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Insecticidal and Fungicidal Activity of Phosphorothioates of Eugenol

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***O*,*O*-Dialkyl thiophosphate esters of eugenol namely, *O*-(4-allyl-2-methoxyphenyl) *O*,*O* dimethyl thiophosphate and *O*-(4-allyl-2-methoxyphenyl) *O*,*O*-diethyl thiophosphate were prepared, characterized and screened for insecticidal and fungicidal activity. The diethyl derivative was found to be more toxic to *Rhizopertha dominica* (Lesser grain borer) than eugenol and the methyl derivative was similar to eugenol in toxicity to this insect. However, these compounds were found to be fungicidal even after eugenol was derivatized to the thiophosphates.**

Key words: Eugenol derivatives, Phosphorothioates, Stored product insects, Antifungal activity.

Pesticides are an indispensable tool to protect agricultural crops, for use in food storage and also in public health like malaria eradication programme. A number of pesticides have become ineffective due to the development of resistance by insect pests. Numerous cases of pest resistance to insecticides have been identified (Taylor and Feyereisen, 1996). This is due to the continuous usage of these compounds over a long period of time. Hence there is need to develop and screen chemical compounds for their biological activity against pests, especially the stored product insects.

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Eugenol (4-allyl-2-mehoxyphenol) is widely used as a general antiseptic in medical and dental practice, agriculture, cosmetics and the food industry, due to its potent fungicidal, bactericidal, anti-oxidant and anti-inflammatory properties (Fujisawa *et al*., 2002; Criddle *et al*., 2003). It is a clear to pale yellow oily liquid extracted from certain essential oils especially from clove oil and cinnamon. It is very slightly soluble in water and soluble in organic solvents. It has a spicy odor and taste of clove. However, not much work has been done on the derivatives of eugenol. We have prepared organophosphate derivatives of eugenol and screened them for their biological activity against stored pests and also fungi.

O,O-Dialkyl phosphorotioate derivatives of eugenol on hydrolysis give eugenol, which is a safer compound that itself is active against insects and fungi. Therefore these compounds were screened for their toxicity against stored product insects. The diethyl thiophosphate of eugenol was prepared by modification of the literature (German patent No. 887814, 1953) and the dimethyl

thiophosphate was similarly prepared (Knowles and Arthur., 1967). They were purified and characterized by ^{1}H and ^{13}C NMR. While these compounds have been screened against mosquito larvae and other insects, no work has been done to find their effectiveness against this stored product insect pest. Therefore, the present work was undertaken.

MATERIAL AND METHODS

Diethyl and dimethyl thiophosphoryl chloride was purchased from Excel Industries Ltd, Mumbai and eugenol from Spectrochem Pvt. Ltd. Mumbai. 4-Amino-*N*,*N*-dimethylaniline dihydrochloride was supplied by Fluka AG, Büchs, Switzerland. Silica gel pre-coated TLC aluminum sheets 20x20 cm, 60 F_{254} were purchased from E. Merck, Germany. NMR spectra were recorded on a 500 MHz Brüker spectrometer with respect to TMS as reference.

Preparation of O-(4-allyl-2-methoxyphenyl) O,O-dimethyl/diethyl thiophosphate

Eugenol (1.5 g) was dissolved in acetone (25 mL) and potassium carbonate (5 g) was added followed by dimethyl thiophosphoryl chloride (1.21 mL) and the mixture was stirred at room temperature overnight. Similarly eugenol (1.5 g) was dissolved in acetone (25 mL) and potassium carbonate (5 g) was added followed by diethyl thiophosphoryl chloride (1.56 mL) and the mixture was stirred as given above.

The reaction was monitored by TLC using petroleum ether and diethyl ether (80+20) as mobile phase and the plate was subsequently sprayed with 4-amino-*N*,*N*-dimethylaniline followed by exposure to bromine vapour (Akmal pasha *et al.,* 1996). Both the dimethyl and the diethyl derivatives were visualized as intense magenta colored spots at Rf 0.27 and 0.30 respectively whereas spot due to eugenol was observed at 0.17. The compounds were subjected to work-up and extracted into dichloromethane, the solvent was evaporated off and purified by passing through basic alumina packed in a glass column and eluted with light petroleum (60-80°C). The un-reacted eugenol was adsorbed onto the column and the pure derivatives eluted out as checked by TLC method given above. Yield: 1.19 gm (45 %) for dimethyl and 1.80 gm (64%) for the diethyl derivative. The structure was characterized by ^{1}H and ^{13}C NMR as given below:

O-(4-Allyl-2-methoxyphenyl) O,O-dimethyl-thiophosphate

^{1}H NMR ($CDCl_3$): δ 3.37 (d, 2H, J=7 Hz, -CH_2- allyl), 3.86 (s, 3H, -OCH_3-Ar), 3.89 (d, 6H, $J_{P,H}$=14 Hz, -$PS(OCH_3)_2$,), 5.12 (m, 2H, =CH_2 allyl), 5.97 (m, 1H, -CH=C allyl), 6.75 (d, 1H, $J_{5,6}$=8 Hz, C-6 Ar), 6.79 (s, 1H, C-3 Ar), 7.10 (dd, 1H, $J_{6,5}$=8 Hz, $J_{5,3}$=1.5 Hz, C-5 Ar) ppm.

^{13}C NMR ($CDCl_3$): δ 39.66 (s, Ar-CH_2-C= allyl), 54.68 (d, -$PS(OCH_3)_2$), 55.64 (s,-OCH_3-Ar), 112.84 (s, C-3, Ar), 115.83 (s, =CH_2 allyl), 120.27 (s, C-6, Ar), 121.65 (d, C-5, Ar), 136.69 (s, -CH=CH_2 allyl), 137.84 (s, C-4, Ar), 150.53 (d, C-1, Ar), 158.50 (d, C-2, Ar) ppm.

O-(4-Allyl-2-methoxyphenyl) O,O-diethyl-thiophosphate

^{1}H NMR ($CDCl_3$): δ 1.38 (t, 6H, J=7 Hz, -$PS(O\text{-}CH_2CH_3)_2$), 3.38 (d, 2H, J=7 Hz, -CH_2- allyl), 3.86 (s, 3H, -OCH_3-Ar), 4.27 (m, 4H, $J_{P,H}$=2.5 Hz, -$PS(O\text{-}CH_2\text{-}CH_3)_2$,), 5.12 (m, 2H, =$CH_2$ allyl), 5.97 (m, 1H, -CH=C allyl), 6.75 (d, 1H, $J_{5,6}$=8 Hz, C-6 Ar), 6.79 (s, 1H, C-3 Ar), 7.10 (dd, 1H, $J_{6,5}$=8 Hz, $J_{5,3}$=1.5 Hz, C-5 Ar) ppm.

^{13}C NMR ($CDCl_3$): δ 39.66 (s, Ar-CH_2-C= allyl), 54.68 (d, -$PS(OCH_3)_2$), 55.64 (s,-OCH_3-Ar), 112.84 (s, C-3, Ar), 115.83 (s, =CH_2 allyl), 120.27 (s, C-6, Ar), 121.65 (d, C-5, Ar), 136.69 (s, -CH=CH_2 allyl), 137.84 (s, C-4, Ar), 150.59 (d, C-1, Ar) ppm.

Fungal cultures

Aspergillus parasiticus, *Aspergillus flavus*, *Fusarium oxysporum*, *Fusarium graminerum* were obtained from Lleida University, Lleida, Spain. Species of *Penicillium monoverticillatum* and *Penicillium rubrum* were locally isolated from cereal samples and identified based on colony characterization and morphological structures under the microscope (Olympus, Japan) according to Raper and Fennell (1949). The other two species of fungi, *Metarhizium anisopliae* and *Beauveria bassiana* were isolated from soil and identified with the help of mycological identification manual Humber *et al*., 1997). All isolated fungal cultures were finally purified using "single spore culture technique" (Johnstone ., 1969).

Fungicidal Activity

O-(4-Allyl-2-methoxyphenyl) *O*,*O*-dimethyl thiophosphate and *O*-(4-allyl-2-

methoxyphenyl) *O*,*O*-diethyl thiophosphate were dissolved in acetone separately to prepare 1000 ppm solution and diluted serially in acetone to obtain 50, 100, 250, 500 ppm. For control plates, 250 ppm of standard fungicide carbendazim (CBZ) was dissolved in minimum quantity of acetone and used. Appropriate concentrations of all above solutions were pour plated to freshly prepared Potato Dextrose agar (PDA) medium. Seven day old pure cultures of all the above individual test cultures grown on PDA plates were cut using cork borer (8 mm size) and aseptically transferred to freshly prepared corresponding medium containing varied concentration of eugenol and its derivatives. Inoculated plates (in triplicate) were incubated at 25±1°C and examined daily for five days using stereoscopic microscope. The increase in size was followed by measuring the colony diameter at right angles to each other and means diameter (in mm) was recorded as given in Table 1.

Insecticidal activity

Eugenol, *O*-(4-Allyl-2-methoxyphenyl) *O*,*O*-dimethyl thiophosphate and *O*-(4-allyl-2-methoxyphenyl) *O*,*O*-diethyl thiophosphate were dissolved in acetone separately to prepare 1000 ppm solution and diluted serially in acetone to obtain 250 and 500 ppm. Clean culture tubes (2.5x10 cm, size) lined with Whatman No.1 filter paper were taken and 1 mL of test sample at different concentration was added uniformly on the filter paper and kept for drying about 10-15 minutes. Twelve days old adult insects (*Rhizopertha dominica*) reared in whole wheat (13% moisture) were released into culture tubes. For each concentration triplicates were maintained (ten insects in each tube). The mouth of inoculated tubes was tied with clean muslin cloth. Controls (without chemical) were also run along with treated tubes. The percent mortality of insects was recorded daily for 4 days and the mean values were calculated (Table 2).

RESULTS AND DISCUSSION

The dialkyl phosphorothioates of eugenol were prepared by modification of the reported methods. Reaction was carried out by stirring the reactants in acetone and no copper salt was used as catalyst (German patent No. 887814, 1953) and the reaction mixture was not refluxed (German patent No. 887814, 1953 and Knowles and Arthur., 1967). The reaction was monitored by TLC and the derivatives were purified by column chromatography instead of distillation at low pressure as in the literature method (German patent No. 887814, 1953) and characterized using ^{1}H and ^{13}C NMR.

Antifungal activity of phosphorothioate

Table 1. Fungicidal activity of phosphorothioate derivatives of eugenol

Test Fungi	Radial Growth (mm)														
	O-(4-allyl-2-methoxyphenyl) dimethyl thiophosphate					O,O-O-(4-allyl-2-methoxy phenyl) *O*,*O*-diethyl thiophosphate					4-allyl-2-methoxyphenol				
	50 ppm	100 ppm	250 ppm	500 ppm	1000 ppm	50 ppm	100 ppm	250 ppm	500 ppm	1000 ppm	50 ppm	100 ppm	250 ppm	500 ppm	1000 ppm
A. parasiticus	30.0	23.0	20.0	18.0	15.0	25.0	24.0	22.0	21.0	20.0	28.0	14.0	9.0	0.0	0.0
A .flavus	27.0	25.0	22.0	18.0	15.0	26.0	26.0	23.0	18.0	15.0	20.0	16.0	9.0	0.0	0.0
F. oxysporum	37.0	35.0	26.0	21.0	20.0	36.0	33.0	30.0	24.0	22.0	22.0	15.0	0.0	0.0	0.0
F. graminearum	28.0	26.0	24.0	20.0	18.0	27.0	26.0	26.0	23.0	16.0	23.0	15.0	0.0	0.0	0.0
P. rubrum	20.0	20.0	15.0	15.0	12.0	19.0	18.0	16.0	15.0	14.0	18.0	10.0	0.0	0.0	0.0
P.monoverticilate	21.0	16.0	15.0	15.0	14.0	21.0	19.0	17.0	16.0	14.0	17.0	12.0	11.0	0.0	0.0
B. bassiana	16.0	14.0	13.0	11.0	9.0	16.0	15.0	14.0	13.0	10.0	15.0	9.0	0.0	0.0	0.0
M. anisopliae	16.0	15.0	13.0	10.0	0.0	18.0	17.0	15.0	11.0	0.0	11.0	0.0	0.0	0.0	0.0
Fungicide standard (CBZ)	12	10	11	11	5	9	12	16	8	4	9	3	0	0	0

*Values are the mean of triplicate plates

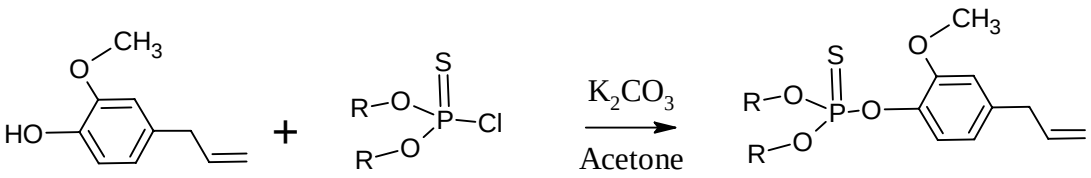

Fig. 1. Scheme of reaction for the preparation of phosphorothioates of eugenol

derivatives along with parent compound eugenol is presented in Table 1. It was found that the radial growth rate (RGR) was directly proportional to the concentration of the compound used. It has been established that a linear increase in size of colony diameter as a function of incubation time for molds in solid medium and found significant differences among RGR (Lopez-Malo *et al.,* 1997; Gonzalez *et al.,* 1987). In this study, RGR was affected by all test compounds compared to control. Eugenol at 250 ppm concentration inhibited most of the test fungi, except a few fungal species such as *A. parasiticus* and *A. flavus* which showed only 9.0 mm growth. Further, slight increase in colony diameter (11.0 mm) was recorded in *P. monoverticilate*. The other test fungi did not show any growth. However, eugenol at 500 and 1000 ppm concentration showed complete inhibition with all the test fungi (Table-1). In general, entomopathogenic fungi *(M. anisopliae* and *B. bassiana*) are more sensitive for fungicidal activity among the organisms tested. The species of *Aspergillus*: *A. parasiticus* and *A. flavus* and also *Fusarium* species *F. oxysporum, F. graminerum* are more resistant as they have exhibited higher radial growth rates (RGR). The species of *Penicillium*: *P. rubrum* and *P. monoverticiate* were moderately affected for fungicidal activity. Therefore, the parent compound is more effective in inhibiting the fungus than its derivatives. Furthermore, not much difference was observed in the fungicidal activity between the two derivatives and this was true with all concentration tested (from 50 to 1000 ppm).

The insecticidal activity of the compounds against *Rhizopertha dominica* is presented in Table 2. The study showed that the insect mortality was directly proportional to the dose of the compounds. Among the three, *O*-(4-allyl-2-methoxyphenyl) *O,O* diethyl thiophosphate has shown higher mortality (66.6% mortality on fourth day). Moderate effectiveness was recorded with eugenol. In most of the cases, ethyl derivative was slightly more toxic than eugenol and the methyl derivative was almost as toxic as eugenol itself. The parent compound eugenol was more fungicidal than the ethyl and methyl phosphorothioate of eugenol.

Table 2. Insecticidal activity of phosphothioate derivatives of eugenol against *Rhizopertha dominica*

Days	Percent Mortality									
	O-(4-allyl-2-methoxyphenyl) *O,O* dimethyl thiophosphate			*O*-(4-allyl-2-methoxyphenyl) *O,O*-diethyl thiophosphate			4-allyl-2-methoxyphenol			Control
	250 ppm	500 ppm	1000 ppm	250 ppm	500 ppm	1000 ppm	250 ppm	500 ppm	1000 ppm	
1st Day	6.6	10.0	16.6	6.6	13.3	23.3	10.0	20.0	20.0	6.6
2nd Day	13.3	16.6	26.6	23.3	33.3	43.3	20.0	30.0	36.6	10.0
3rd Day	26.6	33.3	36.6	36.6	43.3	56.6	26.6	40.0	50.0	13.3
4th Day	40.0	40.0	53.3	46.6	53.3	66.6	36.6	46.6	60.0	26.6

*Values are the mean of triplicate sets

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