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An assay for screening anti-mitotic activity of herbal extracts

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The Herbal Science Trust (HST), Bangalore has developed an effective palliative herbal extract (HST-K) for the management of pain in terminal cancer patients. We were interested in the purification of the anti-mitotic/therapeutic (referred as bioactivity) component in HST-K. In pursuit of this goal we have developed an *in vitro* method for quantification of the bioactivity in HST-K, based upon the observation that the sprouting of green-gram seed was inhibited by HST-K. The inhibition was found to be dose-dependent and was suitable to quantify the bioactivity of HST-K preparations. The method was further extended as an easy screening procedure for anti-mitotic activity of herbal extracts. Synthetic drugs useful for palliation in cancer also inhibited the sprouting of green-gram seeds, whereas other common drugs failed to inhibit sprouting. We have identified a few common vegetables, viz. onion, garlic and capsicum as anti-mitotic using the above screening method.

Keywords: Anti-mitotic activity, herbal extracts, germination, sprouting, inhibition.

THE gift of health to humanity by scientific methods in the war-ravaged European continent in the last 300 years has been unconditionally recognized all over the world today. Despite such achievements, we have not been able to understand the etiology of cancer, and scientific methods can only provide poor palliation. Treatments like chemotherapy, radiotherapy and surgery provide only partial and transient relief. In the 1950s–70s, attempts were made in the West to develop alternate systems for cancer management[1], but these have been inconclusive[2]. Recently, the National Institutes of Health, USA has approved trials of alternate systems of medicine for cancer under 'investigative new drug' development scheme. Under this scheme, treatment by unorthodox medication from alternate systems is legally permitted to be tried on willing patients.

India, the home of Ayurveda, has many references to the management of tumours in texts like *Astanga Hrudaya*[3]. These references are indirect and have to be identified by a careful study of the texts. The Herbal Science Trust (HST), Bangalore is involved in identifying such

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drugs and has successfully utilized them in the management of cancer. Recently, HST was able to remove the toxicity of one of the drugs (derived from *Asteracantha longifolia*) to make it effective for palliation (patent no. GB 2454875 dated 20 November 2007). This formulation was successful for palliation in clinically established terminal cancer cases, and gifted them with a good quality of life (a life without pain, debilitation and discomfort) till the end. The end was always found to be painless in most cases. The palliation was independent of the metastasized primary and the organ which was metastasized.

We were interested in isolating the active principal from this drug. The need for successful isolation is a simple, economical *in vitro* assay system to quantify the bioactivity in HST-K. Many systems are available[4–10], among which inhibition of growth of specified human cancer cell lines in tissue culture is the popular screening procedure adopted today[8,9]. An inexpensive assay using the measurement of mitotic index of sprouting seeds has been recently reported[11]. In the present communication we describe a simple, inexpensive quantitative method for screening herbal extracts for their anti-mitotic activity.

The active drug prepared from *A. longifolia* was a product of HST and will be referred to as HST-K throughout the text. Green-gram seeds were purchased from a grocery store. Genetically cross-bred seeds were a kind gift from Seshagiri, Columbia University, New York, USA. Synthetic chemotherapeutic drugs used in the experiment were purchased from a pharmacy.

For the germination of seeds, 200–500 µl of aqueous solution of the drug was placed in a 24-well microtitre plate and a seed was dropped into each well. It was then covered with a lid and allowed to sprout at room temperature. Depending on the need, the reaction was arrested at different time points by taking the seed out of the well and removing surface water/drug on a dry tissue paper for further studies.

For germination assay, 200–500 µl of aqueous solution of graded doses of the sample was taken in 24-well plates and dry seeds of equal weight were added one each to microtitre wells and the plate was closed with the lid and left at room temperature for 24 h for imbibition of water. At the end of the test period (24 h), the seeds were weighed after drying them on a dry tissue paper. For morphological studies, the time of sprouting was extended to either 72 or 96 h, and photographs were taken.

$$\text{Percentage of inhibition} = \frac{(\text{WtD} - \text{WtE})}{(\text{WtD} - \text{WtHST})} \times 100,$$

where WtD is the wet weight of the seed in distilled water; WtE, the wet weight of the seed in experimental sample and WtHST, the wet weight of the seed in HST-K (100 µl – maximum inhibition).

The various leaf and vegetable extracts were prepared using a simple maceration process. Ten grams of leaf was ground in 10 ml of water and centrifuged, and the supernatant stored in a refrigerator at 4°C. The pH of the extracts was between 6.5 and 7.5.

Germination of green-gram seeds in water in the presence and absence of HST-K is shown in Figure 1. The sprouted seed had developed roots, shoot and two leaves (A, Figure 1). In contrast, seeds that sprouted in the presence of 10 µl HST-K had reduced growth with shortened shoot and small leaves (B, Figure 1), with no visible roots. At 20 µl concentration inhibition of growth was much more pronounced, and at 100 µl, inhibition of germination was complete with no visible sprouting (C and D, Figure 1). These morphological observations indicate the dose dependence of inhibition.

The imbibition of water was associated with increased size of the seed up to 12 h and rupture of the seed coat occurred in the next 8 h. By 48 h, shoots appeared and grew as long as 5–8 cm. The water imbibition and growth were widely different for different seeds from the market. With in-bred seeds the imbibition was consistent (± 4%), and the growth of the shoot was fairly uniform. However, the stalk length could not be measured as it was bent (A and B, Figure 1). Hence, in all experiments in-bred seeds of equal dry weight were used.

Figure 2 presents the dose dependence of inhibition of germination by HST-K. It follows the expected patterns, and the inhibition of imbibition was dose-dependent (Table 1). The errors of estimation in the assay were higher at lower inhibition, but at inhibition above 20% they were satisfactory, with an error of ± 10%. At lower inhibition

Figure 1. Sprouted green-gram seeds in the presence and absence of HST-K. A, Control in water; B, with 10 µl HST-K; C, 20 µl HST-K, and D, with 100 µl HST-K.

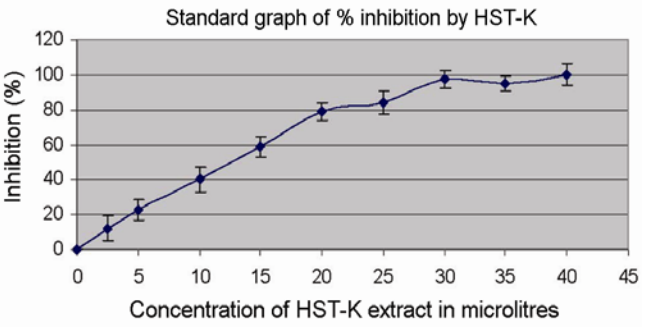

Figure 2. Dose dependence of water inhibition in sprouting assay.

Table 1. Dose dependence of inhibition in the sprouting assay (volume = 500 µl, $N = 8$, $T = 24$ h)

Volume (µl) of HST-K	2.5	5	10	15	20	25	30	35	40	50	100	0
Average seed weight (mg/seed ± SD)	115.71 ± 2.69	111.57 ± 2.22	105 ± 2.82	97.86 ± 2.41	90.00 ± 1.63	88.50 ± 2.56	83.00 ± 1.88	83.85 ± 1.54	82.00 ± 2.44	81.75 ± 2.86	82.28 ± 2.63	120.37 ± 2.97
Reduction in water imbibition (mg/seed ± %SD)	12.06 ± 6.96	22.91 ± 5.78	40.04 ± 7.36	58.66 ± 6.28	78.77 ± 4.24	83.04 ± 6.66	97.053 ± 4.90	95.14 ± 3.97	99.99 ± 6.36	98.87 ± 5.99	100 ± 6.8	00

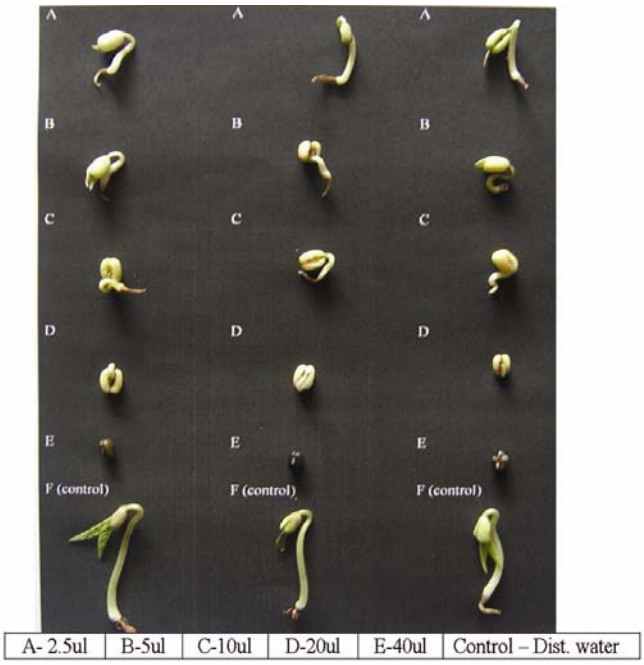

Figure 3. Sprouted seeds with different concentrations of HST-K.

the variation was high and for all quantitative tests the assessment was done at inhibition value more than 40%. Inter-assay variation was done for six sets ($N = 6$) with ± 8%, and intra-assay variation done for $N = 6$ with ± 6%. Figure 3 shows that the morphological changes in the sprouting pattern of HST-K-treated seeds matched qualitatively with the imbibition data (Table 1).

Whether the inhibition is specific for cancer or is incidental to HST-K was found by checking the inhibitory activity of known anticancer drugs. Table 2 shows the effect of several anticancer drugs (synthetic) on germination. All of them showed inhibition to varying extent with a minimum shown by doxorubicin (8%). Morphological data clearly indicated complete inhibition in all, except ifosfamide and dacarbazine (Figure 4 *e* and *f*). In contrast to the inhibition observed with anticancer drugs, other common drugs like paracetamol, antibiotics, vitamin combinations, and pain-killers did not show inhibition in both the modes (< 10%), with the exception of diclofenac (Figure 5 *i*). It is interesting to note that diclofenac is used as an analgesic in cancer management.

The utility of this assay system in screening for anti-mitotic activity of some leaf and vegetable extracts is shown in Table 2, and Figures 6 and 7. Among the leaf extracts, only sandal inhibited completely in both imbibition mode and morphology mode. Others showed > 50% inhibition in the imbibition mode, but none in the morphology mode. Among the vegetable extracts capsicum, onion and garlic showed inhibition in the morphology mode (Figure 7 *c*, *i* and *j*), while all others showed significant inhibition in the imbibition mode (Table 2), indicating the superiority of the morphology mode for screening procedure.

Initial observations clearly established that the herbal extract (HST-K) used in management of cancer in clinical practice did show a positive inhibition in the sprouting of seeds. Water-imbibition studies extended the scope of the method for quantification of the anti-mitotic activity. Herbal extract of *Vinca rosea*, consisting of several anti-mitotic factors[12], inhibited the sprouting of the seeds (Figure 6 *k* and Table 2).

Methods that have been in use are the animal model systems for inhibition of tumour growth implanted in mice[6,7], potato disc tumour induction assay[10,11] and the inhibition of proliferation of cancer cell lines in *in vitro* culture[8,9]. The last method is capable of quantification, and is the most commonly used method. The above assays are expensive and require expensive infrastructure.

The assay reported here is similar to the one recently reported by Kumar and Singhal[11], Primary observation of inhibition in the sprouting of green-gram seeds by cytotoxic/antitumour drugs has been the basis in both cases. Although the method developed by Kumar and Singhal *et al.*[11] was for the preliminary evaluation of cytotoxic effects, we were interested in the purification of the anti-mitotic principal in HST-K. Purification from biological sample requires an easy and inexpensive assay capable of precise quantification. The method developed by Kumar and Singhal[11] has demonstrated inhibition of the following parameters: (a) per cent sprouting of seeds, (b) imbibition of water, (c) length of radical in 24 h and (d) growth (cytotoxicity). The above assay was done using large

Table 2. Inhibition in imbibition assay for drugs, the herbal, vegetable and leaf extracts (% average of triplicates)

Drugs/extract	Anticancer drugs		Non-anticancer drugs		Plant extract		Vegetable extract	
Sample	Drug	Inhibition (%)	Drug	Inhibition (%)	Extract	Inhibition (%)	Extract	Inhibition (%)
A	Methotreaxate	14	Nimesulide	7	Acacia	56	Carrot	71
B	Cytarabine	11	Ibuprofen	9	Teak wood	42	Cabbage	64
C	Leucovorin	43	Ampiclox	2	Sandalwood	100	Capsicum	86
D	Flurouracil	43	Novomax	4	Champaka	63	Radish	79
E	Ifosfamide	22	Crocin	2	Grass	42	Brinjal	75
F	Dacarbazine	25	Ciprofloxin	12	Bougainvillea	84	Potato	71
G	Epirubicin	95	Paracetamol	2	Cleirodendron	72	Tomato	17
H	Cyclophosphamide	36	Cefazolin	4	Ixora	44	Cucumber	39
I	Daunorubicin	8	Diclofenac	0	*Pongamia pinnata*	44	Onion	89
J	Doxorubicin	25	Vitamin B complex	4	Silver oak	70	Garlic	92
K	Etoposide	87	HST-K	100	*Vinca rosea*	100	HST-K	100
L	Vinblastin	94	Distilled water	0	HST-K	100	Distilled water	0
M	Vincristine	99	–	–	Distilled water	0	–	–
N	Cisplatin	100	–	–	–	–	–	–
O	HST-K	100	–	–	–	–	–	–
P	Distilled water	0	–	–	–	–	–	–

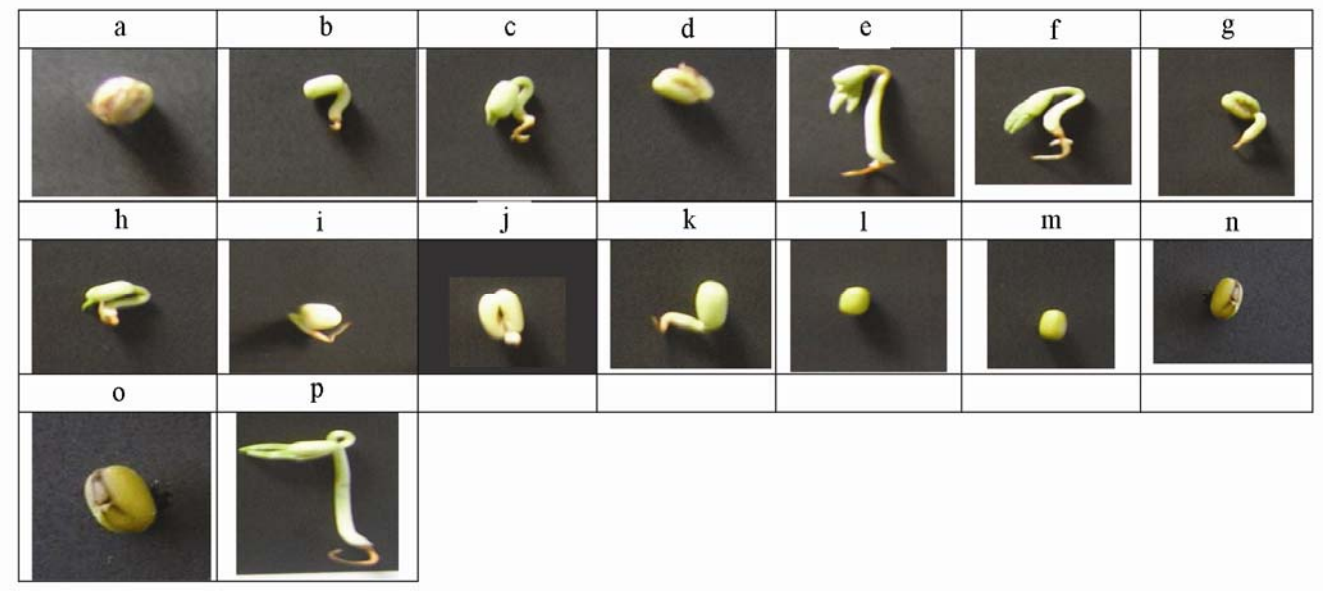

Figure 4. Effect of anticancer drugs on germination of seeds after 72 h (concentration of drugs, 1 mg/ml).

sample numbers (10–35 observations per group and counting nearly 400–500 cells for the determination of mitotic index), which is satisfactory for the study of mitotoxic effects. Statistical evaluations are necessary for validation. Our method reported in this communication is easy to do and is also capable of quantification with fewer observations. Statistical analysis is not required as cross-bred seeds provide consistent results and duplicates are enough for an assay, and in 48 h morphological changes can also be observed. A semi-quantitative assay can be done within 8–12 h, which is an advantage in purification. The length of the shoot showed qualitative dose dependence but required 72–96 h, a disadvantage to develop the shoot length as a means for quantification of the anti-mitotic activity of HST-K. Another difference between the two assay systems is the time of addition of the sample. In the reported assay[11], 24 h germinated seeds were used for all studies. In our system we have used dry seeds for two reasons. (i) For purification, quick assay is an asset and results are obtained as early as 8–12 h. (ii) Any inhibition during the first 24 h would be lost if we start with germinated seeds. A systematic study of the two approaches will be possible when a homogeneous active compound is isolated from HST-K.

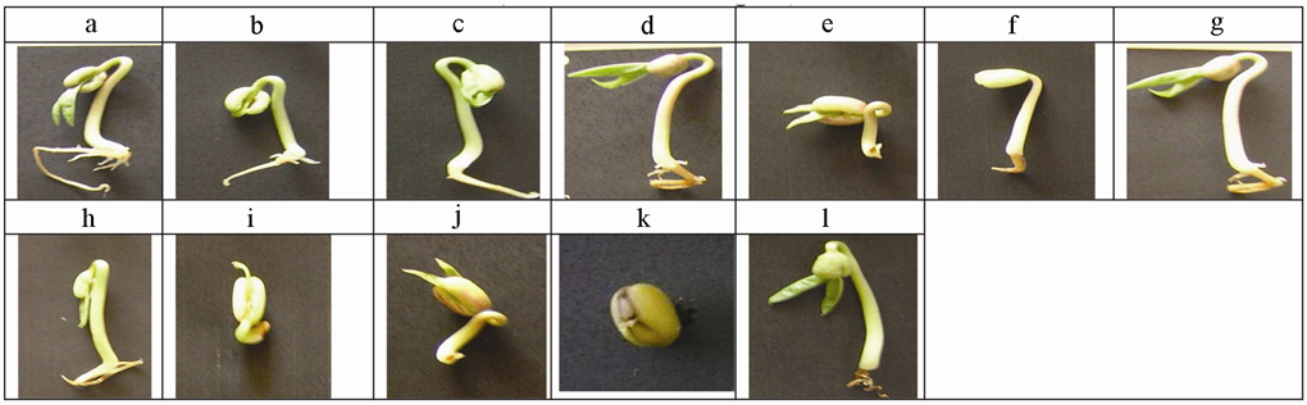

Figure 5. Effect of non-anticancer drugs on germination of seeds after 72 h (concentration of drugs 1 mg/ml).

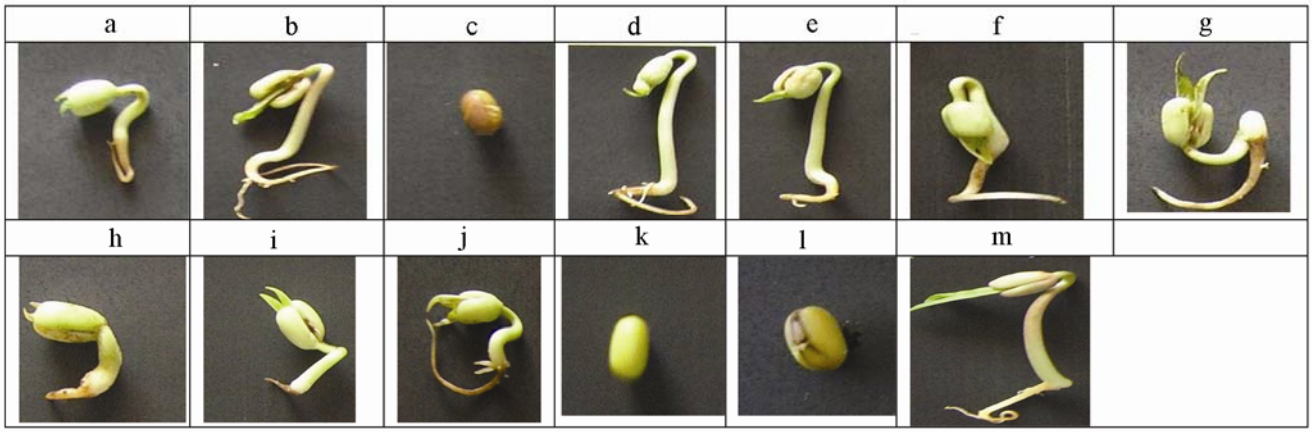

Figure 6. Effect of leaf extracts on germination of seeds after 72 h (concentration of extract 1 g/ml).

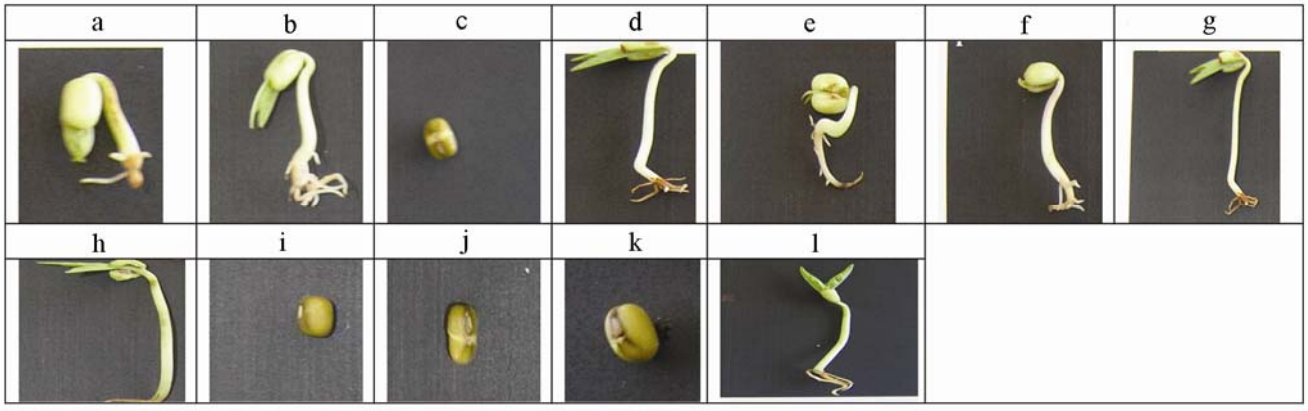

Figure 7. Effect of different vegetable extracts on germination of seeds after 72 h (concentration of extract, 1 g/ml).

An important feature of the assay that has emerged from these studies is its specificity. All synthetic anticancer drugs, few herbal and vegetable extracts (Table 2) completely inhibits sprouting of seeds in the assay[13,14]. All synthetic anticancer drugs (Table 2) with different specificities have shown inhibition, independent of the primary and the organ of metastasis in both the modes, the only exception being diclofenac. In contrast to the synthetic drugs, plant and vegetable extracts showed high inhibition in imbibition mode (Table 2). However, in the

morphological mode inhibition was clearly seen in capsicum, onion, garlic, sandal and *V. rosea*, which incidentally showed highest inhibition in the imbibition mode (Figures 6 *c*, *k*, and 7 *c*, *i*, *j*). Quantification of the activity can be done on the basis of water imbibition (Table 1) and morphological observation (Figure 3). Dozens of samples can be assessed at a time with ease, and the procedure does not involve technically cumbersome operations and analysis. The reproducibility of the results is excellent, and the screening is assessed by two methods in the same experiment, viz. by the increase in weight in 24 h, and the morphological observation of the sprouts after 72 h. Results from the morphological assay are a definite indication of the anti-mitotic activity of the experimental sample, whether it is a synthetic drug or herbal extract. False positive is possible when imbibition alone is measured, as in vegetable extracts (Figure 7) and diclofenac (Figure 5 *i*), but when both methods are used together false positive is absent. Based on the results presented we believe that morphological interpretation is a more reliable method for screening, though the imbibition method is more suitable for quantification.

A possible utility of the above assay is the easy validation of the reported and unreported claims. Since validation can be assessed without any specialized infrastructure, the claims can be easily validated by the morphological method. From this point of view the method is useful in screening the claims and counterclaims in the use of ayurvedic medicines, herbal extracts, etc. This is all the more important since there have been several reports identifying positive effects of botanicals in cancer prevention[13]. In fact, asparagus, which is circulated as an alternate food supplement for cancer cure[15], has inhibited the sprouting of the green-gram seeds (data not shown). For synthetic drugs under study, this could be a convenient screening method. In the limited screening of the anti-mitotic herbals done in the above study four herbals, viz. onion, garlic, capsicum and sandal have anti-mitotic activity (Figures 6 *c* and 7 *c*, *i*, *j*). Thus in the screening of herbals, a significant number could be anti-mitotic and purification of the active principal from them is possible.

Molecular mechanism/s by which sprouting is affected by the anti-mitotic herbals/drugs are not known. It is certain that the effect of the drug in methanol extract of milk weed (*Calotropis procera*), podophyllotoxin, cyclophosphamide and cyprohepatidine is through interference in the cell cycle[11] or through interference in spindle formation in vincristine and vinblastine[12]. It is possible that the herbals which we have studied also follow the same mechanism. An alternate possibility would be through inhibition of synthesis of key enzymes like amylase, etc. Comparative biochemistry of sprouting of seeds and neoplastic growth in humans is to be studied to understand the biochemical basis of specificity of the assay.

In summary, we report an assay method by which antitumour activity of herbal extracts can be quantitatively assessed. This would enlarge the scope of identification and possible purification of the active component/s from the aqueous extracts of herbs. The method can also be used for screening the synthetic compounds for their antitumour activity (either independently or in addition to cell-line assay).

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