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Synthesis of novel thymol derivatives against MRSA and ESBL producing pathogenic bacteria

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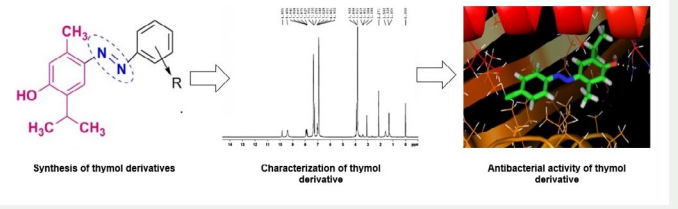
ABSTRACT

Twelve substituted aryl-azo-thymol derivatives (**4a** to **4 l**) were synthesized and characterized by several spectral techniques such as, FTIR, UV-vis, proton NMR, Mass spectrometry and elemental analysis. Antimicrobial activities were evaluated by agar-well diffusion method against isolated MRSA, ESBL-producing pathogenic bacteria and antifungal resistant fungi, *in vitro*. In addition, drug likeness properties of derivatives were assessed through bioinformatic tools such as, PASS prediction, molecular docking and Lipinski rules of five, along with determination of toxic nature and LD_{50} values. Among 12 derivatives, **4a**, **4b**, **4c**, **4 g, 4i, 4j** and **4 k** had significant antibacterial and antifungal activities with minimum inhibitory concentration values, 40 to 80 µg/ml. Moreover, the docking scores of derivatives were −8.27 to −11.44 kcal/mol, against 4 bacterial targets and −9.45 to −12.49 kcal/mol against 2 fungal targets. Thus, from *in vitro* and *in silico* studies, thymol derivatives had control of MRSA, ESBL-producing bacteria and antifungal resistant fungi.



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KEYWORDS
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1. Introduction

Gram-negative (GN) and Gram-positive (GP) pathogenic bacteria cause morbidity and mortality from infections. The first antibiotic penicillin or 6-aminopenicillanic acid (6-APA) could control infections from those bacteria, and later on penicillin was resisted by target bacteria (Chambers and Deleo 2009; Canton and Morosini 2011). Moreover, pathogenic bacteria

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developed further resistance individually to newly introduced antibiotics (Davies and Davies 2010; Canton and Morosini 2011; Swain and Padhy 2015). Thus, multidrug resistant (MDR) bacteria must be controlled with an iron-hand, which has been giving impetus for search for newer antibacterials from conventional and non-conventional sources, apart from chemical modification of antibiotics. Several phytochemicals were reported having antibacterial and antifungal activities and lend themselves to the preparation of mainstream medicine (Cowan 1999; Vázquez-Sánchez et al. 2015).

Thymol, 2-isopropyl-5-methylphenol is a natural monocyclic monoterpene phenol available in several plants such as, *Thymus vulgaris* and other *Thymus* sp., *Cinnamonum zeylanicum, Eugenia caryophyllus,* etc., had been reported as a potent antibacterial, antifungal and antioxidative agent (Falcone et al. 2005; Bollenbach 2015; Vázquez-Sánchez et al. 2015). However, phytochemicals could not be used directly for unknown pharmacological/ chemical constraint/ lack of drug likeness properties (Brown and Dawson 2015; Hughes et al. 2011; Swain et al. 2017). Thus, a solution of this problem to improve the antibacterial activity along with drug likeness property is attempted in the present study. Twelve thymol derivatives (**4a**-**4 l**) were developed through conjugation with several amines for probable control of gruesome human pathogenic bacteria and fungi, isolated from clinical samples; and those were recorded as having improvement of several physico-chemical properties, suitable for further consideration as drugable candidate. Moreover, molecular docking study was carried out using β-lactamase as the bacterial target and sterol α-demethylase along with glucosamine-6-phosphate synthase as fungal targets for evaluating activity of each derivative, *in silico*.

2. Results and discussion

2.1. Chemistry, synthesis and characterization of thymol derivatives

Wave length values of individual aryl-azo-thymol derivatives were observed at a concentration 10^{-5} to 10^{-6} M by UV–visible spectrophotometry using ethanol as solvent. Derivatives had strong bathochromic shifts, as the λ_{max} values ranged from 344 to 361 nm. Similarly, comparative HPLC chromatograms were carried out in reverse-phase Grace Alltima HP Amide analytical column with a solution (v/v) in the ratio, 55:45:: phosphate buffer:acetonitrile; it was evident that the synthesized derivatives had > 93 % purity, for example the derivative **4e** (Figure S1).

Several spectral peaks in structures of 12 synthesized derivatives were confirmed from FTIR and ^{1}H NMR spectral data: peaks at 3225–3150, 1524–1470 and 1610–1600 cm^{-1} in FTIR analysis are the groups of –OH str., –N = N–, and –C = C–aromatic str., respectively. FTIR of **4e** confirms that functionalities were characterized (Figure S2). Moreover, the ^{1}H NMR spectrum exhibited 4 sets of proton signals characteristic of the expected thymol moiety. With the synthesized derivatives, two singlet aromatic proton signals were observed at ranges, δ 7.552–7.853 ppm and δ 6.921–7.253 ppm of H-3 and H-6 positions of thymol, respectively. In addition to the cited protons, gem-dimethyl (isopropyl) and methyl protons of thymol nucleus also appeared at ranges of δ 3.083–3.283 and 1.306–2.271 ppm as multiples and a singlet, respectively. The proton NMR spectrum of **4e** is elucidated (Figure S3).

Table 1. Antibacterial and antifungal activities of synthesized thymol derivatives monitored by agar-well diffusion method *in vitro*.

	Inhibition zone size (mm)					
	Isolated bacterial strains				Isolated fungal strains	
Derivatives/RA	*S. aureus*	*E. coli*	*K. pneumoniae*	*P. aeruginosa*	*A. fumigatus*	*C. albicans*
4a	19.2 ± 0.34	19.3 ± 0.73	18.3 ± 0.73	18.8 ± 0.62	21.3 ± 0.62	20.8 ± 0.62
4b	20.0 ± 0.63	18.6 ± 0.60	21.4 ± 0.58	20.6 ± 0.70	22.8 ± 0.70	21.6 ± 0.70
4c	21.3 ± 0.76	20.8 ± 0.56	19.8 ± 0.61	21.4 ± 0.47	23.2 ± 0.47	22.4 ± 0.47
4d	18.8 ± 0.45	19.3 ± 0.76	18.4 ± 0.56	19.3 ± 0.38	19.9 ± 0.38	20.3 ± 0.38
4e	19.4 ± 0.48	16.9 ± 0.42	16.9 ± 0.42	17.8 ± 0.66	20.8 ± 0.66	21.8 ± 0.66
4f	16.8 ± 0.62	18.0 ± 1.67	18.0 ± 1.67	19.9 ± 0.81	19.9 ± 0.81	20.9 ± 0.81
4 g	19.2 ± 0.86	19.4 ± 0.28	19.4 ± 0.28	18.0 ± 0.44	22.6 ± 0.44	22.2 ± 0.44
4 h	16.8 ± 0.71	16.7 ± 0.78	19.7 ± 0.78	17.9 ± 0.36	21.4 ± 0.36	20.9 ± 0.36
4i	19.5 ± 0.18	19.6 ± 0.38	19.6 ± 0.38	20.4 ± 0.42	24.4 ± 0.42	20.6 ± 0.42
4j	22.9 ± 0.58	19.8 ± 0.54	20.4 ± 0.54	21.6 ± 0.34	25.6 ± 0.34	24.8 ± 0.34
4 k	15.2 ± 1.20	18.8 ± 0.90	19.5 ± 0.90	19.3 ± 0.72	20.3 ± 0.72	21.4 ± 0.72
4 l	18.8 ± 0.82	17.0 ± 0.82	18.2 ± 0.46	17.6 ± 0.56	20.6 ± 0.56	21.2 ± 0.56
*AMP	17.6 ± 0.34	16.0 ± 0.42	17.6 ± 0.28	17.9 ± 0.52	–	–
*FLC	–	–	–	–	19.6 ± 0.34	20.2 ± 0.45

Notes:
*Reference antibiotic; AMP, ampicillin; FLC, fluconazole; –, not done. Data with mean ± standard deviation from 6 replications.

2.2. In vitro *antibacterial and antifungal study*

In vitro antibacterial study against 4 isolated MDR bacterial strains, diameter values of inhibition zones of thymol derivatives ranged from 13 to 23 mm, while the standard antibiotic, amikacin had inhibition zones 16–18 mm (Table 1). Derivatives, **4a, 4b, 4c, 4e, 4i** and **4j** caused inhibition zone sizes as 18–22 mm, and **4j** had the highest inhibition zone as 22.9 mm against MRSA. Similarly, inhibition zone sizes of 12 derivatives were > 20 mm against *E. coli,* and inhibition zones against *P. aeruginosa* were > 22 mm (Table 1). It was clear that **4j** was the most effective derivative against clinically isolated MDR bacterial strains. Furthermore, the recorded antifungal activity of derivatives as diameter values of inhibition zones ranged from 19 to 25 mm, and **4j** had the highest inhibition zones, 25 and 24 mm against *A. fumigatus* and *C. albicans*, respectively (Table 1). Minimum inhibitory concentration (MIC) values of 12 synthesized conjugates were within the range, 40–80 µg/ml; and particularly conjugate, **4a, 4b, 4c, 4e, 4 g** and **4j** had 40–80 µg/ml as the lowest minimum bactericidal concentration (MBC) value against *S. aureus* (Table S1). The recoded MIC and MBC values of the reference antibiotic, gentamicin were 80 and > 100 µg/ml, respectively. Furthermore, **4a, 4d, 4f, 4i, 4j, 4 k** and gentamicin had MIC values 40 µg/ml, and the rest conjugates had MIC values > 80 µg/ml, against *E. coli;* and MIC values of **4b, 4c, 4d, 4i, 4j** and **4 l** were 40 µg/ml and corresponding MBC values were 160 µg/ml against *K. pneumoniae*. Similarly against *P. aeruginosa*, MIC values of thymol derivatives, **4b, 4 g, 4i, 4j** and **4 k** were 40 µg/ml and the corresponding MBC values were within the range, 80 to > 100 µg/ml. (Table S1). Thus, these thymol derivatives could be used as alternatively/ synergistically along with a class of antibiotics for the control MDR bacterial strains. Moreover, the emergence of resistance of pathogenic bacteria to drugs/ antibiotics has now become increasingly commonplace, due to two obvious reasons, the versatility of the microbial genetic system and the lack of stringent antibiotic policy worldwide (Davies and Davies 2010; Canton and Morosini 2011). In this perspective, chemical manipulation of phytochemicals were considered suitable for the use as derivative/

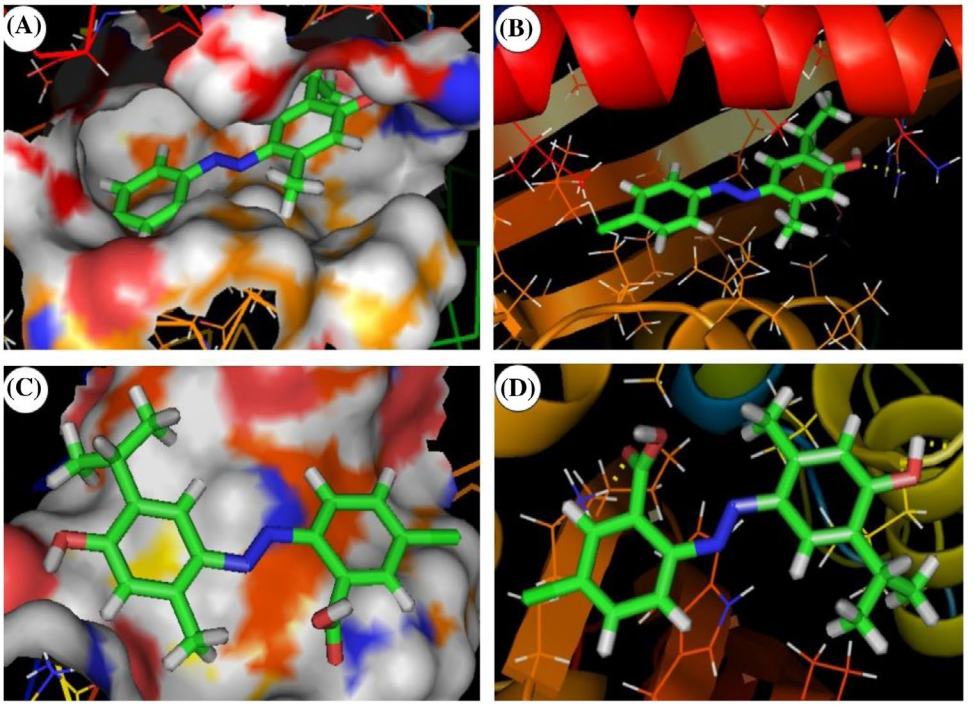

Figure 1. Target-ligand interactions visualized through PyMOL software. **A** and **B** represent the interaction of derivative **4b** with β-lactamase of *K. pneumoniae* (PDB ID: 1N9B); **C** and **D** represent the interaction of derivative **4j** with sterol α-demethylase of *A. fumigatus* (PDB ID: 4UYL) during docking.

semi-synthetic drugs, like attempted examples for malaria and pathogenic bacteria (Brown and Dawson 2015; Cochrane et al. 2009; Machado et al. 2018; Swain et al. 2017).

Moreover, the advance bioinformatics tools are not used only for optimization and selection of effective agents through binding affinity through molecular docking (Table S2; Figure 1), but it could predict biological characteristics, absorption and permeability properties of synthesized thymol derivatives. Herein, the calculated estimates of oral absorption of derivatives are > 80 %, which means chemicals are well soluble and have permeable properties (Table 2). Moreover, it was too clear that except **4b**, computational prediction for LD_{50} values of all derivatives were > 2000 mg/kg body weight, confirming by PASS prediction that the synthesized derivatives are safe as possible antimicrobial agents (Table S3). Thus, the computational study too supports to *in vitro* study and helped to conclude that, thymol derivative have comparatively more effective drug likeness properties to control MDR pathogens. In PASS prediction, a chemical with 'a higher possible active (Pa) value than the corresponding possible inactive (Pi) value' was considered as the possible drug molecule for a specific biological activity (Khurana et al. 2011). Moreover, Lipinski rule of five (RO5), based on molecular properties, molecular weight, numbers of hydrogen acceptors (H-ba), numbers of hydrogen donors (H-bd) and cLogP (partition coefficient between n-octanol and water) values were widely used in the selection-criterion for an active drug molecule (Lipinski et al. 2001); and all derivatives are effective under RO5. Indeed, the 'highest occupied molecular orbitals' (HOMO) energy and the 'lowest unoccupied molecular orbital' (LUMO) energy describe

Table 2. Calculation of molecular properties by Lipinski rule of five with predicted lethal doses, toxicity class, carcinogenicity, mutagenicity nature of thymol derivatives.

	Lipinski rule of five and manually calculation absorbance							Predicted lethal doses, toxicity class				
Derivative	MW (≤ 500 g/mol)	No. of H-ba (≤ 10)	No. of H-bd (≤ 5)	cLogP value (≤ 5)	tPSA (Å)	Stereo centers	Calculated % abs	LD_{50} (mg/kg)	LC_{50} (mmol)	Toxicity class	Carci-no-genicity	Muta-genicity
4a	254.33	3	1	5.05	44.95	0	93.49	4818	0.004985	5	NC	NM
4b	288.77	3	1	5.65	44.95	0	93.49	1250	0.002699	4	NC	NM
4c	332.05	3	2	5.77	44.95	0	93.49	4818	0.004985	5	NC	NM
4d	268.35	3	1	5.39	44.95	0	93.49	4818	0.004403	5	C	NM
4e	284.35	4	1	4.98	54.18	0	90.30	2000	0.003757	4	NC	NM
4f	270.33	4	2	4.70	65.18	0	86.51	4818	0.003757	5	NC	NM
4 g	298.34	5	2	4.53	82.25	0	80.62	2000	0.004612	6	NC	NM
4 h	298.34	5	2	4.53	82.25	0	80.62	7400	0.004783	6	NC	NM
4i	377.23	5	2	5.26	82.26	0	80.62	7400	0.004783	6	NC	NM
4j	332.78	5	2	5.14	82.25	0	80.62	7400	0.002422	6	NC	NM
4 k	268.35	3	1	5.39	44.95	0	93.49	4818	0.004403	5	NC	NM
4 l	284.36	4	1	4.98	54.18	0	90.30	2000	0.003757	4	NC	NM

Notes:
C, carcinogenic; H-ba, hydrogen bond acceptor; H-bd, hydrogen bond donor; MW, molecular weight; NC, non-carcinogenic, NM, non-mutagenic; tPSA, topological polar surface area.

electron donor and accepter regions, respectively of an actively participating ligand during the formation of a protein-ligand complex (Figures S4 and S5). Today, computational tools are used to predict possible effective drug likeness molecule in current drug development method to guide or avoid of synthesis unwanted chemical and save the time and resources in early drug discovery (Cochrane et al. 2009; Hughes et al. 2011; Sliwoski et al. 2013; Swain et al. 2015, 2017).

The molecular docking is used for deciphering the interaction between a small molecule as the ligand with a target enzyme related to a particular disease (Leelananda and Lindert 2016). Advances computational strategies are more beneficial for locating effective drugable candidate or 'lead optimization' thought virtual screening and dynamic techniques in the current drug discovery process in comparison with traditional experimental rather, traditional screening. Particularly, low cost and effective screening is the most advantage of a docking process and it serves as an important tool in pharmaceutical research (Leelananda and Lindert 2016; Swain et al. 2017).

3. Experimental

3.1. Syntheses of thymol derivatives

A series of azoaryl substituted derivatives (**4a**-**4 l**) were synthesized by coupling of diazonium salt of aniline derivatives 2 (**a** to **l**) with thymol in the presence of 10 % sodium hydroxide (see, Supplementary files for characterization reports of 12 thymol derivatives in Scheme-1). The coupling component, thymol was attached by strong diazonium electrophilic N_2^+ to produce azo- substituted thymol, as thymol was the neutral nucleophile agent in the reaction, as described (Sahoo and Paidesetty 2016; Swain et al. 2017). The obtained products were filtered and washed with pre-cooled distilled water, and the final products were dried at 30 °C and were re-crystallized by ethanol. High-performance liquid chromatography (HPLC) study was carried out for checking purity of derivatives, using Shimadzu (Scientific Instruments, Kyoto, Japan), by a reverse-phase Grace Alltima HP Amide analytical column (150 × 4.6 mm; 3 µm) with the injection of an aliquot of 20 µL of each sample. Spectral characterizations of derivatives were interpreted by Fourier transform infrared spectroscopy (FTIR) using KBr discs with JASCO FTIR 4100 Spectrophotometer. Proton nuclear magnetic resonance (^{1}H NMR) spectra of each derivative was studied using tetramethylsilane as the internal standard with Bruker ^{1}H NMR 400 MH_Z using deuterated chloroform ($CDCl_3$) as solvent. Mass spectrometry (MS) using Shimadzu-Mass Spectrometer and differential scanning calorimetry with Mettler Toledo Star^e system were carried out. Furthermore, elemental analyses of derivatives were carried out by Perkin Elmer-2400 CHNO/S analyzer system.

3.2. In vitro *antibacterial and antifungal study*

Antibacterial activities of thymol derivatives were evaluated by the agar-well diffusion method against 4 pathogenic bacterial (Dubey and Padhy 2013; Swain et al. 2017) and 2 antifungal resistant pathogenic fungal strains isolated from clinical samples using Muller-Hinton agar (MHA) and potato dextrose agar (PDA) media, respectively (Fan et al. 2011; [CLSI] Clinical and Laboratory Standards Institute 2013; Swain and Padhy 2015). The MIC and MBC values of individual derivatives were determined. The original stock solution (mg/ml) of individual

derivatives along with the reference antibiotic was prepared in 10% DMSO, thereafter were converted to different concentrations (µg/ml) of each tested sample by serial dilution method. Using 96-well (12 × 6) micro titer plate MIC values as the lowest concentration that completely inhibited bacterial growth by each sample was calculated (Dubey and Padhy 2013; Swain et al. 2017). Bacteria from each well of the microtiter plate were sub-cultured on a nutrient agar plates for the determination of the MBC value of each chemical.

3.3. Computational analysis

In present docking attempts, the bacterial β-lactamase species of *S. aureus* (Protein Data Bank or PDB ID: 1MWS), *Escherichia coli* (PDB ID: 1XPB), *Klebsiella pneumoniae* (PDB ID: 1N9B) and *P. aeruginosa* (PDB ID: 4HEF), while fungal targets sterol α-demethylase of *Aspergillus fumigatus* (PDB ID: 4UYL) and glucosamine-6-phosphate synthase of *Candida albicans* (PDB ID: 2POC) were used as putative drug targets for evaluating docking scores of each thymol derivative (Nichols et al. 2014; Hargrove et al. 2015). Derivatives were designed by the software ChemDraw Ultra for the intended docking study by software AutoDock Vina (Swain et al. 2015, 2017). Moreover, interactions of protein-ligand complexes were visualized by PyMOL software. Possible antiseptic, antiinfective, antiparasitic, antimycobacterial and antiprotozoal activities of thymol derivatives were evaluated using PASS tool (http://www.pharmaexpert.ru/passonline/). Molecular properties of derivatives were standardized in RO5, using the software, ChemDraw Ultra along with Molsoft (http://molsoft.com/mprop/) and Molinspiration (http://www.molinspiration.com/) tools. Additionally, molecular orbital surfaces, HOMO, LUMO and 'energy electrostatic potential charges' plots of 12 derivatives were visualized using the software, Argus Lab.

4. Conclusion

From inhibition zone sizes, MIC and MBC values of all derivatives, **4a**, **4b**, **4c**, **4 g, 4i, 4j** and **4k** were selected as the effective-most antimicrobial agents; nevertheless, all derivatives had controlling capacity on MDR strains of GN bacteria along with fungal strains, *in vitro* and could be used as newer anti-bacterial and antifungal agents after due pharmacologic validation. Furthermore, advanced computational tools, PASS, molecular docking, RO5 supported the *in vitro* work and could help assess several possible drugable information for these synthesized aryl-azo-thymol derivatives. Thus, the computational tools could be considered as effective system in saving time and resources, in modern drug development era that encourages for the synthesis of comparatively more effective antibacterial drugs.

Supplementary material

Synthesis procedure, antibacterial activity procedure with NMR data of thymol derivatives (**4a** to **4 l**) with more figures and tables are available in the supplementary material which are cited in appropriate place in text. https://doi.org/10.1080/14786419.2018.1474465.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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