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Syntheses and antimicrobial activities of amide derivatives of 4-[(2-isopropyl-5-methylcyclohexyl)oxo]-4-oxobutanoic acid

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ABSTRACT

Chiral 4-[(2-isopropyl-5-methylcyclohexyl)oxo]-4-oxobutanoic acid reacts with substituted anilines to produce amides 1-6 in high yields. Resulted amides 1-6 were investigated for their antifungal and antibacterial activities. Compounds 2 (96.5%) against *Aspergillus fumigatus* and 6 (93.7%) against *Helminthosporium sativum* demonstrated excellent activities. However, compounds 3 (37.6%) against *Bacillus subtilis*, 4 (33.2%) against *Pseudomonas aurignosa*, 5 against *Klebsiella pneumonia* demonstrated excellent growth inhibition potential.

Keywords: Chiral 4-[(2-Isopropyl-5-methylcyclohexyl)-oxo]-4-oxobutanoic Acid; Substituted Anilines; Amides; Antimicrobial Activity

1. INTRODUCTION

The growing incidence of bacterial resistance to existing antibiotics poses a serious medical problem in treating pathogenic infections [1,2]. Hence, there is an urgent need for molecules which are more potent and less sensitive to developing resistance properties than currently in use clinical antibiotics [3].

Succinic acid is predicted to be one of the future platform chemicals that can be derived from renewable resources. The amide bond is the most important linkage in organic chemistry and possesses the key functional group in peptides, polymers, in many natural products and pharmaceuticals [4,5]. Amides are synthesized by coupling of carboxylic acids and amines by the use of either a coupling reagent [6] or by prior conversion of the carboxylic acid into a derivative [7]. Alternative procedures involve the Staudinger ligation [8], aminocarbonylation of aryl halides [9] and oxidative amidation of aldehydes [10]. The preparation of amides and their physical and chemical properties are extensively documented [11,12]. The formation of amides on solid support usually involves reactions of amines either with acid halides [13,14] or anhydrides [15] in the presence of base, or with acids in the presence of coupling agents [16-18] such as 1-hydroxybenzotriazole (HOBt), 7-aza-1-hydroxybenzotriazole (HOAt), or their ammonium or phosphonium salts. The formation of amides and analogues such as ureas, urethanes and thioureas, on solid support had also been reviewed [19,20].

Herein, we report improvements in biological propeties of chiral 4-[(2-isopropyl-5-methylcyclohexyl)oxo]-4-oxobutanoic acid amide derivatives. Chiral 4-[(2-isopropyl-5-methylcyclohexyl)oxo]-4-oxobutanoic acid (mono ester of succinic acid) was used as starting material which was derived from succinic anhydride by reacting with *S* (+) menthol. In view of various biological properties are associated with amides therefore, it seemed interesting to synthesize chiral amide derivatives of succinic acid. They were prepared by reaction of different aromatic amines with chiral 4-[(2-isopropyl-5-methylcyclohexyl)oxo]-4-oxobutanoic acid to afford the corresponding amides. Six chiral amides derivatives **1-6** were synthesized and their structure had been characterized by the UV, IR, ^{1}H-NMR, ^{13}C-NMR and mass spectroscopic analysis. All six derivatives were screened for their antimicrobial activities.

2. RESULTS AND DISCUSSION

2.1. Chemistry

Owing to the importance of amides in organic synthe-

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sis, we planned to synthesize some new biologically active amides. In our research work we have selected an inexpensive and commercially available starting material succinic anhydride. Six amides were synthesized by the outlined route as shown in **Scheme 1**.

Chiral menthol ester was synthesized by using our previously reported method [21]. Amide functionality was being introduced by reaction of mono ester with different substituted anilines in $CHCl_3$ in the presence of *N*,*N*'-dicyclohexylcarbodiimide (DCC). All the synthesized compounds were purified by column chromatography and characterized by using spectroscopic techniques like IR, ^{1}H-NMR and ^{13}C-NMR spectroscopy. Compound **1-6** also gave satisfactory elemental analyses.

IR spectra of all six amides displayed C=O (amide) absorption at 1701 - 1728 cm^{-1} beside C=O (ester) absorption at 1649 - 1696 cm^{-1}. In ^{13}C-NMR spectra C=O resonated at 169 ppm for ester and at 172 ppm for amide. In each case, it indicating that an amide linkage has been established. In ^{1}H-NMR protons of -CH_2 groups of succinic acid moiety resonated at 2.65 ppm and 2.73 ppm as triplet integrating for two protons each rather than doublet of triplet indicating the formation of corresponding amide.

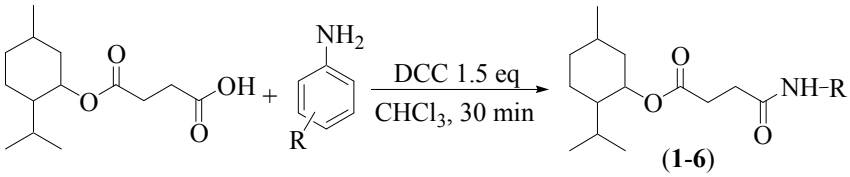
In case of compounds **3** and **6** aromatic protons resonated at 6.84 - 7.44 ppm and 7.11 - 7.41 ppm, respectively, as two doublets indicating the *para* substitution. Complete ^{1}H-NMR and ^{13}C-NMR data is given in experimental section. Elemental analysis values are in acceptable range provide an additional evidence to establish structures of synthesized compounds.

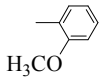
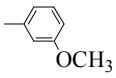
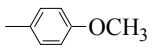
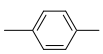
2.2. Bioassay

All six synthetic amides **1-6** were tested to study their antimicrobial activities against three selected fungal strains and four bacterial strains. Following methods were used to check these activities. DMSO was used as control solvent, terbinafin, and penicillium were used as standard drugs for antifungal and antibacterial activities, respectively.

2.2.1. Antifungal Activity

All six synthetic amides **1-6** showed varying degree of growth inhibition against all tested fungal strains and results are collected in **Table 1**.



S. No	*Compound No.*	*R*	*Isolated Yield* (%)
1	**1**		88
2	**2**		81
3	**3**		91
4	**4**		87
5	**5**		84
6	**6**		96

Scheme 1. Synthesis of amides from chiral 4-[(2-isopropyl-5-methylcyclohexyl)oxo]-4-oxobutanoic acid.

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Compound **2** exhibited an excellent percentage growth inhibition (96.5%) against *Aspergillus fumigatus* better than standard terbinafin (96.3%) in the same conditions. Compound **6** also demonstrated an excellent percentage growth inhibition (93.7) against *Helminthosporium sativum* strain also superior to terbinafin (92.7%). Compounds **1** (86.9%), **3** (85.7%), **4** (79.0%) exhibited appreciable percentage growth inhibitions against *Aspergillus fumigatus*, *Fusarium moniliforme*, and *Helminthosporium sativum*, respectively. Compounds **5** (70.7%) and **6** (73.7%) showed considerable percentage growth inhibitions against *Aspergillus fumigatus*. Compounds **2** (68.8%), **4** (66.2%), **5** (68.8%), and **6** (63.6%) were found to be significantly active against *Fusarium moniliforme* fungal strain. However, compounds **1** (55%), **2** (32%), **3** (39%), and **5** (43%) showed moderate to weak activities against *Helminthosporium sativum*.

2.2.2. Antibacterial Activity

All six synthetic amides **1-6** showed varying degree of percentage inhibition against all four selected bacterial strains and results are summarized in **Table 2**.

Table 1. Antifungal activities of the synthesized amides 1-6.

Treatments	Aspergillus fumigatus		Fusarium moniliforme		Helminthosporium sativum	
Compounds (15 mg/mL)	*Mycelial growth* (cm)	*Growth inhibition* (%)	*Mycelial growth* (cm)	*Growth inhibition* (%)	*Mycelial growth* (cm)	*Growth inhibition* (%)
Control	9.9	0	7.7	0	10	0
1	1.3	86.9	5.5	28.6	4.5	55.0
2	5.4	96.5	2.4	68.8	1.8	32.0
3	6.1	38.4	1.1	85.7	6.1	39.0
4	5.1	51.5	2.6	66.2	2.1	79.0
5	2.9	70.7	2.4	68.8	5.7	43.0
6	2.6	73.7	2.8	63.6	6.8	93.7
Terbinafin (*Std.*)	0.37	96.3	0.35	95.5	0.73	92.7

Table 2. Antibacterial activities of the synthetic amides 1-6.

S. No.	*Compounds*	Staphylococcus aureus	Pseudomonas aurignosa	Bacillus subtilis	Klebsiella pneumonia
		% Inhibition	*% Inhibition*	*% Inhibition*	*% Inhibition*
1	1	29.6	10.6	15.3	20.6
2	2	20	10.5	18.3	36
3	3	16.3	12.6	37.6	19
4	4	15.3	33.2	16.3	21.3
5	5	28.3	14.6	24	39.6
6	6	18.3	11	22	19
Penicillium (Std.)		30.3	32.3	34.3	38.6

Concentration = 15 mg/mL.

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Compound **3** exhibited an excellent percentage inhibition (37.6%) against *Bacillus subtilis* superior to standard penicillium (34.3%) in the same conditions. Compound **4** also showed an excellent percentage inhibition (33.2%) against *Pseudomonas aurignosa* better than standard penicillium (32.3%). Compounds **5** demonstrated a strong percentage inhibition (37.6%) against *Klebsiella pneumonia* also superior to penicillium (38.6%). Compound **1** found to have a comparable inhibition against *Staphylococcus aureus* (29.6%) to standard penicillium (30.3%). Compound **2** (36%) demonstrated a appreciable growth inhibition against *Klebsiella pneumonia*. However, compounds **5** also exhibited a good growth inhibition against *Staphylococcus aureus* with a percentage inhibition of 28.3. Compounds **2** (20%), **3** (16.3%), **4** (15.3%), and **6** (18.3%) showed moderate to weak activities against *Staphylococcus aureus*. Compounds **1** (10.6%), **2** (10.5%), **3** (12.6%), **5** (14.6%), and **6** (11%) exhibited moderate to weak growth inhibitions against *Pseudomonas aurignosa*. Compounds **1** (15.3%), **2** (18.3%), **4** (16.3%), **5** (24%) and **6** (22%) were found to have moderate to weak activities against *Bacillus subtilis*. In addition, compound **1** (20.6%), **3** (19%), **4** (21.3), and **6** (19%) showed moderate to weak activities against *Klebsiella pneumonia*.

3. CONCLUSIONS

Compounds **2** (96.5%) against *Aspergillus fumigatus* and **6** (93.7%) against *Helminthosporium sativum* demonstrated excellent activities and may be served as lead compounds for further research on these molecules as useful antifungal agents. However, compounds **3** (37.6%) against *Bacillus subtilis*, **4** (33.2%) against *Pseudomonas aurignosa*, **5** against *Klebsiella pneumonia* demonstrated excellent growth inhibition potential therefore may be served as lead compounds for further research on these compounds in search of better antibacterial agents.

4. EXPERIMENTAL

All reactions were carried out in anhydrous conditions and under static pressure of nitrogen gas using rubber septa and three way stopcock. Solvent like ether was dried and distilled over sodium and benzophenone. Chloroform was dried by refluxing with phosphorus pentoxide and methanol dried with magnesium turnings and iodine crystals. Amines were dried by refluxing over potassium hydroxide. All the reactions were monitored through thin layer chromatography using pre-coated silica gel glass plates (0.25 mm, HF-254, E. Merck). Methanol and chloroform mixture were used as eluent. Chromatograms were visualized using ultraviolet light at λ_{max} 254 or 365 nm. Column chromatography was performed on silica gel (0.063 - 0.200 mm E. Merck).

FTIR spectra were recorded on Schimadzu Fourier Transform Infrared Spectrophotometer Model 270. Solid samples were taken in KBr pellets and oils were used in NaCl cell for recording their spectra. ^{1}H-NMR spectra were recorded on NMR Bruker apparatus at 300 MHz in $CDCl_3$. ^{13}C-NMR spectra were recorded on NMR Bruker apparatus at 75 MHz. Tetramethylsilane (TMS) was used as internal reference. Chemical shifts are given in δ (ppm) and abbreviations s, d, and t have been used for singlet, doublet and triplet, respectively. The optical rotations of the compounds were measured on ATAGO, AP-100 automatic polarimeter.

4.1. General Procedure

In 50 mL conical flask a mixture of 4-(2-isopropyl-5-methylcyclohexyloxy)-4-oxobutan-oic acid and (0.004 mol, 0.418 g) and substituted aniline (1 g 0.004 mol) were taken and added DCC (0.005 mol, 1 g) dissolved in 250 mL of dry $CHCl_3$ and stirred for half an hour. White crystals of urea were filtered off and chloroform was removed under reduced pressure. Residue was dissolved in ethyl acetate, filtered and solvent was evaporated under reduced pressure. The resulting product was purified by column chromatography. The purity of the product was checked with TLC in 10% methanol in chloroform.

4.1.1. (1r,2r,5s)-2-Isopropyl-5-methylcy-clohexyl-4-(2-methoxyphenylamino)-4-oxo-butanoate (1)

Yield 88%, $[\alpha]^{23}_D = -5.94$ (Chloroform, Conc. = 10 mg/20 mL). FTIR: 3337 (NH Str), 2928 (CH Ar), 2855 (CH aliphatic), 1725 (CO ester), 1696 cm^{-1} (CO amide), ^{1}H-NMR (300 MHz,$CDCl_3$): δ 0.76 (d, 3H, J = 9 Hz), 0.91 (dt, 6H, J_3= 6 Hz, J_5 = 3 Hz), 1.48 (m, 6H), 1.69 (m, 1H), 1.88 (m, 1H), 1.99 (m, 1H), 2.71 (t, 2H, J = 6 Hz), 2.31 (t, 2H, J = 6 Hz,), 3.89 (s, 3H), 4.70 (dt, 1H, J_3 = 9 Hz, J_5 = 3 Hz) 6.86 - 7.07 (m, 5H), ^{13}C-NMR (75 MHz, $CDCl_3$): 173.2, 172.4, 119.7 - 154.1, 74.8, 55.6, 46.9, 40.8, 34.2, 32.6, 29.9, 28.9, 26.2, 22.7, 22.0, 16.3; Anal. Calcd for $C_{21}H_{31}NO_4$ (361.48) C, 69.78; H, 8.64; N, 3.87; O, 17.70; Found: C, 69.93; H, 8.71; N, 3.75; O, 17.61.

4.1.2. (1R,2R,5S)-2-Isopropyl-5-methylcy-clohexyl-4-(3-methoxyphenylamino)-4-oxo-buta noate (2)

Yield 81%, $[\alpha]^{23}_D = +0.16$ (Chloroform, Conc. = 10 mg/20 mL). FTIR: 3324 (NH Str), 2927 (CH Ar), 2854 (CH aliphatic), 1701 (CO ester), 1654 cm^{-1} (CO amide), ^{1}H-NMR (300 MHz,$CDCl_3$): δ 0.76 (d, 3H, J = 9 Hz), 0.91 (dt, 6H, J_3 = 6 Hz, J_5 = 3Hz), 1.48 (m, 6H), 1.69 (m, 1H), 1.88 (m, 1H), 1.99 (m, 1H), 2.66 (t, 2H, J = 6 Hz), 2.73 (t, 2H, J = 6 Hz), 3.81 (s, 3H), 4.69 (dt, 1H, J_3 = 9Hz, J_5 = 3 Hz), 7.12 - 7.57 (m, 5H), ^{13}C-NMR (75 MHz,

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$CDCl_3$): 173.2, 172.4, 115.6 - 164.9, 74.8, 55.6, 46.9, 40.8, 34.2, 32.6, 29.9, 28.9, 26.2, 22.7, 22.0, 16.3; Anal. Calcd for $C_{21}H_{31}NO_4$ (361.48) C, 69.78; H, 8.64; N, 3.87; O, 17.70; Found: C, 69.93; H, 8.71; N, 3.75; O, 17.61.

4.1.3. (1R,2R,5S)-2-Isopropyl-5-methylcy-clohexyl-4-(4-methoxyphenylamino)-4-oxo-butanoate (3)

Grey crystalline solid. Yield 91%, m.p = 141˚C, $[\alpha]^{23}_D$ = –1.69 (Chloroform, Conc. = 10 mg/20 mL). FTIR: 3301 (NH Str), 2919 (CH Ar), 2867 (CH aliphatic), 1724 cm (CO ester), 1655 cm^{-1} (CO amide), ^{1}H NMR (300 MHz,$CDCl_3$): δ 0.76 (d, 3H, J = 9 Hz,), 0.91 (dt, 6H, J = 6 Hz, J = 3 Hz), 1.48 (m, 1H), 1.69 (m, 1H), 1.88 (m, 1H), 1.99 (m, 1H), 2.65 (t, 2H, J = 6 Hz), 2.76 (t, 2H, J = 6 Hz), 3.80 (s, 3H), 4.74 (dt, 1H, J = 9 Hz, J = 3 Hz), 6.84 - 7.44 (m, 5H), ^{13}C-NMR (75 MHz, $CDCl_3$): 173.2, 172.4, 114.1 - 121.6, 74.8, 55.5 , 46.9, 40.8, 34.2, 32.6, 30.1, 28.9, 26.2, 22.7, 22.0, 16.3; Anal. Calcd for $C_{21}H_{31}NO_4$ (361.48) C, 69.78; H, 8.64; N, 3.87; O, 17.70; Found: C, 69.93; H, 8.71; N, 3.75; O, 17.61.

4.1.4. (1R,2R,5S)-2-Isopropyl-5-methylcy-clohexyl-4-oxo-4-(2-tolylamino)butanoate (4)

Yield 87%, $[\alpha]^{23}_D$ = –7.43 (Chloroform, Conc. = 10 mg/20 mL). FTIR: 3354 (NH Str), 2922 (CH Ar), 2852 (CH aliphatic), 1725 (CO ester), 1680 cm^{-1} (CO amide), ^{1}H-NMR (300 MHz,$CDCl_3$): δ 0.76 (d, 3H, J = 9 Hz), 0.91 (dt, 6H, J3 = 6 Hz, J = 3 Hz), 1.48 (m, 6H), 1.59 (s, 3H), 1.66 (t, 2H, J = 6 Hz), 1.69 (m, 1H), 1.78 (t, 2H, J = 6 Hz), 1.88 (m, 1H), 1.99 (m, 1H), 4.24 (dt, 1H, J = 9 Hz, J = 3 Hz), 7.28 - 7.74 (m, 5H), ^{13}C-NMR (75 MHz, $CDCl_3$): 172.9, 169.7, 119.8 - 135.3, 74.9, 46.9, 40.8, 34.2, 32.4, 30.1, 28.9, 26.2, 22.7, 22.0, 20.9, 16.3; Anal. Calcd for $C_{21}H_{31}NO_3$ (345.48) C, 73.01; H, 9.04; N, 4.05; O, 13.89; Found: C, 73.16; H, 9.25; N, 3.81; O, 13.78.

4.1.5. (1R,2R,5S)-2-Isopropyl-5-methylcy-clohexyl-4-oxo-4-(3-tolylamino)butanoate (5)

Yield 87%, $[\alpha]^{23}_D$ = –7.43 (Chloroform, Conc = 10 mg/20 mL). FTIR: 3345 (NH Str), 2925 (CH Ar), 2855 (CH aliphatic), 1723 (CO ester), 1649 cm^{-1} (CO amide), ^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.76 (d, 3H, J = 9 Hz), 0.91 (dt, 6H, J = 6 Hz, J = 3 Hz), 1.48 (m, 6H), 1.69 (m, 1H), 1.88 (m, 1H), 1.97 (s, 3H), 1.99 (m, 1H), 2.19 (t, 2H, J = 6 Hz), 2.34 (t, 2H, J = 6 Hz), 4.24 (dt, 1H, J = 9 Hz, J = 3 Hz), 7.26 - 7.73 (m, 5H), ^{13}C-NMR (75 MHz, $CDCl_3$): 172.9, 169.7, 119.8 - 135.3, 74.9, 46.9, 40.8, 34.2, 32.4, 30.1, 28.9, 26.2, 22.7, 22.0, 20.9, 16.3.

4.1.6. (1R,2R,5S)-2-Isopropyl-5-methylcy-clohexyl-4-oxo-4-(4-tolylamino)butanoate (6)

White crystalline solid. Yield 96%, m.p = 76˚C, $[\alpha]^{23}_D$ = –3.47 (Chloroform, Conc = 10 mg/20 mL). FTIR: 3328 (NH Str), 2919 (CH Ar), 2865 (CH aliphatic), 1728 (CO ester), 1660 cm^{-1} (CO amide), ^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.76 (d, 3H, J = 9 Hz), 0.91 (dt, 6H, J = 6 Hz, J = 3 Hz), 1.48 (m, 6H), 1.69 (m, 1H), 1.88 (m, 1H), 1.99 (m, 1H), 2.32 (s, 3H), 2.66 (t, 2H, J = 6 Hz), 2.76 (t, 2H, J = 6 Hz), 4.74 (dt, 1H, J = 9 Hz, J = 3 Hz), 7.11 - 7.41 (m, 5H), ^{13}C-NMR (75 MHz, $CDCl_3$): 172.9, 169.7, 119.8 - 135.3, 74.9, 46.9, 40.8, 34.2, 32.4, 30.1, 28.9, 26.2, 22.7, 22.0, 20.9, 16.3.

4.2. Material and Method

4.2.1. Assay for Antifungal Activity

The agar tube dilution method is used for determination of antifungal activity [22]. Fungal strains *Aspergillus fumigatus*, *Fusarium moniliforme* and *Helminthosporium sativum* were used in this study. Each fungal strain was maintained on Sabouraud's dextrose agar (Oxoid) medium at 4˚C.

The samples for antifungal assay were prepared from initial stock solution of 0.12 g of compound in 1 mL of dimethyl sulfoxide (DMSO). Culture media was prepared by dissolving 6.5 g of Sabouraud dextrose agar per 100 mL of distilled water pH was adjusted at 5.6. Test tubes were marked to the 10 mL mark. The Sabouraud's dextrose agar (Oxoid) dispensed as 10 mL volume into screw capped tubes or cotton plugged test tubes and were autoclaved at 121˚C for 21 minutes. Tubes were allowed to cool to 50˚C and Sabouraud's dextrose agar (Oxoid) agar was loaded with 67 µL of compound pipette from the stock solution. This would give the final concentration of 200 µg/mL of the pure compound in media. Tubes were then allowed to solidify in slanting position at room temperature. Three slants of the compound sample were prepared for each fungus species. The tubes containing solidified media and sample compound were inoculated with 4 mm diameter piece of inoculums, taken from a seven days old culture of fungus. One sample of each compound was prepared, which was used for positive control. Slants without compound were used for negative control.

The test tubes were incubated at 28˚C for 7 days. Cultures were examined twice weekly during the incubation. Reading was taken by measuring the linear length of fungus in slant by measuring growth (mm) and growth inhibition was calculated with reference to negative control and all tests were carried out in triplicate.

4.2.2. Assay for Antibacterial Activity

Antibacterial activity of the methanolic solution of selected compounds was determined by agar well diffusion method [23]. Nutrient broth medium was prepared

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by dissolving 0.4 g of nutrient broth in 50 mL of distilled water. pH was adjusted at 7.0 and was sterilized by autoclaving. Nutrient agar medium was prepared by dissolving 2.3 g agar in 100 mL of distilled water; pH was adjusted at 7.0 and was autoclaved at 121˚C. Four strains of bacteria *Staphylococcus aureus*, *Pseudomonas aurignosa*, *Bacillus subtilis* and *Klebsiella pneumonia* were used in the study. The organisms were maintained on nutrient agar medium at 4˚C. Bacterial pallets obtained after centrifugation of 24 h old culture in nutrient broth of selected bacterial strains were mixed with physiological normal saline solution until a McFarland turbidity standard [106 colony forming unit (CFU) mL^{-1}] was obtained. Then this inoculum was used for seeding the nutrient agar.

Nutrient agar medium was prepared by adding nutriaent agar 2.3 g in 100 mL of distilled water, pH was adjusted at 7.0, and was autoclaved. It was allowed to cool to 45˚C. Petri plates were prepared by pouring 75 mL of seeded nutrient agar and allowed to solidify. Four wells per plate were made with sterile cork borer (5 mm).

Using micropipette, 100 µL of test solutions was poured in respective wells. These plates were incubated at 37˚C. After 24 h of incubation the diameter of the clear zones of inhibitions was measured by a ruler. Antibacterial activity of three dilutions of each compound was determined against three bacterial strains all tests were carried out in triplicate.

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