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ANTITUMOR EFFECT OF THE INFUSION OR PLUMBAGIN METABOLITE OF *PLUMBAGO PULCHELLA* BOISS WHEN ADMINISTERED TO CD-1 MICE WITH L5178Y LYMPHOMA CANCER CELLS

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ABSTRACT

Cancer is a group of diseases with a high incidence worldwide. Since existing treatments have extremely strong adverse effects and are often inaccessible to many patients, it is necessary to seek alternatives. The aim of this study was to explore the effectiveness of infusing *Plumbago pulchella* Boiss and its main metabolite, plumbagin, on L5178Y tumor growth and survival time of the animals. The acute toxicity of *Plumbago. pulchella* Boiss infusion was also evaluated. The results showed that the antitumor effect both infusion and plumbagin was the same, regardless of the concentration or the route of administration (oral / intraperitoneal), nor which is given as a single exposure or every 48 hours. The tumor size was inhibited between days 8 and 14 of treatment; however there was no increase in survival time of the animals. The Infusion of *Plumbago pulchella* shows no toxic effects. No macroscopic alterations were noted in the viscera of the treated rats.

Keywords: Cancer, medicinal herbs, Plumbago pulchella**,** plumbagin.

INTRODUCTION

Cancer is one of the main causes of mortality in the world today. The WHO reported in 2008 that 84 million people will die of cancer between 2005 and 2015 [1] if adequate measures are not taken soon. Although cancer is common in developed countries, over 70% of all deaths due to cancer take place in the developing world, including countries with middle and low average income that have very limited resources to deal with this disorder. According to census data from Mexico in 2008, cancer occupied the third place among the principal causes of death. In 2009, 18% of hospital morbidity in Mexico was due to malignant tumors in hematopoietic organs. [2]

There are several factors that are stimulating the search for alternative treatments for cancer, including the high costs and low efficacy of current treatments, as well as severe adverse reactions. Among the alternatives currently being researched is the treatment of cancer with medicinal plants. In Mexico as in many other countries, medicinal plants are commonly used by many people and especially by those with limited resources. [3] This traditional medicine, which has been used by humans throughout our history, is now in some cases being validated by laboratory research.

However, many medicinal plants have yet to be tested scientifically and among these is *Plumbago Pulchella* Boiss, a species of the *Plumbaginaceae*

family used in the State of Hidalgo, Mexico, as an infusion for the treatment of cancer. [4] This plant is commonly called pañete, cola de iguana, cola de pescado, chililllo, dominguilla, hierba lumbre, hierba del negro, hierba del pescado, hierba del alacrán, tianquiz, tianguiz, tlachichinol, chiricua, curicua, or jiricua. [5]

What makes this plant interesting from a scientific point of view is that it contains a naphthoquinone known as plumbagin (5-hydroxy-2-methylnaphthalene 1,4 dion), which was shown to have toxic effects against tumors cells many years ago [6]; but it is necessary to know the toxicity of the infusion to promote rational use of the plant in the township. This compound has been reported to have different mechanisms of cytotoxicity, [7, 8, 9, 10, 11, 12] proved the cytotoxic effect of the ethanolic extract as well as plumbagin, and suggested that the active agent may well be Cl_{50} because this metabolite has the same effect as the extract at 24 and 48 hours.
The aims of the present study was to explore the possible antitumor effects of the infusion of *Plumbago pulchella* as well as plumbagin , administered in different ways and at different concentrations in CD-1 mice with experimentally induced lymphoma L5178Y; and try out the acute toxicity with a fixed concentration of the infusion of *P. pulchella* B.

2. MATERIALS AND METHODS

2.1 Biological material: *Plumbago pulchella* Boiss was collected in the State of Hidalgo, Mexico, and identified by biologists Miguel Ángel Villavicencio Nieto and Blanca Estela Pérez Escandón. A sample was registered with the Center for Biological Research of university autonomy and the state of Hidalgo (Universidad Autónoma del Estado de Hidalgo, UAEH). The aerial part of the plant was utilized in the current study after being dried in the shade and crushed. Experiments were carried out in the Pharmacology Department of the UAEH.

2.2 Animals: Male CD-1 mice with an average weight of 30 g were housed individually in transparent acrylic cages at room temperature at 22^0 C and maintained on a 12 h light/dark cycle (lights on at 7:00 am), with food and water available *ad libitum*. After undergoing an adaptation period of three days, mice were inoculated intraperitoneally (i.p) with approximately 6.252 X 10^6 viable L5178Y murine lymphoma tumor cells and then randomly divided into 15 groups. Treatments began 3 days later. The handling of animals and all experimental procedures were approved by the Ethics Committee of the institution and were in accordance with the Mexican Official Norm for animal Care and Handling (NOM-062-ZOO-1999).

2.3 Antitumor test: The following 15 groups (n=7) were formed: two control groups, 5 groups to test the infusion, and 8 groups to test plumbagin. The mice of the negative control were administered 0.1 ml /V.O of water, while those of the positive control were given 2 mg/m^2 of vincristine sulphate. [13, 14] The aqueous extract of *P. pulchella* was administered orally to mice in order to test the use of the infusion. [4, 12] This extract was elaborated daily in accordance with the Mexican Farmacopea Herbolaria (2001). [15] The pertinent dilutions were made to obtain the desired concentrations for the experiments, and these were administered through an intragastric cannula in a volume of 0.1 ml per mouse on a daily basis until the animal died. The first concentration utilized was $2X10^{-1}$ mg/kg, which resulted in undesirable effects. Therefore lower concentrations of the extract were subsequently used ($3.9x10^{-4}$, $3.9x10^{-3}$, $7.8X10^{-3}$, $3.9X10^{-2}$ mg/kg). On the other hand, plumbagin obtained from *P. pulchella* [16] was administered orally as well as intraperitoneally at concentrations of 0.1, 0.2 and 0.25 mg/kg, with two different schemes — every 48 h (until the animal died) or in a single dose. Since the experiments were performed in distinct phases, if a particular concentration did not show any effect or caused considerable damage to the animal, it was not used in another group. Mouse weight and survival time were taken as indicators of tumor growth and the antitumor effect of the compound. Therefore, mice were weighed every 48 h and the day of death was registered.

2.4. Acute toxicity test at fixed concentrations: This test was conducted in 5 female nulliparous and 5 male CD-1 mice for each concentration. Animals were fasted for 12 h before and after the single intragastric dose of the infusion on day 0, with 5, 50, 500, or 2000 mg/kg of body weight (BW). The rodents were weighed on days 0, 2, 3, 7 and 14, and were observed daily. Evaluation and interpretation of the results of the observation period was performed according to the literature (Commission of the European Community, 2003). [17] Afterwards, animals were sacrificed and the kidney, liver, spleen and gonads were inspected macroscopically. [18] The sacrifice of the animals used in this research was conducted according to the NOM-062-ZOO-1999; [19] handling and disposal of biological waste products, and cadavers was conducted according to the provisions of NOM-087-ECOL-SSA1-2002. [20]

2.5 Statistical analysis: Analysis of variance (ANOVA) of the data was followed by the multiple comparison test of Tukey. Statistical significance was considered at $p<0.05$.

3. RESULTS AND DISCUSSION

The absence of medical services for marginalized groups has led many people to use medicinal plants and/or prescribe their own medical treatments for grave disorders, including cancer. Thus it is necessary to study plants, as *P. pulchella*, traditionally used to treat cancer in Mexico. One of the secondary metabolites of this plant is plumbagin, a naphthoquinone that has been shown to have cytotoxic effect *in vitro*. [12] The antitumor effect of this compound has been reported in other species of plants, such as *Plumbago rosea* [21] and *Plumbago zeylanica*. [22]

***3.1. In vivo antitumor effect of the infusion of P. pulchella* B.:** An infusion of *P. pulchella* B. was prepared in order to test the antitumor effect of the plant as it is commonly used. A yield of 0.78 mg of extract was obtained for each 100 mg of plant. Inhibition of tumor growth was found at all concentrations tested, especially at $3.9x10^{-3}$ and $7.8X10^{-3}$ mg/kg (these are very similar to the concentrations typically used by the population; see Figure 1). This effect was observed from day 8 to day 14, but was not accompanied by any significant improvement in the overall health of the animals. The concentration of $2X10^{-1}$ mg/Kg, which represents about 10 times the concentration commonly used in infusions), produced emaciation, dense feces, bristly hair, sunken eyes and bad body odor beginning at day 8. These symptoms were not observed for the other concentrations until day 14. The inhibition of tumor growth is lost the day 14, at which time the tumor began to develop again, with great variation according to treatment. However, experimental animals died at the same time as those in the control group (Figure 2). Hence, a dose-dependent antitumor effect of *P. pulchella* was confirmed by the present study. Few reports have been made using the aqueous extract of medicinal plants obtained by infusion or decoction. In one report [23] that used this technique with *Bursera fagaroides*, the antitumor effect popularly assigned to this plant was not found. Contrarily, another study did indeed validate the popular practice of using the infusion of *Trichilia hirta* to treat T-47D breast cancer cells. [24] This positive effect was also found with the methanolic extract of this plant when treating HeLa cells with Cl_{50} at the concentrations of 19.5, 1.5 and 1 mg/kg for 12, 24 and 48 horas. [12]

3.2* In vivo *antitumor effect of plumbagin from* P. pulchella B., *administered orally or intraperitoneally: After observing the effect of the extract of *P. pulchella*, the study of plumbagin was undertaken. This metabolite was been studied in regard to its cytotoxicity [12] and antitumor activity. [21, 25] Different mechanisms of action have been proposed. [7, 9, 11, 21] In the present study this metabolite was administered orally or intraperitoneally at different concentrations.

The administration of 0.2 and 0.25 mg/kg of plumbagin inhibited tumor growth between 8 and 12 days of the assay, whether given orally or intraperitoneally and in a single dose or every 48 h (Figure 3). No significant effect was observed with the concentration of 0.1 mg/kg administered intraperitoneally once (data not shown), nor when given intraperitoneally in a single dose or every 48 hours.

Compared with the other concentrations of plumbagin, that of 0.25 mg/kg administered intraperitoneally every 48 h caused a significant deterioration in animal health. Hence, this or any greater concentration were discarded for further experimentation with the scheme of administration every 48 h. This result is contrary to that found by Hsu (2006) [9] or Kuo (2006), [26] who reported no signs of toxicity with higher concentrations. However, the current results in regard to tumor inhibition are in agreement with those two authors, who reported that when intraperitoneally administered, 2 mg/kg/day of plumbagin in denuded mice with A549 or MDA-MB-231 tumor cells, respectively, induced a reduction in tumor size of 80% or 70% (at days 8 or 10). They also reported that with the same concentration and means of administration, but with a single dose, the reduction was 79% and 64%, respectively. Compared to the negative control, no significant difference was observed in the survival time of the animals with the intraperitoneal administration of plumbagin (Figure 5).

These results do not coincide with those of Uma *et al.*, (1999), [21] who reported an increase in survival time of animals with a concentration of plumbagin between 2 and 6 mg/kg, as well as a complete inhibition of tumor growth with 3 mg/kg administered during 3 days. Compared to that study, in the current contribution a greater deterioration of animal health was observed and without any improvement in survival time when we used a concentration (0.25 mg/kg) 10-fold lower. On the other hand, the same authors observed that when treatment with plumbagin began in the advanced stages of the tumor, there was a lesser effect. This could be an important factor in the current results,

since treatment began 3 days after transplanting tumor cells into the mice. With oral administration of plumbagin at 0.2 and 0.25 mg/kg, we found partial inhibition of tumor growth from day 8 to 12, with great variability among groups (Figure 5). This route of administration produced the greatest variability and the largest number of adverse effects in the animals. A high mortality rate was found with the highest concentration. Only one animal was still alive after day 12. This mouse died on day 32, which represents the longest lifetime of a rodent in any group of the current contribution (Figure 6). The antitumor effect provided by treatment with the infusion or plumbagin was similar in all experimental groups, resulting in an initial reduction in tumor growth without increasing the survival time. After 12 to 14 days the tumors in animals treated with the infusion or plumbagin began to grow again. This effect has been previously reported, [27] which the authors assigned to oncological resistance. Further research is required to confirm this cause-effect relation.

3.3. Acute toxicity test at fixed concentrations: The positive control group, treated only with the extract of *P. pulchella* B., was included in the present study with the aim of evaluating the possible toxicity of the infusion, since such a study has not previously been reported. None of the animals had died 14 days after beginning treatment with the extract, thus indicating the lack of toxicity of this plant. With the macroscopic necropsy of the 5 female and 5 male mice, changes were observed in the color of the liver at all concentrations employed. In some animals alterations were found in the size of the kidneys and testicles (Table 1). The effect of the aqueous extract of *P. pulchella* on the reproductive apparatus coincides with that reported in the literature for plumbagin (10 mg/kg) in regard to dogs and gerbils. [6] However, the present results are not in accordance with the report by Sumsakul *et al*. 2014, [28] who found no change with the acute concentration of 100 mg/kg in the heart, lungs, liver, kidneys and spleen. Considering the adverse effects that are observed with the administration of plumbagin, several researchers have used distinct strategies to reduce the damage. [29]

4. CONCLUSIONS

An inhibition in the growth of the L5178Y lymphoma tumor was herein found between day 8 and 14 of treatment with the aqueous solution of the extract of *Plumbago pulchella* Boiss or the plumbagin metabolite from the same plant. On the other hand, the survival time of the animals was unaffected by these treatments. Toxicity was not observed for the extract, although there were some macroscopic changes in the liver, kidneys and testicles at 5 mg/kg.

Conflicts of interest: All authors have none to declare.

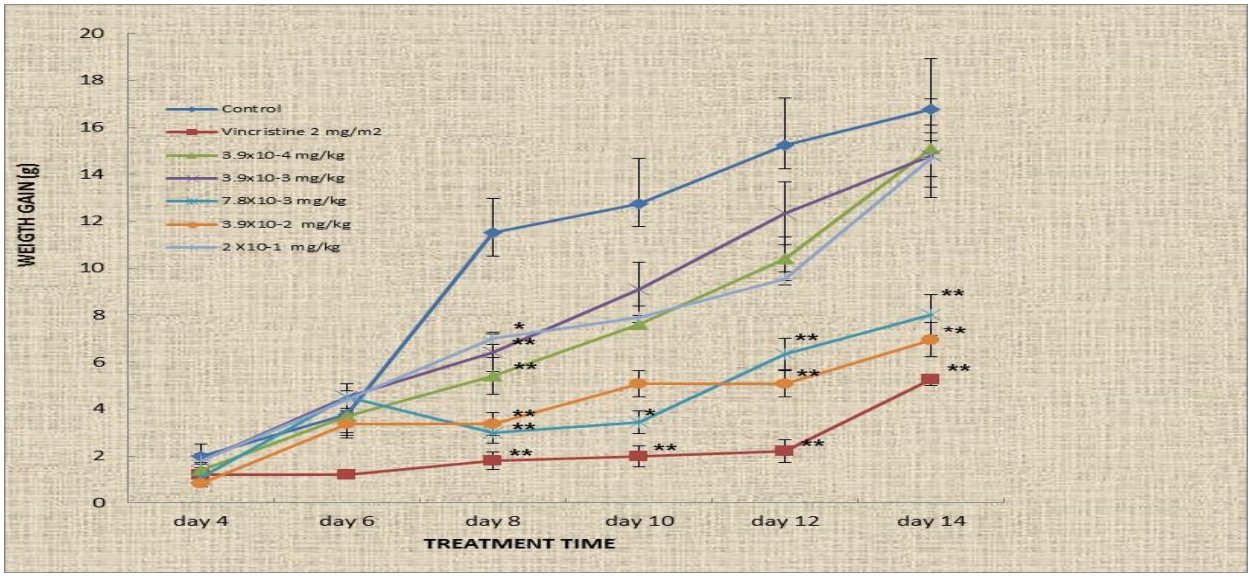

Figure 1. Effect of the oral administration (every 24 h) of the aqueous extract of *P. pulchella* B. on weight gain in CD-1 mice with L5178Y lymphoma. Each bar represents the mean ± standard error (SE) of 7 experiments. *P≤ 0.05; **P≤ 0.01, using the analysis of Tukey.

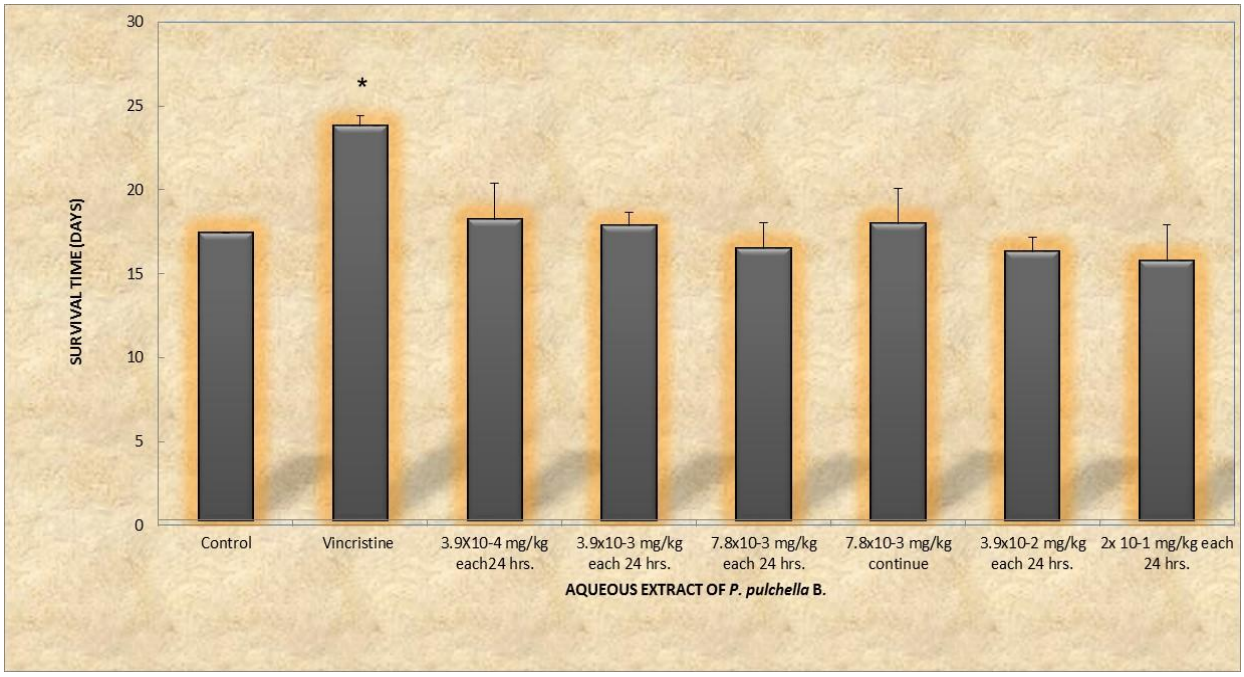

Figure 2. After inoculation of CD-1 mice with L5178Y lymphoma cells, survival time was evaluated during oral administration of the aqueous extract of *P. pulchella* B. Each bar represents the mean ± SE of 7 experiments. *P≤ 0.05; NS= not significant, based on the Tukey test.

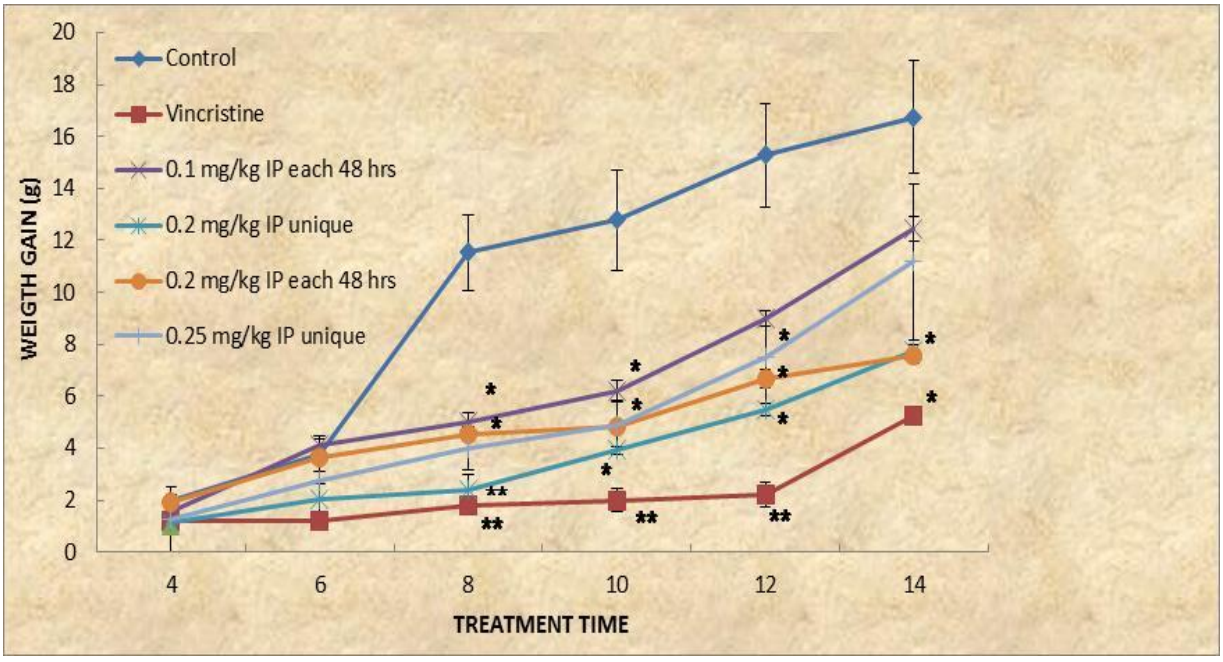

Figure 3. Effect of plumbagin (administered intraperitoneally) on weight gain in CD-1 mice with L5178Y lymphoma. Each point represents the mean ± SE of 7 experiments. *P≤ 0.05; **P≤ 0.01, using the Tukey analysis.

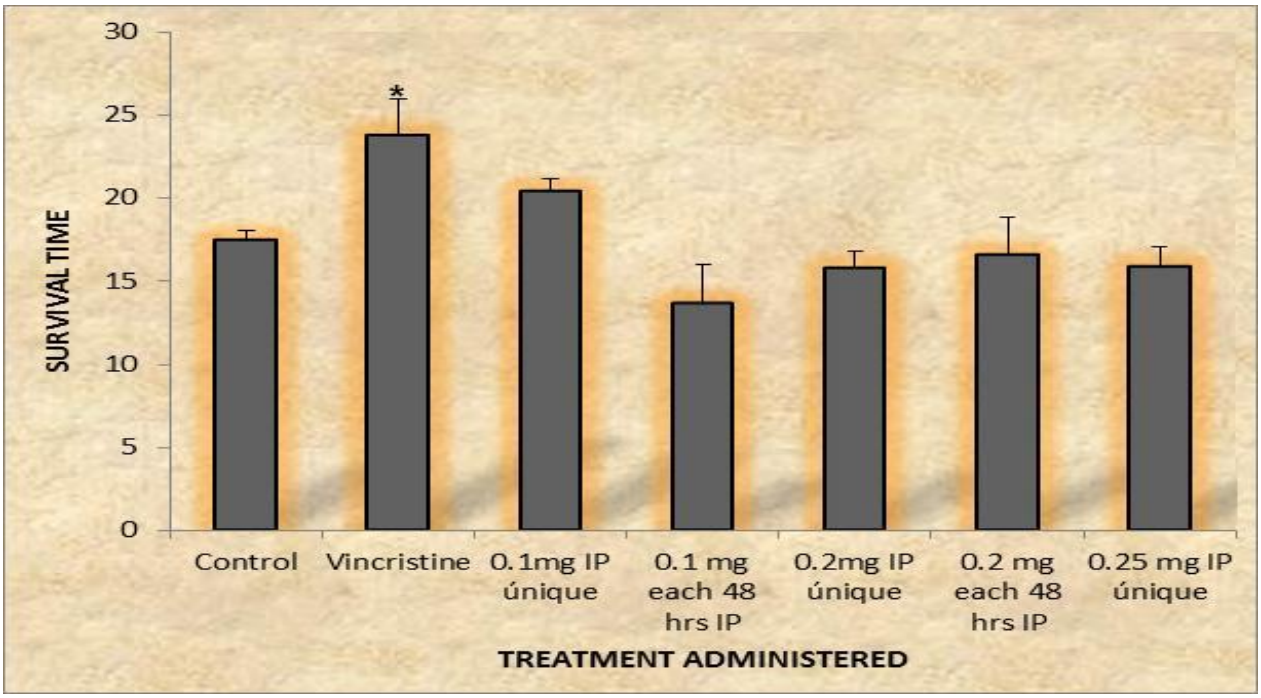

Figure 4. Survival time after transplanting L5178Y lymphoma cells in CD-1 mice and administering plumbagin intraperitoneally. Each bar represents the mean ± SE of 7 experiments. *P≤ 0.05; NS= not significant, using the Tukey analysis.

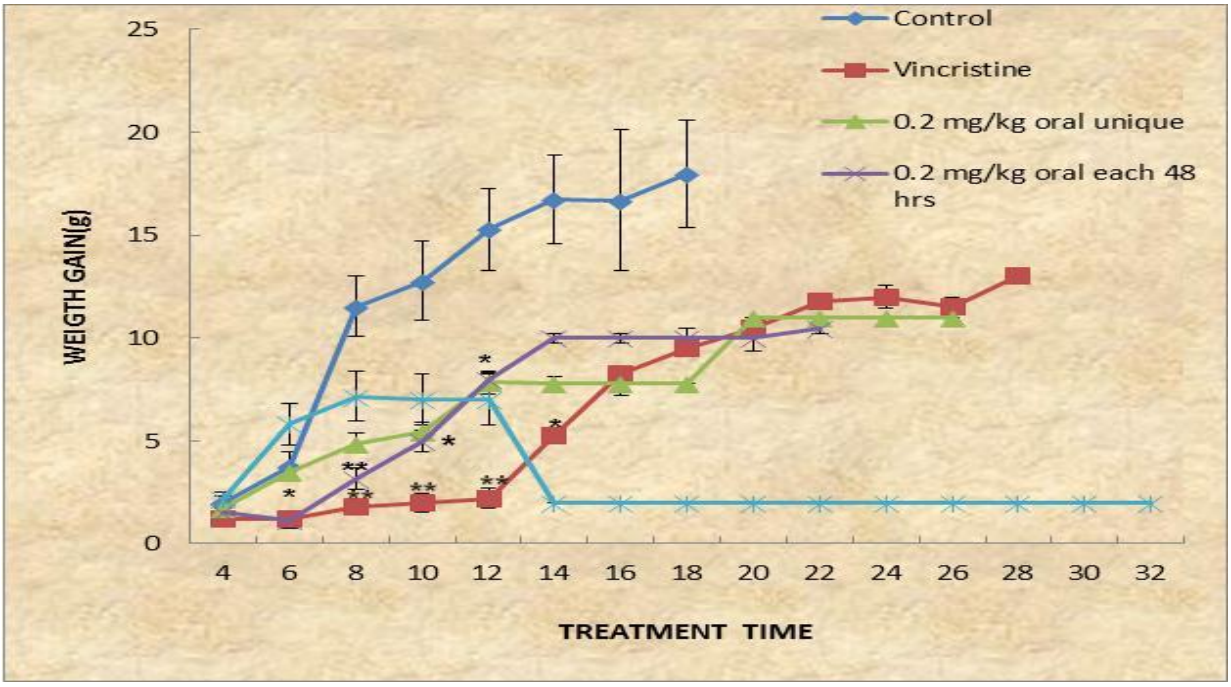

Figure 5. Effect on weight gain in CD-1 mice inoculated with L5178Y lymphoma cells during treatment with plumbagin administered orally in a single dose or every 48 h. Each point represents the mean ± SE of 7 experiments. *P≤ 0.05; **P≤ 0.01, using the Tukey analysis.

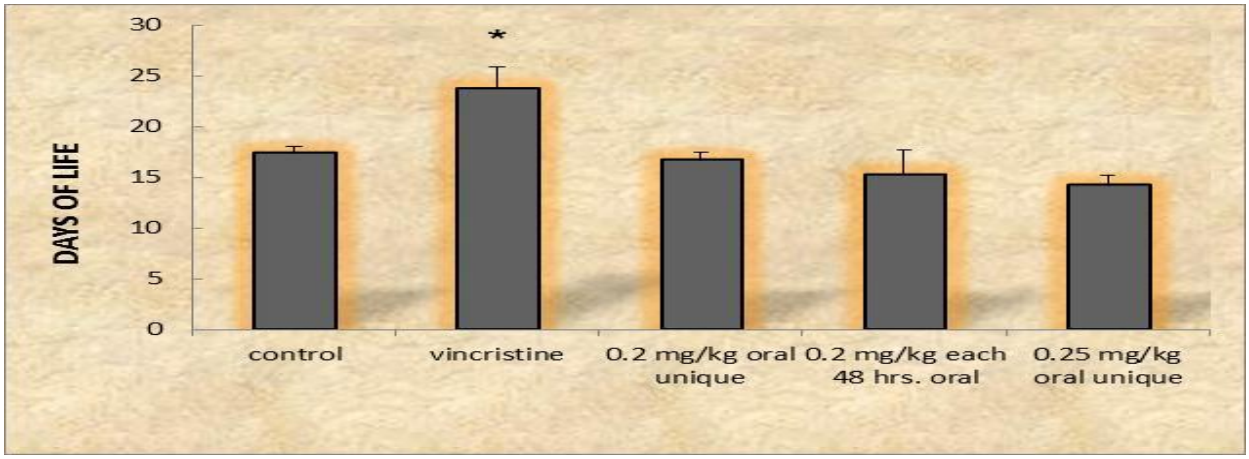

Figure 6. After the transplant of L5178Y lymphoma cells in CD-1 mice, survival time was evaluated during the oral administration of plumbagin. Each bar represents the mean ± SE of 7 experiments. *P≤ 0.05; NS= not significant, using the Tukey analysis.

Table 1. Toxicity at fixed concentrations of the infusion of *P. pulchella* B.

Dose **Organs**	**5 mg/kg**	**50 mg/kg**	**500 mg/kg**	**2000 mg/kg**
Heart	Without any apparent pathological changes	Without any apparent pathological changes	Without any apparent pathological changes	Without any apparent pathological changes
Liver	Areas pale in color	White areas and petechias	White areas and petechias	White areas and petechias
Spleen	Without any apparent pathological changes	1 of 10 animals with congestion; 1 of 10 with splenomegaly	Smaller than normal in 2 of 10 animals	Without any apparent pathological changes
Kidney	Enlarged and pale in 2 of 10 animals	Enlarged in 2 of 10 animals	Smaller than normal in 1 of 10 animals	Enlarged in 2 of 10 animals
Uterus	Without any apparent pathological changes	Without any apparent pathological changes	Without any apparent pathological changes	Without any apparent pathological changes
Testicles	Smaller than normal in 1 of 5 animals	Smaller than normal in 1 of 5 animals	Smaller than normal in all 5 animals	Smaller than normal in all 5 animals

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