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Evaluation of growth and economic parameters of *Bombyx mori* by substituted 1,3,4 –Oxadiazoles

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Abstract: In this study we investigated the growth and economic parameters of Silkworm, *Bombyx mori* (Lepidoptera: Bombycidae) by dietary supplementation of substituted oxadiazoles (Ox1a-c, Ox2a-c and Ox3a-c) in extremely low concentrations. DNA binding assay experiment was conducted in order to find the binding efficiency of the substituted oxadiazoles (Ox1a-c,Ox2a-c and Ox3a-c). *Bombyx mori* larvae supplemented with the diet enriched with the target compounds in milli-molar concentrations increased the growth parameters like larvae weight, silk gland weight and cocoon weight in comparision with control, which was also confirmed by the DNA binding assay. We confirm that diet supplemented with target compounds to *Bombyx mori* silkworm increased the growth percentage and economic parameters.
Key words: Cocoon; DNA binding assay; mulberry leaves; oxadiazoles; silk glands;

Introduction:

Sericulture is one of the oldest industries in India. India is the second largest silk producer in the world. The mulberry silkworm, *Bombyx mori*, is a domesticated and monophagous insect which feeds only on the leaves of mulberry for its nutrition. Growth and size of *Bombyx mori*, silk glands, cocoon and silk filament can be greatly enhanced by the nutritional quality of mulberry leaves. Effect of mulberry leaves enriched with amino acid[1], alanine and glutamine [1,2] and other supplementary nutrients like thiamine or vitamin B1 on the growth of silkworm was studied [3]. Mulberry leaves enriched with nickel chloride [4], potassium iodide [4] and their combinations has increased the cocoon weight even at low concentrations. Amines and amides play an important role in DNA binding [5,6]; affect the DNA synthesis by increasing the rate of the DNA replication fork, repair synthesis of DNA was observed in cells [7]. Polyamines are synthesized in cells via highly regulated pathways. They are polycationic compounds that have a variety of physiological effects in microorganisms and animal cells. They also bind to DNA, affect the DNA synthesis by increasing the movement rate of the DNA replication fork [8]. Polyamines influence the transcriptional and translational stages of protein synthesis in mammalian cells [9]. They stabilize the membranes [10] and alter levels of free calcium [11]. Poly amines are required for cell growth as they are found in higher concentrations in dairy products.

1,3,4 oxadiazoles represent versatile lead molecule as potential bioactive agents [12]. This interesting group of compounds possesses diverse biological activites such as antimicrobial [13], antitubercular [14], anticonvulsant [15], anti-inflammatory [16], anticancer [17], 1,3,4 oxadiazoles are also known to have plant growth regulating activity [18].

A recent rational approach of drug design involves linking two molecules with individual intrinsic activity into a single hybrid molecule. The molecules thus produced were shown to have improved efficacy and minimum toxicity. The effect of Oxadiazoles on growth enhancing properties of *Bombyx mori* was not yet exploited. This encouraged us to synthesise amino substituted oxa diazoles as possible growth enhancers of silkworm, *Bombyx mori*.

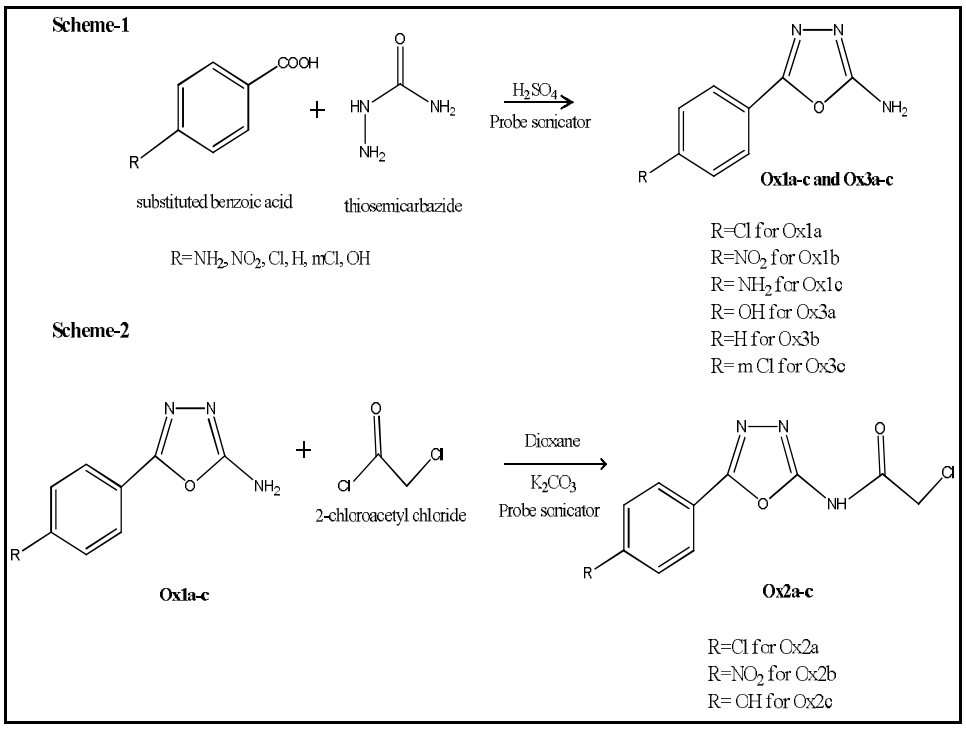
In this study we reported the synthesis of substituted amine and amide derivatives of 1,3,4 oxadiazoles by probe sonicator assisted synthesis with improved yields. The effect of target compounds (Ox1a-c and Ox2a-c) in extremely low concentrations was studied on the growth parameters of *Bombyx mori*. We found that there is an increase in larval weight, silk gland weight, cocoon weight, pupa weight and empty shell weight in comparision with the control. The effect of nitrogen, vitamin and salts supplement on the growth of silkworm has been investigated by many researchers, but the reports on the effect of mulberry leaves enriched with substituted oxadiazoles was not reported. So we aimed to find out the effect of substituted oxadiazoles enriched to mulberry leaves on silkworm growth and economic parameters.

Material and Methods

All the reagents used are of AR grade. The Melting points were determined in open capillary tube. 1HNMR spectra were run on BRUCKER spectrometer (300MHz) using DMSO as solvent and TMS as internal standard. Mass spectra were recorded on Shimaszu, LCMS-2010A spectrometer. IR spectra were recorded on Perkin Elmer FT IR spectrophotometer using KBr pellets and UV spectra were recorded on Perkin Elmer Spectrophotometer using double distilled water as blank at GITAM Institute of science, GITAM University, Visakhapatnam, Andhra Pradesh. The progress of the reaction was monitored by thin layer chromatography on Merck TLC silica gel plates. The solvent system used is Hexane : Ethylacetate (6:4). The spots are visualised under UV chamber. The eggs of *Bombyx mori* (CB Csr2×Csr4 type) larvae were collected from Sericulture department, Visakhapatnam, Andhra Pradesh.

Synthesis of 5-(4-substitutedphenyl)-1,3,4-oxadiazol-2-amine (compounds Ox1a-c and Ox3a-c):

A mixture of semicarbazide (0.01mole), 4-substituted benzoic acid (0.01mole) and concentrated sulphuric acid (15ml) were taken in a round bottomed flask and the reaction mixture was subjected to probe sonicator [19] for 15 to 20 min, the reaction mixture was poured on to crushed ice, and recrystallized from ethanol to give 5-(4-substituted phenyl)-1,3,4-oxadiazol-2-amine (75% yield) (Scheme-1).



Synthesis of 2-chloro-N-(5-(4-substitutedphenyl)-1,3,4-oxadiazol-2-yl)acetamide (compounds Ox2a-c):

A mixture of chloroacetyl chloride (0.4 mL) in dioxane (20 mL) and appropriate 5-(4-substituted phenyl)-1,3,4-oxadiazol-2-amine; Ox1a-c (0.01moles) were taken in a round bottomed flask and the reaction mixture was subjected to probe sonicator for 15 to 20 min,. The reaction mixture was left to cool, poured into crushed ice and the precipitated solid was filtered off, dried, and recrystallized from ethanol. (80% yield) (Scheme-2).

Spectral data:

Compound Ox1a: [5-(4-chlorophenyl)-1,3,4-oxadiazol-2-amine]

Brown colored compound; melting point 184-186ºC. Mass spectra- m/z: 195.02 (100.0%), 197.02 (32.3%), 196.02 (9.8%), 198.02 (2.8%). The 1HNMR (DMSO) aromatic CH protons appeared at δ: 7.55(2H, d, Ar-CH), 7.73(2H, d, Ar-CH) and NH_2 appears at 6.99(2H, s, NH_2). The C13 NMR (DMSO) benzene carbons appeared at 134.3(1C, Ar-C-Cl), 124.2(1C, Ar-C), 129.3(2C, Ar-C), 128.9(2C, Ar-C) and oxadiazole ring carbons appear at 169.3(1C, N=C-O) and 164.5(1C, N=C-O). The FTIR shows bands at 3314-3336(νNH$_2$), 1542(νC=N), 1520(νC=C) and at 648(νC-O).

Compound Ox1b: [5-(4-nitrophenyl)-1,3,4-oxadiazol-2-amine]

Palebrown compound; melting point 218-220ºC. Mass spectra- m/z: 195.02 (100.0%), 197.02 (32.3%), 196.02 (9.8%), 198.02 (2.8%). The 1HNMR (DMSO) aromatic CH protons appeared at δ 8.32(2H, d, Ar-CH), 8.23(2H, d, Ar-CH) and NH_2 at 6.99(2H, s, NH_2). The C13 NMR (DMSO) benzene carbons appeared at 147.9(1C, Ar-C-N), 132.2(1C, Ar-C), 128.8(2C, Ar-C), 130.9(2C, Ar-C) and oxadiazole ring carbons appear at 169.3(1C, N=C-O) and 164.5(1C, N=C-O). The IR shows bands at 3339-3257(νNH2), 1552(νC=N), 1535(νC=C) and at 643(νC-O).

Compound Ox1c: [5-(4-aminophenyl)-1,3,4-oxadiazol-2-amine]

White compound; melting point 212-214ºC. Mass spectra- m/z is: 176.07 (100.0%), 177.07 (10.2%). The 1HNMR (DMSO) aromatic CH protons appeared at δ 7.54(2H, d, Ar-CH), 6.58(2H, d, Ar-CH), NH_2 proton at δ 6.99(2H, s, NH_2) and 6.27(2H, s, Ar-NH_2). The C13 NMR (DMSO) benzene carbons appeared at 145.6(1C, Ar-C-N), 116.1(1C, Ar-C), 115.1(2C, Ar-C), 128.3(2C, Ar-C), oxadiazole ring carbons appear at 169.3(1C, N=C-O) and 164.5(1C, N=C-O). The IR shows bands at 3314-3336(νNH2), 1563(νC=N), 1519(νC=C) and at 645(νC-O).

Compound Ox2a: [2-chloro-N-(5-(4-chlorophenyl)-1,3,4-oxadiazol-2-yl)acetamide]

Pale yellow crystals; melting point 297^{o}C. Mass spectra- m/z is: 253.03 (100.0%), 255.02 (32.0%), 254.03 (11.0%), 256.03 (3.6%), 255.03 (1.3%), 254.02 (1.1%). The 1HNMR (DMSO) aromatic CH protons appeared at δ 7.55(2H, d, Ar-CH), 7.73(2H, d, Ar-CH), NH at δ 9.15(1H, s, NH) and CH_2 at 4.21(1H, s, CH_2-Cl). The C13 NMR (DMSO) benzene carbons appeared at 124.2(1C, Ar-C), 129.3(2C, Ar-C), 128.9(2C, Ar-C), aliphatic CH_2 at 42.7(1C, C-Cl), amide C at 165.4(1C, CO-N) oxadiazole ring carbons appear at 169.3(1C, N=C-O) and 164.5(1C, N=C-O). The IR shows bands at 3171-3296(νNH), 2900(νCH) and at 1670(νC=O).

Compound Ox2b: [2-chloro-N-(5-(4-nitrophenyl)-1,3,4-oxadiazol-2-yl)acetamide]

Pale yellow crystals; melting point 297^{o}C. Mass spectra- m/z is: 253.03 (100.0%), 255.02 (32.0%), 254.03 (11.0%), 256.03 (3.6%), 255.03 (1.3%), 254.02 (1.1%). The 1HNMR (DMSO) aromatic CH protons appeared at δ 8.32(2H, d, Ar-CH), 8.23(2H, d, Ar-CH) NH at δ 9.15(1H, s, NH) and CH_2 4.21(1H, s, CH_2-Cl). The C13 NMR (DMSO) benzene carbons appeared at 132.2(1C, Ar-C), 128.8 (2C, Ar-C), 130.9 (2C, Ar-C), aliphatic CH_2 at 42.7(1C, C-Cl), amide C at 165.4(1C, C-N), oxadiazole ring carbons appear at 169.3(1C, N=C-O) and 164.5(1C, N=C-O). The IR shows bands at 3212-3342(νNH), 2900(νCH) and at 1640(νC=O).

Compound Ox2c: [2-chloro-N-(5-(4-hydroxyphenyl)-1,3,4-oxadiazol-2-yl)acetamide]

Pale yellow crystals; melting point. 297^{o}C. Mass spectra- m/z is: 253.03 (100.0%), 255.02 (32.0%), 254.03 (11.0%), 256.03 (3.6%), 255.03 (1.3%), 254.02 (1.1%). The 1HNMR (DMSO) aromatic CH protons appeared at δ 6.86(2H, d, Ar-CH), 7.96(2H, d, Ar-CH), OH protons appeared at δ 5.35, NH at δ 9.15(1H, s, NH) and CH_2 4.21(1H, s, CH_2-Cl). The C13 NMR (DMSO) benzene carbons appeared at 118.7(1C, Ar-C), 116.4 (2C, Ar-C),

116.3 (2C, Ar-C) aliphatic CH_2 at 42.7(1C, C-Cl), amide C at 165.4(1C, C-N), oxadiazole ring carbons appear at 169.3 (1C, N=C-O) and 164.5(1C, N=C-O). The IR shows bands at 3136-3270(νNH), 2900(νCH), 1663(νC=O) and at 3300-3600(νOH).

Compound Ox3a: [4-(5-amino-1,3,4-oxadiazol-2-yl)phenol]

Red colored compound; melting point 184-186ºC. Mass spectra- m/z: 177.05 (100.0%), 178.06 (8.8%), 178.05 (1.1). The 1HNMR (DMSO) aromatic CH protons appeared at δ: 6.86 (2H, d, Ar-CH), 7.96 (2H, d, Ar-CH), NH_2 appears at 6.99 (2H, s, NH_2) and OH at 5.35 (1H). The C13 NMR (DMSO) benzene carbons appeared at 158.3(1C, Ar-C-OH), 116.3(4C, Ar-C), 116.7(1C, Ar-C) and oxadiazole ring carbons appear at 169.3(1C, N=C-O) and 164.5(1C, N=C-O). The FTIR shows bands at 3314-3336(νNH_2), 3650-3300 (νOH) 1542(νC=N), 1560(νC=C) and at 748(νC-O).

Compound Ox3b: [5-phenyl-1,3,4-oxadiazol-2-amine]

Pale red colored compound; melting point 184-186ºC. Mass spectra- m/z: 161.06 (100.0%), 162.06 (9.8%). The 1HNMR (DMSO) aromatic CH protons appeared at δ: 8.05 (2H, d, Ar-CH), 7.51 (2H, m, Ar-CH), 7.41 (1H, m, Ar-CH) and NH_2 appears at 6.99 (2H, s, NH_2). The C13 NMR (DMSO) benzene carbons appeared at 127.5 (2C, Ar-C), 129.2 (2C, Ar-C) 128.7 (1C, Ar-C) 126.1 (1C, Ar-C) and oxadiazole ring carbons appear at 169.3(1C, N=C-O) and 164.5(1C, N=C-O). The FTIR shows bands at 1542(νC=N), 1520(νC=C) and at 578(νC-O).

Compound Ox3c: [5-(3-chlorophenyl)-1,3,4-oxadiazol-2-amine]

Brown colored compound; melting point 184-186ºC. Mass spectra- m/z: 195.02 (100.0%), 197.02 (32.3%), 196.02 (9.8%), 198.02 (2.8%). The 1HNMR (DMSO) aromatic CH protons appeared at δ: 8.01 (1H, s, Ar-CH), 7.45(2H, m, Ar-CH), 7.93(1H, d, Ar-CH) and NH_2 appears at 6.99(2H, s, NH_2). The C13 NMR (DMSO) benzene carbons appeared at 134.5(1C, Ar-C-Cl), 127.4(1C, Ar-C), 127.5(1C, Ar-C) 129.5(1C, Ar-C), 128.8(1C, Ar-C) 125.6(1C, Ar-C) and oxadiazole ring carbons appear at 169.3(1C, N=C-O) and 164.5(1C, N=C-O). The FTIR shows bands at 3314-3336(νNH_2), 1542(νC=N), 1520(νC=C) and at 648(νC-O).

Feeding Experiment:

Bombyx mori (CB Csr2×Csr4 type) larvae were fed with normal mulberry leaves till the end of the 4^{th} instar larval stage. Mulberry leaves enriched with synthesized compound was given from day one of the 5^{th} instar larva stage. Each compound was separately dissolved in minimum amount of DMSO and diluted with distilled water to obtain 1mM concentration. Concentration of 0.1 mM, 0.5 mM and 0.01 mM was achieved by appropriate dilution with distilled water. Mulberry leaves were treated with the above concentrations by swab method and were fed to the silkworm four times a day. The larvae were divided into three experimental groups for each synthesized compound according to the concentration taken, each group consisting of 45 worms. The treatment was carried out on all the days of 5^{th} instar. The larvae fed with untreated mulberry leaves were maintained as a control group.

Percentage gain in weight of silk worm, percentage gain in weight of silk gland, Cocoon weight, shell weight, shell ratio and pupal weight, silk weight, silk length and reelability were measured according to the formulae from standard procedures [20, 21].

DNA binding assay studies

UV-Spectrophotometric analysis:

The DNA-binding experiment was performed using UV Spectophotometry [22]. Concentration of DNA (silk worm-DNA) was determined from the absorption intensity at 260 nm. Absorption titration experiments were made in a range of 220-400 nm by keeping concentration of silkworm-DNA as constant (100µg/mL) and adding an increment (10µg/mL, 30µg/mL, 50µg/mL 70µg/mL and 90µg/mL) concentration of synthesised compound (Ox1a-c, Ox2a-c and Ox3a-c). Compounds of 100µg/mL concentration without addition of DNA were taken. After the addition of silkworm-DNA to the target compounds, incubation for 10 min in room temperature has been provided, followed by absorbance. DNA mediated hyperchromisim has been observed. The binding constant, *K*b for the synthesized compounds has been determined (Figure-6).

Results:

Growth and economic parameters:

The change in the larvae and cocoon characteristics of silk worm, *Bombyx mori* was studied when the larvae fed on the mulberry leaves enriched with target compound. There was an increase in weight of enriched larvae in comparison with control larvae (Figure-1). Larvae were dissected on 7th day of 5th instar stage and silk glands were separated and weighed. The percentage increase in silk gland weight in comparison with control (Figure-2) was noted.

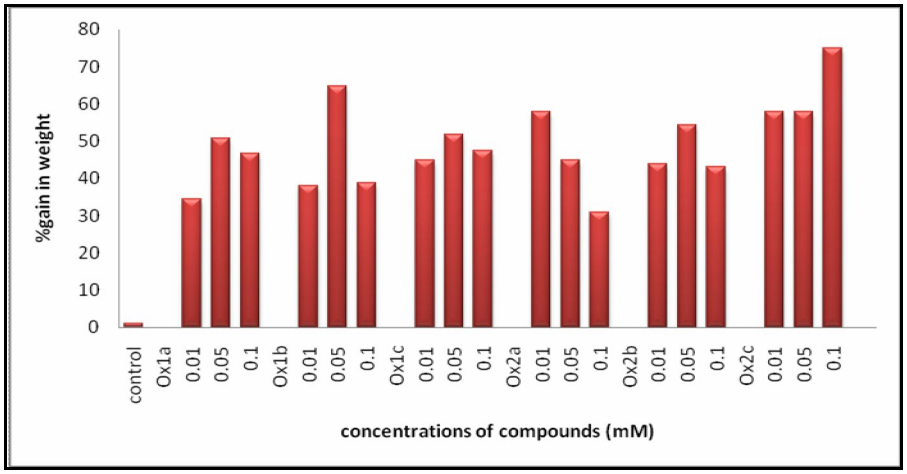


Figure-1: % gain in weight of larvae when treated with target compounds in comparison with control

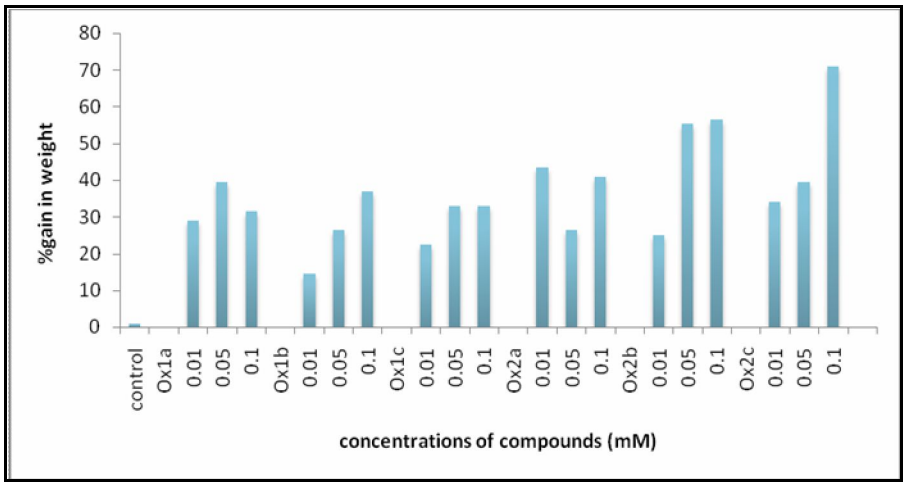


Figure-2: % gain in weight of silk glands of *Bombyx mori* when treated with target compound in comparison with control

Table-1: Economic parameters of *Bombyx mori*

S.No	Compound concentrations in mM	avg wt of coocun (g)	avg wt of pupa (g)	avg wt of empty shell (g)	avg wt of silk (g)	avg length of silk (m)	shell %	reelability %
1	control	0.96±0.04	0.77±0.06	0.2±0.04	0.107±0.005	600±30	19.79	33.33
2	Ox1a							
	0.01	1.16±0.09	0.98±0.06	0.2±0.03	0.165±0.004	640±40	16.94	33.33
	0.05	1.14±0.07	0.96±0.07	0.2±0.04	0.157±0.010	620±20	16.66	50
	0.1	1.22±0.04	1.01±0.06	0.22±0.02	0.167±0.008	690±20	18.18	50
3	Ox1b							
	0.01	1.12±0.06	0.9±0.02	0.22±0.05	0.166±0.006	660±50	19.64	50
	0.05	1.33±0.13	1.09±0.11	0.24±0.02	0.183±0.012	750±10	21.05	33.3
	0.1	1.16±0.05	0.91±0.05	0.26±0.02	0.151±0.010	670±60	20.35	50
4	Ox1c							
	0.01	1.07±0.20	0.89±0.18	0.21±0.04	0.152±0.008	680±40	19.09	33.33
	0.05	1.26±0.04	1.02±0.07	0.25±0.04	0.165±0.006	753±70	20.63	100
	0.1	1.32±0.12	1.06±0.12	0.26±0.01	0.171±0.008	753±20	19.4	50
5	Ox2a							
	0.01	1.43±0.02	1.13±0.04	0.30±0.04	0.225±0.012	720±40	20.97	50
	0.05	1.1±0.15	0.9±0.12	0.2±0.06	0.143±0.010	560±80	18.18	33.33
	0.1	1.21±0.07	0.94±0.03	0.27±0.02	0.179±0.010	700±60	24.1	100
6	Ox2b							
	0.01	1.45±0.04	1.12±0.06	0.25±0.04	0.233±0.008	730±70	25.64	100
	0.05	1.18±0.06	0.93±0.02	0.31±0.10	0.197±0.008	690±60	21.18	50
	0.1	1.43±0.18	1.12±0.17	0.31±0.10	0.206±0.006	720±60	21.67	50
7	Ox2c							
	0.01	1.17±0.01	0.87±0.01	0.28±0.06	0.193±0.010	700±40	22.75	100
	0.05	1.26±0.09	0.98±0.04	0.35±0.02	0.185±0.008	670±30	22.22	50
	0.1	1.5±0.02	1.15±0.04	0.35±0.02	0.257±0.012	760±20	23.3	100
8	Ox3a							
	0.01	0.91±0.09	0.79±0.06	0.1±0.03	0.110±0.004	610±40	15.33	33.33
	0.05	0.99±0.07	0.81±0.07	0.22±0.01	0.115±0.010	620±20	17.16	33.33
	0.1	1.12±0.04	0.92±0.06	0.22±0.01	0.124±0.008	650±20	19.89	50
9	Ox3b							
	0.01	0.97±0.09	0.79±0.06	0.1±0.07	0.103±0.004	600±40	16.85	33.33
	0.05	1.03±0.07	0.85±0.07	0.2±0.01	0.111±0.010	630±20	20.58	50
	0.1	1.09±0.04	0.89±0.06	0.2±0.01	0.117±0.008	650±20	20.99	50
10	Ox3c							
	0.01	0.92±0.09	0.75±0.06	0.1±0.06	0.106±0.004	610±40	15.27	33.33
	0.05	0.98±0.07	0.82±0.07	0.1±0.02	0.114±0.010	620±20	17.56	50
	0.1	1.05±0.04	0.96±0.06	0.1±0.02	0.119±0.008	620±20	17.69	33.33

Post cocoon parameters which are very important for the economic feasibility of silk production were noted. Weight of cocoon, weight of pupa, weight of shell, length of silk filament, weight of silk filament, shell % and reelability are calculated using the formulae for all the synthesised compounds and tabulated (Table-1). All the growth and economic parameters were found to be highest for compound Ox2c.

DNA binding assay:

DNA binding assay results were plotted on graph by taking wavelength (λ) on X-axis and absorption (A^o) on Y-axis. DNA mediated hyperchromisim has been observed for 6 compounds (Ox1a-c and Ox2a-c). The gradual increase in the absorption at 260 nm for compounds Ox1a-c and Ox2a-c indicates strong interaction of these compounds with silkworm-DNA double strand (Figure-3 and Figure-4). Compounds Ox3a-c have no absorption peak at 260 nm which indicates that they are not binding to silkworm-DNA (Figure-5). There was a hyperchromic shift when the graph was plotted by taking compounds (Ox1a-c and Ox2a-c) at a concentration of 90 µg/mL and DNA at 260 nm as the maximum obsorption for compounds was found at 90 µg/mL (Figure-6). Binding constant for target compounds was taken from the graph (Figure-6) and tabulated (Table-2) the binding efficiency of the compound Ox2c was found to be highest (1.5 x 10^3 M^{-1}) among all the tested compounds.

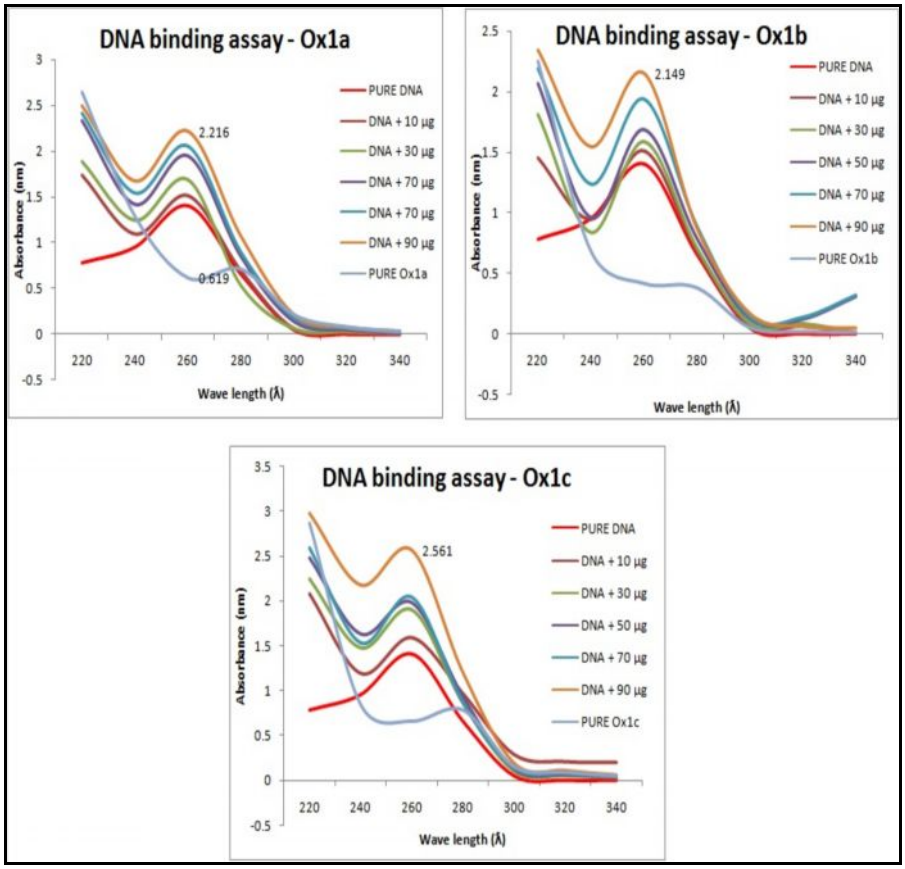


Figure-3: DNA binding assay spectra of compounds Ox1a-c at different concentrations

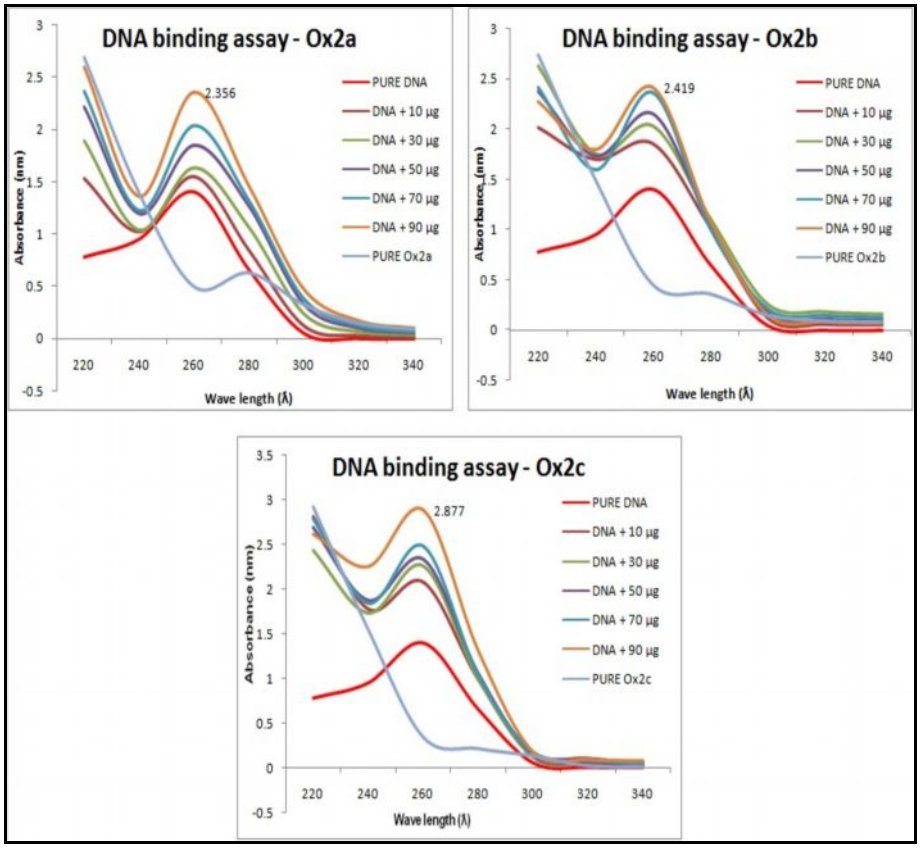


Figure-4: DNA binding assay spectra of compounds Ox2a-c at different concentrations

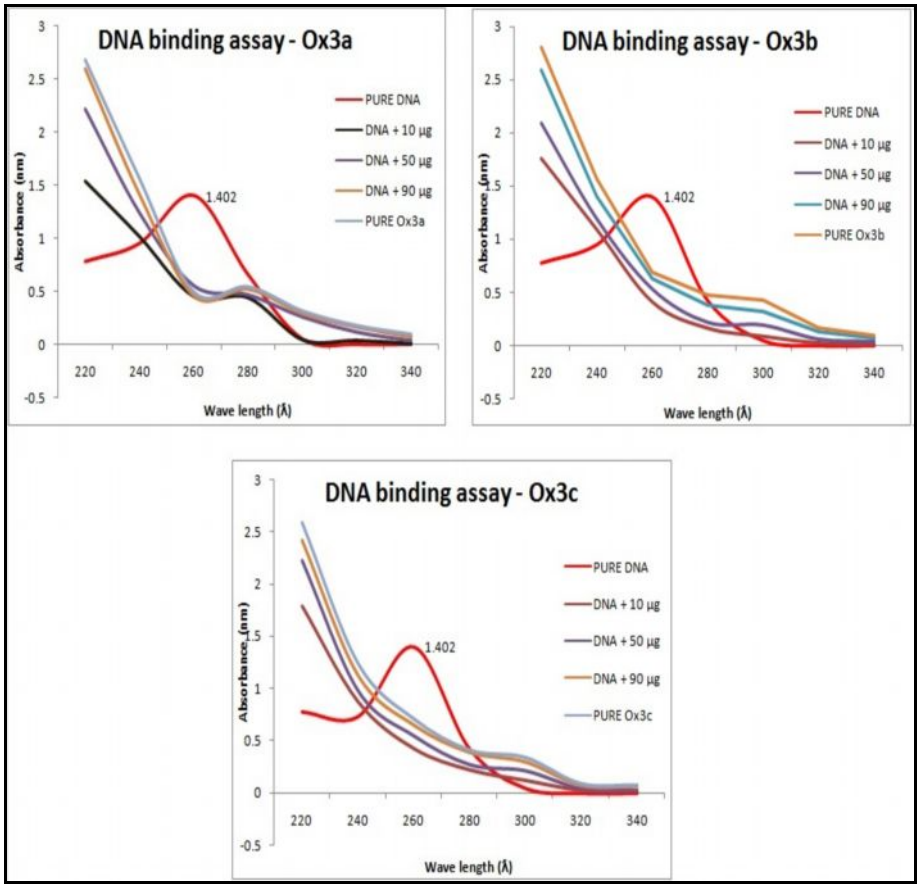


Figure-5: DNA binding assay spectra of compounds Ox3a-c at different concentrations

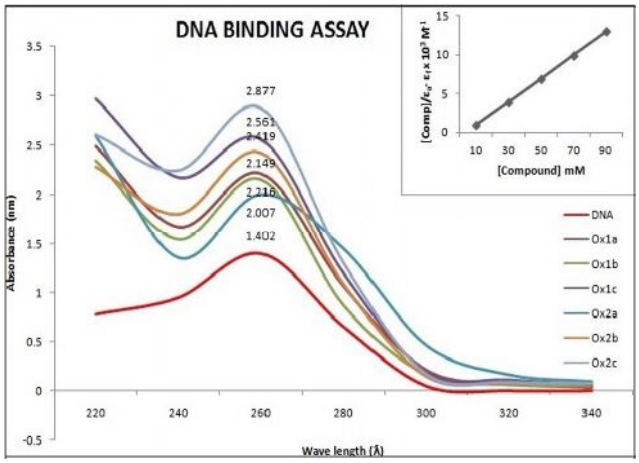


Figure-6: Absorption spectra of target compounds with concentration 90µg bound with DNA. Inset: Plot of [compound]/ ε_a- ε_f x 10^3 M^{-1} vs. [compound].

Table-2: Binding constants (K_b) of synthesised compounds

S.No	Compounds	Binding constant (K_b)
1	Ox1a	0.05 x 10^3 M^{-1}
2	Ox1b	0.09 x 10^3 M^{-1}
3	Ox1c	0.4 x 10^3 M^{-1}
4	Ox2a	0.9 x 10^3 M^{-1}
5	Ox2b	1.1 x 10^3 M^{-1}
6	Ox2c	1.5 x 10^3 M^{-1}

Discussion:

In the present study we have synthesized a series of amine and amide derivatives of oxadiazoles using probe sonicator in improved yields. *Bombyx mori* was fed with the mulberry leaves enriched with the synthesised compounds; there is a significant increase in the weight of larvae and silk glands of the silk worm in comparison with the control group. Among all the synthesised compounds; six compounds (Ox1a-Ox2c) show binding efficiency to the silkworm-DNA. The other three compounds (Ox3a-Ox3c) do not show absorption maxima at 260 nm and thus we concluded that these compounds are not binding to silkworm-DNA. The economic and growth parameters of all the synthesised compounds are tested. The compounds (Ox1a-c and Ox2a-c) which are binding to the DNA are showing better growth in terms of cocoon weight, pupa weight, silk gland weight, silk production and reliability; the other three compounds (Ox3a-Ox3c) which are not binding to the DNA are inferior in terms of growth and economic parameters (Table-1). Oxadiazoles with the amide linkage (Ox2a-c) showed an increase in both the economic and growth parameters in comparison with the amine derivatives (Ox1a-c) and control. Silk worms fed with the compound containing electron donating group on the aromatic ring (Ox2c; OH group) have prominent effect on the weight of the larvae (75% increase in the larval weight) in comparison with the compound containing electron withdrawing group (Ox2b; NO_2 group). The growth parameters are in correlation with the DNA binding assay. The binding constant for Ox2c is 1.5 x 10^3 M^{-1} which shows that the Ox2c have better binding interaction than the other tested compounds. We conclude that DNA binding plays an important role in the growth and economic parameters of *Bombyx mori;* however a molecular mechanism is yet to be studied

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