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One-pot three component synthesis of xanthene by ZnO nanoparticles catalysis and its antifungal effect on soybean seed-borne fungi

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ABSTRACT

A novel 3,3, 6,6- tetra methyl-9-styryl-3,4,5,6,7,9-hexahydro-*2H*- xanthene-1, 8-dione was synthesized by reaction of trans-cinnamaldehyde with the dimedone using zinc oxide nanoparticle as a catalyst. This xanthene molecule was characterized by the spectral analysis and screened for antifungal activity against soybean seed-borne fungi by the posion food method. Among the fungi tested *Macrophomina phaseolina* was found to be more susceptible when compared to *Aspergillus flavus.*

Keywords: *Glycine max* (L.) Merril, Seed-borne fungi, trans-cinnamaldehyde, Dimedone, Zinc oxide nanoparticles, 3,3, 6,6- tetra methyl-9-styryl-3,4,5,6,7,9-hexahydro-*2H*- xanthene-1, 8-dione.

1. INTRODUCTION

Soybean [*Glycine max* (L.) Merrill] have been cultivated in most of the countries for both protein and vegetable oil content [1]. Soybean is frequently defined as the miracle golden bean, the pearl of the Orient, the Cinderella crop of the century, the meat that grows on vines, the protein hope of the future and the salvation crop. Soybean seed is a versatile food plant that is used in various forms and was capable of supplying most nutrients [2]. It can substitute for meat and to some extent for milk too [3-5]. It is a singular food because of its rich nutrient contents [6]. Soybean contains vegetable proteins, oligosaccharides, dietary fibers, phytochemical (especially isoflavones) and minerals [7-8]. Soy proteins are superior among plant proteins because they comprise good supplies of essential amino acids, though they are slightly deficient in some sulphur containing amino acids such as cysteine and methionine. Soybean known to contain higher total digestible nutrient percentage of 91.99% related to cowpea with 79.52%. Consequently, soybean consumption is more supportive in solving nutrition protein-intake problem among the population of third world countries. Nevertheless soybean lack starches and it contain other carbohydrates such as cellulose, pectin and phytic acid. Not only does cellulose promote good elimination together with other indigestible fibre, it helps in maintaining good physical condition and preventing rectal cancer. Soybean is a crop that can end malnutrition if grown as staple food crop and if soya food products are incorporated into local diets in poorer countries. Worldwide production of soybean was 267.02 million metric ton in 2013 and projected to increase to 311.1 million metric ton in 2020.

It has been recognized that under tropical conditions seed deterioration takes place in soybean seeds throughout storage. Storage fungi cause deterioration of stored soybeans consequentially reduced seed germination and downgrading of grain because of damage with mustiness. Seed-borne fungi has attributed to the crop losses by reducing the yields and lower seeds germination, and protein as well as oil content [9]. Chemical fungicides have been a main measure to reduce the incidence of seed-borne fungi [10]. However, the application of chemical fungicides often results in toxic to the environment, to human beings and animal health [11]. The demand for the development of alternative antimicrobial agents has led to research on the plant-based extracts [12]. In recent years there is a great demand for the synthesis of industrially important

and biological molecules, while there are a few methods reported in the literature for the synthesis of 14-aryl-14*H*-dibenzo[*a* ,*j*] xanthene derivatives [13], as there are no methods are available for the synthesis of 3,3, 6,6- tetra methyl-9-styryl-3,4,5,6,7,9-hexahydro-*2H*- xanthene-1, 8-dione. Hence, efforts are made to synthesize the target molecule that can serve as a better agent for the eradication of soybean seed-borne fungi.

Melting point of the prepared xanthene is 101-102 °C and boiling point is 310-312 °C with the chemical formula $C_{13}H_{10}O$ [14]. Xanthene skeleton was known to present in a number of natural products [15]. Xanthene possess biological activities such as antifungal, antibacterial, antiviral, anti-inflammatory and many others [16-17]. In addition, these compounds are used extensively in dyes, in laser technologies and used as pH sensitive fluorescent materials for visualization of biomolecules [18-19]. A group of xanthene derivatives were also reported as anthelmintics specifically with antischistosomal activity. Ehretianone, a quinonoid xanthene was reported to possess antisnake venom activities [20].

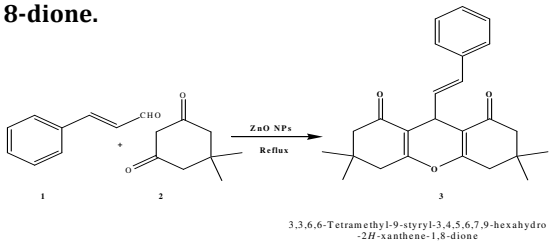
2. EXPERIMENTAL

2.1. Chemicals and reagents

All chemicals are commercial products and were of analytical grade and used without further purification. All the reagents were purchased from Merck (Damrstadt, Germany). The prepared xanthenes was characterized by NMR and Mass spectral analyses. Melting point was measured on a Raaga, Indian make melting point apparatus. NMR spectra were obtained on a 400 MHz and 100 MHz Bruker AMX instruments in $CDCl_3$ using TMS as an internal standard. Electron spray ionization mass spectrometry (ESI-MS) analysis was carried out using ESI-Q TOF instrument (Bruker, United States of America).

2.2. Chemistry

2.2.1. Preparation of 3,3, 6,6- tetra methyl-9-styryl-3,4,5,6,7,9-hexahydro-*2H*- xanthene-1, 8-dione.



Scheme - 1: The synthesis of derivative 3,3, 6,6-tetra methyl-9-styryl-3,4,5,6,7,9-hexahydro-*2H*- xanthene-1, 8-dione catalyzed by ZnO nanoparticles from reactants (1: trans-cinnamaldehyde, 2: dimedone and 3: 3,3, 6,6-tetra methyl-9-phenyl-3,4,5,6,7,9-hexahydro-2H-xanthene-1, 8-dione).

A mixture of dimedone (308 mg, 2.2 mmol), trans-cinnamaldehyde (1 mmol), zinc oxide (ZnO) nanoparticles (NPs) (220 mg, 1 mmol) was refluxed in water (5 mL) for 2-3 hours. The detailed description of the preparation of nano ZnO is been provided in our earlier communication [21]. Completion of the reaction was confirmed by TLC [Hexane: ethyl acetate] (1:4) (Scheme 1). The mixture was filtered and then washed with water. The desired product was obtained with high purity (yields 85% - 90%).

2. 3. Biological activity

Soybean seed variety (JS-335) was surface disinfected with 1% sodium hypochlorite solution for about 2 min. at room temperature and subject to standard blotter method and incubated at room temperature [22]. On the seventh day of incubation seed samples were screened for seed-borne fungi with the help of stereobinocular and compound microscope. Fungi were identified based on their mycelial structure, growth and spore morphology using standard manuals. Associated fungi, which were frequently associated in higher percentage in soybean were further selected for antifungal study [23-24]

2.3.1. Antifungal effect of xanthene

Antifungal activity of xanthene was determined by poison food method [25]. Sabouraud dextrose agar (SDA) media amended with different concentrations of xanthene (8mg/ml, 4mg/ml, 2mg/ml and 1mg/ml) was transferred in to pre-sterilized petriplates and allowed to solidify at room temperature. After solidification, seven day old mycelial disc of test fungi was aseptically inoculated at the center of agar plates. SDA medium, with captan served as positive control (50 µg/ml) and SDA without xanthene and captan served as negative control. All plates were incubated at 28±1^{0}C and radial growth of colony was measured after seven days of incubation. Each test were performed in triplicate and was expressed in terms of per cent mycelial growth inhibition [26].

Per cent growth inhibition= dc-dt /dt ×100

Where dc = Average increase in mycelia growth in the control.

dt = Average increase in mycelia growth in the treatment.

2.3.2. Determination of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of xanthene

MIC and MFC of xanthene was determined by microbroth dilution method [27].

Double fold dilutions of xanthene concentrations ranging from 2 mg/ml to 0.0039 mg/ml was prepared in 96 well microtitre plate with Sabouraud dextrose broth (SDB). Twenty microliters of the test fungal spore suspension (10^6 spores/ml) was added to each well and incubated at 28±1^{0}C for 72 hours. The plates were observed visually for the absence or presence of fungal growth. Treated inoculum from each well was streaked radially on SDA plates and incubated at 28±1^{0}C for 72 hours. The MIC and MFC was recorded for xanthene.

3. STATISTICAL ANALYSIS

Antifungal experiment data were analyzed by using univariate analysis. Observations were expressed as mean ± standard error, (n=3). Means were separated by Tukey's HSD multiple range test at 0.5 significant (P <0.05) using SPSS software (version 19).

4. RESULTS AND DISCUSSION

4.1. Characterization

The synthesized compound 3,3, 6,6- tetra methyl-9-styryl-3,4,5,6,7,9-hexahydro-*2H*-xanthene-1, 8-dione showed spectral and analytical data as follows:

Colorless solid, Mp: 215–218^{0}C.

^{1}H NMR (400 MHz, $CDCl_3$): δ 1.24 (s, 6H, 2Me), 1.26 (s, 6H, 2Me), 2.90 (m, 8H, $4CH_2$), 4.9 (s, 1H, CH), 7.26–7.37 (m, 7H, Ar- H) (Figure 1).

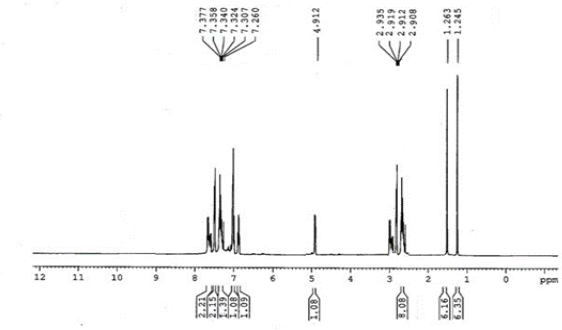


Figure - 1: ^{1}H NMR spectra of 3,3, 6,6- tetra methyl-9-styryl-3,4,5,6,7,9-hexahydro-*2H*-xanthene-1, 8-dione.

^{13}C NMR (100 MHz, $CDCl_3$): δ 27.9, 29.8, 31.9, 33.1, 46.8, 47.4, 77.1, 77.4, 77.8, 112.8, 119.5, 129.4, 130.1115.1, 131.0, 131.8, 140.4, 189.9, 191.4 (Figure 2).

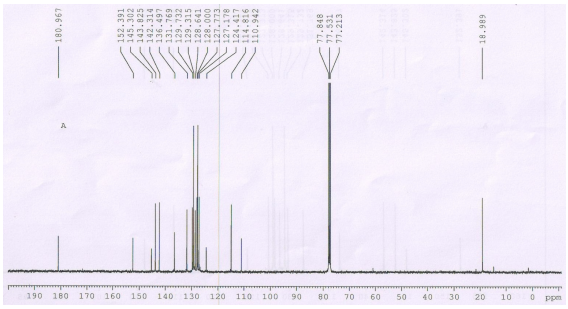


Figure - 2: ^{13}C NMR spectra of 3,3, 6,6- tetra methyl-9-styryl-3,4,5,6,7,9-hexahydro-*2H*-xanthene-1, 8-dione.

ESI-MS: [M+H] 375.7, (Figure 3).

Anal. Calcd. $C_{24}H_{26}N_2O_2$ (%): C, 76.98; H, 7.00; N, 7.48; Found C, 75.34; H, 7.09; N, 8.29.

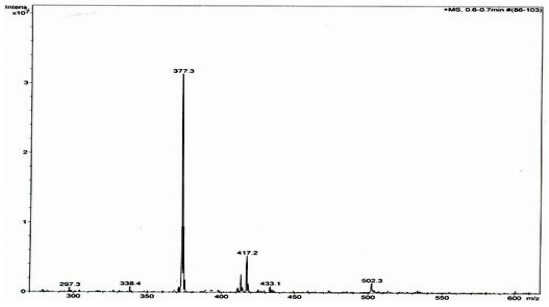


Figure - 3: ESI-MS of 3,3, 6,6- tetra methyl-9-styryl-3,4,5,6,7,9-hexahydro-*2H*- xanthene-1, 8-dione.

4.2. Antifungal activity

Aspergillus flavus, Cladosporium cladosporioides, Fusarium oxysporum and Macrophomina phaseolina occurred in high percent, which served as test fungi for antifungal studies. Antifungal activities of xanthene against soybean seed-borne fungi was assessed by poison food method and were tabulated in table 1 and figure 4. The MIC and MFC for all the text fungi were recorded in the table 2 and figure 5.

Table - 1: Antifungal activity of xanthene on soybean seed-borne fungi.

Organism	Concentration of xanthene in mg/ml				Positive control	Negative control
	8	4	2	1		
A. flavus	88.06±0.51^a	82.90±0.81^b	77.96±0.55^c	72.46±0.42^d	100±0	0
C. cladosporioides	91.77±0.57^a	85.95±0.34^b	81.46±0.65^c	76.03±0.17^d	100±0	0
F. oxysporum	100±0.0^a	100±0.0^a	100±0.0^a	97.70±0.58^b	100±0	0
M. phaseolina	100±0.0^a	100±0.0^a	100±0.0^a	95.03±0.37^b	100±0	0

The above mentioned readings were exclusive of the disc diameter. Observations were expressed as mean ± standard error, (n=3). The values followed by different alphabets differ significantly when subjected to Tukey HSD (row by row analysis) p value ≤ 0.05.

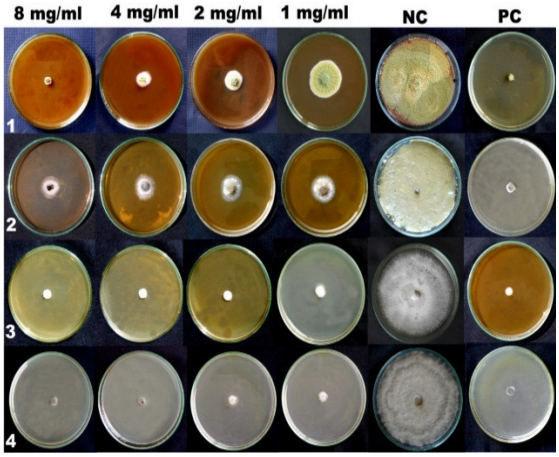


Figure - 4: Inhibitory effect of synthetic xanthene on mycelial growth of soybean seed-borne fungi (**1,** *A. flavus:* **2,** *C. cladosporioides:* **3,** *F. oxysporum:* **4,** *M. phaseolina,* **NC,** Negative control: **PC**, Positive control).

Table - 2: The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of synthetic xanthene against test fungi.

Organism	Xanthene (µg/ml)	
	MIC	MFC
A. flavus	31.25	62.5
C. cladosporioides	15.625	31.25
F. oxysporum	7.81	15.625
M. phaseolina	3.9	7.81l

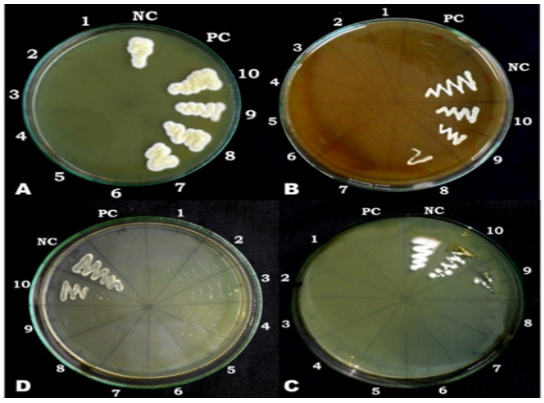


Figure - 5: The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of synthetic xanthene on test fungi.

NC, Negative control: PC, Positive control: A, *A. flavus:* B, *C. cladosporioides:* C, *F. oxysporum:* D, *M. phaseolina.* 1, 2000 : 2, 1000: 3, 500: 4, 250: 5, 125: 6, 62.5: 7, 31.25: 8, 15.62: 9, 7.81: 10, 3.9 µg/ml.

The results indicated that broad spectrum antifungal activity against tested fungi such as *A. flavus, C. cladosporioides, F. oxysporum* and *M. phaseolina*. Xanthene exhibited 100 percent mycelial inhibition at 8mg/ml and 4mg/ml against *F. oxysporum* and *M. phaseolina* respectively. Xanthene exhibited 100 % mycelial inhibition at 2 mg/ml against *F. oxysporum* and *M. phaseolina*. These reports were in concordance with the previous reports [28-29].

MIC was the lowest concentration of compounds resulting in a significant decrease in mycelial growth and MFC was the concentration where 99.9 % or more of the initial inoculum was killed. Xanthene showed an MIC value of 31.25 µg/ml against *A. flavus*, 15.625 µg/ml against *C. cladosporioides*, 7.81 µg/ml against *F. oxysporum* and 3.90 µg/ml against *M. phaseolina*. These findings suggest that xanthene have different effects on the tested fungi. The results obtained signify the prospect of using these semisynthetic compounds as a better antifungal agents against soybean seed-borne fungi [30].

5. CONCLUSION

Better approaches to the synthesis of xanthene molecules as potential antifungal agents have been developed by using zinc oxide nanoparticle as a catalyst. ZnO nano particles have proved to be a greener, simple, highly versatile, efficient catalyst in the synthesis of xanthene diones via multi-component reactions. The present approach is mild, environmentally friendly, inexpensive and highly effective to give the products with excellent yield. The antifungal results showed that most of the soybean seed-borne fungi tests were susceptible to synthesized xanthene molecule comparable with the standard used. Further investigation is required to study the mode of action of the synthesized molecule.

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