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Original Article

Mitotic Block of Human Blood Cells by *Vinca herbacea*, *Catharanthus roseus* and Colchicine Alkaloids

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Abstract

Catharanthus roseus (L.) G.Don is a plant which produces anticancer and anti-mitotic indole alkaloids. Colchicine is an anti-mitotic drug. Anti-mitotic effects of *Vinca herbacea* Waldst. & Kit. indole alkaloids is unknown. The study were evaluated the antimitotic effect of alkaloids *of V. herbacea, Catharanthus roseus* and colchicine on mitosis and microtubule arrangement of human blood cells. In this research, alkaloids were extracted from *V. herbacea* (herba) and *C. roseus* (rose). *In vitro* anti-mitotic and microtubule shortening effects of different concentration of these alkaloids and colchicine were studied on peripheral blood cells. Three alkaloids include herba alkaloid (*V. herbacea* alkaloid) and rose alkaloid (*C. roseus* alkaloid) and colchicine, especially herba alkaloid with increasing concentrations (0, 5, 10 and 20 $\mu g.ml^{-1}$) that induced mitotic block at the metaphase to anaphase transition. Mitotically blocked cells were exhibited aberrant spindles by microtubule dynamics suppression. All three alkaloids strongly suppresses the rate and extent of microtubule shortening *in vitro*. Our results showed *V. herbacea* are a novel source of anti-mitotic and anticancer compounds, probably better of *C. roseus*.

Keywords: Antimititic, Spidle, Microtubule, Anticancer

Introduction

Catharanthus roseus L. and *Vinca herbacea* Waldst and Kit. belonging to the Apocynaceae family. *C. roseus* is a decorative plant of enormous pharmaceutical interest because it is nothing less than a chemical factory, producing more than 130 different terpenoid indole alkaloids, some of which exhibit strong and important pharmacological activities [1-3]. *V. herbacea* produce some indole alkaloids as Majdine, tabersonine, herbadine and some other [4-7]. Majdine and isomajdine are apoptotic, antioxidant and antiradical [8]. The rose alkaloids have generally been used in the treatment of cancer. These compounds repress cell growth because they alter the microtubular dynamics, and ultimately this provokes apoptosis [9]. Alkaloids that disrupt mitotic progression, which are commonly referred to as 'anti-mitotic', are used extensively for the treatment of cancer [10]. Currently, all such alkaloids that have been approved for clinical use target microtubules and much success against a number of cancers showing anti-mitotic effects. Investigating the effects of these agents on microtubule dynamics has revealed much about their mechanism of action. The rose alkaloids, within a concentration range that blocks proliferation. At the lower end of this range, this inhibits microtubule dynamics without altering polymer levels, whereas at higher concentrations, it induces microtubule de polymerization in both situations, mitotic spindle formation is disrupted, and cells therefore fail to complete a normal mitosis [11]. Colchicine is an alkaloid to block mitosis by disrupting the mitotic spindle tubules at metaphase [10]. Peripheral blood mononuclear cells are genetically reprogrammed adult cells that exhibit a pluripotent stem cell-like state similar to embryonic stem cells, thus, they are an invaluable new source of pluripotent cells for drug discovery,

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cell therapy, and basic research [12]. This study was evaluated the antimitotic effect of alkaloids of *Vinca herbacea, Catharanthus roseus* and colchicine on mitosis and microtubule arrangement of human blood cells, to finding anticancer effects of *Vinca herbacea*.

Method and Materials

Alkaloids Extraction

V. herbacea obtained from Siah Bisheh of Iran (36.15 and 52.35) and *C. roseae* from Tehran parks organ green house. Aerial parts of adult plants were used for alkaloid extraction. 100 g of plants powder were mixed in 1000 ml absolute ethanol and were set in bathroom 60 ºC for 2 h. Following filtrated, alkaloids were extracted purified according modified method of Renaudin (1984) as described by Miura *et al*. (1987) [13,14]. For purification, ethanolic extracts were dried by vacuum evaporator at 60ºC and used. Alkaloids isolation stages were: 1. Acidic phase was isolated by sulphuric acid (5%) and diethelic ether (50/50; v/v) in a decanter. 2. Acidic phase separated and made basic (pH 10) with 10 N NaOH and extracted with 60- 100 ml chloroform in decanter. 3. Chloroformic phase were dried by vacuum evaporator at 60 ºC.

Total Alkaloid

Amount of 10 mg of serpentine (Merck) alkaloid standards were placed in a 10 ml flask and dissolved in 10 ml ethanol. Six additional calibrations were prepared by 1:2 serial dilutions with ethanol-water (50:50). Standard solutions were prepared 0 -200 µg ml^{-1}. Total alkaloids were measured at 254 nm by spectrophotometry (UV-Visible Shimatzu) method.

Cell Culture

Human peripheral blood were cultured in RPMI 1640 at 37 °C in the presence of 5% CO2 with 10% fetal bovine serum and 1% penicillin/streptomycin in 250 ml tissue culture flasks or 35-mm six-well plates (68–70 h). Metaphase spreads of human peripheral blood cells were prepared by karyotyping methods by plants alkaloid and colsimid. The effects of 5, 10 and 20 µg.ml^{-1} of herba alkaloid (*V. herbacea* alkaloid) and rose alkaloid (*C. roseus* alkaloid) on human peripheral blood cells proliferation were compared with those of 5, 10 and 20 µg.ml^{-1} colchicine by culturing cells in the absence or presence of alkaloids for one cell cycle and counting the reduce in cell number 2 h later relative to the number of cells present at the time of alkaloid addition [15]. For studies evaluating mitotic block, cells were grown on poly-L-lysine-treated (50 µmg/ml, 2 h, 37 °C, washed once with sterile water) sterile glass coverslips in six-well plates. Plating densities ranged from 1 to 5×10^4 cells/ml to maintain cultures in log growth during plant alkaloid incubation. Cells were incubated with alkaloids by replacing the original medium with an equal volume of medium containing the required concentration of alkaloids, or no alkaloids (control), and incubation was continued at 37 °C for 2 h. Giemsa stain was used to identify chromosomes in the preparation [16,17].

Cell Proliferation

At the time of initiation and termination of vinca alkaloid incubation, duplicate cultures were detached from the culture vessel by incubation with trypsin (0.5 $mgml^{-1}$ in phosphate-buffered saline: 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, 8.1 mM Na2HPO4, and 0.5 mM EDTA, pH 7.2) and live cells were counted using a hemocytometer. Trypan blue dye was used to distinguish living from dead cells [17]. Cell proliferation was calculated from the difference in cell number at the beginning and the end of vinca alkaloids incubation, relative to the increase in cell number for control cultures during the same period.

Mitotic Progression

To evaluate mitotic indices, white blood cells grown for 2 h in the absence or presence of vinca alkaloids in six-well plates were detached by incubation with trypsin, washed with phosphate buffered saline, and fixed with 100% methanol. Cells were incubated with 10% normal goat serum to block nonspecific antibody staining, and then with a rat anti-tubulin monoclonal antibody (Immune Research Laboratories of Pastore institute, Iran) (1 h, 37 °C) followed, after washing, by Rhodamine Red-X-conjugated goat anti-rat IgG (Immune Research Laboratories of institute Pastore, Iran) for 1 h at room temperature. Chromosomes and chromatin were stained with 0.1 to 1 mg/ml 4,6-diamidino-2-phenylindole for 2 to 5 min. Blood white cells were incubated for 18 h in a range of herba alkaloid, rose alkaloid and colchicine concentrations, then both detached and attached cells were collected, fixed, and their microtubules and chromosomes stained. The

numbers of cells in mitosis and in interphase were then determined by microscopy.

To evaluate microtubule and chromosome organization and the stage of mitosis at which the cells were blocked, cells grown on poly-L-lysine-coated coverslips (to enhance cell attachment) were incubated with vinca alkaloids for 20 h, fixed with 10% formalin (25 °C), followed by 100% methanol (4 °C) [10], and stained as described above with the addition of mouse anti-tubulin monoclonal antibody (GTU-88; Sigma Chemical, St. Louis, MO) and fluorescein isothiocyanate- conjugated goat anti-mouse IgG antibody (Sigma Chemicals). Analysis of spindle organization is more accurate using attached cells because the cells attach with their spindle axes lying parallel to the glass surface, and it becomes relatively easy to discern changes in numbers of chromosomes congressed to the metaphase plate and changes in the number of spindle poles. Because only mitotic cells were enumerated, any differential attachment between interphase and mitotic stages weren't important. Stained cells were mounted on slides using Prolong (Molecular Probes, Eugene, OR) anti-fade reagent. Cells were examined using an Eclipse E800 microscope (Nikon, Melville, NY).

Results

Total Alkaloid

The total alkaloid content of *Catharanthus roseus* L. and *Vinca herbacea* shoots were 0.138 and 0.147 mgg^{-1} (FW) respectively. There wasn't significant difference between alkaloid contents of the two plants.

Effects of Herba alkaloid and Rose alkaloid on Blood white Cell Mitotic Progression

Herba alkaloid, rose alkaloid and colchicine were arrested cell division and inhibited cell proliferation at all concentrations. Herba alkaloid treatments had the lowest cell division by all its concentrations. It was very affective on cell division inhibition (Fig. 1).

The relationship between cell cycle progression and inhibition of cell proliferation were examined by determining the mitotic index at a range of all alkaloids concentrations. As shown in Fig. 2, all three alkaloids blocked cell cycle progression in mitosis at the same range of alkaloid concentrations that inhibited proliferation. The effective value was found to be in mitosis were 20 $\mu g.ml^{-1}$.

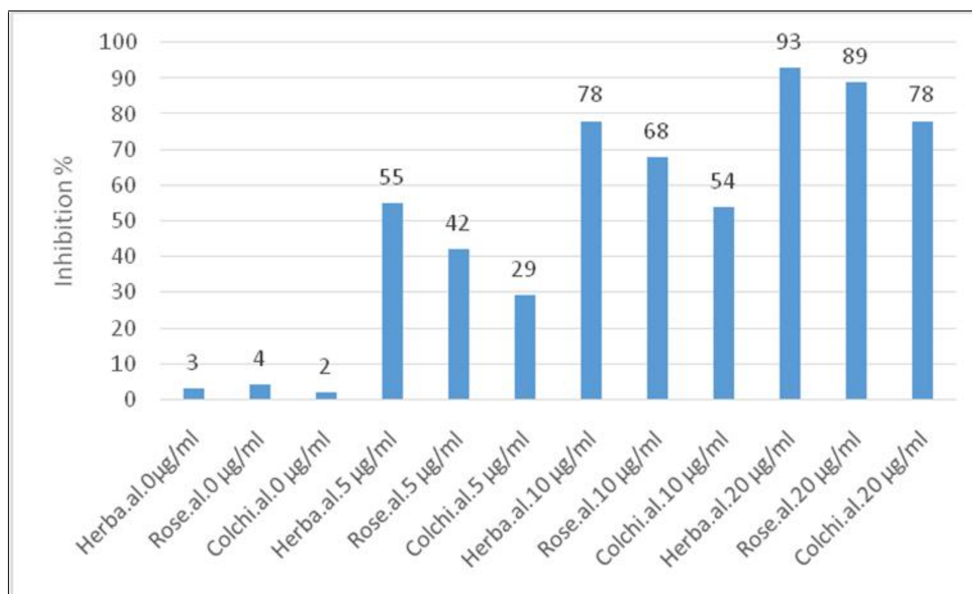

Fig. 1 Concentration dependence for inhibition of blood white cell proliferation by herba alkaloid (her), rose alkaloid (ros), and colchicine (col). The percentage inhibition of cell proliferation is the increase in cell number that occurred during 2 h of continuous incubation with alkaloid compared with the increase in cell number in a parallel culture without alkaloid (0 concentrations).

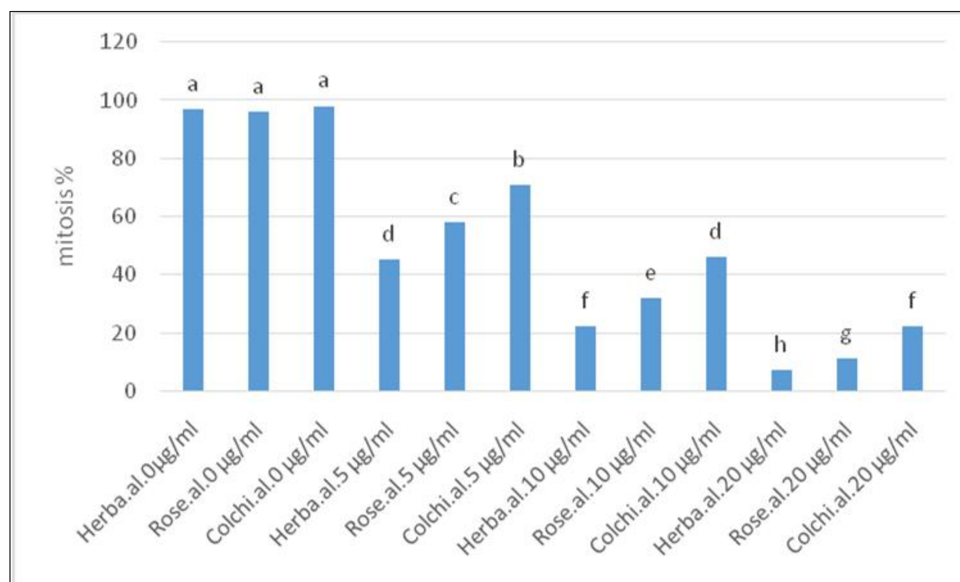

Fig. 2 Concentration dependence for accumulation of blood white cells in mitosis. The mitotic index was determined by counting the percentage of cells in each stage of mitosis (metaphase through telophase) after a 2 h continuous incubation with alkaloid Concentration.

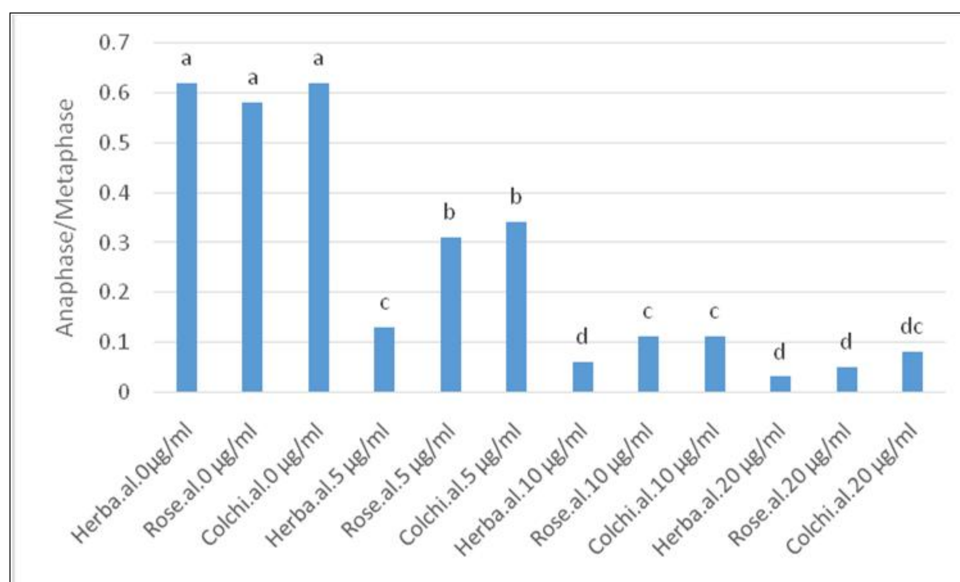

Fig. 3 Concentration dependence of herba alkaloid (her), rose alkaloid (ros) and colchicine(col) for a mitotic block specifically at the transition from metaphase to anaphase. Both floating and attached cells were collected, their microtubules and chromosomes stained and cells counted by fluorescence microscopy. Data are the ratio of the number of cells in anaphase to the number of cells in metaphase; the ratio in the absence of alkaloid was 0.62±0.11 and 0.58±0.07 between 200 and 400 cells were counted for each condition in each experiment.

The stage of mitosis at which the block occurred was determined by counting relative numbers of cells at each stage of mitosis after staining. As shown in Fig. 3, the ratio of the number of cells in anaphase to those in metaphase decreased to zero over the concentration range that induced mitotic block, indicating a block specifically in metaphase. At concentrations of 10 and 20 $\mu g.ml^{-1}$ herba alkaloid, 20 $\mu g.ml^{-1}$ rose alkaloid and colchicine, cells in anaphase were very low. Thus, the block occurred specifically at the transition from metaphase to anaphase (Fig. 4). The potency of the three compounds with respect to both inhibition of cell proliferation and mitotic block was herba alkaloid> rose alkaloid> colchicine.

Effects of Herba Alkaloid and Rose Alkaloid on Spindle Organization

Herba alkaloid, rose alkaloid and colchicine affect microtubule dynamics of cells division. We wanted to determine whether these effects would result in significantly different alterations in spindle organization and might be responsible for the superior antitumor activities of the *V. herbacea* alkaloids to comparison with *C. roseus* alkaloids and colchicine. Thus, we compared the alterations in the arrangement of microtubules, centrosomes, and chromosomes induced by each alkaloid at the 5, 10, and 20 µg.ml^{-1} concentrations for mitotic block, using immunofluorescence microscopy (Fig. 5).

The control cells in mitosis contained well-organized bipolar spindles with two distinct, well-separated spindle poles and a few astral microtubules. At metaphase, all of the chromosomes were organized compact in a equatorial metaphase plate. A small percentage of cells (2.3%) contained multipolar spindles (generally tri polar or quadric polar spindles).

At concentrations of herba alkaloid, rose alkaloid, and colchicine ranging from the 5 to the 20 µg.ml^{-1} for mitosis, spindles were often abnormal or absence. Abnormal spindle of all concentrations consisted of bipolar spindles with few or many un- congressed chromosomes, respectively. Treatment of 20 µg.ml^{-1} consisted of mono-polar spindles enclosed in a ball of chromosomes. Even after alkaloid incubation, some metaphase spindles appeared normal and were bipolar with completely congressed chromosomes; they were indistinguishable from spindles of control cells.

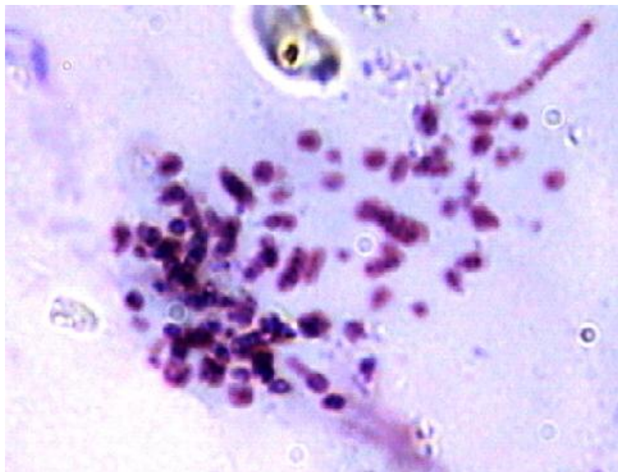

Fig. 4 Prometaphase human blood cells were treated by 10 µg.ml^{-1} herba alkaloid (Gimsa staining).

In mitotic block tests of the 5 µg.ml^{-1} of herba alkaloid, rose alkaloid, and colchicine, 27, 29 and 38% of the spindles appeared normal respectively. At the 5 µg.ml^{-1}, herba alkaloid and rose alkaloid categories of spindles existed in approximately equal numbers cells (73 and 71% abnormal spindles cells respectively), except that the abnormal spindles predominated lower after incubation with colchicine alkaloid (62% abnormal spindles cells). At the 10 µg.ml^{-1} of all categories normal spindles were rare, whereas abnormal spindles predominated (83, 81, 69% abnormal spindles cells).

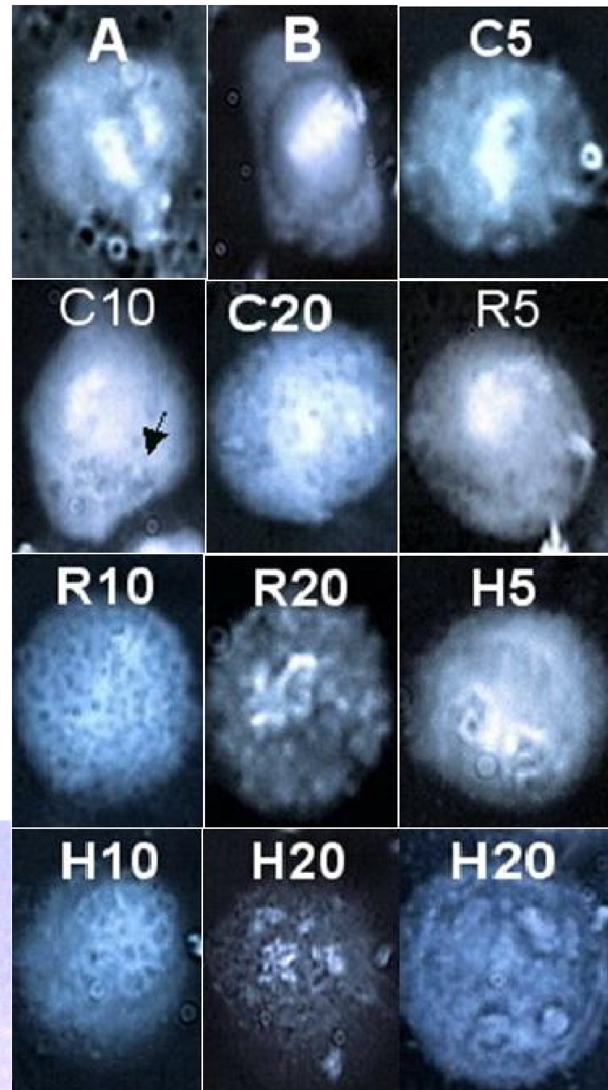

Fig. 5 Mitotic Blood white cells in the absence (A and B) and presence of concentration of 5, 10 µg.ml^{-1} herba alkaloid (H5, H10 and H20), rose alkaloid (R5, R10 and 20) and colchicine (C5, C10 and C20). Cells were fixed and incubated with an anti-b-tubulin antibody to stain microtubules, concentrations of 5 and 10 µg.ml^{-1} abnormal spindles consist of bipolar spindles with uncompressed chromosomes. Alkaloid concentrations of 20 µg.ml^{-1} abnormal spindles consist of monopolar spindles with chromosomes arranged in a ball.

At the 20 µg.ml^{-1} concentration, there were very rare or no normal spindles and 95, 94, 83% of the spindles

were abnormal after incubation with each of the three alkaloids (Fig. 6). Measurement of the distance between poles of bipolar spindles after alkaloid incubation indicated that the pole-to-pole distance was shorter at the 20 µg.ml^{-1} concentrations of the three alkaloids (Table 1).

Herba alkaloid had the greatest effect on the spindle length, reducing it by 37.57%, from 9.45±0.45 µm in controls to 5.9±0.33 µm. The pole-to-pole distance was reduced 29./% and 22.75% by rosa alkaloid (to 6.7±0.2 µm) and by colchicine (to 7.3±0.17µm). Shortening of the spindle may be caused by net microtubule depolymerization or by changes in the balance of dynamics between microtubule subsets in the spindle.

Table 1 Effect of *Vinca* alkaloids at the 20 µg.ml^{-1} concentration for mitotic block on spindle length in blood white cellsSpindle length was measured as the interpolar distance, namely the distance between centrosomes at opposite spindle poles of bipolar spindles after fixing and staining cells with tubulin antibodies (mean ± S.E.M. of at least 50 spindles.

Alkaloids(20 µg.ml^{-1})	Pole-to-pole distance(µm)
Control(no alkaloid)	9.45±0.45
Herba	5.9±0.33
Rosa	6.7±0.2
Colchicine	7.3±0.17

Discussion

We previously determined the alkaloids content of *Vinca herbacea* and *Catharanthus roseus* [18]. Vinca (rose) alkaloids are antimitotic alkaloids that are widely used in cancer treatment [19]. In this research, we have found that herba alkaloid, rose alkaloid and colchicine inhibit proliferation of blood white cells in parallel with mitotic block at the metaphase/anaphase transition. At the concentrations that inhibited mitotic progression, each of the three alkaloids include herba alkaloid (*V. herbacea* alkaloid) and rose alkaloid (*C. roseus* alkaloid) and colchicine,induced similarly aberrant spindle organization. Herba and rose alkaloids treatments had the lowest cell division and the highest cell proliferation inhibition percentage by 20 µg.ml-1 concentrations without significant difference but they have significantly difference with colchicine effect. The herba alkaloid, as rose and colchicine alkaloids, which is a class of compounds that were originally isolated from *Vinca herbacea* (herbaceous periwinkle), interact with microtubule as the rose alkaloid [11]. Herba alkaloid within a concentration range that blocks proliferation, and as good as the vinca alkaloids and colchicine bind to tubulin at the plus-tip of microtubules. At the lower end of this range, this inhibits microtubule dynamics without altering polymer levels, whereas at higher concentrations, it induces microtubule depolymerisation [10]. Colchicine is believed to block mitosis by disrupting the mitotic spindle tubules at metaphase. In comparison, the effective value for colchicine was 5-10 µg.ml^{-1}, as reported previously [15].

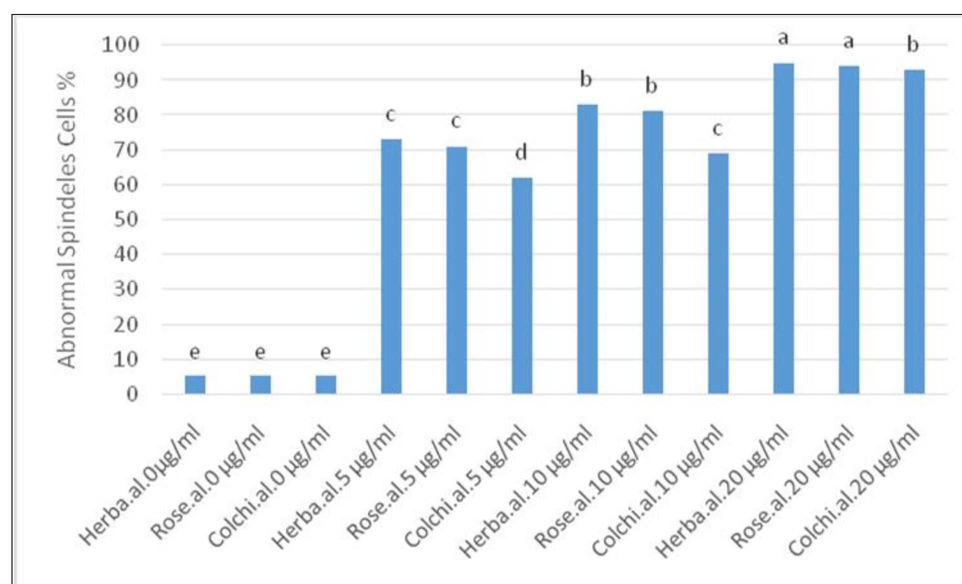

Fig. 6 Frequency of spindle abnormalities induced in blood cells by herba alkaloid (her), rose alkaloid (ros) and colchicine (col) at concentrations of 0, 5, 10 and 20 µg.ml^{-1} for mitotic block.

The action of colchicine on interphase cells has been attributed to a disassembly of cytoplasmic microtubules [11]. In both situations, mitotic spindle formation is disrupted, and cells therefore fail to complete a normal mitosis. At very high concentrations herb and rose alkaloids can induce the aggregation of tubulin into para-crystals; however, this does not occur at clinically relevant concentrations [10]. The potency of the three compounds with respect to both inhibition of cell proliferation and mitotic block was herba alkaloid> rose alkaloid>colchicine.

Taken together, these results indicate that inhibition of cell proliferation by all three alkaloids results primarily from mitotic block induced by suppression of microtubule dynamics. At concentrations of herba alkaloid, rose alkaloid, and colchicine ranging from the 5 to the 20 $\mu g.ml^{-1}$ for mitosis, spindles were often abnormal or absence. Abnormal spindle of all concentrations consisted of bipolar spindles with few or many uncompressed chromosomes, respectively. Treatment of 20 $\mu g.ml^{-1}$ consisted of monopolar spindles enclosed in a ball of chromosomes.

Microtubules (MTs) are components of the cytoskeleton and play very important role in a great numbers of cellular processes. They are involved in chromosome separation during mitosis and meiosis, and are the major constituents of mitotic spindles, besides they are involved in maintaining cell structure, transport and many others cell functions. Any interference with this MT dynamics can provoke a cell cycle arrest and lead to programmed cell death or apoptosis [20]. MT dynamics, and therefore cell division, can also be perturbed by small molecules, which are usually divided into two groups: (i) MT-stabilizing agents that prevent then depolymerization; and (ii) MT-depolymerizing agents that inhibit their formation. Rose alkaloids fall inside the second group, as they arrest tumor cells during mitosis by binding at the surface between two tubulin heterodimers next to the exchangeable guanosine 5-triphosphate-binding site [21] and depolymerizing the MTs. This leads to cell cycle arrest in mitosis [22-24]. At high concentration Vinca alkaloids lead to the formation of large tubulin polymers called “Paracristal” and as a result, the tumoral cells are stopped in mitosis and immediately they die [25]. However, when the levels of Vinca alkaloids are low, the cells are arrested in mitosis and the cells die after a long time of incubation. Drugs that disrupt mitotic progression, which are commonly referred to as ‘anti-mitotic’, are used extensively for the treatment of cancer [26].

Conclusion

All such drugs that have been approved for clinical use target microtubules, with the taxanes, colchicine and rose alkaloids showing much success against a number of cancers. Our results showed antimitotic and cell proliferation inhibition of herba alkaloid is as good as or better from rose alkaloids and colchicine and it can use a new source of anticancer drug. Thus, it will open a new window for the possibility of clinical application of herba alkaloid in severe conditions such as kind of cancer. However, further studies required to highlight the exact underlying determination of kind of effective alkaloids of *Vinca herbaceae* and mechanisms of the observed anti-proliferation effects. As our knowledge improves in this field, therapeutic targeting would be possible in cancer disorders and cytology study.

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