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Effects of Novel Synthetic Pyrazolopyrimidine Compounds against *Pepper Mild Mottle Virus* (PMMoV) Infecting Vegetable Crops and Human Pathogens

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Pepper mild mottle virus **(PMMoV) was obtained from naturally infected pepper (*Capsicum annuum* L.). Plants with mosaic, puckering and mottling yellow or light green symptoms on the upper developing leaves were collected from the Mecca regions, Kingdom of Saudi Arabia. Infected samples were carefully tested by direct enzyme-linked immunosorbent assay (ELISA) with antiserum to *Tobacco mosaic virus* (TMV), *Cucumber mosaic virus* (CMV), *Potato virus Y* (PVY), *Tobacco etch virus* (TEV), *Tomato spotted wilt virus* (TSWV) and *Pepper mild* mottle *virus* and confirmed by electron microscopy and reverse transcription-polymerase chain reaction (RT-PCR) assay. On the other hand, we studied inhibitory effects of the novel synthetic chemical compounds from Pyrazolopyrimidine against PMMoV and important human pathogens. We found that the efficacy of the antiviral agent depends on the capability of the virus to replicate itself by stopping the viral messenger RNA replicate and thus prevent the spread of the virus in infected pepper plants when plants were treated with Pyrazolopyrimidine compounds after 24 h from PMMoV inoculation.**

Key Words: *Pepper mild mottle virus*, PMMoV, Pyrazolopyrimidine, antimicrobial, antiviral

Abbreviations: DAS-ELISA – double antibody sandwich enzyme-linked immunosorbent assay, PMMoV – *Pepper mild mottle virus*, RT-PCR – reverse transcription-polymerase chain reaction, TMV – *Tomato mosaic virus*

INTRODUCTION

Peppers are very important vegetables worldwide. They provide spice and color to foods in addition to essential vitamins and minerals (Green and Kim 1991). Peppers can be infected by a number of viruses such as *Tobacco mosaic virus* (TMV) (Heper 1979), *Cucumber mosaic virus* (CMV), *Tobacco etch virus* (TEV) (Palloix et al. 1994a), *Potato virus Y* (PVY) (Erkan 1991), *Tomato spotted wilt virus* (TSWV) (Yurtmen et al. 1999) and *Pepper mild mottle virus* (PMMoV) (Palloix et al. 1994b). The genus *Tobamovirus* includes PMMoV and consists of a single-strand positive sense RNA of 6357 nucleotides (Garcia-Luque et al. 1990; Kirita et al. 1997; Caglar et al. 2013). PMMoV is transmitted through soil and seeds (Komuro and Iwaki 1996; Lanter et al. 1982). Viruses that belong to the genus *Tobamovirus* are rigid and rod-shaped, and they are transmitted by mechanical inoculation. Pepper growers can prevent virus transmission to plant tissues by taking seeds only from clean plants and by strictly adhering to cleanliness procedures (Baker and Adkins 2000).

This study was carried out to conduct viral identification via symptoms through electron microscopic examination of fresh epidermal cells of healthy pepper plants and those infected by PMMoV. Reverse transcription-polymerase chain reaction (RT-PCR) assay was used for identifying the virus of PMMoV isolated from RNA extracts of infected pepper plants when compared with non-infected plants.

Treatment of plants with novel synthetic chemical compounds from Pyrazolopyrimidine can lead to the induction of resistance agents that are characterized by restriction of virus multiplication and suppression of disease symptoms when

compared with untreated plants (Al-Ani and Hassan 2002; Al-Ani et al. 2002; Al-Ani et al. 2010; Xiao et al. 2015). This study was conducted to evaluate the inhibitory effects of the novel synthetic chemical composites from Pyrazolopyrimidine on virus multiplication and disease development (Al-Ani et al. 2010; Xiao et al. 2015) and on their antimicrobial activity against important human pathogens.

MATERIALS AND METHODS

Pepper (*Capsicum annuum* L.) infected with PMMoV develop mosaic symptoms on the upper developing leaves and symptoms may vary between pepper cultivars. Infected leaves are frequently puckered and mottled yellow or light green.

Infected leaves of pepper showing typical symptoms suspected to be PMMoV infections were collected from the Mecca regions, Kingdom of Saudi Arabia. Detection of PMMoV was checked serologically by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) technique as described by Clark and Adams (1977) against the following viruses: CMV, TMV, PVY, TEV, TSWV and PMMoV. All infected samples were examined in triplicate using the conventional PMMoV-DAS-ELISA kit, alkaline phosphates 500 test according to the manufacturer's instructions (Sanofi-Santi animal, France) with optical density at λ= 405 nm in an ELISA microwell reader (using the Dynatech Immunoassay MR 7000).

PMMoV was isolated from naturally infected pepper plants and biologically purified by the single local lesion technique on *Chenopodium giganteum* plants which produced local lesions surrounded with a little halo edge (Kuhn 1964). It was propagated under controlled conditions on sweet pepper seedlings of cv. Sirtaki, which reacted as mosaic, puckering and mottling yellow symptoms on the upper leaves at least 3 wk after inoculation. After symptoms development, PMMoV presence was confirmed by ELISA test and used as a source of virus in the following experiments.

For ultrathin sections, healthy and infected mesophyll tissues were pieced, fixed and stained according to the standard procedures of Martelli and Russo (1984). Each ultrathin section was viewed with a JOEL-JEA100 CX electron microscope unit.

Total RNA extracted from leaves give a positive reaction with DAS-ELISA (Hadidi et al. 1993). Reverse transcription reactions were functional with 1 µg of total nucleic acid (TNA) extract and 0.5 µg of random primer (Promega, Madison, WI, USA) in the presence of reverse transcription (RT) 5x Buffer and Moloney Murine Leukemia Virus (M-MLV) enzyme, according to the manufacturers' instruction (Invitrogen, Carlsbad, CA, USA). Two pairs of specific primers (CP/s: 5-′ ATGGCATACACAGTTACCAGT-3′ and CP/a: 5-′ TTAAGGAGTTGTAGCCCACGTA3′, designed in this work, and P12/3 and P12/3A) (Velasco et al. 2002) were used in RT-PCR for the amplification of the capsid protein (CP) (474 bp) and partial RNA-dependent RNA polymerase (RdRp) (830 bp) genes, respectively.

All organic solvents were purchased from commercial sources and used as received unless otherwise stated. All other chemicals were purchased from Merck, Aldrich or Acros and used without further purification. Thin-layer chromatography (TLC) was performed on pre-coated Merck 60 GF254 silica gel plates using a fluorescent indicator, and detection was carried out using UV light at 254 and 360 nm. Melting points were measured on a Stuart melting point apparatus and were uncorrected. IR spectra were recorded on Nicolet iS10 FT-IR spectrometer with Smart iTR, which is an ultra-high-performance, versatile attenuated total reflectance (ATR) sampling accessory. The nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance III 400 (9.4 T, 400.13 MHz for 1H, 100.62 MHz for 13C, 376.25 MHz for 19F and 40.56 MHz for 15N) spectrometer with a 5-mm Broadband Fluorine Observation (BBFO) plus probe (Nuclear Magnetic Resonance Spectrophotometer probe type), at 298 K. Chemical shifts (δ in ppm) were given relative to the internal solvent: DMSO-d6 2.50 for 1H, 39.50 for 13C, and for 15N NMR, nitromethane (0.0) was used as an external standard. The standard Bruker pulse sequence hmbcgplpndqf was used for 1H–15N Heteronuclear Multiple Bond Correlation (HMBC) experiments.

Mass spectra were recorded on a Thermo ISQ single quadruple gas chromatography-mass spectrometer. Elemental analyses were conducted on a EuroVector instrument C, H, N, S analyzer EA3000 Series. Microwave experiments were conducted on a CEM Discover & Explorer SP microwave apparatus (300 W), utilizing 35 mL

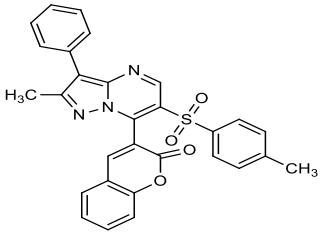
capped glass reaction vessels with automated power control based on temperature feedback (Al-Bogami et al. 2014).

Pepper seeds of the susceptible cv. Sirtaki were germinated and grown in pots (30 × 30 cm) containing sterilized soil under optimum controlled conditions. Pepper seedlings were inoculated with PMMoV after 25 d from planting and spread by solution from chemical synthesis treatment (Pyrazolo[1,5-a]pyrimidine derivatives containing sulfone moiety) diluted to (100 mL/100 µg) 24 h after virus inoculation.

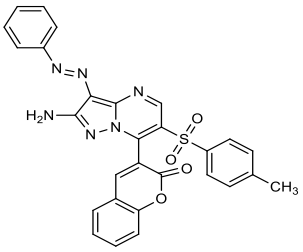
The crude sap from young pepper leaves infected with PMMoV was extracted into potassium phosphate buffer (pH 7.2) with the cooling mortar, and then centrifuged at 10,000 g for 5 min. The supernatant was collected by gently rubbing the dusted healthy pepper seedling. Pots were placed in a growth chamber at 27 °C with 12 h photoperiod and 70–80 % relative humidity in a completely randomized block design with three seedlings per pot, one pot per replicate, and 4 replicates per treatment, giving 3 seedlings per replicate to make a total of 12 seedlings per treatment. The ELISA test was used in addition to symptoms observation for checking the presence of the virus.

The novel synthetic compounds used as treatments against PMMoV were identified as follows: Treatment 1: (CH) control healthy; Treatment 2: (CI) control infected; Treatments 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12: (H and Co.1), (H and Co.2), (H and Co.3), (H and Co.4), (H and Co.5), (H and Co.6), (H and Co.7), (H and Co.8), (H and Co.9) and (H and Co.10), respectively; Treatments 13, 14, 15, 16, 17, 18, 19, 20, 21 and 22: (I and Co.1), (I and Co.2), (I and Co.3), (I and Co.4), (I and Co.5), (I and Co.6), (I and Co.7), (I and Co.8), (I and Co.9) and (I and Co.10), respectively. After 3 wk of PMMoV inoculation, plants were selected and examined for the presence of PMMoV. The proposed novel synthetic compounds were also illustrated as Novel Series of Pyrazolo [1,5-a] pyrimidine derivatives containing Sulfone Moiety, as shown below:

Series: Pyrazolo [1,5-a] pyrimidine derivatives containing Sulfone Moiety



3-(2-methyl-3-phenyl-6-tosylpyrazolo[1,5-*a*]pyrimidin-7-yl)-2*H*-chromen-2-one



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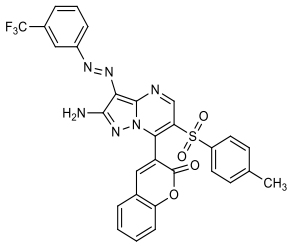
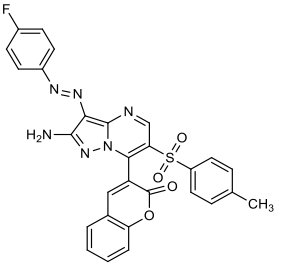


(*E*)-3-(2-amino-6-tosyl-3-((3-(trifluoromethyl)phenyl)diazenyl)pyrazolo[1,5-*a*]pyrimidin-7-yl)-2*H*-chromen-2-one



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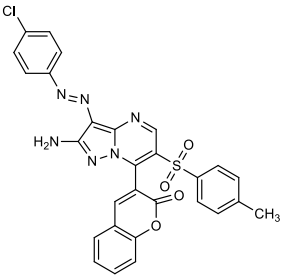

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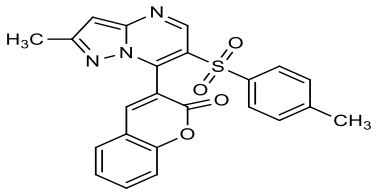
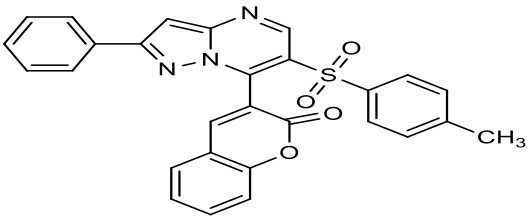
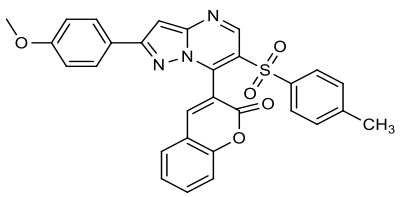


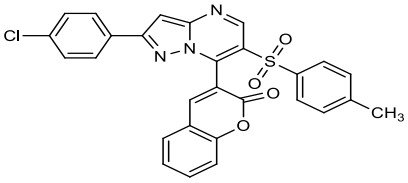
3-(2-methyl-6-tosylpyrazolo[1,5-*a*]pyrimidin-7-yl)-2*H*-chromen-2-one



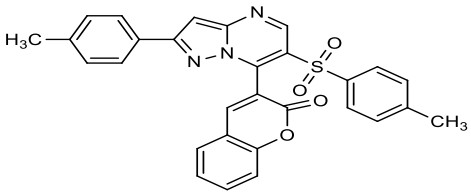
3-(2-phenyl-6-tosylpyrazolo[1,5-*a*]pyrimidin-7-yl)-2*H*-chromen-2-one



3-(2-(4-methoxyphenyl)-6-tosylpyrazolo[1,5-*a*]pyrimidin-7-yl)-2*H*-chromen-2-one



3-(2-(4-chlorophenyl)-6-tosylpyrazolo[1,5-*a*]pyrimidin-7-yl)-2*H*-chromen-2-one



3-(2-(*p*-tolyl)-6-tosylpyrazolo[1,5-*a*]pyrimidin-7-yl)-2*H*-chromen-2-one

PMMoV concentration in infected pepper leaves was measured by ELISA test. After 21 d from virus inoculation, infected upper leaves of the seedlings inoculated were collected and ground in carbonate buffer. Disease severity index was recorded, following a five-point (0–4) disease severity index, where 0 = no visible disease symptoms (highly resistant); 1 = mild mosaic or mottling and leaf stunted (resistant); 2 = moderate mosaic or mottling and fruit deformity (tolerant); 3 = mosaic or mottling or leaf stunted, minor to medium stunting (susceptible); and 4 = mosaic or mottling, leaf deformity, stunting (highly susceptible). The disease severity formula was adopted from Yang et al. (1996), as follows:

$$DS\,(\%) = \frac{(\text{disease grade} \times \text{number of plants in each grade})}{(\text{total number of plants} \times \text{highest disease grade})} \times 100$$

The antimicrobial activity of the novel synthetic compounds from *Pyrazolo[1,5-a] pyrimidine* was in-vitro tested against human pathogens including *Klebsiella oxytoca ATCC 49131*, *Salmonella typhimurium ATCC 14028*, *Staphylococcus aureus* (methicillin-resistant strain) ATCC 43330, *Micrococcus luteus ATCC 49732*, *Candida albicans ATCC 10231*, *Aspergillus niger* (lab isolate) and *Aspergillus flavus* (lab isolate) using the agar well diffusion technique. Specifically, the above strains were inoculated by spreading 100 µL of cells suspension (5 × 10^6 CFU) into Petri dishes containing 20 mL of NA at T = 60 °C. After cooling, wells of 5 mm diameter were opened and 50 µL of each synthetic compound of Pyrazolo[1,5-1] pyrimidine was potted in each well. Following incubation at 35 °C for 18 h, the sensitivity of the organisms was estimated by measuring the diameter of the inhibition zone around each well to the nearest mm.

RESULTS AND DISCUSSION

Naturally infected pepper with PMMoV showed symptoms that usually include various degrees of mottling, chlorosis, curling and dwarfing on the upper developing leaves (Fig. 1A). Similar symptoms have been recorded in previous papers (Lanter et al. 1982; Palloix et al. 1994b; Komuro and Iwaki 1969; Kirita et al. 1997; Caglar et al. 2013). No symptoms were observed in the non-infected leaves. Moreover, PMMoV partial RNA-dependent RNA polymerase (RdRp) (830 bp) genes were detected by using RT-PCR (Fig. 1B) in 14 samples of infected leaves, whereas no symptoms were detected in healthy plants. These results were in agreement with the findings of Hadidi et al. (1993) and Velasco et al. (2002). In ultra-thin sections of pepper leaves inoculated with PMMoV, viral particles that were rigid-rod shaped, as well as inclusion bodies containing laminated aggregates of crystalline particles, typical of *Tobamoviruses*, were observed (Fig. 1C). The genus Tobamovirus includes PMMoV and consists of a single-strand positive sense RNA of 6357 nucleotides (Garcia-Luque et al. 1990; Kirita et al. 1997; Caglar et al. 2013).

The novel series of Pyrazolopyrimidine compounds had no effect on the healthy seedlings (data not shown), which was a prerequisite for the potential use of Pyrazolopyrimidine in agriculture. However, the treatment of infected plants with Pyrazolopyrimidine compounds induced different plant responses, depending on the treatment time after 24 h from virus inoculation (post-viral infection) and on differences in compound structures. In particular, the compounds (1), (2), (6),

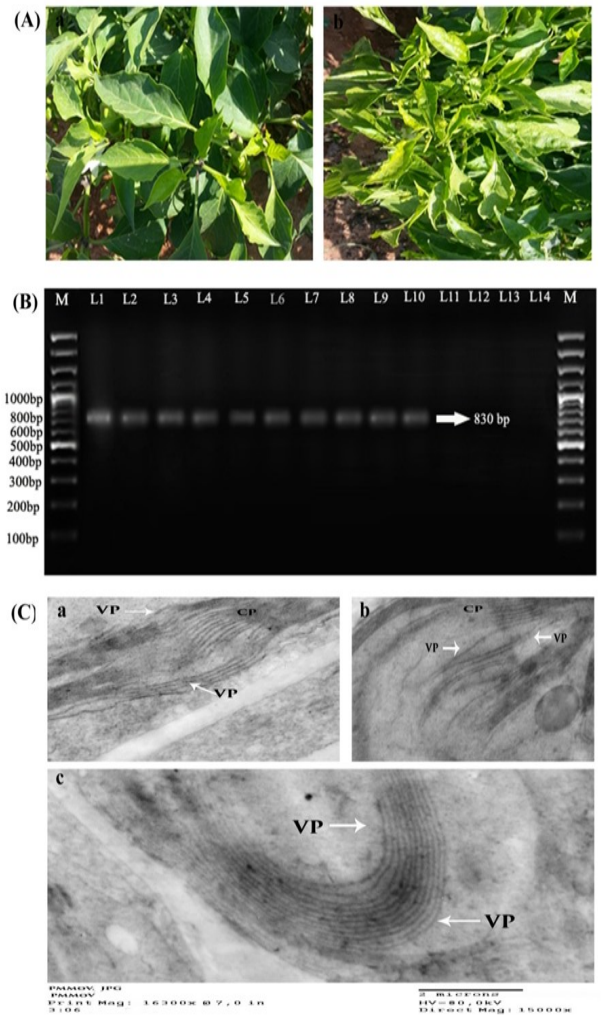


Fig. 1. *Pepper mild mottle virus* (PMMoV) isolation and identification shows (A) symptoms caused by natural PMMoV infection on pepper leaves observed from the Mecca region (a) Healthy plant and (b) showing mosaic symptoms on the upper developing leaves followed by infected leaves that are frequently puckered and mottled yellow or light green. (B) electropherogram showing RNA-dependent RNA polymerase (RdRp)-PCR amplicons primed with P12/3A and P12/3. M: DNA Marker 1 kb; lanes 1–10: representatives of PMMoV-infected samples; lanes 12–13: healthy pepper plant and lane 14: water control reaction. (C) electron micrograph of an ultra-thin section of infected pepper leaves of light green area PMMoV (a, b, c) showing laminated aggregate of crystalline particles (CP) and viral particles (VP).

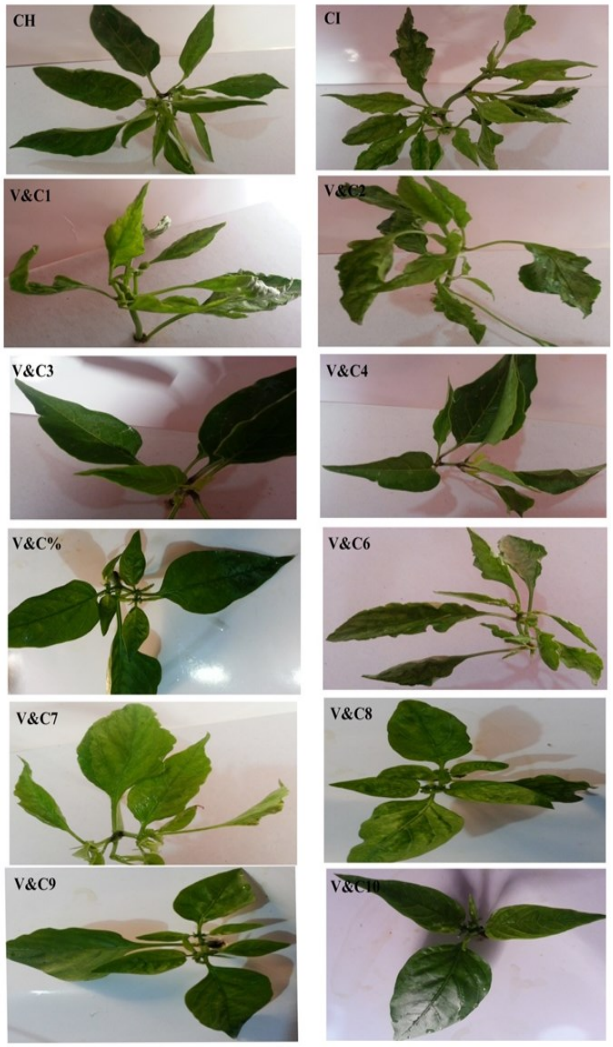


Fig. 2. Effect of different synthetic compounds (100 mL/100 µg) on estimate of symptoms and disease severity of pepper susceptible cultivar, cv. Sirtaki, in the presence of *Pepper mild mottle virus* (PMMoV) under greenhouse condition showing mosaic symptoms on the upper developing leaves and infected leaves that are frequently puckered and mottled yellow or light green. CH: Control healthy leaves. CI: Control infected leaves, and (V & Co.1), (V & Co.2), (V & Co.3), (V& Co.4), (V& Co.5), (V& Co.6), (V& Co.7), (V& Co.8), (V& Co.9) and (V& Co.10) inoculated pepper leaves treated by PMMoV under synthetic chemical treatments.

(7) and (8) had no effect on either the virus concentration or on the severity of the disease (Fig. 2, Table 1). In contrast, remarkable results were observed in the compound (3), (4), (5), (9) and (10) treatments (i.e., 24 h after inoculation) in which each of the Pyrazolopyrimidine compounds prevented all destructive symptoms caused by the virus, while weak PMMoV symptoms were observed when plants were treated by compound (3). Quantitative data on the positive effect of Pyrazolopyrimidine compounds on PMMoV concentration, percentage of infection and disease severity (summarized in

Table 1. Effect of different new synthetic chemicals (100 mL/100 µg) on estimate of virus concentration, percentage of infection and disease severity of pepper susceptible cultivar, cv. Sirtaki, in the presence of *Pepper mild mottle virus* (10^{-3}–10^{-4}) under greenhouse condition.

Treatment	Synthetic Chemicals							
	Virus Concentration M ± SD	Percentage of Infection					Disease Severity %	
		Virus Infectivity (I)						
		R1	R2	R3	R4	(%)	DG	DS %
Control healthy	0.000[a] ± 0.000	0/3	0/3	0/3	0/3	00.00	0	00.00
Control infected	1.436[b] ± 0.005	3/3	3/3	3/3	3/3	100	4	100
Virus & C1	1.422[b] ± 0.013	3/3	3/3	3/3	3/3	100	4	100
Virus & C2	0.998[c] ± 0.006	1/3	3/3	2/3	2/3	66.67	3	50
Virus & C3	0.112[d] ± 0.005	0/3	0/3	1/3	1/3	16.67	1	4.17
Virus & C4	0.020[d] ± 0.006	0/3	0/3	0/3	0/3	00.00	0	00.00
Virus & C5	0.011[d] ± 0.001	0/3	0/3	0/3	0/3	00.00	0	00.00
Virus & C6	1.382[b] ± 0.048	3/3	3/3	2/3	3/3	91.67	4	91.67
Virus & C7	1.157[b] ± 0.022	2/3	3/3	3/3	2/3	83.33	3	62.50
Virus & C8	1.014[b] ± 0.092	3/3	1/3	2/3	3/3	75.00	2	37.50
Virus & C9	0.013[d] ± 0.005	0/3	0/3	0/3	0/3	00.00	0	00.00
Virus & C10	0.059[d] ± 0.009	0/3	0/3	0/3	0/3	00.00	0	00.00

The values are means (M) of four replicates ± standard deviation (SD). Averages followed by the same letter within a column are not significantly different at P ≤ 0.05. In ELISA test for virus concentration, the positive and negative controls are 1.573 and 0.113, respectively. Positive control means infected leaves showed symptoms typically, and negative control means infected leaves showed no symptoms.

Table 1), indicate an important decrease in virus concentration, percentage of infection and disease severity. The positive effects of Pyrazolopyrimidine that enable the virus to replicate itself by stopping the viral messenger RNA replicate tend to stop the spread of the virus in infected pepper plants (Al-Bogami et al. 2014). Xiao et al. (2015 mentioned that the A series of novel pyrazole amide derivatives **3a**–**3p**, which take TMV PC protein as the target, has been designed and synthesized by the reactions of 5 -chloro-1-aryl-3-methyl-1*H*-pyrazole-4-carboxylic acids with 5-amino-1-aryl-1*H*-pyrazole-4-carbonitriles. All the compounds were characterized by 1H-NMR, mass spectroscopy and elemental analysis. Preliminary bioassays indicated that all the compounds acted against the tobacco mosaic virus (TMV) with different *in vivo* and *in vitro* modes at 500 µg/mL and were found to possess promising activity. Most especially important to mention is the compound **3p**, which showed the most potent biological activity against TMV when compared with ningnanmycin.

As shown in Table 2, Pyrazolopyrimidine compounds were the most potent among the 10 compounds in terms of antibacterial activity. The highest inhibition zone against *K. oxytoca* and *S. typhimurium* were detected by Pyrazolopyrimidine compounds 9, 5, and 4 followed by 10 and 3, while 10, 9, 4 and 5 were the most potent Pyrazolopyrimidine compounds against methicillin-resistant *S. aureus (MRSA)*. Compounds 5, 10, 4 and 9 of Pyrazolopyrimidine were the most potent and exhibited the highest inhibition zone against *M. luteus*.

Pyrazolopyrimidine compounds were the most effective among the 10 compounds in terms of antifungal activity (Table 2). None of the Pyrazolopyrimidine compounds 8, 9 and 10 exhibited potency against *C. albicans*. Compounds 5, 2, 1 and 4 of Pyrazolopyrimidine were the most potent and exhibited a high inhibition zone against *C. albicans*, whereas 10, 5, 9 and 4 were the most potent against *A. niger* and *A. flavus*.

Several recent reviews and studies have reported the antimicrobial efficacy of Pyrazolopyrimidine molecules (Nowakowska et al. 2007; Sobolev et al. 2009). The antimicrobial effectiveness of Pyrazolopyrimidine is due to the capability of molecules to form complexes with both extracellular and soluble proteins as well as bacterial membranes (Cowan 1999; Fowler and Koffas 2009). The penetration into the cell and the maintenance of intracellular concentrations in infecting species becomes a critical concern for the development of Pyrazolopyrimidine as the next generation of antibacterial/antifungal agents.

CONCLUSION

PMMoV isolation and identification were done by using viral symptoms, serological test, and ultra-thin section by electron microscopy and confirmed by RT-PCR. We studied the effects of novel synthetic chemical compounds from Pyrazolo-pyrimidine treatments on infected pepper with PMMoV infection. The results showed that the efficacy of the antiviral agent depends on the

Table 2. Inhibition zone (mm) caused by different new synthetic chemicals (Pyrazolo [1,5-a] pyrimidine derivatives containing Sulfone Moiety) on cultures of human pathogen bacteria and fungi.

	K. oxytoca ATCC 49131	*S. typhimurium* ATCC 14028	*S. aureus* (MRSA) ATCC 43330	*M. luteus* ATCC 49732	*C. albicans* ATCC 10231	*A. niger*	*A. flavus*
C1	13.93 ± 0.06	13.67 ± 1.15	15.00 ± 0.00	11.33 ± 0.58	20.33 ± 0.58	12.33 ± 0.58	15.33 ± 0.00
C2	19.27 ± 2.00	20.00 ± 0.00	22.33 ± 1.53	15.60 ± 1.51	21.33 ± 1.15	10.00 ± 1.00	12.00 ± 0.00
C3	23.57 ± 3.39	26.33 ± 0.58	22.00 ± 1.00	15.33 ± 0.58	19.33 ± 0.35	11.67 ± 0.58	12.67 ± 0.58
C4	27.00 ± 1.00	27.00 ± 0.00	29.67 ± 0.58	20.00 ± 1.00	20.00 ± 0.00	12.33 ± 0.58	12.33 ± 0.58
C5	28.67 ± 1.53	29.67 ± 0.58	26.33 ± 0.58	20.67 ± 2.08	22.00 ± 0.00	14.67 ± 0.58	15.53 ± 0.92
C6	14.33 ± 0.58	15.33 ± 0.58	20.00 ± 1.00	11.33 ± 0.58	19.30 ± 0.35	08.67 ± 0.58	10.00 ± 1.00
C7	14.33 ± 1.53	14.00 ± 1.00	20.67 ± 1.53	11.00 ± 0.00	19.30 ± 1.15	10.00 ± 1.15	08.33 ± 0.58
C8	19.67 ± 2.08	20.67 ± 1.15	22.33 ± 0.58	15.67 ± 1.15	00.00 ± 00.0	11.33 ± 0.00	12.00 ± 0.00
C9	28.67 ± 1.53	30.00 ± 1.00	30.00 ± 1.00	19.67 ± 1.53	00.00 ± 00.0	12.33 ± 1.15	12.67 ± 0.58
C10	25.33 ± 1.53	25.00 ± 0.00	30.33 ± 0.58	20.33 ± 1.53	00.00 ± 00.0	14.00 ± 0.00	16.00 ± 1.00

The values are the means of three experiments ± SD.
MRSA – methicillin-resistant *S. aureus*

capability of the virus to replicate itself by stopping the viral messenger RNA replicate and thus prevent the spread of the virus in infected pepper plants. We also studied the inhibitory effects of the Pyrazolopyrimidine compounds on antimicrobial activity against important human pathogens.

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