

# Potential Defensive Effect of Royal Jelly Compared to Cyclophosphamide Induced Hemotoxicity

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## ABSTRACT

**Background:** A major side effect of cyclophosphamide (CPA) during chemotherapy is hemotoxicity. Use of natural products like royal jelly reduced the adverse effect of a drug. A current study was an effort to find out the potential defensive effects of royal jelly against hemotoxicity induced by cyclophosphamide in male albino mice.

**Methods:** Male Swiss albino mice of 20±5 gms were unevenly divided into six groups; G1: normal control group 0.9% saline solution I.P. weekly, G2: royal jelly (100 mg/kg/d) CMC suspended administered by orally, G3: cyclophosphamide (50 mg/kg/week) was injected intra-peritoneally, G4: I.P. cyclophosphamide (50 mg/kg/week) along with royal jelly (100 mg/kg/d), G5: I.P. cyclophosphamide (50 mg/kg/week) with royal jelly (250 mg/kg/d), G6: I.P. cyclophosphamide (50 mg/kg/week) and royal jelly (500 mg/kg/d). The experiment lasted for 12 weeks. The measurement of hematological parameters CBC was performed using automated hematology system. Mean±SEM one way ANOVA followed by Tukey's test were performed to find out the significant difference between groups.

**Results:** Cyclophosphamide treated mice exhibit leucopenia, erythrocytopenia, thrombocytopenia and the significant reduction in hemoglobin (Hb), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) as compared to control group. The administration of royal jelly to CPA treated mice, according to the present experimental plan significantly improves the alterations induced in haemogram.

**Conclusion:** It was suggested that royal jelly ameliorate cyclophosphamide-induced hematological alterations, thus it might be used as a dietary protective natural remedy during the chemotherapy.

**Key-words:** Cyclophosphamide, Erythrocytopenia, Haemotoxicity, Leucopenia, Royal jelly, Swiss albino mice, Thrombocytopenia

## INTRODUCTION

Since the late 1950s the alkylating agent CPA was in general used in chemotherapy because of its curative quality and wide-range of activities against a wide range of cancers [1]. The most common use of cyclophosphamide is in the treatment of different types of hematological and solid malignancies, autoimmune disorders like rheumatoid arthritis, systemic lupus erythematosus, and multiple sclerosis and other medical conditions [2-5].

However, the clinical use of this drug is limited due to the comprehensive side effects and toxicities like nausea, vomiting, alopecia, bone marrow suppression, hepatotoxicity, nephrotoxicity, neurotoxicity, cardiotoxicity, immune-toxicity, mutagenicity, teratogenicity, and carcinogenicity [6-8]. The most harmful effect is genotoxicity related to DNA binding characteristics of CPA [9].

CPA-induced nucleic acid damage causes DNA mutations that result in cytotoxicity, carcinogenicity and teratogenicity [3]. In normal tissues and organs, some toxicity like oxidative stress is stimulated by CPA because of the generation of free radicals [10]. So, the compound consisting antioxidant and cyto-protective properties may be beneficial if given with a CPA as a combined treatment regimen for the protection of normal cells and tissues against CPA-induced toxicities.

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Royal jelly was nothing but a natural milky material, secreted from the hypo-pharyngeal and mandibular glands of worker honeybees which were a complete food source for honeybee larvae <sup>[11]</sup>. Royal jelly containing varieties of nutritional components like proteins, lipids, carbohydrates, vitamins, and minerals <sup>[12]</sup>. It has a lot of properties such as anti-tumor and anti-inflammatory properties, anti-fatigue and hypotensive activity <sup>[13-15]</sup>, antioxidant activities <sup>[16]</sup>, antibacterial effects and enhancement of immune activity. Due to these exclusive properties, Royal jelly has become very important for human beings. So, we considered the beneficial properties of Royal jelly against cyclophosphamide induced hemotoxicity in mice.

Blood is a connective tissue contains RBCs, WBCs, and platelets, which are suspended in a fluid medium; plasma. The main function of blood is to distribute vital materials such as nutrients and oxygen and carry away the waste products from the cells. Complete blood count (CBC) is routinely performed clinical laboratory test which is required to assess or diagnose health problem related to blood.

## MATERIALS AND METHODS

**Chemicals-** The tested compound CPA were bought from Zydus Cadila, (G.Rem). Royal jelly was purchase from the apiculture farm of Hi-tech Natural product (India) Ltd from colonies of *Apis mellifera* in the lyophilized form.

Food pallet was purchased from VRK Nutritional solution, Pune, Maharashtra (India). All other chemicals used in this experiment were of analytical grade from Merck (India) Ltd, Mumbai, India. The dose has been selected on the basis of previously published studies and by acute toxicological study.

**Preparation of Royal jelly and Cyclophosphamide-** At the proportion of 100 mg/kg/d/mice of Royal jelly was dissolved in 5% CMC (carboxymethyl cellulose) suspension administered through an intra-gastric tube through the mouth <sup>[17]</sup>. While CPA was injected through intra-peritoneal rout at the proportion of 50 mg/kg/mice by dissolving in distilled water for combine dose different proportions of Royal jelly as 100 mg/kg, 250 mg/kg, and 500 mg/kg were suspended in CMC. The doses were weighed on SF-400 digital LCD balance.

**Animals-** Total of 36 Pathogen-free male Swiss albino mice with a weight of 20±5 g were obtained from the laboratory of Wockhardt Research Institute Aurangabad

M.S., India. The experimentation performed was in full compliance with the guidelines of the committee for the purpose of control and supervision of experiments on the animal (CPCSEA) Act of 2007 Govt. of India on animal welfare. The study protocol was approved by the Institutional Animal Ethics Committee (IAEC) of Y.B. Chavan College of Pharmacy, Aurangabad, and (MH) India (Ref. No. CPCSEA/IAEC/Pcology-53/2017-18/134).

**Housing conditions-** The mice were housed in standard polypropylene cages having a size of 32x11 cm with stainless steel grill top mesh having facility for holding food palate and a water bottle. The bedding material of the cages was changed daily. Maximum of 6 mice housed per polypropylene cage. All mice were maintained under standard laboratory conditions (25±1°C temperature; 12:12 h light/dark and 50-60% humidity) and isolated 7 days prior to the start of the study. Standard rodent chow diet and water were provided ad libitum to the experimental animals.

**Study Design and Plan-** Total 36 adult male Swiss albino mice of 8 - 9 week age and with 20±5 g weight were randomly allocated into 6 groups; each group consisting of 6 mice and the experiment lasted for 12 weeks. After an acclimatization period of one week (G1-G6):

G1: Normal Control Group (C): served as healthy control. Mice fed only with basal diet and water and were administered with 0.9% Normal saline (10ml/kg/week), for 12 weeks.

G2: Royal jelly group (Royal jelly): mice were administered with Royal jelly (100 mg/kg) CMC suspended orally everyday between 10 AM to 11 AM for each mouse for 12 weeks.

G3: Cyclophosphamide Group (CPA): was injected with CPA 50 mg/kg/week, for 12 weeks (once in a week) by the intra peritoneally.

G4: CPA and Low dose royal jelly Group (CPA+royal jelly1): were injected with CPA (50 mg/kg/week) followed by royal jelly administered orally (100 mg/kg/day) respectively, for 12 weeks.

G5: CPA and Medium dose royal jelly Group (CPA+ royal jelly2): mice were injected with CPA (50 mg/kg/week) followed by royal jelly administered orally (250 mg/kg/day) respectively for 12 weeks.



G6: CPA and High dose royal jelly Group (CPA+ royal jelly<sub>3</sub>): mice were injected with CPA (50 mg/kg/week) followed by Royal jelly (500 mg/kg/day) respectively, for 12 weeks.

**Collection of blood-** After 12 weeks of exposure approximately 1 ml of blood samples were obtained using mice bleeding tubes from retro-orbital sinus plexus in EDTA-containing tubes. All blood samples were labelled and immediately analyzed for a complete blood count.

**Measurements of Hematological parameters-** All hematological parameters like total erythrocyte count, total leukocyte count, total thrombocyte count, % hemoglobin (Hb), PCV, the mean corpuscular values of MCV, MCH and MCHC were analyzed using the automated hematology method with the "Hematology auto-analyzer Sysmex x100. The blood analyzer was periodically calibrated.

**Statistical Analysis-** All Values were expressed as means±SEM. Graph pad PRISM 6.01 for windows

computer program was used for statistical analysis of the results. Data were analyzed using the one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons. The value of \*\*\*P<0.001, \*\*P<0.01, \*P<0.05 were considered to be statistically significant.

## RESULTS

Effect of treatment of royal jelly (G2), cyclophosphamide (G3), and combined treatment of cyclophosphamide with royal jelly at a different proportions low dose (G4) (100 mg/kg), medium dose (G5) (250 mg/kg), and high dose (G6) (500 mg/kg) on male Swiss albino mice on hematological parameters were evaluated. All Groups from (G2 and G3) were compared with normal control (G1) group while combined treatment groups (G4-G6) were compared with cyclophosphamide (G3) group for the period of 12 weeks and obtained results are summarized in Table 1.

**Table 1:** Effect of Royal jelly on CPA-induced changes in Hematological parameters in Male albino mice

Groups Parameters	G1: Cont.	G2: Royal jelly	G3: CPA	G4: Royal jelly <sub>1</sub> +CPA	G5: Royal jelly <sub>2</sub> +CPA	G6: Royal jelly <sub>3</sub> +CPA
WBCs (10 <sup>3</sup> /μl)	5.46±0.38	6.14±0.54	1.49±0.44 ***	2.14±0.52	3.84±0.52†	5.03±0.52††
BCs (10 <sup>6</sup> /μl)	7.16±0.98	9.67±0.47 *	2.34±0.64***	3.85±0.41	5.43±0.40††	7.42±0.50†††
PLT (10 <sup>3</sup> /μl)	277±36.01	398±49.88 *	79±10.57***	175±8.70	239±19.65††	310±23.08†††
Hb (g/dl)	10.46±0.65	12.69±0.78	6.08±0.70***	6.87±0.58	8.65±0.63††	10.23±0.30†††
PCV (%)	34.00±2.64	36.50±3.39	19.28±1.00***	21.17±1.30	26.47±1.83†	32.16±1.42††
MCV (fl)	50.17±2.30	62.17±2.35 *	18.17±1.01***	21.83±3.20	34.67±2.29††	50.33±3.14†††



MCH	23.48	30.00	11.45	12.43	16.19	22.64
(pg)	± 1.10	±2.23*	±1.31**	±1.05	±1.93†	±2.26††
MCHC	33.50	42.83	16.83	19.17	27.33	32.62
(g/dl)	±1.96	±3.38*	±1.92***	±1.92	±2.37†	±2.10††

P<0.05\*, P<0.01\*\*, P<0.001\*\*\* vs. normal control group

P<0.05†, P<0.01††, P<0.001††† vs. CPA injected group

**Total Leucocyte count-** The current study demonstrated that the royal jelly treatment (G2) non-significantly increased the total leukocyte count compared to the control group (G1). However, a significant reduction (P<0.001) in leukocyte count in cyclophosphamide injected mice (G3) was observed as compared with the control (G1). Similarly, there was a non-significant difference in the total leukocyte count was seen in CPA with a low dose (100 mg/kg) RJ group (G4) compared to CPA-injected mice of (G3).

Interestingly, the mice that injected with CPA along with medium (250 mg/kg) and high dose (500 mg/kg) of Royal jelly (G5 and G6) displayed a significant increase (P<0.05, P<0.01) in the total leukocyte count compared to CPA-injected group (G3).

**Total Erythrocyte count-** After experimental period of 12 weeks it was observed that mice of the royal jelly group (G2) exhibited significantly increased erythrocyte count (P<0.05) contrast to control group (G1), whereas mice treated with a cyclophosphamide (G3) showed a significant decline (P<0.001) in the total RBC count as compared to control (G1). Mice of a combined treatment group such as 100 mg/kg of royal jelly with CPA (G4) were significantly similar to CPA injected group, while mice of the group royal jelly 250 mg/kg and 500 mg/kg with CPA (G5 and G6) showed significant improvement in the total RBC count (P<0.01 and P<0.001) as compared with CPA injected (G3) group.

**Total Thrombocyte count-** After completion of 12 weeks, there was a significant rise (P<0.05) for the thrombocyte count were observed in Royal jelly treated groups (G2) against normal control (G1) (Table 1). However significant decline (P<0.001) were recorded in CPA injected mice (G3) contrast to the control (G1) group. The G4 (CPA with 100 mg/kg royal jelly) was significantly not different from CPA injected (G3) group. Though, on the other side, there was significant progress (P<0.01,

P<0.001) found in the platelet count of CPA with 250 mg/kg and 500 mg/kg of Royal jelly group (G5, G6) when compared with CPA injected (G3) group.

**Percent Hb concentration-** (G2) in which Royal jelly given alone by gavages to all the animals showed the non-significant difference in terms of Hb concentration, compared with the control group (G1). Whereas Animals treated with the cyclophosphamide (G3) revealed a significant reduction in the level of Hb (P<0.001, compared to the control (G1) group indicating its toxicity. CPA with Low dose Royal jelly (G4) group exhibited a non-significant difference against CPA (G3) group.

Effect of royal jelly on the toxicity induced by CPA revealed a significant improvement (P<0.01, P<0.001) when CPA and Royal jelly gave in a medium (G5) and high doses (G6) compared with the CPA injected group (G3).

**Hematological indices-** At the termination of the experimental period PCV of the Royal jelly treatment group (G2), were non-significantly increased while the significant elevation (P<0.05) in terms of MCV, MCH and MCHC were recorded as compared to control (G1). significant demotion (P<0.001) in the PCV, MCV and MCHC while the decline was (P<0.01) in the MCH levels were observed in Cyclophosphamide injected mice group (G3) when compared with the control (G1) group.

Correspondingly, there was a non-significant elevation were found in all the indices in CPA with 100 mg/kg of Royal jelly (G4) compared with the CPA-injected mice of (G3) group, even though significantly increased levels (P<0.05) of PCV, MCH and MCHC while (P<0.01) of MCV in (G5) group were observed as compared to (G3) group. Alike the mice that injected with CPA along with a Royal jelly at a proportion of 500 mg/kg (G6) showed a significant increase (P<0.01) in the PCV, MCH and MCHC levels while improvement (P<0.001) in MCV were seen compared with CPA-injected mice (G3).

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## DISCUSSION

Chemotherapeutic agents mostly killed rapidly dividing cells in the body, targeting cancer cells along with normal cells originating from the bone marrow. CPA is a cytotoxic alkylating agent forming reactive oxygen species (ROS), which reacts with DNA. Initially, in the liver the CPA was activated by microsomal oxidation system enzyme cytochrome P450 converting CPA into 4-hydroxy CPA, a cytotoxic metabolite, and then 4-hydroxy CPA was further converted to some other cytotoxic metabolites as phosphoramidate mustard and acrolein [18].

Phosphoramidate mustard, the active metabolite of CPA forms DNA crosslinks which lead to DNA strand breaks and subsequently to chromosomal breaks. Acrolein, the other metabolite interfered with tissue antioxidant defense mechanism, through producing highly reactive oxygen free radicals that further react with DNA causing its damage [19].

In the present study after a chronic administration of CPA for 12 weeks, a severe hemotoxicity had seen in CPA treated mice a contrast to the normal untreated mice. A noticeable decrease was detected in the Hb concentration, leukocytes count, erythrocyte count, thrombocyte count, mean volume of PCV, MCV, MCH and MCHC. Similar results were reported by Elshater *et al.* [20], Bhattacharjee *et al.* [21], and Duggina *et al.* [22].

The results of the study demonstrated that 12 weeks of CPA treatment significantly decreased the total leukocyte count. This might be due to myelosuppression and oxidative stress during CPA treatment.

The main molecular mechanism of CPA was its cytotoxicity. Active metabolites of CPA can interact with the cellular macromolecules such as proteins, membrane lipids, RNA, as well as DNA and render the cell incapable of replication and induce apoptosis [23]. One of its metabolite, namely acrolein, induces oxidative stress that leads to DNA damage of normal cells. Reactive oxygen species are toxic to bone marrow cells and probably can trigger apoptosis and affect cell cycle, causing anemia and a decrease in leukocyte count. Myelosuppression resulting in leucopenia and thrombocytopenia is a frequent and major complication of cancer chemotherapy. Shalit *et al.* [24] observed that the total leukocyte count was decreased after exposure to cyclophosphamide administration. The decrease in WBC count recorded in the CPA-injected rats might be

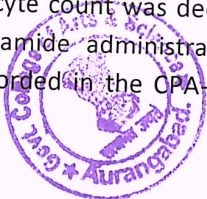
the consequence of the oxidative stress which induced lipid peroxidation and damage of blood cell membranes. De Weerd *et al.* [25] revealed CPA causes myelosuppression, affecting neutrophils and lymphocytes. According to Duggina *et al.* [22] showed significantly decreased counts of neutrophils and lymphocytes in CPA treated rats as compared to control group. Fraiser *et al.* [26] reported cyclophosphamide induced leucopenia and granulocytopenia which increases the patient's susceptibility to pathogenic bacteria or opportunistic microorganisms.

After 12 weeks of cyclophosphamide treatment to mice, the thrombocyte count was significantly decreased compared to the control group. This might be due to CPA inhibiting bone marrow activity or could be due to decreased production of platelets [27]. CPA that produces a transient hemopoietic depression has been reported to cause a persistently low number of stem cells of bone marrow [28].

Although cyclophosphamide is generally considered to be platelet-sparing, thrombocytopenia can also be a significant complication leading to an increased potential for bleeding episodes. Similar results were observed by Langford, revealed Bone marrow suppression is the main toxicity induced by CPA and in turn, decreased in platelets counts [29].

The total erythrocyte count and Hb% After 12 week of CPA treated mice were significantly decreased as compared to control. Similar results were reported by Ahmed *et al.* [30]. The previous results suggested that there was an etiological relationship between anemia and CPA treatment. This relation could be explained through different mechanisms, including the destruction of bone marrow cells or increased osmotic fragility of RBC. Thus, cap therapy might lead to anemia as a result of either suppression of the activity of hematopoietic tissues, impaired erythropoiesis, and accelerated RBCs destruction because of the altered RBCs membrane permeability, increased RBCs mechanical fragility, and/or defective iron metabolism.

Erythrocytes may undergo apoptosis-like suicidal death or eryptosis, which is characterized by cell membrane scrambling [31]. Despite the weak effect of acrolein on cytosolic  $Ca^{2+}$  activity, acrolein exposure leads to decrease of forward scatter reflecting a decrease of cell volume. Eryptotic erythrocyte shrinkage may result from activation of  $Ca^{2+}$  sensitive  $K^+$  channels with subsequent



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K<sup>+</sup> exit, cell membrane hyperpolarization, Cl<sup>-</sup> exit and thus cellular loss of KCl with osmotically obliged water [32] whether or not those channels are activated following exposure to acrolein, remains to be shown.

The Ca<sup>2+</sup> sensitivity of the erythrocyte cell membrane scrambling was enhanced by ceramide. Acrolein treatment indeed resulted in an increase in ceramide formation. Thus, acrolein triggers cell membrane scrambling at least in part by increasing the formation of ceramide, which in turn increases the Ca<sup>2+</sup> sensitivity of cell membrane scrambling. Ceramide was known to stimulate apoptosis of nucleated cells [33].

Emadi *et al.* [34] reported leucopenia, thrombocytopenia, and anemia are common after high dose cyclophosphamide administration. A case of cyclophosphamide-induced methemoglobinemia has also been reported by Shehadeh *et al.* [35] erythropoiesis failure, destruction of mature cells or increased plasma volume were some common causes of erythrocytopenia and decrease hematocrit values.

In mice CPA treatment for 12 weeks, mean values of PCV, MCV, MCH and MCHC values were significantly decreased as compared to the control group. Following the treatment with CPA decreased MCV, MCH and reduced MCHC values suggest that microcytic hypochromic anemia was developed due to the suppression of erythropoiesis [36]. It was reported that ROS increases hemoglobin glycation and erythrocyte fragility and bone marrow can be damaged by direct oxidation [37].

Thus, in the present study, it was observed that the decrease in all hematological indices after exposure to CPA was attributed to the production of erythrocytes with lower MCV, MCH and MCHC and these parameters closely related to Hb. Hb data could be strongly influenced by MCV, MCH and MCHC values [38].

The purpose of chemotherapy should not only to effectively remove tumor cells but also reduced the related immunosuppressive complications that are primarily caused by apoptosis of circulating leukocytes cells (leucopenia). Mice response to CPA chemotherapy caused severe immunosuppressive conditions, as reflected in a lower WBC count.

In the present study, it was observed that after royal jelly treatment the total leukocyte count, total erythrocyte count, hemoglobin % (Hb), thrombocyte count and the mean values of PCV, MCV, MCH and MCHC of

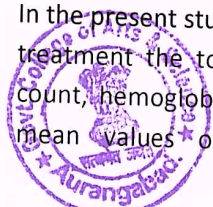
experimental mice were significantly increased as compared to the control group.

Antioxidants can prevent cell damage caused by the action of ROS and free radicals [39]. The antioxidant activities were related to a number of different mechanisms, such as free radical scavenging, hydrogenation, singlet oxygen quenching, metal ion chelation, and acting as a substrate for radicals such as superoxide and hydroxyl. It had been publicized that immunomodulatory compounds along with chemotherapy may reduce myelosuppression and enhance the immune response. Recently, royal jelly had received particular attention as a highly effective antioxidant and has the free radical scavenging capacity [40]. It contains many important compounds with biological activity such as free amino acids, proteins, sugars, fatty acids, mineral and vitamins [41].

In the present study it was observed that after combined treatment of cyclophosphamide along with different concentration 100 mg/kg/d (low dose), 250 mg/kg/d (medium dose) and 500 mg/kg/day (high dose) of royal jelly (G4-G6), the total leukocyte count, total erythrocyte count, hemoglobin % (Hb), thrombocyte count and the mean values of PCV, MCV, MCH and MCHC of experimental mice were significantly increased as compared to animal treated with cyclophosphamide (G3).

In the current experiment, a significant increase of the erythrocyte counts in the RJ-treated groups indicated that RJ may have a stimulatory action on the erythrocytes synthesis and proliferation in the bone marrow. Our findings were harmonious with that reported by El-Tarabany [42]. Royal jelly had a protective role against many drugs [43].

It is a rich source of antioxidants that opposed to myelosuppressive effects induced by CPA. The mechanism might be related to the recovery of hematopoiesis by means of modulating the bone marrow activity, as well as enhanced immune functions. RJ acts as an immune stimulator presumably due to its ability to alter the normal immune cell proliferation thus allowing efficient mobilization of an immune response. Simsek *et al.* [44] reported in their study on rats that an oral RJ application of 300 mg kg<sup>-1</sup> day<sup>-1</sup> increases the number of erythrocytes and their diameters; therefore, it could be used as a supportive antioxidant molecule in anemic patients. Royal jelly was found to increase the







number and diameters of erythrocytes; therefore, it could be used as a supportive antioxidant molecule in anemic patients. MRJPs stimulate cell proliferation by acting as bio-similar or substitutes for growth factors [45]. The royal jelly had a hemato-curative role against azathioprine [46]. Kanbur *et al.* [47] revealed that royal jelly at a dose of 50 mg/kg/d administration to mice ameliorates the oxidative stress in erythrocytes induced by fluoride. Administration of royal jelly to mice ameliorated the effect of radiation that induced oxidative stress and hematological alterations [48].

The present study shown that the improvement in the tested blood parameters as erythrocytes, hemoglobin, leukocytes, platelets and the mean value of packed cell volume (PCV), mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) indicates that royal jelly administration prevented blood cell damage by maintaining the integrity of cells.

### CONCLUSIONS

Cyclophosphamide caused leucopenia, erythrocytopenia, thrombocytopenia and a significant decrease in Hb% and mean values of PCV, MCV, MCH, and MCHC while royal jelly was significantly improved and normalized all hematological parameters altered during cyclophosphamide treatment. These consequences were of clinical importance particularly, use of Royal jelly as an exclusive treatment or in combination to neutralizes cyclophosphamide side effects in cancer.

Further investigations on hem protective effect of royal jelly on cancerous animals would provide a supportive approach for the usage of royal jelly in the reduction of complications of chemotherapy.

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