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Screening of endophytic fungal extract of *Calotropis procera* for insecticidal activity against *Callosobruchus chinensis* L. (Coleoptera: Bruchidae)

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Abstract
The isolation and insecticidal activity of endophytic fungal extract of leaf and seed of *Calotropis procera* against pulse beetle, *Callosobruchus chinensis* were studied. Endophytic fungi were isolated from the freshly collected leaf and seed of *Calotropis procera* and subcultured. The various concentrations of methanol and ethyl acetate crude endophytic fungal extracts were tested against *Callosobruchus chinensis.* The percent mortality was recorded after 96h.The insecticidal activity of the endophytic fungi isolated from leaf of *Calotropis procera* were (LD_{10}= 28.02mg/kg, LD_{50}= 83.89mg/kg) in methanol and (LD_{10}= 30.63mg/kg, LD_{50}= 121.6mg/kg) in ethyl acetate respectively. The fungi isolated from seed of *Calotropis procera* were (LD_{10}= 38.86mg/kg, LD_{50}= 91.64mg/kg) in methanol and (LD_{10}= 51.87mg/kg, LD_{50}= 124.8mg/kg) in ethyl acetate respectively. The mortality increases with increase in concentration of endophytic fungi. The methanol solvent extract showed more insecticidal property against *C. chinensis* due to the secondary metabolites of endophytic fungi. Statistical variance, 95% confidence limits and regression equations are presented.

Keywords: *Callosobruchus chinensis,* endophytic fungi, mortality, *Calotropis procera.*

1. Introduction
The crop and store grain pest problems are nearly as old as the beginning of crop cultivation. With a greater awareness of hazards associated with the use of synthetic organic insecticide there has been an increase need to explore suitable alternative methods of storage pest control [33]. Storage grains are protected by different plant materials from pest infestation. Natural products in their crude form or plant extract provide unlimited opportunities as biopesticide. Heavy infestation of pulse beetle, *Callosobruchus chinensis* causes qualitative and quantitative losses in store grains [3, 21, 34, 36, 41, 46]. Therefore various plants and their derivatives are effective and used for controlling the storage pest [35].
Calotropis procera is the source of ascaricidal [12], schizonticidal, nematocidal [32, 37], anti-microbial [26], antihelmintic, molluscicidal [20], insecticidal, anti-inflammatory, anti-diarrhoeal, larvicidal [19, 22, 24, 30]. There is first report on the insecticidal and larvicidal effects in the latex of *Calotropis procera* [19]. Due to larvicidal compounds of latex of *Calotropis procera* caused 100% mortality in third instar larvae of *Aedesa egypti* after 5 minutes [38].
Endophytes are microorganisms that grow within plants without causing any obvious symptoms of infection or disease [18, 25]. Some of the endophyte microorganisms are thought to protect their host from attack of fungi, insect and mammals by producing secondary metabolites. Therefore plant associated microbes has explore the potential in pest control. The endophytic fungal metabolites showed pesticidal activity against major groundnut defoliator *S. litura* [31]. In several ryegrasses that high fungi infection is correlated with a decrease in the attack frequency of the Argentine stem weevil, *Listronotus bonariensis* [17]. Several authors studied that the role of endophytic fungi in the control of insects [5, 8, 10, 13, 43].
The present study was undertaken for screening of endophytic fungi isolated from leaf and seed of *Calotropis procera* for insecticidal activity of *Callosobruchus chinensis*.

2. Materials and Methods
2.1 Insect culture
Infected seeds were obtained from the Grain market, Aurangabad and culture the insect,

Callosobruchus chinensis in laboratory. The glass jars were cleaned and dried in oven at 60 to 70 ^{0}C. Fresh non-infected grains of *Vigna unguiculata* were purchased from the market and were manually screened to remove the infected or hollow grains. The disinfected grains were then washed and dried in the oven at 60 ^{0}C to kill the stages of life cycle of pests if any. Ten males and ten females were released in 500 grams of these grains and allowed to maintain as stock culture.

2.2 Isolation of endophytic fungi
The plant parts of *Calotropis procera* were collected in month July to October 2015, from local area of Aurangabad and brought to the laboratory. Collected plant parts (leaf, seed) were gently rinsed in running tap water to remove adhered dust and debris. The plant parts were surface sterilized with 0.1% $HgCl_2$ for two minutes followed by washing in 70% ethanol, after that plant parts were washed with distilled water. Small pieces up to 1 cm. were cut and transferred into petridishes containing Potato Dextrose Agar medium (PDA) supplemented with Chloramphenicol antibiotic and incubated at 25 ± 2^{0}C for 3-5 days. The fungus grown out from the plates were subculture in PDA slants. The fungal mycelia growing out of the sample plates were continuously subcultured and maintained in PDA plates [44].

2.3 Preparation of fungal extract
The 100 mg. of mycelium and spores of obtained endophytic fungi were collected separately from leaf and seed and extracted in 100 ml. of methanol and ethyl acetate solvent as stock solution.

2.4 Insecticidal bioassay
The methanol and ethyl acetate extract of endophytic fungi were screened for insecticidal activity against *Callosobruchus chinensis*. For screening 30gm seeds were shaken thoroughly with various concentration of methanol and ethyl acetate endophytic fungal extracts of leaf and seed in each jar. The dose was prepared by mixing the isolated endophytic fungi with respective solvent and was applied to grains. One jar of control containing seeds treated with only respective solvent was maintained. The treated seeds were allowed to evaporate the solvent for 48 hours. 5 male and 5 females emerged in a batch were released in each experimental and control jar containing 30gm. seeds and mortality was recorded after 24h up to 96h of treatment. The percent mortality was calculated at 96h. The calculation of mortality rate was corrected for control mortality according to Abbott's formula [1].

Mc = (Mo-Me/100-Me) X 100

Where, Mo = Observed mortality rate of treated adults (%), Me = mortality rate of control (%) and Mc = corrected mortality rate (%)

2.5 Statistical analysis
The mortality data thus obtained was put into probit/ log concentration transformation so as to plot probit regression lines. These regression lines are plotted for the purpose of calculating the required concentration of endophytic fungal extract to produce 10% mortality and 50% mortality. The standard error of the log LC_{50} (Variance 'V' of the calculated log LC_{50}) and chi square value and fiducial limits to endophytic fungal extract were calculated from regression equation [9, 16].

3. Results
The results showed that the mortality increases with increase in concentration at all doses (Table 1 and 2; Figure 1 and 2).The cumulative percent mortality and corrected mortality of the adult insects released into the jars containing seeds treated with methanol and ethyl acetate endophytic fungal extract are shown in Table 1. The mortality data show that all beetles had died at 96h in the treatment using 6mg. of methanol endophytic fungal extract of leaf and seed while in ethyl acetate endophytic fungal extract of leaf 100% mortality was recorded at 96h of exposure in 8mg. and 6.5mg. of ethyl acetate endophytic fungal extract of seed.

The results of the probit analysis for the estimation of LD_{10}, LD_{50}, variance, 95% confidence limits and regression equation at 96h for the mortality of pulse beetle, *Callosobruchus chinensis* are presented in Table 2. In bioassay of methanol endophytic fungal extract of leaf and seed were LD_{10}= 28.02mg/kg, LD_{50}= 83.89mg/kg and LD_{10}= 38.86mg/kg, LD_{50}= 91.64mg/kg respectively and in ethyl acetate leaf and seed endophytic fungal extract were LD_{10}= 30.63mg/kg, LD_{50}= 121.6mg/kg and LD_{10}= 51.87mg/kg, LD_{50}= 124.8mg/kg respectively. Among the various estimate of regression based probit analysis, the χ^2 values for the regression coefficients showed homogeneity to the data.

Table 1: Mortality percentage of *Callosobruchus chinensis* treated with endophytic fungal extracts isolated from leaf and seed of *Calotropis procera.*

Plant parts	Solvent	Dose (mg/30gm)	No of Insect used	Mortality after 96h.	Percent mortality	corrected mortality
	Control	-	10	-	-	-
Leaf	Methanol	1.5	10	3	30	30
		3.0	10	5	50	50
		4.5	10	8	80	80
		6.0	10	10	100	100
	Ethyl acetate	2	10	3	30	30
		4	10	5	50	50
		6	10	7	70	70
		8	10	10	100	100
Seed	Control	-	10	-	-	-
	Methanol	1.5	10	2	20	20
		3.0	10	5	50	50
		4.5	10	8	80	80
		6.0	10	10	100	100
	Ethyl acetate	2	10	2	20	20
		3.5	10	4	40	40
		5.0	10	7	70	70
		6.5	10	10	100	100

Table 2: LD_{10}, LD_{50} values with variance, 95% confidence limits and probit analysis parameters for adult of *Callosobruchus chinensis* after 96h of treatment.

Plant parts	Solvent	LD_{10} mg/kg	LD_{50} mg/kg	Variance	95% CL		Regression equations	χ^2 (degree of freedom)
					Lower	Upper		
Leaf	Methanol	28.02	83.89	0.007996	1.7484	2.0990	Y= -0.176 + 2.6907x	0.4463 (2)
	Ethyl acetate	30.63	121.6	0.01228	1.8679	2.3023	Y= 0.5383 + 2.1398x	0.0639 (2)
Seed	Methanol	38.86	91.64	0.005140	1.8216	2.1026	Y= -1.4771 + 3.4388x	0.1882 (2)
	Ethyl acetate	51.87	124.8	0.005401	1.9522	2.2402	Y= -2.0446 + 3.3606x	0.2361 (2)

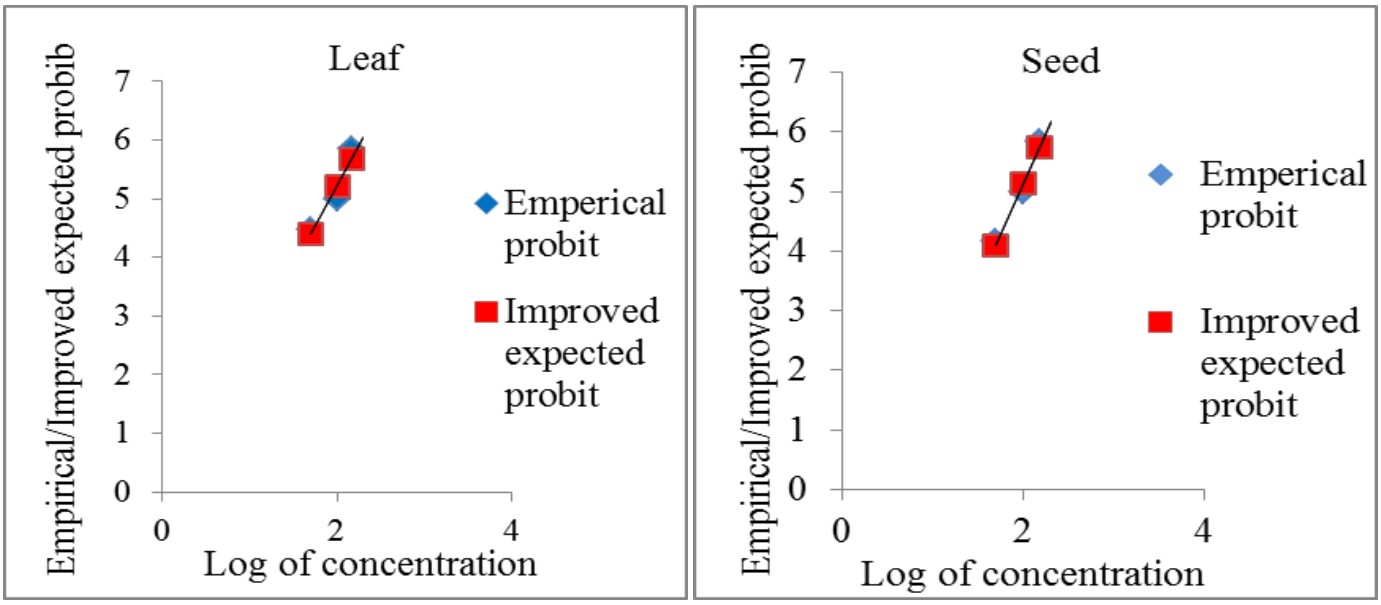

Fig 1: Regression and provisional lines for *Callosobruchus chinensis* exposed to methanol extract of endophytic fungi isolated from leaf and seed of *Calotropis procera* after 96h

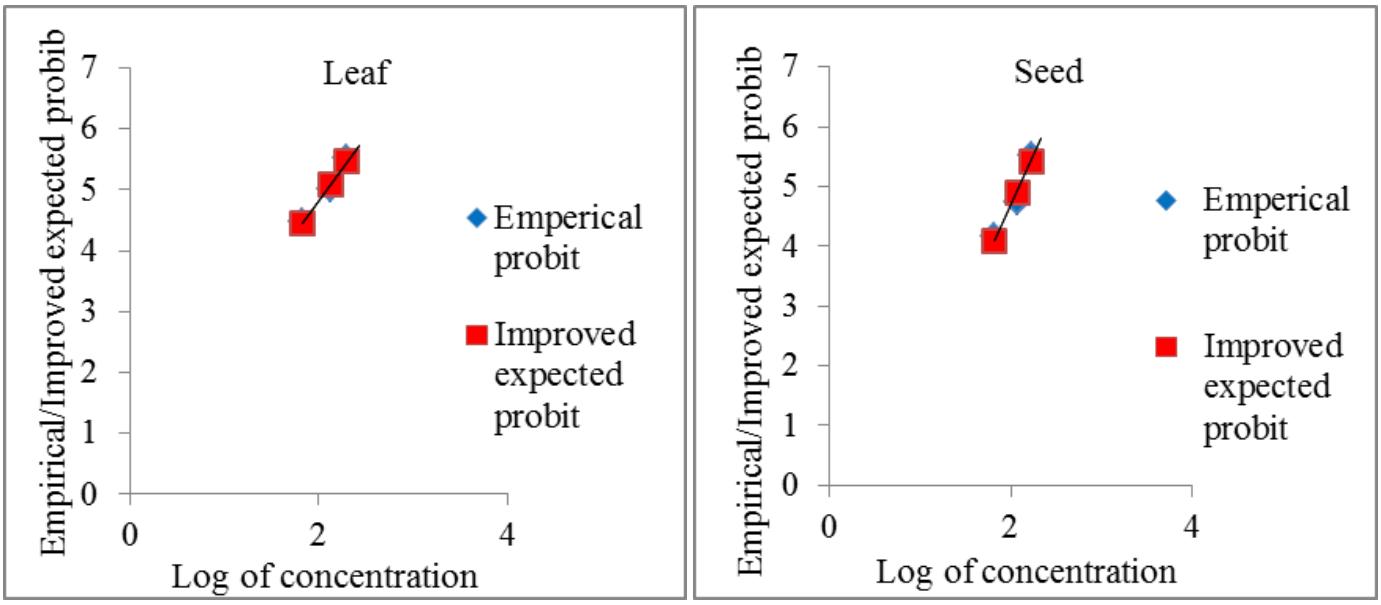

Fig 2: Regression and provisional lines for *Callosobruchus chinensis* exposed to ethyl acetate extract of endophytic fungi isolated from leaf and seed of *Calotropis procera* after 96 h

4. Discussion

In phytochemical screening of the extracts of *Calotropis procera* contains alkaloids, carbohydrates, saponins, phenols, tannins, terpenoids and flavonoids which have medicinal and pesticidal properties [15]. The latex of *Calotropis procera* has been reported to have insecticidal activity against different insects [29, 30]. In the latex of *Calotropis procera* syntheisis two enzymes like chitinases and proteases which act as defensive molecules and are responsible for insecticidal or pesticidal activities [39, 40].

In the present investigation, it was observed that the methanol and ethyl acetate endophytic fungal extract of leaf and seed were more effective in checking mortality than control. Similarly, the methanol crude extract of *Calotropis procera* at 5.0% dosage can protect mung bean seeds from the infestation of *Callosobruchus maculatus* by controlling ovipositions and adult emergence [11]. Several studies documented the growth and development inhibition properties of plant extracts on pulse beetle, *Callosobruchus chinensis*. The *Nerium indicum* bark extract was effective as insecticidal property against pulse beetle, *Callosobruchus chinensis* [33]. The biopesticidal effect of natural saponin isolated from *Acacia concinna*

against pulse beetle, *Callosobruchus chinensis* [7].

The endophytic microorganisms are those that inhabit the interior of plants, especially leaves and branches and stems, showing no apparently harm to the hosts [4]. Endophytic fungi have received considerable attention in the last 20 years because of their capacity to protect hosts against insect's pests and pathogens. Toxic metabolites produced by endophytic microorganisms in many plants can greatly reduce the population of associated insects. The first reported example of plant protection to elm trees by an endophytic fungus, *Phomopsis oblonga* against the beetle, *Physocnemum brevilineum* [47].

In our study the mortality increases with increase in concentration at all doses. Similarly, it is reported that the mortality is depends on concentration [6, 42]. Such a dose dependent mortality with spores of fungi was observed in *Sitophilus zeamais* [2] and *S. litura* [27]. The mortality at 96h had shown that all beetles died in the treatment using 6mg. of methanol endophytic fungal extract of leaf and seed while in ethyl acetate endophytic fungal extract of leaf 100% mortality was recorded at 96h of exposure in 8mg. and 6.5mg. of ethyl acetate endophytic fungal extract of seed. Similarly, the ethyl acetate extract of endophytic *Alternaria alternata* induced significant inhibitory effects on survival and reproductive potential of *Spodoptera litura* [23].

The extracts of foliar fungal endophytes isolated from *Picea rubens* Sarg. (red spruce) needles were toxic to the forest pest *Choristoneura fumiferana* Clem. (Eastern spruce budworm) in dietary bioassays [28, 45]. Toxic metabolites produced by endophytic fungi (*Epichloe* and *Neotyphodium* species) in fescue grasses greatly reduce the populations of associated herbivorous insects. These fungi produce various alkaloids that affect herbivore growth [14].

The finding of the present investigation revealed that, the endophytic fungal extract isolated from leaf and seed of *Calotropis procera* possess remarkable insecticidal activity against *Callosobruchus chinensis*. The study needs further investigation to find out active ingredients responsible for insecticidal properties against wide range of store grain pest and to reach any final recommendations.

5. Conclusion

The results of the study have confirmed that the endophytic fungi have explore the potential of biopesticide and grain protecting activity against store grain pest.

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