Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
www.entomoljournal.com
JEZS 2022; 10(2): 200-205

Received: 10-01-2022
Accepted: 14-02-2022

Medhini
Etho-biomolecules Laboratory, Department of Microbiology & Biotechnology, JB Campus, Bangalore University, Bengaluru, Karnataka, India

Karthik V Rao
Etho-biomolecules Laboratory, Department of Microbiology & Biotechnology, JB Campus, Bangalore University, Bengaluru, Karnataka, India

Manjulakumari D
Etho-biomolecules Laboratory, Department of Microbiology & Biotechnology, JB Campus, Bangalore University, Bengaluru, Karnataka, India

Corresponding Author:
Manjulakumari D
Etho-biomolecules Laboratory, Department of Microbiology & Biotechnology, JB Campus, Bangalore University, Bengaluru, Karnataka, India

Ovicidal activity of factors from *Calendula officinalis* L. extracts against a generalist herbivore, *Spodoptera litura* Fab. (Lepidoptera: Noctuidae)

Medhini, Karthik V Rao and Manjulakumari D

DOI: https://doi.org/10.22271/j.ento.2022.v10.i2c.8989

Abstract
The leaf and flower extracts of the *Calendula officinalis* prepared using various solvents of varying concentrations served as test solutions for bioassays against the eggs of *Spodoptera litura* F. The promising results of reduced egg hatchability (98.02%) at a 5% concentration of chloroform leaf extract during our preliminary bioassays with topical application to the eggs facilitated further fractionation of extract with different combinations of solvents that yielded eleven fractions. Following this was the conduction of bioassays with each of these fractions (0.1% concentration) against eggs of different age groups (freshly laid, 1-day and 2-day old), which revealed fractions F-2 and F-9 to show poor egg hatchability (14.6±8.71%, 21.3±8.78%; 31.67±6.03%, 45.3±1.78% and 38.33±3.58%, 45.3±1.78%) respectively among all age groups consistently followed by other fractions and controls. The fractions were subjected to UV, 1HNMR, 13CNMR, IR and EI Mass spectroscopy and their spectral data confirmed the probable compounds to be α-antiarin and Oryzanol-A in the fractions F-2 and F-9 respectively. Incidentally, both being known ones procured from other plant sources, this happens to be the first report not only to detect their presence in Calendula but also to report their ovicidal activity against *Spodoptera litura.* The results from the present investigation clearly advocate that the active fractions or the isolated compounds could probably be used to develop a novel pesticidal formulation to control economically important agricultural pests.

Keywords: Calendula, oviposition, egg hatchability, chloroform extracts

1. Introduction
Plant volatiles is associated with insect orientation and ultimate recognition of host plants for feeding, mating and oviposition in many phytophagous insects [Pare *et al*., 1999] [17]. Similarly, non-host plants act as deterrents and prevent them from feeding and oviposition. These non-nutritional molecules, produced by the plants modify the behavior or the biology of insects through diverse modes of action. They can express several properties such as growth retardation, feeding deterrence, digestive enzyme and substrate inhibition, oviposition deterrence, reduced reproductive capacity, etc. These secondary plant products, broadly classified as alkaloids, polyphenolics, terpenes, isoprenoids, or cyanogenic glucosides, have been used in pest management for a long time [Harborne 1973] [11]. The most promising botanical groups to source these allelochemicals are Meliaceae, Rutaceae, Asteraceae, Annonaceae, Labitae, Aristolochiaceae and Malvaceae. The members of Asteraceae are more pronounced as they are known to possess characteristic secondary metabolites with distinct anti-herbivore effects [Hassan 2010] [12].
In the present investigation, a common garden plant Pot marigold, *Calendula officinalis* L. (Asteraceae) was evaluated for its impact on the egg hatchability of a lepidopteran pest, *Spodoptera litura* Fab. *Calendula officinalis* well known for its ornamental and medicinal purposes has also been given a lot of credit as a pest deterrent. Intercropping with marigold, a traditional agricultural practice has, time and again, proved the plant to be the most effective pest control agent. Earlier work on cabbage aphid *Brevicoryne brassicae* L., flea beetles, *Phyllotreta*, Small White *Pieris rapae* L., Large White *P. brassicae* L., cabbage moth *Mamestra brassicae* L., diamondback moth *Plutella xylostella* L. and *Liriomyza helianthi* Spencer [Dimock 1991, Claudio *et al*. 1998] [5, 4] has demonstrated the larvicidal, ovicidal, repellent action, and oviposition deterrent effects of this plant extracts.

Spodoptera litura Fabricus, is one of the most important polyphagous insect pests of agricultural crops, of the late, occupying new habitats and substituting other pest species across the globe [Regnault Roger, 1997] [20]. It is known to infest more than 120 species of plants belonging to 44 families, of which about 40 species are reported from India alone [(Chari & Patel, 1983] [3]. Application of broad-spectrum insecticides at high dosages and repeated use of the same active ingredients eventually resulted in *S. litura* developing resistance to all classes of insecticides *viz*., organochlorines, organophosphate and synthetic pyrethroid [Prakash, 1997] [19]. As *C. officinalis* has been shown to have a larvicidal effect on some of the insects, it would be worthwhile investigating the chemical constituents of this plant and their effect on *S. litura* to negate the pesticide resistance.

2. Materials and Methods

2.1 Laboratory mass culture of *Spodoptera litura*.

Spodoptera litura larvae were reared in the laboratory on an artificial diet modified [D], until pupation and adult emergence. The mass culture and the experiments were carried out at 28±2 ºC, 70±5% relative humidity, with a 12:12 L: D cycle.

2.2 Preparation of extracts

The extracts of shade dried leaves and flowers (500 g each) of *C. Officinalis* in petroleum ether, benzene, chloroform, methanol and water in succession in increasing order of their polarity were prepared using the Soxhlet apparatus [Martin Rathi *et al*., 2006] [14]. The crude extract was dissolved in a minimum quantity of the respective solvent to obtain stock. Dilutions of 0.5%-5% from the store were prepared in acetone after completely evaporating the respective residual solvent and emulsified with a few drops of sandovit.

2.3 Ovicidal activity by dip method

To assess the ovicidal activity, the freshly laid egg mass was collected. A fine brush was used to carefully remove the scales from the egg masses. In each of the aforesaid extracts and the controls, 50 eggs were dipped for five seconds, air-dried, placed in petri plates lined with moist filter paper and observed for egg hatchability in due course. The percentage of ovicidal activity was calculated using the formula (No. of eggs hatched/No. of eggs laid) x 100.

2.4 Fractionation of chloroform leaf extract

The chloroform leaf extract was subjected to fractionation on TLC (precoated Merck®Silica gel 60 F254 chromatographic plates of 1 mm thickness) using several solvent systems individually as well as in combination till very good separation into bands was achieved. The solvent systems such as hexane, petroleum ether, chloroform, acetone, methanol, chloroform: petroleum ether (4:1), benzene: acetone (9:1), chloroform: methanol (99:1), benzene: methanol (9:1), and benzene: ether (2:3) were used. The best suitable solvent system which gave distinct separation was chosen for collect the ion of the active fraction and scale-up.

2.5 Preparative Thin Layer Chromatography

A 10 mm thickness of silica gel on a 20 X 20 cm glass plate was used for preparative TLC. The chloroform leaf extract (10 ml) was applied as a streak with a capillary tube onto the plate and allowed to dry. This plate was placed in the TLC chamber, which was saturated for two hours with Benzene: acetone: ethyl acetate: methanol: water in the ratio of 4:1:1:1:2 that served as a mobile phase. The plate was run till the bands got separated distinctly. Thick and darker bands were marked and scraped off the plate and silica gel. Several plates were developed in the same manner to harvest more significant quantities. Each band was dissolved in chloroform and methanol and centrifuged at 3000 rpm for 10 minutes, later the supernatant was decanted and dried at room temperature. The residues thus collected were dissolved in acetone to get a 0.1% concentration and used for ovicidal bioassays against freshly laid, 1-day old and 2-day old eggs of *S. litura*. The ovicidal bioassays, as well as the calculations, were conducted as described in the section 2.3.

2.6 Statistical Analysis

Data of the three trials were expressed as the Mean values with Standard Deviation (SD) for each measurement. The data were also analyzed by one-way analysis of variance (one-way ANOVA). Tukey's Multiple Range Test was used to determination of the significance of the difference ($p< 0.05$). Analysis was performed with SPSS 11.0 (SPSS, Inc., Chicago, IL).

3. Results and Discussion

3.1 Ovicidal activity of the crude extracts

Among different concentrations tested, a statistically significant reduction in the egg hatchability was recorded in 5% concentration chloroform leaf extract ($p\leq0.05$) and petroleum ether flower extract. Considerable decrease in hatchability was observed with the increase in the concentration, as shown in Fig 1.

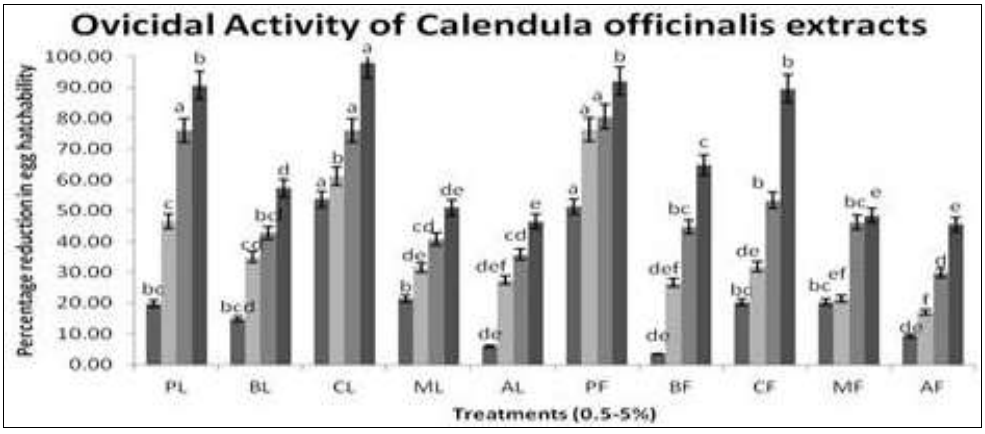


Fig 1: Ovicidal activities of C.officinalis leaf and flower extracts (0.5-5%)

All the measurements were expressed as Mean ± SD (n = 50). The ovicidal activity was expressed as a percentage reduction in egg hatchability. The bars without common letters differ significantly ($p \leq 0.05$).

Ovicidal activity correlated with poor hatchability of eggs has shown in *Earias vittella* F., *Dysdercus koenigii* F. and *Helicoverpa armigera* (Hubner) [Gurusubramanian and Krishna (1996)] [10], *S. litura* [Packiam *et al.*, (2005)], *Caphalocrocis medinalis* [Prabal Saikia, 2002] [18] and *L.orbonalis* [Yashoda and Natarajan, 2007] [26] as a result of exposure to volatile compounds of plants. The volatile components of plant extracts mainly constitute alcohols, aldehydes and terpenoids, especially monoterpenoids that exhibit toxic activity on insects diffuse into the eggs through the micropile [Keane and Ryan, 1999; Enan, 2001] [13, 8] and bring about several alterations in the vital physiological and biochemical processes such as inhibition of the critical enzymes involved in the embryonic development, *anti-juvenile* hormone activity [Abo-el-Ghar, *et al.*, 1996] [1] leading to incomplete blastokinesis and abnormal breakage of extra embryonic membranes in the embryo [Enslee and Riddiford (1977)] [9]. However, these alterations also depends upon the concentration of the extract, degree of diffusion, [(Papachristos and Stamopoulos, 2004] [16] and distribution of the phytoconstituents (through the egg chorion) to different parts of eggs etc [Enslee and Riddiford 1977] [9]. Respiratory appendages spread across the eggshell facilitate gas exchange across the eggshell supplying oxygen to the developing embryo. The waxy layer imparted by the lipophilic components of the extracts might block these appendages thereby causing respiratory distress to the developing embryo [Papachristos and Stamopoulos, 2004] [16] In addition to this, under development or cessation of the embryogenesis may be an outcome of acute toxic effects of triterpenoids, saponins, glycosides and other components present in the extracts [Packiam *et al.*, (2012] [25].

3.2 Fractionation of chloroform crude extract

Chloroform leaf extract subjected to fractionation on TLC yielded three bands with chloroform solvent system but the resolution was very low. Combination of Benzene and Acetone (9:1) improved the separation with five bands. Further modification of this solvent system with a combination of benzene: acetone: ethyl acetate: methanol: water (4:1:1:1:2) yielded 11 bands with good resolution.

Fig 2: TLC of Chloroform leaf crude extract of *Calendula officinalis* showing poorly separated bands in Benzene: Acetone (9:1)

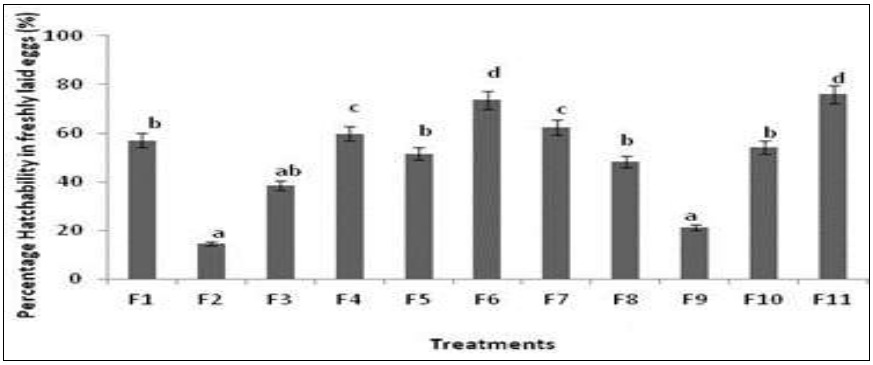
3.3 Ovicidal activity of the fractions

Egg hatchability decreased with all the eleven fractions tested compared to controls (97.1%) in freshly laid eggs (0-day old). Poor hatchability was observed with the fractions F2 (14.6±8.71% i.e., 85.4% reduction in egg hatchability), and F9 (21.3±8.78% i.e., 78.7% reduction in egg hatchability) while the minimum reduction was seen with F6 (26.4%) and F11 (24.1%) as represented in Fig 3a.

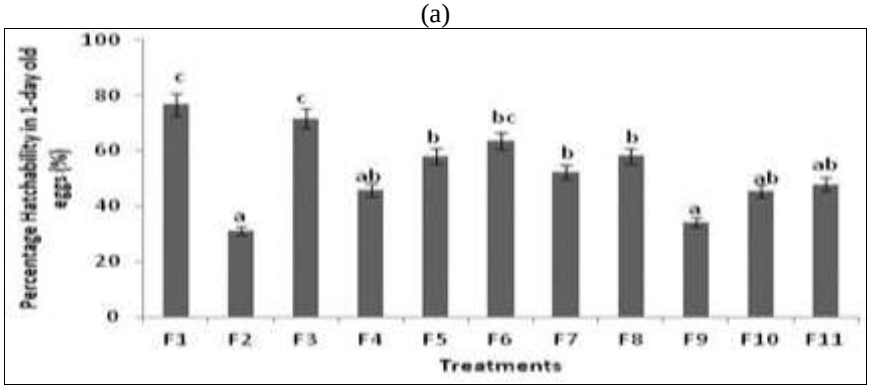
When 1-day-old eggs were exposed to different fractions, poor hatchability was observed with the F2 (31.67±6.03%), F9 (45.3±1.78%), F10 (34.2±9.1%), and F11 (47.9±4.54%) whereas higher hatchability was observed in F1 (76.67±5.55%) and F3 (71.67±6.03%). However, the activity of all the fractions was significant upon comparison with that of the controls (Fig 3b).

Upon exposure of 2-day-old eggs to the fractions, hatchability was not that affected in most of the fractions but for the fractions F2 (38.33±3.58%) and F9 (45.3±1.78%). The hatchability was more or less the same among most of the remaining fractions that are more or less comparable to the controls (Fig 3c).

Fractions F2 and F9 reduced the egg hatchability significantly in all age groups unlike other fractions. A similar reduction in egg hatchability upon exposure to different plant extracts in eggs of different age groups was observed by Elango *et al*., (2010) [7] against *Anopheles subpictus*, Shadia *et al*., (2007) [23] against *Spodoptera littoralis*, and Roni *et al*., (2013) [21] against *Anopheles stephensi* Liston. Age of the embryos plays a crucial role in egg hatchability upon treatment and the efficient penetration of allelochemicals. The failure of egg hatchability when freshly laid eggs were directly exposed to the fraction indicated more penetration of the chemical inside the eggshell, which affected embryogenesis (Broadbent *et al.*, 1984). These results are also comparable to alkaloids of *A. squamosa* [Saxena *et al*., 1993)] [22], ethyl acetate fractions (seeds) of *Calophyllum inophyllum*, petroleum ether fractions of *Rhinacanthus nasutus* and ethyl acetate fractions of *S. suratense* [Pushpalatha and Muthukrishnan, 2001] [6] against *A. stephensi* Liston.



(a)



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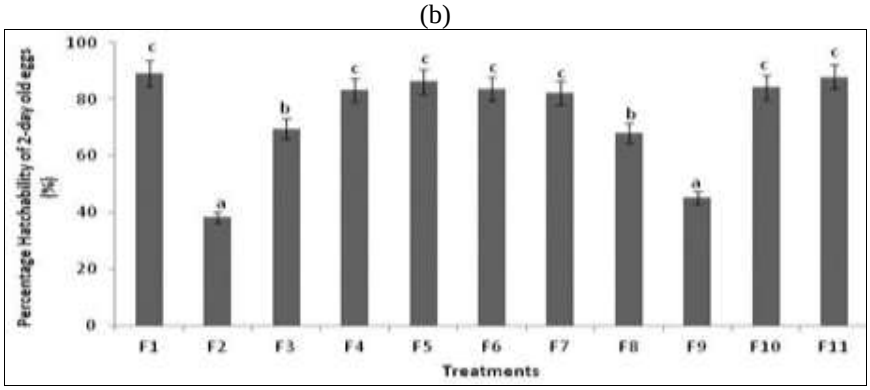

(c)

Fig 3: Egg hatchability of eggs (a. freshly laid, b. 1-day old, c. 2-day old) upon treatment with TLC fractions

With the advancement of the age of the eggs (0-day old, 1-day old, 2-day old) an increased hatchability was seen with all the fractions tested indicating the freshly laid (0-day-old) and 1-day-old eggs were more sensitive compared to the older eggs. Fractions F2 and F9 showed poor hatchability in all age groups of eggs.

3.4 Spectral analyses of the active fractions

The probable structure of isolated compounds was determined by physical and spectral analyses (UV, FT-IR, LC-MS). The spectral data of both these active fractions agreed with those in the literature.

The compound in fraction 2 showed maximum absorbance at 217 nm under UV with IR ν_{max}^{KBR} (cm^{-1}) 3397, 1610, 1106. LC-MS analysis revealed the mass peak at m/e 565 [M+] with RT of 0.759 indicating the molecular weight of the probable compound to be 566. When compared with that of the databases like Metlin and Mass bank database, it was observed that the probable compound could be alpha-antiarin (Fig 4). In general, this cardiotonic glycoside compound is a potent toxin previously isolated from *Antiaris toxicaria* and is known to possess ATPase inhibitory properties. However, no known reports about its pesticidal properties are available.

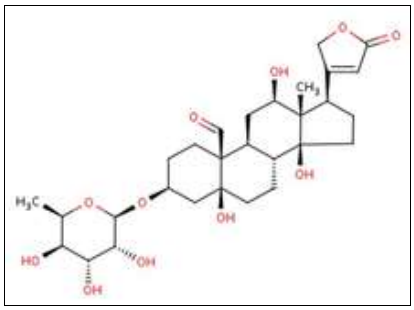

Fig 4: Alpha-antiarin

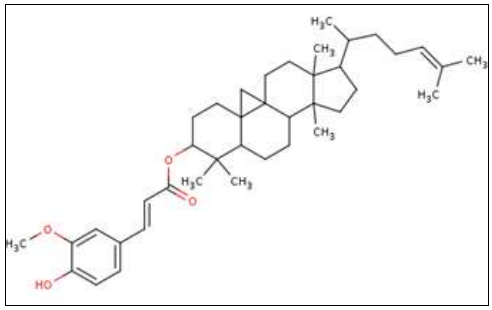

Fig 5: Oryzanol-A

The compound in fraction 9 showed maximum absorbance at 216 nm under UV with IR $_{vmax}^{KBR}$ (cm-1) 3413, 816-789. LC-MS analysis revealed the mass peak at m/e 603 [M+] with RT of 0.638 indicating the molecular weight of the probable compound to be 496. When compared with that of the databases like Metlin and Mass bank database it was observed that the probable compound could be Oryzanol-A, a steroidal aromatic compound (Fig 5). Oryzanol-A a derivative of Tocopherol has been isolated majorly from rice bran oil than any other sources. Though, no known reports about the feeding deterrent activity of Oryzanol-A per se are available so far, the insect feeding deterrent activity of its parent compound tocopherol is well established [Mohammed, *et al*., 1997].

4. Conclusion

In the current study, we investigated the potential uses of leaf and flower extracts from *Calendula officinalis* L., which is the first attempt of this kind against a phytophagous lepidopteran pest. Results obtained from ovicidal bioassays demonstrate that the chloroform leaf extract of this plant has strong ovicidal activities against the pest. Furthermore, the fractions of this extract also showed a marked reduction in egg hatchability upon treatment. In conclusion, these groups of phytochemicals may have either individual effects or cumulative effects on various activities against *S. litura* and provide insights to develop a new product to control this notorious pest.

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