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ORIGINAL ARTICLE

Melia azedarach seed oil EC formulation and evaluation of its antifungal activity against *Rhizoctonia solani* and S*clerotium rolfsii* pathogens.

Rajmani Prajapati[1], LK Thakur[2], Upma Singh[1]*
1. Department of Applied Chemistry**,** Gautam Buddha University, Greater Noida, UP, India
[2] Analytical Division, Institute of Pesticide Formulation Technology, Gurgaon, Haryana, India
*drupmagbu@gmail.com

ABSTRACT
Rhizoctonia solani and Sclerotium rolfsii are common soil borne fungi which causes yield loses in crops and vegetables. Melia azedarach *plant belongs to* Meliaceae family *well described for their fungicidal properties. Present work focuses on the development, optimization and evaluation of antifungal activity of Emulsifiable Concentrate (EC) from seed oil of* M. azedarach *using methyl oleate and surfactant (Tween-80 and TritonX-100) against selected fungi. GC-MS analysis confirmed the presence of fatty acids, fatty acids derivatives and other phytochemical constituents in seed oil. Physicochemical parameters like thermal stability, emulsion stability, acidity, inflammable test, and pH of developed ECs were investigated. The formulation EC-4 (20% oil) showed LD_{50} values 58.06 and 570.02 ppm against R. solani and S. rolfsii respectively. The seed oil observed high LD_{50} values 138.70 and 16949.3 ppm against R. solani and S. rolfsii respectively. The formulations passed all test parameters and were more effective than seed oil for controlling fungi. Therefore, this formulation may be used as bio-fungicide.*
Key Words: Melia azedarach**;** *Emulsifiable Concentrate; Antifungal Activities; Plant Pathogens.*

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INTRODUCTION

The injudicious use of synthetic pesticides is being discouraged owing to their toxic effects on non-target organisms and adverse effect on the environment [1]. Due to persistent use of synthetic pesticides, pathogens develop resistance against various chemical fungicides [2].Botanical products are biodegradable and have been studied to be effective against pests without harming beneficial insects [3]. Plants produce a great deal of secondary metabolites, many of them with antifungal activity. Phenols, flavonoids, phenolic glycosides, unsaturated lactones, saponins, cyanogenic glycosides and glucosinolates are the phytochemicals produced by plants which have antifungal activities [4]. Natural products derived from plants have been shown to be promising alternatives against plant pathogens than conventional pesticides [5, 6].

R. solani and *S. rolfsii* are common soil-borne plant pathogenic fungi that cause disease and damage to the plants.*S. rolfsii* has wide host range due to abundant growth of the pathogen and its capability of producing excessive *sclerotia* that may persist in soil for several years [7,8]. This fungus is of great importance especially when the disease severity is high in the fields. The crop loss may be between 10-25% or even more than 81% in some fields [9].Various diseases were reported caused by it like, stem rot disease in garlic,stem rot in tomatoandcollar rot disease of chickpea [10-12]. *R. solani* (teleomorph: *Thanatephorus* spp.) is also a plant pathogenic fungus with a wide host range and worldwide distribution. *R. solani* attacks its hosts when they are in their juvenile stages of development such as seeds and seedlings, which are typically found in the soil. *R. solani*is a causal fungus for potato (*Solanum tuberosum* L.) that causes the most serious diseases called stem canker and black scurf, leading to direct reduction of

tuber yield and quality. This fungus causes various diseases like seedling blight of longleaf pine, damping off and root rot diseases to wide range of vegetable and crop plants, black scurf on tubers and shoot/stolon canker on young plants [13, 14].

In present work, the aim is to develop and formulate Emulsifiable Concentrates (ECs) from seed oilof ***M. azedarach*** by using biodegradable oil (methyl oleate) replacing petroleum based solvent and nontoxic surfactants (Tween-80 and Triton X-100) to minimize the toxicity of formulation. GC-MS of seed oil analysis was carried out for confirmation of active phytochemicals. Physicochemical parameters were investigated for their standardization of formulation. *In-vitro* fungicidal activity of developed EC was evaluated against *R. solani* and *S. rolfsii* plant pathogenic fungi.

MATERIAL AND METHODS

Materials

M. azedarach seed oil was procured from Tilak Bazar, Delhi. Methyl oleate purchased from Mohini Organics Pvt. Ltd, Mumbai. Tween-80 and Triton X -100 were procured from Sd Fine, Mumbai. Potato-dextrose-agar (PDA) was supplied by SRL Pvt. Ltd. Mumbai.

GC-MS Analysis

Initially, GC-MS (Gas chromatography coupled with mass spectrometry) analysis of ***M. azedarach*** seed oil was carried out with Simadzu GC-2010 instrument coupled with GC-MS-QP2010 detector. The detector voltage was 0.05kV, ion-source temperature 160˚C and interface temperature 160˚C.A column DV-5MS, 30m × 0.25mm diameter and 0.25µm were used. Injection temperature was 225˚C with split injection mode. The oven temperature was programmed as follows: from 80˚C, 2 min. hold raised at 2˚C/min. up to 180˚C hold for 2 min. total run time was 62.0 minute.The identification of compounds was performed by comparing their mass spectra with data from NIST and WILEY8 mass spectra library.

Preparation of Emulsifiable Concentrates

For preparation of Emulsifiable Concentrates, seed oil was dissolved in fixed quantity of solvent (methyl oleate) and blend of emulsifiers in specific ratio was added to obtain a clear solution. Details of developed ECs are given in Table 1.Different compositions of ECs were prepared and their emulsion properties like blooming and stability were checked as per standard method for optimization and standardization of formulation [15, 16].

Table 1: EC formulations *of* ***M. azedarach*** seed oil

Formulation Code	***M. azedarach*** oil (%w/w)	Surfactants	Emulsifiers Percentage (%w/w)	Ratio of Surfactants	Methyl Oleate (%w/w)	Appearance
EC-1	05	Tween-80 and TritonX-100	16	75:25	79	Transparent
EC-2	10				74	√
EC-3	15				69	√
EC-4	20				64	√
EC-5	30				54	√

Physico-chemical studies of EC formulations

Physicochemical parameters of developed Emulsifiable Concentrates (EC) like accelerated temperature stability (ATS), blooming, emulsion stability, cold stability, pH values measurements and flash point were carried out as per Bureau Indian Standard (BIS).

Emulsion stability/ Blooming Test

Dispersion of above developed ECs in hard water (342 ppm) gave excellent blooming followed by milky white emulsion formation. Formed emulsions were kept undisturbed at room temperature for two hour to check phase separation and sedimentation.

Thermodynamic stability

The thermodynamic stability of developed ECs was carried out at accelerated temperature stability (ATS-54˚C) with storage for 14 days while low temperature stability at 10˚C for 1 hour.

Measurement of pH

The pH of optimized and developed EC was determined using a digital pH meter (Eutech) at room temperature**.**

Viscosity measurement

The viscosity of the developed formulations was determined at 100 rpm by using Fungi lab viscometer R model using spindle TR-10 at 25 °C.

Bio-efficacy evaluation

Plant pathogenic test fungi *Rhizoctonia solani* ITCC 5563 and *Sclerotium rolfsii* ITCC 6181 were procured from Indian Type Culture Collection (ITCC) centre, Indian Agricultural Research Institute, New Delhi-110012, India. Cultures of the test fungi were maintained on Potato Dextrose Agar (PDA) slant at 26°C for six days and were sub-cultured in Petri dishes prior to testing. The test fungi were routinely grown on fresh slant tubes of PDA and stored at 4°C.

Preparation of media

39 g PDA was suspended in 1000 mL distilled water. This was boiled to obtain uniform media. 100 mL media was transferred to each of the 100 mL conical flasks and the flasks were plugged with surgical grade cotton. The media were sterilized in an autoclave at 15 psi for half an hour prior to use.

Preparation of test concentrations

The best formulation (EC-4), seed oil and surfactants used in formulations were selected for their *in-vitro* antifungal study against plant pathogens by food poison technique. A 5 mL stock solution of 50,000 ppm was prepared from developed EC-4 by dissolving in distilled water. A 2 mL stock solution of 1,00,000 ppm in acetone was prepared from seed oil of ***M. azedarach and*** 2 mL stock solution of 1,00,000 ppm each of non-ionic surfactants Tx-100 and Tween-80 was prepared in distilled water. To prepare 1000, 500, 250, 125, 62.5 31.25 and 15.625 ppm concentration of ECs in PDA medium 2, 1, 0.5, 0.25, 0.125 mL, 62.5 µL and 31.25µL were added to 100 mL of prepared sterile media. In case of seed oil and surfactant, 1 mL of stock solutions (1,00,000 ppm) were added in to 100 mLmedia and diluted sequentially with acetone and distilled water respectively to make resulting solutions of 1000, 500, 250, 125, 62.5 31.25 and 15.625 ppm concentration. 30 mL media of each concentration was poured into Petridish with three replications under aseptic conditions in a laminar flow chamber and allowed the media to solidify. Similar method was followed for second fungi.

Inoculation and Incubation

A 5 mm thick disc of fungus (spores and mycelium) cut from earlier sub-cultured fungus was inoculated aseptically to the centre of the Petriplate. Treated, acetone control and control Petri dishes were kept in Biological Oxygen Demand (B.O.D.) incubator at 27˚C ±1 till the fungal growth in the control petri dish was almost complete. The incubation period was observed to be 2-3 and 3–4 days for *R. solani* and *S. rolfsii* respectively.

Recording of Observations

The mycelial growth of fungi in treated and control was measured diametrically (mm) in three different directions and growth inhibition (I) was calculated using the formula:

Inhibition (%) = (C- T) × 100/ C

C = control/ acetone control growth, T = Treated growth

The corrected percent inhibition (IC) was calculated as

$$IC = [(I\ \% - CF)/(100 - CF)] \times 100$$

CF is the correction factor obtained from the equation CF = ((90-Co)/Co) **×** 100, where 90 is the diameter (mm) of the Petri dish and Co is the growth of fungus (mm) in control/ acetone control. LD_{50} values (effective dose for 50% inhibition) were calculated for inhibition of growth using GW basic software.

RESULTS AND DISCUSSION

GC-MS analysis

Analytical chromatogram and spectra of seed oil obtained by GC-MS analysis (qualitative) revealed the presence of several compounds. Components among them were confirmed as major compounds by comparing data with the NIST & WILEY library. Twenty two phyotochemicals were detected in ***M. azedarach*** seed oil. The major phytochemicals detected in seed oil were 9-hexadecenoic acid-methyl ester (2.53%), Hexadecanoic acid (14.02%), Methyl octadeca-9, 12- dienoate (2.04%), 9- Octadecanoic acid methyl ester (3.3%), Octadecanoic acid methyl ester (36.07%), 9, 12-octadecadinoic acid (7.07 %),9, 12-octadecadieoyl chloride (1.68%) and 9-Octadecanoic acid 2,3-dihydroxypropyl ester (1.98 %). Details of chemical constituents are given in Table 2.

TABLE 2: PHYTO-CHEMICALS IN *M. AZEDARACH* SEED OIL.

Compound name	Molecular weight	Retention time (min)	% Area	Base ion (m/z)	Other fragments ion(m/z)
DECANAL-2	154	7.84	0.07	55	70, 98, 136
2, 4- DECADIENAL	152	8.14	0.03	81	67, 96, 152
TETRADECANE	198	9.95	0.01	57	71, 198
1 H CYCLOPENTA [1, 3] CYCLOPROPA [1, 2] BENZENE	204	10.24	0.01	161	55, 105, 133
2 H BENZOPYRAN-6, 7 DIETHOXY 2, 2 DIMETHYL	220	11.95	0.04	205	69, 161
CYCLOPENTANETRIDECENOIC ACID	214	10.18	0.01	60	57, 143, 214
9-OCTADECENIC ACID	282	12.84	0.02	55	74, 97, 282
NONADECANE	268	13.26	0.01	57	71, 99, 269
TETRATETRACONTANE	618	14.82	0.01	57	71, 99, 155
9-HEXADECENOIC ACID-METHYL ESTER	268	14.82	2.53	55	74, 143, 270
HEXADECANOIC ACID-METHYL ESTER	270	15.19	0.91	74	55, 143, 270
HEXADECANOIC ACID	256	16.63	14.02	73	60, 129, 256
METHYL OCTADECA-9, 12- DIENOATE	294	18.42	2.04	67	81, 96, 294
9- OCTADECANOIC ACID METHYL ESTER	296	18.57	3.3	55	74, 97, 296
OCTADECANOIC ACID METHYL ESTER	298	20.61	36.07	74	55, 143, 298
9, 12-OCTADECADINOIC ACID	280	20.75	7.07	55	81, 96, 280
HEXADECANOIC ACID-2- HYDOXY	330	28.16	0.58	57	74, 98
9, 12-OCTADECADIEOYL CHLORIDE	298	31.98	1.68	55	81, 98
9-OCTADECANOIC ACID 2,3, DIHYDROXYPROPYL ESTER	356	32.08	1.98	55	81, 98, 264
1-MONOARAHIDIN	386	32.57	0.26	57	74, 98, 134
SQUALENE	410	34.83	0.60	69	81, 341
STIGMAST 5-ENE 3-OL (3 BETA, 245)	414	44.78	0.27	55	81, 107, 414

Physicochemical parameters

The results of physico-chemical parameters like blooming, emulsion stability, ATS, flash point and viscosity are given in Table 3. Blooming of developed 5, 10, 15, 20, and 30% EC showed good blooming when dispersed in water.After dispersion of ECs in water no creamy layer was observed and all formulations were stable for two hours. After passing these parameters ECs were kept at 54°C for 14 days and at low temperature of 10˚C for 1 hour. At both the temperatures, no phase separation occurred in all formulations. The pH values of developed ECs from seed oil were found in range of 6.26 to 5.44. All developed formulations did not catch fire till 54˚C thus these formulations complied flash point test.Viscosities of formulations fromseed oil were found in range of 51.3 to 92.2 cps. Physico-chemical parameter results indicated that developed ECs were thermodynamically stable that pass all test parameters.

Table 3: Physicochemical parameters of ***M. azedarach*** seed oil (%w/w) EC formulations

Sample Code	**Blooming**	**Emulsion Stability (1Hour)**	**Temperature Stability**		**pH**	**Flash Point**	**Viscosity (cps)**
			ATS[b] (at 54˚C±1˚C for 14days)	**Cold Test (at 10˚C for 1 hour)**			
EC-1	Excellent	Stable	Stable	Stable	6.26 ±0.2	Complies	51.3 ±1.3
EC-2	Excellent	√	√	√	6.08 ±0.03	√	59.2 ±1.2
EC-3	Very good	√	√	√	5.96 ±0.01	√	68.4 ±2.1
EC-4	Good	√	√	√	5.76 ±0.02	√	82.7 ±1.4
EC-5	Average	√	√	√	5.44 ±0.03	√	92.2 ±1.3

ATS[b] –Accelerated temperature stability

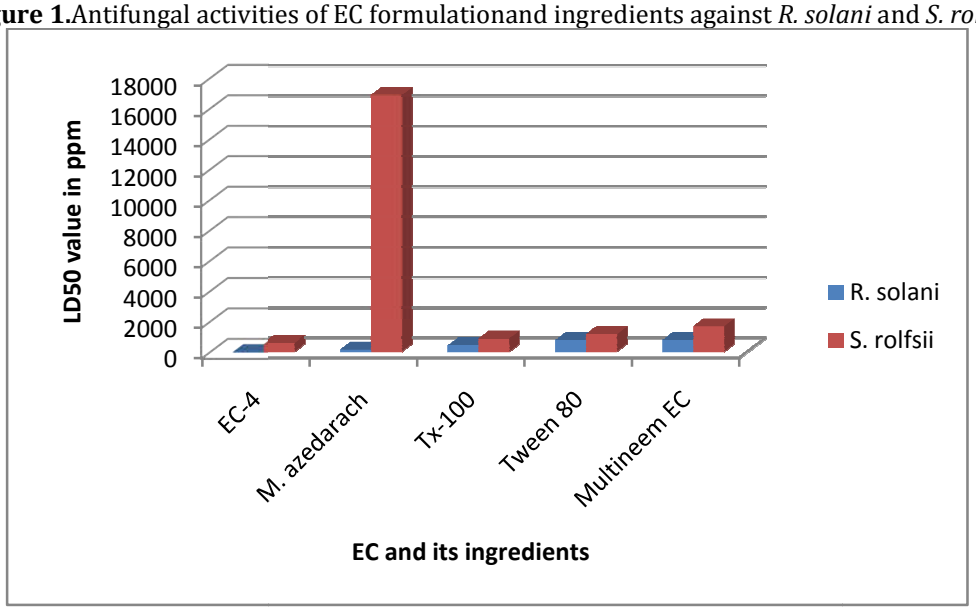
Bio-efficacy evaluation

In-vitro antifungal activities were evaluated by poisoned food technique. The developed formulation EC-4, seed oil, surfactants and multineem oil EC showed antifungal activities against *R. solani* and *S. rolfsii*. Fungicidal activities and their LD_{50} values and fuducial limits are reported in Table 4. LD_{50} values in histogram are presented in Figure **1**. The formulation EC-4 showed LD_{50} values 58.06 and 570.02 ppm against *R. solani* and *S. rolfsii* respectively. The seed oil of ***M. azedarach*** observed high LD_{50} values 138.70 and 16949.3 ppm against *R. solani* and *S. rolfsii* respectively. Surfactants used in formulation Tween-80 and Triton X-100 showed LD_{50} values 1150.29 and 866.71ppm against *S. rolfsii* while 756.29 and 409.99ppm against *R. Solani*fungus. Commercial neem oil EC (multineem) chosen as positive control showed LD_{50} values782.89 and 1670.41ppm against *R. solani*and *S. rolfsii* respectively.

Table 4. Antifungal activities of EC formulations against *R. solani* and *S. rolfsii*

Formulation, seed oil and surfactants	*R. solani*		*S. rolfsii*	
	LD $_{50}$ value (ppm)	Fuducial limit (ppm)	LD_{50} value (ppm)	Fuducial limit (ppm)
EC-4	58.06	41.93 – 80.39	570.02	441.12 – 736.58
M. azedarach	138.70	98.22 – 195.87	16949.3	2360.58 – 121697.9
Tx-100	409.99	360.39 – 466.45	866.71	383.98 – 567.26
Tween-80	756.29	625.58 – 915.12	1150.29	935.40 – 1414.56
Multineem EC	782.89	633.66 – 967.24	1670.41	5040.18 – 16078.03

Figure 1. Antifungal activities of EC formulationand ingredients against *R. solani* and *S. rolfsii*



The results of ATS and cold stability test, no phase separation and no sedimentation indicated that formulated ECs have thermodynamic stability. When the oil concentration increases in formulation after a particular concentration a slight creamy layer was observed in EC dispersion in water, this phenomenon limits the oil concentration. It was found that with the increasing concentration ofseed oil, the pH of formulations decreases, the viscosity of developed formulations increases with increase in the concentration of seed oil this phenomenontakes place due to high viscosity of seed oil.

In GC-MS analysis it was found that seed oil contains mainly fatty acid and their derivatives.The total area 71.52% detected as known phytochemicals compounds in seed oil in which some are reported as bioactive compounds. Hexadecanoic acid has the properties of antioxidant and nematicidal and pesticidal activities[17-18]. 9, 12-octadecadienoic acid (z,z) exhibits a broad spectrum of biological activities which can be used as nematicide, antibacterial, fungicidal agent [19]. Squalene was reported as antimicrobial, antioxidant, antitumor and fungicidal and pesticidal activities [20]. 2-decenal and 9-octadecenoic acid were responsible for fungicidal, pesticidal and nematicidal activity [21].

Methanolic fruit extract at different concentration of *M. azedarach* were found effective in controlling *S. rolfsii* pathogens [22]. β-sitosterol, β-amyrin, ursolic acid, benzoic acid, 3,5 dimethoxybenzoic acid and

maesol were isolated from the chloroform fraction of leave extract against Ascochytarabiei fungi [23]. Methanolic fruit extract has been found effective in suppressed growth of *R. solani, S.rolfsii* and other soil borne pathogens [24-25]. Methanol shoot extract of *M. azedarach* was found effective against *R. solani* and other fungi [26]. Present work is designed on development and standardization of EC from seed oil for controlling fungi. The antifungal activity data showed that the developed EC formulation was found more effective than seed oil, non-ionic surfactant used in formulation and commercial formulation (multineem) against selected plant pathogens. The LD_{50} value of developed formulation was compared with LD_{50} values of multineem (commercial formulation) which were found very lower than multineem (commercial formulation), indicating that developed formulation is much more effective than multineem (commercial formulation) against these plant pathogens. Multineem was found least effective, it may be due to low content of ***Azadirachtin*** (0.03%). Bio-efficacy results indicating that *R. solani* is more susceptible than *S. rolfsii* against developed formulations as well as seed oil. In present study, bio-efficacy and physicochemical parameters showed that developed formulations are stable and passes all parameters and very much effective for controlling plant pathogenic fungi. The EC is cost effective and most widely used formulation than other agrochemical formulation. On the basis of results it can be concluded that this research works would be helpful in organic farming and sustainable agriculture.

CONCLUSION

EC was successfully prepared from *M. azedarach* seed oil by using non-toxic surfactants (Tween-80 and Tx-100) and methyl oleate (biodegradable solvent). Developed ECs are thermodynamically and physically stable that passes all physicochemical parameters. The bio-efficacy study data shows that developed EC-4 wasmuch more effective than seed oil and multineem (commercial EC) against selected plant fungi. Since no toxic chemicals were used in the formulations, this effort would further the development of environmental friendly fungicides from seed oil (natural ingredient).

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