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Larvicidal and Antimicrobial Activities of Some Novel Triazinone Derivatives

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Abstract: *A series of novel 3-tert-butyl-7-benzyl-4H-[1,3,4]thiadiazolo[2,3-c][1,2,4]triazin-4-ones(5a-h) were prepared by condensing 4-amino-6-tert-butyl-3-sulfanyl-1,2,4-triazin-5(4H)-one (1) with various substituted phenyl acetic acids in the presence of phosphorus oxychloride at 150^0 C (Scheme 2). 4-amino-6-tert-butyl-3-sulfanyl-1,2,4-triazin-5(4H)-one (1) was prepared by treating 3,3-dimethyl-2-oxobutanoic acid (trimethyl pyruvic acid) (1) with thiocarbohydrazide (2) in ethanol as solvent for 12h (Scheme 1). The products obtained were confirmed by ^{1}H-NMR, FTIR and Mass Spectral studies. The novel derivatives were evaluated for the growth inhibition (antimicrobial) property against certain bacterial and fungal pathogens. The derivatives have also been screened for their potency of mosquito larvicidal activity.*

Keywords: *Antimicrobial, Triazinone, thiadiazolotriazinones; Antimicrobial property; Larvicidal activity;*

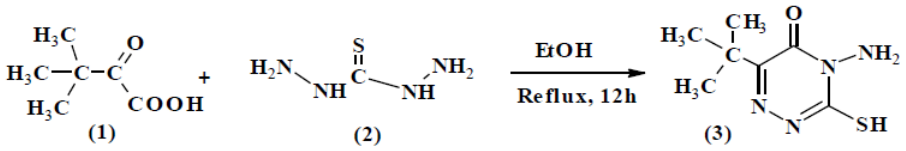
1. INTRODUCTION

Various substituted 1,2,4-triazin-5-one derivatives are known to play important roles possessing various activities in medicinal and agricultural fields[1-7]. The advancement in technology has made availability of voluminous literature and helped to boost the progress of research directed towards this class of compounds, particularly 4-amino-1,2,4-triazin-5-one derivatives, which have been shown to exhibit significant activities, including antimicrobial[8-10]and anticancer[11,12]. It was observed in the literature that these triazinone derivatives also possess antifish parasitic[13-15], anticonvulsant[16,17], antitumour[18] and antimetastatic[19] properties. In addition to the activities of triazinones, thiadiazoles are useful as anti-tuberculosis [20], antitumor[21-24], anti-microbial[25], antidepressant[26], pesticidal [27, 28] and larvicidal[29] agents. In view of various pharmacological importance of triazinones and 1,3,4-thiadiazoles, we planned to synthesize some novel bridge head heterocycles such as thiadiazolotrazinones (5a-5h) and screen them for antimicrobial activities. Taken into consideration of high rate of vector-mediated contagious diseases and development of resistance to existing insecticides, the novel derivatives have also been studied for their possible mosquito-larvicidal activity.

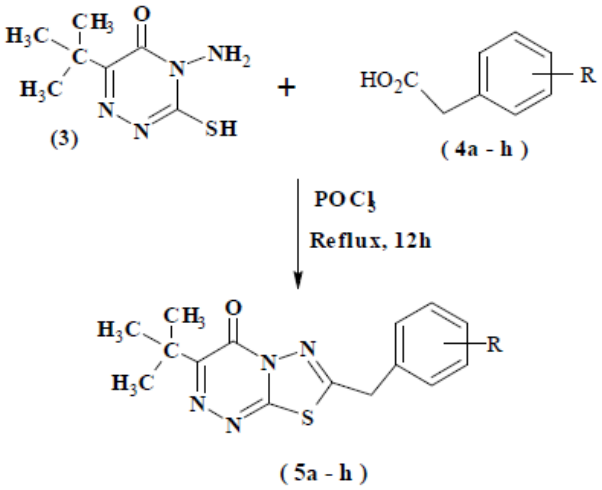
2. MATERIALS AND METHODS

The chemicals 3,3-dimethyl-2-oxobutanoic acid, hydrazine hydrate, carbondisulfide, phenylacetic acids and phosphorus oxychloride used for the synthesis of novel thiadiazolotriazin-4-ones were procured from Sigma-Aldrich, Bengaluru, India. Melting points of the compounds (5a-5h) were determined in open capillary tubes and were uncorrected. The purity of the synthesized compounds was checked by TLC showing a single spot on Merck silica gel 60 F_{254} coated alumina plates. The structures of thiadiazolotriazin-4-ones (5a- 5h) were confirmed by spectral studies. The IR spectra (cm^{-1}) were recorded on a Shimadzu-FTIR 577 Infrared spectrophotometer in KBr pellets. The ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Brucker AMX-400(400 MHz) spectrometer using $CdCl_3$-*d* as solvent and TMS as the internal standard. The Mass spectra were recorded on Perkin-Elmer 018444–Y, Triple Quadrupole LC/MS Spectrometer.

2.1. Reaction Schemes



Scheme1.



Scheme2.

Table1. *Characterization data of Triazinone Derivatives [5a-h]*

Compd.	R	Mol. Formula	M. W	M. P (^{0}C)
5a	4-NO_2	$C_{15}H_{15}N_5O_3S$	345.35	140-146
5b	4-CH_3	$C_{16}H_{18}Cl_4N_4OS$	314.41	190-196
5c	4-Br	$C_{15}H_{15}BrN_4OS$	379.28	184-190
5d	4-OCH_3	$C_{16}H_{18}N_4O_2S$	330.41	162-164
5e	4-OH	$C_{15}H_{16}N_4O_2S$	316.38	175-178
5f	4-F	$C_{15}H_{15}NF_4OS$	318.37	190-195
5g	4-Cl	$C_{15}H_{15}N_4ClOS$	334.82	148-156
5h	2-Chloropyridyl	$C_{14}H_{14}N_5ClOS$	335.81	130-136

2.2. General Procedure for the Synthesis of 4-Amino-6-Tert-Butyl-3-Sulfanyl-1,2,4-Triazin-5(4h)-One (3). (Scheme 1)

3,3-dimethyl-2-oxobutanoic acid (trimethyl pyruvic acid) **(1)** (6.5g, 0.05mol) and thiocarbohydrazide**(2)** (5.3g, 0.05mol) was refluxed in ethanol for 14h. The completion of the reaction and purity was monitored by running thin layer chromatography in ethyl acetate and petroleum ether mixture. After 10 hours the reaction mixture was poured into crushed ice. The solid product obtained was filtered under vacuum and recrystallized from hot ethanol. The yield after recrystallization was 75%.

2.3. General Procedure for the Synthesis of 7-Benzyl-3-Tert-Butyl-4h-[1,3,4]Thiadiazolo[2, C][1,2,4]Triazin-4-One (5a - H) (Scheme 2)

4-amino-6-*tert*-butyl-3-sulfanyl-1,2,4-triazin-5(4*H*)-one**(3)** (0.005mol) and substituted phenyl acetic acids**(4)** (0.005mol) were condensed in $POCl_3$ at 90^{o}C for 10 hours. The reactions were carried out in dry condition. Reaction mixture was then cooled and the solid product was separated by pouring the reaction mixture into crushed ice. The solid product obtained was filtered, washed, dried and recystallized from ethanol. The structures were characterized. The characterization data are summarized in **table 1**.

2.4. Analyses of 7-Benzyl-3-Tert-Butyl-4h-[1,3,4]Thiadiazolo[2,3-C][1,2,4]Triazin-4-One Derivatives (5a-H)

5b: IR (KBr, cm^{-1}) : 3087 (Aromatic C-H), 2963(-CH_3 group of t-butyl), 1709 (>C=O), 1538 (-C=N stretch), 1470 and 1556 (C=C), 1450(-N=N-), 1278 (C-S). ^{1}H-NMR ($CDCl_3$, δ, ppm): 1.41 (9H, s,

$3CH_3$), 4.70 (2H, s, $-CH_2$), 7.4(2H, d, J= 8.4Hz, 4-methylphenyl), 7.56(2H, d, J = 8.8Hz, 4-methylphenyl). ^{13}C-NMR (δ, ppm): 27.8 ($3CH_3$ of t-butyl), 37.4(t-butyl C-atom), 38.2 ($-CH_2$), 26($-CH_3$), 129.0,133.4, 134.0,136.82, 137.89, , and 139.34 (4-methylphenyl), 148.0, 152.0 and 158.9(3 C-atoms of thiadiazolotriazin-4-one) and 170.45 (-C = O of thiadiazolotriazin-4-one). LC-MS [M+], (m/z): 314.40. Anal. cald for $C_{16}H_{18}N_4OS$: C,61.12; H,5.77); N,17.82. Found: C,61.46); H,5.89; N,17.95%. m.p.192-194^{0}C, yield 71%.

5c: IR (KBr, cm^{-1}) : 3069 (Aromatic C-H), 2955($-CH_3$ group of t-butyl), 1692 (>C=O), 1522 (-C=N stretch), 1491 and 1416 (C=C), 1456(-N=N-), 1189 (C-S), 505(Ar-Br). ^{1}H-NMR ($CDCl_3$, δ, ppm): 1.38 (9H, s, $3CH_3$), 4.45 (2H, s, $-CH_2$), 7.6(2H, d, J= 8.4Hz, 4-bromophenyl), 7.4(2H, d, J = 8.4Hz, 4-bromophenyl). ^{13}C-NMR (δ, ppm): 27.1 ($3CH_3$ of t-butyl), 38.12(t-butyl C-atom), 39.4 ($-CH_2$), 120.13, 131.6, 132.8 132.9, 133.41, , and 139.83 (4-bromophenyl), 146.8, 163.0 and 167.32(3 C-atoms of thiadiazolotriazin-4-one) and 168.32 (-C=O of thiadiazolotriazin-4-one). LC-MS [M+], (m/z): 378.9 and 176.9. *Anal. calcd* for $C_{15}H_{15}BrN_4OS$: C,47.50; H,3.99; N,14.77. Found: C,47.13; H,3.35; N,14.89%. m.p.184-188^{0}C, yield 80%.

5d: IR (KBr, cm^{-1}) : 3095 (Aromatic C-H), 2955($-CH_3$ group of t-butyl), 1690 (>C=O), 1498 (-C=N stretch), 1490 and 1440 (C=C), 1472(-N=N-), 1259 (C-S), 1290(C-O). ^{1}H-NMR ($CDCl_3$, δ, ppm): 1.38 (9H, s, $3CH_3$), 4.62 (2H, s, $-CH_2$), 4.23(1H, s, -OMe) 6.94(2H, d, J= 8.8Hz, 4-methoxyphenyl), 7.2(2H, d, J = 8.4Hz, 4-methoxyphenyl). ^{13}C-NMR (δ, ppm): 29.8 ($3CH_3$ of t-butyl), 40.0(t-butyl C-atom), 41.2 ($-CH_2$), 64.9(-OMe), 116, 121.2, 129.2, 132.0 138.65, and 157.7(4-methoxyphenyl), 148.8, 151.0 and 163.98 (3 C-atoms of thiadiazolotriazin-4-one) and 169.46 (-C=O of thiadiazolotriazin-4-one). LC-MS [M+], (m/z): 330.40. *Anal. calcd* for $C_{16}H_{18}N_4O_2S$: C,58.16; H,5.49; N,16.96. Found: C,58.75; H,5.32; N,17.00%. m.p.162-165^{0}C, yield 69%.

5e: IR (KBr, cm^{-1}): 3100(-OH), 3009 (Aromatic C-H), 2940($-CH_3$ group of t-butyl), 1704 (>C=O), 1450(-C=N stretch), 1560 and 1503 (C=C), 1432(-N=N-), 1295 (C-S),. ^{1}H-NMR ($CDCl_3$, δ, ppm): 1.39 (9H, s, $3CH_3$), 4.69, (2H, s, $-CH_2$), 5.5 (1H, s, -OH), 6.88(2H, d, J= 8.2Hz, 4-hydroxyphenyl), 7.1(2H, d, J = 8.8Hz, 4-hydroxyphenyl). ^{13}C-NMR (δ, ppm): 28.43 ($3CH_3$ of t-butyl), 37.9(t-butyl C-atom), 38.8 ($-CH_2$), 115.9, 123.45, 128.0 130.28, 131.3 and 148.8(4-hydroxyphenyl), 147.6, 156.93 and 166.93 (3 C-atoms of thiadiazolotriazin-4-one) and 173.8 (-C=O of thiadiazolotriazin-4-one). LC-MS [M+], (m/z): 316.37. *Anal. calcd* for $C_{15}H_{16}N_4O_2S$: C,56.94; H,5.10; N,17.71. Found: C,56.74; H,5.25; N,17.83%. m.p.175-177^{0}C, yield 81%.

5f: IR (KBr, cm^{-1}) : 3080 (Aromatic C-H), 2964($-CH_3$ group of t-butyl), 1704 (>C=O), 1509 (-C=N stretch), 1458 and 1391 (C=C), 1364(-N=N-), 1225 (C-S), 1022(Ar-F). ^{1}H-NMR ($CDCl_3$, δ, ppm): 1.38 (9H, s, $3CH_3$), 4.45 (2H, s, $-CH_2$), 4.23(1H, s, -OMe) 7.24(2H, d, J= 5.2Hz, 4-fluorophenyl), 7.68(2H, d, J = 5.6Hz, 4-fluorophenyl). ^{13}C-NMR (δ, ppm): 29.30 ($3CH_3$ of t-butyl), 38.2(t-butyl C-atom), 44.0 ($-CH_2$), 118.3, 119.78, 145.56, 148.85 153.9, and 164.0(4-fluorolphenyl), 151, 155.41 and 166.33 (3 C-atoms of thiadiazolotriazin-4-one) and 168.32 (-C=O of thiadiazolotriazin-4-one). LC-MS [M+], (m/z): 319.0. *Anal. calcd* for $C_{15}H_{15}FN_4OS$: C,56.59; H,5.97; N,17.60. Found: C,56.43; H,5.22; N,17.58%. m.p.190-194^{0}C, yield 69%.

5g: IR (KBr, cm^{-1}) : 3072 (Aromatic C-H), 2955($-CH_3$ group of t-butyl), 1692 (>C=O), 1521 (-C=N stretch), 1494 and 1455 (C=C), 1388(-N=N-), 1168(C-S), 823(Ar-Cl). ^{1}H-NMR ($CDCl_3$, δ, ppm): 1.31 (9H, s, $3CH_3$), 4.46 (2H, s, $-CH_2$), 7.44(4H, d, J= 8.2Hz, 4-chlorophenyl). ^{13}C-NMR (δ, ppm): 28.30 ($3CH_3$ of t-butyl), 38.6(t-butyl C-atom), 40.9 ($-CH_2$), 128.8, 133.56, 135.28, 137.0 139, and 144.95(4-chlorolphenyl), 152, 156.14 and 159.33 (3 C-atoms of thiadiazolotriazin-4-one) and 166.15 (-C=O of thiadiazolotriazin-4-one). LC-MS [M+], (m/z): 334.82. *Anal. calcd* for $C_{15}H_{15}ClN_4OS$: C,53.81; H,10.59; N,16.73. Found: C,53.61; H,10.42; N,16.85%. m.p.148-151^{0}C, yield 65%.

5h: IR (KBr, cm^{-1}): 3018 (Aromatic C-H), 2865($-CH_3$ group of t-butyl), 1710 (>C=O), 1510, 1498 and 1411 (-C=N stretch), 1419 (C=C), 1433(-N=N-), 752(-Cl). ^{1}H-NMR ($CDCl_3$, δ, ppm): 1.38 (9H, s, $3CH_3$), 4.5 (2H, s, $-CH_2$), 8.1(2H, d, J= 8.4Hz, 2-chloropyridyl), 9.2 (2H, d, J = 8.8Hz, 2-chloropyridyl). ^{13}C-NMR (δ, ppm): 26.1 ($3CH_3$ of t-butyl), 38.6(t-butyl C-atom), 45.97 ($-CH_2$), 124.5, 131.4, 139.3, 146, and 158.15(2-chloropyridyl), 159, 162.71 and 168.42 (3 C-atoms of thiadiazolotriazin-4-one) and 170.68 (-C=O of thiadiazolotriazin-4-one). LC-MS [M+], (m/z): 335.81. *Anal. calcd* for $C_{14}H_{14}ClN_5OS$: C,50.07; H,4.20; N,20.85. Found: C,50.8; H,4.15; N,20.03%. m.p.130-132^{0}C, yield 75%.

3. LARVICIDAL ACTIVITY

3.1. Methodology

Larvicidal bioassay was conducted in accordance with the WHO standard protocol (World Health Organization, 1981 were used as the test The test[30] was carried out using a batch of 20 late third or early fourth instar larvae of *Aedes aegypti*, *Culex quinquefasciatus and Anopheles stephensi*. Concentration ranging from 50 ppm to 800 ppm were prepared from the 1% stock solution (1% acetone + 0.001% Tween – 80). The test was performed in a 250-mL glass beaker, with 1 mL of each of the test chemicals and 99 mL distilled water. 1 mL acetone with 0.001% Tween-80 was used as the negative control, kept with each set of the experiment. The mortality rate was recorded after 24 hours of exposure. Median lethal concentration (LC_{50}) with 95% confidence limit was calculated using Abbott's formula (1925) and Log probit analysis, and results are expressed as ppm. Each test chemical was evaluated in triplicates and mean (±SD) values were taken. The results obtained are tabulated in Table 2 and Fig. 1).

Table2. *Results obtained for larvicidal activity of Thiadiazolotrizinones against three disease vector mosquitoes*

Test compounds	*A. aegypti*		*C. quinquefasciatus*		*A. stephensi*		Average LC-50
	LC-50	LC-90	**LC-50**	LC-90	**LC-50**	LC-90	
Control	-		-	-	-	-	-
5a	248	538	223	482	276	612	249
5b	182	399	196	426	234	504	204
5c	239	521	210	454	247	529	232
5d	320	634	246	492	293	636	286
5e	236	511	184	413	221	485	214
5f	251	533	196	422	235	506	227
5g	184	402	179	392	269	582	211
5h	269	584	239	478	310	660	273
Ref.std. (Temephos)	0.019	0.0612	0.016	0.049	0.017	0.078	-

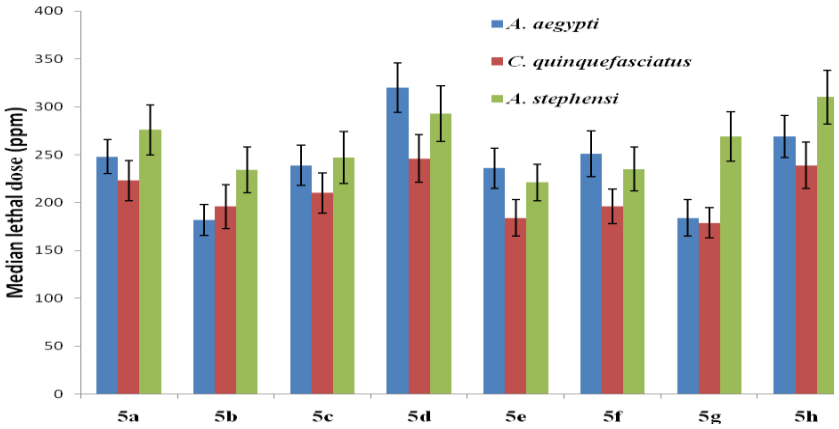


Fig1. *Bar diagram showing the larvicidal activity of Thiadiazolotrizinones against three pathogenic vectors (mosquitoes)*

3.2. Antimicrobial screening assay

The antimicrobial activity of the samples was tested *in vitro* against four bacterial pathogens and two fungal pathogens by disc diffusion method[31,32]. The bacteria *Staphylococcus aureus* MTCC 7443 (BP1), *Bacillus subtilis* MTCC 441 (BP2), *Escherichia coli* MTCC 725 (BP3), *Proteus vulgaris* MTCC 426 (BP4), the yeast *Candida albicans* MTCC 183 (FP1) and mycelial fungi *Aspergillus niger* MTCC 281 (FP2) were used as the test organisms. 100 µL suspensions of bacterial and fungal strains prepared in physiological saline (0.85% NaCl) were spread on Muller-Hinton agar (MHA) and Sabouraud dextrose agar (SDA) respectively. Sterilized filter paper discs (6.5 mm in diameter) were impregnated with 50 µL of the samples dissolved in dimethyl sulfoxide (DMSO; 1mg/mL), and placed on the inoculated agar plates. Negative control was prepared using DMSO. Gentamycin and Ketoconazole were used as the positive controls for antibacterial and antifungal activity studies. The inoculated plates were kept at room temperature for 10 min and incubated at 37°C (24 hours) for the bacterial strains and at room temperature (48 hours) for the fungal strains.

Antimicrobial activity was evaluated by measuring the zone of inhibition against the test microbial pathogens and the results were incorporated in Table 3 and 4.

4. ANTIBACTERIAL ACTIVITY

Table3. *Antibacterial Activity (Zone Of Inhibition) Data of Compounds (5a-H)*

Control/Sample	Zone of inhibition in mm			
	S. aureus MTCC 7443	*B.subtilis* MTCC 441	*E. coli* MTCC 725	*P. vulgaris* MTCC 426
Gentamicin(Positivecontrol)	21.17±0.28	29.0±0.0	23.67± 0.28	10.33 ±0.28
Negative control (DMSO disc)	7.33±0.28	-	-	7.67± 0.28
Parent compound	8.4± 0.36	9.17± 0.28	-	10.0± 0.0
5a	8.33± 0.28	9.4± 0.40	-	8.33± 0.28
5b	-	10.9± 0.46	-	-
5c	-	9.33± 0.28	-	-
5d	8.17± 0.28	11.07± 0.51	-	-
5e	-	10.83± 0.28	10.67± 0.29	-
5f	9.27± 0.25	9.17± 0.28	-	9.37± 0.32
5g	8.5± 0.5	8.7± 0.28	-	9.5± 0.00
5h	9.47±0.28	9.0± 0.0	-	10.5 0.00

5. ANTIFUNGAL ACTIVITY

Table4. *Antifungal Activity (Zone Of Inhibition) Data Of Synthesized Compounds (5a-H) And Ketoconazole (Ref. Standard).*

Control/ Sample	Zone of inhibition in mm	
	Candida albicans MTCC 183	*Aspergillus niger* MTCC 281
Ketoconazole(Positive control)	21.0±0.0	9.67 ±0.28
Negative control(DMSO disc)	7.43 ±0.40	-
Parent compound	8.3 ±0.26	-
5a	8.17± 0.28	-
5b	11.0 ±0.00	-
5c	10.5±0.00	-
5d	11.33 ±0.15	-
5e	10.4 ±0.38	-
5f	12.83± 0.29	-
5g	12.63±0.41	-
5h	8.4 ±0.36	-

6. RESULTS AND DISCUSSION

After confirmation of the novel derivatives through spectroscopic analyses, they have been screened for larvicidal activity against *A. aegypti*, *C. quinaquefasciatus* and *A. stephensi*. All the novel derivatives exhibited the larvicidal activity, but with different LC50 values, ranging between 179 and 320 ppm. Lesser the LC50 values, greater is the potency of the activity. The results indicate that comparatively, C. quinquefasciatus larvae were found to be more sensitive to the novel derivates as indicated by the lesser IC50 values compared to those to those of the other two species..

Taken into account of LC-50 values less than 200 ppm as the substantial activity, among the test compounds, 5b is effective against *A. aegypti* (182 ppm) and *C. quinquefasciatus* (196 ppm), 5e and 5f were effective against *C. quinquefasciatus (*184 ppm and 196 ppm respectively), 5g was effective against *A. aegypti* (184 ppm) and *C. quinquefasciatus* (179 ppm).

The average LC-50 values indicate that 5b is more effective (204 ppm) followed by 5g, 5e, 5f, 5c, 5a, 5h and 5d in the order of efficacy. Thus, among the test compounds, **5b** can be considered as an agent with potential broad spectrum larvicidal activity. The presence of methyl group at 4^{th} position on the phenyl ring may be possible reason for the larvicidal activity with higher efficacy. Having obtained encouraging results, the compounds may further be studied for toxicities in non-target organisms; If they found to be relative non-toxic and environment friendly they may be recommended as a larvicidal agent. The reference standard temephos exhibited a very high larvicidal activity compared to the novel derivatives. However, it has greater limitations in terms of its toxic effects on non-target organisms and efficacy of the test compounds cannot directly be compared.

The results derived from antibacterial study revealed that among the novel derivatives, those which bear hydroxyl substituent at 4th position of the phenyl ring **(5e)** exhibited a broad-spectrum antibacterial activity against both Gram positive and Gram negative species. Considering zone of inhibition >10 mm as the good activity, 5b, 5d, 5e and 5h were found to be sensitive against *B. subtilis*, both *B. subtilis* and *E. coli* and *P. vulgaris*, respectively. These may contribute to the development of potential antibiotics against bacterial infection. Relatively 5d exhibited the maximum activity against *B. subtilis,* which may be due to the presence of methoxyl substituent at 4th position. Compared to the parent compound all the novel derivatives exhibited more values of antibacterial activity.

With reference to the antifungal activity, interestingly, all the novel derivatives exhibited moderate activity but only against *C. albicans*. None of the compounds gave the positive results against *A. niger*. The differential effects may be due to the species difference, such that each species has its own sensitivity or resistance to foreign agents depending on the availability of the target molecules. Comparatively, the compounds exhibited the growth inhibition activity against C. albicans in the decreasing order as follows: 5f, 5g, 5d, 5b, 5c, 5e, 5b, 5h and parent compound. Thus, 5f and 5g can be considered as agents with more activity, followed by 5d and 5b, which exhibited moderate activity.

7. CONCLUSION

Compound 5b exhibited a potential broad-spectrum larvicidal activity. The derivative 5e was found to possess broad-spectrum antibacterial property. 5f showed a good antifungal activity against *C. albicans* Thus, further studies may be taken up to give insight into the potential activity by respective novel compounds leading them to commercial exploitation as an antimicrobial and/or a larvicidal agents.

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