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Formulation of 2-methyl-3,1-(4H)-benzoxazin-4-one and evaluation its antifungal activity against some pathogenic fungi

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Abstrcat:

Methyl benzoxazineone was formulated as wettable powder formulation (54% WP). The new local formulation passed successfully all physico-chemical properties of wettable powder formulation. The antifungal activity of both active ingredient and its local formulation was evaluated under laboratory conditions. There are a regration relationship was found between tested concentration of active ingredient and their percentages of inhibition against *Sclerotium rolfsii* Sacc. whereas this indication was not found in case of *Rhizoctonia solani* Kühn. On contrast the above relationship was found with both tested fungi in case of local formulation. Depending on EC_{50} values the effectiveness of active ingredient increased by 37.6% and 100% in case of *Sclerotium rolfsii* and *Rhizoctonia solani* respectively as resulting of formulation.

Introduction

Fungi can grow in almost all habitats, including soil, air, seas, rivers, as well as on organic matter, including food, and other organisms, such as plants, animals, and even human skin (Jampilek, 2016). Many fungal genera including *Fusarium*, *Alternaria*, *Botrytis*, *Helminthosporium*, *Penicillium*, and *Rhizoctonia* have proved harmful pathogenic fungi and cause huge loss of crop yield world-wide (Boyraz and Ozcan, 2006). In Pakistan, all major crops are frequently infected by fungal plan pathogens and cause loss of yield in quality and quantity. Among these diseases, *Rhizoctonia* black scurf and stem canker caused by the fungus *Rhizoctonia solani* Kühn are a severe problem in all potato producing zones of the country (Sneh *et al.,* 1991; Ahmad *et al.,* 1995 and Khan *et al.,* 1995).

Sclerotium rolfsii Sacc. is a soil-borne pathogen that commonly occurs in the tropics, sub-tropics and other warm temperate regions of the world causing root rot, stem rot, wilt and foot rot on more than 500 plant species including almost all the agricultural and horticultural crops (Aycock, 1966;

Domsch *et al.,* 1980 and Farr *et al.,* 1989).

Application of fungicides is the most convenient and predominant way for disease control. Their use has made it feasible to enhance crop yields and food production. The efficacy of fungicides is influenced by many biological and environmental factors that directly influence the metabolic activities of fungal cells (Reinprecht, 2010). Sometimes critical concentrations are not effective long-term, as the fungus can become resistant to the fungicide (Neely, 1969 and Brent and Hollomon, 2007). Therefore, it has become an important issue to find alternative control strategies are effective as synthetic pesticides (Javed *et al.*, 2006).

Infectious diseases caused by bacteria and fungi affect millions of people world-wide. Concerted and systematic progresses to discover and develop new antibiotics are always done due to the development of resistance by the microorganisms to the drugs commonly used against them. The rapid rise in bacterial resistance to the traditional antibiotics such as penicillins and tetracyclines had encouraged a continuing search for new classes of compounds with novel modes of antibacterial activity. Quinazolines are considered a very important class of compounds that show a diversity of activities, most prominent of which are antimicrobial and antifungal (Grover and Kini ,2006 ; Girija and Hemalatha, 2010 and Bartroli *et al.,* 1998).

2-methyl-3,1-(4H)-benzoxazin-4-one (Figure,1) was used for the synthesis of quinazolinone derivatives. Quinazolinone and its derivatives are inhibitory to several fungal pathogens of plants, including *Helminthosporium turcicum, Stagonospora nodorum, Microdochium nivale, Fusarium moniliforme, Fusarium culmorum*, *Gaeumannomyces graminis* and some isolates of *G. graminis, F. culmorum* and *F. moniliforme* (Friebe *et al.*,1998).

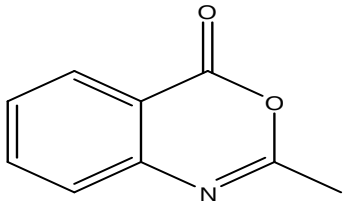

Figure (1): Structure of 2-methyl-3,1-(4H)-benzoxazin-4-one.

Quinazoline derivatives are a class of chemical compounds that have been proved to have antimicrobial activity. The biological activities of quinazoline derivatives such as antitumor, anti-inflammatory, anti-HIV, antihypertensive, anthelminitic and antituberclusis activity and the derivatives of 2-methyl-3- amino-quinazoline-4(3H)-one were synthesized. The antimicrobial activity has been evaluated by micro dilution method. The antifungal activity of the compounds was quite lower than their antibacterial activity (Rezai *et al.,* 2012).

4H-3,1-benzoxazin-4-ones have attracted considerable attention as inhibitors of serine proteases by enzyme acylation due to the nucleophilic attack of the active site serine on the lactone carbon (Gilmore *et al*., 1996 and Gutschow and Neumann, 1997). Benzoxazinone derivatives are also used as antiphlogistic drugs, antifungal and antibacterial agent (Segarra *et al.*, 1998). If a vinyl or phosphate functional group is connected to an aromatic ring located at the position two of the heterocycle, the resulting compounds possess antimuscular contraction properties and can be used as a hypnotic drug. This special reactivity allows this class of compounds to be useful as antimicrobial (Mathew *et al.*, 2010), anti-platelet aggregation (Pritchard *et al.*, 2007),

human leukocyte elastase inhibitors (Pei-Wen Hesieh *et al.*, 2005 and Arcadi *et al.*, 1999), receptor antagonist active (Ward *et al.*, 2007 and Bromidge *et al*., 2009), pesticides (Shakil *et al*., 2010), tissue culture protective and *in vivo* pharmaceuticals 2011, 41033 model of neurodegeneration (Wang *et al.*, 2010) and improve the umbilical vein endothelial cells (Dong *et al.*, 2010).

The successful use of any active ingredient depends on its correct formulation into a preparation which can be applied for crop protection with safety to those applying materials to animal life and to the environment. In general formulation plays an important role in spread over a very large area. Also, it facilitates penetration of the active ingredient to reach its target and achieve its action (El-Kady *et al.*, 2010).

The aim of this is study: 1- Evaluation of fungicidal activity of (methyl benzaxazineone) as active ingredient. 2- Formulation of (methyl benzaxazineone) in suitable formulation form and evaluation their fungicidal activity.

Material and methods

1. Tested chemicals:

1.1. Fine chemicals:

1.1.1. Acetic anhydrid (1), molar mass 122.12 g/ mol^{-1}.

1.1.2. Anthranilic acid (2-amino benzoic acid) (2), molar mass 137.14 g/ mol^{-1} were purchased from Obour Pharmaceutical Industrial Company.

1.2. Surface active agents: Tween 80, polyethylene glycol 600 diolate (PEG 600 diolate), Toximol, Toximol H and Toximol R were supplied by EL-Gomhoria Co., Cairo, Egypt.

1.3. Solvents: Xylene, acetone, absolute ethanol, DMSO (dimethyl sulfoxide) and DMF (dimethyl formamide) were supplied by EL-Gomhoria Co., Cairo, Egypt.

2. Physico-chemical properties of the basic formulation ingredients:

2.1. Physico-chemical properties of active ingredient:

2.1.1. Solubility: It was determined by measuring the volume of distilled water, acetone, xylene, DMSO and DMF for complete solubility or miscibility of one gram of active ingredient at 20 ^{o}C (Nelson and Fiero, 1954). The % solubility was calculated according to the following equation:

$$\% \text{ solubility} = W/V * 100$$

W: active ingredient weight

V: volume of solvent required for complete solubility.

2.1.2. Free acidity or alkalinity: It was determined according to CIPAC MT 31.1 (2002).

2.1.3. Melting point: It was determined by using electro thermal melting point apparatus 9200A.

3. Surface active agents:

3.1. Solubility: It was determined as mentioned before.

3.2. Free acidity or alkalinity: It was determined according to World Health Organization (WHO) (1979).

3.3. Hydrophilic-Lipophilic balance (HLB): The solubility of surfactant in water was considered as approximate guide to their HLB and usefulness **(**Lynch and Griffin, 1974).

3.4. Critical micelle concentration (CMC): The concentration in which the surface tension of solution doesn't decrease with further increase in surfactant concentrations, (CMC) of the tested surfactants was determined according to Osipow (1964).

3.5. Surface tension: It was determined by using Cole- Parmer surface tension 21 for solutions containing 0.5 % (W/V) surfactant according to ASTM- 1331 (2001).

4. Carriers: Aswanly clay:

4.1. Free acidity or alkalinity: It was determined according to **WHO specification (1979)**.

4.2. Wettability: It was determined according to CIPAC MT 53.3 (2002).

5. Bulk density: This property was determined according to CIPAC MT 33 (2002).

6. Preparation of methyl benzoxazinone as wettable powder (WP).

This type of formulation is suitable for the active ingredients that did not soluble in water or xylene; several trials were carried out as follow: Different weights from active ingredient were added to other different weights from carrier then mixed together to make a homogenous powder and wetting or dispersing agent single or mixed together with different percentages was added to the mixtures and stirred well using glass rod to ensure homogeneity. After drying, the mixtures were sieving through 590 my sieve to ensure that all particles have the same size. Suspensibilty test was carried out according to CIPAC MT 185 (2002) for all prepared formulations to judge on the success of formulation.

7. Determination of the physico-chemical properties of the local formulated wettable powder:

7.1. Suspensibility: It was determined according to CIPAC MT 184 (2002).

7.2. Wattability: It was determined according to CIPAC MT 53.3 (2002).

7.3. Free acidity or alkalinity: It was determined according to CIPAC MT 31.1 (2002).

8. Determination the physico-chemical properties of the spray solution of the local formulation at the field dilution rate:

8.1. Surface tension: It was determined by using Cole- Parmer surface tensiometer 21, where dyne/cm is the unit of surface tension measurements.

8.2. Viscosity: It was determined by using Brookfield viscometer model DV Π + Pro, where centipoise is the unit of measuring viscosity according to ASTM D- 2196 (2005).

8.3. PH value: It was determined by using Cole-Parmer pH Conductivity meter 1484-44.

8.4. Electrical conductivity: It was determined by using Cole-Parmer pH Conductivity meter 1484-44, where µ/mohs is the unit of electrical conductivity measurement according to Dobrat and Martijn (1995).

9. Fungal strains used:

Pure cultures of *Sclerotium rolfsii* and *Rhizoctonia solani* were supplied from the department of Fungicides, Bactericides and Nematicides, Central Agricultural Pesticides Laboratory (CAPL), Agricultural Research Center (A. R. C.)

10. Effect of active ingredient (methyl benzoxazineone) and its wettable powder formulation on pathogenic fungi:

Antifungal activity of active ingredient (methyl benzoxazineone) and its formulated form (WP 54%) were determined by food poisoned technique (Mohanty *et al.,* 2012). Active ingredient was dissolved in DMSO at concentration 33.3%. Both active ingredient and its wettable powder formulation 54% were added separately to get the required concentrations. The tested concentrations were mixed with 50ml of sterilized PDA medium and transferred equally into three Petri dishes. The media could solidify. Then seven-day old fungal culture disk of 5-mm diameter was taken and inoculated to the center of Petri dishes containing active ingredient (methyl benzoxazineone) and formulated form WP in separate manner. Instead of PDA medium without

active ingredient (methyl benzoxazineone) and formulated form served as control. All dishes were incubated at 27±2°C and radial growth of colony was measured when the mycelia of control had almost filled the Petri dishes. Each test was performed in triplicate.

The fungal growth inhibition which was calculated due to treatment against control using the following formulaaccrding to Satya *et al.* (2014):

Inhibition of growth (%) = R-r/R *100

R is the radial growth of fungal mycelia in the control plate.

r is the radial growth of fungal mycelia in the treated plate.

11. Statistical analysis:

The concentration inhibition regression lines were drawn according to the method of Finney (1971).

Results and discussion

1.Physico-chemical properties of 2-methyl-3,1-(4H)-benzoxazin-4-one as active ingredient:

Data in Table (1) showed that, 2-methyl-3,1-(4H)-benzoxazin-4-one was medium soluble in DMF and DMSO (25 and 33.3%) consequently but completely insoluble in Water, Acetone and xylene. It showed acidic property which appeared from its free acidity (19.6). Taking these results into account, it could be prepared as wettable powder and needs acidic surface-active agents for complete compatibility.

Table (1): Physico-chemical properties of 2-methyl-3,1-(4H)-benzoxazin-4-one as active ingredient.

Solubility % (W/V)					Free acidity as % H_2SO_4
Water	Acetone	Xylene	DMSO	DMF	
N. S	N. S	N. S	33.3	25	19.6

N.S means insoluble.

2.Physico-chemical properties of surface-active agents.

Data in Table (2) showed the physico-chemical properties of toximol, toximol R, toximol H, toximol 500 and Tween 80 as surface active agents. All toximols showed the slight changes in surface tension values, their values were between 36 to 39.2 dyne/cm while for Tween 80 and sds it was 39.2 and 31. Also all the tested surfactants had the same hydrophilic-lipophilic balance, except sds. Also, there was no differences between toximol and sds in CMC values that showed 0.3 while Tween 80 showed 0.5 %. On the other hand for the free acidity or alkalinity, all tested surfactant showed acidic property, Tween 80 showed the highest value (0.61), followed by Toximol R (0.49), toximol 500 (0.39) then toximol H (0.2) at the finally toximol has lowest value (0.03). Depending on the values of free acidity for the five surface active agents, any of them can be used for formulating this active ingredient in the form of wettable powder, but the main factor that determined the best surfactant for this formula was their stability and compatibility with the required properties of required formulation.

Data in Table (3) showed that physico-chemical properties of aswanly clay as carriers were 7.86 wettabillity per second, 0.87 density and 0.8 bulk density.

Table (2):Physico-chemical properties of the tested surface-active agents.

Surface active agent	Surface tension dyne/cm	HLB	CMC %	Free acidity as % H_2SO_4	Free alkalinity as NaOH
Toximol	36	8-10	0.3	0.03	-
Toximol R	37.02	8-10	0.3	0.49	-
Toximol H	39.2	8-10	0.3	0.2	-
Toximol 500	36	8-10	0.3	0.39	-
Tween 80	39.2	8-10	0.5	0.61	-
Sds	31	>13	0.3	-	0.026

Table (3):Physico-chemical properties of carriers.

	Wettabillity Second	Density	Bulk density
Aswanly clay	7.86	0.87	0.8

3.Physico-chemical properties of the local % wettable powder formulation before and after accelerated storage.

Table (4) show that physico-chemical properties of the 54 % Wettable powder formulation before and after accelerated storage (50 ±3 °C for three days). All physico-chemical properties of the formulation did not show any valuable changes, it showed acidic property before and after storage by relatively close values; in addition, it was completely suspensibility in both cases. Generally, there were no effective changes in the physico-chemical properties of the new formula before and after accelerated storage.

Table (4): Physico-chemical properties of local formulation before and after accelerated storage.

Before storage			Cold storage	After storage		
Suspensibility		Free acidity as H_2SO_4		Suspensibility		Free acidity as H_2SO_4
Hard water	Soft water			Hard water	Soft water	
100%	100%	21.56	pass	100%	100%	22.54

4.Physico-chemical properties of spray solution at field dilution rate.

The biological activity of a pesticide to the target pest species is greatly influenced by its physical and chemical properties. The physical properties of a pesticide determine the pesticide mode of action, dosage, mode of application and the subsequent environmental chemodynamics. The physical properties of pesticides vary greatly according to their chemical nature and formulation. The spray solution showed a decrease in surface tension and pH, while an increase in electrical conductivity and viscosity was observed (Table,5) . Decreasing in surface tension of spray solution cause improving in wettability and spreading on the treated surface then increasing deposit and activity of pesticide (Osipow, 1964). The decrease in PH value with increasing electrical conductivity can result in an increase in pesticide efficacy according to Tawfik and El-Sisi (1987) who stated that, retention and effectiveness of pesticides spray solution increased with decreasing in pH values with increasing its conductivity. The relation between increasing viscosity and increasing the pesticidal efficiency could be explained according to Richardson (1974) who reported that, increasing viscosity of spray solution caused a reduction in drift and an increase in the retention and sticking of spray solution on the surface of plant.

Table (5): Physico-chemical properties of spray solution at field dilution rate 0.5%.

Viscosity centipoises	Electrical conductivity µ/mhos	PH	Surface tension dyne/cm
12.72	720	5.05	39.5

5.Effect of active ingredient (methyl benzoxazineone) on pathogenic fungi:

Data in Table (6) indicated that there are a regration relationship was found between tested concentration of (methyl benzoxazineone) and their inhibition effect against *S. roffsii*. On contrast no inhibition effect was found with all tested concentration in case of *R. solani.* The effect of the active ingredient may be due to it mode of action (Gilmore *et al*., 1996) told that 4H-3,1-benzoxain-4-ones have attracted considerable attention as inhibitors of serine proteases by enzyme acylation due to the nucleophilic attack of active serine on the lactone carbon. On the other hand, *R. solani* may be possessed barriers prevent the active ingredient from reach to location effect.

Table (6): Effect of active ingredient (methyl benzoxazineone) on pathogenic fungus.

Concentration of (ppm)	*Sclerotium rolfsii*		*Rhizoctonia solani*	
	Radial growth (mm)	% of inhibition	Radial growth (mm)	% of inhibition
1000	32.6	67.3	90.0	0.0
500	52.3	47.6	90.0	0.0
250	60.3	39.6	90.0	0.0
125	79.6	20.3	90.0	0.0
62.5	90.0	0.0	90.0	0.0
*Control no solution	90.0	0.0	90.0	0.0
**Control DMSO	90.0	0.0	90.0	0.0

Each number represents the mean of 3 replicates.
***Control without active ingredient (medium free of any solvent and discs were cut from the pathogen only on PDA).**
****Control DMSO (medium mixed DMSO and discs were cut from the pathogen grown on PDA).**

6. Effect of formulated (methyl benzoxazineone) 54% WP on pathogenic fungus:

Data in Table (7) indicated that there is a positive relationship were found between tested concentrations of local formulation and its inhibition percentages. On the other hand, local formulation increased the activity of active ingredient against *R. solani*. This indication may be due to the wetting and depressing agents that used in formulation.

Table (7): Effect of formulated (methyl benzoxazineone) 54% WP on pathogenic fungus.

Concentration of (ppm)	*Sclerotium rolfsii*		*Rhizoctonia solani*	
	Radial growth (mm)	% of inhibition	Radial growth (mm)	% of inhibition
2000	-	-	0.87	99.1
1500	-	-	7.6	92.4
1250	-	-	15	85
1000	26	74	55.3	44.7
500	41	59	90.0	0.0
250	52	48	90.0	0.0
125	68	32	90.0	0.0
62.5	77	23	90.0	0.0
*Control	90.0	0.0	90.0	0.0

Each number represents the mean of 3 replicates, (-): disappear

***Control without active ingredient (medium free of any solvent and discs were cut from the pathogen only on PDA).**

Data in Table (8) showed that clearly the percent of EC_{50} and EC_{90} values and slop value for the tested fungi *S. roffsii* and *R. solani*. Depending on EC_{50} values, local formulation increased the effectiveness of active ingredient against both tested fungi by 37.6% and 100%. Also *S. roffsii* was more sensitive to local formulation in case of *S. roffsii* than *R. solani*. On the other hand. the slope values showed the nearest value with both tested fungi.

Table (8): The EC_{50}, EC_{90} and slope values for *Rhizoctonia solani* and *Sclerotium rolfsii* with the active ingredient and formulated (methyl benzoxazineone) 54% WP.

Treatment	*Sclerotium rolfsii*			*Rhizoctonia solani*		
	EC_{50}	EC_{90}	Slope	EC_{50}	EC_{90}	Slope
Active ingredient	477.5	4401	1.3±1989	-	-	-
Formulation	298.1	3880	1.1499± 0.1420	1018	1390	9.4745± 1.2275
Increased effectiveness	37.6%			100%		

(-): disappear

It is concluded that methyl benzoxazineone was formulated as wettable powder formulation. Both active ingredient and local formulation were evaluated as fungicidal against *S. rolfsii and R. solani* under laboratory condition. The local formulation increased the effectiveness by 37.6% and 100% respectively.

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