological studies showed that diffractaic acid at low dose has more protective effect and more safety than the higher doses. We suggested that many more investigation has to be carried out with *U. longissima* lichens and its derivatives using various cancer cell lines and higher animal models to use an alternative antitumoral agent.

## REFERENCES

- Agrawal, S.S., S. Saraswati, R. Mathur and M. Pandey, 2011. Cytotoxic and antitumor effects of brucine on ehrlich ascites tumor and human cancer cell line. *Life Sci.*, 89: 147-158.
- Bayir, Y., F. Odabasoglu, A. Cakir, A. Aslan, H. Svleyman, M. Halici and C. Kazaz, 2006. The inhibition of gastric mucosal lesion, oxidative stress and neutrophil-infiltration in rats by the lichen constituent diffractaic acid. *Phytomedicine*, 13: 584-590.
- Cansaran, D., D. Kahya, E. Yurdakulola and O. Atakol, 2006. Identification and quantitation of usnic acid from the lichen *Usnea* species of anatolia and antimicrobial activity. *Zeitschrift fur Naturforschung C*, 61: 773-776.
- Choudhary, M.I., Azizuddin, S. Jalil and Atta-ur-Rahman, 2005. Bioactive phenolic compounds from a medicinal lichen, *Usnea longissima*. *Phytochemistry*, 66: 2346-2350.
- Einarsdottir, E., J. Groeneweg, G.G. Bjornsdottir, G. Harethardottir, S. Omarsdottir, K. Ingolfsdottir and H.M. Ogmundsdottir, 2010. Cellular mechanisms of the anticancer effects of the lichen compound usnic acid. *Planta Med.*, 76: 969-974.
- Engel, K., U. Schmidt, J. Reuter, S. Weckesser, B. Simon-Haarhaus and C.M. Schempp, 2007. *Usnea barbata* extract prevents ultraviolet-B induced prostaglandin E<sub>2</sub> synthesis and COX-2 expression in HaCaT keratinocytes. *J. Photochem. Photobiol. B: Biol.*, 89: 9-14.
- Fenninger, L.D. and G.B. Mider, 1954. Energy and nitrogen metabolism in cancer. *Adv. Cancer Res.*, 2: 229-253.
- Garg, D.K. and R.N. Goyal, 1992. Haematological and hepatotoxic effects of silken styles of corn in albino rats. *J. Applied Toxicol.*, 12: 359-363.
- Guldur, M.E., M. Ozaslan, T. Aytekin, I.H. Kilic and I.D. Afacan, 2006. Toxicity effect of zinc supplementation on the liver tissue. *Pak. J. Biol. Sci.*, 9: 1139-1142.
- Halici, M., F. Odabasoglu, H. Suleyman, A. Cakir, A. Aslan and Y. Bayir, 2005. Effects of water extract of *Usnea longissima* on antioxidant enzyme activity and mucosal damage caused by indomethacin in rats. *Phytomedicine*, 12: 656-662.
- Islam, F., H. Khatun, S. Ghosh, M.M. Ali and J.A. Khanam, 2012. Bioassay of *Eucalyptus* extracts for anticancer activity against Ehrlich Ascites Carcinoma (EAC) cells in Swiss albino mice. *Asian Pac. J. Trop. Biomed.*, 2: 394-398.



- Islam, F., S.M. Ali, J.A. Khanam and S.R. Kabir, 2013. Hepatoprotective effect of acetone semicarbazone on Ehrlich ascites carcinoma induced carcinogenesis in experimental mice. *Asian Pac. J. Trop. Biomed.*, 3: 105-110.
- Jaiswal, J.V., P.A. Wadegaonkar and S.W. Hajare, 2012. The bioflavonoid galangin suppresses the growth of ehrlich ascites carcinoma in Swiss albino mice: A molecular insight. *Applied Biochem. Biotechnol.*, 167: 1325-1339.
- Kim, M.S. and H.B. Cho, 2007. Melanogenesis inhibitory effects of methanolic extracts of *umbilicaria esculenta* and *Usnea longissima*. *J. Microbiol.*, 45: 578-582.
- Kruskal, W.H. and W.A. Wallis, 1952. Use of ranks in one-criterion variance analysis. *J. Am. Stat. Assoc.*, 47: 583-621.
- Kumar, S. and K. Muller, 1999. Lichen metabolites. 2. Antiproliferative and cytotoxic activity of gyrophoric, usnic and diffractaic acid on human keratinocyte growth. *J. Nat. Prod.*, 62: 821-823.
- Lee, K.A. and M.S. Kim, 2005. Antiplatelet and antithrombotic activities of methanol extract of *Usnea longissima*. *Phytother. Res.*, 19: 1061-1064.
- Nishitoba, Y., H. Nishimura, T. Nishiyama and J. Mizutani, 1987. Lichen acids, plant growth inhibitors from *Usnea longissima*. *Phytochemistry*, 26: 3181-3185.
- Odabasoglu, F., A. Aslan, A. Cakir, H. Suleyman, Y. Karagoz, M. Halici and Y. Bayir, 2004. Comparison of antioxidant activity and phenolic content of three lichen species. *Phytother. Res.*, 18: 938-941.
- Odabasoglu, F., A. Cakir, H. Suleyman, A. Aslan, Y. Bayir, M. Halici and C. Kazaz, 2006. Gastroprotective and antioxidant effects of usnic acid on indomethacin-induced gastric ulcer in rats. *J. Ethnopharmacol.*, 103: 59-65.
- Ozaslan, M., I.D. Karagoz, M.E. Kalender, I.H. Kilic, I. Sari and A. Karagoz, 2007. *In vivo* antitumoral effect of *Plantago major* L. extract on balb/c mouse with erlich ascites tumor. *Am. J. Chinese Med.*, 35: 841-851.
- Yamamoto, Y., Y. Miura, Y. Kinoshita, M. Higuchi and Y. Yamada *et al.*, 1995. Screening of tissue cultures and thalli of lichens and some of their active constituents for inhibition of tumor promoter-induced Epstein-Barr virus activation. *Chem. Pharm. Bull. (Tokyo)*, 43: 1388-1390.