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Dose Response of *Viola odorata* on Meiotic and Mitotic Chromosomes of *Vicia faba*

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Authors' contributions

This work was carried out in collaboration between all authors. Author MA designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author AK managed the experimentations, analyses of the study and the literature searches. Both authors have read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: *Viola odorata* is a medicinally important plant. It contains bioactive principle viz violin and probably methyl salicylic ester. The present study was designed to analyze the effect of different doses of this medicinal plant extract on meiotic and mitotic chromosomes of *Vicia faba*.
Study Design: In this study, different concentrations (5, 10, 20, 30, 40, & 50 %) of *Viola odorata* extract were applied for 4, 8, 12 and 24h on *Vicia faba* plant. Effect of these concentrations was studied on the meiotic and mitotic chromosomes. Gesticulate negative activity of herbal medicine was observed under the effect of different concentrations of the extract.
Place and Duration of Study: The extract of *Viola odorata* was prepared in the laboratory. Study was performed from January, 2011 to December, 2012.
Methodology: Flower bud treatment method was applied for meiotic analysis and seed soaked & root tip treatment methods were used for mitotic analysis. Mitotic and meiotic chromosome indicated various abnormalities against different doses. Cytotoxicity was inferred when the Mitotic index (dividing cells/1000 scored) of treated cells was significantly different ($P<0.05$) from control.
Results: The percent of their abnormalities increase with increasing dose (5-50%) and time (4-24 hours) dependent. Low dose treatment of buds caused increase in mitotic

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index. Treated roots were found negatively affected with no exception and found more harmful and effective compared to seed soaked treatment. Stickiness was more frequent than other abnormalities.
Conclusion: *Vicia faba* chromosome analysis is indicative of clastogenic action of *Viola odorata*. Thus, results of the present study suggest the doses of *Viola odorata* extract to verify the toxic properties before using for treatment.

Keywords: Medicinal plant; chromosome; plant extract; anomalies; stickiness; cytotoxicity.

DEFINITIONS

Chromatin Bridges*: A bridge between two newly forming daughter cells made of chromatin. When cell division occurs, the two centromeres of a broken chromosome may be pulled to the opposite spindle poles of the cell, forming an irregular, long Chromosome Bridge between the two newly forming daughter cells. Eventually, the abnormal chromatid breaks in two or may be left behind during cell division. Or Simply chromosome bridges are abnormalities at anaphase that appear when rearranged chromosomes frequently fail to segregate in an orderly manner, forming bridges between the spindle poles instead. At the anaphase-telophase transition, these bridges may subsequently break, resulting in novel chromosomal variants in the daughter cells.*

Swelling of Chromosome*: An increase in diameter of chromosome due to the effect of some chemical or reagents. The hydration produces the swelling of the cytoplasmatic proteins that in turn gives raise to the chromosome spreading. In the case of the chromosomes, the same hydration process causes the swelling of the proteins that increases the three-dimension size of the chromosomes; consequently, more or less condensed areas along the chromosome itself (that will be seen as bands after staining) are originated.*

Stretching and Thinning of Chromosomes*: Stretching and thinning are due to change in viscosity and surface tension of the lipid contents in chromosomes with treatment.*

Chromosome Stickiness*: The chromosomes appeared as a dense chromatin clump almost losing their identity. The stickiness in chromatin material persisted even during anaphases and telophases, causing difficulties in segregation of chromosomes.*

1. INTRODUCTION

Many plant extracts and their active principles have been described and utilized as therapeutic agents. There is considerable interest in determining the risk that these products may pose to health, because many of these plants contain compounds which are known to cause diseases in animals and humans. Thus, an assessment of their cytotoxic and mutagenic potential is necessary to ensure a relatively safe use of medicinal plants [1]. *Viola odorata* (family: Violaceae) is a medicinally important plant that have active principle named violine. Violine is believed to resemble emetine, the alkaloid of the emetic drug ipecacuanha in some of its characters. Viola also contains methyl salicylic ester [2]. Flowers are astringent, diaphoretic, diuretic and aperients. In France syrup of Violets is a medicine for cough and hoarsness. Flowers also contain traces of volatile oil [3]. Plant extract could be potent mutagens and thus selection of proper dose is required before using it as a treatment.

Vicia faba L. (2n = 12), of the family Fabaceae, have homozygous genotype because of self pollination [4]. Their chromosomes are useful to chromosome anomaly studies because of large and easily visible size. Eukaryotic cells have similar type of nucleic acid materials hence one can study an initial indication of effects of plant extracts on plant chromosomes, before a trial on human beings.

2. MATERIAL AND METHODS

2.1 Methanolic Extraction

A quantity of 30gm of grounded whole plants (shoots, leaves, flowers and roots) of *Viola odorata* was put in thimble tube in Soxhlet apparatus. Aliquots of 200ml methanol were used for extraction at 45°C for 5-6 hours per day for three successive days after that solvent was evaporated by keeping the extraction in an incubator (37°C) until the solvent totally evaporated. The extract was collected and weighed then diluted with methanol to prepare 5%, 10%, 20%, 30%, 40%, and 50% weight/volume.

2.1.1 For meiotic analysis – Flower bud treatment

Growing plants of *Vicia faba* (35days old) were sprayed at the flowering time with varying concentration (5, 10, 20, 30, 40 & 50 %). The spraying of plant was done for two consecutive days. Now the young buds were collected before anthesis at early morning and fixed in acetic alcohol (1:3) mixed with few drops of ferric acetate solution for 6-15 hrs. After fixation the material was washed with distilled water and stored in 70% alcohol. To study meiotic behavior, anthers were macerated in one drop of 2% acetocarmine [5].

2.1.2 For mitotic analysis – Seed Soaked treatment

Healthy seeds were selected and presoaked in distilled water for (6 to 12 hrs) and then subjected to 6 concentration (5%, 10%, 20%, 30%, 40% and 50%) for 24hours treatment. Treated seeds were planted in pots till germination. Young roots were excised for mitotic study.

2.1.3 For mitotic analysis – Root tip treatment

To analyze root tip, healthy seeds of *Vicia faba* were germinated on moist filter paper in petri-dishes without soaking in extract solutions. Then germinated seeds having root tips of about 0.5 to 1 cm length were cut from seeds and transferred into varying concentration (5% to 50%) of *Viola odorata* extracts for 4, 8, 12 and 24 hrs duration [5].

Excised tips of both treatments were fixed in freshly prepared fixative solution acetic alcohol (1:3) for 24hrs. Feulgen squash technique was used according to Darlington and La Cour [6] and examined microscopically for mitotic analysis.

Mitotic Index was calculated by using the formula given below

$$\text{Mitotic index} = \left[\frac{\text{number of dividing cell}}{\text{number of dividing + non-dividing cell}} \right] \times 100$$

2.1.4 Statistical analysis

The percentage of aberrations at each dose of extract was compared with that of the negative control using the one way ANOVA. One-way ANOVA followed by Turkey's multiple comparisons test was performed using GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com to differentiate means. A dose of extract was considered to be toxic if Turkeys's comparison test was significant at $P \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1 Meiotic Study

Varying concentration of *Viola odorata* induced various abnormalities viz stickiness, stretching, multivalent, chromatin bridges, etc (Table 1) in the pollen mother cells of *Vicia faba*. The treated plants exhibited various degrees of abnormalities varying with different concentrations applied.

3.1.1 Multivalents

Meiosis of untreated control plant is normal showing six bivalents (2n = 12). Treated plants were showed sticky multivalents in the form of tri, quadri, hexa and octavalent (Fig. 1. A,B) and that was more frequent at higher concentrations. In spray treatment, percentage of abnormalities ranged from 0.65% to 1.43%. This is probably caused by cryptic structural changes in the chromosome forming genetic differences restrict pairing with other homologous ones [7].

3.1.2 Stickiness

The stickiness of chromosomes at diplotene, diakinesis, metaphase I & II (Fig.1: C, D) were observed. Frequency was high in metaphase I in *Vicia faba* (4.02%). This effect was not observed for aberrated cells only but for many of the normal ones also. The stickiness of chromosome was believed to be the primary effect induced by *Viola odorata* which depolymerize DNA or disrupt the bonds between protein and nucleic acids constituents of chromosomes [8]. The thick bridges may be due to the stickiness in chromosomes. This stickiness interfered in the normal arrangement of chromosomes at metaphase and further led to their inability to separate, thus leading to sticky bridges. The spindle fibers pulled the chromosomes towards the poles these bridges were broken into fragments, which either moved towards the poles or formed laggards and micronuclei. During stickiness chromosomes formed a compact mass and the identity of individual chromosomes was lost.

3.1.3 Chromatin bridges

The chromatin bridges with or without fragment (fig. 1. D) was noticed in *Vicia faba*. Chromatin bridges were observed in high frequency at Anaphase I and low frequency at Anaphase II (Fig. 1. E). The bridge was formed due to sticky nature of chromosomes. The bivalent could not move at anaphase I to their respective poles due to inactivation of spindle and sticky nature got stretched up due to attracting forces of the poles [9,10].

Single or double bridges were known to result from asymmetrical length of chromatids or chromosomes inter changes [11].

Table 1. Effects of different concentration of *Viola odorata* on pollen mother cells (PMCs)

Concentration (in percentage)	No. of PMCs	Type of abnormalities (in percentage)				Total percent of abnormality
		Sticky	Multivalents	Bridge	Others (Stretching)	
Control	57	0.08	-	-	-	0.08
5	52	3.12	0.65	0.45	-	4.22
10	55	3.56	0.81	0.97	-	5.34
20	60	3.78	0.98	-	0.23	4.99
30	51	4.01	1.20	-	0.61	5.82
40	44	4.56	1.35	-	-	5.91
50	38	5.12	1.43	1.03	0.87	8.45
Total	300	24.15	6.42	2.45	1.71	34.81

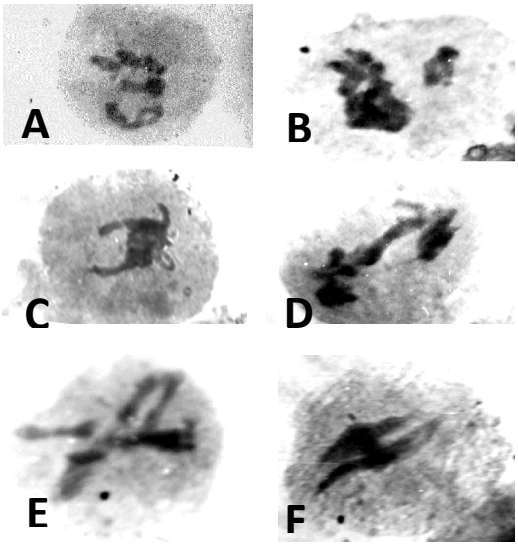

Fig. 1. Effect of different doses of *Viola odorata* extract on meiotic chromosome- A. Diakinesis: Association of bivalents into hexavalent, Quadrivalent & Bivalent B. Diakinesis: Clumping of 5 Bivalents and one free C. Sticky metaphase chromosome D. Late sticky anaphase with chromatin bridge E. Telophase II with thick chromatin bridge F. Stretched chromosomes.

3.1.4 Swelling, stretching and thinning of chromosomes

The abnormalities were also observed but in low percentage (Fig. 1: F). It is possibly the result of change in viscosity and surface tension of the lipid contents in chromosomes with treatment. These anomalies revealed that the extract concentration affects on both, chromosome structure and spindle material [12].

The mean ± standard deviation was considered to investigate order of various abnormalities in all concentrations. The order of frequency was: stickiness (4.25% ± 0.72) >multivalent (1.07% ± 0.31) > bridges (0.817% ± 0.32) > stretching (0.570% ± 0.32).

3.2 Mitotic Study

The data obtained has been summarized in table (2 & 3) with detail study of the effect of plant extract on mitotic index. The extract of *Viola odorata* had a strong mitostatic effect on *Vicia faba* root as evident by the mitotic index which decreases with increase in duration 4-12 hrs and concentration from 5 to 50 %. The reduction in mitotic index might be due to turbagenic changes induced by the extract in nuclear chromatin [13]. The mean percent of mitotic index (MI) was much reduced in root tip treatment as compared to seed soaked treatment.

Table 2. Type and distribution of somatic chromosomal abnormalities induced by different concentrations and durations of *Viola odorata* (root-tip treatment) in *Vicia faba* –

	Conc. %	DC	NDC	TOC	MI	Cond SC	Frag	CB	La	Total %ab
4h	Control	109	701	810	13.50	1.42	-	-	-	1.42
	5	109	709	818	13.33	8.84	1.5	-	-	10.34
	10	110	720	830	13.21	9.04	1.34	0.98	-	11.36
	20	109	720	829	13.09	10.88	0.93	1	-	12.81
	30	106	709	815	12.95	11.51	0.72	1.1	-	13.33
	40	102	703	805	12.65	12.03	0.51	1.12	-	13.66
	50	100	700	800	12.48	12.89	0.28	1.32	0.5	15.49
8h	Control	111	694	805	13.75	0.97	-	-	-	0.97
	5	105	694	799	13.10	8.95	1.75	-	0.5	11.2
	10	92	611	703	13.05	9.86	1.5	0.91	-	12.27
	20	102	688	790	12.88	10.99	1.25	1.05	-	13.29
	30	99	676	775	12.75	11.63	1.02	1.11	-	13.76
	40	98	683	780	12.50	12.5	0.98	1.14	-	15.3
	50	98	702	800	12.23	13.01	0.75	1.23	1	17.79
12h	Control	108	687	795	13.60	2.05	-	-	-	2.05
	5	104	696	800	13.01	9.03	2.01	1.07	-	12.11
	10	102	683	785	12.95	10.08	1.83	1.1	-	13.02
	20	100	690	790	12.68	11.78	1.51	1.2	-	14.42
	30	96	679	775	12.43	12.51	1.32	1.41	-	16.24
	40	97	698	795	12.20	13.01	1.08	1.63	-	16.95
	50	92	676	768	12.00	13.86	1.02	1.8	-	18.69
24h	Control	107	694	801	13.31	1.46	-	-	-	1.46
	5	103	692	795	12.91	8.8	1.65	0.68	-	11.13
	10	100	680	780	12.78	9	1.38	0.79	-	11.17
	20	100	698	798	12.50	10.13	1.01	0.81	-	11.95
	30	96	707	803	12.01	10.51	0.92	0.98	-	12.41
	40	94	695	789	11.96	11.62	0.23	1.13	-	12.98
	50	94	706	800	11.81	12.01	-	1.23	-	13.24

Note: Du. = Duration; DC= dividing cells; NDC= none dividing cells; TOC = total observed cell; Con % = Concentration in percentage; MI = Mitotic index (Number of dividing cell/1000 scored); Cond SC = Condensed and sticky chromosomes; Frag = Fragments; CB = Chromatin bridges; La = Laggards; Total % ab = Total of abnormalities. All values for 24hrs observations were found extremely significant at p value P ≤0.05 in comparison to control

Table 3. Somatic chromosomal abnormalities induced by different concentration of *Viola odorata* (seed-soaked treatment of 24 hrs duration) in *Vicia faba*

	Conc %	DC	NDC	TOC	MI	CondSC	Frag	CB	La	Tot %ab
24hrs	Control	122	653	775	15.68	0.18	-	-	-	0.18
	5	85	635	720	11.82	5.48	0.17	0.3	-	5.95
	10	82	657	739	11.03	6.03	0.61	0.43	-	7.04
	20	83	695	778	10.64	6.97	1.35	0.53	-	8.85
	30	78	687	765	10.15	7.51	-	0.79	-	8.3
	40	75	683	758	9.91	8.25	-	0.98	-	10.18
	50	68	681	749	9.05	9.06	-	1.04	-	12.01

Note :Conc % = Concentration in percentage; DC= dividing cells; NDC= none dividing cells; TOC = Total observed cell; MI = Mitotic Index; Cond SC = Condensed and sticky chromosomes; Frag = Fragments; CB = Chromatin bridges; La = Laggards; Total % ab = Total of abnormalities. All values were found extremely significant at p value $P \leq 0.05$ comparison to control

The percentage of abnormalities recorded at different treatment, increased as the concentration increased and time prolonged. Strongly we could conclude that there is strong negative relationship between MI and the percentage of abnormalities as previously recorded for many extracts [14,15].

Various types of abnormalities were observed viz., condensed and sticky chromosomes, disturbed anaphase, fragments, chromatin bridges and laggards. These observations may be due to nucleotoxic action of extracts or the disturbance of the formations of spindle fibers during cell division, which leads to chromosomal aberration.

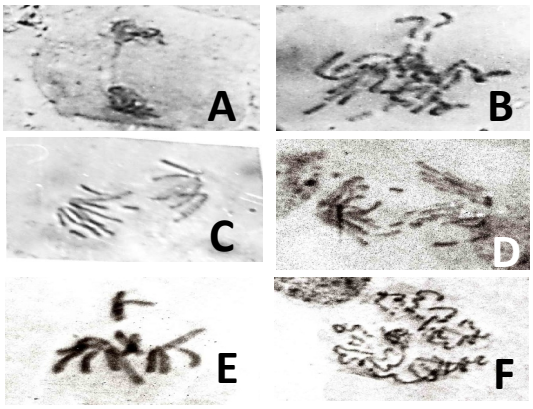

Fig. 2. Effect of different doses of *Viola odorata* extract on mitotic chromosome- A. Telophase: chromatin bridge B. Anaphase : Disturbed and fragmented C. Anaphase : Lagging chromosome D. Anaphase: laggard and fragments. E. Metaphase: Sticky and Un-oriented chromosome F. Prophase: Fragmented

3.2.1 Sticky chromosome

Stickiness and clumping of chromosomes were the most frequent effect of extract. Stickiness usually leads to formations of anaphase and telophase bridges. Stickiness might

be due to DNA depolymerisation and partial dissolution of nucleoprotein proteins and the stripling of the protein covering of DNA in chromosomes as also observed by Onyenwe, [16]. Numerical and structural changes in chromosomes were attributed to spindle failure, leading to fragmentation, lagging chromosomes [17] (Fig. 2. E).

3.2.2 Chromatin bridges

Bridge was observed due to sticky nature of chromosomes [18] (Fig. 2. A).

3.2.3 Fragments

Fragments were noticed either due to terminal breaks in the chromosome or failure of chromosome thread to rejoin (Fig. 2. B, D, F).

3.2.4 Lagging chromosome

The lagging chromosome was possibly formed due to the inhibition of centromeric or spindle activity which inhibits chromosome movements and due to presence of acentric fragment or the interaction of extract with protein of spindle apparatus [19] (Fig. 2. C, D).

The mean ± standard deviation was considered to investigate order of various abnormalities in all concentrations at 24h. The order of frequency was: stickiness (10.345 ± 1.32) >fragment (1.038 ± 0.54) > bridges (0.937 ± 0.21) > laggard (0.00% ± 0.0), in root tip treatment method. The abnormalities in seed soaked method was observed with same order with different values i.e. stickiness (7.217 ± 1.34) >fragment (0.710 ± 0.60) > bridges (0.678 ± 0.30) > laggard (0.00% ± 0.0).

Both methods (seed soaked treatment and root tip treatment) indicate significant abnormalities at $P \leq 0.05$ (Table 2 & 3) that was subjected to varying concentration of extract. The MI values were comparably lower ($P \leq 0.05$) than control. Types of abnormalities were same in root tip treatment and seed soaked treatment; in all cases sticky and bridge types anomalies were frequent. The possible mechanism for the induction of above aberrations can be directly or indirectly concerned with the action of higher doses of *Viola odorata* extract on DNA. Other herbal drugs may also concern with the action of the plant extract on DNA. Therefore herbal extracts must be checked for correct doses before administration.

4. CONCLUSION

Viola odorata are used for treatment of various diseases [20] however, at this time there is not enough scientific information to determine an appropriate range of doses for *Viola odorata*. Based on the information provided in this work, it is concluded that the cytotoxic effect of *Viola odorata* extract depends on their concentration. The detractive effect of higher dose of *Viola odorata* extract's on mitosis and meiosis of *Vicia faba* shows that it has a clastogenic effect on root tip cells as well as buds. It is known that plant cytotoxic bioassays have a good correlation with mammalian cell based assays [21-28]; therefore present study also suggests that inadvisable higher dose of *Viola odorata* posing definite risk if it contains toxic substances.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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