Enzyme profiles of green gram seeds pre-treated with the herbal drug ‘Kokilaksha’ followed by restoration of conditions favouring germination

Mamtha, M., Priya Mohan,., Mrinalini Menon

Abstract — Treatment of green gram seeds with Kokilaksha (also termed as the HST – K drug) over a 24 – 120 hour period reduced growth parameters such as water imbibition, appearance of radicle, plumule, etc apart from inhibiting different enzymes including amylase and alkaline phosphatase. This was described as a cost-effective means for initial identification of potential antiproliferative compounds, whose therapeutic efficacy could be further studied. Extending the scope of this study, we sought to explore whether treatment of the seeds with HST-K for a shorter duration of time, say 24 hours would suffice for irreversible inhibition. Thus, in our present study, green gram seeds were treated with the 1:5 and 1:10 v/v diluted HST-K drug for 24 hours after which they were transferred to distilled water for 120 hours. Amylolytic and alkaline phosphatase activities in these seeds were restored to the extent of 35.6±0.6% and 32.4±4.6% respectively, vis-à-vis controls following treatment with the 1:5 diluted HST-K drug. In respect of both enzymes, pre-treatment with the 6.3-fold diluted HST-K drug followed by transfer to distilled water yielded restoration of 50% activity. These observations strengthen the view that the duration of exposure and related parameters deserve to be explored as part of the K-drug’s efficacy studies against human disorders wherein amylase and alkaline phosphatase are overexpressed.

Index Terms — assay, green gram, growth inhibition, irreversible, K-drug

1 INTRODUCTION

The herbal drug formulation Kokilaksha (termed as the ‘K’-drug or HST-K drug; Patent No.GB2454875 dt.20th Nov, 2007) derived from Asteracantha longifolia prevented sprouting in green gram (Murthy et al, 2011), besides altering amylolytic and alkaline phosphatase activities in a dose dependent fashion (Deepthi and Menon, 2015). This approach had been used as a cost-effective method for the identification of compounds having anti-proliferative properties as a step towards exploring their anti-cancer potential (Kumar and Singhal, 2010; Murthy et al, 2011). It may be pointed out in the above studies (Kumar and Singhal, 2010; Murthy et al, 2011; Deepthi and Menon, 2015), germinating green gram seeds had either been continuously exposed to the drug for 24-120 hours (Deepthi and Menon, 2015) or the observations themselves as a whole had been confined to 24 hours (Kumar and Singhal, 2010; Murthy et al, 2011).

These observations stimulated us to explore whether the inhibition of germination in green gram by HST-K drug was irreversible. Therefore we undertook the present study wherein seeds were treated with different concentrations of the HST-K drug for 24 hours, at the end of which they were transferred to distilled water that had been used as the control in all our previous studies. Observations were based upon the parameters used by us in our previous studies namely:-

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

2 MATERIALS AND METHODS

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

2.1 Drugs used

The K-drug formulation was diluted 1:5 v/v and 1:10 v/v with distilled water as previously described by Murthy et al, 2011. As observed by us, previously, the pH of all these solutions was found to be 6.5-7.0 (Deepthi and Menon, 2015).

2.2 Seed treatment

Green gram seeds (0.5 gms) were treated with different
concentrations (1:5 and 1:10 v/v) of the water-diluted HST-K drug for 24 hours, after which the seeds were transferred to distilled water for various time durations ranging from 24-120 hours and allowed to germinate. Correspondingly, controls devoid of HST-K drug treatment were also subjected to germination in distilled water for the same period of 24-120 hours (Table 1).

### Table 1: Study design with regard to the type of treatments followed

<table>
<thead>
<tr>
<th>S.No</th>
<th>Type of sample (n=8)</th>
<th>Drug dilution (v/v)</th>
<th>Seed treatment prior to germination</th>
<th>Conditions for germination</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>None</td>
<td></td>
<td>Transferred to distilled water where incubated for 24-120 hours</td>
</tr>
<tr>
<td>2</td>
<td>Test (K-drug treated)</td>
<td>1:5</td>
<td>24 hours</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:10</td>
<td></td>
<td>24 hours</td>
</tr>
</tbody>
</table>

#### 2.3 Weight profiles
Alterations in weight were measured at the end of each incubation in distilled water ranging from 24-120 hours, following treatment with/without the HST-K drug. The seeds were removed from the petri plates at the end of each incubation period and briefly dried on the surface of paper towels to remove the excess moisture, after which the fresh weights of the seeds was estimated using an Electronic Balance (Model No: BSA 224S-CW).

#### 2.4 Preparation of enzyme extract
Seedlings were extracted and homogenized in the cold, 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 used for our further studies.

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

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

#### 2.7 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.

#### 2.8 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.

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

Half maximal drug concentration of the drug or the IC-50 was defined as that amount of drug required to sustain 50% reduction in the specific activity of enzymes with reference to the control, despite restoration of germination-favourable conditions after 24h of drug exposure.

#### Statistical tools
The results obtained have been expressed as an average of 8 trials ± standard error unless otherwise stated. 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 as significant while ≤ 0.01 was termed as ‘highly significant’.

### 3 RESULTS

#### 3.1 Alterations in seed weight
The weight of seeds pre-exposed to 1:5 v/v water diluted HST K- drug for 24 hours prior to germination in distilled water was observed to be 67.6 ± 3.1% vis-à-vis control. However the weight of seeds treated with 1:10 v/v diluted HST K- drug for 24h followed by 120h incubation in distilled water exhibited no reduction, but was 1.4 fold greater than that of the control group (Fig 1).

![Fig 1. Green gram seeds (0.5 gms) were treated with different concentrations (1:5 and 1:10 v/v) of the HST-K drug for 24 hours, after which the seeds were transferred to distilled water for various time durations ranging from 24-120 hours and allowed to germinate. Correspondingly, controls devoid of HST K drug treatment were also subjected to germination in distilled water for the 24-120 hour period.](image-url)
3.2 Altered morphogenesis

Restoration of morphological differentiation akin to control was observed in the 1:10 v/v drug treated seeds observed over the 24-120h period (Fig 2).

In the case of the 1:5 v/v drug-treated seeds, only a slight protuberance of hypocotyl was evident at the end of 120 h whereas seedling formation was complete in a majority of the 1:10 v/v drug treated seeds. The individual stages of growth at the end of each incubation period are indicated in Table 2.

Table 2: Morphological alterations

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Particulars of the 24h Seed Treatment</th>
<th>Morphological alterations over a 24-120 h incubation period*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S++H+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H++</td>
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<tr>
<td></td>
<td></td>
<td>R++</td>
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<td></td>
<td>C++</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L++</td>
</tr>
<tr>
<td>2</td>
<td>Test (K-drug treated)</td>
<td>1:5 v/v</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S++</td>
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<td></td>
<td></td>
<td>S++</td>
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<tr>
<td>2</td>
<td></td>
<td>1:10 v/v</td>
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<td></td>
<td></td>
<td>S+</td>
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<td>S++H+</td>
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<tr>
<td></td>
<td></td>
<td>R++</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C++L+</td>
</tr>
</tbody>
</table>

Abbreviations
S: Seed coat; H: hypocotyl; R: radicle; C: cotyledon; L: leaves
+: just appearance; ++: development complete

iii) Alterations in specific amylolytic activity: The specific activity of amylase at the end of 120 hours was equal to 120.4 ± 2.6 U/mg in the control. In comparison, the specific activity of seeds pre-exposed to 1:5 v/v water-diluted HST-K drug for 24 h prior to germination in distilled water, was only 42.8 ± 0.3 U/mg at the end of 120 hours, thus indicating that only 35.6±0.6 % of the control was restored (Fig 3).

Pre-exposure to the 1:5 v/v water-diluted HST-K drug followed by incubation in distilled water thus resulted in 64.4±0.6% inhibition (p<0.01) of specific amylolytic activity when observed at the end of 120 hours. In contrast, the 1:10 v/v diluted drug pre-treatment had no significant inhibitory
effects (p>0.05) vis-à-vis control at the end of 120 hours.

These observations prompted us to measure the half maximal inhibitory concentration (EC-50) value of the K-drug. Different fold-dilutions of the drug were thus used for the 24 hour pre-exposure and the amylase inhibition units calculated as discussed under Materials and Methods. The amylase inhibition values were plotted as a function of the dilution factor of the drug wherein we observed that a pre-treatment with a 6.3 fold water-diluted K-drug was required to reduce the amylase activity by 50% (Fig 4).

iv) Alterations in specific alkaline phosphatase activity:
Specific activity of alkaline phosphatase in the control, was equal to 15.0 ± 1.6 U/mg at the end of 120 hours. In comparison, the seeds pre-exposed to 1:5 water-diluted HST-K drug for 24 h prior to germination in distilled water, had a specific activity of only 4.1± 0.2 U/mg at the end of 120 hours. Thus the inhibition corresponded to 67.6±4.6% (p<0.01) at the end of 120 hours. Seeds pre-treated with 1:10 v/v water-diluted HST-K drug exhibited specific activity of 18.8 ± 5.7U/mg at the end of 120 hours which was not significantly different from the control (p > 0.05). As indicated in Fig 5, significant differences were observed only in the case of seeds pre-treated with the 1:5 water-diluted HST-K drug for 24 h.

Upon plotting the % inhibition as a function of the dilution factor values for the drug used in pre-treatment we observed that the 6.3 fold water-diluted K-drug reduced the alkaline phosphatase activity half maximal inhibitory concentration (EC-50) value of the K-drug was calculated.
DISCUSSION
Dose-dependent inhibition of water imbibition and the specific activities of amylase and alkaline phosphatase in response to continuous exposure to HST-K drug treatment for 24-120 hours was previously reported by Deepthi and Menon (2015) in the same laboratory. Our present observations suggest that a 24 hour exposure to the 1:5 v/v water-diluted HST-K drug followed by restoration of germination-favouring conditions, irreversibly altered the morphological development of green gram seeds (*Phaseolus radiatus* syn. *Vigna radiata*). However, if we consider reduction in seed weight as a parameter for inhibitory efficacy as expressed by Kumar and Singhal, 2010 or Murthy et al, 2011, it may be observed that no significant difference was observed in terms of weight differences between the seeds treated with 1:5 v/v water-diluted drug vis-à-vis control, when measured at the end of 120 hours. However it needs to be emphasized that these studies have not addressed what would be the response in case of a short exposure to the inhibitor followed by restoration of germination-favouring conditions akin to the control. We however wish to state that inhibition-based weight reduction alone as a measure of inhibitory efficacy needs to be considered with caution, since the removal of inhibitory conditions could result in a reversal.

Although weight profiles of drug-treated seeds did not significantly differ from the control, significant inhibition of amylolytic activity was reported following 24h treatment with the 1:5 v/v diluted drug. Sudha et al, 2011 have reported that various plants used in Ayurveda such as Cassia fistula, Ocimum tenuiflorum, Morus alba and Linum usitatissimum, have amylase inhibitory properties. Interestingly, it may be noted that all these plants are also known for their anti-diabetical properties. *Asteracanthalangifolia*, whose Extract forms the HST-K or Kokilaksha formulation has also been reported to have hypoglycemic properties (Muthulingam, 2010; Subramoniam, 2016), although no reports of amylase inhibitors characterized are available. In accordance with the observations of Murthy et al, 2011, who have indicated the role of the HST-K Drug in palliation and management of cancer, the investigations on specific hyperamylasemiccancers such as lung adenocarcinoma or ovarian carcinoma is needed in order to substantiate the possibility that amylase elevation could be controlled by this drug. Interestingly, benign disorders such as drug-induced pancreatitis also have elevated serum amylase levels as markers (Zhang et al, 2013) wherein the efficacy of HST-K and similar herbal would merit investigation.

Likewise, alkaline phosphatase activity was also significantly retarded upon treatment with the 1:5 v/v diluted drug. Alkaline phosphatase elevation has been reported as an important marker in malignancies of the nasopharyngeal tract and bone (Jin et al, 2015; Kim et al, 2017), besides various benign disorders such as jaundice (Fang et al, 1980) and cholecystitis (Thapa et al, 2010). Alkaline phosphatase has been described by Rashida and Iqbal, 2014 as a potential drug target, inhibited by natural compounds such as theophyline from cocoa bean. Considering the ability of Kokilaksha root to restore the levels of other serum enzymes to normalcy in liver-damaged rats as reported by Dattatreya 2012, the question could be asked as to whether serum alkaline phosphatase levels could likewise be controlled.

The 6.3-fold water diluted HST-K formulation irreversibly retarded the selected enzymes’ activity by 50%. We wish to state that such an approach maybe followed for the identification of lead compounds with anti-proliferative potential.

Reducing the chemotherapy/radiotherapy dosage followed by administration of herbals has been described as a possible approach in cancer treatment by Yin et al 2013. These authors report that this approach significantly lessened damage to healthy tissues. The HST-K drug deserves to be addressed in this connection as well. Moreover, its efficacy in relation to hyperamylasemic and hyperphosphatasemic malignancies also deserves to be explored. It is anticipated that our observations would serve as a starting point to substantiate the efficacy of this antimitotic herbal drug as an anticancer medication.

7.2 Acknowledgment
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References


