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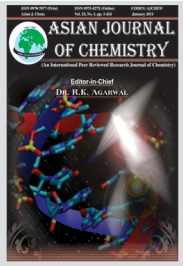

Synthesis and Biocidal Effect of New Steroidal Heterocyclic Compounds

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The preparation of new steroidal heterocyclic compounds derived from testosterone and pregnenalone was described. The structure of new products was elucidated by spectroscopic techniques. The products were examined for their biocidal effect against bacteria and fungi and their results compared with standard antibiotics.

Key Words: Steroidal heterocyclic, Biocidal effect, Standard antibiotics.

INTRODUCTION

It is well known from the literature that both steroids and heterocyclic compounds have medicinal and biological activities[1]. They are also used as drugs, antibacterial and antiviral agents. The presence of heterocycles in all kinds of organic compounds of interest in biology, pharmacology, optics, electronics and material sciences[2]. Among them sulfur and nitrogen-containing heterocyclic compounds have maintained the interest of researchers through decades of historical development of organic synthesis. The ground of this interest was due to their biological activity and unique structure that led to several applications in different areas of pharmaceutical and agrochemical research[3]. On the other hand steroids have great effects as antiinflammatory, antibacterial and antiviral drugs[4].

Nonsteroidal antiinflammatory drugs (NSAIDs) are an inhomogeneous family of pharmacologically active compounds which are widely used in treatment of acute and chronic inflammation, pain and fever, However, long-term clinical employment of NSAIDs is associated with significant side effects. Therefore, the discovery of new safer drugs is a challenging goal for research[5]. Attention has been devoted in the literature to synthesis of several steroidal heterocyclic derivatives that exhibit marked medicinal activities[6-8]. In recent years, heterocyclic pregnane derivatives have been found to possess a variety of interesting pharmacological and biological activities[9]. Recently, reactivity of some steroidal hormones towards lawessons's reagent as sulfur moiety produced acyclic and cyclic sulfur compounds containing a steroidal unit which have an antibacterial and antifungal activities[10]. These observations led us to attempt to make new heterocyclic systems bearing steroidal moieties.

EXPERIMENTAL

Melting points were determined by Thomas-Hoover capillary melting point apparatus and are uncorrected. IR spectra were recorded using KBr disks on a Nicolet Magna 520 Fourier transform spectrometer. ^{1}H NMR spectra were determined in $CDCl_3$ with TMS as an internal standard reference at 600 MHz on a Brucker Avance DPX 600 spectrometer while ^{13}C NMR spectra were recorded in deuteriochlo-roform at 150 MHz with a Brucker Avance DPX 400 spectrometer. Mass spectra were recorded on a VG Autospec. Micro-analysis were carried out using Perkin-Elmer.

3β-Hydroxypregn-5-en-20-dithioicformic acid hydrazone (II): Equimolar amounts of dithioic formic acid hydrazide (0.85 g, 10.4 mmol) and 3β-hydroxypregn-5-en-20-one (**I**) (3 g, 9.5 mmol) in DMF (20 mL) was refluxed for 2 h, cooled then poured onto ice. The solid product was filtered off to afford 3β-hydroxypregn-5-en-20-dithioicformic acid hydrazone (3.1 g, 80 %) (**II**) which was crystallized from DMF as needles, m.p. 203-205 °C, HRMS calculated for $C_{22}H_{34}N_2OS_2$ 406.2113 found 406.2118; FTIR ν_{max}/cm^{-1}: 3412 (OH), 3386 (NH) and 1639 (C=N); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.54 (3H, s, 18-H), 1.01 (3H, s, 19-H), 1.7 (3H, s, 21-H) 3.53 (1H, tt, J = 11.2 and 5.6Hz, 3α-H), 4.35, (1H, brs, NH) 5.3 (1H, s, 6-H).

3β-Hydroxypregn-5-en-20-(1,2,3,4-tetrahydro-2-thioxo-3-iminothiazoldine-4-one) (III): A mixture of chloroacetic acid (0.01 mol), 3β-hydroxypregn-5-en-20-dithioic formic acid hydrazone (**II**) (0.01 mmol) and anhydrous sodium acetate (5 g) in ethanol (50 mL) was refluxed for 4 h, cooled and then poured onto ice. The solid product was filtered off to give 3β-hydroxypregn-5-en-20-(1,2,3,4-tetrahydro-2-thioxo-3-iminothiazoldine-4-one (**III**) (0.92 g, 83 %) which

was recrystallized from mthanol as white plates; m.p. 215-218 °C; HRMS calculated for $C_{24}H_{34}N_2O_2S_2$ 446.2062 found 446.2053; FTIR ν_{max}/cm^{-1}: 3505 (OH), 1702 (C=O) and 1643 (C=N); ^1H NMR ($CDCl_3$, 400 MHz) δ 0.81 (3H, s, 18-H), 1.12 (3H, s, 19-H), 1.7 (3H, s, 21-H), 3.62(1H, tt, *J* = 11.2 and 5.6 Hz, 3α-H), 3.56 (2H, s, S-CH_2-CO) 5.3 (1H, s, 6-H)

3β-Hydroxypregn-5-en-20-(1,2,3,4-tetrahydro-2-thioxo-3-iminothiazolidin-5-one) (IV): Chloroacetyl chloride (0.01 mol) was added dropwise to a solution of 3β-hydroxypregn-5-en-20-dithioc formic acid hydrazone (**II**) (0.01 mol) in 50 mL DMF. The mixture was warmed for 2 h, cooled and then poured onto ice. The solid product was filtered off to give 3β-hydroxypregn-5-en-20-(1,2,3,4-tetrahydro-2-thioxo-3-thioxo-3-iminothiazolidin-5-one (**IV**) (0.83 g, 82 %) which was recrystallized from ethanol as white plates; m.p. 224-227 °C; HRMS calculated for $C_{24}H_{34}N_2O_2S_2$: 446.2062 found 446.2071; FTIR ν_{max}/cm^{-1}: 3512 (OH), 1711(C=O) and 1670 (C=N); ^1H NMR ($CDCl_3$, 400 MHz) δ 0.83 (3H, s, 18-H), 1.11 (3H, s, 19-H), 1.62 (3H, s, 21-H), 3.64 (1H, tt, *J* = 11.2 and 5.6 Hz, 3α-H), 3.81 (2H, s, N-CH_2-CO), 5.37 (1H, s, 6-H).

3β-Hydroxypregn-5-en-20-(hexahydro-2-thioxo-3-imino-1,3-thiazin-4,6-dione) (V): Equimolar amounts of 3β-hydroxypregn-5-en-20-dithioic formic acid hydrazone (**II**) (0.01 mol) and malonic acid in glacial acetic acid (50 mL) was refluxed for 4 h, cooled then poured onto ice. The solid product was filtered off to give 3β-hydroxypregn-5-en-20-(hexahydro-2-thioxo -3-imino-1,3-thiazin-4,6-dione (**V**) (0.97 g, 83 %) which was recrystallized from ethyl acetate as yellow prisms; m.p. 194-197 °C; HRMS calculated for $C_{25}H_{34}N_2O_3S_2$: 444.2011 found 474.2005; FTIR ν_{max}/cm^{-1}: 3491 (OH), 1703 and 1711 (2C=O) and 1653 (C=N); ^1H NMR ($CDCl_3$, 400 MHz) δ 0.79 (3H, s, 18-H), 1.03 (3H, s, 19-H), 1.56 (3H, s, 21-H),3.59 (1H, tt, *J* = 11.2 and 5.6 Hz, 3α-H), 3.41 (2H, s, OC-CH_2-CO), 5.53 (1H, s, 6-H).

3β-Hydroxypregn-5-en-20-thiocarbohydrazone (VI): A mixture of thiocarbohydrazide (0.84 g, 7.9 mmol) in dil. HCl, 10 %, 20 mL and 3β-hydroxypregn-5-en-20-one (**I**) (3 g, 9.5 mmol) in 20 mL ethanol) was refluxed for 2 h. The reaction mixture was cooled and poured onto ice then neutralized with aqueous Na_2CO_3. The solid product was filtered off to give 3β-hydroxypregn-5-en-20-thiocarbohydrazone (**VI**) (3.2 g, 88 %) which was crystallized from ethanol as white prisms; m.p. 240-242 °C; HRMS calculated for $C_{22}H_{36}N_4OS$ 404.2610 found 404.2605; FTIR ν_{max}/cm^{-1}: 3422 (OH), 3358, 3223 (NH_2) 1601 (C=N); ^1H NMR ($CDCl_3$, 400 MHz) δ 0.55 (3H, s, 18-H), 0.98 (3H, s, 19-H), 1.8 (3H, s, 21-H), 3.53 (1H, tt, *J* = 11.2 and 5.6 Hz, 3α-H), 4.52 (2H, brs, NH_2), 5.53 (1H, s, 6-H).

3β-Hydroxypregn-5-en-20-(4-amino-1,2,3,4-tetrahydro-2-thioxo-3-thioxo-1,2,4-triazin-5-one) (VII): A mixture of chloroacetic acid (0.01 mol), 3β-hydroxypregn-5-en-20-thiocarbohydrazone (**VI**) (0.01 mmol) and anhydrous sodium acetate (5 g) in ethanol (50 mL) was refluxed for 4 h, cooled and then poured onto ice. The solid product was filtered off to give. 3β-Hydroxypregn-5-en-20-(4-amino-1,2,3,4-tetrahydro-2-thioxo-3-thioxo-1,2,4-triazin-5-one (**VII**) (0.77 g, 70 %) which was recrystallized from methanol as reddish needles; m.p. 210-213 °C; HRMS calculated for $C_{24}H_{36}N_4O_2S$ 444.2559 found 444.2547; FTIR ν_{max}/cm^{-1}: 3524 (OH), 1715 (C=O) and 1615 (C=N); 1H NMR ($CDCl_3$, 400 MHz) δ 0.91 (3H, s, 18-H), 1.08 (3H, s, 19-H), 1.59 (3H, s, 21-H), 3.59 (1H, tt, *J* = 11.2 and 5.6 Hz, 3α-H), 3.65 (2H, s, HN-CH_2-CO), 5.37 (1H, s, 6-H).

3β-Hydroxypregn-5-en-20-(4-imino-1,2,3,4-tetrahydro-3-thioxo-1,2,4-triazin-6-one) (VIII): Chloroacetyl chloride (0.01 mol) was added dropwise to a solution of 3β-hydroxypregn-5-en-20-thiocarbohydrazone (**VI**) (0.01 mol) in DMF 50 mL. The mixture was warmed for 2 h, cooled and then poured onto ice. The solid product was filtered off to give 3β-hydroxypregn-5-en-20-(4-imino-1,2,3,4-tetrahydro-3-thioxo-1,2,4-triazin-6-one (**VIII**) (0.75 g, 68 %) which was recrystallized from ethanol as white needles; m.p. 174-177 °C; HRMS calculated for $C_{24}H_{36}N_4O_2S$ 444.2559 found 444.2542; FTIR ν_{max}/cm^{-1}: 3519 (OH), 3345, 3419 (2NH), 1703 (C=O) and 1630 (C=N); ^1H NMR ($CDCl_3$, 400 MHz) δ 0.91 (3H, s, 18-H), 1.05 (3H, s, 19-H), 1.61 (3H, s, 21-H), 3.63 (1H, tt, *J* = 11.2 and 5.6 Hz, 3α-H), 4.16 (2H, s, N-CH_2-CO), 5.50 (1H, s, 6-H).

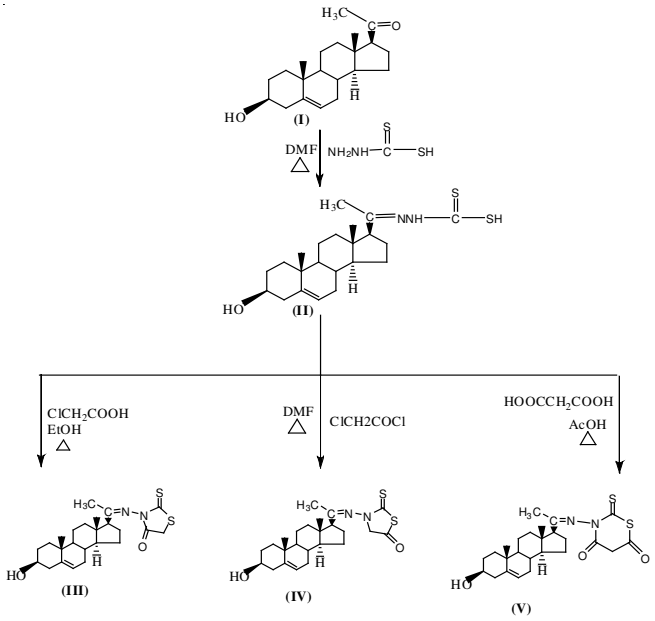
3β-Hydroxypregn-5-en-20-(4-imino-1,2,3.4-tetrahydro-1,2,4-triazol-2-dithione) (IX): Carbon disulfide (0.02 mol) was added dropwise to a solution of 3β-hydroxypregn-5-en-20-thiocarbohydrazone (**VI**) (0.01 mol) in DMF, 50 mL the mixture was then refluxed for 4 h, cooled and poured onto ice. The solid product was filtered off to give 3β-hydroxypregn-5-en-20-(4-imino-1,2,3.4-tetrahydro-1,2,4-triazol-2-dithione (**IX**) (0.68 g, 61 %) which was recrystallized from methanol as red needles; m.p. 189-188 °C; HRMS calculated for $C_{23}H_{34}N_4OS_2$: 446.2174 found 446.2188; FTIR ν_{max}/cm^{-1}: 3510 (OH), 3291, 3276 (2NH) and 1663 (C=N); ^1H NMR ($CDCl_3$, 400 MHz) δ 0.91 (3H, s, 18-H), 1.15 (3H, s, 19-H), 1.62 (3H, s, 21-H), 3.58 (1H, tt, *J* = 11.2 and 5.6 Hz, 3α-H), 3.84, (2H, brs, NH), 5.54 (1H, s, 6-H).

RESULTS AND DISCUSSION

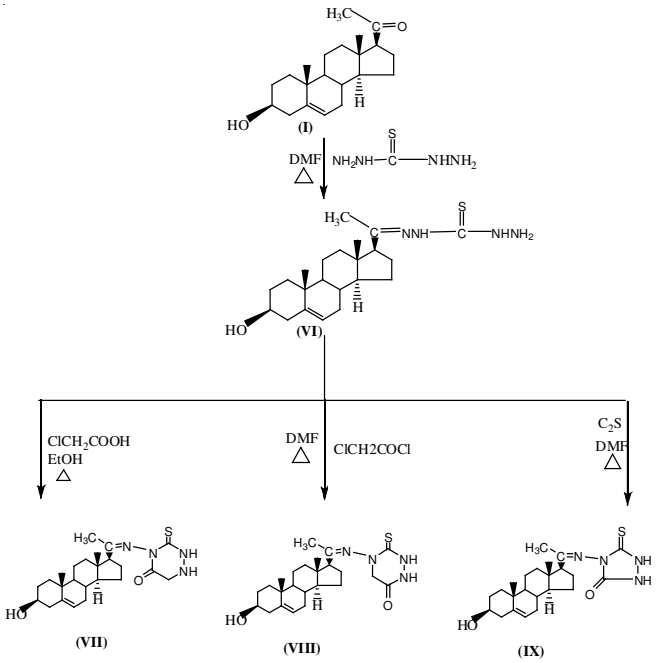
Pregnenalone (**I**) was refluxed with dithioic formic acid hydrazide in DMF to yield 3β-hydroxypregn-5-en-20-dithiocformic acid hydrazone (**II**). The ^1H NMR spectrum of the product (**II**) contained a signal at δ 4.35, (1H, brs, NH) which was not present in the spectrum of the starting material. The IR spectrum showed a new signal at 1639 cm^{-1} (C=N) instead of the carbonyl signal at 1702 cm^{-1}. The ^{13}C NMR revealed two new signals at δ 171.2 and δ 207.8 which confirmed the presence of (C=N) and (C=S) groups. This suggested that the compound was 3β-hydroxypregn-5-en-20-dithioc formic acid hydrazone (**II**) (**Scheme-I**).

3β-Hydroxypregn-5-en-20-dithioic formic acid hydrazone (**II**) was refluxed with chloroacetic acid to give 3β-hydroxypregn-5-en-20-(1,2,3,4-tetrahydro-2-thioxo-3-iminothiazoldine-4-one (**III**). The IR spectrum contained a new band at 1702 cm^{-1} (C=O). The ^1H NMR spectrum of the product contained a new signal at 3.86 (2H, S, S-CH_2-CO). The ^{13}C NMR revealed two new signals at δ 44.9 and δ 180.1 which were confirmed the presence of (CH_2) and (C=O) groups. These results confirmed that the product was 3β-hydroxypregn-5-en-20-(1,2,3,4-tetrahydro-2-thioxo-3-iminothiazoldine-4-one (**III**) (**Scheme-I**).

Chloroacetyl chloride was refluxed with 3β-hydroxypregn-5-en-20-dithioc formic acid hydrazone (**II**) to afford



Scheme-I



Scheme-II

3β-hydroxypregn-5-en-20-(1,2,3,4-tetrahydro-2-thioxo-3-iminothiazolidin-5-one (**IV**). The IR spectrum contained a new signal at 1711 cm^{-1} (C=O). The ^1H NMR spectrum of the product contained a new signal at 3.81 (2H, s, N-CH_2-CO). The ^{13}C NMR reviled two new signals at δ 69.9 and δ 199.6 which were confirmed the presence of (CH_2) and (C=O) groups. These results confirmed the structure of the product (**IV**) (**Scheme-I**).

3β-Hydroxypregn-5-en-20-dithioic formic acid hydrazone (**II**) was refluxed with malonic acid to yield 3β-hydroxypregn-5-en-20-(hexahydro-2-thioxo-3-imino-1,3-thiazin-4,6-dione (**V**). The ^1H NMR spectrum of the product contained a new signal at 3.41(2H, s, OC-CH_2-CO). The ^{13}C NMR revealed three new signals at δ 52.9, δ 173.5 and 178.1 which confirmed the presence of (CH_2) and 2(C=O) groups. The IR spectrum showed two new signals at 1703and 1711 cm^{-1} (2C=O). These results confirmed the structure of the product (**V**) (**Scheme-I**).

Pregnenalone (**I**) was refluxed with thiocarbohydrazide in hydrochloric acid and ethanol to give 3β-hydroxypregn-5-en-20-thiocarbohydrazone (**VI**). The IR spectrum showed a new signal at 1601 cm^{-1} (C=N) instead of the carbonyl signal at 1702 cm^{-1}. The ^1H NMR spectrum contained a signal at δ 4.52 (2H, brs) assigned for NH_2 group. The ^{13}C NMR gave new signals at δ 167.2 and δ 195.8 which were assigned to groups (C=N) and (C=S). These results confirmed the structure of the product (**Scheme-II**).

3β-Hydroxypregn-5-en-20- thiocarbohydrazone (**VI**) was refluxed with chloroacetic acid to give 3β-hydroxypregn-5-en-20-(4-amino-1,2,3,4-tetrahydro-2-thioxo-3-thioxo-1,2,4-triazin-5-one (**VII**). The IR spectrum contained a new signal at 1715 cm^{-1} (C=O). The ^1H NMR spectrum of the product contained a new signal at 3.65 (2H, s, HN-CH_2-CO). The ^{13}C NMR reviled two new signals at δ 60.2 and δ 177.1 which were confirmed the presence of (CH_2) and (C=O) groups. These results confirmed that the product was 3β-hydroxypregn-5-en-20-(4-amino-1,2,3,4-tetrahydro-2-thioxo-1,2,4-triazin-5-one (**VII**) (**Scheme-II**).

Chloroacetyl chloride was refluxed with 3β-hydroxypregn-5-en-20-thiocarbohydrazone (**VIII**) to give 3β-hydroxypregn-5-en-20-(4-imino-1,2,3,4-tetrahydro-3-thioxo-1,2,4-triazin-6-one (**VIII**). The IR spectrum showed two new peaks at 3345 and 3419 cm^{-1} (NH_2), 1703 cm^{-1} (C=O). The ^1H NMR spectrum of the product contained a new signal at 4.61 (2H, s, N-CH_2-CO). The ^{13}C NMR revealed two new signals at δ 66.9 and δ 180.1 which confirmed the presence of (CH_2) and (C=O) groups. These results confirmed the structure of the product (**VIII**) (**Scheme-II**).

3β-Hydroxypregn-5-en-20-thiocarbohydrazone (**VI**) was refluxed with carbon disulfide to afford 3β-hydroxypregn-5-en-20-(4-imino-1,2,3.4-tetrahydro-1,2,4-triazol-2-dithione (**IX**). The IR spectrum showed two new signals at 3291 and 3276 cm^{-1} (2NH). The ^1H NMR spectrum of the product contained a new signal at 3.84 (2H, brs, NH). The ^{13}C NMR revealed a signal at δ 173.5 which confirmed the presence of (C=S) group. These results confirmed that the product was 3β-hydroxypregn-5-en-20-(4-imino-1,2,3.4-tetrahydro-1,2,4-triazol-2-dithione (**IX**) (**Scheme-II**).

Antimicrobial assay: Some new synthesized compounds were tested *in vitro* using the agar diffusion disk method[3,4].

The antimicrobial potentialities of the tested compounds were estimated by placing the pre-sterilized filter paper disks (6 mm in diameter) impregnated with 50 mg/disk. DMF which showed no inhibition zone, was used as solvent for dissolving the tested compounds. Inhibition zone (IZ) of the tested compounds (mm) were measured after 24-28 h incubation at 37 °C for bacteria and after 5 days incubation period at 28 °C for fungi (Table-1).

The minimal inhibitory concentration (MIC) (Tables 2 and 3) method of the biologically active compounds was applied using different concentrations per disks against bacteria and fungi using naliolixic acid and nystalin as reference drugs.

TABLE-1
PRELIMINARY SCREENING ANTIMICROBIAL ACTIVITY OF SOME SYNTHETIC COMPOUNDS

Compd.	Gram-positive bacteria		Gram-negative bacteria			Fungi	
	Bacillus subtilis	*Staphylococcus aureus*	*Escherichia coli*	*Pesudomonas aeuruginosa*	*Klebsiella pneumonia*	*Candida albicans* (Aucc 1720)	*Aspergillus funigatus* (Aucc 1924)
VII	16	20	14	18	20	10	10
VIII	15	22	14	20	24	12	6
IX	15	24	14	24	22	12	6
III	16	19	14	22	24	12	6
IV	18	20	14	20	22	12	8
V	20	22	14	22	24	12	10
Nalidixic acid, 30 mg/disk	32	30	30	12	22	6	6
Nystatin	6	6	6	6	10	10	32

Inhibition zones: ≥ 12 mm (highly active); 9-12 mm (moderately active); 6-9 mm (slightly active); 6 mm = not sensitive.

TABLE-2
MIC OF THE BIOLOGICALLY ACTIVE COMPOUNDS TOWARDS GRAM NEGATIVE BACTERIA

Compd.	Inhibition zone (mm)														
	Escherichia coli					*Pesudomonas aeuruginosa*					*Klebsiella pneumonia*				
	50	40	30	20	10	50	40	30	20	10	50	40	30	20	10
VII	14	12	10	8	6	18	15	11	8	6	20	15	12	8	6
VIII	14	11	10	8	6	20	16	12	8	6	24	15	11	8	6
IX	14	12	9	8	6	24	15	12	8	6	22	16	12	8	6
III	14	13	11	8	6	22	15	11	8	6	24	19	10	8	6
IV	14	14	10	8	6	20	15	11	8	6	22	18	10	6	6
V	14	13	10	8	6	22	15	12	8	6	20	16	10	6	6

Inhibition zones: ≥ 12 mm (highly active); 9-12 mm (moderately active); 7-9 mm (slightly active); 6 mm = not sensitive.

TABLE-3
MIC OF THE BIOLOGICALLY ACTIVE COMPOUNDS TOWARDS GRAM POSITIVE BACTERIA

Compd.	Inhibition zone (mm)									
	Bacillus subtilis					*Staphylococcus aureus*				
	50	40	30	20	10	50	40	30	20	10
VII	16	16	12	9	7	20	17	9	6	6
VIII	15	15	10	8	6	22	14	6	6	6
IX	15	15	12	8	6	24	14	8	6	6
III	16	16	12	9	6	19	13	9	6	6
IV	18	16	14	12	7	20	13	8	6	6
V	16	16	14	10	8	20	14	9	6	6

Inhibition zones: ≥ 12 mm (highly active); 9-12 mm (moderately active); 7-9 mm (slightly active); 6 mm = not sensitive.

Conclusion

From above results (Tables 1-3), it is concluded that: (i) The tested compounds were very active towards the tested microorganisms. (ii) The tested compounds were more effective than standard antibiotics at MIC evaluations. (iii) After using UV-visible light, the tested compounds showed a high effect especially towards Gram-ve bacteria (*Pseudomonas aeuruginosa*) and also fungi (*Candida albicans* and *Asperigllus funigatus*). (iv) Only compounds a_3 and a_4 showed a very highly effect than other compounds towards all the tested compounds. (v) All the tested compounds showed a high effect when compared with the antibiotic nystatin.

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