Isolation of de-oiled flower extract of *Swertia densifolia* and study of its bioactivity towards *Apis florea*

Pradnya Deshpande[1] and Dattatraya Naik[2]

[1]*Assistant Professor, School of Chemistry, MIT-WPU, Maharashtra, India*
[2]Project Coordinator, *Biodiversity Department, Abasaheb Garware College, Maharashtra, India*

ABSTRACT

Flower extract of Swertia densifolia (Griseb.) (Gentianaceae) is known to show dose dependent attractant and repellent properties towards Apis cerana indica F. and A. florea. The essential oil of these flowers has been shown to possess repellent properties towards A. florea. As the next logical step in this programme, we now report screening results of de-oiled flowers extract of S. densifolia on A. florea. The extract showed dose dependent attractant properties at lower concentrations and repellent properties at higher concentrations, a behaviour similar to an attractant pheromone. TLC and HPLC analyses of the extract indicated the presence of six constituents.

Keyword: - Swertia densifolia, honey bees, Apis florea, bee attractants, bee repellents, round table bioassay, de-oiled flowers

1. INTRODUCTION

In our pursuit for honey bee attractants, the superiority of plant based lures has been shown. In this programme, the chemical constituents of Nasonov gland pheromone of the Indian honey bee *Apis cerana indica* was investigated (Naik et al., 1988) and attractant formulations were developed (Naik et al., 1989). Lures to attract the other Indian honey bee, *A. florea* were also eventually developed (Naik et al., 2001), but the attractant properties of both of these lures were limited to a very narrow range of concentrations. For this reason, alternate attractant formulations were researched.

An extract of Fagara budrunga (Rutaceae) was shown to attract *A. cerana* over a much wider range of concentrations (Naik et al., 2003). Application of lemon grass Cymbopogon citratus (Poaceae) extract in attracting A. mellifera (Malerbo-Souza et al., 2004) supported our findings. Observations by Reinhard et al. (2004 a, b) demonstrated that floral scents induced recall of navigational memories in honey bees and indicated a strong possibility of getting an attractant formulation from extracts of plants frequently visited by honey bees. Earlier we have reported the properties of a leaf extract of the Indian medicinal plant Swertia densifolia (Griseb.) (Gentianaceae). Crude ethanolic extract of leaves was shown to possess dose dependent attractant and repellent properties towards *A. cerana indica* (Naik et al., 2005) and *A. florea* (Naik et al.,2007). Subsequently the essential oil from these leaves was isolated and studied. Its major constituents were procured and their formulations were screened. It was shown that the essential oil as well as the major constituents possessed repellent properties towards *A. cerana indica* (Naik et al., 2008). We have also evaluated and reported the properties of the ethanolic extract of de-oiled leaves that is leaves recovered after separation of the essential oil, on *A. cerana indica* (Naik et al., 2010).

As a part of this ongoing programme, the essential oil from these flowers was isolated and studied. Its major constituents were procured and their formulations were screened. It was shown that the essential oil as well as the major constituents possessed repellent properties towards *A. florea* (Naik et al., 2015). Also the properties of a flower extract of the plant *Swertia densifolia* (Griseb.) (Gentianaceae) were evaluated. Like the crude ethanolic extract of leaves, the crude ethanolic extract of flowers was shown to possess dose dependent attractant and repellent properties towards *A. florea* (Deshpande et al., 2019). These observations prompted us to evaluate properties of the ethanolic extract of de-oiled flowers that is flowers recovered after separation of the essential oil, on *A. florea*.

2. MATERIALS AND METHODS

2.1 Honey bees *A. florea*

Two colonies of *A. florea* were maintained at the experimental apiary at the Agharkar Research Institute (ARI), Pune, were used for the bioassay.

2.2 Plant material

Whole plant *S. densifolia* (5 kg) was collected from three locations: (i) near 'Table Land', Wai (17° 57'N, 73° 53'E; 1371 m above msl), (ii) near 'Venna lake', Mahabaleshwar (17° 56'N, 73° 39'E; 1439 m above msl) and (iii) near 'Arthur Seat', Mahabaleshwar (17° 58'N, 73° 38'E; 1340 m above msl) from Mahabaleshwar region, Western Maharashtra, India and taxonomically identified by the Botany Group of Agharkar Research institute (ARI, Voucher No. WP - 115).

Flowers of *S. densifolia* were separated from the plant. 125 g of flowers were washed with distilled water and cut into small pieces. Hydrodistillation of the material was carried out using a Clevenger apparatus to separate the essential oil. The de-oiled flowers were filtered, shade dried and powdered. 80 g of powdered deoiled flowers were extracted with 1 liter of ethyl alcohol in a Soxhlet extraction apparatus for 20 h. The ethanolic solution obtained was then concentrated in vacuuo to yield the crude ethanolic de-oiled extract.

2.3 Characterization of the crude ethanolic flower de-oiled extract

The extract was characterized by recording its Thin Layer Chromatogram (TLC) on a pre coated TLC plate (10 x 6.5 cm) of 60 F 254 (Merck 1.0554.0007). The optimum resolution was obtained using the solvent system ethyl acetate: hexane (2: 8 by volume).

High Performance Liquid Chromatography (HPLC) was run on a ZORBAX, Eclipse, XDB – C8, 4.6 mm x 150 mm, 5 µm column using Agilent 1100 high performance pump and Agilent 1100 variable wavelength UV detector (254 nm) using methanol : water (80 : 20, by volume) at a flow rate of 1 ml / min.

3. BIOASSAY OF THE EXTRACT

Twelve test formulations of increasing concentrations were prepared by mixing the de-oiled flower extract (5 – 165 mg / ml) with liquid paraffin. Formulation for the bioassay was selected randomly. Bioassay of each of the test formulations was carried out in the campus of ARI using the rotating table bioassay (Naik et al., 2007).

A set of 10 observations was recorded for each formulation. The mean difference (Δ) between the number of bees visiting the test formulation and that visiting the control recorded for each concentration was estimated as a measure of attractant or repellent nature of the test formulation. The data generated were graphically analyzed in Microsoft Excel 2010.

3.1 Statistical analyses

All statistical analyses were carried out using SPSS version 11.0. Mann Whitney Test and Kruskal Wallis Test were used to determine the significance of the observations.

4. RESULTS

Soxhlet extraction of de-oiled flowers yielded the extract as a dark green, viscous liquid (15 g, 19%). Examination of the extract by TLC showed presence of six distinct spots. The R_f values were 0.67, 0.54, 0.43, 0.30, 0.21 and 0.10. The HPLC analysis indicated six signals indicating the presence of six components in the de-oiled extract.

4.1 Results of Bioassay

Twelve test formulations of increasing concentrations were prepared by mixing the ethanolic extract of de-oiled flower (5 – 165 mg / ml) with liquid paraffin. Formulation for the bioassay was selected randomly. Bioassay of each of the test formulations was carried out in the campus of ARI using the round table bioassay.

The average number of honey bees visiting the control and test dishes during 5 minutes was determined. The mean difference (Δ ± std. dev.) between the number of bees visiting the test formulation and that visiting control recorded for each concentration was calculated (Table 1).

Table – 1: Average number of honeybees visiting the formulations of the ethanolic extract of de-oiled flower and that visiting control at different concentrations.

Sr. No.	Conc. (mg / ml of liq. paraffin)	Average no. of bees visiting test formulations*(a)	Average no. of bees visiting control*(b)	Mean difference* (Δ) (a – b)	Standard deviation
1	0	4.8	4.4	0.4	± 0.52
2	5	12.7	6.6	6.1	± 1.10
3	10	17.9	6.1	11.8	± 1.32
4	25	24.1	4.3	19.8	± 2.62
5	37	24.3	9.5	14.8	± 1.55
6	50	20.6	7.5	13.1	± 0.32
7	75	17.5	7.4	10.1	± 1.10
8	87	13.9	17.2	-3.3	± 0.82
9	100	14.2	20.5	- 6.3	± 0.95
10	125	17.5	22.2	- 4.7	± 0.95
11	140	13.3	17.5	- 3.6	± 0.70
12	150	12.9	16.1	-3.2	± 0.63
13	165	16.7	19.9	-3.2	± 0.79

*Average of 10 observations.
a) Average number of bees visiting the test formulation counted during a period of 5 min (n=10),
b) Average number of bees visiting control counted during a period of 5 min (n=10).

Analyses by Mann Whitney Test yielded $p < 0.01$ and analyses using Kruskal Wallis Test yielded $\chi 2 = 84.262$, $\rho = 0.0001$, indicating that observations were significant. The plot of the difference (Δ ± std. dev.) recorded for each of the formulations against the concentration of the test formulations is shown in Figure 1. The standard deviation computed for each observation (Table 1) is also presented in figure 1.

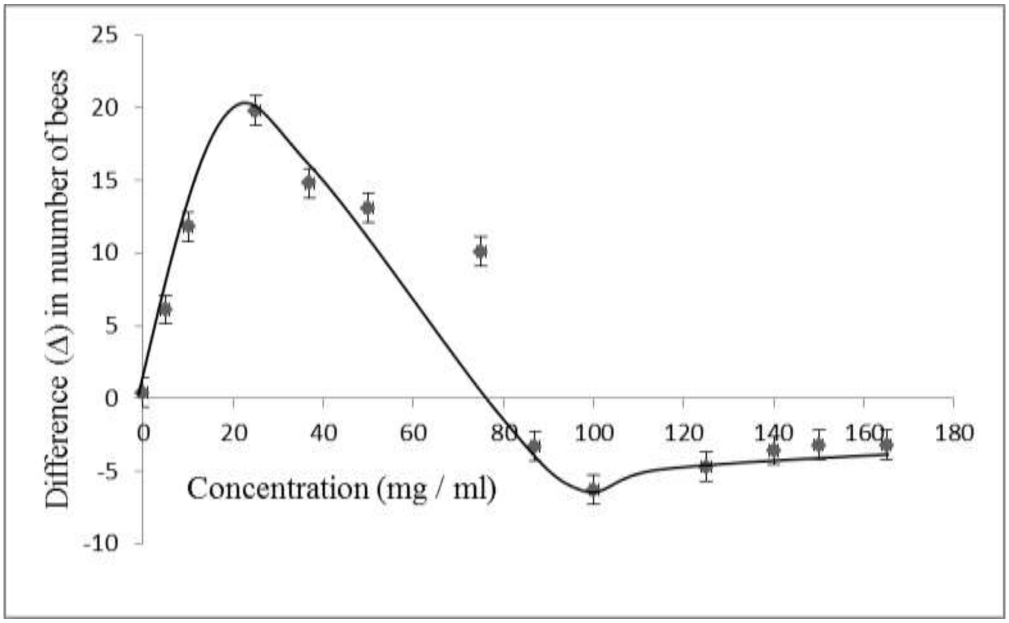

Figure – 1: Graph of the difference (Δ ± std. dev.) between the average number of bees visiting the dish containing the test formulation of de-oiled flower extract and that visiting liquid paraffin control. Standard deviation is shown at each point.

Examination of Table 1 and Figure 1 clearly brings out the properties of the test formulations. It is seen that there is almost no difference in the average number of honeybees visiting both the dishes when only liquid paraffin is kept in them (Sr. No.1, Table 1) indicating the inert nature of liquid paraffin towards honeybees. The results of screening of formulations of lower concentrations (Sr. Nos.2-7, Table 1) show that number of honeybees visiting the dish containing the test formulation was more than that visiting the dish containing the liquid paraffin (control). This indicated attractant property of the test formulation since the number of honeybees visiting the test formulation was more than that visiting the control. The dose dependent nature of the attractant property is clearly shown in Figure 1. It is further seen that the formulations of concentrations higher than 75 mg/ ml of liquid paraffin (Sr. No.8-13, Table 1) show the exactly opposite behavior. The number of honeybees visiting the dish containing the liquid paraffin (control) was more than that visiting the dish containing the test formulation. This demonstrated repellent property since the number of honeybees visiting the control was more than that visiting the test formulation. Further examination of Figure 1 shows the variation in the repellent nature of the test formulation with concentration. Thus the dose dependent nature of the attractant / repellent properties is clearly demonstrated.

Examination of Figure 1 indicates that the attractant nature of the test formulations of lower concentration gradually changes with the concentration and eventually the formulations of higher concentrations become repellent. The graph crosses X-axis at the concentration 78 mg of extract / ml of liquid paraffin. To ascertain this, the formulation of the concentration 78 mg of extract / ml of liquid paraffin were made and its bioassay was carried out. In complete agreement with the graph, the screening results indicated no attractant or repellent nature of this formulation, a behavior similar to liquid paraffin.

5. DISCUSSION

The results show that the yield of ethanol extract of de-oiled flowers was 19 %. This was much less than the crude extract obtained from flowers before hydrodistillation (32.5%).

Results of the bioassay of formulations prepared from extracts of fresh leaves, flowers of *S. densifolia* are already reported (Naik et.al., 2005, 2007 and Deshpande et. al. 2019). Examination of the observations shows that the crude extract of fresh flowers exhibited dose dependent repellent and attractant properties towards honeybees *A. florea*. Maximum repellency was found for the formulation of concentration 50 mg / ml. In due course, the essential oil from the flowers was removed and the response of honeybees to its formulation was recorded. The formulations of essential oil exhibited repellent properties at all the concentrations (*Naik et. al.* 2015). The flowers left after removal of essential oil, referred as 'de-oiled flowers', were then extracted and response of honeybees to the formulations of de-oiled flower extract was studied. These formulations also showed dose dependent repellent and attractant properties. It is interesting to note that the maximum repellent properties were found for the formulation of concentration 100 mg / ml, more than the corresponding formulation of extract of fresh flowers. This indicated the absence of constituents possessing repellent properties removed in the essential oil. Further, the attractant properties also reflected the absence of essential oil. The maximum attractant property was found for the formulation of concentration 100 mg / ml for the extract of fresh flowers while the concentration of the corresponding extract from the de-oiled flowers was found to be 25 mg / ml. Similar trend was also observed for the extracts of leaves of the plant *S. densifolia*.

At the same time concentration of formulations showing maximum attractant properties seems to get reduced due to the removal of essential oil from the plant material

Our earlier results indicate that the crude ethanolic extracts of leaves and flowers of *S.densifolia* shows dose dependent repellent / attractant properties towards *A. florea*. Our present observations are seen to follow an opposite trend.

The dose dependent repellent and attractant properties of ethanolic flower extract of *S. densifolia* were due to the presence of compounds having attractant as well as repellent properties. The essential oil isolated from these flowers possessed only repellency, implying that constituents having repellent properties were isolated in the essential oil. It was, therefore, envisaged that extract of de-oiled flowers might contain constituents having attractant properties. Our present observations confirm this hypothesis. The pattern of activity observed is very similar to that seen in bioassay of pheromone constituents (Naik et al., 1989).

6. CONCLUSION

Availability of attractant formulations can help beekeepers to direct honey bees to visit plants of interest, thereby improving pollination efficiency. Our field trials have shown applications of lures developed earlier to improve seed yield in sunflower. Our results may help beekeepers to select appropriate formulations of *S. densifolia* de-oiled flowers to be employed for attracting or repelling honeybees.

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8. REFERENCES

[1] Malerbo-Souza, D T; Nogueira-Couto, R H; Couto, L A (2004) Honey bee attractants and pollination in sweet orange, Citrus sinensis (L) Osbeck, var. pera-rio. Journal of Venomous Animals and Toxins Including Tropical Diseases 10(2): 144-153.

[2] Naik, D G; Gadre, R V; Kapadi, A H; Singh, M K; Suryanarayana, M C; Kshirsagar, K K (1988) Nasonov gland pheromone of the Indian honey bee, Apis cerana indica F. Journal of Apicultural Research 27(4): 205–206.

[3] Naik, D. G.; Kapadi, A. H.; Singh, M. K.; Suryanarayana, M. C.; Kshirsagar, K. K. (1989). Lure development for Indian hive bees. Indian Bee Journal 51(2): 47–50.

[4] Naik, D G; Kapadi, A H; Kshirsagar, K K (2001) Developing a honey bee attractant useful to attract Indian honey bees Apis florea. Indian Patent No.185391, sealed on 14 September 2001.

[5] Naik, D G; Banhatti, P; Chawda, S S; Thomas, D (2003) Fagara budrunga fruit extract as an attractant for Apis cerana. Journal of Apicultural Research 42(1-2): 49-50.

[6] Naik, D. G.; Dandge, C. N.; Puntambekar, H. M.; Patil, T (2005) Attractant and repellent properties of *Swertia densifolia* leaf extract towards *Apis cerana*indica. Journal of Apicultural Research 44 (3): 116–118.

[7] Naik, D. G.; Dandge, C. N.; Puntambekar, H. M. (2007) *Swertia densifolia* leaf extract as a dose-dependant attractant or repellent for *Apis florea*. Journal of Apicultural Research 46 (1): 15–18.

[8] Naik, D. G.; Dandge, C. N.; Puntambekar, H. M.; Deshpande, P. V. (2008). Chemical examination of *Swertia densifolia* leaf essential oil and its repellent activity towards *Apis cerana indica* F. Journal of Apicultural Research and Bee World 47(1): 63-67.

[9] Naik, D. G., C. Dandge, and P. Deshpande. 2010. 'Pheromone-like' properties of de-oiled leaf extract of Swertia densifolia towards Apis cerana indica F. J. Apic. Res. 49: 149–153.

[10] Naik, D. G. ; Vaidya-Kannur, H.; Deshpande, P. V.; Dandge, C. N.; Reddy, G. V. P. (2015). Potential Use of an Essential Oil From the Flower of *Swertia densifolia* as a Repellent for *Apis florea* (Hymenoptera: Apidae). Annals of Entomological Society of America 108(1): 18-25.

[11] Pradnya Deshpande, Dattatraya Naik **. "**Attractant and repellent nature of ethanolic extract of *Swertia densifolia* flowers towards *Apis florae*". International Journal of Social Sciences & Management Studies (Peer Reviewed & Refereed Research Journal) I.J.S.S.M.S. Vol. 5 (1), pp. 206-211: (2019). ISSN : 2454 – 4655.

[12] Reinhard, J; Srinivasan, M V; Guez, D; Zhang, S W (2004a) Floral scents induce recall of navigational and visual memories in honey bees. Journal of Experimental Biology 207: 4371–4381.

[13] Reinhard, J; Srinivasan, M V; Zhang, S W (2004b) Scent triggered navigation in honey bees. Nature 427: 411.