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Research Article

# BIOEFFICACY OF PODOPHYLLOTOXIN AGAINST *Plutella xylostella* AND *Pieris brassicae*

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**Abstract:** Podophyllotoxin was extracted and purified from the roots of *Podophyllum hexandrum* Royle. and evaluated for repellent, antifeedant as well as for its toxicity against *Plutella xylostella* and *Pieris brassicae*, in a feeding deterrence assay, 93.47 % feeding rejection was observed in the treatments with the 3rd instar larvae of *P. xylostella* at 1200 ppm whereas at the same concentration, the feeding inhibition in the larvae of *P. brassicae* was 76.11 %. In a no-choice assay both the insects *viz. P. xylostella* and *P. brassicae*, consumed minimum area of 0.94 and 1.71 $cm^2$ after one day of treatment, respectively whereas in the untreated control, the leaf area consumption was 4.16 and 4.00 $cm^2$. The $LC_{50}$ values of 210.51 ppm and 11.25 ppm was observed for both the insects *viz. Plutella xylostella* and *Pieris brassicae*, respectively.

**Keywords:** Biological activity, Antifeedant, Cabbage, Cauliflower, *Plutella xylostella, Pieris brassicae*, Podophyllotoxin, *Podophyllum hexandrum*

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## Introduction

Many plant species are capable of synthesizing secondary metabolites with biological properties that are important in the fight against insect pests [1]. Such natural chemicals obtained from these plants are known to exhibit selective action against pests through a variety of biological activities, including the repellent, antifeedant, direct toxicant and growth regulators especially for lepidopteran insect pests [2]. These selective agents found in many plants have many added advantages such that they can reduce the application of hazardous chemicals, selective (not harmful to the natural enemies), safe with no residual build-up in food and environment friendly. In today's scenario the pest management in agriculture has often relied on toxic chemicals which intern results in negative impacts on natural enemies, pollinators and other non-target organisms. The world over concern to human health and environmental awareness has necessitated the need for evolving effective and safe methods to manage such pests. Under such circumstances the plant-derived extracts and phyto-chemicals have been a subject of research as an alternative to the conventional insecticides.

Several medicinal plants have been perceived to have insecticidal and insect deterrent properties [3]. *Podophyllum hexandrum* Royle, known as Indian May Apple is one such perennial herb belonging to *Berberidaceae* family which grows in the inner ranges of the Himalayas from Kashmir to Sikkim at altitudes of 3,000-4,200 m. [4]. The plant yields podophyllotoxin as principal active compound which is isolated as a sustainable natural bioresource from the roots of rhizomes *Podophyllum hexandrum* Royle and there are reports which indicate that lignans of this plant have strong insecticidal activities [5,6]. The other important lignans present in the roots of this plant are deoxypodophyllotoxin, β-peltatin and α-peltatin are also reported to have insecticidal activities. In view of this, insecticidal activities of podophyllotoxin were studied against the diamondback moth, *Plutella xylostella* (*Yponomeutidae*) and the cabbage butterfly, *Pieris brassicae* (Pieridae), the two important insect pests of cauliflower in Himachal Pradesh, a hilly state of India where this is grown as cash crops. Since, farmers use synthetic insecticides in abundance which leave harmful residues and also cause environmental pollution. The studies thus will not only help in defining the active role of plants as an insecticide but will also help in exploiting such plant species for their use against the harmful insect species thus reducing the pesticidal residue.

## Materials and Methods

### Plant Material

Roots of two-year-old *P. hexandrum* were collected from Kalatop Reserve Forest in the Kalatop area of Himachal Pradesh (India) with prior permission of the Department of Forests, Himachal Pradesh, India. Isolation of podophyllotoxin The isolation of podophyllotoxin was done as per the methods discussed by various researchers [7, 8]. The oven dried (600C for 8 hours) and finely powdered roots (100 g) were extracted 3 times with 250 ml methanol. All the extracts were combined and evaporated using vacuum evaporator till 10-15 ml of brown viscous residue was obtained. This extract was ice cooled and slowly poured into the ice cooled 1 percent hydrochloric acid (HCL) in distilled water with constant stirring. On formation and setting down of pale brown precipitates (after 10 hours) it was filtered through Whatman filter paper No.1 along with washing with distilled water to make it free from HCL traces. These filtrates (acid free) were allowed to dry to get dark brown colour solid podophyllin (a resin from *Podophyllum hexandrum*) with a yield of 14.0 g. A small amount (90-93 mg) of this solid mass (podophyllin) was dissolved in 5 ml of methanol and thin layer chromatography (TLC) was developed along with the standard of podophyllotoxin (Fluka-Biochem, USA) in chloroform:methanol (90:10) which confirmed the presence of podophyllotoxin as active ingredient having RF value of 0.71 (on aluminum pre-coated 1 mm thick TLC) developed and spot visualization was done with iodine. The above mixture of lignans called podophyllin (10 g) was dissolved in methanol and thoroughly mixed with 15 g of silica gel (60-120 mesh) and dried in vacuum desiccators till free flowable. The adsorbed mixture was loaded on a glass column (4 cm x 60 cm) packed with silica gel (200 g, 60-120 mesh) with the help of benzene. The column was then eluted with solvents in step gradient using benzene, benzene:chloroform (50:50), chloroform and finely with chloroform: methanol (90:10). Fractions obtained with last solvent mixture (total fractions-20) were collected in 100 ml flasks and monitored using TLC for the presence of podophyllotoxin. All fractions containing the podophyllotoxin were combined and after concentration, the concentrated solution was again monitored using TLC. The crude podophyllotoxin was then crystallized with the help of benzene to get the fine needle shaped crystals of podophyllotoxin of 286.9 mg. The crystallization process was repeated three times to remove the impurities.

Table-1 Percent antifeedance of podophyllotoxin against 3rd instar larvae of *P. xylostella* and *P. brassicae*

| Concentration (ppm) | *P. xylostella* | | Mean | *P. brassicae* | | Mean |
|---|---|---|---|---|---|---|
| | Antifeedance (%) at indicated day of treatment | | | Antifeedance (%) at indicated day of treatment | | |
| | 1 | 2 | | 1 | 2 | |
| 25 | 21.82(27.33) | 55.52 (49.21) | 38.67 (38.27)$^{a}$ | 27.66 (31.68) | 19.80 (24.04) | 23.73 (21.86)$^{a}$ |
| 50 | 21.76 (27.39) | 66.09 (59.65) | 43.92 (43.52)$^{a}$ | 38.89 (38.47) | 51.84 (47.54) | 45.36 (43.00)$^{bc}$ |
| 100 | 30.56 (33.48) | 65.95 (56.45) | 48.25 (44.96)$^{a}$ | 26.48 (30.52) | 62.72 (52.95) | 44.60 (41.74)$^{bc}$ |
| 200 | 34.30 (35.82) | 69.41 (61.65) | 51.85 (48.74)$^{a}$ | 19.83 (26.31) | 72.22 (63.55) | 46.02 (44.93)$^{c}$ |
| 400 | 42.08 (40.42) | 80.64 (73.45) | 61.36 (56.94)$^{b}$ | 34.34 (35.87) | 100.0 (90.00) | 67.17 (62.94)$^{d}$ |
| 800 | 88.18 (70.32) | 98.77 (86.31) | 84.50 (74.40)$^{bc}$ | 49.25 (44.57) | 93.60 (81.34) | 71.42 (62.96)$^{d}$ |
| 1200 | 94.61 (82.10) | 74.39 (66.71) | 93.47 (78.31)$^{c}$ | 57.06 (49.10) | 95.17 (82.54) | 76.11 (65.82)$^{d}$ |
| Mean | 47.61 (45.27)$^{a}$ | 72.97 (64.78)$^{b}$ | | 36.22 (36.65)$^{a}$ | 70.76 (63.14)$^{b}$ | |

Figures in parentheses are arc sine transformed and the figures with similar alphabets do not differ significantly

CD $_{(p=0.05)}$ for *Plutella xylostella*
Concentration 21.08
Day = 11.27
Concentration x Day = 29.82

CD $_{(p=0.05)}$ for *Pieris brassicae*
Concentration = 15.80
Day = 8.45
Concentration x Day =22.34

Table-2 Leaf area (cm$^2$) consumed by 3rd instar larvae of *Plutella xylostella* and *P. brassicae* due to the treatment of podophyllotoxin

| Concentration (ppm) | *P. xylostella* | | Mean | *P. brassicae* | | Mean |
|---|---|---|---|---|---|---|
| | Leaf area (cm$^2$) consumed at indicated day of treatment | | | Leaf area (cm$^2$) consumed at indicated day of treatment | | |
| | 1 | 2 | | 1 | 2 | |
| 25 | 3.01 | 1.39 | 2.20$^{b}$ | 2.77 | 1.08 | 1.92$^{b}$ |
| 50 | 3.02 | 1.13 | 2.08$^{bc}$ | 2.43 | 0.67 | 1.55$^{bc}$ |
| 100 | 2.79 | 1.23 | 2.01$^{bc}$ | 2.91 | 0.43 | 1.67$^{bc}$ |
| 200 | 2.56 | 0.87 | 1.72$^{cd}$ | 3.19 | 0.33 | 1.76$^{b}$ |
| 400 | 2.50 | 0.63 | 1.56$^{de}$ | 2.62 | 0.12 | 1.37$^{cd}$ |
| 800 | 1.33 | 0.61 | 0.97$^{ef}$ | 2.03 | 0.11 | 1.07$^{de}$ |
| 1200 | 0.94 | 1.38 | 1.16$^{f}$ | 1.71 | 0.06 | 0.89$^{e}$ |
| 0.0 (Control) | 4.16 | 3.01 | 3.58$^{a}$ | 4.00 | 1.41 | 2.70$^{a}$ |
| Mean | 2.54$^{a}$ | 1.28$^{b}$ | | 2.71$^{a}$ | 0.53$^{b}$ | |

Figures in parentheses are arc sine transformed and the figures with similar alphabets do not differ significantly

CD $_{(p=0.05)}$ for *Plutella xylostella*
Concentration =0.41
Day = 0.22
Concentration x Day = 0.61

CD $_{(p=0.05)}$ for *Pieris brassicae*
Concentration = 0.38
Day = 0.19
Concentration x Day =0.53

The purity of crystallized podophyllotoxin was confirmed using Co-TLC with reference compound.

**Test Insects**

Two lepidopteran pests, namely the diamondback moth, *Plutella xylostella* (L.) (*Yponomeutidae*) and the cabbage butterfly, *Pieris brassicae* (L.) (Pieridae) were used as test insects to study the effect of podophyllotoxin for its biological activities. Both the species of test insects were reared under laboratory conditions as per the methods given below:

***Plutella xylostella:*** The stock culture of this species was maintained under laboratory conditions throughout the year. For this purpose, larvae, pupae as well as adult moths were collected from cauliflower fields of the University vegetable farm and were kept in wooden rearing cages (36 cm x 34 cm x 26 cm) with glass pans on three sides under laboratory conditions. Fresh cauliflower leaves with their petiole dipped in plastic vials (10 cm length x 4 cm dia) were also kept inside the rearing cages. Cotton swab soaked in sugar solution (10%) was kept inside each rearing cage to stimulate egg laying. The eggs thus laid were shifted to the rearing cages to obtain eggs and subsequently the larvae for further maintenance of the culture and carry out biological studies.

***Pieris brassicae:*** The egg masses of this species were collected from cauliflower and cabbage crops in the month of February from the vegetable farm of the University. The eggs so collected were kept over a wet tissue paper lying in Petri plates (diameter 10 cm). Newly hatched larvae were transferred to the cabbage/cauliflower leaves with their petiole dipped in plastic vials (10 cm length x 4 cm dia) having water. These vials were put inside the wooden rearing cages with glass pans on three sides (36 cm x 34 cm x 26 cm). Fresh leaves were provided daily to caterpillars (or when exhausted). The larvae of the 3rd instar were used to carry out various biological studies.

**Biological activity**

**Antifeedant activity:** The antifeedant activity was observed by determining the leaf area fed by the test insect species after 24 and 48 hours using a leaf area meter (LI-COR, Model-3100 Area Meter, Licor Inc. USA). The percent antifeedance for each treatment was calculated as (100 – T/C) x 100 where T and C represent the consumption on treated and untreated disks, respectively as per the method [9].

**Intrinsic toxicity:** The intrinsic toxicity of podophyllotoxin was evaluated under laboratory conditions against the 3rd instar larvae of *P. xylostella* and *P. brassicae*, respectively. The test material was dissolved by using minimal quantity of the solvent (used for the extraction) to make it flowable and a stock emulsion of the required concentration was prepared using emulsified distilled water containing 2 percent Triton-X 100. Working concentrations were derived using emulsified water from this stock solution by single dilution method. The bioassay studies were carried by using leaf-dip treatment method. Each treatment was replicated 5 times with 10 larvae of required stage in each replicate. Simultaneously, an untreated control was also maintained by using emulsified water. For all the treatments five effective concentrations giving mortality range of 20-80 percent were selected. The $LC_{50}$ values were calculated by Probit analysis [10].

**Results**

**Isolation of podophyllotoxin**

Podophyllotoxin from the roots of *P. hexandrum* was extracted using methanol

(AR). The presence of the podophyllotoxin in the methanol extract was confirmed and qualified by high-performance liquid chromatography (HPLC). The total quantity of podophyllotoxin obtained was 2.75 %.

**Antifeedant activity**

**Plutella *xylostella*:** The antifeedant activity of podophyllotoxin was evaluated against the 3rd instar larvae of *P. xylostella* and *P. brassicae*. The data presented in [Table-1] reveal that the maximum antifeedance over control was observed at 1200 ppm (93.47%) which decreased to 84.50, 61.36, 51.85, 48.25, 43.92 and 38.67 percent at 800, 400, 200, 100, 50 and 25 ppm, respectively. When the leaf area consumption was observed it was found that the minimum consumption of 1.16 and 0.97 $cm^2$ was at 1200 and 800 ppm concentrations, respectively and was statistically at par with each other [Table-2]. The leaf consumption increased to 1.56, 1.72, 2.01, 2.08 and 2.20 $cm^2$ with the decrease in concentration and was statistically different from the leaf consumption in control (3.58 $cm^2$).

***Pieris brassicae:*** The antifeedant effect of podophyllotoxin was studied against the 3rd instar larvae of *P. brassicae* under laboratory conditions. A perusal of data presented in [Table-1] reveals that the maximum antifeedance (76.11%) was observed at 1200 ppm and was statistically at par to the percent antifeedance of 71.42 and 67.17 percent at 800 and 400 ppm concentrations, respectively. The minimum leaf area of 0.89 $cm^2$ was consumed at 1200 ppm followed by the leaf consumption of 1.07, 1.37, 1.76, 1.67, 1.55 and 1.92 $cm^2$ at 800, 400, 200, 100, 50, and 25 ppm concentrations, respectively. The maximum leaf area was consumed in control (2.70 $cm^2$) which was statistically different from to the leaf area consumed in all other treatments [Table-2].

**Intrinsic toxicity**

***Plutella xylostella:*** The mortality response data from the 3rd instar larva of *P. xylostella* (50 number pre-starved for 8 hrs) to podophyllotoxin was studied at 25, 50, 100, 200, 400 and 800 ppm which gave mortality ranging from 18 to 76 percent. The data were corrected for control mortality and then subjected to statistical analysis to get the regression equation which had a slope of 0.16. The homogeneity of the test population was revealed by the calculated value of $\chi 2$ as 0.30, being lower than the tabulated value of 11.07 (p=0.05 at 4 df.). However, the $LC_{50}$ value obtained through probit analysis was 210.51 ppm with fiducial limits of 147.32 and 300.80 ppm [Table-3].

***Pieris brassicae:*** A mortality range of 24 to 84 percent was obtained when fifty larvae (third instar) of *P. brassicae* (pre-starved for 8 hrs) were fed on treated leaves of cauliflower at concentrations ranging from 3.12 to 50 ppm of podophyllotoxin [Table-3]. The data when subjected to statistical analysis gave regression equation with the slope value of 0.21. The homogeneity of the test population was ascertained as the calculated value of $\chi 2$ was 1.04, lower than the tabulated value of 9.49 at p=0.05 and 4 df. The $LC_{50}$ value was calculated from the probit analysis as 11.25 ppm with fiducial limits of 8.81 and 14.37 ppm [Table-3].

**Discussion**

The podophyllotoxin was evaluated against the 3rd instar larvae of *P. xylostella* and *P. brassicae* at 25, 50, 100, 200, 400, 800 and 1200 ppm. At 1200 ppm, the antifeedance in *P. xylostella* was 93.47 percent and was at par with the antifeedance observed at 800 ppm (84.50%) whereas in *P. brassicae*, the antifeedance at 1200, 800 and 400 ppm were 76.11, 71.42 and 67.17 percent and all these were at par with one another. The minimum antifeedance was observed at 25 ppm in both the test insects. Not much literature is available on the antifeedant effect of methanol extract of the root of *P. hexandrum*, podophyllin and podophyllotoxin. But as per the reports *P. hexandrum* roots contain a number of bioactive limonoids like desoxypodophyllotoxin, podophyllotoxin, α and β-paltatin and 4'- demethylpodophyllotoxin which are found to be toxic to insects. Limonoids are highly oxygenated, modified terpenoids with a prototypical structure either containing or derived from a precursor with a 4,4,8-trimethyl-17-furanylsteroid skeleton [11]. The bis-epoxylignans, kobusin and sesamin have also been reported to inhibit the growth of silkworm (*Bombyx mori*) larvae [12]. Whereas [13, 14] reported ρ-benzolactone as an insect feeding inhibitor in this plant and methoxylenedioxy which gives inhibitory effect due to mixed function oxidase, the enzyme system that is responsible for oxidation and inactivation of most toxins. The antifeedant effect of podophyllotoxin against *P. brassicae* has been observed where the total area consumed by the larvae of *P. brassicae* was 1.29, 1.43 and 1.62 $cm^2$ at 1000, 500 and 250 ppm which is in agreement to the present studies whereas the total leaf consumption at 1200, 800 and 400 ppm of podophyllotoxin was 0.89, 1.07 and 1.37 $cm^2$, respectively. Similar trend has also been observed in *P. xylostella* where the area fed was 1.16 $cm^2$ at 1200 ppm in comparison to the control (2.20 $cm^2$) [6].

**Intrinsic toxicity**

The $LC_{50}$ value of podophyllotoxin has been reported for *P. xylostella, Spodoptera litura*, Mythimna separate and *Epilachna sparsa* larvae [5]. Podophyllotoxin resulted in 55.0 percent mortality at 1000 ppm concentration using leaf-dip test after two days, whereas the *S. litura* larvae were not affected by the podophyllotoxin. Another study [15] reported the delayed mortality of *P. brassicae* larvae due to different derivatives of podophyllotoxin. In one of the derivatives of podophyllotoxin these workers reported 94.95 percent mortality after 15 days of the treatment. The efficacy of *P. hexandrum* roots with methanol has also been reported [6]. The rhizome extracts with ethanol when used at 0.5, 1.0, 2.5, 5.0 and 10.0 percent concentrations resulted in a mortality of 16.7, 29.4, 41.0, 71.7 and 73.3 percent with a $LC_{50}$ value of 2.34 percent against the second instar larvae of *P. brassicae* which was slightly higher than the mortality observed in the present studies. The results of the present studies revealed the importance of *P. hexandrum* as an insecticide. Further studies need to isolate other active principles which further can be exploited for its use against a vast group of insect species.

**Application of research**

Roots of *P. hexandrum* can be used for the eco-friendly management of these two pests of cole crops. They can reduce the application of hazardous chemicals, selective (not harmful to the natural enemies), safe with no residual build-up in food and environment friendly.

**Research Category:** 1,2 Keywords

**Abbreviations:** $LC_{50}$- Lethal Concentration 50, TLC- Thin layer chromatography, HCL- hydrochloric acid, RF- Reference Factor, HPLC- high-performance liquid chromatography, AR- Analytical Reagent.

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