Effect of Zizyphus Leaves Extract on Mice Suffering from Ehrlich Ascites Carcinoma

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Abstract: The present study was concerned with the pharmacological potency of zizyphus leaves extract towards antitumor in the form of Ehrlich ascites carcinoma (EAC) model in female albino mice. Ascites tumor was introduced into mice by inoculation of $2 \times 10^6$ viable tumor cells/mouse. After 10 days of transplantation, the extraction of zizyphus leaves was given daily for 21 days via intraperitoneal route at a dose level of 200mg/kg body weight (b.w.) to mice bearing EAC cells. Then, the blood samples and tissues of liver, kidney, spleen, small and large intestine were collected from treated and control animals for biochemical and histopathological examination.

The therapeutic role of zizyphus leaves extract against Ehrlich ascites carcinoma appeared, to a great extent, in retardation of animal body weight as well as improvement of corticoesterone level and immune markers such as monocytes chemoattractant protein-1 (MCP-1), vascular endothelial growth factor (VEGF), interleukin-10 (IL10). The enhancement of antioxidant status in extract treated animals was appeared in restoration of serum thiobarbituric acid reactive substance (TBARs) and total antioxidants values. Regarding to histopathological results, treatment with zizyphus leaves extract diminished most of the pathological alterations induced by EAC cells in mice and confirmed the biochemical results. Thus, zizyphus leaves extract may be utilized to reduce EAC tumor and it could be recommended with attempts to integrate from animal studies, and considers their possible application in human.

Keywords: Effect; Zizyphus; Leave; Extract; Mice; Ehrlich Ascites Carcinoma

1. Introduction:

An extremely promising strategy for cancer prevention today is chemoprevention, which is defined as the use of synthetic or natural agents (alone or combination) to block the development of cancer in humans. Surgery, radiotherapy and chemotherapy, the established treatment modalities for various cancers are costly, mutilating, having serious side effects and associated with residual morbidity as well as frequent relapses. During the last few years, novel chemopreventive agents of natural origin have been targeted with fruits and vegetables being a key interest due to high content of bioactive compounds (Rafter, 2002). Emerging evidence suggests that a number of plants are known to be the source of useful drugs in modern medicine (Sadiq et al., 2009) and have been accepted currently as one of the main source of cancer chemoprevention drug discovery and development (Gonzales and Valerio, 2006) due to their diverse pharmacological properties including cytotoxic and cancer chemopreventive effects (Gupta et al., 2004 and Dahiru et al., 2005). There are a lot of pathological factors, including reactive oxygen species (ROS) involved in the process of cancer initiation, promotion and progression (Marnett, 2000). First, an oxidative stress can induce DNA damages that lead to genomic instability and possibly stimulate cancer progression (Oberley, 2002). Second, elevated ROS levels are responsible for constant activation of transcription factors and the progression of the disease (Gupta et al., 1999). For these reasons, the search for antioxidants as cancer chemopreventive agents is a continued process. Another aspect of antioxidant administration in cancer patients is that these could affect antineoplastic efficacy or the development of side effects of anticancer drugs (Conklin, 2004).

Despite widespread use of plant resources in traditional medicines, few recent studies focused on the potential role of the plant leaves extraction as prevention or regression agent affecting the growth of certain tumors (Kunwar et al. 2009). Zizyphus, a member of the family Rhamnaceae, is used traditionally as tonic and aphrodisiac and sometimes as hypnotic-sedative and anxiolytic, anticancer (Melanoma cells), antifungal, antibacterial, antiulcer, anti-inflammatory,cognitive,antispastic, antinephritic, cardiotonic, antioxidant, immunostimulant, and wound healing properties (Godini, 2009). Since,
pharmacological screening studies indicated that the extract of zizyphus leaves contains beutic acid and ceanothic acid, cyclopeptides, as well as flavonoids, lipids, protein, free sugar and mucilage (Adzu, et al., 2003) and four saponin glycosides: christanin A, christianin B, C and D (Glombitza et al., 1994 and Mahran et al. 1996). It well reported that the extract of zizyphus exhibited anti-nociceptive potency (Adzu et al.,2001) and may prevent chronic alcohol-induced liver injury by enhancing the levels of total antioxidant status and inhibiting hepatic lipid peroxidation (Dahiru and Obidoa 2007). The present study was undertaken to predict the potential anti-tumor activity of zizyphus leaves extract using Ehrlich ascites carcinoma (EAC) model in Swiss Albino mice.

2. Materials and methods

Preparation of zizyphus leaves extract:
Zizyphus leaves were collected from a piece of land at Anshas and the extraction procedure was done in the department of biochemistry, Faculty of Agriculture, Banha University. The leaf samples washed by petroleum ether and wear air-dried at room temperature and blended to a mesh size of 1 mm. The blended samples (1kg) in 4 liters of 70% ethanol for 48 h filtered and concentrated to dryness using rotary evaporator. The ethanolic extract was kept in the refrigerator until usage (Seyyednejad et al., 2001 and Moazedi et al., 2007).

Acute toxicity study:
Preliminary study was conducted on a large number of animals (about 75 mice) with widely different doses of zizyphus leaves extract under the same environmental conditions and the same manner to select the optimal safety dose which could be used.

Animals:
Based upon preliminary results, zizyphus leaves extract at a dose of 200 mg/kg b.w. was optimize to inject intraperitoneally into 45 albino female mice for 21 consecutive days starting from day 10 of EAC cells transplantation. The animals were housed in controlled environmental conditions (temperature 25 ± 2 °C and 12 h dark/light cycle) with standard diet and water ad-libitum. General health condition was observed daily and development of ascites was monitored by recording the change in animal body weight throughout the experiment. Following the last injection, blood samples were allowed to clot, centrifuge and separated serum samples were stored at -20°C until assayed for biochemical analysis. Thereafter, the animals of control and treated groups were dissected and the tissues of liver, kidney, spleen, small and large intestine were removed and stored in 10% formalin saline for histological study.

Biochemical study:
Preparation of tumor cells:
Ehrlich ascites carcinoma (EAC) cells collected from donor mice (Swiss albino) of 18–20 g body weight obtained and suspended in sterile isotonic saline. A fixed number of viable cells (usually 2×10⁶ cells/20 g body weight) were implanted into the peritoneal cavity of each recipient mouse (Gothoskar and Ranadive, 1971). The tumor cells multiplied relatively freely within the peritoneal cavity. The cells were withdrawn by sterile disposable syringe and diluted with physiological saline. The viability of the cells was 99% as judged by trypan blue exclusion assay

Serum monocytes chemoattractant protein-1 (MCP-1), vascular endothelial growth factor (VEGF), and interleukin-10 (IL10) activity these using tests are based on a solid phase enzymelinked immunosorbent assay (ELISA) from (Boster Biological Technology LTD, Malden, MA 02148 USA), were determined according to the methods of Hsu et al., (2007), (Ferrara, 2001) and Chan and Perlstein (1987) respectively. Corticoesterone was estimated using RIA technique according to (AL-Dujaili et al., 1981) Also, total antioxidant and thiobarbutric acid reactive substances (TBARs) were measured spectrophotometry (Unicam UV-Visible Spectrometry HeXios, United Kingdom) according to the reported methods by Okawa et al. (1979) and Buege and Aust, (1978).

Histopathological estimation:
Tissue specimens of liver, kidney, spleen, small and large intestine were fixed in 10% formalin saline. Trimming was done on the fixed tissue specimens and washed in tap water for 12 hours. Serial alcohol (methyl, ethyl and absolute) were used for dehydration of the tissue samples. Tissue specimens were cleared in xylene and embedded in paraffin. The paraffin blocks were sectioned at 3 microns thickness by slide microtome. The obtained tissue sections were collected on the glass slides and stained by hematoxylin and eosin stain for histopathological examination by the light microscope (Banchroft et al., 1996).

Statistical Analysis:
All values were expressed as mean ± SEM. Statistical analysis was performed with one way analysis of variance (ANOVA) followed by Duncan's test. P values < 0.05 were considered to be statistically significant.
3. Results

Body weight:

As shown in table (1), there was no difference between animals body weight of control and *zizyphus* leaves extract treated mice. But the mice bearing EAC revealed a significant increase (p< 0.05) in body weight reached to 53.01% of the initial weight. Injection of *zizyphus* leaves extract into mice bearing EAC caused significant reduction (p<0.05) in their body weight reached approximately to control mice weight.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control group</th>
<th>Zizyphus-treated group</th>
<th>EAC bearing group</th>
<th>EAC bearing-treated group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body wt (gm)</td>
<td>20.63±0.22</td>
<td>21.05±0.23</td>
<td>21.24±0.34</td>
<td>21.0±0.54</td>
</tr>
<tr>
<td>Final body wt (gm)</td>
<td>21.71±0.16</td>
<td>20.80±0.38</td>
<td>32.50±1.04</td>
<td>21.80±0.54</td>
</tr>
<tr>
<td>change</td>
<td>+ 5.23%</td>
<td>- 1.19%</td>
<td>+ 53.01%</td>
<td>+ 3.81%</td>
</tr>
</tbody>
</table>

Values represent means ± S.E.
Values with same superscript in the raw are not statistically different.

Biochemical results:

Table (2) clarified that administration of *zizyphus* leaves extract reaveled slightly decrease in serum MCP-1 and IL10 activity as compared to control. On the other hand, EAC bearing mice showed significant increase (p<0.05) in MCP-1, VEGF and IL10 activity as compared to both control and *zizyphus* extract administered mice. Treatment with *zizyphus* leaves extract improved to a great extent, the activity of MCP-1, VEGF and IL10 in EAC bearing mice towards the control level.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Groups</th>
<th>Control group</th>
<th>Zizyphus-treated group</th>
<th>EAC bearing group</th>
<th>EAC bearing-treated group</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCP-1 (pg/ml)</td>
<td>178.23±1.24</td>
<td>175.91±2.14</td>
<td>379.13±2.61</td>
<td>186.69±3.80</td>
<td></td>
</tr>
<tr>
<td>VEGF (pg/ml)</td>
<td>Zero</td>
<td>Zero</td>
<td>435.75±4.09</td>
<td>206.93±1.27</td>
<td></td>
</tr>
<tr>
<td>IL10 (pg/ml)</td>
<td>68.36±1.90</td>
<td>60.68±0.92</td>
<td>114.79±1.12</td>
<td>61.22±1.05</td>
<td></td>
</tr>
</tbody>
</table>

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Values with same superscript in the raw are not statistically different.

Table (3) demonstrated that administration of *Zizyphus* leaves extract to normal mice decreases TBARS level and did not affect on total antioxidant concentration. On the other hand, EAC bearing mice showed significant decrease (p<0.05) in serum total antioxidant concentration and significant increase (p<0.05) in TBARS level as compared to control. The enhanced antioxidant status in extract treated animals was evident in decline of TBARS level and increased (p<0.05) of total antioxidant activity to approximate to control.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Groups</th>
<th>Control group</th>
<th>Zizyphus-treated group</th>
<th>EAC bearing group</th>
<th>EAC bearing-treated group</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. antioxidant (mM/L)</td>
<td>1.07±0.01</td>
<td>1.06±0.04</td>
<td>0.80±0.025</td>
<td>1.07±0.096</td>
<td></td>
</tr>
<tr>
<td>TBARS(µmol/ml)</td>
<td>16.63±0.215</td>
<td>14.52±0.51</td>
<td>20.28±0.15</td>
<td>15.00±0.27</td>
<td></td>
</tr>
</tbody>
</table>

Values represent means ± S.E.
Values with same superscript in the raw are not statistically different.

Table (4) depicted that, when *zizyphus* leaves extract injected into normal mice, the level of serum corticoesterone did not affected. When it was injected into mice bearing EAC, the concentration of serum corticoesterone decreased significantly (p<0.05) as compared to mice bearing tumor.
Table (4): Effects of *zizyphus* leaves extract and/or EAC on corticosterone level in female mice.

<table>
<thead>
<tr>
<th>Groups parameters</th>
<th>Control group</th>
<th>Zizyphus- treated group</th>
<th>EAC bearing group</th>
<th>EAC bearing- treated group</th>
</tr>
</thead>
<tbody>
<tr>
<td>corticosterone (ng/ml)</td>
<td>58.31b ± 0.31</td>
<td>57.50b ± 0.45</td>
<td>72.81a ± 0.71</td>
<td>59.72b ± 0.47</td>
</tr>
</tbody>
</table>

Values represent means ± S.E.
Values with same superscript in the raw are not statistically different.

Histopathological results:
Liver tissue:
The group of normal mice administrated *zizyphus* leaves extract showed dilation and congestion in the central and portal veins of liver (Fig.2). With respect to Ehrliched mice, microscopical examination of liver revealed thickening in hepatic capsule with inflammatory and pigmented cells (P) as well as diffuse kupffer cells(D), proliferation in between the degenerated cytomegalic hepatocytes(C) as shown in Fig. (3 & 4). Interestingly, treatment with *zizyphus* leaves extract reduced most of the pathological alterations induced by EAC cells in mice. Since, liver section showed few focal inflammatory cells infiltration in the hepatic parenchymal associated with slight congestion in the central vein (CV) (Fig.5)

Kidney tissue:
According to histopathological findings, cortical blood vessels (V) between the glomeruli and renal tubules in kidney section of normal mice administered the leaves extract (Fig.7) were observed. Section of kidney of mice bearing EAC cells showed mild dilatation and congestion in the cortical blood vessels (V) and vacuation in the lining endothelium of the glomerular tuft (G) (Fig.8 & 9) as well as focal extravasation of red blood cells in between the degenerated tubules at the corticomedullary junction was observed (Fig.10). *Zizyphus* leaves extract administration restored most of normal intact histological structure in kidney section (Fig. 11).

Spleen tissue:
Light microscopic observation of normal mice administered *zizyphus* leaves extract spleen showed lymphoid hyperplasia in white pulps (W) (Fig.13). Regarding to mice bearing EAC, there was a multiple numbers of immature megakaryocytes in the white pulps (Fig. 14). On the other hand, mild lymphoid hyperplasia in the white pulps (W) was found in spleen section (Fig. 15).

Small and large intestine:
There was a focal inflammatory cells infiltration with oedema in the lamina propria (L) appeared in large intestine section of normal mice administered the leaves extract (Fig.17). Meanwhile, small intestine of this group showed normal histological structure (Fig. 19). With respect to mice bearing EAC, large intestine revealed oedema inflammatory and pigmented cells infiltration in serosal layer (Fig.20) but, the small intestine showed normal intact histological structure (Fig.21).

Apparently, the treatment with *zizyphus* leaves extract diminished most of the pathological changes in Ehrliched mice. Since focal oedema with few inflammatory cells infiltration in serosal layer (M) of small intestine was observed (fig.22), as well as few inflammatory cells infiltration with mild oedema in focal manner at the lamina propria of the mucosal layer of large intestine (Fig. 23).
Liver tissue:
1- Section of liver obtained from control mice.
2- Section of Liver collected from normal mice administrated *zizyphus* leaves extract.
3&4: Section of Liver obtained from mice bearing EAC cells.
5- Section of liver obtained from mice bearing EAC and treated with *zizyphus* leave extract.
Kidney tissue:
6-Section of kidney obtained from control mice
7- Section of kidney collected from normal mice administrated zizyphus leaves extract.
8, 9 &10: Section of Kidney obtained from mice bearing EAC cells.
11-Section of kidney obtained from mice bearing EAC and treated with zizyphus leave extract.

Spleen tissue:
12- Section of spleen obtained from control mice.
13-Section of spleen collected from normal mice administrated zizyphus leaves extract.
14-Section of Spleen obtained from mice bearing EAC cells.
15- Section of spleen obtained from mice bearing EAC and treated with zizyphus leave extract.
Small and large intestine:
16- Section of large intestine obtained from control mice.
17- Section of large intestine collected from normal mice administrated zizyphus leaves extract.
18- Section of small intestine obtained from control mice.
19- Section of small intestine collected from normal mice administrated zizyphus leaves extract.
20- Section of large intestine obtained from mice bearing EAC cells.
21: Section of small intestine obtained from mice bearing EAC cells.
22-Section of small intestine obtained from mice bearing EAC and treated with zizyphus leave extract.
23- Section of large intestine obtained from mice bearing EAC and treated with zizyphus leave extract.
4. Discussion:

Ascites fluid is the direct nutritional source for tumor cells. So, a rapid increase in ascites fluid with tumor growth would be a mean to meet the nutritional requirement of tumor cells (Rajeshwar et al., 2005). This hypothesis was evident in the present study, since inoculation of EAC cells into mice caused significant increase in the mice body weight reached to 53.01% after one month of inoculation. Such increase was due to accumulated ascites fluid of EAC in peritoneal cavity (Altun and Ozalpan, 2004). On the other hand, the soluble factors associated with tumor growth may mediate certain hormonal changes since the serum level of corticosterone was observed to increase following injection of tumorous ascites into normal mice (Mandal et al., 2007). Elevated level of corticosterone is a major contributing factor to anti-inflammation induced by tumorous ascites injection and constitute the principal mechanism of anti-inflammation following tumor transplantation (Normann et al., 1988). So, the physiological corticosterone levels rather stimulate than suppress immunity (Kanchev et al., 2006). The impairment of host immune function by tumor may be related to several strategies of tumor escape from immunosurveillance. Therefore, MCP-1 induction associated with VEGF secretion observed in the present study, in concert with the direct chemotactic effects of VEGF on monocytes (Clauss et al., 2001) and may contribute to the angiogenic processes stimulated by VEGF in vivo. However, VEGF-induced MCP-1 expression in vascular endothelial cells, elevated endothelial permeability changes in vivo (Lee et al., 2006), that the process is considered to be important for tumor growth (Loberg et al. 2007). Since, MCP-1 reported as an important chemokine that is necessary for maximal recruitment of monocytes in a number of different pathologies (Stathopoulos et al., 2008) and for induction of new vessels formation required for tumor growth (Stathopoulos et al. 2008). On the other hand, in EAC bearing mice, the tumor cells secrete immunosuppressive cytokines, interleukin-10 (IL-10) that was identified as a key immunomodulatory cytokine on T cells activity (Ye et al., 2007). The production of IL-10 within the tumor microenvironment can be sustained by malignant cells and tumor-infiltrating macrophages and lymphocytes (Ghosh et al., 2004). In this context, provided evidence for the involvement of free radicals production in MCP-1 mRNA induction (Marumo et al., 1999) and considered the reactive oxygen species as mediator growth factor--induced responses in tumor bearer (Lander, 1997).

However, lipid peroxidation/oxidation process plays a key role in tumor growth invasiveness (Chakraborty et al. 2009). Accordingly, the decrease of total antioxidants value concomitant with increase in TBARs concentration observed in the present study could be considered as onslaught of free radicals resulted from carcinomous tissues (Ahmet and Suleyman, 2006).

Interestingly, intraperitoneal injection of zizyphus leaves extract realized an effective role in treatment of EAC tumor which was manifested in reduction of body weight, improvement of MCP-1, VEGF and IL-10 levels and modulation of antioxidant status in mice bearing tumor. Such results referred to the ability of this extract in inhibition of accumulated ascites fluid (Ganachari and Shiv, 2004) and recovery of associated immune and oxidants risk factors. It was established that the extract of zizyphus leaves stimulate chemotactic, phagocytic and intracellular killing potency neutrophils (Ganachari and Shiv, 2004). On the other hand, the blockage of VEGF activities was considered as inhibitor factor of malignant ascites (Manenti et al., 2005) because VEGF level correlates with tumor burden, ascites formation and dissemination in the organs of the peritoneal cavity (Loberg et al. 2007). Therefore, this model may serve to monitor tumor progression and response to zizyphus leaves extract treatment (Manenti et al., 2005). However, phytochemical analysis of this extract showed a highest level of antioxidant contents at all level of antioxidants (Pisha et al., 1995) namely, betulinic acid which is a known anticancer agent induced successive activation of caspase 9 and caspase 3 (Pokrovskii et al., 2006) and flavonoids which could be expected to be responsible for its bioactivity. In this regard, the decreased VEGF-induced MCP-1 inhibition by antioxidants was strongly suggest that a signaling pathway stimulated by VEGF may involve production of ROS (Yan et al., 1994). It could be suggested that the therapeutic role of flavonoids ultimately was the induction of apoptosis in cancer cells (Vahedi et al., 2008), inhibition of cell proliferation (Shimmyo et al., 2008) and modulation of a number of cellular signaling pathways activity (Williams et al., 2004). Further, the inclination in endogenous antioxidant activities in EAC bearing mice after treatment with zizyphus leaves extract was an indication of immune and structural integrity (Das et al., 1995). However, the abundance of antioxidant compounds content in the extract (Shaiban et al.,2006) particularly, tannins, saponins and phenolic compounds (Adzu, et al., 2003) were probably together prevented excess releasing of free radicals (Das et al., 1995) or converted free radicals to more stable products or stimulate the antioxidant enzymes directly (Dahiru and Obidoa 2008).
The mentioned results were further supported by the histopathological examination of mice bearing EAC and/or administrated zizyphus. There was a diminishing in pathological structure, to a great degree, towards normal intact histological structure. Mosaad et al. (2007) showed that animals treated with Zizyphus extract had a significant improvement in histological picture of liver and kidney because the containing antioxidant principles were cytotoxic towards tumor cells (Ruby et al., 1995).

In conclusion, treatment of mice with extract of Zizyphus leaves (200 mg/kg body wt) has a therapeutic role against EAC cells. It is possible to propose that the additive and synergistic antioxidant activity of phytochemicals such as flavonoids, tannins, saponins, etc, present in zizyphus leaves extract are responsible for the its potent antitumor activity which could be inferred from the improvement of immune markers and antioxidant status.

ACKNOWLEDGMENT:

The authors are grateful to Dr. Adel M. Bakeer Kholoussy, Professor of Pathology, Faculty of Veterinary Medicine, Cairo University for his helping in the examination of the histopathological slides and for his valuable comments.

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5. References:


9/12/2010