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CONTACT BIOASSAY OF AN ENDEMIC PLANT TO ETHIOPIA ON THREE APHID SPECIES

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ABSTRACT: *Millettia ferruginea* (Hochst.) Baker (Abaca: Papilionoideae) is endemic to Ethiopia and is a multipurpose tree which is known locally as *birbira.* Although it was confirmed effective particularly on the bean bruchids, *Chalosobruchus chinensis* L., its potential as a botanical for the control of insect pests has received little attention. Different workers had done extraction and characterization of the allelochemicals present in *birbira.* However, none looked into the possible usages of what they extracted such as insecticidal potency. In order to achieve applied meaning and significance, natural product chemistry must incorporate bioassays. Based on this, extracts from *birbira* with water or chloroform were assayed on three aphid species. Substance extracted both by water and chloroform caused significantly higher mortality on the three aphid species than the residue in chloroform and the untreated control. The LC_{50} amounts of water and chloroform extracts, for the respective aphid species, were verified under field conditions and similar toxicity was observed for the pea aphid and the brassica aphid whereas reduced mortality was noted for the barley aphid, which was apparently due to the leaf rolling habit of the aphid, which prevented contact between the aphids in the enrolled leaves and the extracts. Therefore, it can be concluded that *birbira* is an effective aphicide with contact activity and hence could be developed for practical use by small scale farmers.

Key words/phrases: Allelochemical; *Birbira*; Bioassay; Chloroform; Extracts; Residue; Water.

INTRODUCTION

Millettia ferruginea (Hochst.) Baker (Fabaceae: Papilionoideae) is endemic to Ethiopia and its natural habitat is rather diverse and it commonly grows between 1,000 and 2,500 m above sea level (Legesse Negash, 1995). *Birbira* is a relatively fast growing tree, up to 12 to 15 m in height, and is found at the edge of forests or in forests as an under story tree (Amare Getahun, 1976, unpublished). It is a multipurpose tree: provides shade for coffee in the coffee-growing regions; its pods are a good source of fuel and the wood is used to make tool handles and household utensils. Traditional fishers have been using *birbira* for mass fishing in rivers (Azene Bekele *et al*., 1993). The bark and mature fruit (pod and seed) are ground into powder

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and spread over the water. The fish stunned by the effects of the drug start to come close to the surface, thus enabling an easy catch. Nevertheless, there has not been any report of human death related to the consumption of *birbira* intoxicated fish. It is also a nice ornamental tree and is sometimes planted in towns lining streets (Amare Getahun, 1976, unpublished).

Botanicals are gaining a wider acceptance as alternatives to the use of synthetic insecticides to control insect pests. Although the potential of *birbira* as a botanical for the control of insect pests has received little attention, it has been confirmed effective particularly, on a storage pest of grain legumes, the bean bruchids, *Chalosobruchus chinensis* L. (Bayeh Mulatu and Tadesse Gebremedhin, 2000). In their study, the powder and oil from its seed cotyledons were evaluated. The oil in particular was found very effective in preventing oviposition by the bean bruchids on faba bean.

The use of *birbira* for mass harvesting of fish in rivers and its proven efficacy as botanical to control storage pests may imply that it contains biocidal chemicals that are active when dissolved in water and/or organic solvents, thus, adding to the possibility of widening the potential use of *birbira*. Therefore, evaluating it for the control of field insect pests was thought important. Extending the use of this botanical to control field insect pests requires an appropriate way of delivering the active chemicals contained in *birbira* to the target inset pests. For practical reasons, the solvent of choice for field application can only be water. What is important is that the allelochemicals in *birbira* that may have insecticidal potency should be able to disperse well in water and are readily delivered to the target insect in crop fields. This may be confirmed through bioassay of extracts from *birbira* that might be recovered with different solvents.

Different workers had done extraction and characterization of the allelochemicals present in *birbira*, and their results are described below. Clark (1943) extracted rotenone from the ground seeds of *M. ferruginea*. The seeds yielded 1% rotenone by direct crystallization. Further studies done on the extraction, separation and characterization yielded more rotenoids and flavones (Highet and Highet, 1967; Ermias Dagne *et al*., 1990; Ermias Dagne and Amha Bekele, 1990). However, none of these work tried to evaluate the possible usages of the allelochemicals extracted from *birbira* such as insecticidal potency. In general, studying the chemistry of natural products has been going on for a very long time with limited consideration for bioassay (McLaughlin *et al*., 1998).

In order to achieve applied meaning and significance, natural product chemistry must incorporate bioassays. Extracts from botanicals must be screened for biological activity, the active extracts selected, fractionations directed to bioassays, and the bioactive compounds identified and then exploited for the intended purpose (McLaughlin *et al*., 1998), in this case for the control of field insect pests. These require extracting with different solvents and subsequently confirm the presence of potent chemicals in the different extracts from *birbira* that may kill insect pests. The biocidal effects of these extracts can only be confirmed through bioassays. The objective of this study was, therefore, to determine through bioassays, the ability of different extracts from *birbira* to kill field insect pests, particularly aphids.

MATERIALS AND METHODS

1. *Ex-situ* contact bioassay of *birbira*

Test insects used

Colonies of three aphid species: the barley aphid (*Diuraphis noxia* (Mordwilk)); the brassica aphid (*Brevicoryne brassicae* L.) and the pea aphid (*Acyrthosiphon pisum* (Harris)) were collected within the premises of the Holetta Agricultural Research Center from barley, cabbage and field pea plants, respectively. Laboratory colonies were established in a parasitoid-free environment created using protective cylinder cages. Colonies of the three aphids were maintained on their respective host plants for the entire study period. The rearing was carried out at room temperature and relative humidity. The contact bioassay was conducted on progeny of similar age group, i.e., young adults.

Botanical preparation

Seeds of *birbira* were collected from the premises of the Holetta Research Center and put in polythene bags and preserved at 4^{o}C. The seed coats were removed and the cotyledons were crushed with mortar and pestle and the powder was kept in polythene bags at 4^{o}C. There were three preparations made: (1) *birbira* seed cotyledon powder dissolved in water, (2) chloroform extracted cream from the seed cotyledon powder, and (3) residue left after organic soluble content in *birbira* seed cotyledon powder was extracted with chloroform. This residue was separated, dried at room temperature, crushed in similar way and made ready for use. Stock solutions were prepared from the three preparations by weighing 20, 40, 60, 80 and 100 mg and dissolving each one of them in 100 ml water in separate Pyrex glass flasks. The mixtures were stirred using magnetic stirrer (Stuart Scientific, UK)

continuously at room temperature until the time when no sediments remained in the flasks and homogeneous solutions were obtained. All were transferred into a refrigerator at 4^{o}C until use. The three extracts produced uniform and lemonade water looking solutions. Stock solutions were prepared for one time use only.

Contact bioassay setting

The contact bioassay was conducted in glass Petri dishes of diameter 9 cm, at room temperature in an insectary. Ten adult apterous aphids of similar age, from one of the three species described earlier, were transferred into a Petri dish. The three stock solutions prepared as described above at 5 rates: 0.2, 0.4, 0.6, 0.8 and 1.0 mg extract/ml water were removed from the refrigerator and placed at room temperature. Before use, they were stirred again to create uniform dispersion. They were topically applied using a micropipette at 5 µl per aphid on the abdominal dorsum of each aphid. All the aphids in a Petri dish were treated in a similar way. Plain water was included as a blank control. There were 24 replications or runs for each rate in each of the extracts. The experiments were initiated at 10:00 hrs in the mornings and terminated after 5 hrs. The evaluation was made after 5 hrs to note aphid mortality that took place at the lowest rates. They were not provided with food after treatment in order to avoid mortality that might be caused while transferring them to food sources. The replications were run divided into four on successive days by starting and ending at similar time of the day. The aphid species were treated in succession. Data were collected on the number of aphids that died after 5 hrs of application. The lack of response by an aphid to the gentle touching and rubbing of its abdomen using camel brushes confirmed death of the aphid. Based on this, all aphids that were sessile and those which were seemingly alive, but hardly tried to move about when touched, were proclaimed dead.

2. On-farm verification of LC_{50} amounts of *birbira* extracts

On-farm verifications were made on the effectiveness of the LC_{50} amounts (Table 4), obtained from the Probit analyses, of water and chloroform extracted contents of *birbira*, on the three aphid species. For the pea aphids, the verification was done in three pea grown fields, which were randomly selected around Adade Mariam, SW Shewa. Each site was divided into four equal plots of 5 m x 5 m. The plots were randomly assigned to receive chloroform or water extracted *birbira*, Pirimor or left untreated. Fifteen plants were randomly picked per plot and tagged with colored acrylic thread. On each plant the number of aphids present was counted and the

whole plot was sprayed or left untouched in the case of the control. The spray volume was prepared by calibrating the amount of water needed to cover $1m^2$ areas, which was found to be 24 ml of water. After 24 hrs, post spray counts were made again on the tagged plants. The difference in aphid counts between the pre- and post-sprays were computed to get the mortality data. Similarly, at Chacha, in North Shewa, on-farm verification was made on the barley aphid. This was done in one big farm, which was divided into three blocks and each block divided to accommodate the four treatments on 5 m x 5 m plots. The verification on the brassica aphid was done in a similar way as for the barley aphid, in Holetta, W Shewa.

Data analysis

The percent survival of aphids after treatment with water (blank control) was used to correct for the natural death of aphids in the three treatments and to compute percent mortality due to treatment effect. The correction was done using Abbott's formula (Abbott, 1925 cited in Matsumura, 1985). One-way and multi-way ANOVAs (JMPIN, 2000) were carried out on the corrected percent mortality data. Probit analyses (SPSS 10 for windows, 2003) were made to produce the dosage-mortality curves for the three aphid species due to the application of different doses of the three extracts (Sokal and Rohlf, 1995). Moreover, the lethal concentrations of the respective extracts that killed 50% of each of the three aphid species and their confidence intervals were also determined using the Probit model, SPSS 10 for windows (SPSS, 2003). The data obtained from the on-farm verification of the LC_{50} amounts of the respective extracts on the respective aphid species were also analyzed using one-way ANOVA. The means ±SE values obtained from the laboratory and on-farm evaluations are all reported here and Tukey Kramer honest significant difference test was done for mean separations.

RESULTS

1. Contact bioassay of *birbira* in the laboratory

The multi-way ANOVAs computed on the percent mortality data from the three treatments showed that the first-degree interaction between aphid species and treatment and the main effects of aphid species, treatments and dosages resulted in highly significant aphid mortality (Table 1). Among the aphid species, mean mortality was, in general, significantly lower for the brassica aphid. From the different extracts, the residue in chloroform caused significantly lower mortality on all species. However, the three aphid species responded differently to this extract and the barley aphid was by far

the most sensitive. Mortality caused by *birbira* extracts in water and chloroform was not significantly different for the barley and brassica aphids whereas for the pea aphid, *birbira* extracts in water brought forth significantly more death than extracts in chloroform (Table 2).

Table 1 Sum of squares, F-ratios and P-values for the different factors and their interactions that affected aphid mortality.

Source of variation	DF	Sum of squares	F-ratio	Prob>F
Aphid species	2	287632	447	0.0001
Treatment	2	164891	256	0.0001
Aphid species*Treatment	4	26137	20	0.0001
Dosages	4	92758	72	0.0001
Aphid species*Dosages	8	2390	0.93	0.4900
Treatment *Dosages	8	1277	0.49	0.8600
Aphid species*Treatment *Dosages	16	7524	1.46	0.1000

Table 2 Mean ±SE percent mortality recorded on the three aphid species caused by the three different extracts from *birbira* seed cotyledon topically applied dissolved in water.

Aphid species	Mean percent mortality in *birbira* extracts				F-ratio (3,428)	P-value
	In water	In chloroform	Residue[§]	Control		
Barley aphid	78.10±1.44Ab	82.97±1.58Aa	66.46±1.86Ba	4.58±0.71Cb	391	0.0001
Brassica aphid	53.52±1.94Ac	48.13±2.78Ab	17.35±1.76Bc	8.75±1.31Ba	98	0.0001
Pea aphid	88.63±1.02Aa*	76.57±1.74Ba	52.25±1.97Cb	5.41±0.84Db	428	0.0001
F-ratio (2,357)	142	78	183	4.98		
P-value	0.0001	0.0001	0.0001	0.007		

*The upper cases and the lower cases are for along rows and down columns comparison, respectively.
[§]Residue left after extraction with chloroform

The rate dependent mortality values of the three aphid species are presented as dosage-mortality curves obtained through Probit analysis. In Probit analysis the log dose dependent mortality is supposed to assume linearity. It is when this situation is fulfilled that lethal concentration 50% (LC_{50}) could be predicted with confidence (Finney, 1964 cited in Sokal and Rohlf, 1995). All the dosage-mortality curves that showed effects of the three treatments on each species assumed linearity (Fig. 1) and the R^2 values for all these fittings were very high (Table 3).

Table 3 Parameter estimates of the Probit (p) models for the three extracts obtained after repeated iterations.

Aphid species	Extract type	Regression coeff.	Intercept	R^2-value
	Water extracted	0.39	-0.34	0.96
Barley aphid	Chloroform extracted	0.39	-0.29	0.84
	Residue in chloroform	0.39	-0.48	0.98
	Water extracted	0.77	-0.56	0.77
Brassica aphid	Chloroform extracted	0.77	-0.64	0.80
	Residue in chloroform	0.77	-1.28	0.89
	Water extracted	0.47	-0.20	0.98
Pea aphid	Chloroform extracted	0.47	-0.34	0.99
	Residue in chloroform	0.47	-0.65	0.91

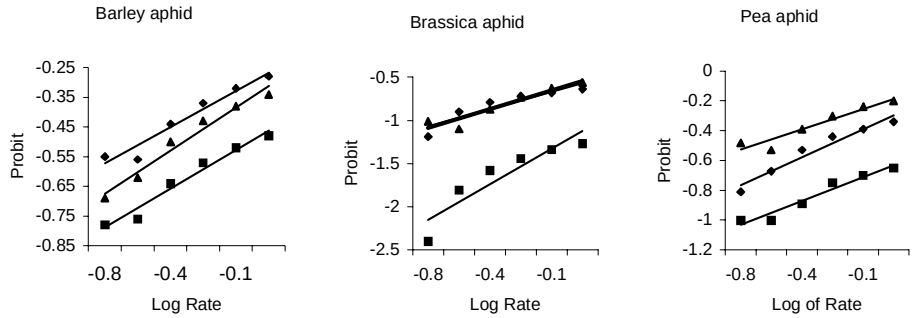

Fig. 1. Probit transformed mortality responses of barley, brassica and pea aphids to the biocidal effect of three extracts from *birbira* seed cotyledon topically applied dissolved in water. (■ Water extract, ◆ Chloroform extract and ▲ Residue in chloroform).

For the brassica aphid, mortality caused by extracts with water and chloroform was very similar at all rates. In contrast, the extracts from the residue in chloroform caused significant aphid kill only at the higher rates. On the other hand, for the barley aphid, the rate dependent mortality, due to even the extracts from the residue in chloroform was higher than that on the brassica aphids at all rates. Nevertheless extracts in water and chloroform killed significantly more barley aphids than extracts from residues in chloroform. The pea aphid showed very similar response trends with the barley aphid for the three extracts at all rates (Fig. 1). Therefore, the lethal concentration (mg *birbira* extract/ml of water) that kill 50% of the test aphids by contact for the extracts obtained by dissolving *birbira* in water and chloroform and the residue in chloroform were determined for the three aphid species and are described in Table 4. The concentration of the extracts

in water that caused 50% mortality was lower for the pea aphid, intermediate for the brassica aphid and higher for the barley aphid. In contrast the concentration of the extracts in chloroform for the same effect was similar for barley and pea aphids but higher for the brassica aphid.

Table 4 Mean and 95% confidence limits of lethal concentration 50% (LC_{50} mg/ml) of the three extracts from *birbira* seed cotyledons on the three aphid species topically applied dissolved in water.

Aphid species	LC_{50} of *birbira* extracts by solvent					
	Mean			95% confidence limits		
	Water	Chloroform	Residue*	Water	Chloroform	Residue
Barley aphid	7.38	5.31	16.68	3.04-65.32	2.40-36.56	5.44-277.7
Brassica aphid	5.31	6.72	45.91	2.41-40.18	2.85-62.00	10.87-2059
Pea aphid	2.69	5.32	24.25	1.56-8.01	2.63-23.34	8.10-262

* Residue of *birbira* left after chloroform extracted and dissolved in water

2. 1. On-farm verification

In the on-farm verification, the mortality differences between the three treatments and the blank control were highly significant for the three aphid species (Table 5). For the pea aphids, the mortality differences were not significant between chloroform and water extracted *birbira* and the standard aphicide, Pirimor. On the other hand, the number of aphids in the control plots increased by about 5% in 24 hrs after spray. For the brassica aphid, the standard chemical gave 100% control, which however was not significantly different from the cream of *birbira*, which in turn was not different from the powder. In this treatment, there was a 22% increase in the number of aphids in the control in the post spray count. In contrast, for the barley aphid, the difference between the standard aphicide and *birbira* extracts was highly significant. For the barley aphid, Pirimor gave significantly higher control than the *birbira* extracts, which were significantly different from the blank control. The barley aphid population increase in the blank control was 6% in the post spray count.

Table 5 Mean ±SE mortality of the three aphid species under field condition after sprayed with chloroform and water extracts from *birbira* seed cotyledon and the standard aphicide, Pirimor.

Treatment	Rates used	Mean percent mortality
Barley aphid		
Chloroform extracted	5.32 mg/ml	45.41±5.37b
Water extracted	7.38 mg/ml	29.48±3.67b
Pirimor 50% WP	1kg/ha	95.25±1.58a
Control	---	-5.24±6.66c
P-value		0.0001
Brassica aphid		
Chloroform extracted	6.72 mg/ml	65.33±7.28ab
Water extracted	5.31 mg/ml	47.46±5.33b
Pirimor 50% WP	1kg/ha	100a
Control	---	-21.89c
P-value		0.0001
Pea aphid		
Chloroform extracted	5.32 mg/ml	98.78±1.21a
Water extracted	2.69 mg/ml	88.02±3.96a
Pirimor 50% WP	1kg/ha	99.89±0.10a
Control	---	-6.14±9.51b
P-value		0.0001

Values followed by same letter along a column are not significantly different at $p<0.05$ according to Tukey Kramer honest significant difference test (HSD).

DISCUSSION AND CONCLUSION

The consistently recorded higher survival of the aphids in the blank controls indicated the validity of the assay setting used in this study. Besides this, the highest survival of aphids in the controls relative to those treated with the different extracts indicated that showering the aphids with water had minimal effect on the aphids.

For the aphids treated with the three extracts, mortality was confirmed to be significantly dependent on the rates of application of each extract. Therefore, mortality found after the correction for the natural death of the aphids in the three treatments is attributable to the concentration gradient dependent biocidal effect of the extracts by the two solvents and the residue in chloroform on the three aphid species. But, the lethal concentration that killed 50% of the aphids was different for the three treatments for each species. These values were much lower for the extracts by water and chloroform than the residue in chloroform, which had a much lower killing effect. Thus, the significant insecticidal potency of the allelochemicals in *birbira* extracted by chloroform was proven by the low efficacy of the residue remaining after extraction in chloroform. The extracts that were contained in the residue in chloroform and were soluble in water caused significant aphid mortality only at very high concentrations. This might have been because the active chemicals contained in the residue in

chloroform might have been similar to those extracted by chloroform, but remained in the residue during the extraction process and hence could only be expressed at very high concentrations.

The significant insecticidal potency of the allelochemicals extracted by water is interesting because water is the cheapest solvent to use to deliver the potent insecticides in *birbira* to the target insects. Those chemicals that were able to dissolve in water and kill aphids might be the same as those which intoxicate fishes. Based on these results either of the following scenarios might be possible: (1) Although both extracts have significant insecticidal potency, the allelochemials extracted by water might be different from those extracted by chloroform, thus indicating the possibility of producing synergy to better control aphids by contact; (2) On the other hand, the potent compounds that were extracted by the two solvents might be of the same group of allelochemicals, but, because of their nature, might dissolve in solvents of different characteristics. These assumptions are subject to further investigation. They need to be proven by isolating the different fractions that dissolve in water or chloroform, characterizing them for their insecticidal potency through bioassay, identification and structural elucidation of the potent fractions.

The significant response difference among the aphid species to the effect of *birbira* is an important phenomenon to notice. Although the LC_{50} value for brassica aphid was not pronouncedly different from the values for the other two species, it sustained the lowest mortality. The brassica aphid is specialized to feed on *Brassicaceae*, which are all endowed with allelochemical glucosinolates that are even toxic to humans. This adaptation might have helped it reduce its susceptibility to the effect of the allelochemicals extracted from *birbira* seeds, although *birbira* is a leguminous plant, which has not been reported so far to contain glucosinolates (Nigusie Alemayehu, 2001). On the other hand, the body of the brassica aphid is covered with grayish white mealy wax, which is also secreted onto the surface of the plant and extends through the colony (Blackman and Eastop, 2000). This wax layer might act as an impediment to the penetration of cuticle of the brassica aphid by the biocidal chemicals in the different extracts. The latter assumption might hold true because the LC_{50} values of extracts in water and chloroform for the brassica aphid were not pronouncedly different from the values for barley and pea aphid. It can be concluded that *birbira* contains potent insecticides soluble either in water or chloroform and effective to kill aphids, which are major field insect pests in Ethiopia.

The results obtained from the on-farm verification of the LC_{50} amounts under field condition demonstrated the efficacy of *birbira* extracts with water and chloroform in controlling the three aphid species. The lowered killing effects of the two extracts in the on-farm condition for the barley aphid was due to the feeding habit of the aphid, which is inside rolled barley leaves that create a protected feeding niche. It was mainly the aphids that were not in the enrolled barley leaves that were killed. In fact all the exposed barley aphids were found dead after 24 hrs. The other two species feed exposed on the leaf surfaces of their respective host plants and hence were not difficult to reach by contact. In general, however among the three aphid species, the pea aphid was by far the most affected by the extracts from *birbira*. Therefore, it can be concluded that *birbira* is an effective aphicide with contact effect and hence could be developed for practical use by small scale farmers.

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