Cytotoxic Effect of *Vinca rosea* Aqueous Extracts on (L20B) Cell Line *In Vitro*

Hind H. Obaid[1], Liqaa H. Saqban[2], Lamia Y. Mohammed[3]

[1] Dep. of Biology, College of Science, Baghdad University, Baghdad, [2]Dep. of Biology, College of Education Pure Science, Karbala University, Karbala, [3]Middle Technical University, Baghdad, Iraq

Abstract

The present study investigated the cytotoxic effects of aqueous crude extracts of *Vinca rosea* leaves , flowers and seeds on cell line (L20B Cell line)*in vitro ,* by using double dilution series (concentration between 1.95 – 1000 µg/ ml.

The results showed that the cytotoxic effect of extract is dependent on type of extract, amount of dose and exposure time. The concentration 1000 µg/ml gave the highest growth inhibition (IR) (74 and 74%) to leaves and flowers respectively compared with control 100% after 24 hours exposure time, but seeds were (49%) after 48 hours. However, low concentrations of aqueous extracts were found to induced the L20B cells growth and proliferation (PR), which recorded (122, 123) % by treatment with flowers extract in 1.95 µg/ml after 72 hours. Crude aqueous extract of *Vinca rosea* had hormetic effect (Hormesis), because it also induced the proliferation of cancer cells by using low concentrations of the extract.

KeyWords: *L20B,* ***Vinca rosea****, cytotoxicity, Inhibitory Rate.*

Introduction

Cancer emerged as a leading cause of death in the world. It is a serious disease that kills millions of people every year, and during lifetime, it affects one in two men, one in three women, while it causes death of one in four women[1].Cancer remains a problem for scientists despite the existence of several methods of treatment. There are chemotherapy, physiotherapy and surgical therapies, but all of them were not convincing for doctors or the patient himself. Thus, research centers and researchers turned to find other alternative therapies for the existing treatments and took another approach that may have great hope to eliminate the disease. Commercial drugs cost large amounts to import, and their effectiveness is gradually lost by continuous use due to the resistance of cancer cells to these medicines [2]. Therefore, many countries worldwide paid much attention to their plants as the natural source of drugs [3]. The discovery of anticancer effect of alkaloids of *Vinca rosea* plant gave it a great medical importance, because these alkaloids are chemotherapeutical agents for different types of human cancers [4,5].

About 75 types of alkaloids have been discovered, some of which have anticancer effects, including vinblastine and vincristine [6], as well as the use of this plant for the treatment of diabetes [7]Several studies were also carried out to use the plant extract in the treatment of microbial diseases such as skin diseases[8].Mitosis inhibitors such as Vinca alkaloids, derived from the *Vinca rosea (Cathathanthus roseus)* occupy a special place among chemotherapeutical drugs used in the treatment of many types of cancer [4,9].In this sense, and in order to enhance the study of the effect of natural plant extracts found in the Iraqi environment against some cancerous and transformed cell lines as a first step to explore their counter effects, this study was designed (as a part of an extensive study of different cell types) to investigate the effect of crude extracts of the *Vinca rosea* plant in the local Iraqi environment in inhibiting the growth of the L20B mouse fibroblast cell line.

Materials and Method

• **Plant collection**: The *Vinca rosea* was collected as an ornamental plant in the gardens of the College of Education/University of Karbala, and the plant was classified by the Iraqi National Herbarium/

Public authority for the examination and certification of seeds of the Ministry of agriculture. After the plant was collected and cleaned, it was washed thoroughly by tap water, and the plant parts (leaves, flowers and seeds) were separated and left to dry in the dark and at room temperature in a well ventilated dry environment to prevent damage of the samples. After drying of these three parts, they were finely grinded with an electric mill, then preserved in clean plastic containers away from light, heat and moisture until use.

Preparation of crude aqueous extracts of *Vinca rosea*: The cold aqueous extract was prepared according to the method used by Harborne *et al*[10], by taking 50 g of the dry powder for each part of the plant and adding 250 ml of distilled water and left the mixture on the magnetic stirrer at room temperature for 3 days, then the mixture was filtered by gauze then by filter paper (Whatman No.1). After that, the supernatant was dried to get the dry powder from which the required concentrations were prepared.

Indicative chemical detection of effective compounds: The types of secondary chemical metabolites found in the studied plant extracts (alkaloids, terpenes, flavonoids and glycosides) were determined, depending on what is stated in [10].

Study toxic effects of *Vinca rosea* extracts on the growth of (L20B) cell line

Type of the studied cancer cells: The (L20B) mouse fibroblast cell line was used by passage (18). The cells were grown on tissue culture medium with Minimum Essential Media (MEM), supplied by Sigma (USA) company with 5% (Fetal Calf Serum / FCS) supplied by the company itself.

Cytotoxic effects: Multiple (96) tissue culture microtiter plates, flat bottom were used to perform this experiment, which included three stages:

Cell seeding:

- After the growth and multiplication of cells, the containers with monolayer were taken, then cells were harvested using Trypsin-Versin (T.V) solution.

- Twenty ml of the serum-containing culture medium was added to each container and mixed well. Then, the cells were counted by the (Haemocytometer) using the (1%) trypan blue dye according to what indicated by Freshney [11].

- By a micro pipette, (0.1) ml from the cell suspension was taken and placed in each well of the plate. Each well contained (1×10^4) cell / well. The surface of the plate was then covered with a special sterile transparent adhesive paper for this purpose and the plate was moved gently, after that incubated at ($37C^0$) until the next day to allow (cell attachment).

2- Exposure of cancer cells to the plant extract:

The next day of seeding, serial dilutions were done in sterilized test tubes for each type of plant extract using the MEM-Serum free media, and dilutions from 1/2 to (1/1024) started gradually, which yielded the concentrations from 1000 to 1.95 µg/ml) respectively, taking into account to prepare the dilutions simultaneously at work. The culture media was poured from the wells after lifting the adhesive paper. The column No. (1) was considered as a negative control, as 0.2 ml of the serum free culture medium was added to it, while to the columns from 2 to 12 the dilutions of the extract, which were prepared as (0.2 ml / well / concentration) were added, and then a new layer of adhesive paper was re-placed on the surface of the plate.

The plates were incubated at (37C), while exposure times were (24,48 and 72) per hour.

Cytotoxicity assay:

Crystal violet stain was used to detect the cytotoxic effect of the extracts on cells pursuant to the following:

After the end of each incubation period, the plates were taken and their contents were poured and then washed with Phosphate Buffer Saline (PBS) solution, and 0.1 ml of crystal violet stain.

was added to each well, and left for (20) minutes. The cells were then washed with PBS solution several times until the excess stain disappeared. After the plates were dried completely, the results were read using the ELISA microplate spectrophotometer at a wavelength of 492 nanometers. The mean inhibitory Rate / I.R was measured according to the equation indicated by [12], and the mean proliferation rate / PR was calculated according to[13].

Finding

Indicative detection of active chemical

compounds: Detection of chemical compounds in the *Vinca rosea* extract showed that they contained (alkaloids, terpenes, flavonoids and glycosides)**.**

Cytotoxic effect of *Vinca rosea* extract on (L20B) Cell Line

Cytotoxic effect of the leaf extract: The toxic effect of leaf extract was studied in the L20B cell line by passage (18). The results shown in figure (1), revealed that the inhibitory toxicity effect was on the first day of exposure because it gave the best results, when the highest percentage of inhibitory rate (IR) growth inhibition was 74% at the concentration of 1000 µg/ ml of the extract after the first 24 hours of exposure. After that, the inhibition decreases as the concentration decrease, however, it remains effective. The lowest concentration used of 1.95 µg / ml gave a 41% growth inhibition. It is also noticed that the inhibitory effect 48 to 72 hours after treatment with plant extract was generally similar to the treatment after 24 hours, although it began to decline over time, because the highest rate of growth inhibition was 61% and 40% (with a viability rate of 39% and 60%) at 1000 µg/ml concentration after 48 and 72 hours of treatment respectively.

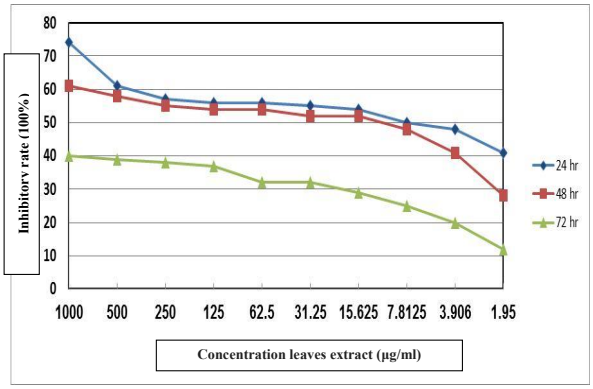

Figure (1): Effect of aqueous extract of the *Vinca rosea* leaves on the percentage of inhibitory rate on L20B cell line after different exposure time.

Cytotoxic effect of the flower extract: The results of the treatment of the L20B cell line with the *Vinca rosea* flower extract revealed that the inhibitory effect was similar for the three periods of time and gave a similar growth inhibition rates (IR) when using the first three high concentrations, reaching (74, 74, 65)% after 24 hours and (74, 73, 58)% after 48 hours and (71,62,56)% after 72 hours at the concentrations (1000, 500 , 250) µg / ml respectively. After that, the inhibition gradually began to decline with decreasing the concentration reaching to (8.3)% at 1.95 µg/ ml after 24 and 48 hours of exposure respectively as shown in figure (2). While the rate of cell growth was gradually increased beginning from 62.6 µg / ml making the proliferation rate reach to 123% at 1.95 µg / ml after 72 hours of treatment compared with control (100%).

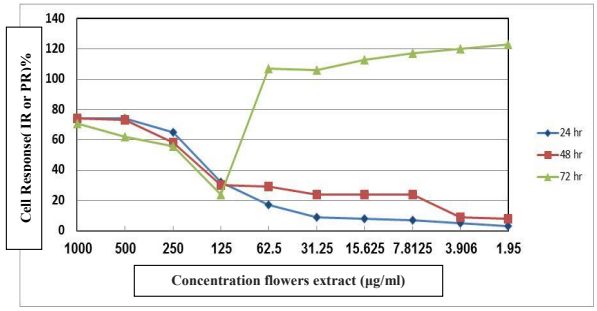

Figure (2): Effect of aqueous extract of the *Vinca rosea* flowers on the percentage of inhibitory rate onL20B cell line after different exposure time.

Cytotoxic effect of seed extract: There was less inhibition effect of the extract of *Vinca rosea* seeds on the L20B cell line compared with the two previous extracts (leaves and flowers). The best results were observed after 48 hours of exposure and then the rate of inhibition of growth decreased after 72 hours (increased cell viability). The toxic effect reached to 42% (cell viability 58%) after 24 hours of treatment at the highest used concentration of 1000 µg/ ml. In addition, the growth-stimulating effect appeared after 72 hours of treatment only, ranging from (110-122)%, and began to appear at the concentration of 7.8125 µg/ ml reaching to a minimal concentration of 1.95 µg/ ml respectively. figure (3).

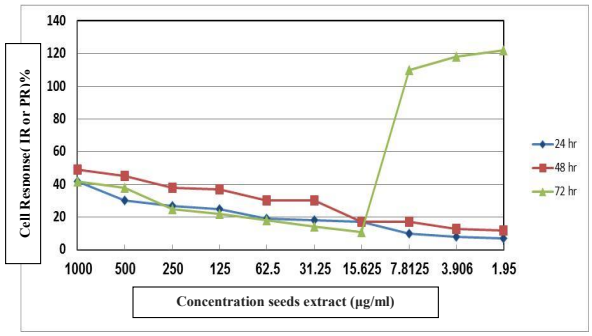

Figure (3): Effect of aqueous extract of the *Vinca rosea* seeds on the percentage of inhibitory rate onL20B cell line after different exposure time.

Comparison effect of the exposure time on the viability of the L20B cell line of the three studied extracts.

When a comparison between the viability of the three studied extracts (leaves, flowers and seeds) at each time of exposure, it was found that the first 24 hours of treatment showed the highest growth inhibition rates, as the viability of the cells was (26)%, (growth inhibition rate was 74%) for leaf and flower extracts and at the highest treated concentrations with 1000 µg/ ml (Fig. 4). In addition, results of 48 hour exposure were generally different from the 24 hour exposure, since the inhibition rate of leaves and flowers declined, while the highest rate appeared when the seed extract was used reaching to (49%), (51% viability) at 1000 µg/ ml concentration, and these rates decreased for all used concentrations compared with the exposure time of 24 hours (figure 5). The cell inhibitory rate within 72 hours of exposure was (40%, 71% and 42%) at 1000 µg/ml, (the viability rate was 60%, 29% and 58%) respectively, compared to control 100% (figure 6). It is observed that the viability rate of these cells begin to increase when low concentrations of flower and seed extracts are used to reach the highest viability rate (122 and 123)% respectively at 1.95 µg/ ml concentration. The results obtained showed that L20B cells were more sensitive to leaf and flower extracts and less sensitive to seed extract due to the high rate of cell viability.

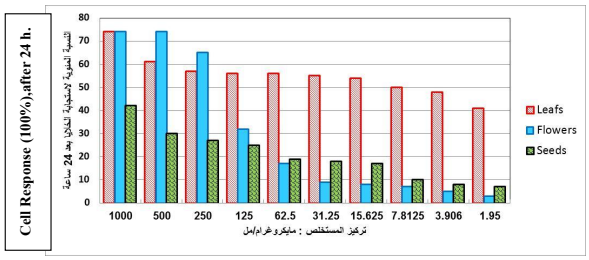

Figure (4): Comparison the effect of aqueous extracts of *Vinca rosea* on L20B cell viability after 24 h. exposure time.

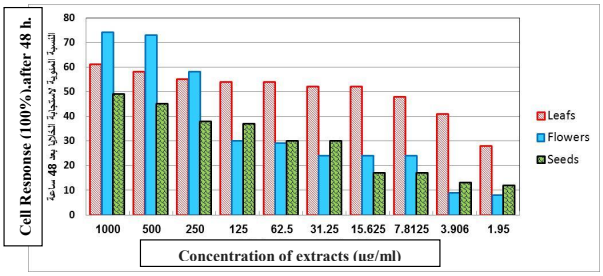

Figure (5): Comparison the effect of aqueous extracts of *Vinca rosea* on L20B cell viability after 48 h. exposure time.

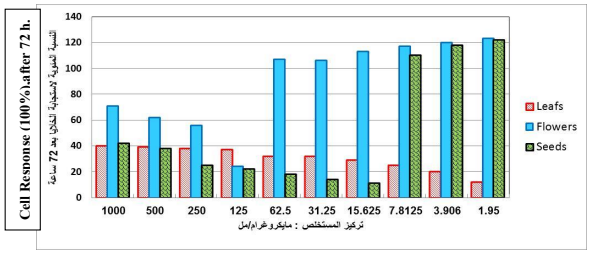

Figure (6): Comparison the effect of aqueous extracts of *Vinca rosea* on L20B cell viability after 72 h. exposure time.

Due to the importance of finding effective substances against cancer and finding more types of plants that possess these substances, the *Vinca rosea*, one of the locally available medicinal plants with different therapeutic properties, was [selected to identify the effects of crud aqueous extracts on L20B cell line] (as part of an extensive study in this field in many cancer cell lines) and the extent to which these extracts can be used as anticancer medical treatment in the future.The extracts of *Vinca rosea* contain many compounds and, as shown in the indicative chemical detection, the presence of alkaloids, terpenes, flavonoids and glycosides which may contribute to the better killing of cancer cells as a result of synergistic effect between them, which may reduce the toxicity of the used pure compounds.

The results showed that the crud extracts played a role in killing LB20 cells and inhibiting their growth and their division *in vitro*. The results indicated that the toxic effect of *Vinca rosea* on L20B cells was based mainly on the concentration used, exposure time and type of extract. The leaves extract had the best effect. On the other hand, it is observed that this type of cell, according to the results of this study, is sensitive to the prepared therapeutic aqueous extracts, as it inhibited the growth of cells, on contrary to the seed extract, which did not exceed the percentage of 49% inhibition after 48 hours of treatment using the high concentration of 1000 µg/ml, then the inhibitory effect is reduced when the exposure time is increased for all types of extracts. These results indicate that the effect of leaves and flower extracts is during the first 24 hours of exposure and at high concentrations, so when its effect is diminished, the living cells begin to reactivate and divide, showing the importance of giving repeated and continuous high doses to ensure the killing of all remaining cancer cells. The same thing occurs with the seed extract, which gives the best results after 48 hours of exposure and then the inhibition decreases and the cells reactivate.

A study performed by Yaseen *et al*[14]who compared between the two types of alcohol and aqueous extracts, and found that alcohol extracts are more effective than their aqueous counterparts on Hep-2 cells. This may be due to the fact that the ratio of the active substance extracted with ethanol (70%) is greater when using the aqueous extract, and this is reported by Harborne *et al*[10]. In two local studies on the same extracts in two cancer cell types - human cervical cancer cells (Hela-cells) [15] and human brain cancer cells (AMAG) [16], the results showed that both types of cancer cell lines were resistant to the aqueous *Vinca rosea* extracts (Leaves, flowers and seed), where low inhibition rates were recorded (not exceeding 46% in Hela cells and 64% in AMAG cells) using the highest concentrations (1000 µg/ml), while the L20B cells were shown to be sensitive to the same extracts. This is likely due to different receptors and antigenic determinants on the surface of each of the cancer and transformed cells.

The crud extracts of *Vinca rosea* contain a high percentage of alkaloids, which contain more than 75 types [17], as well as the presence of terpenes, phenols [18] and many mineral elements [19]. The proportion of secondary metabolic products in the plant varies according to the type of plant organ (leaves, flowers or seeds), and this is also affected by surrounding environmental factors[18]. Alkaloids are themost important and most effective substances in these extracts. The mechanism of their action is to inhibit the mitotic division, to keep the cells in the metaphase by inhibiting the polymerization of the protein Tubulin which is responsible for the formation of spindle fibers [9,20]. In addition, alkaloids inhibit the building of nucleic acids *in vitro*[21]. Several previous studies have also indicated that *Vinca rosea* alkaloids are effective against cancer cells, including human cervical cancer cells (Hela cells), because low concentrations cause inhibition to spindle fibers action [22,23]. On the other hand, Parekh and Simpkins [24] confirmed that these alkaloids affect on cancerous lymphocyte cells of rat and on human ovarian cancer cell line that are resistant to commonlychemotherapies used such as Cisplatin, as well as its being more effective than Taxol and Adriamycin.

Regarding the effectiveness of phenolic compounds, including flavonoids, which have an antioxidant effect by removing the generated free radicals and they direct the cell to enter apoptosis stage [25].

Many effective compounds effect on opposite directions depending on the concentration used. As noted by the above results, high concentrations inhibited the growth of L20B cells, while the low concentrations stimulated growth of these cells, increasing the viability by (122-123)% compared to the control (100%), indicating that the extract under study has a Biphasic effect [26], or Hormetic effect. There are many chemical therapeutical compounds, antibiotics, and toxins whose action is governed by the Hormesis phenomenon (abiological phenomenon common in toxicology), which act at low concentrations to stimulate, and may be useful to the organism, especially when the immune cells are activated, while high doses cause partial or total inhibition to the cells [27].

It is worth mentioning that the extract used in this study is crud extract, it contains many types of active compounds whose effectiveness was previously indicated or not mentioned, which supports the results of the emergence of antagonism in the influence on L20B cells depending on the concentration used. It is likely that its effect on the genetic material is in two directions, the first causes the inhibition of certain genes, while the other stimulates growth and multiplication.

Conclusion

The results showed that L20B cells were more sensitive to leaf and flower extracts and less sensitive to seed.

Conflict of Interest: Non

Funding: Self

Ethical Clearance: Non

References

1. American Cancer Society . Cancer Facts and Figures 2015 American Cancer Society, Atlanta.2015.
2. Murray RK. Cancer, Cancer Genes, and Growth Factors. In: Harper's Biochemistry.(24th Ed). Murray RK, Granner DK,Mayes PA, Rodwell VW (Eds.), Appleton and Lange, Stamford1996 . pp. 757-778.
3. Safarzadeh E, Shotorbani SS, Baradaran B. Herbal Medicine as Inducers of Apoptosis in Cancer Treatment. Adv Pharm Bull 2014;4(1):421-427.
4. Aslam JHK, Sheba HS, Zahid F, Zohra M,Mukthar A. *Catharanthus roseus* Don. Lan Important Drug

IT'S Applications and Production, Pharmacie Globale (IJCP) 2010;4:12.

5. Al-Azawee NHI. The cytotoxic effect of some chemotherapti drugs & functional activity of breast cancer patients peripheral blood lymphocytes. *International Journal of Advanced Research*2015; 3(1): 81-86
6. Almagro L, Fernandez-Perez F, Pedreno MA.Indole Alkaloids from *Catharanthus roseus*: Bioproduction and Their Effect on Human Health, *Molecules*2015;*20*: 2973-3000.
7. Ahmed MF, Kazim SM, Ghori SS, Mehjabeen SS, Ahmed SR, Ali SM, Ibrahim M. Antidiabetic activity of *vinca rosea* extracts in alloxan – induced diabetic rats.Int. J. Endocrinology 2010;6:81-90.
8. AL-makhzumi AA, AL-dulaimy HH, Jawaad AM.*In vivo*effect of *Catharanthus roseus crude* extracts on pathogenic bacteria isolated from skin infections. Iraqi Journal of Science2015; 56(1) : 656-664.
9. Mohammed IH. Effect of Vincristine and Vinblastine from *Vinca rosea* on Microtubules of Tumor H22 Cell line.Diyala Journal of Medicine2012; 3 (1):97–104.
10. Harborne JB. Phytochemical Methods . (2nd ed.) Chapman and Hall, H. p. London 1984;pp193 .
11. Freshney RI. Culture of animal cells: A manual for basic technique. 4th ed. Wiley-Liss, A John Wiley and Sons, Inc. Publication, New York 2000.
12. Gao S, Yu B, Li Y, Dong W, Luo H. Antiproliferative effect of Octreotide on gastric cells mediated by Inhibition of Akt/PKB and telomerase.World J. Gastroenterol, 2003; 9:2362-5.
13. Chumchalova J, Smarda J. *Human Tumor Cells are Selectively* Inhibited by Colicins. Folia Microbiol. 2003; 48: 111-5.
14. Yaseen Ny, Husan HR, Saqban LH. Study the Effect of *Vinca rosea*Crud Extracts onMammalian Cancer and Normal Cell Lines Growth *In vitro*. Journal of Karbala University (A special issues of researchesof the fifth scientific conference of the University of Karbala),2009.
15. Obaid HH, Saqban LH.Al-Darraji MNJ, karim RM,Passat DN, Ahmed DA.Cytotoxicity Effect of *Vinca rosea*Aqueous Extracts onHuman CervixUteri Epitheloid CarcinomaCell Line (Hela) *In Vitro*. Iraqi Journal of Biotechnology2015;14(2): 388-402.
16. Saqban LH, Obaid HH,Al-Darraji MNJ, karim RM, Ahmed DA. Passat DN.Cytotoxicity Effect of *Vinca rosea*Aqueous Extracts onHuman BrainCarcinomaCell Line (AMAG) *In Vitro*. Engineering and Technology Journal 2016;34 parte B(3):129-140.
17. Mukherjee AK, Basu S, Sarkar N, Ghosh CA. Advances in cancer therapy with plant based natural products.Current Medicinal Chemistry 2001;8:1467-1486.
18. Vazquez-Flota F, Carrillo-Pech M, Minero-Garcia Y, Mirand-Ham M. Alkaloid metabolism in wounded *Catharanthus roseus* seedlings . Plant Physiology and Biochemistr2000; 42:623-628.
19. Sahito SR, Kazi TG, Kazi GH, Jakhrani MA, Shaik MS. Trace elements in two varieties of indigenous medicinal plant *Cathranthus roseus* (*Vinca rosea*). The Science 2001;74-77.
20. Ngan VK, Bellman K, Hill BT, Wilson L, Jordan MA. Mechanism of mitotic block and inhibitionof cell proliferation by the semisynthetic vinca alkaloids vinorelbine and its new derivative vinflunine. Molecular Pharmacology2001; 60:225-232.
21. Gascoigne KE,Taylor SS. How do anti-mitotic drugs kill cancer cells?. *Journal of Cell Science*2009; 122: 2579-2585.
22. Becvarova P, Skorp Ikova J, Janisc HR, Novy J. Avinca Alkaloids Effect on Microtubules of HeLa Cells. SCRIPTA MEDICA (BRNO)2006; 79 (1):19–34.
23. Parekh HR, Simpkins H. Cross-resistance and collateral sensitivity to natural product drugs in cisplatin- sensitive and resistant rat lymphoma and human ovarian carcinoma cells. Cancer Chemother. Pharmacol. 1996; 37:457-462 .
24. Sathiya S, Karthikeyan B, Jaleel CA, Azooz MM, Iqbal M. Antibiogram of *Catharanthus roseus* extracts. Global J. Mol. Sci. 2008; 3(1) : 1-7.
25. Pedra M, Ferreira MM, Cidade H, Kijjoa A, Bronze– da – rocha E, Nascimento M. SJ.*Artelastin is a cytotoxic prenylated Flavone that disturbs microtubules and interferes with DNA replication in MCF -7 human breast cancer cells* . Life Sciences 2005; 77 : 993 – 311 .
26. Calabrese EJ, Baldwin LA. *Hormesis :* the dose - response revolution . Annu . Rev .Pharmacol . Toxicol. N2003; 43 : 175 – 197