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LARVICIDAL CAPABILITY OF CHLOROFORM EXTRACTS OF C*alophyllum inophyllum* l. AND C*assia fistula* L. ON O*ryctes rhinoceros* (LINN.) LARVAE *in vitro*

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Owing to the fact that the application of chemical pesticides cause adverse effects on beneficial organisms, pollute the environment and are detrimental to human health, plant based products are being tested for the control of variety of insect pests. The present study was carried out to test and compare the bioefficacy of chloroform solvent extracts of *Cassia fistula* and *Calophylluminophyllum* against *Orychtesrhinocerous* larvae The leaves of these two plants were dried, powdered and extracted in a Soxhlet apparatus using chloroform as the solvent. These leaf extracts exhibited dose-dependent mortality. The rate of mortality also increases with the increasing hours of exposure indicates more sensitivity.The plant extracts alters the Total Hemocyte Count and Differential hemocyte count. The increased mortality may be due to its antifeedant activity or may be due to its altering the physiological activity and endocrine glands which secretes regulatory hormone.Of these two plants *C. inophyllum* is more effective than *C. fistula*.Thus from the work it is concluded *C. fistula* and *C. inophyllum* showed varying degree of toxicity and can be used in Integrated Pest Management of *O.rhinocerous* and minimise use of chemical insecticides.

Keywords:*Cassia fistula; Calophylluminophyllum; Orychtesrhinocerous*; hemocyteinsecticides.

1. INTRODUCTION

Oryctes rhinoceros Linn.is one of the serious pests of coconut palm *Cocosnucifera*L.The adult beetle bores the unopened fronds and inflorescence of the palm. Decaying organic matter and cowdung are the breeding sites of adult beetle. Adult *O. rhinoceros* attacks the deeper, soft parts of the crown of coconut trees and results in stunted growth of the plants and decreased nut production brings about great economic

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loss to the farmers. Hence this pest has an important status in economic entomology [1].Treatment of breeding sites with insecticides is oneof the methods to control this pest which causes adverse effects like toxicity to non-target organisms, development of insecticide resistance, pest resurgence, environmental pollution and health hazards.Emphasis is now being given to integrated pest management which pays way for the minimal use of chemical insecticides [2]. Biopesticides are considered to be safe to natural enemies and free from residue problem on the crop and in the environment [3]. Secondary metabolites are capable of producing multiple effects in insects such as antifeedancy, growth regulation fecunditysuppression, sterilization, ovipositional changes,repellency and change in the biological fitness like reduced life span, loss of flying ability,low absorption of nutrients, high mortality, immunodepression, enzyme inhibition and disruption of biological synthesis" is using fitness in a faulty way. As sterilization, decreased fecundity are also decreasing fitness. The definition is: Fitness describes individual reproductive success and is equal to the average contribution to the gene pool of the next generation that is made by individuals of the specified genotype or phenotype. Which mean if the animal weekend by immunosuppression it can decrease fitness.

Many workers reported that plants are considered as one of the richest sources secondary metabolites that can be used as pest control agents [4]. Secondary metabolites are capable of producing multiple effects in insects such as antifeedancy, growth regulation fecundity suppression, sterilization, ovipositional changes, repellency and change in the biological fitness like reduced life span, loss of flying ability,low absorption of nutrients, high mortality, immunodepression, enzyme inhibition and disruption of biological synthesis [5].In many countries plants are used as biopesticides [6,7].

Indian literature has documented the use of much plant as biopesticide [8, 9].The chemical literature of *C. inophyllum and C. fistula* leaves shows the presence of diverse biomolecules such as flavonoids triterpenes [10] and have assorted biocidal activity such as anti-microbial cytotoxic, larvicidalactivity was shown by several authors [11,12].Thus the present work is an attempt to evaluate alteration in physiological function of the pest *O. rhinocerous* caused by *Cassiafistula*belongingtothe family Caesalpiniaceaeand*Calophylluminophyllum*ofCalophyllaceae

2. MATERIALS AND METHODS

2.1 Collection and Extraction of Plants

The leaves of *Calophylluminophyllum* and *Cassia fistula* were collected from the Vizhunthayambalam of Kanniyakumari District. The fresh leaves were washed with tap water, chopped into small pieces and ground to fine powder in an electric blender after shade dried for 12 days. Fifty grams of powdered leaves were extracted with 300ml of chloroform in soxhelet apparatus for 8 hours. The extract was then concentrated at reduced vacuumpressure of about350 mbarusing rotary evaporator and stored in vials at 4°C until further analysis.The concentrated extract was stored for a maximum period of 30 days. The extract was weighed and mixed with other components in various proportions and *O.rhinoceros* grubs were allowed.

2.2 Phytochemical Analysis

Table1. Qualitative analysis of thechloroform extracts of *C.inophyllum* and *C.fistula*

S.No.	Experiments	Observations	Phytochemical
1	Plant extract + Wagner's reagent	Reddish brown precipitate	Alkaloid
2	Plant extract + Benedict's reagent	Reddish brown precipitate	Carbohydrate
3	Plant extract + Glacial acetic acid and Ferric chloride solution +Sulphuric acid.	Appearance of violet or a greenish ring	Glycosides
4	Plant extract + Sodium hydroxide solution	Appearance of intense yellow colour and colourless solution on addition of dilute hydrochloric acid	Flavanoids
5	Plant extract + Ferric chloride	Appearance of deep blue or black colour	Phenol
6	Plant extract + Ninhydrin solution	Purple colour	Proteins and aminoacids
7	Plant extract + Ferric chloride solution.	Appearance of Blue or greenish color	Tannins
8	Plant extract + Water. The mixture was shaken vigorously	Formation of persistent foam	Saponins
9	Plant extract + Hydrochloric acid.	Appearance of Red precipitate	Phloba tannins
10	Plant extracts + Chloroform + Sulphuric acid	Appearance of Reddishbrown precipitate	Terpenoids

Table 2. Composition of medium for *O. rhinocerous* grubs

Materials (in g)	Extract concentration (in %)					
	Control	3.33	6.66	10	13.33	16.66
Leaf extracts +Straw powder adjuvant	0	3+7	7+13	10+20	14+26	17+33
Cow dung	200	193	187	180	174	167
Paddy Straw	100	97	93	90	86	83
Total	300	300	300	300	300	300

2.3 Preparation of Culture Medium

The culture medium was prepared by mixing dried cow dung and paddy straw in a ratio of 2:1 and is sterilized in an autoclave at a pressure of about 15 lb/sq. inch at temperature 121- 124°C for about 15 minutes.

2.4 Rearingof Larvae and Lethal Concentration

The egg masses of *Oryctesrhinocerous* were collected from the manure pits surrounding area of Vizhuthayambalam, Kanniyakumari District. The collected eggs were reared in a plant pot of 500gm capacity contains300 gm culture medium. After hatching the larvae were transferred to a culture medium in a ratio of 3 larvae per container with 300gms medium. Third instar larvae,size varying from 96$\pm$ 0.903 cm length and 3.0 $\pm$ 0.2 cm width and weight 10:83 $\pm$ 0.9 gm were selected for the present investigation.

10,20 30,40 and 50 grams of *C. inophyllum* and *C. fistula* leaf powder was mixed separately with a substratum of cowdung and paddystraw (2:1 ratio). Exactly10,20, 30,40 and 50 g of thechloroform extracts of *C .inophyllum* and *C. fistula* leaves were mixed with equal amount of powdered paddy straw and was finally mixed separately with a respective substratum(cowdung and paddy straw)in such a way to make up 300 grams/ experiments . The composition of medium and the concentration of straw powder with plant extracts were given in Table 2.

The result was recorded till the emergence of adult. If any mortality was observed in the control then the mortality data was subjected to Abbot's formula [13] in order to find out corrected present mortality using the following formula. The lower and upper fiducial limit (LC_{30}), (LC_{90}) median lethal concentrations (LC_{50}) were calculated using probit analysis in excel 2007 [14].

$$Percentage\ mortality = \frac{Number\ of\ dead\ larvae}{Number\ of\ larvae\ introduced} X100$$

$$Corrected\ percentage\ mortality = \frac{(1 - Number\ in\ T\ after\ treatment\ )}{Number\ in\ C\ after\ treatment} X100$$

Where, T- Experimental and C- Control

2.5 Total Haemocyte Count (THC) and Differential Haemocyte Count (DHC)

Five 3rd instar larvaeof *O. rhinocerous* from the stock culture was reared separately in 300 gms culture medium containing 1:1, 1:3 and 1:5 dilution of LC_{50} concentration of chloroform solvent extracts of *C. inophyllum* and *C. fistula*. After 72 hours of exposure the haemolymph was collected by amputating the prolegs. Oozing haemolymph was collected in a prechilledeppendorf tube having few crystals of phenyl thiourea. Total haemocyte was counted in treated and control groups [15] using haemocytometer [16]. Differential haemocyte count (DHC) was estimated by counting different haemocytes from the population of 200 cells, based on the morphological features as described by Nittono [17]. THC and DHC was determined in at least 10 larvae both in control and experimental animal separately.

2.6 Data Analysis

The data collected were represented as mean ± SD.The mortality (%) was corrected by Abbott's formula and subjected to probit analysis to derive 50% mortality (LC_{50}). Analysis of Variance (ANOVA) was carried out to understand the impact of treatment withthe two leaf extracts on *O.rhinoceros* larvae over different periods of exposure.

3. RESULT

3.1 Phytochemical Analysis

The phytochemical analyses showed the presence ofCarbohydrates and Flavanoids both the leafextracts. Inaddition, *C.inophyllum* contains saponins and phlobatannins and *Cassia fistula* containstannins and glycosides (Table 3).

Alkaloids, glycosides, phenols, proteins, amino acids, tannins, terpenoids were absent in the chloroform extracts of *C.inophyllum*. Similarly,alkaloids, phenol, proteins, tannins, saponins and phlobatannins were absent in *C. fistula* extracts.

Table 3. Availability ofphytochemicals in thechloroform extracts of the two selected plants

Secondary metabolites	*C. inophyllum*	*C. fistula*
Alkaloid	-	-
Carbohydrate	+	++
Glycosides	-	+
Flavnoides	+	+
Phenol	-	-
Protein	-	-
Amino acid	-	-
Tannins	-	+
Saponins	+	-
Phloba Tannins	+	-
Terpenoids	-	-

3.2 Acute Toxicity Assay

The study conducted on lethal toxicity revealed that the chloroform solvent extracts of *C. inophyllum* and *C.fistula* were toxic to *O.rhinocerous*. Medial lethal concentration (LC_{50}) is considered the most accepted basis to determine the toxicity of the plants. In this study, the 100% mortality and the survival of experimental larvae was decreased compared to controls.indicatedthetoxicityof the plant extract. A dose-dependent increase in mortality rate was observed in all experimental categories.A decrease in LC_{50} values of selected plants was observed at 24, 48 and 72 hoursas21.1, 8.9 and 4.3 mg/ml of chloroform solvent leaf extracts of *C. inophyllum* and 50.8 and 35.4 mg/ml *C.fistula* respectively.Requirement of less concentration of plant extracts with the increasing hours of exposure indicates that the larvae were more sensitive with the increasing hours of exposure (Fig. 1).

Compared to *C. inophyllum*, *C. fistula* is less toxic, for instance after 24 hrs of the treatment, no mortality was observed in the groups of *O.rhinoceros*grubs treated with all the fiveconcentrations of the chloroform extract of *C. fistula*.On the other hand, 16 ± 1.06 mortality was observed in *C. inophyllum* treated categories. In *C. fistula* 100% mortality was observed at 72 Hrs of exposure at 50 gms (16.6%) concentration where as same mortality per cent was observed at 40gm (13.3%) of extracts 48 hrs of exposure time for *C. inophyllum* (Fig. 2). Highly significant mortality was observed on the 96 hour of exposure df -1; P (0.001)< 0.005; F= 82.96 F crit = 7.71 and df-1; P (0.001) < 0.005 ; F= 69.71 ; F crit+ 7.71 for *C. inophyllum* and *C.fistula* respectively (Table 4) .A perfect positive correlation between LC_{50} and exposure time indicates larvae are more sensitive with increasing exposure time (Fig. 2).

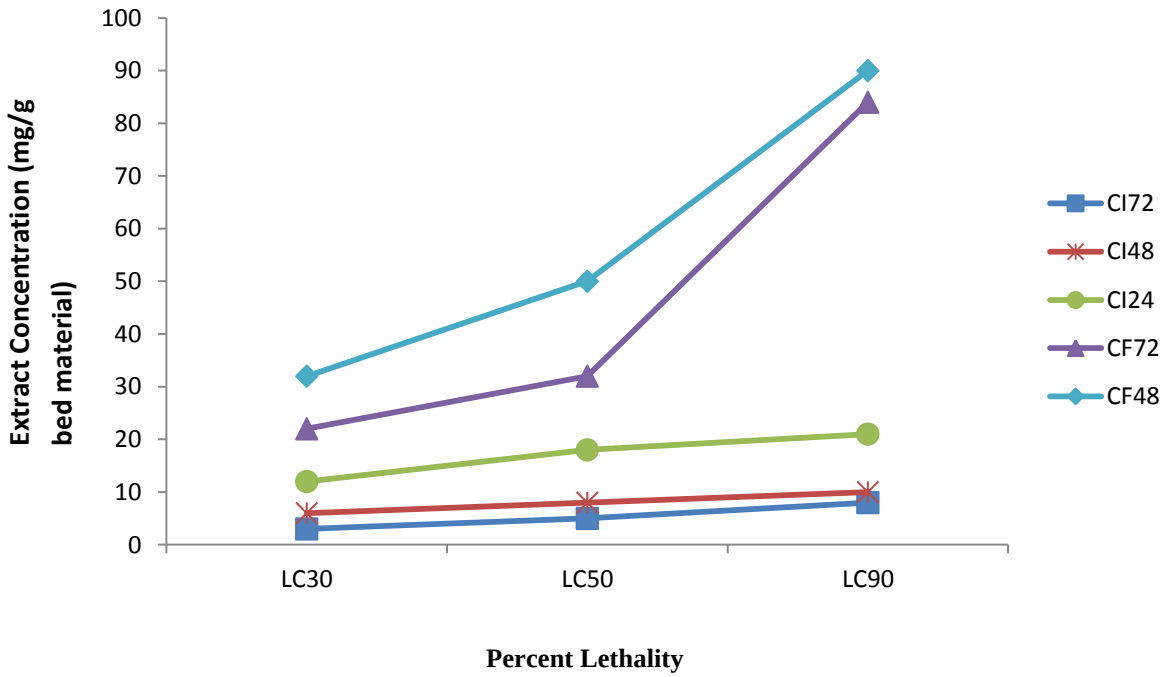

Fig. 1. nhrLethal Concentration values of the chloroform extracts of *C.inophyllum*(CI) and *C.fistula* (CF)

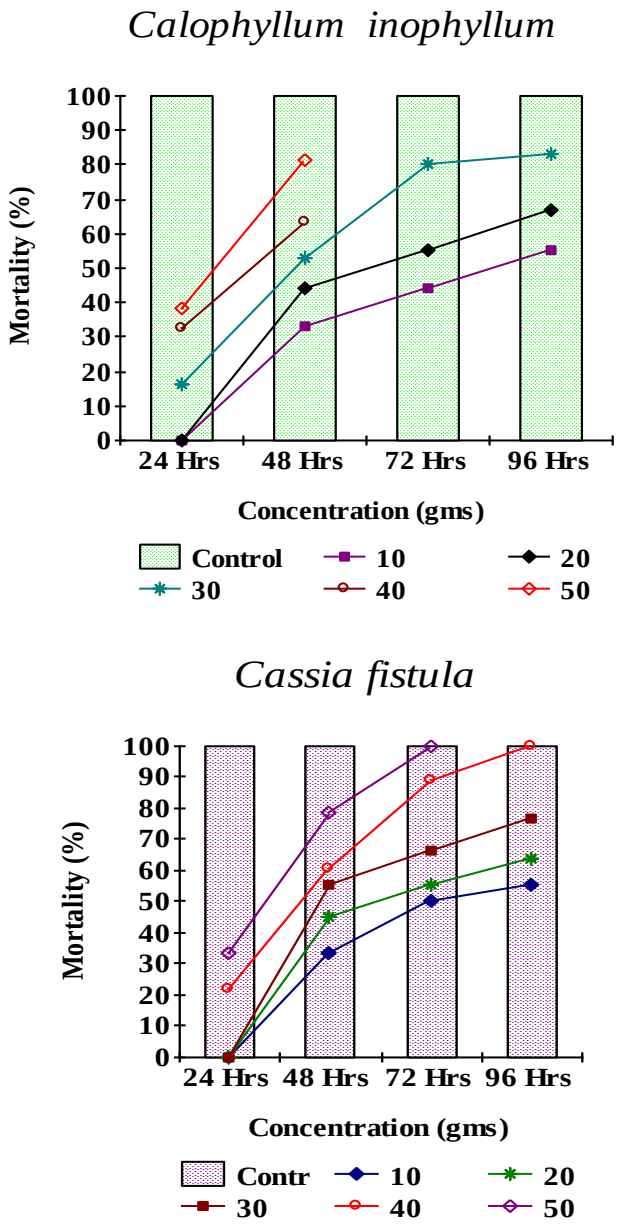

Fig. 2.Percent mortality of *O.rhinoceros* treated with different concentrations of chloroform extracts of the experimentalplants at different time intervals

Table 4. Statistical significance of *C.inophyllum* and *C. fistula* treated categories for different exposure time

Treatment hrs	*C. inophyllum*		*C. fistula*		*F crit*
	F	*P-value*	*F*	*P-value*	
24	4.82	0.10	2.50	0.19	7.71
48	44.38	0.003	42.15	0.003	
72	44.68	0.003	65.76	0.001	
96	82.96	0.001	69.71	0.001	

Table 5. Total Hemocyte count on *O. rhinoceros* third instar grub treated with different concentration of chloroform solvent extract of *C. fistula* (CF) and *C. inophyllum* (CI)

Treatment	LC_{50} dilution		
	1:1	**1:3**	**1:5**
CF	6000 ± 234 (16.96)	6650± 260 (7.96)	6950±180 (3.8)
CI	5950 ± 190 (17.65)	6450 ± 222 (10.73)	6750± 193 (6.57)
Control	7225 ± 200	-	-

Values in parentheisis represent percent increase/decrease

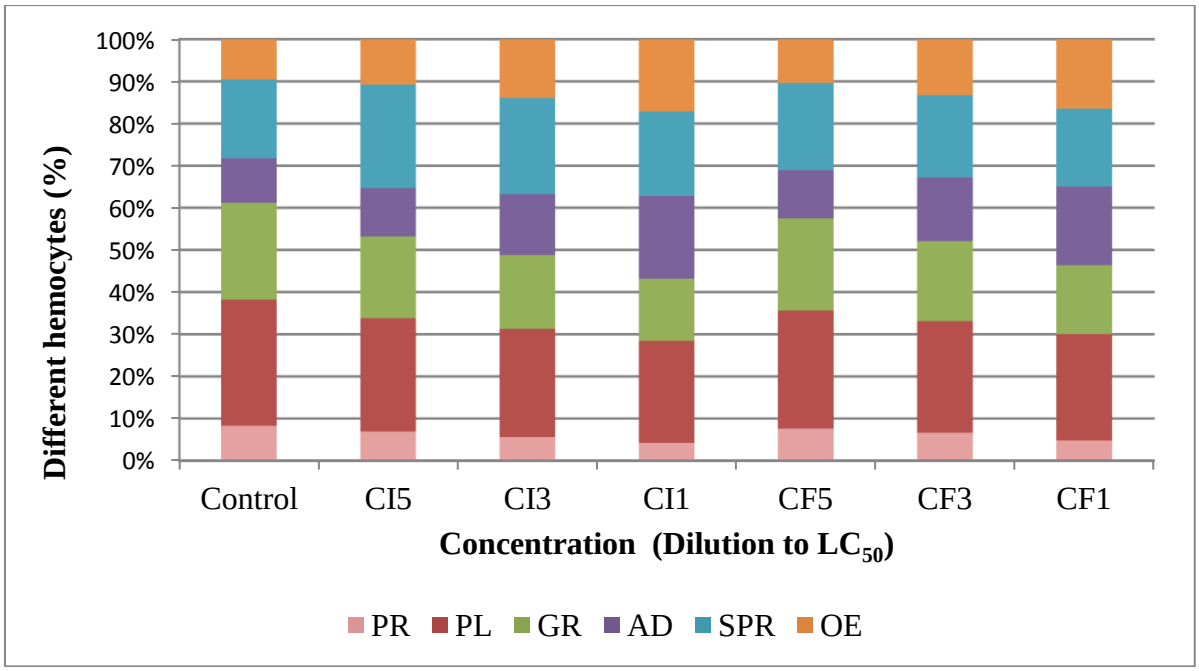

Fig. 3. Differential hemocytes of *O.rhinocerous* treated with 1:1; 1:3; 1:5 dilution of LC_{50} concentration of chloroform solvent extracts of *C. fistula* (CF) and *C. inophyllum* (CI)

CI – C.inophyllum; CF – C. fistula; 1- 1:1 dilution; 3- 1:3 diulution; 5- 1:5 dilution

3.4 Total Haemocyte Count (THC) and Differential Haemocyte Count (DHC)

The Total Haemocyte Count (THC) countin all experimental categories were declined compared to control(7225 ± 200). The decrease in number of hemocytes is concentration dependent as 16.96 and 17.65 % was observed as maximum in 1:1 dilution of *C. fistula* and *C. inophyllum* respectively.

Six different types of haemocytesnamely Prohaemocytes (PR), Plasmatocytes (PL), Granulocytes (GR), Adipohaemocytes (AD), Spherulocytes (SP) and Oenocytoids (OE) were observed in both experimental and control categories of *O. rhinocerous*. In control categories maximum percent of hemocytes were observed in the order **PL**(29.9%)>**GR** (23%)>**SP** (18.8%)>**AD** (10.5)>**OE** (9.5%)>**PR**(8.3%).Changes in this order of percent of cells were observed in all experimental categories. For instance in 1:1 dilution of *C. fistula* treated category the percent of cells were as PL(24.2 %) >**SP** (20.1%) >**AD** (19.6) >**OE** (17.1%) >**GR** (14.8%) >**PR** (4.2%). In total a decreasing trend was observed Plasmatocytes, Granulocytes and Prohemocyteandan increasing trend was observed in Adipocytes, Sperulocytes and Oenocytescompared to control (Fig. 3).

4. DISCUSSION

The present study showed the larvicidal capability of *C. inophyllum and C. fistula* leaves as a novel source against *O.rhinocerous* grubs. A dose-dependent increase in mortality rate observed in the present study wasattributed to their primary and secondary

metabolite compositions selected plants. Plant derivatives reduce the survival rates of larvae and pupae as well as adult emergence [18]. The leaves of *C. fistula* showed the highest total phenolic, flavonoid and proanthocyanidin contents [19]. According to(Tukimin and Heri ,2012)*C. inophyllum* had antifeedant, repellent and larvicidalactivity.Mortality was caused by caused by inhibition of feeding activity or poisoning of hormone-producing organs that regulate insect development.According to [20] ecdysone and 20-hydroxyecdysone is a hormone that regulates the growth and changes occur in the cuticle during the developmental process. Mortality will be caused by the disturbed hormonal regulation. In addition to hormonal disorders, physiological disorders can also inhibit the growth of larvae such as protease and invertase enzymes that can interfere with the food digestion of insects [6].

Six different types of hemocytes, prohemocytes (PRs),plasmatocytes (PLs), adipohemocytes (ADs), granulocytes (GRs), oenocytoids (OEs)and spherulocytes (SPs) were observed in the hemolymph of the normal 3rd instar grub of *O. rhinoceros*. The total hemocyte count (THC) in the experimental categories of larvae showed a significantdecreasein Plasmocytes , Granulocytes and Prohemocytes whereas increase in Adipocytes, Oenocytes and spherulocytescompared to control .Reduction in THC in the experimental categories substantiates the finding of other workers, who worked on neem based formulations [21,22].The reduction may be due to toxic effects of secondary metabolites or inhibitory effects of the extracts on endocrine glands and their secretions [22,23] The production, multiplication and differentiation of hemocytes were controlled by the hormone [22] and it is assumed the disturbed hormonal regulation affects the production of cells. Reduction in PR number is attributed either to the inhibition of mitotic division, conversion of other types of cells or the inhibitory activity on hematopoietic organs.Plasmatocytes and Granulocytes are phagocytosis in function may be attracted to other compounds which enter the body. Oenocytes are resisting the penetration of plant metabolites thus remain unaffected [24].

5. CONCLUSION

We hereby summarizethat the two chosen plant *C. fistula* and *C.inophyllum*has pronounced effect on the mortality in the of the III instar grub at different hours of exposure. The larvae were exhibiting increasing sensitivity with increasing hours of exposure. The larvicidal effect of the plant extracts may be due to its antifeedant nature or alterations in the physiological function or may be due to its inhibitory effect on the hormone producing organ which leads to the absence of regulatory hormones. The plant extracts also alter the immune function by increasing or decreasing the number of immunocytes either inhibiting its proliferation or inducing phagocytic activity of immune cells.Thusfromtheworkitisconcludedthat *C.fistula*and*C.inophyllum*showedvaryingdegreeof toxicityto *O.rhinoceros* larvae andcan beusedin theIntegratedPestManagement(IPM)toreduce theuseofhazardous chemicalinsecticides.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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