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Formulation and Bio-efficacy of Emulsifiable Concentrates of *Pongammia pinnata* and *Jatropha curcas* Seed Oils against Plant Pathogens

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Abstract : In present work, developed Emulsifiable Concentrates (ECs) from *Pongammia pinnata, Jatropha curcas* seed oil and their mixed oilin biodegradable solvent, nontoxic surfactants and evaluated their *in-vitro* antifungal activity against *Rhizoctonia solani* and *Sclerotium rolfsii* fungi.GC-MS analysis confirmed the presence of phytochemical constituents in both seed oils. Developed ECs were passes physico-chemical parameters. Formulations, PPEC-30 (30% oil), JCEC-30 (30%) and mixed oil EC (MEC-30)showed LD_{50} values 197.32,185.4 and 41.69 ppm against *R. solani* while LD_{50} values 437.38, 849.29 and 369.2 ppm against *S. rolfsii* respectively. LowLD_{50}value of developed ECs than other ingredient revealed that formulations were found more effective against pathogens so these formulations may be used as bio-fungicides.
Key Words : Antifungal Activities, Emulsifiable Concentrate, *J. curcas*, *P. pinnata*, Plant Pathogens.

Introduction

The injudicious use of synthetic pesticides is being discouraged owing to their toxic effects on non-target organisms, adverse effect on the environment[1]. Due to persistent use of synthetic pesticides, pathogens develop resistance against various chemical fungicides[2]. Studies have shown that botanical pesticides in general possess low mammalian toxicity,no adverse effect on plant growth, minimal residual effects,no environmental pollution, less hazards to non-target organisms, less expensive and easily available because of their natural occurrence[3]. Natural products derived from plants have been shown to be promising alternatives against plant pathogens than conventional pesticides[4,5].

R. solani and *S. rolfsii* are common soil-borne phyto-pathogenic fungi that cause various disease and damage to the plants.*R. solani* (teleomorph: *Thanatephorus* spp.) is a soil borne plant pathogenic fungus has a wide host range and worldwide distributions.This fungus attacks its hosts when they are in juvenile stages of developments and causes serious diseases like seedling blight of longleaf pine[6], damping off and root rot diseases to wide range of vegetable and crop plants[7], black scurf on tubers and shoot/stolon canker on young plants[8], Tobacco leaf spot and root rot[9]. *S. rolfsii* also 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[10,11].The study of this fungus is 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[12].

Extract of *J.curcas*has been studied to possesantifungal effect at 5%, 8% and 10% against *Aspergillus flavus, A.fumigatus, A.niger, A.terreus, Fusarium oxysporium* seed borne fungi and *Trichoderma harzanium* and *Rhizopussp*[13,14].Antifungal activity has been evaluated of curcin isolated from *J.curcas* seeds[15].Leaf extracts, stem extract, roots extract, latex and oil of *J. curcas*, are found effective for controlling plant pathogenic fungi *Alternaria alternata, A. flavus, A. niger, F. oxysporum,* and*R. solani*and plant pathogenic bacteria[16]. The plant possesantifungal activity against *A. solani* and *Helminthosporium turcicum* fungi[17].

In present work focus on formulating and optimization of Emulsifiable Concentrates (ECs) from seed oilof *P. pinnata* and *J. curacas* by using methyl oleate and Tween 80 and Triton X-100 as surfactantsto minimize the toxicity of formulationthat can be a saferbotanical pesticide. GC-MS analysis confirmed the presence of active chemical constituents.*In-vitro* bio-efficacy of developed ECswas evaluated against plant pathogenic fungi.

Material and Methods

Materials

P.pinnata and *J. curacas* seed oil were procured from Rakesh Products, Kanpur (U.P.)and Jatropha Vikas Sansthan, New Delhi respectively. Methyl oleatewas purchased from Mohini Organics Pvt. Ltd, Mumbai. Tween 80 and Triton X -100 were procured from Sd Fine, Mumbai. Potato-dextrose-agar (PDA) was procured fromSRL Pvt. Ltd. Mumbai. Multineem contains 0.03% azadirachtin (commercial bio pesticide) was purchased from Multiplex Agricare Pvt. Ltd.

Methods

GC-MS Analysis

GC-MS (Gas chromatography coupled with mass spectrometry) analysis of *P. pinnata, J. curcas* seed oil was carried out with SimadzuGC-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, 30 m × 0.25 mm diameter was 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 60.0 minute. The identification of compounds was performed by comparing their mass spectra with data from NIST and WILEY8 mass spectra library.

Preparation of ECs

ECswere developed by dissolving seed oils in methyl oleate with selected mixer of surfactants. Seed oil was dissolved in fixed quantity ofsolvent;blend of emulsifiers inspecific ratioafter screening was added to obtain a clear solution. Details of developed ECs are given in Table 1.

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 and flash point were carried out as per Bureau of Indian standard[18, 19].The thermodynamic stability of developed EC was carried out at 54˚C with storage for 14 days while low temperature stability at 0˚C for 24 hrs. Developed ECs were dispersed in hard water (342ppm) for checking blooming followed by milky white emulsion formation.Formed emulsions were kept undisturbed at room temperature for two hours to check phase separation and sedimentation.The pH of optimized and developed EC was determined using a digital pH meter (Eutech) at room temperature**.** The viscosity of the developed formulations was determined at 100 rpm by using Fungi lab Viscometer R model using spindle TR-10 at 25 ℃.

Table 1.EC formulations of *P.*pinnata, *J. curcas* and mixture (50:50) of both seed oils.

Formulation code	% of *P. pinnata* seed oil (w/w)	Surfactants	Percentage (%w/w)	Ratio of Surfactants	% of Methyl oleate (w/w)	Appearance
PPEC*-1	05	Tween 80 and TritonX-100	16	75:25	79	Transparent
PPEC-2	10				74	√
PPEC-3	15				69	√
PPEC-4	20				64	√
PPEC-5	30				54	√
JCEC*-1	05	Tween 80 and TritonX-100	16	75:25	79	Transparent
JCEC-2	10				74	√
JCEC-3	15				69	√
JCEC-4	20				64	√
JCEC-5	30				54	√
MEC*-1	05	Tween 80 and TritonX-100	16	75:25	79	Transparent
MEC-2	10				74	√
MEC-3	15				69	√
MEC-4	20				64	√
MEC-5	30				54	√

PPEC*- *P. Pinnata* EC, JCEC*- *J. curcas*EC, MEC*- mixed oil EC

Bio-efficacy Evaluation

Phyto-pathogenic fungi *Rhizoctonia solani* ITCC 5563 and *Sclerotium rolfsii* ITCC 6181 were procured from Indian Type Culture Collection (ITCC) centre, Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi, India. Cultures of the test fungi were maintained on PDA slant at 26°C for six days and were sub-cultured in petri dishes prior to testing.

Preparation of Media

3.9% PDA culture solutions was prepared in distilled water and 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 formulations(PPEC-30, JCEC-30 and mixed oil EC-30), seed oils, seed oils mixture and surfactants used in formulations were selected for their antifungal study against *R. solani* and *S. rolfsii* plant pathogens. 5 mL stock solution of emulsions of 50,000 ppm were preparedfrom developed PPEC-30, JCEC-30 and mixed oil EC-30 by dissolving in distilled water. 2 mLstock solution of 1,00,000 ppm in acetone was prepared from each seed oilof *P. pinnata,J. curcus* and mixed oil, while 2 mL stock solution of 1,00,000 ppm in distilled water of non-ionic surfactants Triton X-100 and Tween 80 was prepared. 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, 0.125mL, 62.5 µL and 31.25µL were added to each 100 mL 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 from conical flask was poured into petri dish with three replications under aseptic conditions in a laminar flow chamber and allowed the media to solidify. Similar procedurewas followed for second fungi.

Inoculation and Incubation

A 5 mm thick disc of fungus (spores and mycelium) cut from earlier sub-cultured fungus, in a petriplate was inoculated aseptically to the center of the petriplate. Treated, acetone control and controlpetri 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)] × 100

CF is the correction factor obtained from the equation CF = ((90-Co)/Co) × 100, where90 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 GWbasic software.

Results

In *J. curcas* and *P. pinnata* seed oil 20 and 21 phytochemicals were detected respectively by GC-MS. Details of chemical constituents are given in Table 2 and 3.

Table 2.Phytochemicals in *J. curcus* seed oil.

Compound name	Molecular weight	Retention time	% Area	Base ion (m/z)	Other fragments ion(m/z)
Decenal	156	7.73	0.01	55	70, 96, 22
Decanal-2	154	7.85	0.37	55	70, 98, 136
2, 4 –decadienal	152	8.14	0.09	81	67, 96, 152
Bicycle [7,2,0] undecan-4-ene 4,11,11 –trimethyl	204	9.41	0.13	41	93, 105, 133 204
Nepthalene	204	10.1	0.01	105	69, 93, 204
Undecanoic acid	214	10.18	0.01	60	57, 143, 214
α-Cadinol	222	10.96	0.08	95	55. 134,
Pentadecanoic acid methyl ester	256	12.29	0.02	74	55, 143, 256
Hexadecanoic acid methyl ester	256	15.2	1.99	74	55, 97, 143, 270
Methyl octadeca 9, 12- dinoate	294	18.41	2.53	67	81, 96, 123, 294
9 -Octadecanioc acid methyl ester	296	18.58	8.30	55	74, 97, 123, 264
Octadecanoic acid (stearic acid)	298	19.79	5.87	74	55, 97, 143, 298
Methyl dihydromelvate	296	22.85	0.16	55	83, 97, 152
Eicosonoic acid methyl ester	326	23.45	0.38	74	55, 97, 143
9, 12- Octadecadien-1 ol	266	26.7	0.2	67	81, 96
9-Octadecanoic acid	282	26.84	0.35	55	69, 83
Docosonoic acid	354	28.22	3.52	340	25, 129, 143
9 -Octadecenoic acid	356	28.51	4.80	55	81, 98, 264
Squalene	410	34.81	0.18	69	81, 341
Stigmasta 5, 22 –dien-3-ol acetate	454	43.62	0.35	43	55, 97

Table 3.Phytochemicals in *P. pinnata* seed oil.

Compound name	Molecular weight	Retention time	% Area	Base ion (m/z)	Other fragments ion(m/z)
Dodecane	170	7.26	1.01	57	71, 98
1 -Octanal 2- methyl	186	7.33	0.14	59	71, 97
Decenal-2	154	7.85	0.85	43	55, 70
2, 4-Decadienal	152	8.14	3.14	81	67, 96, 152
1-Eicosanal	298	8.59	0.28	43	41, 57, 81

Tetradecane	198	9.05	0.93	57	71, 99, 198
Hepatadecane	240	9.95	0.90	57	71, 99
Eicosane	282	11.97	0.56	57	71, 282
Nonadecane	268	14.76	0.39	57	71, 268
Pentadecanoic acid 14-methyl ester	256	15.18	1.94	74	55, 143, 256
9-Octadecanoic acid	282	16.05	5.15	55	69, 83
Undecanoic acid	214	16.43	0.62	60	57, 143, 214
Methyl octadeca 9, 12-dinoate	294	18.33	3.86	67	81, 96, 123, 294
9 -Octadecanoic acid methyl ester	296	18.45	2.13	55	74, 97, 123, 264
9, 12-Octadecadinoic acid	280	19.54	6.29	67	55, 97
1, 2-Benezenedicarboxylic acid	390	28.45	1.90	104	57, 71 279
Linoleic acid methyl ester	308	28.53	1.48	67	81, 96, 263
Tetratetraacontane	619	28.64	0.73	57	43, 71, 85
Squalene	410	43.53	1.29	69	81, 341
Stigmast-5-ene-3-ol	414	44.84	14.86	43	81, 107, 145

Physico-chemical Parameters

The results of physico-chemical parameters are given in Table 4. Blooming of developed 5%, 10%, 15%, 20% and 30% ECs showed good result 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 low temperature (10°C) for one hour. At both high and low temperature, no phase separation occurred in all formulations. The pH of developed ECs from seed oil of *P. pinnata,J. curcus*andmixture oilwere found in the range of 6.56 to 5.02, 6.95 to 4.88 and6.72 to 4.99 respectively.All developed formulations did not catch fire till 54°C thus these formulations comply flash point test. Viscosity of developed ECs from seed oil of *P. pinnata,J. curcus* and mixture oil were found in range of 31.3 to 63.2 cps, 33.1 to 64 cps and 32.2 to 63.6 cps respectively.Physico-chemical parameter results indicated that developed ECs were thermodynamically stable that passes all test parameters.

Table 4. Physicochemical parameters of *P. pinnata* seed oil EC formulations (w/w)

Sample Code	**Blooming**	**Emulsion stability**	**ATS[b] test**	**Cold test**	**pH**	**Flash point**	**Viscosity (cps)**
PPEC-5	Excellent	Stable	Stable	Stable	6.56±0.2	Complies	31.3±1.3
PPEC-10	Excellent	√	√	√	6.16±0.03	√	39.2±1.2
PPEC-15	Very good	√	√	√	5.73 ±0.01	√	49.4±2.1
PPEC-20	Good	√	√	√	5.55±0.02	√	51.7±1.4
PPEC-30	Good	√	√	√	5.02±0.03	√	63.2±1.3
JCEC-5	Excellent	√	√	√	6.95±0.02	√	33.1±1.6
JCEC-10	Excellent	√	√	√	5.69±0.03	√	37.3±1.7
JCEC-15	Good	√	√	√	4.98±0.03	√	52±1.9
JCEC-20	Very good	√	√	√	4.94±0.01	√	54±1.2
JCEC-30	Good	√	√	√	4.88±0.02	√	64±1.3
MEC-5	Excellent	√	√	√	6.72±0.02	√	32.2±1.1
MEC-10	Very good	√	√	√	5.92±0.03	√	37.3±1.5
MEC-15	Good	√	√	√	5.38±0.02	√	50.6±1.9
MEC-20	Good	√	√	√	5.21±0.04	√	53.2±1.8
MEC-30	Good	√	√	√	4.99±0.03	√	63.6±1.9

b: ATS –Accelerated temperature stability

Bio-efficacy Evaluation

In-vitro antifungal activities of formulations and seed oil were evaluated are in Table 5. The formulation PPEC-30 and JCEC-30 showed LD_{50} values 197.32 and 185.4 ppm against *R. solani* while these formulations showed LD_{50} values 437.38 and 849.29ppmagainst *S. rolfsii*.LD_{50} values of EC developed from mixed seed oil (MEC-30) were observed to be 41.69 and 369.62ppm against *R. solani*and *S. rolfsii* respectively. The seed oil of *P. pinnata*, *J. curcas* and mixture of oil observed high LD_{50} values 4377.26, 13435.9 and 1574.18ppm against *S. rolfsii* while 821.64,1031.79 and 571.51 ppm against *R. solani* respectively. Tween 80 and Triton X-100 surfactants used in formulation 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 (mutineem) chosen as positive control showed LD_{50} values 782.89 and 1670.41ppm against *R. solani*and *S. rolfsii* respectively.

Table 5.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)**
J. curcus	1031.79	877.72 – 1212.89	39435.89	18049.06 – 86164.50
P. Pinnata,	821.64	696.98 – 968.62	4377.26	2877.92 – 6357.02
Multineem	782.89	633.66 – 967.24	1670.41	5040.18 – 16078.03
Tween 80	756.29	625.58 – 915.12	1150.29	935.40 – 1414.56
Mixed oil	571.51	487.15 – 670.48	1574.18	1223.76 – 2024.94
Tx-100	409.99	360.39 – 466.45	866.71	383.98 – 567.26
PPEC30	197.32	175.48 – 221.88	437.38	392.62 – 509.78
JCEC30	185.40	165.93 – 207.16	849.29	719.43 – 1002.60
MixEC30	41.69	41.69 – 74.24	369.62	323.45 – 408.12

Discussion

In ATS and cold stability tests, there was no phase separation and sedimentation which indicates that formulated ECs are thermodynamically stable. 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. The viscosity of developed formulations increases with increase in the concentration of seed oil.This phenomenontakes place due to high viscosity of seed oil, 163±1.4 and 173±1.5cps of *J. curcus* and *P. pinnata* respectively.

In GC-MS analysis, it has been found that both seed oil contains mainly fatty acid and their derivatives.The major constituents in *J.curcas* seed oil aremethyl ester of hexadecanoic acid (1.99%), methyl octadeca 9,12-dinoate (2.53%), 9 -octadecanioc acid methyl ester (8.30%), octadecanoic acid (5.87%), docosonoic acid (3.52%), 9 -octadecenoic acid (4.8%). The major components detected in *P. pinnata* seed oil are dodecane (1.01%), 2,4-decadienal (3.14%), pentadecanoic acid 14-methyl ester (1.94%), 9-octadecanoic acid (5.15%), methyl octadeca 9, 12-dinoate (3.86%), octadecanoic methyl ester (2.13%), 9,12-octadecadinoic acid (6.29%) 1,2-benezenedicarboxylic acid (1.90%), linoleic acid methyl ester(1.48%), squalene (1.29%) and stigmast-5-ene-3-ol (14.86%).Hexadecanoic acid,squalene and naphthalene have properties of antioxidant and nematicidal and pesticidal activities[20, 21]. 9,12-octadecadienoic acid (z,z) exhibits a broad spectrum of biological activities which can be used as nematicide, antibacterial, fungicidal and insectifuge agent[22].Stigmast-5-ene-3-ol (14.86%) and 9,12-octadecadinoic acidhas been reported as bioactive compounds[23].

In literature, various reports are available on fungicidal and insecticidal activities of *P. pinnata* and *J. curcas* seed oils and to the best of our knowledge, there is no report on antifungal activity of their ECsagainst *R. solani* and *S. rolfsii.* Seed oil contains secondary metabolites which are responsible for biological activities. These properties make seed oil products safer and effective for controlling the pest. The EC is cost effective and most widely used formulation than other agrochemical formulation.On the basis of previous study, present work is designed on formulation and optimization of EC from seed oil for controlling fungi. The antifungal activity data showed that the developed EC formulations were more effective than seed oil, non-ionic surfactantused in

formulation, and commercial formulation (multineem) against selected plant pathogens. In case ofseed oils and developed ECs,mixture of oil and developedmixed ECs, it has been observed that efficacy increased in both cases due to synergistic effect. *P. pinnata* oil and developed formulation(PPEC-30)showed better efficacy against both fungi than *J. curcas* seed oil as well as its developed formulation (JCEC-30).TheLD_{50} values of developed formulations were compared with LD_{50} values of multineem (commercial formulation) which were found lower than multineem (commercial formulation), indicating that developed formulations are much more effective than multineem(commercial formulation) against these plant pathogens. Multineem used as supplied by was found least effective which may be due to low content of Azadirachtin(0.03%).Bio-efficacy results indicated that *R. solani* is more susceptible than *S. Rolfsii* against developed formulations as well as seed oil.

The literature supports that formulation provides better efficacy than the used ingredients[24]. In present study, bio-efficacy and physicochemical parameters showed that developed formulations are stable and passes all parameters and very effective for controlling plant pathogenic fungi and these formulations are potential candidates to be used as natural fungicides and are excellent alternatives to synthetic pesticides.

Conclusion

To conclude, stable ECs and Mixed ECs were successfully prepared from *P.pinnata* and *J. curacas* seed oil by using non-toxic surfactants (Tween 80 and Tx-100) and methyl oleate (biodegradable solvent). Developed ECs were observed to be thermodynamically and physically stable that passes all physico-chemical parameters. The bio-efficacy study data shows that developed ECs were found much more effective than both seed oils and multineem EC (commercial EC) against selected soil borne plant pathogens.This effort will be helpful for the development of environment friendlybio-fungicides from seed oils (natural ingredient) as an alternative to toxic synthetic pesticides to minimize negative impact on human health and environment.

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