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Vol. 11(2), pp. 32-37, 15 January, 2017
DOI: 10.5897/AJPP2016.4401
Article Number: B2C5D0C62388
ISSN 1996-0816

http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Screening of some pyrazole derivatives as promising antileishmanial agent

Abdu Tuha[1], Adnan A. Bekhit[2,3] and Yimer Seid[1*]

[1]Department of Pharmacy, College of Medicine and Health Sciences, Wollo University, Dessie, Ethiopia.
[2]Department of Pharmaceutical Chemistry, School of Pharmacy, Addis Ababa University, Addis Ababa, Ethiopia.
[3]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Alexandria University, Alexandria 21521, Egypt.

Received 30 June, 2015; Accepted 2 August, 2015

Pyrazole derivatives (I-VII) were prepared in good yields using aldol condensation followed by cyclization and were characterized by elemental analysis, IR and ^{1}H NMR spectroscopy. *In vitro* antileishmanial activity test was conducted using Alamar blue reduction method. The test revealed that the synthesized compounds (except compound IIb) exhibit better antileishmanial activity than the standard drug miltefosine and lower antileishmanial activity (except compounds III and IIIb) compared to the standard drug amphotericin B deoxycholate. Compound IIIb, phenyl pyrazoline with propanoyl side chain, 1-(3-phenyl-5-(1-phenyl-3-p-tolyl-1H-pyrazol-4-yl)-4,5-dihydropyrazol-1-yl)propan-1-one, was found to be the most active (IC_{50}= 0.0112 µgml^{-1}) than the standards miltefosine (IC_{50} = 0.3±0.04 µgml^{-1}) and amphotericin B deoxycholate (IC_{50} = 0.2±0.02 µgml^{-1}) for *Leishmania donovani*. Compound III was found to be the most active (IC_{50} = 0.28±0.03 µgml^{-1}) and has comparable antileishmanial activity to the standard miltefosine (IC_{50} = 0.3±0.04 µgml^{-1}) and amphotericin B deoxycholate (IC_{50} = 0.2±0.02 µgml^{-1}) on *Leishmania aethiopica* amastigote.

Key words: Pyrazole derivative, biological screening, antileishmanial agent.

INTRODUCTION

Leishmaniasis is a group of vector-borne diseases caused by species of the genus *Leishmania,* a compulsory intra-cellular parasite of the mammalian host cell (dos Santos et al., 2011; Luiz et al., 2012). *Leishmania* parasites exist in two forms: amastigote in the mammalian host and a flagellated promastigote in the insect vector (dos Santos et al., 2011). Clinical manifestation of leishmaniasis occur in four major forms in humans: (i) visceral, the most severe and life-threatening form; (ii) cutaneous, originating as nodules and ulcers that may persist for years; (iii) diffuse cutaneous leishmaniasis, which is a long-lasting disease due to a deficient cellular-mediated immune response; and (iv) mucocutaneous, causing permanent lesions in the mouth, nose or genital mucosa (dos et al., 2011; Luiz et al., 2012; Sa´nchez-Moreno et al., 2012). This life-threatening disease that affects about 12 million people worldwide with 1.5 million to 2 million new cases of cutaneous leishmaniasis (CL) and 500,000 new cases of visceral leishmaniasis (VL) each year is endemic in the tropical and sub-tropical regions (Desjeux,

*Corresponding author. E-mail: yimer69@gmail.com.

1996). Endemic human leishmaniasis is reported in 88countries, majority of them are low-income countries (Desjeux, 1999). East Africa is one of the world's main endemic areas for VL, and over the last 20 years has gained dramatic increase in the number of VL cases, due to a complexity of factors (Reithinger et al., 2007). Several studies have convincingly shown that malnutrition, HIV and genetic susceptibility are individually responsible for VL (Bucheton et al., 2002). The epidemiological pattern of *Leishmania* species is changing, with a tendency to urbanization and geographic expansion. Despite the high worldwide prevalence, no vaccine for Leishmaniasis and complex vector control, few advances were made in the treatment of this disease ((dos et al., 2011; Marra et al., 2012).

The difficulty to control this parasitic disease remains a serious problem mainly due to the diversity of mammalian reservoirs (wild and domestic animals), species of vectors and *Leishmania* species (dos et al., 2011). Chemotherapy for leishmaniasis is generally ineffective mainly due to the emergence of drug-resistant strains and toxicity of the therapeutic agents (Marra et al., 2012) The pentavalent antimonials compounds, such as sodium stibogluconate (pentostan) and meglumine antimoniate (glucantime) are widely used as primary therapy, but they induce toxic side effects together with drug resistance (dos et al., 2011; Braga et al., 2007).

Amphotericin (AmBisome) is now the treatment of choice. Its failure in some cases to treat visceral leishmaniasis (*Leishmania donovani*) has been reported in Sundar (Sundar et al., 2007); but this may be related to host factors such as co-infection with HIV or tuberculosis rather than parasite resistance. Miltefosine (Impavido) is a new drug for visceral and cutaneous leishmaniasis. Paromomycin drug is thought to be an inexpensive and effective treatment for leishmaniasis (Mueller et al., 2007).

Pyrazole derivatives were found to possess various important biological activities, such as antibacterial (Samir et al., 2011; Nilesh and Manish, 2011), antiinflammatory (Adnan et al., 2008; Lingaiah et al., 2011), antioxidant (Ramesh and Chetan, 2011) ACE inhibitory (Marco et al., 2010), anti-cancer (Hai-Jun et al., 2010), MAO-B inhibitory (Nesrin et al., 2007), antidepressant (Mohamed et al., 2009), antiviral (Guiping et al., 2008), antimycobacterial (Ramaiyan et al., 2010; Daniele et al., 2008), antileishmanial (Bernardino et al., 2006; Naresh et al., 2006), and antimalarial (Katiyar et al., 2005; Cunico et al., 2006) activities.

These reports have been useful for biologist, chemists and pharmacists engaged in the development of new drugs and/or synthetic routes from pyrazoline derivatives. Pyrazoline derivatives were reported to possess significant *in vitro* anti-leishmanial activity (Bekhit et al., 2014). This has prompted the synthesis and investigation of safe, effective and cheap antileishmanial agent from pyrazoline derivatives containing phenyl or thiophenyl moiety in this research laboratory.

METHODOLOGY

^{1}H NMR spectra were recorded in Bruker Avance DMX400 FT-NMR spectrometer and IR spectra using Shimadzu 8400SP Spectro-photometer. For melting point and elemental analysis, Eelectro thermal IA9100 hot storage melting point apparatus and Perkin Elmer 2400 elemental analyzer were respectively used. Haemocytometer was used for counting leishmania parasites. Purity of the reaction products were checked by means of thin layer chromatography (TLC) using silica gel plate with fluorescent indicator, melting points, IR and ^{1}H NMR spectra.

Chemicals and reagents

Acetophenone, 2-acetylthiophene and hydrazine hydrate (Sigma Aldrich), ethanol, glacial acetic acid, propanoic acid, hydrochloric acid, KOH, absolute methanol, acetonitrile, chloroform, ethyl acetate, benzene, sodium citrate, distilled H_2O, dimethyl sulfoxide (BDH, England), alamar blue, RPMI 1640 were used throughout the experiments.

Parasite strain

L. donovani, a leishmanial parasite that causes visceral leishmaniasis in Africa and *L. aethiopica* the leading cause of cutaneous leishmaniasis in Ethiopia were used for the antileishmanial testing.

Standard drugs

Amphotericin B deoxycholate (Fungizone®, E R Squibb, UK) and miltifosine/hexadecylphosphocholine (A G Scientific, San Diego, CA, USA) were used as standard drugs in the determination of the antileishmanial activity of the synthesized compounds.

Synthesis of target compounds

The intermediate α, β unsaturated ketones (II and III) were synthesized by aldol condensation of 1-phenyl-3-p-tolyl-1H-pyrazole-4-carbaldehyde I with 2-acetylthiophene and acetophenone in alcoholic KOH. The target thienyl and phenyl pyrazolines (Figures 1 and 2) were synthesized by cyclization of the intermediate α, β unsaturated ketones (II and III) with hydrazine hydrate in ethanol or the appropriate aliphatic acid (Tuha et al., 2014)

Culture conditions

L. donovani and *L. aethiopica* were cultured in tissue flasks containing RPMI 1640 medium supplemented with 10% HIFCS and 100 IU penicillin and 100 μgml^{-1} streptomycin solution at 26°C (Tariku et al., 2010; Habtemariam, 2003; Seifert et al., 2010).

Stock solution and working concentration preparation

All the compounds tested (II, IIa, IIIa, IIb, III, IIIb, IIc) were dissolved in DMSO to a final concentration of 1 $mgml^{-1}$. Both test and standard solutions were serially diluted to appropriate

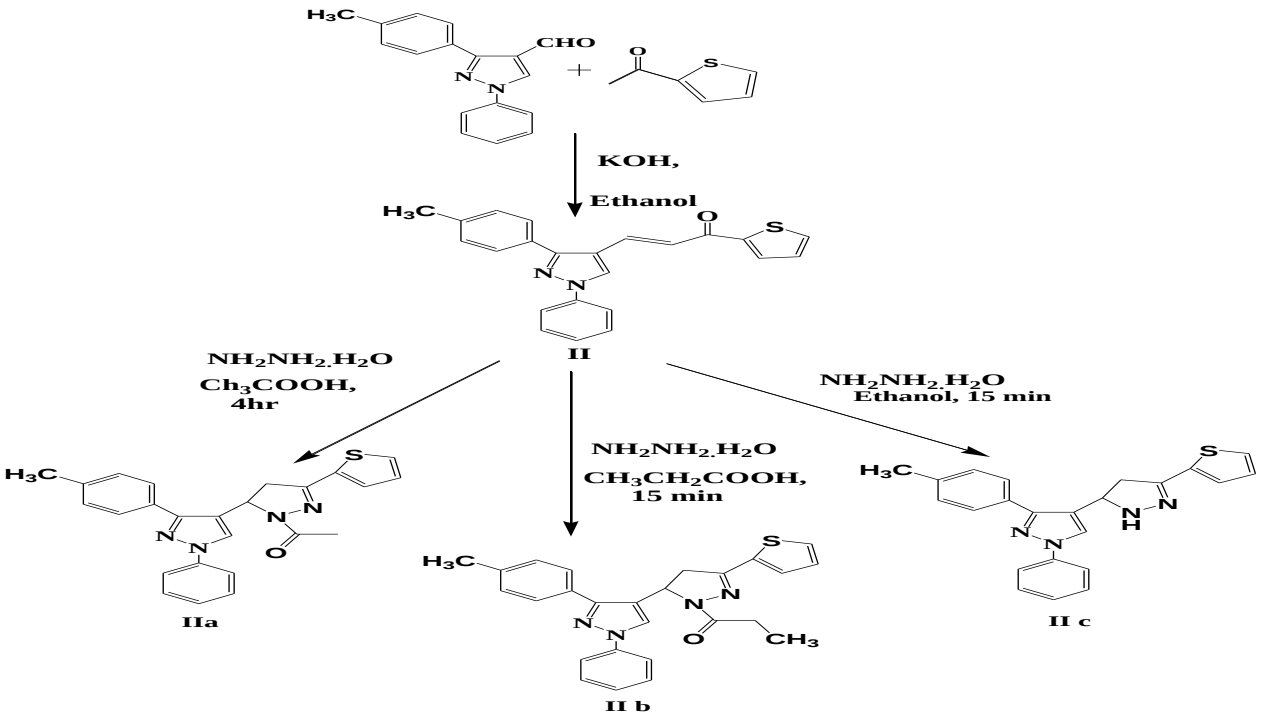

Figure 1. Scheme of synthesis of intermediate α, ß unsaturated ketone (II) and thienyl pyrazoline derivatives.

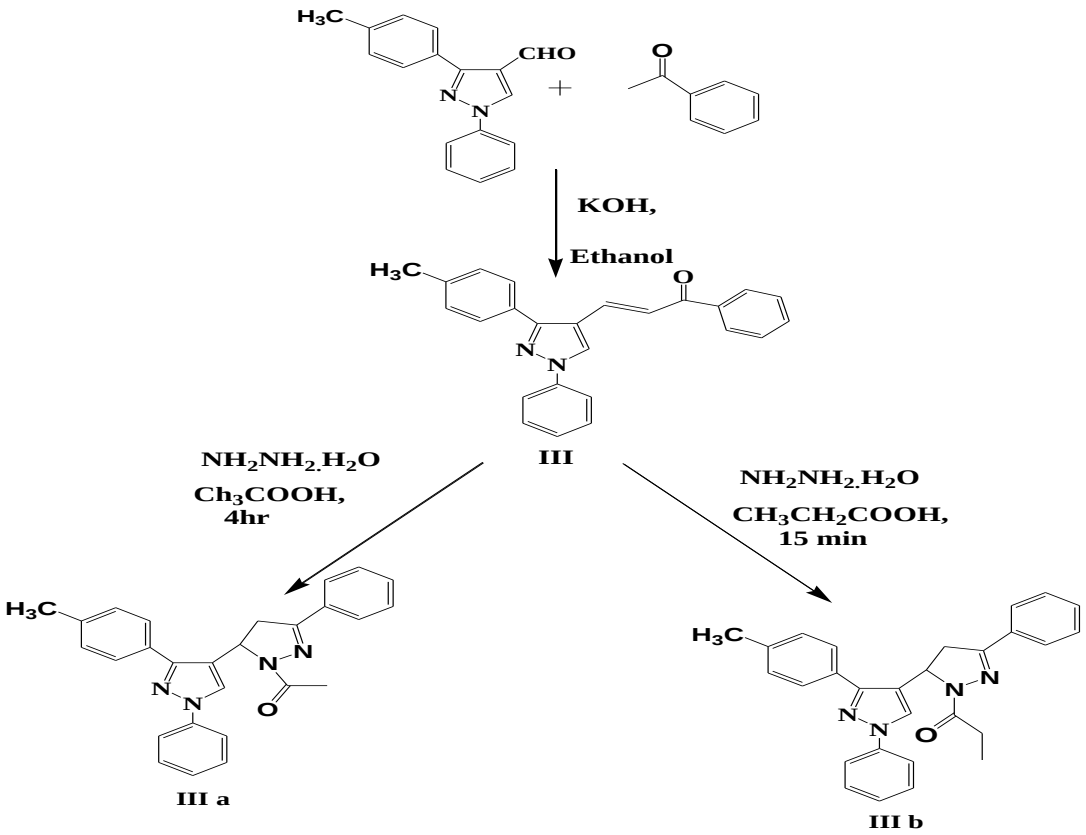

Figure 2. Scheme of synthesis of intermediate α, ß unsaturated ketone (III) and phenyl pyrazoline derivative.

Table 1. Antipromastigote and antiamastigote activity (IC_{50} µgml^{-1}) of the test compounds and reference standards.

Test compound	Activity IC_{50} (µgml^{-1})	
	Antipromastigote	Antiamastigote
Compound II	3.1143	1.84 ± 0.08
Compound IIa	2.0730	1.08 ± 0.14
Compound IIb	6.5310	1.29 ± 0.24
Compound IIc	0.1673	2.24 ± 0.34
Compound III	0.0422	0.28 ± 0.03
Compound IIIa	1.3076	2.861 ± 0.16
Compound IIIb	0.0112	4.22 ± 0.03
Miltefosine	3.1911	0.3 ± 0.04
Amphotericin B deoxycholate	0.0460	0.2 ± 0.02

IC_{50}: values indicate the effective concentration of a compound required to achieve 50 % growth inhibition in µg/ml.

concentrations using complete media. The test compounds were prepared by three fold serial dilutions from 10 µgml^{-1} to 0.04 µg ml^{-1}. Amphotericin B deoxycholate and miltefosine which were used as a positive control for comparison of the antileishmanial activities of the test compounds, were also made in three fold serial dilutions (Foroumadi et al., 2005)

Biological activity test

In vitro antipromastigote assay

Promastigote forms of *L. donovani* and standard drugs Amphotericin B deoxycholate and miltefosine were used for the assay. 3×10^6 promastigotes of *L. donovani* in 100 µl were seeded to each well in a 96 well flat bottom plate. Various dilutions (10, 3.33, 1.11, 0.37, 0.12, and 0.04 µgml^{-1}) of test compounds were added to the parasites. The tests were done in duplicates. Some of the wells contained only the standard drugs and served as a positive control. The media and DMSO alone were used as a negative control. The plates were kept at room temperature. After 24 h, 20 µl of Alamar blue (12.5 mg of resazurin dissolved in 100 mlof distilled water) (Yang et al., 2010) was added to each of the wells. Absorbance of the resulting mixture was measured after 48 h at a wavelength of 540 and 630 nm using Enzyme Linked Immuno Sorbent Assay (ELISA) plate reader (Al-Nasiry et al., 2007). A quantitative colorimetric assay using the oxidation-reduction indicator Alamar Blue was developed to measure cytotoxicity of the synthesized compounds against the protozoan parasite *Leishmania donovani*. The Alamar Blue assay permits a simple, reproducible and reliable method for screening antileishmanial drugs (Judith and Dietmar, 2001; Shimony and Jaffe, 2008; Nakayama et al., 1997).

***In vitro* antileishmanial activity on *L. aethiopica* amastigotes**

In a 96-well microtitre plate, test substances were serially diluted to final test concentrations of 0.04 to 10 µgml^{-1} in 50 µl culture medium and 50 µl suspensions of axenic amastigotes containing 2×10^7 cells/ml were added to each well. Contents of the plates were then incubated in humidified atmosphere containing 5% CO_2 at 31°C for 72 h. After 68 h of incubation, 10 µl of fluorochrome resazurin solution (12.5 µg dissolved in 100 ml of PBS, pH=7.2) was added into each well and the fluorescence intensity was measured after a total incubation time of 72 h using 37 Victor 3 Multilabel Counter at excitation wavelength of 530 nm and emission wavelength of 590 nm. The IC_{50} (µgml^{-1}) values for each extract were evaluated from sigmoidal dose-response curves using computer software Graph pad prism 3.0 and values expressed as mean + standard [SD] of triplicate experiments with each test concentration in duplicate. Assays with standard antileishmanial drugs and negative controls (medium alone and 1% DMSO) were also performed to have reference values. Also the background fluorescence intensity of each extract, essential oil and reference drug were measured (Habtemariam, 2003).

Data analysis

The IC_{50} values for synthesized compounds tested for their *in vitro* antileishmanial activity were evaluated from sigmoidal dose-response curves using non linear regression software (GraphPad Prism®; GraphPad Software, Inc., San Diego, CA).

RESULTS AND DISCUSSION

Biological assays

In vitro antipromastigote activity

The antipromastigote assay of the synthesized compounds was carried out according to the method described in the experimental part. The results obtained were analyzed and IC_{50} (µgml^{-1}) for each test compound was calculated using Graph pad prism software (Table 1). The result revealed that the synthesized compounds except compound IIb possess better antileishmanial activity than the standard drug miltefosine which has IC_{50} value 3.1911 µgml^{-1}. However, synthesized compounds except for compounds III and IIIb exhibited lower antileishmanial activity compared to the standard amphotericin B deoxycholate (IC_{50} = 0.0460 µgml^{-1}). Compound IIIb, the phenyl pyrazoline with propanoyl side chain, was found to be the most active (IC_{50} = 0.0112 µg ml^{-1}) compound as compared to the standard miltefosine (IC_{50} = 3.1911 µg ml^{-1}) and amphotericin B deoxycholate

(IC_{50} = 0.0460 µg ml^{-1}). Compared to study done by Vikramdeep et al. (2014) and Manuel et al. (2012), this research reveal that the phenyl pyrazoline derivative compound III and IIIb have better antileishmanial activity with IC_{50} value of 0.0422 and 0.0112 µgml^{-1}, respectively. This might be due to the formation of hydrogen bonding between its carbonyl group and backbone of certain receptor active site in the former compound III, and the presence of propanoyl group in the latter compound IIIb, may play a role in the interaction with vital important biochemical process. The thienyl pyrazoline derivative, 1-phenyl-4-(3-(thiophen-2-yl)-4,5-dihydro-1H-pyrazol-5-yl)-3-p-tolyl-1H-pyrazole (compound IIc) the non-substituted compound IIc (IC_{50} = 0.1673 µgml^{-1}) seems to affect positively the biological activity leading to the better antileishmanial activity when compared with ethanyol (CH_3CO-) compound IIa and propanoyl (CH_3CH2CO-) compound IIb substituted compound and study done by Pinheiro et al. (2012).

The phenyl derivative displayed better activity than the corresponding thienyl derivatives for leishmania activity. Regarding the thienyl derivatives of the pyrazolines, activity decreased with the increase in the carbon number of aliphatic substitution at pyrazoline N_1 from H to propanoyl group. However, the activity increased with increasing the length of the side chain in the phenyl pyrazolines. This could be attributed to the associated increase in hydrophobicity of the compounds that increases hydrophobic interaction with the molecular target site.

In vitro antiamastigote activity

The antiamastigote assay of the synthesized compounds, standard drug miltefosine and amphotericin B deoxycholate on *L. aethiopica* was carried out according to the method described in the experimental part. The results obtained were analyzed and IC_{50} for each test compound was calculated using Graph pad prism software (Table 1). The result showed that the synthesized compounds except for compounds III (IC_{50} = 0.28±0.03 µgml^{-1}), possess lower antileishmanial activity than the standard drug miltefosine and amphotericin B deoxycholate that have IC_{50} value 0.3±0.04 and 0.2±0.02 µgml^{-1}, respectively. Compound IIIb which exhibited the highest antipromastigote activity, has shown the least antiamastigote activity, while compound III has almost comparable activity with the standard drug miltefosine and amphotericin B deoxycholate.

Conclusion

Seven pyrazole derivatives were synthesized using aldol condensation and subsequent cyclization reactions in a good yield (71.39 to 95.20%). The compounds were purified with recrystallization method and were characterized by elemental microanalysis, IR, and ^1H NMR spectroscopy. *In vitro* antileishmanial activity was conducted using Alamar blue reduction method and the results revealed that synthesized compounds showed better antileishmanial activity than the standard drug miltefosine. But all the synthesized compounds except for compounds III and IIIb exhibited lower antileishmanial activity compared with the standard amphotericin B deoxycholate.

Moreover, the phenyl pyrazolines showed better antileishmanial activity compared with the thienyl pyrazolines and their activity increased with increased number of carbons in the side chain. Compound IIIb, 1-(3-phenyl-5-(1-phenyl-3-p-tolyl-1H-pyrazol-4-yl)-4,5-dihydropyrazol-1-yl)propan-1-one phenyl pyrazoline, is found to be the most active (IC_{50} = 0.0112 µgml^{-1}) and this compound could represent a fruitful matrix for the development of antileishmanial agents that would deserve further derivatization and investigation. Among seven synthesized compounds, compounds III is found to be the most active (IC_{50} = 0.28±0.03 µgml^{-1}) and has comparable antileishmanial activity to the standard miltefosine and amphotericin B deoxycholate *on L. aethiopica* amastigotes.

Conflict of Interests

The authors have not declared any conflict of interests.

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