Preparation and *in vitro* antifungal evaluation of carbendazim nanoemulsion for effective translocation in fungal cell

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Received October 22, 2018 and Accepted January 5, 2019

ABSTRACT : The present study has aimed to investigate the effective permeability of antifungal carbendazim by formulating as o/w nanoemulsion (NE). The NE was prepared by spontaneous emulsification technique using oleic acid, tween 20 and acetone as oil, surfactant & cosurfactant, respectively, after optimization. They were characterized for transparency, drug content, compatibility, pH, viscosity, stability and size (by transmission electron microscopic analysis). *In vitro* antifungal assay were conducted against two phytopathogenic fungi *viz. A. alternata* and *D. oryzae*. Transparent NE of narrow size distribution, suitable pH and viscosity were obtained. TEM analysis revealed the formation of discrete nanosized droplets with size varying between 80-140 nm. The NEs were stable under variable temperature conditions and have better permeation through fungal spore cells leading to better results as compared to the marketed Wettable Powder or suspension of Carbendazim.

Key Words : Nanoemulsion (NE), Carbendazim, tissue permeability, *in vitro* characterization, TEM, antifungal screening.

Organic molecules have low water solubility and therefore low tissue uptake. For bioactivity assays and field applications they are generally mixed with surfactants and used in the form of micro emulsions which generally leads to inflict higher doses of potentially active ingredient than actually required. To combat this problem, nanotechnology can be employed that immensely improves the bioactivity of the insoluble organomolecules in low concentrations. Just reduction in size has known to increase the biological properties of the molecules. It involves the conversion of the effective molecules into nanoemulsions, or coating them on metal nanoparticles or on other inorganic carriers, which leads to augmented bio-potential than the materials in bulk (Vijaya *et al.*, 2015).

Nanoemulsions are isotropic novel drug delivery systems consisting of emulsified oil and water systems with mean droplet size less than 300 nm (El-Aasser and Sudol, 2004; Anton and Vandamme, 2011). In the last few decades, these o/w nanoemulsions have found enormous application in the field of healthcare, cosmetics, food, agrochemicals, pharmaceuticals and biotechnology (Janjic and Ahrens, 2009; Sheikh *et al.*, 2007; Shakeel *et al.*, 2007; Mustafa *et al.*, 2009; Teo *et al.*, 2010). It represents the environment friendly formulations with low organic solvent contents, increased water dispersibility, penetration ability resulting in more efficiency in pesticide delivery and reduced rate of application of active ingredients than required in bulk.

Carbendazim (Methyl 1H-benzimidazol-2-ylcarbamate) is a well-known antifungal agent that can be used to control a broad range of diseases on field crops, fruits, and vegetables, including sclerotinia rot of canola, wheat head blight, peanut leaf spot, and SB on rice (Zhang *et al.*, 2007). Another study revealed that carbendazim was the most effective fungicide for inhibiting the mycelial growth of *Moniliophthora pernicious* (Gea *et al.*, 2010). It is easily degraded through chemical, physical and biological processes, such as UV, H_2O_2 and microorganisms (Helweg, 1977; Mazellier *et al.*, 2003; Lin *et al.*, 2011). It has been officially registered

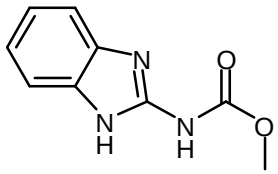

Fig.-1 : Structure of Methyl 1H-benzimidazol-2-ylcarbamate

in several countries. Unfortunately, its solubility in water is 6.11 mg/mL, making it difficult to apply as water soluble formulation (Ni *et al.*, 2002).

So, the present paper was aimed to prepare an o/w carbendazim nanoemulsion formulation using Tween20 as surfactant and to see the effect of nanosization on antifungal potential against various phytopathogenic fungi. So, we have reported the standardization, preparation, stability and antifungal assay of carbendazim nanoemulsion against two phytopathogenic fungi *viz. A. alternata* and *D. oryzae*.

Material and Methods

Chemicals

Carbendazim was obtained from the See Ciba crop

sciences (India) as a gift sample. Tween 20 (polyoxyethylene (20) sorbitanmonolaurate), acetone, methanol, chloroform, acetic acid, oleic acid were obtained from S.D Fine Chemicals, Mumbai. All the chemicals were of analytical grade. Deionized water was used for all experiments.

Micro-organism

The isolates of phytopathogenic fungi for the *in vitro* antifungal evaluation were provided by the Plant Pathology Department of the Punjab Agricultural University, Ludhiana. The cultures were maintained on potato dextrose agar medium (PDA: potato, 200 g; dextrose, 20 g; agar, 20 g; deionized water, 1 L) and kept at literature suggested temperatures.

Screening of oil and surfactant

50 mg of pure carbendazim was mixed with 1ml of different oil. The solubility was checked at different temperature. The oil that had shown maximum solubility was selected for subsequent experiments.

In order to select the most effective surfactant, the preparation of carbendazim nanoemulsions were made using two different surfactants *viz.* Span 20 and Tween 20. The quality of each surfactant was then determined according to appearance of the sample, the one in which transparency was maintained along with stability was selected for further screening.

Preparation of carbendazim nanoemulsions

The homogeneous organic phase (S1) composed of 40 mg of Carbendazim in different concentrations of oleic acid and volume was made 10 ml by addition of water–miscible solvent acetone. The homogeneous aqueous phase (S2) composed of Tween20 in 50 ml with distilled water. The organic phase was injected into the aqueous phase under sonication for 15 minutes. The water miscible solvent was removed by evaporation. The prepared nanoemulsion was stored in screw caped vials and kept at room temperature. The transparency was observed visually.

Transmission electron microscopy

Transmission electron microscopic analysis was carried to observe the shape of dispersed oil droplets. A drop of diluted nanoemulsion was applied to a copper grid and was left for 1min. Excess of nanoemulsion was removed by absorbing on filter paper and the grid was analyzed using the Hitachi TEM System operated at 100 kV.

Viscosity and pH determination

The viscosity of the nanoemulsions was determined by Ostwald viscometer, method based on Poiseuille's law. The results for different emulsions so formed have been calculated relative to water. The pH value of the formulations (NE) was measured using a calibrated digital pH meter by MAX electronics.

Drug content determination

One ml of an optimal nanoemulsion was mixed with 10ml of suitable solvent (chloroform-acetic acid mixture in 3:2 volumetric ratio) sonicated and filtered. For making standard curve, aliquots of different concentrations (0, 1, 2, 5, 10 and 20 mg) were prepared by solubilizing the content in 10 ml of solvent mixture. The drug content present in per ml of nanoemulsion was calculated by plotting against respective absorbance in standard curve. All the absorbance were measured at λ_{max} of 280 nm (Jain *et al.*, 2011).

Physiochemical Stability

Three Physiochemical aspects of the optimal nanoemulsions *viz.* stability after dilution, low temperature stability and stability above room temperature were determined. Two types of nanoemulsion formulations were assayed, differing in the type of water used: distilled water and a standard hard water solution (342 mg/L total dissolved solids) (Leng *et al.*, 2012; Leng *et al.*, 2013).

Antifungal Assay

The *in vitro* antifungal activity of carbendazim nanoemulsion formulation and non-formulated carbendazim were made against two phytopathogenic fungi *viz. A. alternata* and *D. oryzae* by Poisoned food technique (Devi and Chhetry, 2012) with some modifications. Mixture of nanoemulsion with carbendazim concentration equivalent to 1000, 500, 250, 100 and 50μg/ml and PDA was taken as test compound. Similar water solutions of carbendazim at same concentrations 1000, 500, 250, 100 and 50 μg/ml was taken as positive control and PDA with no carbendazim was taken as negative control.

PDA (Potato dextrose agar) media was taken in the round bottom flasks, each test concentration was added to different flasks by multiplication with appropriate dilution factor and the contents were mixed thoroughly. The contents of the flask were poured aseptically into the petriplates. Test compound was, however, replaced by an equal amount of tween 20 and oleic acid mixture only in the control set. After the media solidified, one inoculum disc of mycelium of the test fungus was aseptically inoculated to each petriplate and incubated at 15±1°C and 24 ± 1°C in case of *A. alternata* and *D. oryzae*, respectively. The average diameter of fungal colonies was measured on the 7th day after inoculation. The Percentage inhibitions of various solutions were calculated using below formula:

Percentage of inhibition =

$$\frac{\text{Diameter in negative control} - \text{Diameter in treatment}}{\text{Diameter in negative control}}$$

Statistical Analysis

The SAS 9.3.1 statistical software was used for analysis of the results recorded for antifungal evaluation. The results recorded in triplicates were subjected to one way analysis of variance (ANOVA) followed by post hoc Duncan's test to confirm its effective demarcation, from control sets. The paired student's t-test was used to analyze the inhibitory effects of the carbendazim nanoemulsion and solutions. $P < 0.05$, i.e., statistical significance at 5% level of significance was chosen as criterion for compilation of all the results.

Results and Discussion

Preliminary Screening

Carbendazim usually had limited solubility in most of the oil phases. However, from our analysis we had selected oleic acid as the best oil phase by virtue of its maximum carbendazim solubility (50 mg/ml) compared to castor oil and groundnut oil that have high density and form a non-transparent solution above 20 mg/ml carbendazim concentration.

Tween 20 selected as surfactant lowers the necessary energy to form the nanoemulsion that consequently improves the drug solubility and have high miscibility with oil phase. It has the HLB (Hydrophillic-Lypophillic balance) value of 16.7. Hence, the required HLB value *i.e.* >10 could be achieved in the formation of o/w nanoemulsion (Modi and Patel, 2011).

Different combinations of oil and surfactant mixture (1:9, 1:8, 1:7, 1:6, 1:5, 1:4, 1:3, 1:2, 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1) were employed. The ratio of 1:2 was considered to be best owing to its appropriate viscosity, pH and particle size for the nanoemulsion.

The addition of cosurfactant acetone enhances the NE stability by reducing the droplet size. This reduction in size may be due to the rapid diffusion of cosurfactant by way of evaporation.

Addition time and comparative evaluation of sonication and stirring

Different experiments were carried out to standardize the effect of addition time with stirring and sonication techniques. Oil and Surfactant mixture in ratio of 1:2 was selected for the preparation of nanoemulsion. Nanoemulsions of suitable size and stability were obtained under sonication in a time period of 15 min with maximum addition of 10 droplets of optimum size oil

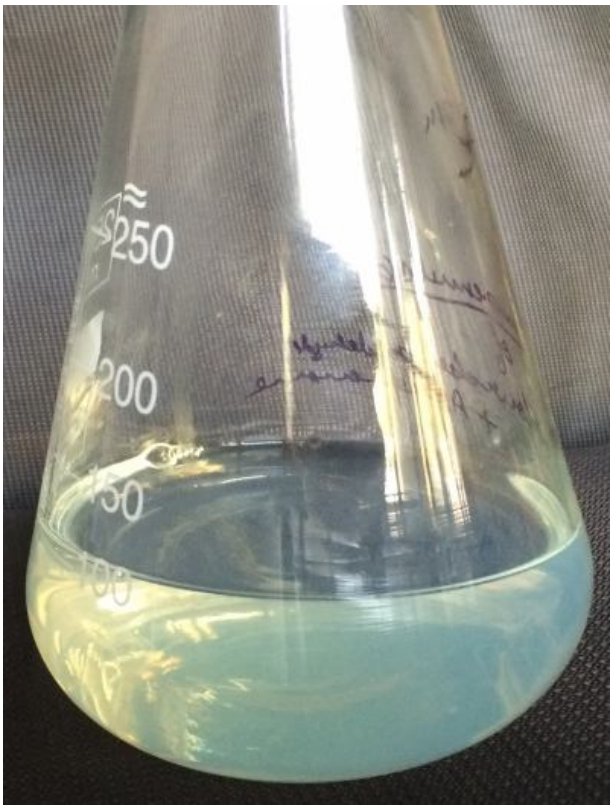

Fig.-2 : Transparent nanoemulsion with 1:2 oil to surfactant concentration.

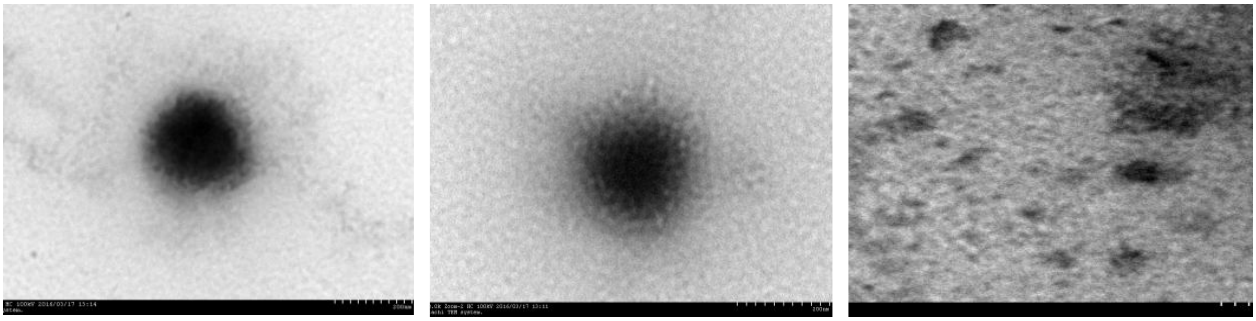

Fig.-3 : TEM images of nanoemulsions with different S_{mix} ratio.

carbendazim mixture per minute. These conditions were kept constant in the preparation of NE.

Transparency of nanoemulsion

The transparencies of the nanoemulsion so formed had been observed over a dark background. The result in terms of clarity is shown in Fig.-2.

pH and Viscosity determination

The pH values of all prepared formulation ranged from 5.62 to 6.08 which are considered as acceptable to pH avoid any risk to soil and crops. The pH values have indicated that higher oleic acid concentration decreases the pH value. For the best selected Smix ratio (1:2, oil:surfactant) the pH was found to be nearly equal to 6. The calculated viscosity coefficient for the optimal nanoemulsions was found to be 1.34.

Drug content determination

Drug content was determined at λ_{max} of 280 nm which had ranged from 0.835 to 0.931 mg per ml of solution.

Physiochemical Stability Assay

The prepared optimal carbendazim nanoemulsion was found to be clear and no phase separation had been observed in a study period of 2 months indicating the physical stability of NE at room temperature for longer period of time. Further the physiochemical stability of nanoemulsion under different conditions was as follows:

Dilution stability : Carbendazim nanoemulsion was diluted with standard hard water to concentrations of 5%, 2%, 1% and 0.5%. All samples remained transparent and uniform; no floating oil or precipitate was observed.

Low temperature stability : After storage at 0°C for 14 days, there was no precipitate formation or phase separation; no significant change in the mobility or stability was observed.

Stability above room temperature: No phase change or loss of stability was observed by heating the formulation up to the boiling point of water *i.e.* 100°C.

Antifungal screening by Poisoned food technique

Inhibition of *A. alternata*: The zone of inhibition that remains free from fungal growth was appeared around the fungal inoculum on the agar plate after 7 days of incubation at required temperatures. 48% of growth was inhibited in presence of 100 µg/ml of carbendazim in nanoemulsion formulation, which was significantly higher than that of a 100 µg/ml aqueous solution of carbendazim (30%) ($P< 0.05$). Further the inhibition was more enhanced with increase in the concentration of carbendazim in nanoemulsion formulation that reached up to 100% for concentration equivalent to 250 µg/ml and above. The calculated ED_{50} values for the formulation was 100 µg/ml whereas the non-formulated aqueous solution had the ED_{50} value of 250 µg/ml, which were significantly different from each other ($P<0.05$).

Inhibition of *D. oryzae:* Growth kinetics had shown that *D. oryzae* takes 12 days for complete growth as observed in control plate. But cultivation with the presence of 100 µg/ml of carbendazim nanoemulsion resulted in inhibited mycelial growth upto 40% that was significantly higher than that of 25% inhibition with aqueous solution of carbendazim ($P < 0.05$). Further, the increase in concentration leads to increased inhibition upto 80% at 250 µg/ml in nanoemulsion formulation whereas, the inhibition by aqueous carbendazim solution with same concentration is only 45%. The calculated ED_{50} values for carbendazim nanoemulsion and aqueous solution were 125 and 300 µg/ml, respectively.

The above mentioned results confirmed that the carbendazim nanoemulsion formulation had a significantly stronger inhibition than the aqueous solution of the same. And the reason for the same can be considered to be the better permeation of the nanodroplets through the fungal spore cells.

In this work, carbendazim nanoemulsion was prepared and it was found that varying parameters such as

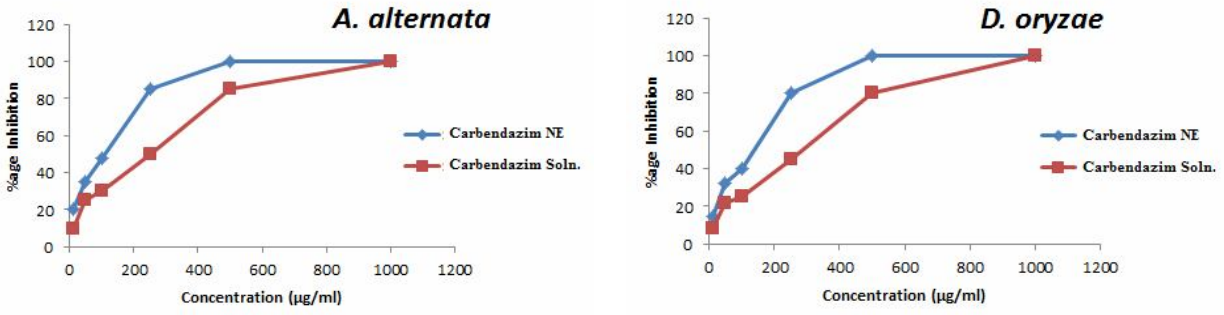

Fig.-4 : Comparisons of percentage inhibition by carbendazim nanoemulsion and solution against the two test fungi at different concentrations.

using different oils, surfactant, their concentration, agitation time and agitation speed influences the formation of NE. There was a significant enhancement in antifungal potential of carbendazim against phytopathogenic fungi, when applied as nanoemulsion due to more effective penetration through the fungal cell surface. Overall, it could be concluded that antifungal carbendazim can have a better translocation in plant system if applied as nanoemulsion as it is more effective compared to conventional method under *in vitro* studies.

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