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ORIGINAL ARTICLE

Water based (o/w) Microemulsions from *Pongammia pinnata* Seed oil and their *In-vitro* Bio-efficacy against Plant Pathogens.

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ABSTRACT
The aim of present study is to develop water based (o/w) microemulsions (ME) from seed oil of Pongammia pinnata known as P. pinnata in non-ionic surfactant and n-BuOH as co-surfactant, and evaluate in-vitro bio-efficacy against Rhizoctonia solani and Sclerotium rolfsii plant pathogens. This selected plant belongs to Fabaceae family which is well described for their fungicidal and insecticidal properties. Oil in water microemulsions were formulated in Tween- 80 and Triton X-100 surfactant using n-butanol as co-surfactant. Physico-chemical parameters like thermodynamic stability, physical stability, viscosity, conductivity, total dissolve salt (TDS) and pH measurements were carried out for developed MEs. Oil in water (o/w) microemulsion regions were investigated in pseudo ternary phase diagrams at different surfactant and co-surfactant ratios (2:1, 4:1, 9:1 and 19:1) in both surfactants. Out of total six formulations, LD_{50} values of selected two formulations F-3 (1.8 % oil) and F-6 (2.5% oil) were observed to be 215.31 mg/L and 48.82 mg/L against R. solani, and 602.17 mg/L and 80.22 mg/L were observed against S. rolfsii. LD_{50} values of developed formulations were found to be much lower than P. pinnata seed oil, surfactants (Tween-80 and Tx-100) and multineem (positive control). Transmission Electron Microscope (TEM) analysis result showed that particle size of the developed F-3 and F-6 formulations were in the range 91.61 nm to 115.51 nm and 48.19 nm to 127.06 nm respectively. The developed formulations F-3 and F-6 were found effective for controlling selected soil borne plant pathogens and these bio-fungicides may be potential alternatives to synthetic fungicides.
Key Words: *Microemulsion; Pongammia pinnata; Phase behavior; Antifungal activities; Plant pathogens.*

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INTRODUCTION

The injudicious use of synthetic pesticides is being discouraged because of their toxic effects on non-target organisms and adverse effect upon the environment due to their long term persistence [2]. Natural products derived from plants have been shown to be promising alternatives to control plant pathogens [8]. Therefore, there is a need to develop bio pesticides which are effective, biodegradable and do not leave any harmful effect on environment.

S. rolfsii and *R. solani* are important soil-borne phyto-pathogenic fungi that cause disease and damage to the plants. *S. rolfsii* has wide host range due to its abundant growth and capability of producing excessive *sclerotia* that may persist in soil for several years [5]. *S. rolfsii* is a soil-borne fungal pathogen that causes diseases in a wide range of horticultural and agricultural crop plants. It has over 500 species hosts in 100 plant families [7]. *R. solani* is also a soil borne pathogenic fungi that causes damping-off disease in more than 200 crops globally resulting in yield losses [11].

Microemulsion is spontaneously forming single-phase colloidal dispersion of either oil-in-water (o/w) or water-in-oil (w/o) stabilized by an interfacial film of surfactant(s) and co-surfactant(s) [2]. It is isotropic and dispersed micro- heterogeneous systems composed of water, oil and amphiphile. ME system has

several advantages including enhanced drug solubility, ease of manufacturing, good thermodynamic stability and enhancement effect on transdermal ability over conventional formulation [16].
Available literature showed that *P. pinnata* has fungicidal and insecticidal properties. Plant possessed antifungal activity against *A. solani* and *Helminthosporium turcicum* fungi [12, 13]. Plant extract was found effective to control *Alternaria alternata, A. flavus, A. niger, A. fumigates* and *Rhizopus sp* fungi [9]. This seed oil was found to provide maximum protection against fungal disease of pea [4].
The present work focuses on the formulation of oil-in-water microemulsions from seed oil of *P. pinnata* in non-ionic surfactant and n-BuOH as co-surfactant, and investigation of oil-in-water (o/w) microemulsion regions at various Smix ratios by pseudo-ternary phase diagram. Physicochemical parameters and TEM analysis were carried out for characterization of formulations. Bio-efficacy of developed MEs was evaluated against *R. solani* and *S. rolfsii* plant pathogens.

MATERIAL AND METHODS

Materials

Seed oil of *P. pinnata* was procured from Rakesh Products, Kanpur (U.P.). Tween-80, Triton X -100 surfactants and n-butanol (n-BuOH) were procured from Sd Fine, Mumbai. Potato dextrose-agar was supplied by SRL Pvt. Ltd. Mumbai. Multineem containing 0.03% *azadirachtin* (commercial neem based bio pesticide) was purchased from Multiplex Agricare Pvt. Ltd.Tiumur, Karnataka.

Methods

Solubility study

Solubilization study of seed oil was carried out in 10%, 20% and 30% micellar solution (w/w) of Tween-80 and Triton X-100 as surfactant in distilled water at room temperature (Table 1). Due to high viscosity of oil, desired quantity of n-BuOH (co-surfactant) was mixed for easy solubilization in micellar solutions.

Microemulsion preparation

Based on solubility study of seed oil in surfactant and n-BuOH, microemulsions were formulated using *P. pinnata* seed oil as active ingredient, Tween-80 or Tx-100 as surfactant, n-BuOH as co-sufactant and distilled water. The required quantity of seed oil was mixed with surfactant and n-BuOH thoroughly. The clear blend mixture of seed oil, surfactant and co-surfactant was slowly added in fixed quantity of distilled water using magnetic stirrer at room temperature till the clear solution was obtained. After this step, the clear solution was sonicated for 30 minutes by using ultra probe sonicator. The details of developed *P. pinnata* oil microemulsions are given in Table 2.

Preparation of Pseudo Ternary Phase diagram

Titration method was used in construction of pseudo-ternary phase diagrams. The physical state of the micro emulsion was marked on a pseudo three component phase diagram with one axis representing aqueous phase (distilled water), the other representing oil (*P. pinnata* seed oil) and the third representing mixture of surfactant and co-surfactant at fixed weight ratios (Smix ratio). Surfactant (Tween-80 or Triton X-100) and co-surfactant (n-BuOH) ratio were grouped in four different combinations for phase studies i.e. 2:1, 4:1, 9:1 and 19:1 which were presented in text R ratio (co-surfactant : surfactant) as R = 0.5, R = 0.25, R = 0.11 and R = 0.053 respectively. These Smix ratios are chosen in increasing concentration of surfactant w.r.t. co-surfactant. For each phase diagram, Smix and water were mixed thoroughly in different weight ratios from 0:10 to 10:0 (0:10, 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, 9:1 and 10:0) in different glass vials. Slow titration at room temperature with oil phase was done with each weight ratio of aqueous and Smix; and visual observation was carried out for transparent and clear microemulsion until the appearance of persistent turbidity. The weight percent ratio of Smix, *P. pinnata* oil and water of each system was calculated and results were plotted on triangular co-ordinates to construct the ternary phase diagram by using Originpro8 software.

Characterization of microemulsion

Physicochemical parameters of formulated microemulsion, and formulation without seed oil (control formulation) were performed. Appearance of microemulsions was observed to be clear and homogeneous. The prepared microemulsions were dispersed in tap water and were observed to form clear solution. After passing above step further parameters were carried out as discussed below.

Thermodynamic stability

The thermodynamic stability of developed microemulsions was carried out at accelerated temperature stability (ATS-54^{0}C) with storage for 14 days, while low temperature stability was observed at 0^{0}C for 7 days. Those formulations which were stable at these temperatures were subjected to centrifugation test.

Centrifuge test

The developed formulations were centrifuged at 3500 rpm for 30 minutes (Remi-12, Japan) and those formulations that did not show any phase separation were taken for further test.

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.

Measurement of pH

The pH of developed microemulsions was measured using a digital pH meter (Eutech) at room temperature**.**

Conductivity measurement

Conductivity measurements were carried out to demonstrate the effect of water dilution on microemulsion. The conductivity of developed microemulsion formulations were measured using conductivity meter (Eutech) at room temperature.

Total dissolved salt (TDS)

The TDS of developed microemulsion formulations were measured using TDS meter (Eutech) at room temperature.

TEM analysis

Morphology and particle size of developed micro-emulsions (F-3 and F-6) were studied using TEM technique. To perform the TEM observations, 2 µL of formulation was taken and put on var-coated grid. After 2 min, 2µL of uranyl acetate was added and dried for 1hour. After 1 hour grid was taken and observed for the image.

Bio-efficacy evaluation

Phytopathogenic test 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-110012, India. Cultures of the test fungi were maintained on potato dextrose agar (PDA) slant at 26°C for at least 4–7 days and were sub-cultured in Petri dishes prior to testing. The test fungi were routinely grown on fresh PDA media in petridish and stored at 4˚C.

Preparation of media

39 g of potato dextrose agar was suspended in 1000 mL distilled water and was boiled to obtain uniform media. 100 mL media was transferred to each of the 100 mL conical flasks and then 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

20 mL stock solution each of 15,000 mg/L concentration of developed microemulsions (F-3) and (F-6) were prepared by dissolving in distilled water. 5 mL emulsion solution of 50,000 mg/L was prepared by dissolving multineem (commercial EC) in distilled water. 2 mL stock solution of 100,000 mg/L of *P. pinnata* oil was prepared in acetone while the 2 mL stock solution each of 100,000 mg/L of non-ionic surfactants Tween-80 and Tx-100 were prepared in distilled water.

For preparing test solutions of 500, 250, 125, 62.5 31.25 15.625 and 7.81 mg/L concentration, 6670, 3330, 1670, 833, 417, 208 and 104 µL of stock solution of developed MEs was added in conical flasks each containing 100 mL of sterile media respectively. For preparing test solutions of multineem of 500, 250, 125, 62.5 31.25, 15.625 and 7.81 mg/L concentration, 1000, 500, 250, 125, 62.5, 31.25 and 15.625 µL stock solution of multineem was added in conical flasks each containing 100 mL sterile media respectively.In case of *P. pinnata* oil and surfactants, 1 mL of stock solution (100,000 mg/L) was added in 100 mL media to make 1000 mg/L, the remaining stock solutions were further diluted by 1 mL of acetone and distilled water respectively. 1 mL of diluted stock solution was further added to 100 mL media to make 500 mg/L. For preparing 250, 125, 62.5, 31.25 and 15.625 mg/L media, the stock solutions were serially diluted with acetone and water respectively and 1-1 mL stock solutions were added in conical flasks each containing 100 mL sterile media. About 30 mL media from each conical flask was poured into petriplate with three replications under aseptic conditions in a laminar flow chamber and allowed the media to solidify. Similar method was followed for the second fungus.

Inoculation and incubation

A 5 mm thick disc of fungus (spores and mycelium) was cut from petriplate of earlier sub-cultured fungus and inoculated aseptically to the center of the petriplate containing solid media of different concentrations. All the treated petriplates, control of each fungus and acetone control (containing 250 µL acetone in each petriplates) in replicates were kept in Biological Oxygen Demand (B.O.D.) incubator at 27 ˚C (± 1˚C) till the fungal growth was almost completed in the control petriplate. 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 fungus 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

Solubility of P. pinnata oil

In order to screen appropriate concentration of surfactant for the preparation of microemulsions, the solubility of *P. pinnata* oil with n-butanol in single micellar solution was measured. It was observed that the solubility of *P. pinnata* oil increased with the increasing concentration of the surfactant. Maximum solubilisation of *P. pinnata* oil was found 2.56 % in Tx-100 micellar solution while 1.83% in Tween-80 micellar solution (Table 1).

Phase behavior and co-surfactant effect of the system

Phase diagrams of pseudo ternary system composed of Smix (surfactants: Tween- 80 and Tx-100, co-surfactant: n-butanol), *P. pinnata* oil and water at room temperature were constructed. Based on visual identification, regions corresponding to clear isotropic systems were considered microemulsion areas. The main objective was to determine the oil in water (o/w) microemulsion region.

Tween-80 and n-BuOH/ P. pinnata oil /water system

In case of Tween-80 surfactant, four phase diagrams were constructed as shown in Fig. 1a to 1d at same Smix ratio. All phase diagrams were found in adjoining stage. It was observed that solubilisation of oil increased with increasing concentration of Smix in all four R ratios. Solubilisation of oil was found very low at initial concentration of Smix as compared to water. When the concentration of Smix exceeds with respect to water, solubilisation of oil increases very fast, resulting in expansion of microemulsion region in all R ratios. With increasing concentration of Tween-80 with respect to n-BuOH in Smix, solubilisation of oil increases at low concentration as well as higher concentration of Smix. Maximum solubilisation of oil was found R = 0.053 and minimum solubilisation was found at R = 0.5 at low concentration of surfactant as well as higher concentration of surfactant with respect to water as shown in Fig 1d and Fig 1a respectively. It was noticed that solubilisation of *P. pinnata* oil increased with decreasing concentration of n-BuOH w.r.t. to Tween-80 and thus expansion in oil in water region was observed. Microemulsion region (o/w) were found in the order R=0.053>R=0.11>R=0.25> R=0.5.

Tx-100 and n-BuOH/ P. pinnata oil/ water system

Four phase diagrams (R= 0.5, R= 0.25, R = 0.11 and R = 0.053) were constructed in presence of Tx-100, n-butanol, *P. pinnata* oil and water for investigating microemulsion regions which are shown in Fig. 2a-d. All phase diagrams showed adjoining pattern and were similar to some extent. At R=0.5, solubilisation of oil was very low at initial concentration but at higher concentration of Smix as compared to water, solubilisation of oil was increased which resulted in expansion of microemulsion region as shown in Fig 2a. At R = 0.25 area of microemulsion (o/w) region (Fig.1b) was more expanded than others due to dramatically increased consumption of oil at higher concentration of Smix with respect to water phase. When the concentration of Tx-100 increased with respect to n-BuOH at R = 0.11 and R 0.053, solubilisation of oil decreased at higher concentration of Smix and the resulting o/w region was reduced (Fig. 1c and Fig.1d). In case of R=0.053 and R=0.11Smix ratio, at low concentration of Tx-100 w.r.t. to water it was observed that solubilisation of oil slightly increased as compared to R=0.25 and R= 0.5. In case of Tx-100, the order of oil in water ME regions was found R=0.25 >R= 0.5>R=0.053≥R=0.11.

Characterization of microemulsion

Stability

The formulations which were developed using non-ionic surfactant were found stable, no phase separation occurred at ATS (54 °C) with storage for 14 days. The developed MEs were stable at 0°C for 7 days and neither phase separation nor sedimentation was observed (Table 3).

Centrifuge test

No phase separation occurred in all the developed formulations when they were centrifuged at 3500 rpm for 30 minutes which indicating kinetic stability of all the formulations (Table 3).

Viscosity measurement

The minimum viscosity of the developed formulations was 9.2 cps while maximum viscosity was 179.2 cps determined at 100 rpm. In case of control formulation viscosity range was noticed from 7.2 to 96.5 cps at room temperature (Table 4).

Measurement of pH

The pH of formulations was observed to be in the range 5.17 to 5.82, while in control formulation pH range was 5.17 to 6.36 (Table 4).

Conductivity measurement

Electrical conductivity measurements can be used to determine the microstructure of microemulsion. The conductivity of formulation in Tween-80 surfactant was observed in the range 91.2 to 138 424 µS/cm while in control formulation it was ranging from 113.2 to 162 µS/cm. The conductivity of formulation with Tx-100 surfactant was observed in the range from 229 to 424 µS/cm while in control formulation it was found to be 196 to 381 µS/cm (Table 4).

TDS measurement

The total dissolved salt of developed microemulsion formulations was measured using TDS meter at room temperature. The TDS value of formulations was determined from 44.6 to 213 ppm while in control formulation this value was observed from 55.3 to 194 ppm (Table 4).

TEM analysis

TEM is one of the important techniques used to measure the size of microemulsion droplets. In this study, microemulsion samples (F-3 and F-6) containing seed oil of *P. pinnata* had TEM images at 200 nm scale (Fig. 3.a and 3.b). TEM analysis result showed that particles size of developed F-3 formulation was in 91.61 to 115.51 nm range while in formulation F-6 droplet size was in range 48.19 to 127.06 nm. TEM images are shown in Fig 4a and 4b.

BIO-EFFICACY STUDY

Bio-efficacy (antifungal activity) of developed microemulsions (F-3 and F-6), seed oil, surfactants used in formulations and multineem EC were evaluated against *R. solani* and *S. rolfsii* in *in-vitro* condition by the poisoned food technique. The LD_{50} values of formulated microemulsions F-3 and F-6 were observed to be 215.31 and 48.82 mg/L concentration against *R. solani* respectively while 602.17 and 80.22 mg/L against *S. rolfsii* respectively. LD $_{50}$ values of *P. pinnata* seed oil were observed to be 821.64 and 4377.26 mg/L against *R. solani* and *S. rolfsii* respectively. LD_{50} values of surfactants Tween-80 and Tx-100 that are used in formulation were found to be 756.29 and 709.99 mg/L against *R. solani* while these surfactants showed LD_{50} value of 1150.29 and 466.71 mg/L against *S. rolfsii*. LD_{50} value of Multineem EC (positive control) was observed to be 782.89 and 1670.41 mg/L against *R. solani* and *S. rolfsii* respectively. The LD_{50} values and fiducial limits of formulations and other components are given in Table 5 and graphical presentation is given in Fig 5.

Table 1. Solubility of *P. pinnata* oil (w/w %) in aqueous micellar solutions.

Surfactant	N-BuOH (w/w %)	Aqueous micellar solutions (w/w %)	Solubility (w/w %) of *P. pinnata* oil
Tween-80	1.0	10	0.5 ± 0.03
	2.0	20	1.24 ± 0.03
	3.0	30	1.83 ± 0.02
Tx-100	0.5	10	0.63 ± 0.01
	1.0	20	1.53 ± 0.03
	1.5	30	2.56 ± 0.04

Table 2: Microemulsions of *P. Pinnata* oil (w/w %).

Chemical reagents	F-1	F-2	F-3	F-4	F-5	F-6
Tween- 80	10	20	30			
Tx-100	-	-	-	10	20	30
n-BuOH	1	2	3	0.5	1	1.5
P. pinnata seed oil	0.5	1.2	1.8	0.6	1.5	2.5
Make up distilled water	Up to 100	Up to 100	Up to 100	Up to 100	Up to 100	Up to 100

Table 3. Stability of developed formulations

Formulation Code	Stability at 54 °C	Stability at 0 °C	Centrifuge test at 3500 rpm	Appearance
F-1	Stable	Stable	Stable	Clear
F-2	√	√	√	√
F-3	√	√	√	√
F-4	√	√	√	√
F-5	√	√	√	√
F-6	√	√	√	√

Table 4 Physicochemical parameters of developed formulations and controls.

Code	Viscosity (cps)		pH		Conductivity (µS/cm)		TDS (ppm)	
	Formulation	Control	Formulation	Control	Formulation	Control	Formulation	Control
F-1	9.2 ± 1.6	7.2 ± 1.4	5.82 ± 0.02	6.36 ± 0.03	91.2	113.2	44.6	55.3
F-2	18.2 ± 1.5	15.6 ± 1.5	5.82 ± 0.02	5.95 ± 0.02	137.2	152	68.2	77.3
F-3	179.2 ±1.3	96.5 ± 1.4	5.79 ± 0.03	5.83 ± 0.03	138	162	69.7	83.4
F-4	15.4 ± 1.4	10.69 ± 1.3	5.69 ± 0.02	5.58 ± 0.01	229	196	114	101
F-5	22.3 ± 1.7	19.6 ± 1.7	5.30 ± 0.01	5.29 ± 0.02	357	310	183	155
F-6	63.9 ± 1.8	48.2 ± 1.4	5.17 ± 0.02	5.17 ± 0.02	424	381	213	194

Table 5 Antifungal activity of microemulsions, surfactant and seed oil against *R. solani* and *S. rolfsii.*

Formulation Seed oil and surfactants	*R. solani*		*S. rolfsii*	
	LD_{50} value (mg/L)	Fiducial limit (mg/L)	LD_{50} value (mg/L)	Fiducial limit (mg/L)
F-6	48.82	36.96 – 64.49	80.22	62.45 – 103.05
F-3	215.31	192.89 – 240.34	602.17	527.31 – 687.67
Tx-100	709.99	660.39 – 766.45	466.71	383.98 – 567.26
Tween- 80	756.29	625.58 – 915.12	1150.29	935.40 – 1414.56
*P. pinnata*oil	821.64	696.98 – 968.62	4377.26	2877.92 – 6357.02
Multineem EC	782.89	633.66 – 967.24	1670.41	5040.18 – 16078.03

Fig 1a-1d Ternary phase diagram using Tween-80/n-BuOH/ *P. pinnata* seed oil/ water system with Smix (2:1, 4:1, 9:1 and 19:1) ratios.

Fig 1a

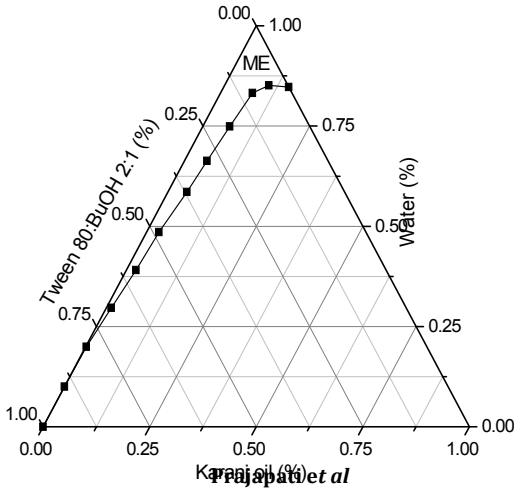
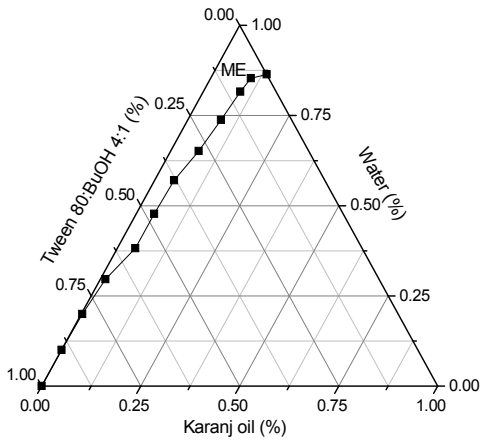


Fig 1b



Fiig 1c

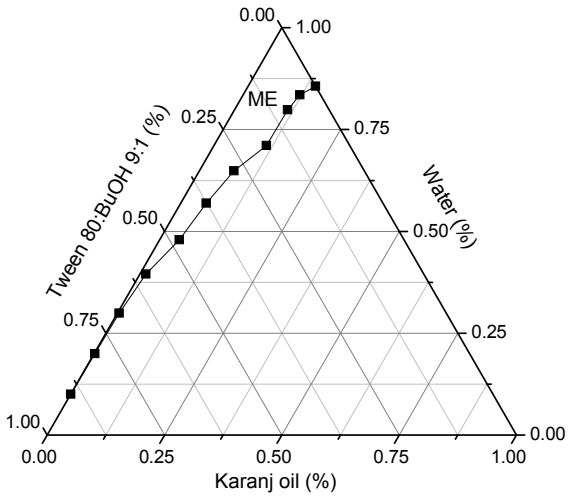


Fig 1d

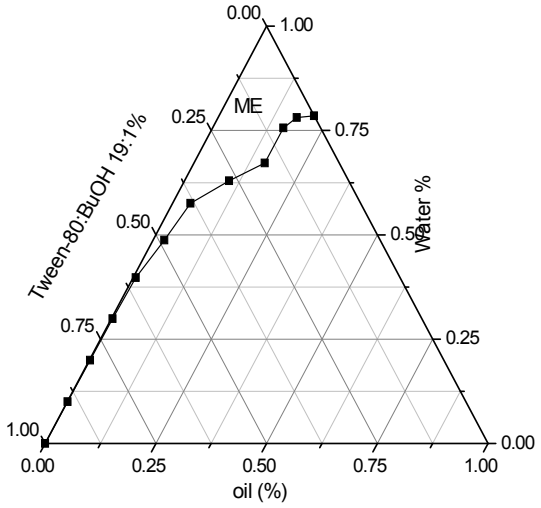


Fig 2a-2d Ternary phase diagram using Tx-100/n-BuOH/ *P. pinnata* seed oil/ water system with Smix (2:1, 4:1, 9:1 and 19:1) ratios.

Fig 2a

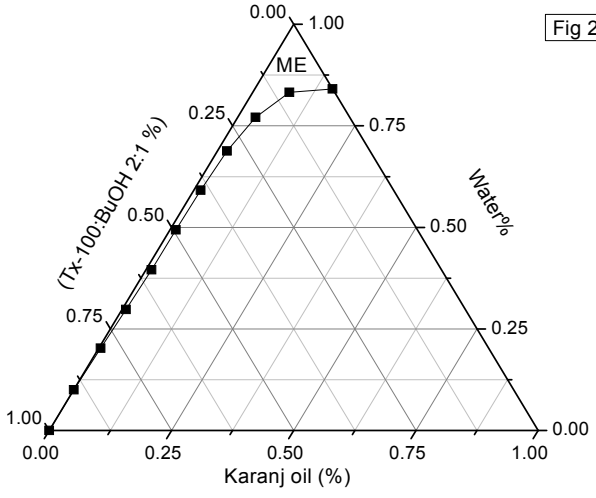


Fig 2b

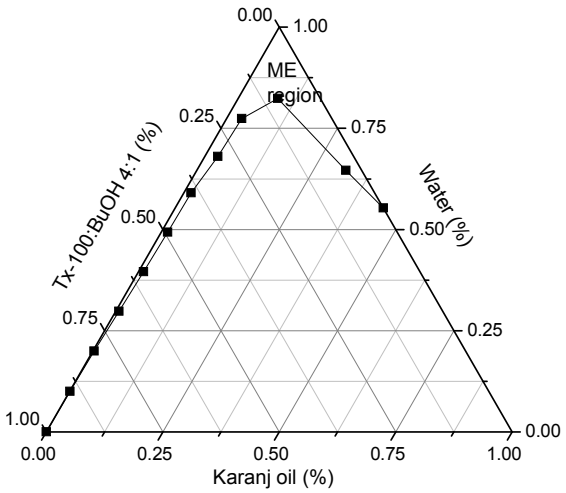


Fig 2c

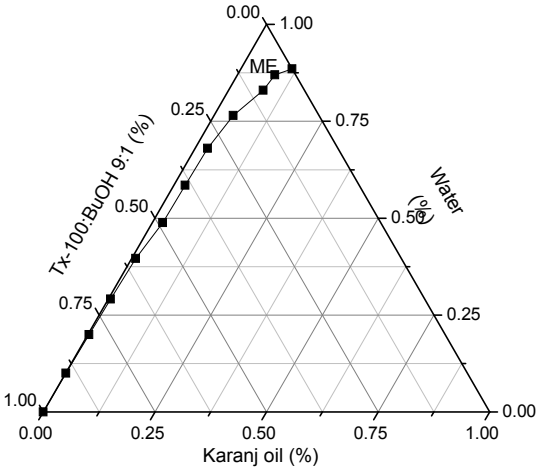
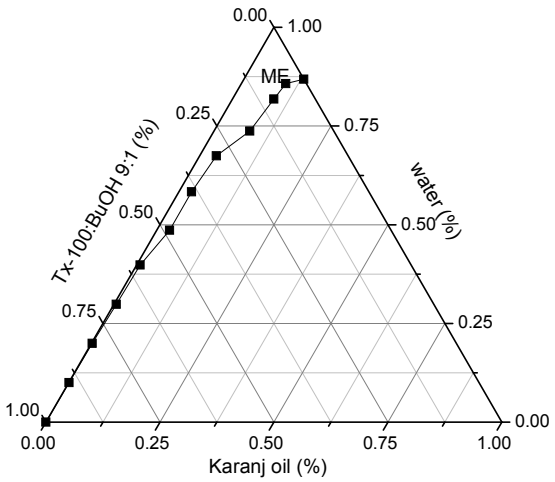
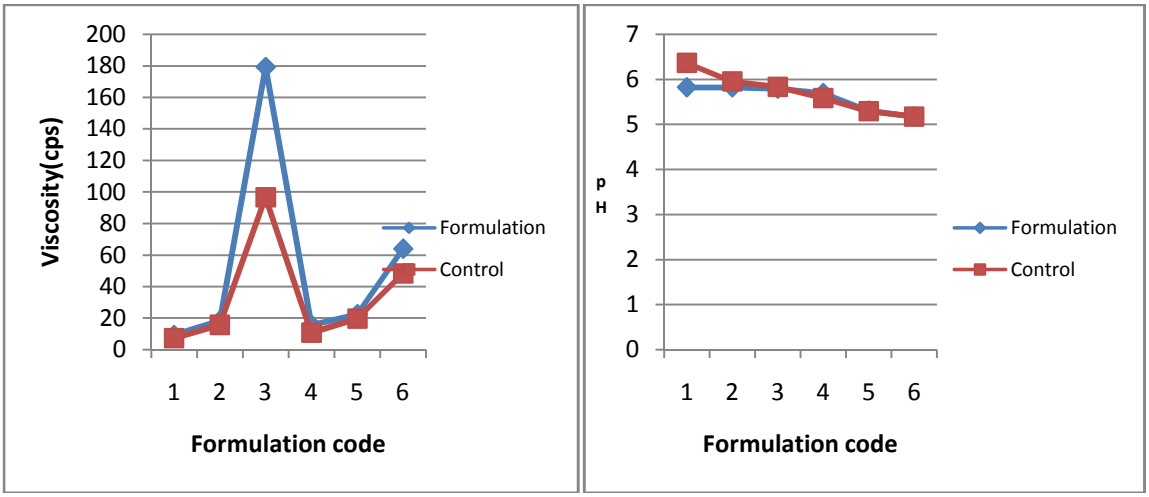


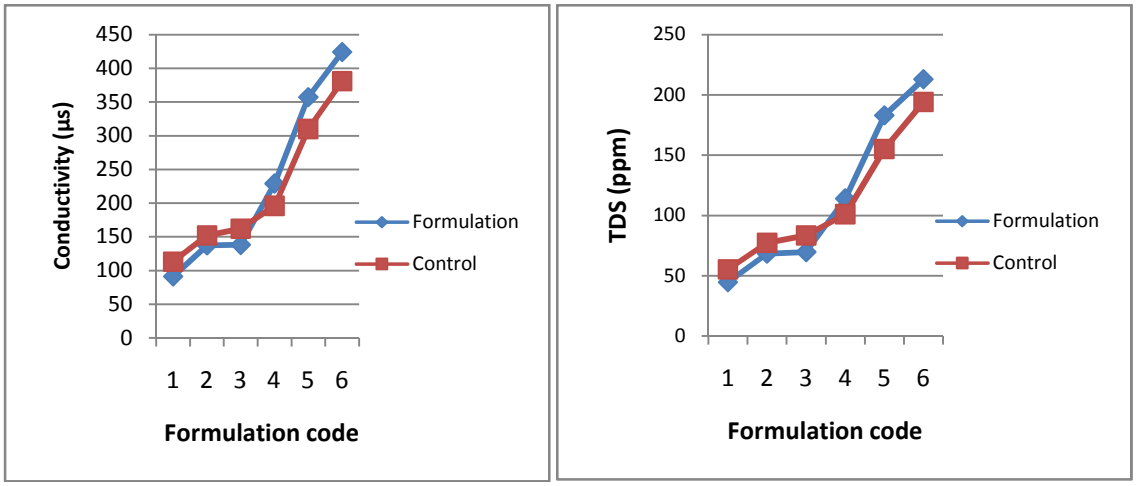
Fig 2d





(a) **(b)**

Fig 3.a Comparison of viscosity of formulations and controls.
Fig 3.b Comparison of pH of formulations and controls.



(c) **(d)**

Fig 3.c Comparison of conductivity of formulations and controls.
Fig 3.d Comparison of conductivity of formulations and controls.

Fig 4.a TEM image of F-3 formulation at 200 nm scale.

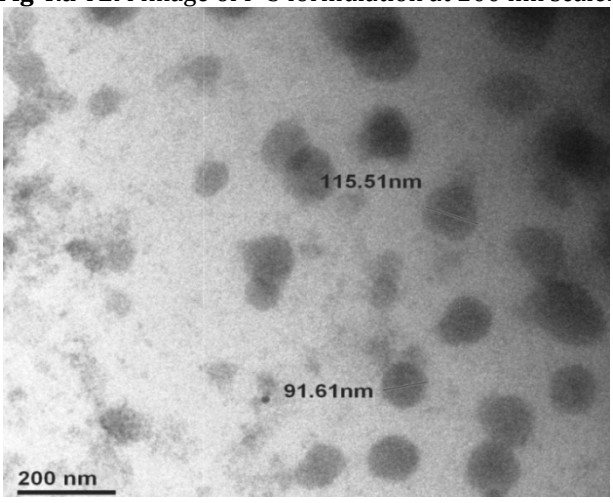


Fig 4.b TEM image of F-6 formulation at 200 nm scale.

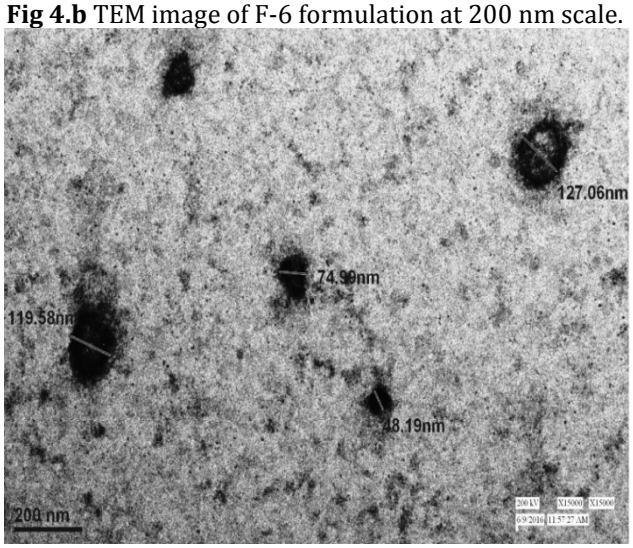
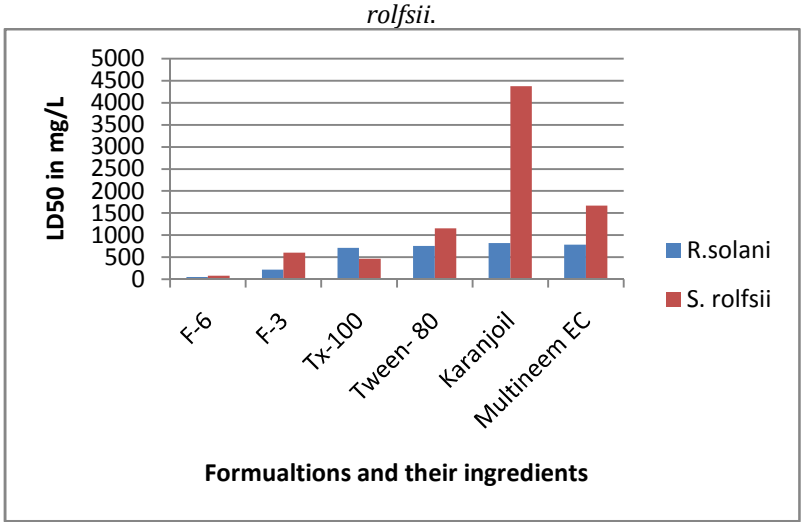


Fig 5 Antifungal activities of *P. pinnata* oil microemulsion and other ingredients against *R. solani* and *S. rolfsii*.



DISCUSSION

Developed MEs from non-ionic surfactants were found stable at ATS as well as low temperature. The viscosity of formulations increases with increasing concentration of both the surfactants. It was also observed that higher concentration of *P. pinnata* oil increased viscosity of formulations. It is well known that increase of volume fraction of dispersed phase in microemulsion, increases the viscosity of the system [6]. The pH of formulations was slightly lower than control may be due to low pH of *P. pinnata* oil. The conductivity of formulations was found lower than control in case of Tween-80 surfactant while high conductivity value was found in case of Tx-100 surfactant. Conductivity is a function of weight fraction of aqueous component. In all developed formulations, it was noticed that TDS value have same pattern like conductivity. It means that TDS and conductance are directly proportional to each other.

In both surfactants all phase diagrams were found in adjoining pattern. Solubilisation of *P. pinnata* oil was found concentration dependent in both surfactants so pattern of phase diagrams were also same. In case of Tx-100 surfactant, it has been found that at initial stage of titration when concentration of Smix was low w.r.t. water, consumption of *P. pinnata* oil was very low. Solubilisation of *P. pinnata* oil increases at higher concentration of Smix w.r.t. to water so more expansion in ME region were found in all phase diagrams. Maximum oil in water (o/w) region was observed at R=0.25 ratios so maximum solubilisation of *P. pinnata* oil at this ratio make this more useful for oil in water microemulsions. The amphiphilic nature of low molecular co-surfactant can also distribute between the aqueous and oil phase, altering the chemical composition and the relative ratio of hydrophilicity/lipophilicity resulting in increase of solubilisation [1, 18]. In case of Tween-80 surfactant, with decreasing concentration of n-butanol w.r.t. to surfactant, solubilisation of *P. pinnata* oil increases. So maximum oil in water ME region was found at

R=0.053 ratio. At R-ratios, R=0.11 and 0.053, at higher concentration of Smix w.r.t. water solubilisation of *P. pinnata* oil did not increase with increasing concentration of surfactant. The droplet sizes of both formulations were found in nano range of microemulsion system. In F-3 formulation microemulsion droplets were more spherical and denser than F-6 formulation, while nano range droplet size of F-6 formulation was found wider.

Various reports are available on biological activities of different part extract of this seed oil. Leaf extracts of *P. pinnata* oil was reported as antifungal agent against *R. solani* [10]. Aqueous leaves extract of *P. pinnata* was found effective against *S. rolfsii* at 5, 10 and 15% concentration [15]. On the basis of previous study, present work focused on the development of water based microemulsions that can be used as a bio fungicide. The antifungal activity data showed that the developed microemulsions were found more effective than seed oil, non-ionic surfactant that was used in formulation and commercial formulation (multineem) against selected plant pathogens. In both the formulations, the F-6 formulation was observed to have low LD_{50} value indicating that this formulation was more effective against selected fungi, may be due high toxicity of Tx-100 than Tween-80 surfactant. The LD_{50} values of developed formulations were compared with LD_{50} values of multineem (commercial formulation) which were found very low than multineem (commercial formulation), indicating that developed formulations are much more effective than multineem (commercial formulation) against these plant pathogens. Multineem was found least effective, it may be due to low contain of $Azadirachtin$ (0.03%) and EC formulation. The LD_{50} values of Tween-80, Tx-100, multineem and seed oil were almost similar against *R. solani* while in case of *S. rolfsii* LD_{50} values are not similar. Bio-efficacy study indicated that *R. Solani* is more susceptible than *S. Rolfsii* against developed formulations and other tested components.

Developed formulations showed better efficacy possibly due synergistic effect, and formulation generally improve efficacy of ingredient [14]. In present study, bio-efficacy, physicochemical parameters and TEM analysis of F-3 and F-6 showed that these developed formulations have droplet size in microemulsion range, thermal stability, pass all parameters, very effective for controlling plant pathogens and may acts as potential natural fungicide which is less toxic than synthetic pesticides. Seed oil contains secondary metabolites which are responsible for biological activities [3]. On the basis of results it can be said that present research works would be helpful in organic farming, pre and post harvesting of vegetable and fruits, and integrated pest management (IPM).

CONCLUSION

Microemusions were successfully prepared from *P. pinnata* seed oil by using non-toxic surfactants (Tween-80 and Tx-100) and n-BuOH as co-surfactant (no toxic chemicals were used in formulations). The developed microemulsions are thermodynamically and physically stable and pass all physicochemical parameters. The droplet size of microemusions (F-3 and F-6) were observed in 91.61 to 115.51 nm and 48.19 to 127.06 nm nano range. The investigated oil in water microemulsion regions were used in formulating a numbers of oil in water microemulsions. The bio-efficacy study data showed that developed formulations were much more effective than seed oils and multineem (commercial EC) against selected plant pathogens (fungi). These *P. pinnata* oil formulations may be used as bio-fungicide for controlling selected plant fungi. This effort will be helpful for the development of environmental friendly formulations from seed oil (natural ingredient) which may be a part of integrated pest management system.

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