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## RESEARCH ARTICLE

### ALTERED ENZYME ACTIVITY IN GREEN GRAM SEEDS TREATED WITH ANTIMITOTIC HERBAL DRUGS

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#### ABSTRACT

Seed germination inhibition in green gram (*Phaseolus radiatus*) by compounds inhibitory to cell division has been previously shown to be an *in vitro* assay mechanism for the screening of antimetabolic drugs with anticancer potential. While these studies sought to demonstrate the retardation of cell growth and proliferation as a function of drug treatment, the inhibition of selected hydrolytic enzymes in germinating seeds as a means to further substantiate the biochemical efficacy of these drug molecules forms the objective of the present study. The enzymes selected include amylase and alkaline phosphatase- both of which are enhanced during seed germination as well as in different human metabolic disorders. Treatment with *Kokilaksha* (abbreviated as K-drug), a herbal drug derived from *Asteracantha longifolia*, resulted in significant dose-dependent reduction in the specific activities of both the hydrolytic enzymes within the germinating seeds. Further, significant inhibition of water uptake by imbibition as well as delayed morphogenesis was observed in the K-drug treated seeds, monitored over 24-120h. Similarly the effects of vincristine against these parameters were also studied, for comparison. Since the K-drug shows promise in treatment of human metabolic disorders, involving the elevation of amylolytic and alkaline phosphatase enzymes, the same deserves clinical investigation.

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#### INTRODUCTION

The ability of antimetabolic compounds to inhibit seed germination in green gram enabled the use of this method as a cost and time-effective assay system for screening potential anticancer drugs (Kumar and Singhal, 2010 ; Murthy *et al.*, 2011). Using the seed germination assay, the antimetabolic efficacy of *Kokilaksha* or the K-drug among other drugs was quantified in terms of weight gain by water imbibition at the end of 24h (Murthy *et al.*, 2011). In this study, it was also hypothesized that inhibition of cell proliferation, by the K-drug, whether in seeds or in human disorders could involve altered enzyme profiles. Elevated activity of hydrolytic enzymes has been reported during seed germination (Rahman *et al.*, 2007, Sivakumar *et al.*, 2013). Moreover, various benign as well as malignant human disorders involve the elevation of serum hydrolases such as amylase (Tossiti *et al.*, 2001; Tomita, 1988; Tsukawaki, 1992) and alkaline phosphatase (Singh *et al.*, 2013). We therefore considered it worthwhile to investigate the hypothesis put forth by Murthy *et al.*, 2011 that enzyme profiles

could be altered upon treatment with the K-drug, which could account for the inhibition of seed germination, thus paving the way for investigations into the use of the K-drug in human disorders involving these enzymes, as well.

Thus, we chose to investigate the effects of *Kokilaksha* (abbreviated as the K-drug) on the following parameters over a 24-120h period:-

- Seed weight and onset of morphogenesis.
- Specific activity profiles of amylase and alkaline phosphatase.

#### MATERIALS AND METHODS

All chemicals were either obtained from standard manufacturers such as Sigma, Hi-Media and Merck or were of analytical/reagent grade. Vincristine was obtained from the Kidwai Institute of Oncology Bangalore while the *Kokilaksha* formulation (Patent No.GB2454875 dt.20<sup>th</sup> Nov, 2007), was obtained from the Herbal Science Trust Bangalore. Seeds of *Phaseolus radiatus* (green gram) were obtained from the local markets.

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## Seed treatment

Seeds of green gram (0.5 gm) were immersed in distilled water (which served as control) or different concentrations of the test drugs contained in petri plates. All the plates were incubated for varying periods of time ranging from 24, 48, 72, 96 and 120 h respectively at room temperature. At the end of each incubation, the seedlings were removed from the petri plates and the excess moisture removed by drying on paper towels, after which the fresh weight of the seedlings was recorded.

## Preparation of crude enzyme extract from seeds

The seedlings were harvested at the end of each incubation, homogenized at 4°C and clarified by centrifugation at 10,000 *X* g at 4°C for 10 min to obtain the supernatant which constituted the source of the enzyme estimated.

## Drugs used

The K-drug was diluted 1:5 v/v and 1:10 v/v with distilled water as previously described by Murthy *et al.*, 2011. Since vincristine was also reported by the same authors to exhibit an activity comparable with the K-drug, we chose vincristine for comparison. The commercially available stock solution of vincristine (1.0 mg/mL) was diluted to final concentrations of 0.1 mg/mL and 0.2 mg/mL with distilled water respectively. The pH of all the solutions was found to be 6.5-7.0, thus ruling out any possibility of pH-induced alterations.

## Amylase assay

Amylase activity was determined by quantifying the amount of residual starch by a suitable modification of the starch-iodine method described by Zhizhuang *et al.*, 2006. Suitable blanks, one devoid of starch and another with acid-denatured enzyme, were also set up. One unit of amylase activity is defined as the decrease in absorbance of the starch-iodine complex by 0.01A at 580 nm at 37°C under the assay conditions.

## Alkaline Phosphatase assay

Alkaline phosphatase activity was determined by the method described by Nigam and Aiyyagari, 2008. One unit of activity is defined as the amount of enzyme required to produce 10µM of p-nitrophenol under the assay conditions

## Protein estimation

Protein content was routinely performed using the Coomassie G-250 dye-binding method of Bradford, 1976 using bovine serum albumin as the standard.

## Units of comparison

Specific enzyme activity (expressed in Units/mg) was calculated by dividing the total units of enzyme activity by the total protein content as estimated by the Bradford method.

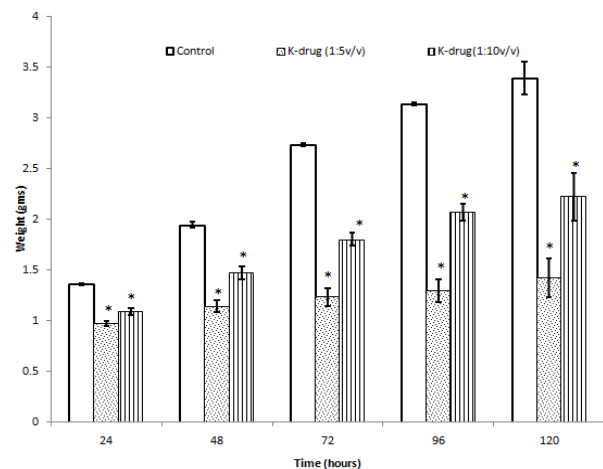
$$\text{Specific enzyme activity} = \frac{\text{Total enzyme activity (units)}}{\text{Total protein content (mg)}}$$

## Statistical tools

The results obtained were expressed as an average of 4 trials ± standard error in all cases except weight profiles, where an average of 5 trials ± standard error was calculated. ANOVA followed by two sided Dunnett analysis was used for determining the statistical differences between specific enzyme activities of the control and the test categories. Linear regression analysis was also used for determining the extent of causal relationships of altered enzyme profiles vis-a-vis drug concentration and altered seed weight. P values ≤0.05 were considered significant.

## RESULTS

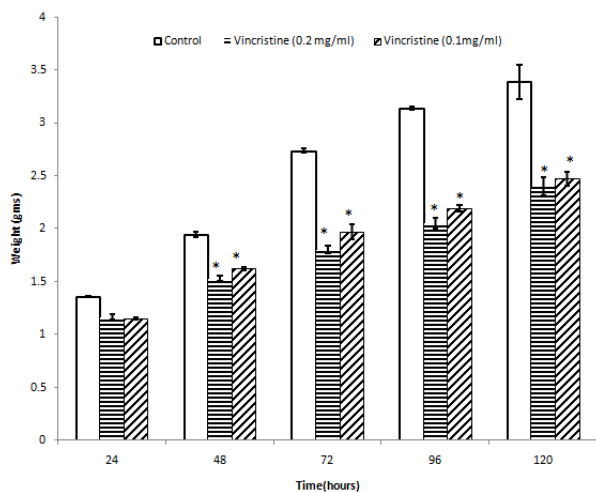
**i) Alterations in seed weight:** Increase in seed weight between 24-120h in the K-drug treated seeds was observed to be significantly inhibited. Seeds germinated in distilled water exhibited a 2.5±0.1-fold increase in weight. By contrast, the fold-increase in the 1:5 v/v diluted K-drug treated seeds was only 1.5±0.2, while treatment with the 1:10 v/v diluted K-drug yielded a 2.03±0.2 fold increase (Fig 1) over the same period. Seeds treated with 0.1mg/ml and 0.2 mg/ml vincristine yielded 2.15±0.05 and 2.05±0.04-fold increases in weight respectively (Fig 2). Statistical analysis indicated that treatment with the K-drug as well as vincristine yielded significant retardation of water imbibition over the 24-120h vis-à-vis control (p < 0.05).



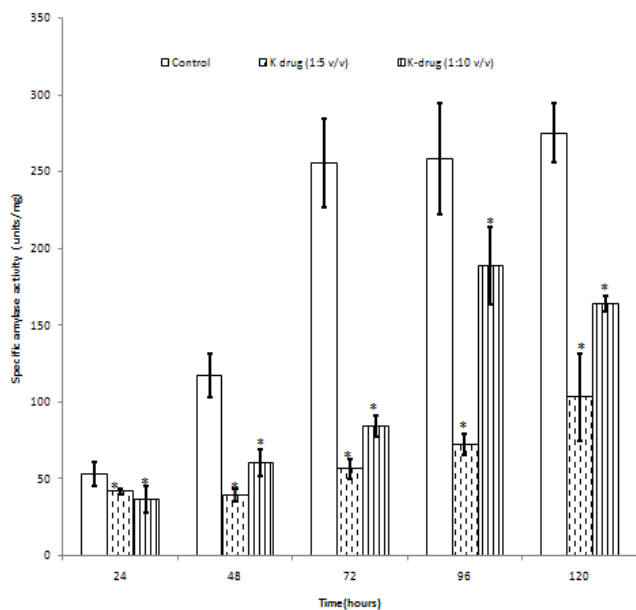
**Figure 1.** Effect of K-drug treatment on seed weight increase by water imbibition: Seeds germinated under control (distilled water), 1:5 w/v and 1:10 w/v of the K-drug for varying periods of time ranging from 24-120 h as indicated under Materials and Methods. At the end of each incubation, fresh weight of seeds was measured. Readings represent the average of 5 trials ± S.E. Significant statistical differences in the fresh weights of the drug-treated seeds with reference to control are indicated by an asterisk (\*)

**ii) Alterations in specific amyolytic activity:** The specific amyolytic activity of 1:5 v/v diluted K-drug treated green gram seeds exhibited an increase of 2.5 ± 0.5 fold over 24-120h unlike the control wherein a fold increase of 5.2 ± 0.8 was observed. At the end of 120h, only 37.5± 7.6% of the activity in the control was retained in the 1:5 v/v diluted K-drug treated seeds, while the 1:10 diluted K-drug treated seeds displayed specific amyolytic activity which was only 59.6±5.6% of the control (Fig.3). Statistical analysis revealed that the K-drug yielded significant inhibition of

amylolytic activity ( $p < 0.05$ ). Vincristine treatment failed to inhibit specific amylase activity over the 24-120h period ( $p > 0.05$ ) with the vincristine-treated seeds at both concentrations exhibiting a higher specific amylolytic activity than the control at the end of 120h (Fig.4).



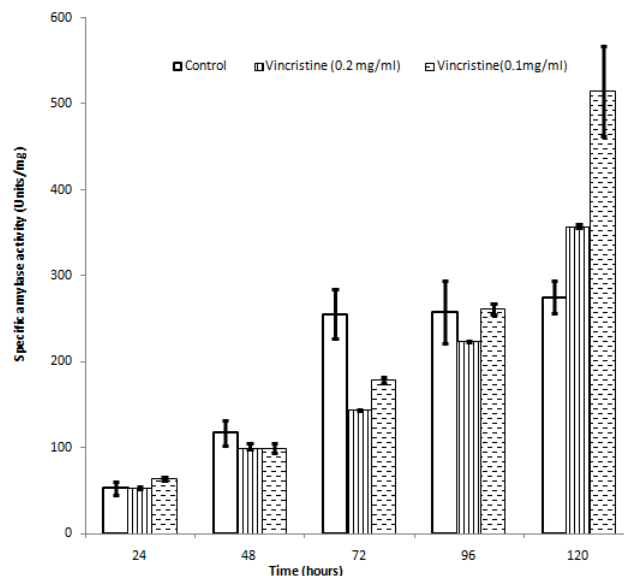
**Figure 2. Effect of Vincristine treatment on seed weight increase by water imbibition:** Seeds germinated under control (distilled water), 0.1mg/ml and 0.2mg/ml of vincristine for varying periods of time ranging from 24-120 h as indicated under Materials and Methods. At the end of each incubation, fresh weight of seeds was measured. Readings represent the average of 5 trials  $\pm$  S.E. Significant statistical differences in the fresh weights of the drug-treated seeds with reference to control are indicated by an asterisk (\*).



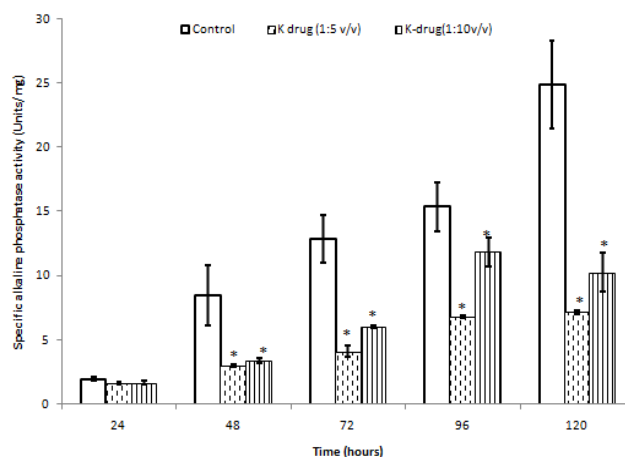
**Figure 3. Effect of K-drug treatment on specific amylolytic activity :** Seedlings germinated under control (distilled water), 1:5 w/v and 1:10 w/v of the K-drug for varying periods of time ranging from 24-120 h harvested as indicated under Materials and Methods. The clarified supernatant was used for amylolytic enzyme assay by the method of Zhizhuang et al (2006). Readings represent the average of 4 trials  $\pm$  S.E. Significant statistical differences in the fresh weights of the drug-treated seeds with reference to control are indicated by an asterisk (\*)

The value of Pearson correlation coefficient for specific amylase activity vis-à-vis weight alterations was calculated as 0.96 and 0.68 for treatments with the K-drug and vincristine respectively.

**iii) Alterations in specific alkaline phosphatase activity:** Green gram seeds germinated in distilled water exhibited a  $12.6 \pm 1.4$  fold increase in specific alkaline phosphatase

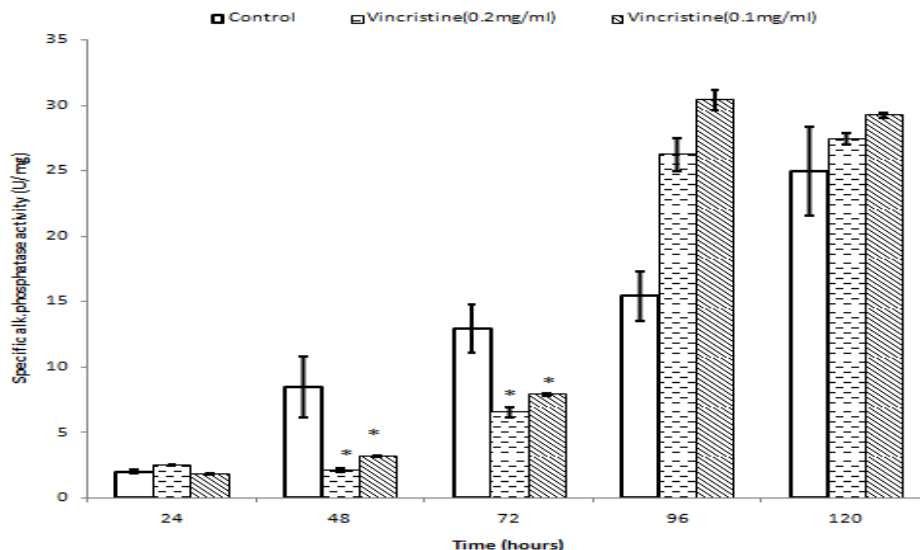


**Figure 4. Effect of Vincristine treatment on specific amylolytic activity :** Seeds germinated under control (distilled water), 0.1mg/ml and 0.2mg/ml of vincristine for varying periods of time ranging from 24-120 h as indicated under Materials and Methods. The clarified supernatant was used for amylolytic enzyme assay by the method of Zhizhuang et al (2006). Readings represent the average of 4 trials  $\pm$  S.E. Significant statistical differences in the fresh weights of the drug-treated seeds with reference to control are indicated by an asterisk (\*)



**Figure 5. Effect of K-drug treatment on specific alkaline phosphatase activity:** Seedlings germinated under control (distilled water), 1:5 w/v and 1:10 w/v of the K-drug for varying periods of time ranging from 24-120 h harvested as indicated under Materials and Methods. The clarified supernatant was used for amylolytic enzyme assay by the method of Nigam and Aiyagari (2008). Readings represent the average of 4 trials  $\pm$  S.E. Significant statistical differences in the fresh weights of the drug-treated seeds with reference to control are indicated by an asterisk (\*)

activity over the 24-120h period while only a  $4.3 \pm 0.2$  fold increase was observed in 1:5 v/v diluted K-drug treated seeds over the same period. Likewise the 1:10 v/v diluted K-drug treated seeds exhibited a  $6.2 \pm 0.6$  fold increase in specific alkaline phosphatase activity over the 24-120h period (Fig.5). At the end of 120h, the specific alkaline phosphatase activity of the 1:5 v/v diluted K-drug treated seeds was  $29 \pm 4.6\%$  of the control while in the 1:10 v/v diluted K-drug treated seeds, only  $41.3 \pm 4.1\%$  of the activity remained. Statistical analysis revealed that the K-drug significantly inhibited alkaline phosphatase activity at both the concentrations tested ( $p < 0.05$ ).



**Figure 6. Effect of Vincristine treatment on specific alkaline phosphatase activity:** Seeds germinated under control (distilled water), 0.1mg/ml and 0.2mg/ml of vincristine for varying periods of time ranging from 24-120 h as indicated under Materials and Methods. The clarified supernatant was used for amyolytic enzyme assay by the method of Nigam and Aiyagari (2008). Readings represent the average of 4 trials ± S.E. Significant statistical differences in the fresh weights of the drug-treated seeds with reference to control are indicated by an asterisk (\*)

**Table 1. Variations in morphological traits following drug treatment**

S.No.	Particulars of Seed Treatment	Morphological alterations over a 24-120 h incubation period*					
		24	48	72	96	120	
1	Control	S+++H+	H++	R++	C++	L++	
2	K-drug	1:5 v/v	S-	S++	S++	S++	S++
		1:10 v/v	S+	S+++H+	H++	R++	C++L+
3	Vincristine	0.2mg/mL	S+	S++	H+	H++	R+
		0.1 mg/mL	S++	H+	H++	R++	C+

**Key**

R :Root; S: shoot; H: hypocotyl ; C: cotyledon; L: leaf

++ : appears in >50% of seeds

+ : appears in <50% of seeds

However, vincristine failed to inhibit alkaline phosphatase ( $p>0.05$ ) despite some initial differences seen at 48h and 72h. Increased activity vis-à-vis control was observed at the end of 96h and 120h of vincristine treatment (Fig.6)

The value of Pearson correlation coefficient for specific alkaline phosphatase activity vis-à-vis weight alterations was calculated as 0.95 and 0.66 for treatments with the K-drug and vincristine respectively.

**iv) Altered morphogenesis:** Seeds in the control group germinated at the end of 120h to yield seedlings bearing well- differentiated root and shoot. Treatment of green gram seeds with the 1:5 diluted K-drug resulted in seed coat rupture at 24-48 h with hypocotyl emergence at 72-96 h. Further elongation of hypocotyl was inhibited. Seed treatment with the 1:10 diluted K-drug caused characteristic stunting of radicle and plumule (Fig 7 I-III).

Vincristine (0.2 mg/mL) treatment delayed morphogenesis with hypocotyl formation alone evident after 120 h. At 0.1 mg/mL, vincristine-treated seeds exhibited persistent elongation of the radicle, while onset of cotyledon development was evident at the end of 120 h (Fig 8, I-III).

The morphological development profile is indicated in Table 1.

**DISCUSSION**

Our results reveal that the K-drug inhibited water uptake by seeds. This inhibition was dose-dependent over the 24-120h incubation period. Similarly, vincristine also effected a significant inhibition of water uptake. While Murthy *et al.*, 2011 have reported that vincristine exhibited 99% of the water imbibition inhibitory activity of the K-drug, it may be noted that their data is based upon a 24h observation. We report that treatment with the K-drug significantly altered seed weight, morphogenesis and the specific activity of the selected enzymes. However, treatment with vincristine resulted in reduced seed weight and altered morphological differentiation alone. While seed weight alterations positively correlated with enzyme activity in both cases, it was observed that the degree of correlation was greater in the case of the K-drug when compared to vincristine. It was also observed that the activity of both the enzymes tested was significantly lower than the control up to 72h incubation in the case of vincristine-treated seeds and that the sharp increase vis-à-vis control occurred only towards 96-120h.

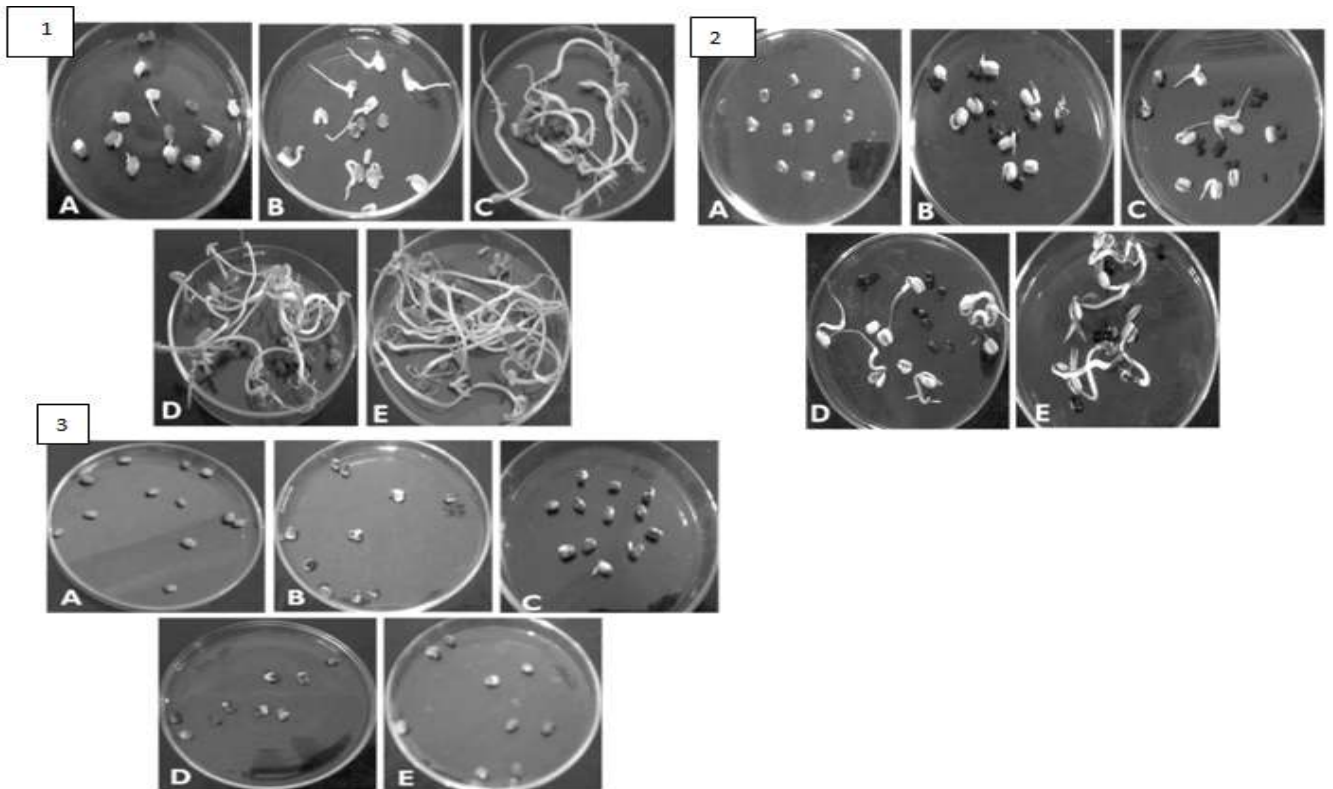


Figure 7. Effect of K-drug treatment on morphological development: Morphological changes in green gram seeds treated with: I: distilled water; II: 1/5 v/v diluted K-drug; III: 1/10 v/v diluted K-drug. A:24h B:48h C:72h D:96h E:120h

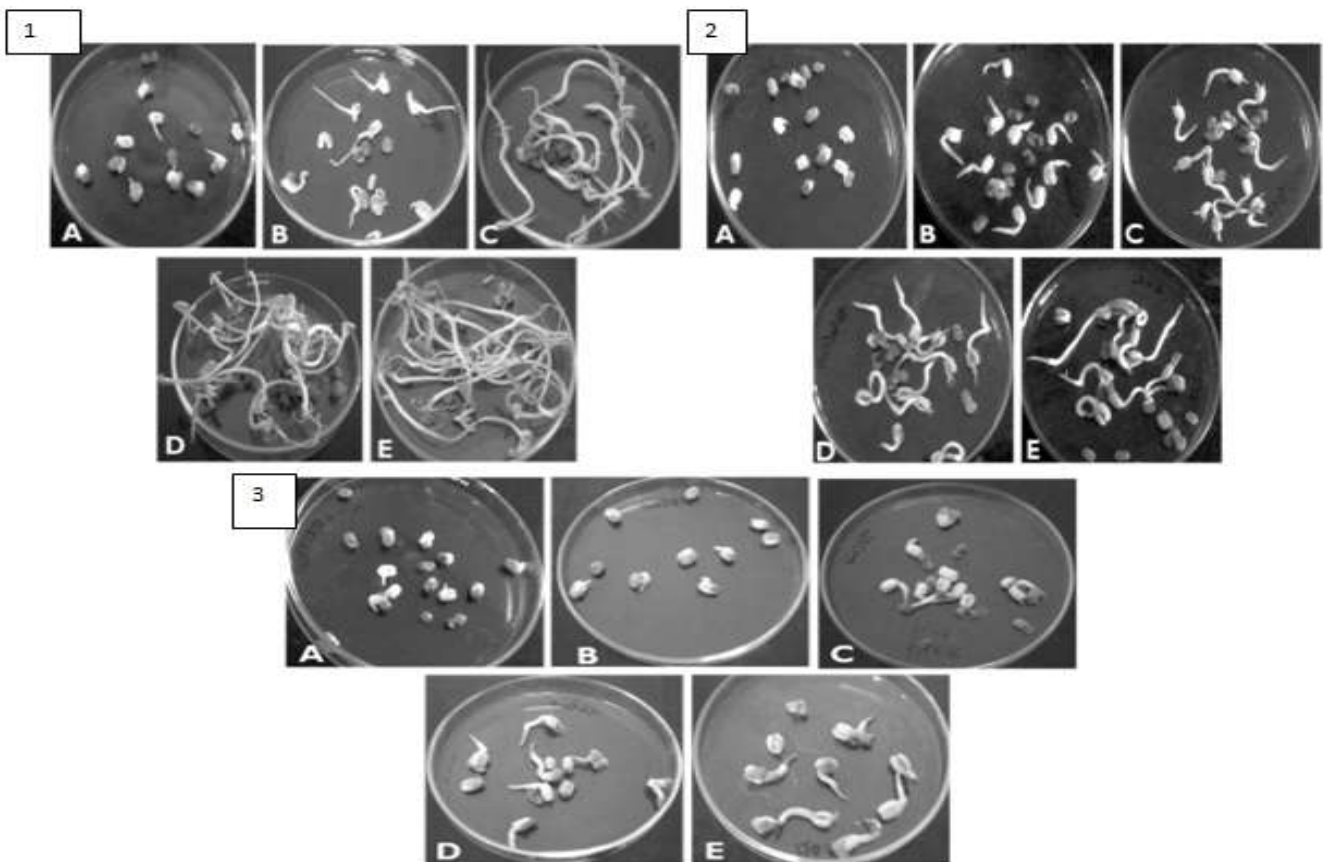


Figure 8. Effect of vincristine treatment on morphological development: Morphological changes in green gram seeds treated with: I: distilled water; II: 0.2 mg/ml vincristine; III: 0.1 mg/ml vincristine. A: 24h B:48h C:72h D:96h E:120h



Since the stimulation of hydrolytic enzymes is controlled by phytohormones such as gibberellins, it could be possible that the K-drug exhibited greater antagonism to these phytohormones than vincristine. Previous seed germination studies have reported that tannins could function as gibberellin antagonists but failed to inhibit endogenous phytohormone activity (Corcoran *et al.*, 1972). Active principles in the K-drug extract appear to inhibit endogenous phytohormone activity also, as evident from our current observations.

In case of human and veterinary metabolic disorders, also, the occurrence of hyperamylasemia following the administration of vincristine as part of remission induction therapy for acute lymphoblastic leukemia has been reported by Quintanilla *et al.*, 2014. Tsukawaki *et al.*, 1992 have reported that vincristine administered as part of a multi-drug chemotherapy regimen only resulted in short-term reduction in amylolytic activity, with fluctuations. Acute pancreatitis, which is characterized by increased serum amylase activity, has also been reported by Toprak *et al.*, 2012, as an adverse effect of VAD chemotherapy which included vincristine as one of the drugs.

Vincristine also enhanced serum alkaline phosphatase activity through hepatocyte destruction in albino rats (Upamanyu *et al.*, 2009). Dose dependent elevation of serum alkaline phosphatase owing to drug-induced cholestasis following administration of the drug into rabbits has also been reported (James *et al.*, 2007). By contrast, the aqueous extract of the Kokilaksha root has been reported to have restored increased serum enzyme levels to normalcy in liver-damaged rats (Dattatraya, 2012). While these studies did not specifically include alkaline phosphatase or amylolytic enzymes, the hepatoprotective effects of Kokilaksha have been reported.

Similarly, Ahmed *et al.*, 2001 have reported that seed extracts of *Asteracantha longifolia* exhibited anti-tumor properties with significant inhibition of enzymes like gamma-glutamyl transpeptidase. Dattatraya, 2012 has also stated that Kokilaksha could serve as a potentially safe herbal remedy for various disorders and thus could be advantageous in treatment of disorders wherein conventional methods of therapy are besought with adverse side-effects.

Considering the significant dose-dependent reduction in the amylolytic and alkaline phosphatase activity evident in seed germination, the efficacy of Kokilaksha in human disorders involving alkaline phosphatase or amylolytic enzyme elevation needs to be investigated since the drug could offer a potentially safer alternative to existing therapies. Moreover, the therapeutic efficacy of the herbal drug could be quantified in terms of dose-dependent inhibition of such enzyme biomarkers as indicated by Sumantran, 2010. It may also be stated that such human disorders need not be confined to malignancies but could include benign disorders such as pancreatitis or chronic gastritis as well.

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**Conflict of interest:** None

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