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Isolation and Identification of Insect Antifeedant Compound from Ethanol Extract of *Hemidesmus indicus* Root

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ABSTRACT

Phytochemicals with insect antifeedant potential can be used as a safer alternative to harmful chemicals that are used as grain protectants. The insect antifeedant effect of the extracts and fractions of *Hemidesmus indicus* root were tested against the stored grain insect pest *Corcyra cephalonica* Stainton. Bioactivity-guided study of ethanol extract of *Hemidesmus indicus* root led to isolation and identification of a triterpenoid, Lupeol with insect antifeedant potential. Although Lupeol showed insect antifeedant potential the ethanol extract was found to be more effective as an antifeedant. This implies that the synergistic action of compounds present in the ethanol extract of *H. indicus* root is responsible for the higher antifeedant potential.

Key words: Antifeedant, Biopesticide, *Corcyra cephalonica*, Integrated pest management strategies, Lupeol, Post-harvest-storage, Stored grain pest.

INTRODUCTION

Post-harvest storage of agricultural products is a matter of concern to farmers. Insect pests are a major challenge to stored grains and other food commodities. *Corcyra cephalonica* is a destructive insect pest of almost all stored food products and damage it by spinning web and converting it into a webbed mass; ultimately rendering unfit for human consumption. The insect pest management system often relied upon toxic broad-spectrum synthetic chemical insecticides. Controlling them with chemical pesticides is a serious concern as it leads to adverse environmental impact and health hazards.

Phytochemicals with insect antifeedant potential can be used as a safer alternative to harmful chemical pesticides. The identification of deterrent factors present in plants that could be isolated in sufficient quantities or synthesized for use as crop protectants should be considered for controlling insect pests.

Hemidesmus indicus commonly known as Anantamool or Indian Sarsaparilla is a slender laticiferous twining shrub distributed all over South East Asia, India, Srilanka Malaysia, etc. It is widely used in various traditional medicines as tonic, demulcent, diaphoretic, blood purifier and diuretic. The present study is the first of its kind to analyse the antifeedant potential of *H. indicus* root ethanol extract against stored grain insect pest. The study was undertaken to isolate and characterise the bioactive compound present in ethanol extract of *Hemidesmus indicus* root *against C. cephalonica* larvae.

MATERIALS AND METHODS

Hemidesmus indicus (Anantamool or Indian Sarsaparilla) root were washed, shade dried and powdered. The powder was subjected to fractional extraction on Soxhlet apparatus, using acetone, ethanol and water as solvents. As our earlier studies showed that ethanol extract is effective in controlling *Corcyra cephalonica*, the ethanol fraction was used for the study. The solvent from the extract was completely evaporated using rotary evaporator followed by vacuum evaporation and stored for further use.

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Fractionation by column chromatography

The ethanol fraction was subjected to fractionation by column chromatography and the elution was monitored by TLC (Silica gel G; visualization: methanol-sulphuric acid reagent heated at 110°C and identical elutes (TLC monitored) were combined and concentrated and kept in a refrigerator. The fractionation by column chromatography resulted in the isolation of two compounds which were identified by spectral studies like IR, NMR and Mass Spectrometry.

HPLC analysis

Preparation of sample solutions

Accurately weighed 6mg of powdered ethanol extract of *H. indicus* root was taken in a 10 ml volumetric flask. The extract

was dissolved in 7 ml of the HPLC grade acetonitrile. The volume was made up to 10 ml and the sample solution was sonicated using ultrasonicator for 10 min. Standard protocols were followed for HPLC analysis.

The ethanol extract was weighed and dissolved in ethanol. The extract (20 µl) in ethanol was injected onto the HPLC column at a temperature 30°C. The peaks were recorded at a wavelength 200nm using DAD detector. HPLC of different known concentrations of the standards α-amyrin and lupeol were also performed. The results obtained from the ethanol extract were compared with the standard. The quantification of the compounds present was done by plotting standard curve against known concentrations of α-amyrin and lupeol on X-axis and Peak area on the Y-axis.

Bioactivity-guided identification of antifeedant compound

Insect Rearing

The eggs of *Corcyra cephalonica*, (National Accession No: NBAII-MP-PYR-01) was obtained from the ICAR-National Bureau of Agricultural Insect Resource (NBAIR), Bangalore, Karnataka were reared in the laboratory conditions in standard rearing medium.

Ten pre-starved fourth instar larvae were introduced into the treated rice at different dosages of the fractions, subfractions and compounds. The minimum amount of acetone was used to dissolve all fractions, subfractions and compound along with water. Doses of fractions, subfractions and compounds were determined by the ratio of their presence in the ethanol extract of the corresponding doses. All the treatments were serially diluted to apply on rice at required doses. After 72 hours, changes in food consumed (weight change in rearing medium) and the difference in larval body mass were noticed. Two experimenatl sets having distilled water and acetone-distilled water mixture was used as controls. Equal numbers of starved larval replicates were also used to analyse and compare the weight loss after starving for three days.

Calculations

Nutritional indices and weight loss were calculated using (Ho *et al*., 2003; Isman *et al*., 1990) with some modifications. The following parameters were calculated using standard formulae

$$\text{Mean weight gain WL} = (FW\text{-}IN)/N$$

FW= Weight after 3 days.
IN= Initial weight.
N = Initial number of larvae.

Antifeedant activity or the grain protection or loss of protection due to the application of plant extracts was evaluated by calculating the Feeding Deterrence Index (FDI %)

$$FDI\% = (C - T) / C \times 100$$

C is the consumption of control rice kernels and T is the consumption of treated rice kernels.

Percentage of starvation was calculated according to the formula (Abdel-Rahman and Al-Mozini, 2007).

$$\% \text{ Starvation} = (C\text{-}E)/(C\text{-}S) \times 100$$

Where,
C = Mean weight gain of control larvae after three days.
E = Mean weight gain of treated larvae at each tested concentration after three days.
S = Mean weight gain of starved control larvae after three days.

The EC_{50} dose which induced 50% starvation was calculated using probit analysis.

Data analysis

The data were tested for normality using the Shapiro–Wilk test and homogeneity of variance using the Levene test. Since the data were normally distributed with homogeneous variances, a significant treatment effect was determined using the one-way ANOVA followed by Duncan post hoc test at $P < 0.05$ using IBM SPSS statistics 20 software for windows and tables and the graphs were produced accordingly.

RESULTS AND DISCUSSION

Isolation of compounds by column chromatography

The fractionation of the ethanol extract of *H. indicus* root lead to the isolation of two compounds. NMR and IR studies of the isolated compounds revealed that the compounds were α-amyrin and lupeol.

HPLC analysis

From the HPLC-Chromatogram of the ethanol extract of *H. indicus* root, the peaks were seen at Rt-value 3.134 min and 3.42 min by using solvent system acetonitrile: water using gradient elution and the ratio of the solvents as given above. The peaks which could be identified from the graph were lupeol and α-amyrin respectively. A standard graph was drawn by taking the concentration of standard for each compound on the X-axis and peak area on Y-axis. From the graph, the concentration of compounds in the extract was calculated. From the calculations, it was found that α-amyrin was present at a concentration of 740 µg/g and lupeol at a concentration of 4mg/g (Fig 1).

Bioactivity-guided fractionation studies

Isolation of ethanol extract initially yielded two fractions, hexane fraction 275mg and ethyl acetate fraction 4g and residual aqueous fraction 3.65g (Fig 1). The ethyl acetate fraction was the active fraction among the three. There was 15, 25.69, 44.47 and 62.13% feeding deterrence at the doses corresponding to 1, 2, 4 and 6% ethanol extract. The FDI for ethanol extract was 15.54, 26.15, 47.64 and 65.3% respectively for the dose tested (Table1).

Further isolation of ethyl acetate fraction gave five fractions (Fractions 1-5). These fractions were concentrated and weighed and studied for antifeedant potential. Fraction 3 showed the highest antifeedant activity at the doses studied. There was a dose-dependent increase in feeding deterrent index (FDI) also. Fraction 3 gave FDI% of 13.96, 25.53, 49.15 and 61.66% respectively for the doses corresponding to 1, 2, 4 and 6% of ethanol extract (Table 2).

Table 1: Mean percentage feeding deterrent index of fourth instar larvae of *C. cephalonica* after 72 hours of feeding on Hexane, ethyl acetate and aqueous fractions of *H. indicus* root ethanol extract treated diet.

Hexane (Dose mg/10g rice)	Ethyl acetate (Dose mg/10g rice)	Aqueous (Dose mg/10g rice)	Ethanol (Dose %w/w) (Dose mg/10g rice)
2.60± 0.94[a] (acetone control)	2.60±0.94[a] (acetone control)	2.60±0.94[a] (acetone control)	2.60±0.94 a (acetone control)
7.91± 1.64[b] (2.75)	15.00±1.4[b] (40)	7.08±1.2[b] (36.55)	15.54±2.32 (1%) (100)
10.84±1.23[c] (5.5)	25.69±1.56[c] (80)	10.66±0.34[c] (73)	26.15±3.9 (2%) (200)
16.98±1.43[d] (11)	44.47±2.37[d] (160)	15.98±1.5[d] (146)	47.64±3.17 (4%) (400)
17.72±1.89[d] (16.5)	62.13±1.75[e] (240)	17.05±1.5[d] (219)	65.30±2.9 (6%) (600)

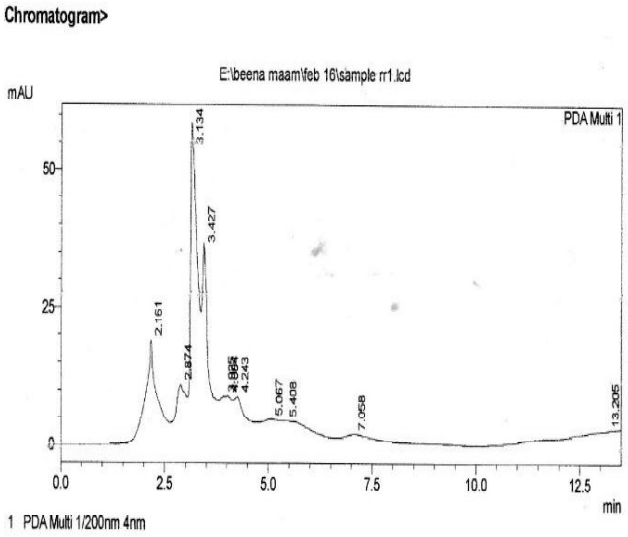
The result is expressed as mean ± SD followed by the same letter in each drug group do not differ significantly using Tukey's test.

Fraction 3 was further isolated to subfractions A and B. The subfraction B showed the highest level of activity. The FDI% for the most active subfraction (SubFrB) was 13.2, 24.16, 46.13 and 60.38% respectively (Table 3) for the doses corresponding to the dose used in ethanol extract.

Lupeol was more active than alpha-amyrin as an antifeedant. The bioactivity of the compound was not at par but was close matching with that of ethanol extract. The FDI% was 12.32, 22.68, 43.57 and 59.08% for the doses studied (Table 3). Percentage Starvation was calculated for each of the test group. The percentage of starvation for ethyl acetate, Fraction 3, subfraction B lupeol and amyrin were 80.69%, 78.74%, 74.55%, 72.58% and 3.9% respectively. Amyrin-lupeol combination showed 75.08% starvation and for ethanol fraction, it was 95.89%. The EC_{50} for lupeol, which is the major component responsible for the antifeedant activity, was calculated as 3.45% (Probit analysis). The combination of amyrin and lupeol showed a small increase in indices when compared to lupeol alone.

The antifeedant effect of the non-volatile fractions isolated showed that the fraction 3 of ethyl acetate fraction of ethanol extract was most effective in inducing feeding deterrence. Further fractionation of fraction 3 yielded two subfractions (A and B). The subfraction B was found to be most effective and further isolation and characterization led to the identification of two triterpene compound, lupeol and α-amyrin. The compound lupeol has also shown antifeedant potential against fourth instar larvae of *Corcyra cephalonica*, while α-amyrin was less effective.

Numerous secondary products are generated by various metabolic pathways of plants. These plant secondary metabolites such as polyphenols and steroids have gained utmost attention in recent years due to their diverse pharmacological potential and benefits rendered to different industries (Atanasov, 2015). These natural compounds have shown to be effective in agricultural pest management, as they function as antifeedant, growth inhibitors, toxic, repellent, fumigant, attractant *etc*. and also causes moulting disruption, respiratory inhibition, pheromone-based behavioural adaptations, oviposition deterrence and fecundity reduction against target pest populations (AlJabr *et al*., 2017; Koul, 2008; Nawaz *et al*., 2017). Majority of plant secondary metabolites are untapped and are of particular interest in insecticide development (Isman, 2006; Miresmailli and Isman, 2014).



Peak Table

PDA Ch1 200nm 4nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.161	390864	18819	15.917	11.791
2	2.874	185389	10708	7.549	6.709
3	3.134	666950	58259	27.159	36.501
4	3.427	432381	36443	17.607	22.833
5	3.925	84700	8545	3.449	5.354
6	4.004	89644	8573	3.650	5.371
7	4.243	189417	8442	7.713	5.289
8	5.067	142430	4299	5.800	2.693
9	5.408	200835	4000	8.178	2.506
10	7.058	67110	1461	2.733	0.915
11	13.205	5978	58	0.243	0.037
Total		2455699	159607	100.000	100.000

Fig 1: HPLC chromatogram of ethanol extract of *H. indicus* root.

The pharmacological activities of natural triterpenoids and their therapeutic potentials are well documented (Dzubak *et al*., 2005, 2006a, 2006b; Mahato *et al*., 1992; Shanmugam *et al*., 2012; Zhou *et al*., 2017). Triterpenes are part of the terpenoid family, an extensive group of natural products, which are abundant in the plant kingdom. Triterpenes are reported having anti-inflammatory activity. Similarly, these compounds are reported to be antioxidant (Fiorentino *et al*., 2007), antiparasitic (Danelli *et al*., 2009), antiviral (Kuo *et al*., 2009; Zhu *et al*., 2014), antifungal (Yuan *et al*., 2009), antibacterial (Yuan *et al*., 2009), antitumor,

Table 2: Mean percentage Feeding deterrent index of fourth instar larvae of *C. cephalonica* after 72 hours of feeding on fractions of ethyl acetate fractions of *H. indicus* root ethanol extract treated diet.

F1(Dose in mg /10g rice)	F2 (Dose in mg /10g rice)	F3 (Dose in mg /10g rice)	F4 (Dose in mg /10g rice)	F5 (Dose in mg /10g rice)
2.60±0.94^{a} (Acetone control)	2.60 ± 0.94^{a} (Acetone control)	2.60±0.94^{a} (Acetone control)	2.60±0.94^{a} (Acetone control)	2.60±0.94^{a} (Acetone control)
7.78±1.8^{b} (0.96)	10.92±2.21^{b} (2.2)	13.96±1.7^{b} (11.7)	6.81±2.28^{b} (0.83)	6.56±2.74^{b} (14)
10.55±1.24^{c} (0.192)	15.95±3.76^{b} (4.4)	25.53 ±4.07^{c} (22.34)	8.32 ±1.68b^{c} (1.66)	7.87±2.25^{b} (28)
16.57±1.73^{d} (0.384)	26.39±3.8^{c} (8.8)	49.15±1.06^{c} (44.68)	10.90±1.28^{c} (3.32)	8.72±1.18^{b} (56)
17.1±1.47^{d} (0.558)	31.31±4.15^{c} (3.2)	61.66±2.28^{e} (67.02)	16.77±1.58^{d} (4.98)	12.62±1.91^{c} (84)

The result is expressed as mean ± SD followed by the same letter in each drug group do not differ significantly using Tukey's test.

Table 3: Mean percentage Feeding deterrent index of fourth instar larvae of *C. cephalonica* after 72 hours of feeding on subfractions of fraction 3 of *H. indicus* root Lupoel, α-amyrin and ethanol extract treated diet.

SubFrA (Dose mg/10g)	SubFrB (Dose mg/10g)	Lupeol (Dose mg/10g)	α-amyrin (Dose mg/10g)	α-Amyrin + lupeol (Dosemg/10g)
2.60±0.94^{a} (Acetone control)	2.60±0.94^{a} (Acetone control)	2.60±0.94^{a} (Acetone control)	2.60±0.94^{a} (Acetone control)	2.60±0.94^{a} (Acetone control)
5.70±1.6^{b}(2.19)	13.20±1.86^{b}(63.4)	12.32±0.94^{b} (0.4)	4.45±1.1^{a} (0.074)	13.96±1.71^{b} (0.474)
8.90±1.72^{c}(4.38)	24.16±4.87c(126.8)	22.68±2.1^{c} (0.8)	7.52±1.4^{b} (0.148)	24.99±4.55^{c} (0.848)
10.75±1.61^{c}(8.76)	46.13±3.97^{d}(253.6)	43.57±2.62^{d} (1.6)	13.35±1.8^{c} (0.296)	47.87±5.38^{d} (1.896)
12.72±2.25^{d}(13.14)	60.38±4.31^{e}(380.4)	59.08±1.39^{e} (2.4)	19.35±2.9^{d} (0.444)	62.7±2.5^{e} (2.844)

The result is expressed as mean ± SD followed by the same letter in each drug group do not differ significantly using Tukey s test.

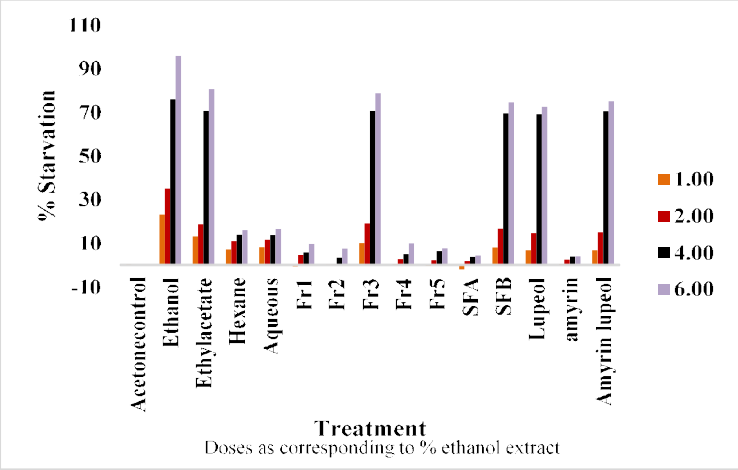


Fig 2: Mean percentage starvation of fourth instar larvae of *C. cephalonica* after 72 hours of feeding on extract, fractions, subfractions and compounds isolated from *H. indicus* root ethanol extract treated diet.

The result is expressed as mean ± SD followed by the same letter in each drug group do not differ significantly using Tukey's test.

anticarcinogenic (Chen *et al*., 2010; Gordaliza, 2010; Kuo *et al*., 2009), antidiabetic (Castellano *et al*., 2013; Nazaruk and Borzym-Kluczyk, 2015; Patil *et al*. 2011), antiulcerogenic (de Andrade *et al*., 2008), hepatoprotective (Li *et al*., 2017; H. Wu *et al*., 2016), neuroprotective (Koneri *et al*., 2014), analgesic (Nieto *et al*., 2013; C.-R. Wu *et al*., 2010) *etc*.

The antifeedant activity of ethanol extract of *Hemidesmus indicus* root on the fourth instar larvae of *Corcyra cephalonica* could be mainly due to the triterpenoid compound lupeol. Triterpenes have been reported to be active against the leaf miners *Ctenopseustis obliquana* (Walker) (Lepidoptera: Tortricidae) in feeding deterrence bioassays (Thoison *et al*., 2004). Insect antifeedant and phytotoxic effects of several pentacyclic triterpenes of plant origin on *Spodoptera littoralis*, *Leptinotarsa decemlineata* have been reported by (Caballero *et al*., 2001; Pavela, 2010).

Even though lupeol showed antifeedant effect, the ethanol extract was found to be the most effective when compared to fractions and compound isolated. This could be due to the synergistic effect of some other compound present in the extract along with lupeol. Most of the well-documented plant-derived insect antifeedants are triterpenoids. These are compounds have a 30-carbon skeleton and occurs mostly as glycosides and are often highly oxygenated. The limonoids from the neem (*Azadirachta indica* and chinaberry (*Melia azedarach*) trees, contains azadirachtin and toosendanin respectively and limonin from *Citrus* species are very well documented as insect antifeedants. Other antifeedants belonging to the triterpenoids include cardenolides, steroidal saponins and withanolides (Isman, 2002).

Thus along with the identified compound, one or more factors present in ethanol extract of *H. indicus* root are involved in the induction of feeding deterrence in the fourth instar larvae of *C. cephalonica.* Combinations of the two were also tested against the larvae. The results reveal that lupeol is the major bioactive component responsible for the antifeedant potential of the ethanol extract of *H. indicus* root.

From the chemical studies, it is evident that the volatile, as well as the non-volatile components of the *Hemidesmis indicus*, have contributed to the antifeedant effect of the plant against the rice pest *Corcyra cephalonica*. Since ethanol extract is more effective as a grain protectant than lupeol further ecotoxicological studies were carried out in the ethanol extract to ensure its safety.

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