Egypt. J. Rad. Sci. Applic., Vol. **27**, No. 1-2, pp. 1-15 (2014)

Effect of Egyptian Propolis Extract as an Adjuvant with Irradiated Cancer Vaccine against Ehrlich Ascites Carcinoma in Mice

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PROPOLIS is a non-toxic natural substance with multiple pharmacological properties including anti-cancer and antioxidant. The objective of this study was to investigate the effect of Egyptian propolis extract (Prop) as an adjuvant co-injected with irradiated tumour cell lysate vaccine (Irr-V) against Ehrlich ascites carcinoma (EAC) in mice. Animals were divided into five equal groups (n=10). Control group. EAC group; injected with viable EAC ($2x10^5$/mouse) in the right thigh. EAC-Prop group; injected subcutaneously (Sc) with Prop (0.4mg/ mouse) weekly for three times, then after 2 weeks mice were received EAC viable cells (the day of challenge). Irr-V group; vaccinated with irradiated EAC cell lysate weekly for three times at a dose of 0.2µl in the right thigh. Prop-Irr-V group; vaccinated as Irr-V group, and treated with Prop as EAC-Prop group. Two weeks post the last treatment; animals of groups 4 and 5 were challenged with normal viable EAC ($2x10^5$/mouse) in the opposite thigh. Results: The results revealed a decrease in red blood cells (RBC) count, haematocrite value (Hct) and haemoglobin content (Hb) and an increase in total leucocytes, absolute lymphocyte and neutrophil counts in EAC-bearing mice. Furthermore, oxidative stress identified by a decrease in glutathione (GSH) content and superoxide dismutase (SOD) activity associated with an increase in the content of advanced oxidation protein products (AOPP) and malondialdehyde (MDA) were recorded in the liver and blood tissues of EAC-bearing mice. Propolis, Irr-V as well as Irr-V-Prop treatment improved haematological toxicities and oxidative stress in EAC-bearing mice. However, improvement was more pronounced in Irr-V-Prop group and the cell viability assay, the tetrazolium dye;3-(4,5-dimethylthiazol-2-yl)-2,5-iphenyltetrazolium (MTT) showed a significant decrease in viable cells compared to each treatment alone. It could be concluded that Prop extract might be used as an adjuvant for irradiated cancer vaccines in cancer therapy.

Keywords: Egyptian propolis, cancer vaccine, Ehrlich ascites carcinoma, mice.

Propolis is a mixture of plant resins and bee secretions collected by honeybees and characterized by multiple pharmacological properties and healthcare functions (Zhang *et al.,* 2013). Bees gather propolis from diverse resinous plant parts and in different phytogeographic regions (Popova *et al*., 2013). The biological properties of propolis are related to its chemical composition and more specifically to the phenolic compounds that vary in their structure and concentration depending on the region of production, availability of sources to collect plant resins, genetic variability of the queen bee, the technique used for production, and the season in which propolis is produced (Toreti *et al*., 2013). Propolis possesses beneficial biological effects, including antimicrobial (Scazzocchio *et al*., 2006), antioxidant (Valente *et al.,* 2011) anti-inflammatory (Tan-No *et al.,* 2006), anticancer (Orsolic *et al*., 2006) and immunostimulant (Sforcin, 2007).

A combination of immunotherapy with chemotherapy, radiotherapy and surgery for cancer is an emerging challenge and an emerging paradigm, in contrast to the concept that defines most standard treatments as immunosuppressive (Ridolfi *et al*., 2010). Anti-cancer vaccination strategies are already focused on combination with other immunotherapeutic strategies (Andersen *et al*., 2008). Cancer vaccines act for stimulating an immune response as a mean of combating cancer encompasses a number of approaches, including whole tumour cell vaccines, tumour-extracted protein vaccines, tumour antigen vaccines, dendritic cells (DCs)-mediated vaccines, and tumour antigen-encoding virus vaccines. Most cancer vaccines are designed to be therapeutic vaccines (Pecorino, 2005). Cancer cellular vaccines can be packed in at least three forms; DCs, Autologous or allogenic tumour cells and Tumour-APC hybrids (Delgado, 2002). These vaccines can be delivered alone or with adjuvants (Li *et al*., 2009). The first cancer vaccines were composed of irradiated tumour cells, being modelled after successful attenuated pathogen vaccines (Pecorino, 2005). There is no standard dose of gamma-irradiation with which it could be pre-treat tumour cells in vitro prior to their use in a vaccine. Previous studies have used gamma radiation ranging from 25 to 200 Gy. By using gamma irradiation, the tumour cells lose their proliferative function (Deacon *et al*., 2008). Propolis has been suggested to be a promising adjuvant substance in duck inactivated vaccines (Cai *et al*., 2001). Total ethanolic

extracts of green propolis have been shown to improve humoral and cellular immune responses in mice inoculated with an inactivated vaccine against bovine herpes-virus type 5 (Fischer *et al.,* 2007).

The study aims to investigate the effect of propolis co injected with Irr-V against EAC in mice. Cell viability assay, haematological profile, GSH contents and SOD activities in blood and liver were determined. AOPP, MDA concentrations were assessed in serum and liver.

Materials and methods

Experimental animals

Fifty adult male mice, weighing (24± 2 g) were obtained from the Nuclear Research Centre (NRC), Atomic Energy Authority, Anchas, Egypt. Mice were allowed to acclimate in metal cages inside a well-ventilated room for 2 weeks prior to the commencement of the experiment. They were maintained under standard laboratory conditions, were fed a standard commercial pellet diet and water *ad libitum*. Animals were randomly divided into five equal groups (n=10). Control group; normal healthy mice. EAC group; injected with viable EAC (2x105/mouse) in the right thigh. EAC-Prop group injected Sc with Prop (0.4mg/mouse) weekly for three times, then after 2 weeks mice were received EAC viable cells (the day of challenge). Irr-V group; vaccinated with irradiated EAC cell lysate weekly for three times at a dose of 0.2µl in the right thigh. Prop Irr-V group; vaccinated as Irr-V group and treated with Prop as EAC-Prop group. Two weeks post the last treatment animals of groups 4 and 5 were challenged with normal viable EAC ($2x10^5$/mouse) in the opposite thigh.

Ethanol extraction of Prop

Prop was purchased from the Local market. The ethanol extraction was performed according to Najafi *et al.* (2007) with modification. In the first step Prop was extracted by ethanol, Prop (90g) was added into 400 ml of 96% ethanol and mixed for 18 h at 15°C. The mixture was then centrifuged at 7000 rpm for15 min at 20°C. The supernatant was collected and the pellet was re-extracted with 100 ml ethanol. After pooling the supernatants of both steps, they were used for the experiments.

Cell Line of EAC; Mus muscles which is a fibroblast like in shape (Sato *et al*., 1961) was obtained from the National Cancer Institute (NCI), Cairo university. The cells were propagated as ascites in female Swiss albino mice after intra peritoneum inoculation.

Radiation facility

Different doses of gamma rays (2KGy, 4KGy, 6KGy or 8KGy) were examined and 8 KGy dose was chosen to precede the experiment and given at a dose rate of 0.49 Gy/min. Irradiation was performed at NCRRT, using a Gamma Cell-40 (^{137}Cesium) biological irradiator manufactured by the Atomic Energy of Canada Ltd, Ottawa, Ontario, Canada.

Vaccine prepared from tumour cell lysate

EAC cells were lysed according to method Schnurr *et al*. (2001) with modification. Briefly, Cells were incubated with 0.01 % EDTA solution for 10 min. The cells were washed twice in PBS, and re-suspended at a density of $5x10^6$/ml in serum-free medium .The cell suspensions were frozen at 80 °C and disrupted by four freeze-thaw cycles. For the removal of crude debris, the lysate was centrifuged for 10 min at 300xg.The mice were injected by supernatant cell lysate.

MTT assay

MTT assay was assessed according to Freimoster *et al.* (1999) with modification. Briefly, 300µl of MTT solution (0.5 mg MTT/ml) were added to each well and incubated in 5% CO_2 incubator for 4hrs. Cells were pelleted by centrifugation (15,000xg) for 5 min. The media were removed. 500 µL of mixture Isopropanol /HCl were added (2 ml of 0.1 N HCl in 23ml isopropanol). The samples were vortexed vigorously and the absorbance (abs) was measured at 560 nm. The viability was calculated. The percentage of viable cell = sample abs/control absx 100. Spleen was removed from animal immediately after dissection; each spleen was placed into RPMI medium. Then spleen was disaggregated. The spleenocytes were poured from strainer to remove any debris then centrifuged for 7min at 600xg at room temperature. The pellet was flicked gently and re-suspended in 10 ml RPMI media (Rowland-Jones and Mc-Michael, 2000).

Blood sample collection and haematological and oxidative stress assays

Two blood samples were immediately collected by heart puncture. The first sample was collected in heparinised tube (2.25µL heparin/5 ml blood) for haematological assays. The second blood sample was centrifuged at 1000xg for 10 min, to collect the serum which was stored at -20°C until analyzed. The liver was dissected out and washed. For oxidative stress biomarkers, a known weight of the organ was used to obtain a 10 % tissue homogenate in 0.15M KCl. Blood picture was investigated using an automated haematology analyzer (BC-2800vet; Mindray, China). GSH content was measured by spectrophotometer at 412 nm (Beutler *et al*., 1963). SOD activity was determined according to the method of Minami and Yoshikawa (1979). Lipid peroxidation is based on the determination of MDA which reacts with thiobarbituric acid in acidic medium to yield a pink coloured trimethine complex at 532 nm (Yoshioka *et al*., 1979). AOPP were measured by spectrophotometer on a microplate reader at 340 nm (Witko-Sarsat *et al*., 1998).

Analysis of data

The results are presented as percentage and mean± S. E. Statistical analysis was performed using one-way analysis of variance (ANOVA), statistical package of social science (SPSS) version 15.0 for windows. Individual difference among groups was analysed by Duncan's test. Significance was accepted at $P< 0.05$.

Results

EAC-bearing mice showed a significant decrease in Hb content and Hct value and RBC count compared with normal control group. In Irr-V Group, Hb content and Hct value and RBC count were increased significantly ($P< 0.05$) compared with EAC- group. Prop (0.4 mg/mouse) co-injected Irr-V (0.2µl of cell lysate, 3 weeks) in EAC-bearing mice elevated significantly the decrease in Hb content and Hct value and RBC count compared to EAC and Irr-V groups (Table 1). EAC-bearing mice showed a significant increase in total leukocyte, neutrophil, and lymphocyte counts compared to control group. In Irr-V Group, total leukocyte, neutrophil and lymphocyte counts were decreased significantly as compared with EAC group. Prop co-injected with irradiated tumour cell lysate vaccine in EAC-bearing mice decreased significantly ($P< 0.05$) the

increase in total leukocyte, neutrophil, lymphocyte counts as compared to EAC and Irr-V groups (Table 1).

TABLE I. Leucocyte, neutrophil, lymphocyte ($x10^3/mm^3$) and RBC ($x10^6/mm^3$) counts and Hb content and Hct values in the different groups.

Groups	Control	EAC	Irr-V	EAC-Prop	Prop-Irr-V
RBC	6.3± 0.5	3.8± 0.2^{acd}	4.8± 0.3^{bd}	4.5± 0.3^{d}	5.9± 0.3^{bc}
Hb (g/dl)	14.4± 0.9	8.1± 0.5^{acd}	10.4± 0.7^{bd}	9.7± 0.7^{d}	12.7± 0.7^{bc}
Hct (%)	32.6± 1.5	19.4± 1.2^{acd}	23.9± 1.6^{bd}	23.2± 1.7^{d}	29.9± 1.6^{bc}
Leucocytes	6.1± 0.5	11.1± 0.8^{acd}	8.8± 0.5^{bd}	8.5± 0.8^{b}	6.8± 0.5^{bc}
Neutrophils	1.3 ± 0.1	3.2± 0.2^{acd}	1.9± 0.1^{b}	2.4± 0.2^{b}	1.5± 0.1^{bc}
Lymphocytes	4.8± 0.4	7.9± 0.5^{acd}	6.6± 0.4^{bd}	5.9± 0.6^{b}	4.9± 0.4^{bc}

a:significance with control, b: significance with EAC, c: significance with Irr-V, d: significance with Prop-Irr-V.

Data in Table 2. showed that EAC-bearing mice showed a significant decrease in blood GSH concentration and SOD activity and significant ($P< 0.05$) increase in serum AOPP and MDA contents compared to control group. Irr-V Group showed significant decrease in contents of serum AOPP and MDA as well as significant increase GSH level, SOD activity as compared to EAC-bearing mice. Combination treatment of Prop (0.4 mg/ mouse) as an adjuvant with Irr-V (0.2µl of cell lysate, 3 weeks) against EAC in mice reduces significantly AOPP, MDA concentrations and enhances significantly GSH content and SOD activity as compared to EAC and Irr-V group.

TABLE 2. Blood GSH content, SOD activity and serum MDA and AOPP levels in the different groups.

Groups	Control	EAC	Irr-V	EAC-Prop	Prop-Irr-V
GSH (mg/dl)	35.8± 1.4	21.7± 1.8^{ad}	25.9± 1.0^{bcd}	25.1± 1.0^{d}	30.3± 1.5^{bcb}
SOD (U/ml)	5.3± 0.3	2.6± 0.2^{ad}	3.2± 0.3	3.8± 0.4^{bd}	4.5± 0.5^{bc}
AOPP (µmol/l)	26.3± 2.1	39.2± 2.4^{acd}	33.4± 1.8^{bd}	31.0± 1.6^{b}	27.6± 1.8^{bc}
MDA (nmol/l)	46.1± 1.6	70.7± 2.3^{acd}	53.9± 1.8^{bd}	56.2± 1.9^{bd}	47.9± 1.3^{bc}

Legends as in Table 1.

Data in Table 3. showed that EAC-bearing mice showed a significant decrease in liver GSH concentration and SOD activity and significant increase in liver AOPP and MDA as compared to control group. Irr-V Group showed significant decrease in contents of serum AOPP and MDA as well as significant increase GSH level, SOD activity as compared to EAC-group. Combination treatments EAC in mice inhibit significantly ($P< 0.05$) AOPP, MDA

concentrations and enhances significantly GSH content and SOD activity as compared to EAC and Irr-V groups.

TABLE 3. Hepatic GSH & MDA levels (nmol/ g tissue) and SOD (U/ g tissue) activity and AOPP (µmol/g tissue) in the different groups.

Groups	Control	EAC	Irr-V	EAC-Prop	Prop-Irr-V
GSH	24.8± 1.5	14.5± 1.3^{acd}	19.2± 1.4^{b}	19.4± 1.1^{bcd}	23.4± 1.7^{bc}
MDA	185.4± 3.1	223.6± 4.3^{acd}	204.1± 4.7^{bd}	197.9± 4.5^{b}	190.9± 3.6^{bc}
SOD	126.0± 3.1	103.8± 3.0^{acd}	118.2± 3.4^{bd}	122.0± 2.6^{b}	128.8± 2.8^{bc}
AOPP	206.1± 6.7	267.3± 4.7^{acd}	243.2± 4.8^{bd}	246.3± 6.1^{bc}	225.7± 5.2^{bc}

Legends as in Table 1.

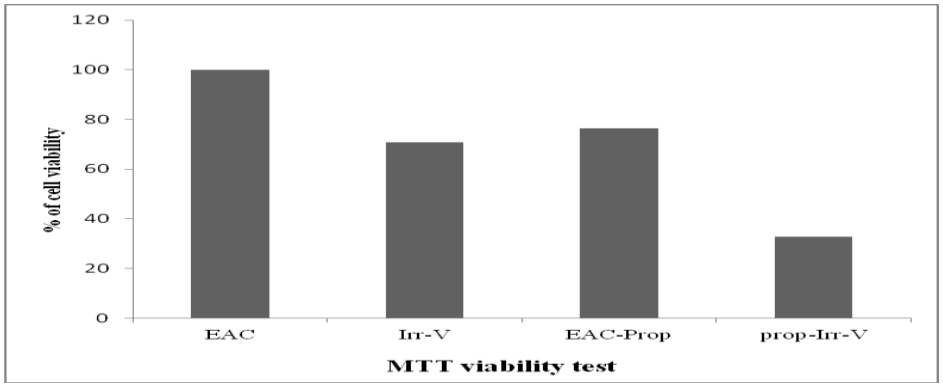

Fig.1. MTT viability test in different groups.

The MTT results in Fig.1. showed a decrease in the cell viability in animals treated with Prop, irradiated tumour vaccine alone as compared to EAC-group. In combination treatment, MTT showed a significant decrease in viable cells compared to each treatment alone.

Discussion

In the present study, anaemia occurs in tumour-bearing mice represented by significant reduction in RBC count, Hb content and Hct value. These findings corroborate previous results (Badr *et al*., 2011 and Pandya *et al*., 2013). This may occur either due to iron deficiency or due to haemolytic or other myelopathic conditions (Shivhare *et al*., 2011) such as suppressive effect of EAC cells on bone marrow erythropieosis (DeGOwin and Gibson, 1978). Granulocytic leucocytosis that was observed might be due to the acute inflammatory response or stress due to the proliferation of EAC cells (Hashem *et al*., 2004).

EAC-induced oxidative stress represented by significant decrease in the SOD activity and GSH level in blood and liver tissues and increased in AOPP and MDA in serum and liver of EAC bearing mice. Gupta *et al.* (2001) demonstrated that reduction in several antioxidant defence mechanisms correlates with the emergence of the malignant phenotype. The implication of free radicals in tumours is well documented (Ravid and Korean, 2003). The observed decrease in the SOD activity in EAC bearing mice might be due to the loss of the Mn SOD activity in EAC cells and loss of mitochondria, leading to a decrease in the total SOD activity in the liver (Sun *et al*., 1989). SOD-anions (O_2 -) to H_2O_2 and protects the cells against (O_2-) mediated lipid peroxidation. MDA, the end-product of lipid peroxidation, was reported to be higher in tumour tissues than in non diseased organs (Yagi, 1987). GSH is a major non-protein thiol required for the proliferation and metabolism of tumour cells (Guruvayoorappan and Girija Kuttan, 2007). GSH is regulator of protein synthesis, DNA synthesis and cell proliferation (Suthanthiran *et al*., 1990). Moreover, some amino acid precursors for GSH synthesis have been shown to be essential in cancer metabolism, e.g. glutamine (Moreadith and Lehninger, 1984) and methionine (Hoffman, 1985). The decrease in GSH content in EAC-bearing mice occurred through impaired GSH synthesis, which is associated with an increase in oxidized glutathione. Peroxide production by tumour cells–can lead to GSH oxidation within the red blood cells and different tissues (Badr El-Din, 2004). This may occur due to the decreased transport activity of the oxidized GSH through membranes with a decrease in the activity of glutathione reductase which is augmented by riboflavin deficiency (Abou-Bedair *et al*., 2002).

The Prop-treated mice bearing EAC at the doses of 0.4 mg/mouse ameliorated the haematological parameters. The extract also reduced the elevated levels of lipid peroxidation and AOPP and increased the GSH content and SOD activity in EAC-treated mice. It was reported that Prop-containing antioxidant principles depends mainly on the presence of flavonoids (Nakajima *et al*., 2009) that have been reported to enhance immune system (Wleklik *et al*., 1987). Two main immune potent chemicals of propolis have been identified as caffeic acid phenethyl ester (CAPE) and artepillin C. Propolis, CAPE, and artepillin C have potential antitumor properties by different postulated mechanisms such as suppressing cancer cells proliferation via its anti-

inflammatory effects; decreasing the cancer stem cell populations; blocking specific oncogene signaling pathways; exerting antiangiogenic effects and modulating the tumour microenvironment (Chan *et al*., 2013). The decrease of live cells could be due to the interference of propolis constituents with the growth of EAC cells directly during the early phase of treatment and leading to a considerable elimination of these cells (Orsolic *et al*., 2005). Increased lymphocyte proliferation lead to enhance macrophage activation and thus an amplification of general immunological responses (Stuehr and Nathan, 1989). Antitumor activity of these antioxidants is either through induction of apoptosis (Ming *et al*., 1998) or by inhibition of neovascularization (Keshavarz *et al*., 2009). The free radical hypothesis supported the fact that the antioxidants effectively inhibit the tumour (Ravid and Korean, 2003).Thus, the present findings indicate that the ethanol extracts of propolis contain components that may have anticancer activity (Ishihara *et al*., 2009). phenolic acid esters are present (72.7 %) in a major quantity in Prop (Abdel-Hady, 1994).

Irradiated cancer vaccine treated animals bearing EAC showed significant decrease in cell viability compared to control. The total leukocyte, neutrophil, lymphocyte counts were increased as compared with EAC group. Vaccines would seem to trigger immunologic memory and thus subsequent treatments that are capable of up regulating tumour-associated antigen expression (Ridolfi *et al*., 2010). From an immunologic perspective, many cancer vaccines have been shown to induce significant specific humeral or T-cell responses to the tumour antigens being targeted, and some have induced levels of CD8 T cells approaching those seen in response to foreign pathogenic viruses (Berinstein, 2007).

Prop co injected with irradiated cancer vaccine by restoring significantly the haematological parameters towards normal, exhibits its therapeutic role on haematopoietic system. Also, the results showed that significantly restored the MDA, AOPP as well as GSH and SOD in blood and liver tissues of EAC bearing mice as compared to Irr-V group. Cytotoxic effect of Prop treated group and irradiated tumour cell lysate vaccine made a decrease in the viability of EAC cells, whereas the cytotoxicity effect of the co injected vaccine with prop made a significant decrease in the viability of EAC than each treatment alone.

Stegel *et al.* (2006) demonstrated that the mature DCs could be efficiently maturated in vivo through vaccination of mice with irradiated tumour cells in combination with C-class CpG oligo-deoxynucletides (CpG ODN). Also they confirmed that processes triggered by this vaccination included the activation of the effectors of native immunity (phagocytes) as well as the activation of Cytotoxic T lymphocytes. However, the main cells influenced by the vaccine appeared to be the phagocytes, including the DCs and the main process their maturation (Novakovic *et al*., 2007). Cerkovnik *et al.* (2011) demonstrated that by the tumour vaccine composed of CpG ODN and irradiated tumour cell the antigen presenting cells, including the DCs are manipulated in vivo. By this kind of vaccine, the differentiation and maturation of antigen presenting cells (APCs) is triggered primarily in the spleen and is subsequently followed by the migration of these APCs to the bone marrow. Once in the bone marrow, these APCs (especially the DCs) play a crucial role in the development and maintenance of long-lived memory T cells capable of preventing a relapse of malignant disease.

In conclusion: Prop extract as an adjuvant with irradiated tumour cell lysate vaccine reduces haematological toxicities, decrease Lipid peroxides and AOPP levels in serum and liver , improves blood and liver GSH content , SOD activity induced by EAC, restrains oxidative stress and prevents injuries. The present study provides details about the effect of prop extract as an adjuvant with irradiated tumour cell lysate vaccine in EAC-bearing mice, suggesting that it could be a potential therapeutic agent for cancer therapy, though the mechanism of the thought effect is not yet clear.

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(Received: 06/04/2014;

accepted: 03/06/2014)

تأثير مستخلص صمغ النحل المصرى كمادة محفزة مع لقاح اجزاء الخلايا السرطانية المشعة ضدالفئران المحملة بخلايا ارليخ السرطانية

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قسم بيولوجيا الإشعاع ، المركز القومي لبحوث وتكنولوجيا الإشعاع ، ص. ب. ٢٩ مدينة نصر ، مصر.

صمغ النحل هو مادة لزجة تجمع بواسطة النحل من النباتات .وكانت تستخدم قديما كدواء . الهدف من هذه الدراسة هو بحث تأثير صمغ النحل كمادة محفزة مع لقاح اجزاء الخلايا السرطانية المشعة ضد خلايا اورام ارليخ فى الفئران .
تم قياس السمية الخلوية ،صورة الدم ، كما تمت دراسة الاجهاد التأكسدى. وقد اظهرت النتائج ان الفئران الحاملة لورم ارليخ حدث بها انخفاض فى عدد خلايا الدم الحمراء و نسبة الهيموجلوبين كذلك فقد انخفض محتوى الجلوتاثيون والسوبر اكسيد ديسميوتيز . وقد زادت نسبة نواتج البروتين حديث التأكسد وخلايا الدم البيضاء ونسبةاكسدة الدهون. اما بالنسبة للمجموعة الحاملة لورم ارليخ و المعالجة بصمغ النحل ٤ مجم/ فأر لمدة٤ ايام فقد لوحظ تحسن الانخفاض فى عدد خلايا الدم الحمراء و نسبة الهيموجلوبين . كما لوحظ التحسن فى زيادة عدد خلايا الدم البيضاء .ان استخدام صمغ النحل المصرى فى علاج الفئران المحملة بورم ارليخ قلل من زيادة نواتج البروتين حديث التأكسد و اكسدة الدهون وحسن نسبة الجلوتاثيون والسوبر اكسيد ديسميوتيز . كما اظهرت النتائج ان استخدام صمغ النحل المصرى فى الفئران المحملة بورم ارليخ يعدل السمية الدموية المتمثلة فى نقص العد الكلى لخلايا الدم الاحمر والابيض والاجهاد التأكسدى من خلال زيادة نواتج البروتين حديث التأكسد و اكسدة الدهون وحسن نسبة الجلوتاثيون والسوبر اكسيد ديسميوتيز والاضطرابات الحيوية وكذلك يمكن ان يحسن الاستجابة الخلوية فى الفئران الملقحة باجزاء الخلايا السرطانية المشعة. وهذه النتائج اظهرت ان صمغ النحل عند استخدامه كمادة محفزة مع لقاح اجزاء الخلايا السرطانية المشعة يعطى نتائج افضل من استخدام اللقاح فقط .