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Antitumor Activity of Natural Compounds Against Ehrlich Ascites Carcinoma in Swiss Albino Mice

KEYWORDS

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ABSTRACT *Natural products, from different biological origins, represent the main source of compounds used to synthesize drugs. For example, azurin, from Pseudomonas aeruginosa, and extracts from Viscum album, have been reported to possess anticancer properties. They induce apoptosis, natural immunity in ill individuals, respectively. The antitumor activity of azurin and water extract of Viscum album were evaluated against Ehrlich ascites carcinoma (EAC) tumor inoculated in mammary glands of Swiss albino mice. The effect of treatment were performed either separately or in combination. Azurin was administered daily and the Viscum album was given three times a week, for 21 days. After administration of the last dose and 18h fasting ,animals were sacrificed and blood were collected for analysis. Several parameters were measured to evaluate the effect of azurin and Viscum album on the growth of tumor: life span of EAC bearing hosts and simultaneous alterations in the oxidative stress, superoxide dismutase (SOD) Catalase (CAT) reduced glutathione (GSH) and Malondialdehyde (MDA) , tumor necrosis factor (TNF-α), Interleukin-10 (IL-10). Each of the two compounds increased the life span of EAC tumor-bearing mice, (SOD) , (GSH) and (CAT) levels. However, the MDA. IL-10 and TNF-α were decreased in the studied groups relative to the negative control group. A combination of azurin and Viscum album extract greatly improved all measured parameters after infected by EAC.*

Introduction

Cancer is caused by a group of uncontrollably dividing cells that can invade surrounding and distant tissues. Cancer is the world's second killer after cardiovascular disease, it killed 7.6 million people in 2005 alone of them three quarters were from in low and middle income countries. That number is expected to increase up to 9.0 million in 2015 and rise further to 11.5 million in 2030, *(WHO, 2011).*

Many options do exist for cancer treatment including: surgery chemotherapy, radiation therapy, biological therapy. The use of any of these treatments depends upon the location, the grade and/or the stage of the tumor, as well as the general state of a person's health *(WHO, 2011).* Chemotherapy is always applied after surgery, it also affects normal cells which is more devastating for so many patients. The Radiotherapy or the use of ionizing radiation may be used before surgery to shrink the tumor or after surgery to destroy any remaining cancer cells, all of these treatments have inherited drawbacks which may complicate and exaggerate the suffering of the patients. Therefore, the search for a biological, relatively safer, alternative such as biological therapy was highly sought throughout the history of such hard-curable disease. (*Sawadogo ,et al.,2012).* It is widely accepted in the literature, folk and traditional medicines that some bacteria, vegetables, herbs and plants exhibited anticancer potentials by producing some specific compounds that were able to interfere with molecules involved in tumor growth *(Lambert et al., 2010).* An emerging and promising strategy for cancer prevention today is the chemoprevention in which synthetic or natural agents (alone or combination) are encouraged to be used to block the development of cancer in humans *(Abdullaev, 2000).* Taxol, vincristine and camptothecin are just examples of medicinal plants derived anticancer drugs (*Saluja et al., 2011).*

Many studies in vitro and in vivo have examined the antitumor properties of Viscum album (European mistletoe) extracts or certain constituents isolated from these extracts on cancers (Cebovic et al., 2008,). Extract of Viscum album has been used in carcinoma treatment like colon, rectum, or stomach; and breast, (Maticek ,et al.,2001) ovarian (Maticek ,et al.,2007), and urinary bladder carcinoma (Urech, et al.,2006) .

Some studies have shown that some pathogenic microorganisms associated with tumors may produce specific peptides which proliferate inside hypoxic cancer lesions, simultaneously stimulating the immune system of the host during infection, leading to inhibition of cancer progression. (Harvey, 2010). Vaccine strain Mycobacterium bovis Calmette-Guerin (BCG), used in the treatment of superficial bladder cancer (Alexandroff et al., 1999). Azurin from Pseudomonas aergunosa is another bacterial compound that stabilized the tumor suppressor protein p53 leading to apoptosis of cancer cells (Chakrabarty, 2003) and could across the cell surface of host cells enables to distinguish the normal cells from cancerous cells and selectively kill breast cancer cell. (Yamada, et al., 2002,Yamada, et al.,2004 and Punj, et al.,2004) .

The objectives of this study was to explore the therapeutic potentials of *Viscum album* and azurin solely and/or in combination in treatment of Ehrlich ascites carcinoma (EAC) implantation in Swiss albino mice. These two compounds administered to mice either pre or post inoculated with EAC Leeds to improvement in their survival rates.

Materials and methods

Materials:-

Azurin preparation. Azurin was extracted from P. aeruginosa according to the method of *Sankar, and Mandal. 2011,* where the bacterial cell were grown overnight at 37^0C and azurin was precipitated from the supernatant by ammonium sulfate. The crude azurin was obtained after

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dialysis against 0.02M potassium phosphate buffer pH7.4 .

Preparation of aqueous extract of Viscum album: The crude extract was prepared according to the methods of *Handa, et al. 2008* . The dried plant materials were pulverized into powder (50 g) and extracted by maceration with 200 ml of distilled water at room temperature in a rubber-corked bottle for 4 days. The crude extract was filtered, dried in an electric oven at 35-40°C for 6 days and kept at 4°C tell used. The injection solution was prepared and filtered through a Minisart single use, non-pyrogenic filter unit.

Animals. Adult Swiss female albino mice (22-25g) used throughout this study were obtained from National Cancer Research Institute (Cairo, Egypt). They been kept in cages under controlled environment (temperature 25±2°C and 12 h dark and light cycle), and standard veterinarian care *(Lawal, ,et al.,2013).* All methods described here were approved by institutional Animal Ethical Committee ,Mansoura University, Mansoura ,Egypt.

Methods:-

Induction of Ehrlich ascites carcinoma (EAC). Induction of carcinoma was established by injecting 0.2ml of $1x10^6$ cells/mouse obtained from a 25g female albino mouse. *(Lawal, et al.,2013).*

Effect of Viscum album and Azurin on EAC-Bearing Mice : Swiss albino mice were divided into Seven groups(n=15) : Group(I) is a negative control (without EAC inoculation) and Group (II) is a positive control that inoculated with 0.2 ml of EAC (1x106 cells) Intraperitoneal. The treated groups were as follow: groups III were injected with 1.0 ml Viscum album (50mg/kg) in the 1st , 3rd and 6th , and group IV were injected with 1.0 ml of azurin (122 ug/ml) daily, for one week and after 24h of last dose the two groups (III, IV) were inoculated with 0.2 ml of EAC cells (1×106 cells) Intraperitoneal (IP) ; groups V, VI and VII injected with 0.2ml of ECA cells (1×106 cells) Intraperitoneal (IP) first ,and seven days after the inoculation ,they were treated: group V with 1.0 ml Viscum album (50mg/kg) and group VI with (1.0ml) azurin (122 ug/ml), group VII was treated with a combination of Viscum album (50mg/kg) and azurin (122 ug) .

After the last dose followed by 18-h fasting, all mice were sacrificed and blood was collected from each group. The following parameters and markers were measured in the serum of all groups, The activity of superoxide dismutase (SOD) and catalase (CAT) where determined, and the level of reduced glutathione (GSH), Malondialdehyde (MDA), tumor necrosis factor (TNF-α) and interleukin-10 (IL-10) were measured . Our analyses were carried out with the SPSS program. The mean survival time (MST) and percentage increased life span (% ILS) were calculated according to the method of Lawal , et al.,2013 using the following

equation:-

MST (days)= (Day of first death + Day of last death) ÷ 2

ILS (%) =[ )MST of treated group / MST of control group(-1]× 100

Statistical analysis: The results of all measured parameters were statistically expressed as the mean ± S.E.

Results

Mean survival time and life span of animals

Table-(1):Effect of *Viscum album* and azurin treatment on mean survival time and life span of EAC-bearing mice:-

Parameters	Control groups n=15		Pre treatment n=15		Post treatment n=15		
	Group I no EAC	Group II with EAC	Group III VIS+EAC	Group IV AZ+EAC	Group V EAC+VIS	Group VI EAC+AZ	Group VII EAC+AZ+VIS
Mean survival time/day M± S.E	20.80±0.18***	9.75±1.04	17.10±0.9***	14.30±1.50**	14.00±2.27**	13.10±2.70*	18.5±1.50***
Increase life Span% M± S.E	113.33±1.00***		75.36±8.80***	47.14±15.60**	44.17±20.20**	43.59±23.31*	89.72±15.40***

Results are expressed as M ± Standard error of n=15 animals compared with EAC control group(*p<0.05 ,p<0.01 ,***p<0.001). EAC: (Ehrlich ascites carcinoma),VIS:(Viscum album), AZ :(azurin) , MST (Mean Survival time), ILS% (Increased life span%).**

In the control group (II) bearing mice, a regular rapid increase in ascetic tumor volume was observed. The Mean Survival time (MST) of animals was found to be 9.75±1.04 days, But in group III and IV found to be 17.1±0.9 and 14.3±1.5 respectively (P<0.001) group V and group VI the life span increases and the average was 14.0±2.27 and 13.1±2.7 respectively (P<0.001). whereas very highly significant increase was in group VII 18.5±1.5 (p<0.001). Viscum album has a 75.36% increased lifespan in group(III) pre EAC inoculation , and it was 44.17% in group(V) post inoculation, while injection of azurin to group(IV) pre EAC inoculation,increase lifespan with 47.14 % and and 43.59% in group(VI) with post EAC inoculation. A combination of the two therapeutic agent has the greatest effect, it was 89.72% The reliable criteria for judging the value of an anticancer treatment cancer was prolongation of the life of all treated animal groups.

Table (2): Effect of treatment on superoxide dismutase (SOD) , catalase (CAT) serum activity, Malondialdehyde (MAD)and reduced glutathione (GSH) serum level, in of EAC -bearing mice .

Parameters	Control n=15		Pre treatment groups n=15		Post treatment group n=15		
	Group I Without EAC	Group II With EAC	Group III Vis+EAC	Group IV AZ+EAC	Group V EAC+VIS	Group VI EAC+AZ	GroupVII EAC+AZ+VIS
SOD(u/l) M± S.E	27.8± 4.20***	2.38±0.26	13.38±1.27***	20.53±0.19***	9.85 ± 0.70***	8.44 ± 0.71+++	22.66±4.97***
CAT(mmol/l) M± S.E	45.20±6.59***	10.62 ± 0.82	22.43±1.54***	24.44±2.66***	19.62 ± 1.40***	15.26± 1.32*	43.53±1.74***
MDA(mmol/l) M± S.E	17.49±1.70***	183.34±0.08	45.35±14.99***	45.75±3.02***	65.59±16.63***	108.30±20.14***	21.77±4.73***
GSH (mg/dl) M± S.E	14.87±1.93***	3.49 ± 0.15	7.99± 0.55***	8.57 ± 0.21***	6.10 ± 0.97***	4.82 ± 0.85*	10.22±0.87***

Results are expressed as :Mean ± Standard error (M± S.E) of n= 15 animals compared with EAC control group(*p<0.05 ,p<0.01 ,***p<0.001). EAC: (Ehrlich ascites carcinoma),VIS:(Viscum album),AZ: (azurin),superoxide dismutase (SOD), catalase (CAT) reduced glutathione (GSH), malondialdehyde (MDA),**

The EAC development was responsible for significant decrease in serum level activity of superoxide dismutase (SOD) catalase (CAT) reduced glutathione (GSH), but Malondialdehyde (MDA) serum level was highly increased . After treatment with Viscum album and azurin, (SOD) (CAT) activity, (GSH) level were increased ,and (MDA) level was decreased in all groups (III, IV, V , VI VII). Superoxide dismutase (SOD) were very highly significant increased p<0.001 in all groups(III, IV,V,VI, VII,) , comparing to group II.

Catalase (CAT) were very highly significant increased (p<0.001) in groups (III,IV,V,VII). While it was significant p<0.05 in group VI,. Malondialdehyde (MDA) were very highly significant decreased (p<0.001) in all groups(III ,IV ,V ,VI ,VII) compared to group II. Where glutathione (GSH) were

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very highly significant increased (p<0.001) in group (III,IV,V,VII) but there were significant increased p<0.05 in groups (VI)

Superoxide dismutase (SOD) was shown in Fig (1), catalase (CAT) in Fig (2),malondialdehyde (MDA) in Fig (3), and Glutathione (GSH) in Fig (4), Respectively.

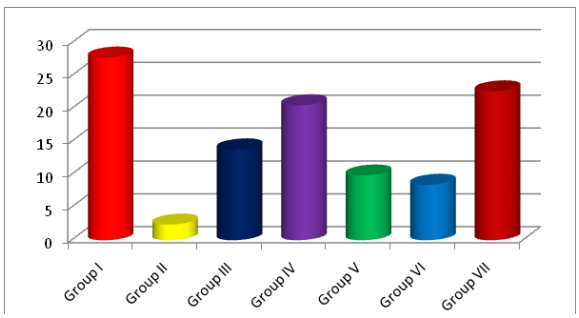

Fig. (1):Effect of Viscum album and azurin treatment on the activity of Super oxidase dismutase (SOD) serum level in EAC bearing mice .

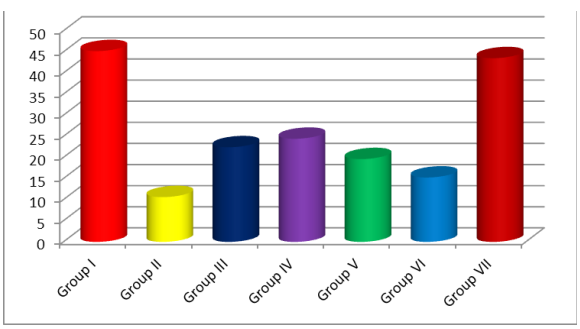

Fig. (2): Effect of Viscum album and azurin treatment on the activity of Catalase (CAT) serum level in EAC bearing mice .

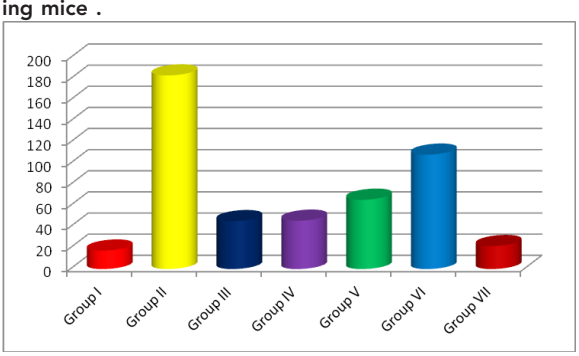

Fig.(3): Effect of Viscum album and azurin treatment on the serum level of malondaialdehyde (MDA) in EAC bearing mice .

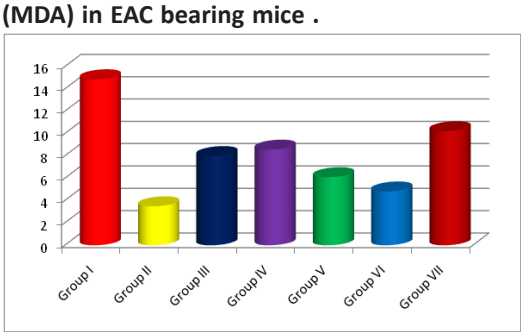

Fig. (4): Effect of Viscum album and azurin treatment on the serum level of reduced glutathione (GSH) in EAC bearing mice.

Table (3) The effect of Viscum album and azurin on Interleukin (IL-10) and Tumor necrosis factor TNF-α serum level, in of EAC -bearing mice :-

Parameters	Control n=15		Pre treatment groups n=15		Post treatment group n=15		
	Group I without EAC	Group II with EAC	Group III VIS+EAC	Group IV AZ+EAC	Group V EAC+VIS	Group VI EAC+AZ	Group VII EAC+AZ+VIS
IL10(pg/ml M±S.E	15.46±0.70*	127.81±1.40	30.22±3.19***	54.23±4.60***	55.38±5.19***	73.0±9.20***	35.79± .41***
TNF-α (pg/ml) M±S.E	10.24±0.70*	40.98±1.48	24.83±2.59***	27.33±0.20***	32.05 ± 2.30***	33.74±3.00***	14.46±1.59***

Results are expressed as Mean ± Standard error (M± S.E) of n= 15 animals: P*<0.05, P<0.01 ,P***<0.001. EAC :(Ehrlich ascites carcinoma),VIS:(Viscum album), AZ:(azurin),tumor necrosis factor :(TNF-α),Interleukin-10:(IL-10).**

The levels of interleukin 10 (IL-10) in serum was significantly higher in group I as compared to that of group II (p<0.05), while, there was highly significant decreased in group (III, IV, V ,VI and VII) after treated with *Viscum album* and azurin.The pre and post treatment effect of *Viscum album* and azurin on interleukin 10 (IL-10) serum level was represented in Fig.(5).

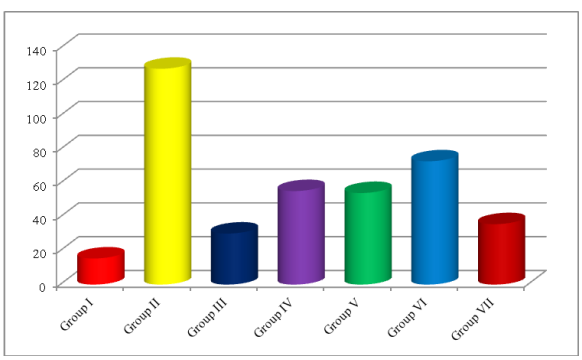

Fig.(5): Effect of Viscum album and azurin treatment on interleukin 10 (IL-10) activity serum level of EAC bearing mice .

The effect of Viscum album and azurin treatment on TNF-α serum level was shown in table (3).It showed that the levels of tumor necrosis factor (TNF-α) in serum was significant increased in control group II as compared to the group I (P<0.05). While, it was very highly significant decreased in groups (III, IV, V , VI and VII) as compared to that of group II .The comparison between different groups before and after treatment on TNF-α level was shown in Fig.(6).

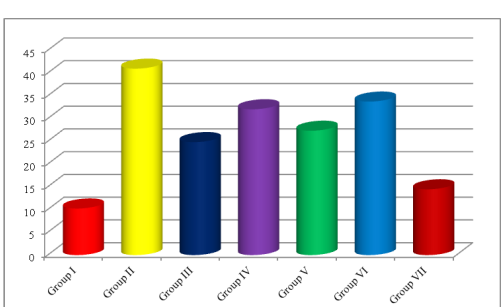

Fig.(6): Effect of Viscum album and azurin treatment on tumor necrosis factor (TNF-α) activity in serum level of EAC bearing mice .

Discussion:

The present study was to explore the therapeutic potentials of *Viscum album* and azurin solely and/or in combi-

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nation in the treatment of Ehrlich ascites carcinoma (EAC) in Swiss albino mice. These two compounds administered to mice either before or after EAC inoculation. It was observed that both Viscum album and azurin separately or in combination were increased the life span of EAC-bearing mice treated , this was in agreement with *Adreani et al., 1983* who has suggested that an increase in the lifespan of ascites-bearing animals by 25% is considered indicative of significant drug activity.

Our results showed that there was highly decrease in activity of CAT, SOD and GSH level in all inoculated EAC groups (II,III,IV,V,VI,VII) at the end of our experiment, but after treatment with Viscum album and azurin, the activity of CAT, SOD and GSH level were increased in all treated groups,(III ,IV ,V ,VI , and VII), this result was in agreement with the results of Sarpataki ,2014 and Bhattacharya, 2011 .These increase of activity of antioxidant enzymes was due to phenolic content of Viscum album and its antioxidant ability, (Alali et al., 2007, Tosun et al., 2009,Kılıcgun and Altıner, 2010, and Song et al., 2010). Regarding azurin ,the studies proved that azurin is anticancer agent, by acting in three different forms: 1) entering into the cancer cell and stabilizing the tumor suppressor protein p53, thus inducing apoptosis; 2)interfering extracellularly in the ephrin/Eph signaling system that is involved in tumor progression, angiogenesis, migration and invasion (Chaudhari et al., 2007); and 3) inhibiting the vascular endothelial growth factor A involved in angiogenesis (Mehta et al., 2011).

Malondialdehyde (MDA) is used as an indicator of tissue damage involving a series of chain reactions in cancer status *(Salzman,et al.,2009).* In our study, tumor bearing mice showed a highly significant increase level of MDA in all inoculated groups and it was highly decreased after treated with our therapeutic agent lonely or in combination ,but combination were giving better result. This prove that our agents have anticancer effect and in agreement with other studies, *(Vadivel, et al., 2011 Punith, et al.,2011 and Saroja,et al.,2012).*

Interleukin 10 (IL-10) and tumor necrosis factor (TNF-α) level were very high in all inoculated EAC-bearing mice groups (II,III,IV.V,VI,VII), while it was decreased after treated with our agents, It was reported that in all experimental models, IL-10 can inhibit protective immune response during infection (Moore et al., 2001), and it is overexpression induced immunodepression in the major surgery which predispose to infectious complications, (Kobayashi et al., 2001).

The pro-inflammatory and tumor sustaining effects of TNF-α are diminished by blocking one of the major tumor pro-survival pathways. Inflammatory cell involvement and cytokine release was evoked as an important factor in tumor growth inhibition, *(Hsu et al., 2011)* . However, the data here indicate that both azurin and Viscum album directly and/or indirectly induces immune cell proliferation and cytokine production , and may function was to reverse tumor-induced immunosuppression and may delay tumor outgrowth through immunotherapeutic mechanisms , and this agreement with study of*(Licastro,et al.,1993 and Yamamoto, et al.,1995).*

The immunomodulating and anti-cancer activities of Viscum album are caused by biologically active components like lectins (glucoproteins), viscotoxins, alkaloids and polysaccharides, (Hajto et al.,1989) . The reduction of TNF-α and IL-10 in treated experimental models lead to suggests an effective inter-relation among cytokines and inhibition of Ehrlich ascites cell growth . IL-10 was produced in higher quantities than TNF-α, so we believe that IL-10 is the main cytokine associated with Ehrlich ascites growth, either as a growth factor or a host suppressor factor .

Conclusion

Azurin and Viscum album were found to be cytotoxic to tumor cells and they also reduced the tumor development in Ehrlich ascites tumor model. Viscum album extract and azurin had been proved to exhibit immunomodulatory and anti-inflammatory activities and had antioxidant effect, also the present data suggested that pre-treatment was more significantly with the establishment of ascites, than post-treatment.

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