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Preparation,Analysis and Bioefficacy of Water Soluble Botanical Pesticide Formulation from Neem and Sweet Flag

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Abstract: Azadirachtin (AZA) and β-asarone (BAS) which are lipophilic in nature were made into water soluble formulation by encapsulating them in to β- methyl cyclodextrin (BMCD). This formulation was found to effective against plant feeding insects and plant pathogenic fungi. A simple and fast HPLC method was developed for simultaneous estimation of azadirachtin and β-asarone in the formulation. AZA and BAS were separated on Nova Pak (150 x 0.39 mm, 4 µm) column using water and acetonitrile as mobile phase by gradient elution. Peaks were monitored at 210 nm using photo diode array (PDA) detector. The developed method was validated for linearity, accuracy, precision and sensitivity. Linear regression analysis revealed an excellent correlation between peak responses and concentrations (R^2 values of 0.999) for the AZA and BAS. The developed HPLC method was applied for estimation of AZA and BAS in multi component water soluble biopesticide formulation. The recoveries of AZA and BAS were in the range of 94.37-99.98% and 99.26-100.08% respectively, and precision values were less than 4.5%
Key words : Azadirachtin, β-asarone, β- methyl cyclodextrin, HPLC, pathogenic fungi.

Introduction

Biopesticides are plant protectants derived from natural materials such as animals, plants, bacteria, and minerals. Secondary metabolites of certain plants are used as biopesticides. They are now being widely used all over the world as plant protectants due to their ecofriendly nature. Neem tree *(Azadirachta Indica*) is a tropical plant that is well known for its pesticidal properties.[1] It has been traditionally used for its medicinal properties as antimalarial, antihelmentic, antiseptic and antimicrobial.[2] Neem leaves are used to protect grains and cloths from insects and the seed oil has been used as insecticide. Many studies demonstrated that neem seed contains limonoids and terpenoids responsible for their biological activities.[3,4] Among these limonoids, azadirachtins are considered to be the most important active principle due to its various effects on insects.[5] Mainly Azadirachtin-A (Figure 1) inhibits feeding in the Desert Locust at concentration as low as 0.001 ppm.[6] Azadiractin interrupts metamorphosis in insects, causing pesticidal effects.[7] Its activity at low concentrations and eco-friendly nature makes it a perfect alternative to synthetic pesticides such as organochlorines and orgnophosphorus molecules.[1] Water soluble neem insecticide formulation was used to control the coconut black-headed caterpillar.[8]

Sweet flag (*Acorus calamus L.*) is a widespread, semi-aquatic plant of temperate to sub-temperate regions. The ancient peoples of China used it to lessen swelling and for constipation. In Ayurvedic medicinal practice in India, the rhizomes have been used to cure several diseases like fever, asthma and bronchitis, and as a sedative. β-Asarone (Figure 1) is the major active ingredient present in *Acorus calamus L.* The rhizomes of Nepalese *Acorus calamus* L. yields 0.74% of volatile oils, containing mainly β-asarone(46.78%).[9] The volatile oil from the tetraploid "Indian" form of *A. calamus.L* contains β-asarone as a major component (70 to 80%),

whereas, the oil from the "European" or triploid form contains less than 10%.[10] Extracts of sweet flag rhizome and leaves shows antimicrobial activity.[9] Anti-candida potential of *Acorus calamus* rhizome containing BAS, was evaluated against the human fungal pathogen, *Candida albicans* which exhibited promising growth inhibitory activity at 0.5 mg/ml and fungicidal activity at 8 mg/ml.[11] Since β-asarone is highly lipophilic, water soluble formulations from sweet flag were made and *in vitro* activity against some fungal plant pathogens studied.[10] β-Asarone completely inhibited mycelial growth of some plant pathogenic fungi, *Cladosporium cucumerinum, Colletotrichum orbiculare, Magnaporthe grisea, and Pythium ultimum*, in a range of 0.5-30 ppm.[12]

In order to make a formulation having insecticidal and anti-fungal activity, azadirachtin and β-asarone were combined and made into water soluble material. Literature survey revealed that cyclodextrins have been widely used to prepare inclusion complexes to improve the stability, solubility, and bioavailability of many lipophilic substances.[13,14] In this study we have used β- methyl cyclodextrin to encapsulate extracts of *Azadirachta Indica* containing Azadirachtin and *Acorus calamus L* containing β-asarone and subjected the resulting formulation for its bio-efficacy on plant feeding insects and plant pathogenic fungi. Also an HPLC method was developed for simultaneous estimation of active ingredients viz., azadirachtin and β-asarone.

Literature survey revealed several methods available for estimation of azadirachtin[15-19] and β-asarone[20–27] individually using various photometric, chromatographic and electrophoresis techniques. There is no reported HPLC method for simultaneous estimation AZA and BAS. When the above reported AZA method was used for estimation of both AZA and BAS, BAS eluted at higher retention time. When reported BAS method was used, AZA peak merged with co-eluting peaks. To overcome this problem, a simple, fast and accurate gradient HPLC method was developed for simultaneous estimation AZA and BAS in biopesticide formulation.

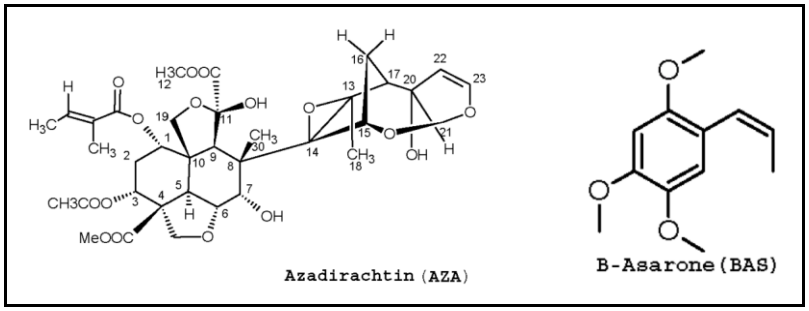

Figure1 Structure of azadirachtin and β-asarone

Experimental

Preparation of water soluble biopesticide formulation (WSP) from neem and sweet flag

Reagents and chemicals:

The BMCD was procured from Wacker Chemie (Germany), 60% sweet flag extract was obtained by extraction of *Acorus calamus* rhizomes using ethyl acetate,[10] azadirachtin technical 45% was obtained from in-house facility of Vittal Mallya Scientific Research Foundation and high-purity water was prepared using a Millipore Milli Q Plus purification system (Millipore Corporate Headquarters, Billerica, MA).

Inclusion of azadirachtin and β-asarone into BMCD:

Acorus calamus L. extract containing about 60% of β-asarone was added to a 20% aqueous solution of BMCD in different ratios as mentioned in table 1. The contents were continuously stirred using magnetic stirrer at room temperature for 8 h,[10] to this solution different concentrations of azadirachtin technical powder (45%) was added and stirred for 4 h, then filtered through celite bed. The clear filtrate was lyophilized to get dried off-white colored powder having differing amounts of β-asarone and azadirachtin. The BAS and AZA content in the inclusion complex is estimated by using the HPLC method. The percentage encapsulation of BAS and AZA into the BMCD was done by comparing the theoretical content and actual content in the complex.

$$\%\ \textbf{Encapsulation of BAS and AZA in BMCD} = \frac{\textbf{Actual content of BAS and AZA in complex}}{\textbf{Theoritical content of BAS and AZA in complex}} * 100$$

***In vitro* analysis using fungal plant pathogens**

The water soluble powder formulation (WSP) prepared by combining neem and sweet flag extracts were evaluated *in-vitro* against plant pathogenic fungi *Phytophthora capsici* and leaf eating caterpillar *Spodoptera litura* in the laboratory following standard bioassay procedures with three replications. Two dosages (1 g/l and 2 g/l) of the WSP formulation were tested vis-à-vis individual formulation developed from either neem seed kernel extract (containing 6% Azadirachtin) or sweet flag rhizome extract (containing 1% β-asarone) against both the plant pathogen and pest insect.

The *P.capsici* culture was revived using 20% carrot agar from the stocks maintained at VMSRF and fungal discs of 10 days old were used for bioassay using poison plate technique. The plates were incubated at 28°C. The colony diameter in different treatments and untreated controls were recorded after 7 days of fungal inoculation. Growth inhibition (%) in different treatments was worked out in comparison to the same in untreated control plates.

For the bioassay against insects, weighed quantity of the each test formulation as per the treatments was added in 50 ml the standard artificial diet and poured uniformly in to 12 well of multicavity plates. Early second instar Spodoptera larvae reared on castor leaves in the Entomology laboratory at VMSRF were released in to each well @ 3 larvae per well. All the plates were kept at room temperature. One 12 well plate with only artificial diet was maintained as untreated control. For observation purpose, 4 wells of each plate were treated as one replication making 12 larvae for each replication of all treatments. Insect larval mortality and weight were observed and recorded after 3 days (72 hr) of treatment imposition. Per cent mortality and weight reduction was worked out for treatment comparison. The data were analyzed following standard statistical procedures.

Analysis of AZA and BAS in WSP by HPLC

Reagents and chemicals:

Reference standards of β-asarone and azadirachtin were obtained from Sigma Aldrich, (Lahnau 2, Germany). HPLC grade acetonitrile was supplied by Merck, (Darmstadt, Germany). High-purity water was prepared using a Millipore Milli Q Plus purification system (Millipore Corporate Headquarters, Billerica, MA).All other chemicals were of analytical reagent grade.

Apparatus:

The HPLC system consist of two LC-10AT VP pumps, an SPD-M10A VP photo-diode array detector, a CTO-10AS VP oven and SCL-10A VP controller (all from Shimadzu, Japan). A reverse-phase Nova Pak (150 × 3.9 mm i.d.; 4 μm) column (Waters, Ireland) was used for separation, and chromatograms were integrated using Class vp software. An Ultrasonic cleaner model 8890 (Cole Parmer, India) was used for preparation of sample and standard.

Chromatographic conditions:

The mobile phase was water and acetonitrile; before delivering into the column it was filtered through a 0.45μm Nylon filter (Millipore) and degassed. The analysis was carried out under gradient condition using a flow rate of 1.0 ml / min at 30°C. Chromatograms were recorded at 210 nm using a PDA detector.

Preparation of standard and sample solutions:

Solutions of (1000 μg/ml) AZA and BAS were prepared by dissolving known amounts in acetonitrile. These solutions were further diluted with diluents solution (acetonitrile: water, 50:50, v/v) to determine the accuracy, precision, linearity and sensitivity.

A 25 mg of the sample was taken in to 50 ml volumetric flask, dissolved using diluent and made up to the mark. The solution was filtered through 0.45 μm nylon syringe filter and injected to HPLC.

Results and discussion

The inclusion complex of azadirachtin and β-asarone in β-methyl cyclodextrin was prepared by stirring the *Acorus calamus* extract and technical azadirachtin in 20% BMCD aqueous solution. The percentage inclusion of both BAS and AZA(equal amounts, w/w) into the BMCD is less when their ratios are 1:2, but their

concentration in complex is more (BAS is 4.22%, AZA is 12.31). As the amount of BMCD increases the percentage of encapsulation of AZA and BAS increases, this trend continues till 1:30 ratio, where the encapsulation of BAS is 90.79% and AZA is 98.67%. In the ratios 1:50, 1:60 and 1:70 there is drastic decrease in the encapsulation of BAS, but slight decrease in the encapsulation of AZA was observed. The encapsulation trend is shown in table 1

Table 1 Trend of encapsulation of BAS and AZA into BMCD

(AZA+BAS) : BMCD	% of BAS in complex	% of BAS utilized	% of AZA in complex	% of AZA utilized
1:2	4.22	6.98	12.31	52.38
1:5	3.41	12.52	8.10	90.1
1:10	3.11	21.25	4.36	96.86
1:20	2.81	70.56	2.21	98.23
1:30	2.60	90.79	1.48	98.67
1:50	1.21	81.26	0.86	95.52
1:60	0.81	62.18	0.70	93.33
1:70	0.65	55.13	0.59	92.15

***In vitro* analysis against fungal plant pathogens**

The WSP formulation of neem and sweet flag (Aza 1.25% + β Asarone 1%) was found to have significant fungicidal and insecticidal properties (Table 2). It is found to inhibit the wilt fungus *P.capsici* to the tune of 39 and 29 per cent when treated *in-vitro* @ 2 g/l and 1 g/l dosages, respectively when compared to untreated control. The WSP formulation from sweet flag containing β Asarone 1% alone, recorded significantly higher inhibition of the plant pathogenic fungus (52 and 44% @ 2 g/l and 1 g/l dosages, respectively).

Table 2 Bio-assay data

Treatments	Formulation	Dosage (g/l)	Inhibition (%) of *P.capsici*	Spodoptera larval mortality (%)	Insect larval weight loss (%) against untreated control
1. Neem + Sweetflag (Aza 1.25% ; β Asarone 1%)	WSP	1	28.8 d	20.0 cd	43.5 c
2. Neem + Sweetflag (Aza 1.25% ; β Asarone 1%)	WSP	2	38.7 c	26.5 c	47.8 c
3. Neem alone (Aza 6%)	WSP	1	05.0 e	68.0 b	72.0 b
4. Neem alone (Aza 6%)	WSP	2	08.0 e	84.7 a	92.0 a
5. Sweetflag alone (β Asarone 1%)	WSP	1	43.8 b	10.0 de	28.5 d
6. Sweetflag alone (β Asarone 1%)	WSP	2	51.8 a	10.0 de	33.5 d
7. Untreated control	--	--	0.30 ef	00.0 e	-
	S Em +/-		**1.54**	**3.76**	**1.68**
	CD (5%)		**4.75**	**11.59**	**5.18**
• **Values followed by same alphabet are not significantly different**					

However, the WSP showed significantly enhanced effectiveness both in terms of Spodoptera larval mortality (20-26.5 % mortality) or larval weight loss (43.5- 48%) when compared to the treatment with only sweet flag extract.

The purpose of the WSP was to have a duel effect against both insect pests and plant pathogenic fungi. In majority of the field situations the insect pests and fungal pathogens infest together and farmers have to resort frequent sprays to manage both types of pests. Off late both non-availability of labors for spraying the crop and their high charges, make the farmers to mix and spray fungicides and insecticides. Botanicals which are used for ages proved to be very safe and well documented. Combination of botanicals could be more

practical with wide spectrum of action, convenient to apply, pack and store and to develop as a green pesticide for use in many crop production systems where diseases and insect pests occur together.

It is evident from the experiment that individually neem did not prove to be a good fungicide or sweet flag to be an insecticide. When neem and sweet flag extracts were combined, both insecticidal and fungicidal properties were observed. Though there is reduction in fungicidal ability of the combi-formulation ie WSP , probably due to certain reactions in the combination, 29-39 per cent inhibition against the fungus at the tested dosages are quiet significant and practical. Similarly against the leaf feeding insect, the effect of the WSP was much higher than sweet flag alone. The insecticidal action of the WSP was much lesser vis-à-vis straight formulation of neem tested in this experiment and that is because of higher concentration of azadirachtin at 6%. Unfortunately in the present experiment we do not have data to compare a treatment of 1 per cent formulation of neem alone, but if the mathematical logic is applied, the WSP formulation with 1.25 per cent of Azadirachtin, has caused about 40 per cent more effect than the straight formulation at the same dosage (say 1g/l). Similar is the case with the larval weight loss (43-48%), suggesting that the larvae might not develop normally, even if they get normal food immediately.

Analysis of AZA and BAS by HPLC

Optimization of chromatographic conditions:

Azadirachtin and β-asarone were separated using reverse-phase HPLC columns of different make and dimension and using different solvents. The separation and peak shapes were found to be good on Nova pak (150 X 3.9 mm, 4 µm) column using water and acetonitrile as mobile phase. Since AZA and BAS possess wide difference in the polarity, they have wide retention gap when isocratic elution was carried. Hence the gradient elution was applied for separation. Initially linear gradient programme was run by keeping solvent B concentration at 35% for 6 minutes, until more polar AZA elutes. After that, solvent B concentration was increased to 90% from 6 min to 10 min and retained there up to 15 min. Then solvent B concentration was brought to initial level after 16 mins and stabilized for two minutes before next injection. The gradient elution programme is recorded in Table 3. Under above chromatographic condition, AZA and BAS were eluting at 5.0 min and 11.0 min respectively. The UV wavelength at 210 nm was chosen for the detection and quantification, since the AZA and BAS have good absorption at that wavelength.

Table 3 Gradient elution programme

Time/min	Solvent A, %	Solvent B, %
0.01	65	35
6.0	65	35
10.0	10	90
15.0	10	90
16.0	35	35
18.0	35	35

Solvent A: water, Solvent B: Acetinitrile

Specificity:

The specificity of the method was demonstrated by spiking 5 µg/ml of AZA and BAS to BMCD. Chromatographic peak purity and homogeneity were evaluated with PDA detector. There was good separation between AZA and BAS, and also peak impurity index >0.999 indicating that there was no impurity embedded in it.

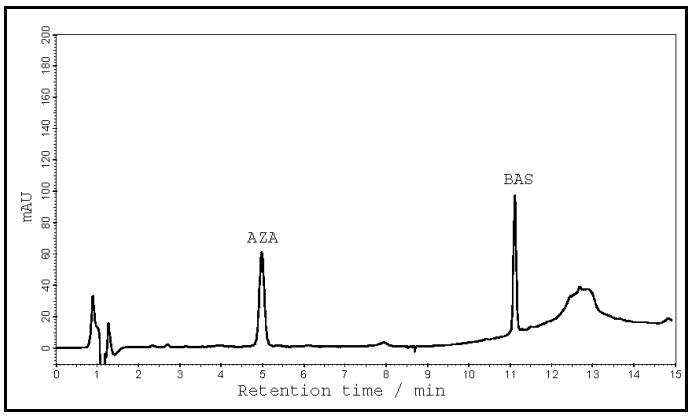

Fig. 2. Typical chromatogram of AZA and BAS

Limit of detection and Limit of Quantitation:

Limit of detection (LOD) and Limit of quantitation (LOQ) represent the concentration of the analytes that would yield signal-to-noise ratio of 3 for LOD and 10 for LOQ, respectively. LOD and LOQ were determined by measuring the magnitude of analytical background by injecting blank samples and calculating the signal-to-noise ratio for both compounds and injecting series low concentrations solutions until the *S/N* ratio is 3 for LOD and 10 for LOQ. The LOD and LOQ of AZA and BAS were 0.035, 0.012 and 0.11, 0.04 respectively.

Linearity:

The linearity of the detector response to different concentrations of AZA and BAS was studied by analyzing BMCD solution containing AZA and BAS at ten different concentration levels ranging from 0.05 – 100 µg/ml. AZA and BAS showed a linear response over the concentration range tested, with correlation coefficient values 0.999 and regression equations y = 20798x - 997.8 and y = 15948x – 47147 respectively.

Accuracy:

Accuracy of the analytical method is defined as the similarity of the results obtained by the analytical method to the true value[28]. Accuracy of the method was studied at four concentration levels (0.1, 1.0, 25 & 100 µg/ml) by spiking the AZA and BAS to BMCD at specification level (1000 µg/ml). Analysis was done in triplicate in all specified levels. Recovery values of AZA ranges from 94.37 – 99.98% and RSD values ranges from 0.15 – 2.73%. Similarly, the recovery values of BAS ranges from 99.26 – 100.08% and RSD values were in the range 1.04 – 2.38%. Results indicate that the developed method has good accuracy for estimation of AZA and BAS. Values are recorded in Table 4.

Table 4 Accuracy data[a]

Sample Name	Added quantity, µg/ml	Recovered quantity, µg/ml ± SD	%, Recovery	RSD, %
AZA	0.1	0.0943 ± 0.0025	94.37	2.73
	1	0.9740 ± 0.0155	97.4	1.59
	25	24.9950 ± 0.6971	99.96	1.2
	100	99.9814 ± 0.1550	99.98	0.15
BAS	0.1	0.0993 ± 0.0010	99.3	1.04
	1	0.9925 ± 0.0236	99.26	2.38
	25	25.0210 ± 0.7064	100.08	1.84
	100	99.3494 ± 1.8145	99.34	1.82

[a] (n=3) average of three determinations

Precision:

The precision of the method was estimated at three concentrations (0.1, 1.0 and 25 µg/ml) by repeatability (intra-day) and intermediate (inter-day) precision. Repeatability is the intra-day variation obtained at three different concentration levels and is expressed in terms of RSD, % calculated for each day. The RSD

values at each level were below 3% for AZA and 3.5% for BAS indicating good intraday precision. The intermediate precision is the inter-day variation at the same concentration levels determined on two different days with two different analysts. The RSD values at each level were <3.5% for AZA and <4.5% for BAS indicating good intermediate precision. The precision values are recorded in Table 5.

Table 5 Precision data

Name	Added quantity, µg/ml	Intraday precision, RSD%(n=3)		Inter-day precision, RSD%(n=6)
		Day 1	Day 2	
AZA	0.1	2.73	2.53	3.13
	1	1.59	1.37	1.86
	25	1.20	1.08	2.11
BAS	0.1	1.04	3.38	4.19
	1	2.38	1.30	1.72
	25	1.84	1.96	2.29

Sample analysis:

Accurately weighed 25 mg of sample in to 50 ml volumetric flask, dissolved in diluent by sonication and made up to the mark. The solution was filtered through 0.45 µm Nylon filter and injected to HPLC. Four different water soluble formulations were analyzed, each formulation contains different concentrations of AZA and BAS. The AZA and BAS content in formulations are recorded in Table 6.

Table 6 Sample analysis data[a]

Sample name	AZA, % w/w ± SD	BAS, % w/w ± SD
Formulation-1	4.48 ± 0.04	2.91 ± 0.06
Formulation-2	3.57 ± 0.10	1.85 ± 0.08
Formulation-3	1.05 ± 0.02	1.28 ± 0.12
Formulation-4	5.13 ± 0.09	2.60 ± 0.05
Formulation-5	1.56 ± 0.06	2.50 ± 0.11

[a] (n=3) average of three determinations

Conclusion

Water soluble powder (WSP) formulation was prepared from neem extract and sweet flag extract by encapsulation into BMCD. The encapsulation was maximum at the ratio of 30:1[BMCD:(BAS+AZA equal amounts,w/w)] with 90.79%(BAS) and 98.23%(AZA). The WSP which is prepared for the first time was found to exhibit duel effect with significant fungicidal and insecticidal properties. Also HPLC method was developed and validated for simultaneous estimation of BAS and AZA present in WSP which was found to be sensitive, precise and accurate.

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