Evaluation of traditional anthelmintic herbs as substitutes for the endangered *Embelia ribes*, using *Caenorhabditis elegans* model

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Vidanga, *Embelia ribes* Burm. f. (Myrsinaceae) is a top-traded (>500 mt/yr) Ayurvedic herb, which is endangered and in short supply forcing substitutes in the markets. A comparative anthelmintic study was undertaken between *E. ribes* and substitutes, *E. tsjeriam-cottam* (Roem. & Schult.) A. DC., *Myrsine africana* L. and *Maesa indica* (Roxb.) DC. Evaluation of anthelmintic activity was done using eggs, L1 larvae and adults of *Caenorhabditis elegans*. Anthelmintic activity of ethyl acetate extracts of all four candidates and their respective purified marker components (embelin or kiritquinone) was evaluated. Amongst the extracts, bioactivity of *E. tsjeriam-cottam* was found to be comparable to *E. ribes*, followed by *M. africana* and *M. indica*. None of the extracts displayed ovicidal effect. Additionally, crude extracts of all candidates were found to be 2–3 times better than their respective purified marker components. Results from this study indicate that usage of *E. tsjeriam-cottam* as a substitute anthelmintic drug for endangered *E. ribes* can be a good conservation strategy.

Keywords: Anthelmintic activity, *Caenorhabditis elegans*, *Embelia ribes*, Vidanga.

HELMINTIC infestations are one of the major health problems, especially in under-developed and developing nations. Helminthiasis is caused by various parasitic worms like roundworms (*Ascaris lumbricoides*), whipworms (*Trichuris trichiura*) or hookworms (*Necator americanus* and *Ancylostoma duodenale*). Several worms reside in the gastrointestinal tract causing problems in assimilation of food and essential micronutrients. Hookworm or whipworm infestations can cause chronic blood loss either from the gastrointestinal or urinary tract[1], which may lead to further complications like anaemia. Helmintic infestations are not limited to human beings; they infect a wide range of livestock leading to reduced productivity and mortality[2]. Thus, they have major health as well as economic implications.

The World Health Organization (WHO) has recommended various drugs such as mebendazole, albendazole, ivermectin and levamisole to tackle helminthiasis[3]. A major setback in the use of these drugs is development of genetic resistance[4] in the parasites as seen in case of levamisole[5], albendazole, ivermectin[6] and mebendazole[7]. Thus, there is a need to develop alternative drugs that can tackle worm infestations effectively. Analyses of various traditional medical practices have indicated the use of herbal preparations for worm infestations[8], which need to be scientifically explored.

Vidanga is an Ayurvedic Rasayana herb which is a drug of first choice for deworming, particularly in treating conditions like anaemia (Pandu)[9]. *The Ayurvedic Pharmacopoeia of India* correlates Vidanga to *Embelia ribes* Burm. f. (ER) (family Myrsinaceae) and *E. tsjeriam-cottam* (Roem. & Schult.) A. DC. (ET) as a substitute[9,10]. However, at least two other plant species of the family Myrsinaceae, namely *Myrsine africana* L. (MA) and *Maesa indica* (Roxb.) DC. (MI), are also used as Vidanga in different parts of India[11]. ER, a redlisted species[12], is a dioecious woody climber which grows sporadically in the northeastern states of India and in pockets of the Western Ghats[13].

ER which is authenticated as Vidanga is an essential ingredient of many formulations in Ayurveda[9]. The demand for ER, a Red-listed species, is >500 mt/yr, which is much more than its availability from sources[12]. This leads to the use of substitutes/adulterants to meet the demand. Non-use of an appropriate herb or improper substitution can compromise the medicinal value of the drug and efficacy of the treatment. However, scientific evaluation of bioactivity of probable substitutes could help identify alternative medicinal plants.

Anthelmintic activity of ER[14] and MA[15] has been previously reported, while that of ET and MI is unknown. Embelin, a benzoquinone, is one of the bioactive compounds found in fruits of ER, ET[16] and MA[17], but it is absent in MI[18]. Embelin is reported to have anthelmintic[19], antifertility[20] and antimicrobial[21] activities. A new benzoquinone was isolated from the fruits of MI and named kiritiquinone[18]. Bioactivity of kiritiquinone has not yet been reported. *Caenorhabditis elegans*, a soil-borne non-parasitic nematode (round worm), is one of the best model organisms to study anthelmintic activity[22].

The aim of the present study is to evaluate the anthelmintic activity of ET, MA and MI, used traditionally as substitutes for the endangered ER, using *C. elegans* as a model system.

Embelin (98% purity) was procured from Sigma, and kiritiquinone was prepared in-house[18] (97% purity, as determined by HPLC), Levamisole (Dicaris tablet, Johnson and Johnson, Mumbai) was procured from a local vendor. Solvents and other routinely used chemicals were of AR grade (Spectrochem, India).

Mature fruits of ER, ET, MA and MI were collected from various geographical locations of India by qualified field botanists and authentication of the plant material

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was done by in-house plant taxonomists and Ayurvedic physicians. Voucher specimens (ER-L/06/08/018, ET-L/05/07/037, MA-L/07/10/30 and MI-L/10/07/015) are deposited in the Foundation for Revitalization of Local Health Traditions (FRLHT) herbarium and raw drug repository (National Herbarium).

For preparation of the herbal extract, well-dried fruits of ER, ET, MA and MI were powdered using an electric grinder (Kenstar, MF 0204, Aurangabad). The powder was passed through a sieve (BIS, mesh no. 85).

Extraction of the powder was carried out in a Soxhlet apparatus placed in a water bath maintained at 80°C using ethyl acetate as solvent, until the solvent showed no colouration under UV when spotted on TLC plate. Ethyl acetate extracts were used for testing the anthelmintic activity as the yield of embelin, which was used as a marker compound in this study, in ER was maximum in ethyl acetate extract[23].

For HPTLC quantification of marker compounds, all stock solutions were prepared in ethyl acetate (1 mg/ml of all four Vidanga candidates, 10 mg/ml of embelin and 1 mg/ml of kiritiquinone). From these stock solutions, working solutions were prepared as required in ethyl acetate.

Next, 20 and 25 mg/ml of ethyl acetate extracts of all four plant species were prepared in ethyl acetate for anthelmintic assay and ovicidal assay respectively. Then 20 mg/ml of embelin was prepared in ethyl acetate by briefly (~ 10 sec) heating at 50°C, and 20 mg/ml of kiritiquinone was also prepared in ethyl acetate. Levamisole solution was prepared by dissolving a single powdered Dicaris tablet, equivalent to 150 mg levamisole, in 1 ml distilled water. Clear solution was obtained by separating insoluble material by centrifugation at 10,000 *g* for 2 min. All working solutions were freshly prepared by diluting the stock solution in 1× phosphate buffer saline (PBS). For 1 litre of 10× PBS: 80 g NaCl, 2 g KCl, 11.1 g Na_2HPO_4, 2 g KH_2PO_4 pH 7.4; to prepare 1× PBS, dilute 1 : 10 in distilled water.

Ethyl acetate extracts of all four species were analysed by HPTLC along with marker compounds embelin and kiritiquinone. HPTLC fingerprint was generated on TLC silica gel 60 F_{254} plates (Merck, Darmstadt). Mobile phase used was toluene–ethyl acetate–formic acid (5 : 5 : 0.5 v/v/v).

Quantification of marker compounds was done by HPTLC method. Analysis was performed on TLC silica gel 60 F_{254} plates. Appropriate aliquots of standard embelin and kiritiquinone were taken from the working solutions along with known amounts of the ethyl acetate extract working solutions of ER, ET, MA and MI and were loaded on the plate using Linomat 5 (Camag, Muttenz) automated spray-on band applicator equipped with a 100 µl CAMAG Linomat syringe. Development of the plate was carried out in a twin-trough chamber (Camag, Muttenz), saturated for 10 min with the mobile phase. The migration distance was 80 mm. After development, the plate was air-dried and the chromatograms were evaluated by measuring peak area after scanning at 339 nm (for embelin) and 383 nm (for kiritiquinone). The images were recorded using CAMAG TLC visualizer (Camag, Muttenz) under visible light, 254 nm and 366 nm wavelength. R_f values of the markers and the compounds of interest were noted. The amount of embelin and kiritiquinone in the four extracts was determined by the software from the calibration curve of standards.

Wild type (N2 strain) *C. elegans* was a kind gift from Sandhya Koushika (TIFR, Mumbai). The worms were cultured on nematode growth medium (NGM) using standard procedure[24]. Synchronized culture of worms from eggs was obtained as described previously with minor modifications[24]. Briefly, the eggs were harvested from a plate having high number of gravid worms by bleaching in alkaline medium. The bleached worms were placed on fresh NGM seeded with *Escherichia coli* OP50 to obtain synchronized L1 larval population after 16 h incubation at 20°C. Synchronized L1 population was harvested by washing the plates thoroughly with M9 buffer (for 1 l: 3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl and 1 ml 1 M $MgSO_4 \cdot 7H_2O$ sterilize by autoclaving at 121°C and 15 psi for 20 min). The number of worms was determined in 5 µl by counting under a 4× objective of a stereomicroscope (Labomed, CSM2, Culver City).

The anthelmintic assay on L1 larvae was performed in 1.5 ml microfuge tubes. The worm suspension containing ~500 worms was used per group. To pellet the worms, the worm suspension was centrifuged at 1400 *g* for 5 min (Ependorf 5810R, Hamburg). L1 larvae were treated to different concentrations of extracts, embelin, kiritiquinone (200–500 µg/ml) and levamisole (200–500 µg/ml and 15 mg/ml) for 3 h at 20°C. Treatment was followed by recovery in 1× PBS for 18–20 h at 20°C. Appropriate negative (PBS), positive (levamisole) and solvent controls (ethyl acetate/water) were maintained under similar experimental conditions. The treatment volume was maintained at 100 µl. Each experiment was repeated at least three times.

After recovery, the worms were harvested by centrifugation at 1400 *g* for 5 min at 18°C. The pellet was resuspended in a small volume (10 µl) of supernatant and the worms were placed on a slide. A cover slip was placed on the drop and the worms were allowed to settle for 2 min. Live and dead worms were counted under a bright field microscope (Olympus BX41, Tokyo) fitted with a camera (Olympus DP72, Tokyo) and connected to a computer, using 4X objective. Image was processed using Image Pro Express software. For each treatment group ≥ 200 worms were counted with the help of digital image appearing onscreen. Live worms are curve-shaped and moving, while the dead worms appear straight (Figure S2; see Supplementary data, online) and show no movement[25]. A dose response curve, for the extract

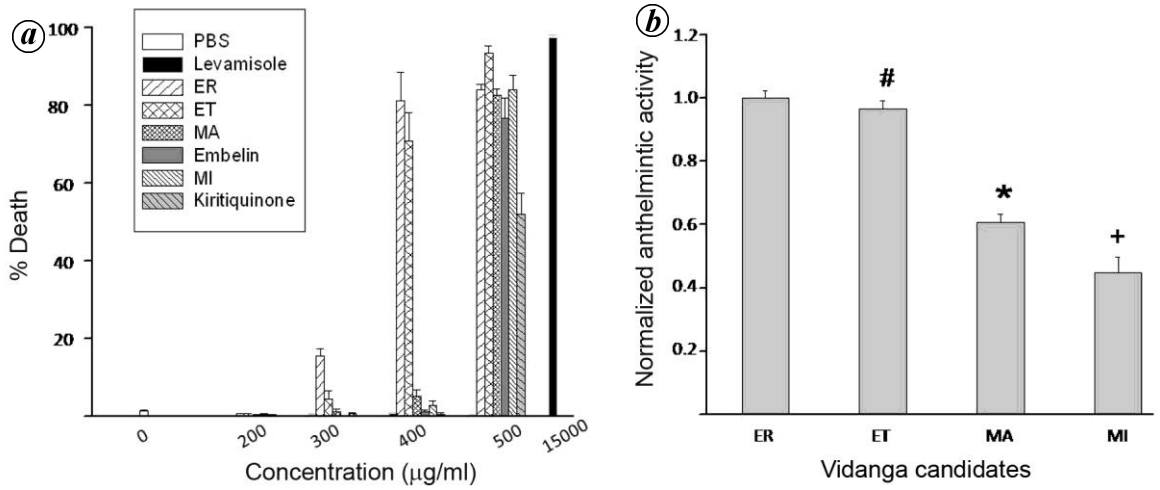

Figure 1. Effect of Vidanga treatment on L1 larvae. ***a***, Anthelmintic activity of different concentrations of ethyl acetate extracts of crude drugs of Vidanga candidates and their respective marker compounds. ***b***, Relative anthelmintic activity of crude drugs of *Embelia tsjeriam-cottam* (ET; #, statistically non-significant), *Myrsine africana* (MA; *, $P < 0.01$) and *Maesa indica* (MI; +, $P < 0.01$) as normalized against the original drug, *Embelia ribes* (ER).

concentration between 200 and 500 µg/ml, could not be established, as there was a steep increase in lethality at 500 µg/ml.

Anthelmintic assay for adults was performed similar to those for L1 larvae with minor modifications. Briefly, adults were harvested 48 h after bleaching. About 100 worms were used per group. Working solutions (200–400 µg/ml) of all four extracts were prepared by diluting the stock solution in distilled water. Treatment, recovery and counting procedures were similar to the ones used for L1 larvae. A dose response curve for the extract concentration between 200 and 400 µg/ml, could not be established as there was a steep increase in lethality at 400 µg/ml.

Anthelmintic activity of crude drugs of ER, ET, MA and MI was calculated by multiplying activity of ethyl acetate extract at highest concentration used and per cent yield of the extract. The activities of ET, MA and MI were normalized against ER, the original drug.

Statistical analysis of comparative anthelmintic activity was done by using one-way ANOVA followed by Tukey's HSD test. $P < 0.05$ was considered significant.

For ovicidal assay, gravid *C. elegans* adults were allowed to lay eggs on a fresh NGM plate seeded with *E. coli* OP50, incubated overnight at 20°C. The following day, eggs were harvested. First, 1 ml of distilled water was added to the NGM plate. The plate was gently swirled and the suspension, containing only gravid adults, was aspirated out. Finally, the eggs, stuck to the bacterial lawn, were collected using distilled water (2–3 ml) in a 15 ml conical tube. The number of eggs was quantified in 10 µl of the egg suspension by counting under a 4× objective of a stereomicroscope. The assay was performed in 1.5 ml microfuge tubes. The suspension containing ~100 eggs was used in each treatment group. To pellet the eggs, the suspension was centrifuged at 1400 *g* for 5 min. Eggs were treated with 500 µg/ml of each of the four plant extracts and levamisole (15 mg/ml), for 3 h at 20°C. Appropriate negative control (PBS) and solvent control (ethyl acetate) were also maintained under similar experimental conditions. The treatment volume was maintained at 100 µl. Percentage hatching for each group was analysed after incubation in 1× PBS for 18–20 h at 20°C by microscopically counting the number of newly hatched worms.

Yield of ethyl acetate extract was 17.48%, 15.15%, 10.77% and 7.85% (w/w) of dried fruits of ER, ET, MA and MI respectively. HPTLC analysis of the extracts showed 5.94%, 4.32% and 1.85% (w/w) embelin in the fruit powders of ER, ET and MA respectively, and 4.4% (w/w) kiritiquinone in MI. No embelin was detected in MI and no kiritiquinone was detected in ER, ET and MA (Figure S1a and b; see Supplementary data, online).

All four Vidanga extracts showed nonlinear anthelmintic dose response. The minimum inhibitory concentration for ER and ET (15% and 4% respectively) was 300 ug/ml, while that of MA and MI (5% and 3% respectively) was 400 ug/ml. All extracts showed maximal activity (80–90% death), at 500 µg/ml, when treated for 3 h (Figure 1 *a*).

Treatment with ≥ 2.5% of ethyl acetate in 1× PBS for more than 3 h was found to be toxic to L1 larvae. Therefore, the worms were exposed to non-lethal concentration of ethyl acetate (≤ 2.5%).

Analysis of anthelmintic activity of the fruits of the three substitute Vidanga candidates showed ET to be most comparable, to the original drug, ER, followed by MA and MI. These observations were statistically valid as shown by ANOVA analysis (Figure 1 *b*). Levamisole

caused 90% lethality at 15 mg/ml concentration and no effect was observed in the range 200–500 μg/ml.

Quantification of marker compound indicated that 500 μg/ml of ER, ET, MA ethyl acetate extracts contained 169.95, 142.65, 86 μg/ml embelin respectively. Also, 500 μg/ml of MI extract contained 287.5 μg/ml of kiritiquinone (Figure S1c and d; see Supplementary data, online). The concentration of embelin in ER, ET and MA was <200 μg/ml and that of kiritiquinone was <300 μg/ml. Therefore, 200 μg/ml of embelin and 300 μg/ml of kiritiquinone were used for comparative activity analysis. Also, 200 μg/ml of standard embelin showed no lethality, whereas extracts of ER, ET and MA, containing equivalent concentrations of embelin showed 80–90% lethality (Figure 2). Similarly, 300 μg/ml of kiritiquinone showed no lethality, whereas extract of MI containing equivalent concentration of kiritiquinone showed close to 80% lethality (Figure 2). At higher concentrations (500 μg/ml) embelin showed 76% lethality, while kiritiquinone showed 51% lethality.

Ethyl acetate extract (400 μg/ml) of all four Vidanga candidates showed maximum activity against one-day-old *C. elegans* adults. ET had the highest activity (>75% kill) followed by ER (>65% kill), MA (>40% kill) and MI (>30% kill, Figure 3 *a*). Treatment with levamisole (15 mg/ml) resulted in >95% kill, under similar experimental conditions, whereas 200–500 μg/ml showed no effect. Comparative activities of ER and ET were similar followed by MA and MI (Figure 3 *b*). The worms were exposed to non-lethal concentrations of ethyl acetate. In case of adult worms, it was found to be >2%. Thus, anthelmintic effect of concentrations higher than 400 μg/ml could not be assessed in this case.

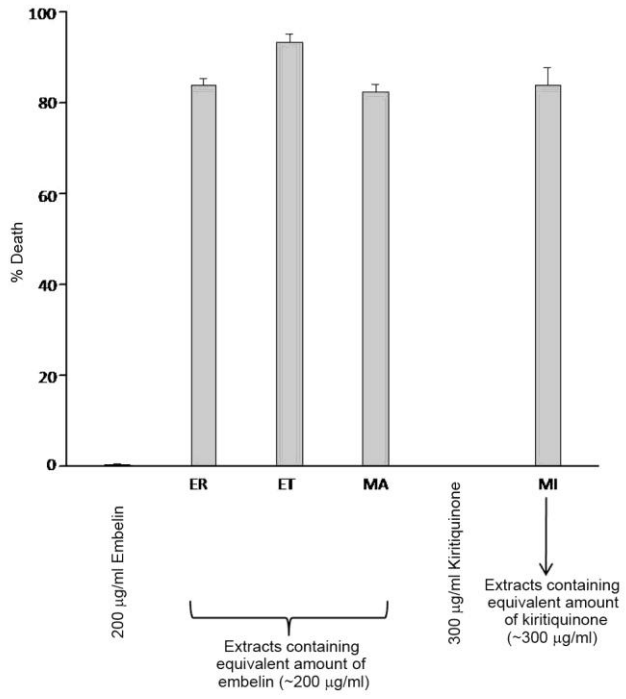

Figure 2. Comparative anthelmintic activity of Vidanga candidates along with their respective marker compounds. Anthelmintic activity of 500 μg/ml of ER, ET and MA compared with equivalent amount of embelin and anthelmintic activity of 500 μg/ml of MI compared with equivalent amount of kiritiquinone.

None of the four Vidanga species showed ovicidal effect on *C. elegans* eggs at maximum usable concentration (500 μg/ml) after treatment for 3 h. Concentration higher than 500 μg/ml could not be used because of solvent toxicity (ethyl acetate at ≥2%). Levamisole at 15 mg/ml also did not show any ovicidal effect after treatment for 3 h.

In Ayurveda, Vidanga is given importance, especially as a part of treatment for health problems like anaemia (Pandu). We have investigated the anthelmintic potential of fruit extracts of four species used as Vidanga. Ethyl acetate extracts of all four species were lethal to L1 larvae and adults of *C. elegans* (Figures 1 *a* and 3 *a*). Amongst the four plant species studied for their anthelmintic activity, the commercially available ET demonstrated anthelmintic activity similar to ER, followed by MA and MI (Figures 1 *b* and 3 *b*).

Critical conservation status of ER is because of heavy and improper harvesting from natural resources coupled with problems in natural propagation[12]. Amongst the three other plant species used as Vidanga, conservation status of ET is vulnerable while that of MA and MI is not of known concern[13]. Natural propagation and cultivation of ET (commonly traded Vidanga species) is less challenging compared to that of ER. Our study has brought out an approach for conservation of medicinal species of conservational concern through identification of legitimate substitutes[26]. Herbal drugs with 30–40% kill have been considered as anthelmintics[27]. Thus, MA and MI can also be explored as an anthelmintic substitute, albeit at higher dosages.

Comparison of anthelmintic activity of the marker compounds, embelin and kiritiquinone, with respective extracts showed that activity of the crude extracts was significantly (2–3 times) higher than the purified active components when used at equivalent concentrations (Figure 2). These results indicate that fruit extracts of all four Vidanga candidates which are multi-component drugs are more effective than isolated compounds. Interestingly, studies have shown that plant extracts have molecules with complementary actions leading to better efficacy than single molecules[28]. In case of anti-plasmodial activity, evidence suggests that plant extracts are more potent than isolated bioactive components at an equivalent dose[29]. It is interesting to note that levamisole, a nicotinic receptor agonist[30], requires 15 mg/ml to be effective, whereas the crude plant extracts were bioactive at a concentration of 500 μg/ml. Traditional knowledge has been useful in the identification of new and effective bioactive plant products and compounds[18]. Understanding the mode of action is warranted for explaining high efficacy of multi-component drugs.

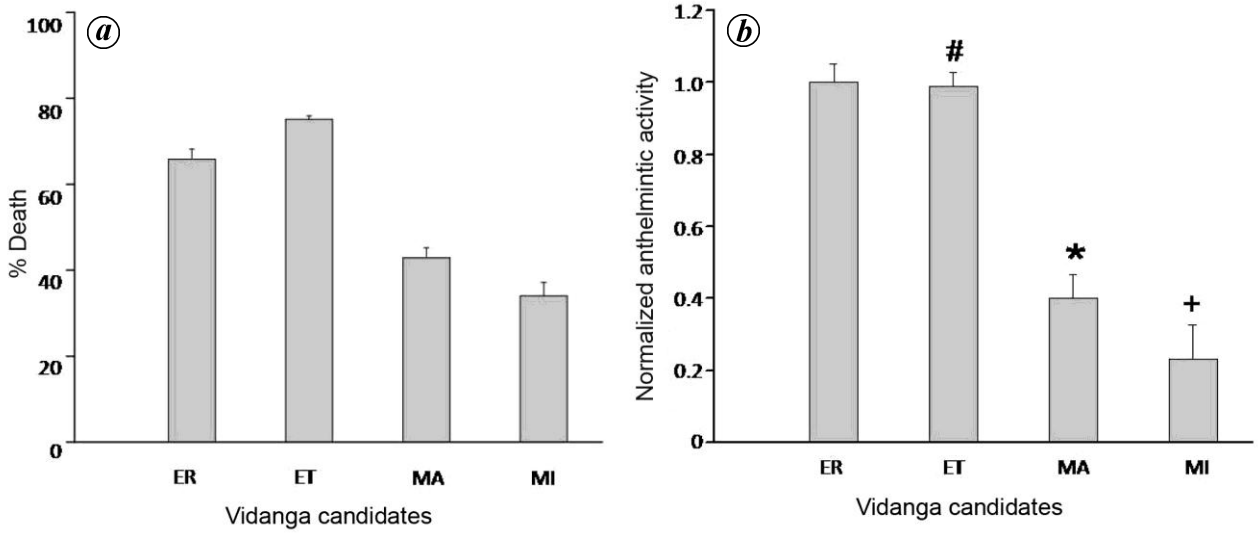

Figure 3. Effect of Vidanga treatment on adult *Caenorhabfitis elegans*. ***a***, Anthelmintic activity of 400 μg/ml ethyl acetate extracts of the four Vidanga candidates. ***b***, Relative anthelmintic activity of extracts (400 μg/ml) of (ET; #, statistically non-significant), (MA; *, $P < 0.01$) and (MI; +, $P < 0.01$) as normalized to that of ER.

This study has demonstrated that traditional practices provide leads to identifying potential substitute species for those of conservational concern. Efficacy and toxicity of the studied species need to be further studied using higher animal models.

All four Vidanga candidates in this study possess anthelmintic activity as tested on *C. elegans*. ET is similar in anthelmintic activity to the authentic Vidanga, ER, followed by MA and MI.

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Reliability of earthquake nucleation model in Koyna region

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We question the inferred downward nucleation of hypocentres that forms the basis for short-term earthquake prediction in the Koyna region. A careful relocation of foreshocks/aftershocks for a *Mw* 4.4 main shock sequence in 1996, hitherto believed to have occurred at 8 km depth, reveals that the main shock occurred at a depth of only 1.8 km, and formed part of a clear and almost instantaneous northward migration of micro-earthquakes along a narrow zone that could only be a fault plane at 4–8 km depth. This finding requires the current hypothesis for forecasting deep main shocks from shallow foreshocks in Koyna region to be discarded.

Keywords: Earthquake nucleation, micro-earthquakes, short-term prediction.

THE study of the earthquake nucleation process is one of the important problems in earthquake mechanics. While the precise earthquake prediction is a distant dream, efforts have been made towards short-term prediction using diagnostic features of earthquake patterns occurring before the main shock. The concept emerged from laboratory experiments that suggest 'during the nucleation, the energy is exclusively consumed in and around nucleation zone… Accordingly, dynamic instabilities of small scales (micro-seismicity) are induced and activated during the nucleation process… The model shows that immediate foreshock activity is a part of main shock earthquake nucleation…'[1]. This concept along with continuous downward depth migration of earthquakes prior to the main shock occurrence has been patented and used as a model for short-term earthquake prediction in Koyna region[2,3]. The results suggest that fracture nucleates at shallow depth (<1 km) and gradually deepens to cause the main shock at a depth of 8–11 km.

The above result of depth migration from shallow to deeper level is at variance with the well-known physical basis that due to the progressive homogenization of the crust with depth, the rupture that nucleates in the shallower region is inhibited from propagating due to presence of small inhomogeneities, while those nucleating in deeper regions (high stress drop) have the probability of growing into a main shock and propagate over the entire fault plane[4,5]. We study here the validity of the proposed nucleation model presented for Koyna region earthquakes

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