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ANTI-LEISHMANIAL ACTIVITY INVESTIGATION OF SOME NEW QUINAZOLINONE DERIVATIVES

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration among all the three authors. All authors read and approved the final manuscript.

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ABSTRACT

In this study, some new 3-aryl, 2-substituted quinazoline-4 (3H) - one derivatives were synthesized using a three-step synthetic route. They were obtained in a good yield (59.5-85%) by applying different chemical reaction like cyclization and condensation reaction. The chemical structure of the final compounds was also verified by spectroscopic methods (IR, 1HNMR) and elemental microanalysis. The synthesized compounds (IVa, IVb, IVc, IVd, IVe and IVf) were screened for their in vitro anti-leishmanial activity against L. aethiopica isolate (CL/039/09). All tested compounds (IVa (0.03766 µg/ml), IVb (0.00538 µg/ml, IVc (0.00412 µg/ml, IVd (0.00110 µg/ml), IVe (0.03017 µg/ml) and IVf (0.03894 µg/ml)) showed excellent potency that is much better than the standard drug, amphotericin B (IC50 = 0.04359 ug/ml).
The results of acute toxicity indicated that all test compounds (IVa, IVb, IVc, IVd, IVe and IVf) proved to be non-toxic and well tolerated by the experimental animals up to 300 mg/kg in oral and 140 mg/kg in parental studies.

Keywords: Quinazolin-4(3H)-one derivative; *In vitro* antileishmanial activity; acute toxicity; three step synthetic rout.

1. INTRODUCTION

1.1 Leishmaniasis

Leishmaniasis is an illness caused by protozoon parasites that belong to the genus leishmania and is transmitted by the bite of certain species of sands fly (subfamily Phlebotominae). Although it is not a domestic name like malaria, the diseases caused by infection with Leishmania continue to have a major impact on important of the world's

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population and are presently considered to be an arising illness with high morbidity and mortality in the tropics and subtropics [1].

1.2 Clinical Manifestation of Leishmaniasis

Leishmaniasis is characterized by both diversity and complexity. Depending on strain (s) of the parasite involved in pathogenesis and the immune response established by the host it can cause clinical symptoms that range from mild self-limiting cutaneous lesion to fatal visceral disease [2,3].

a. Localized Cutaneous Leishmaniasis (LCL)

Cutaneous leishmaniasis is the most common form of leishmaniasis [2]. The disease is caused by *L. Tropica*, *L. major* and *L. aethiopica* in the Old World (Africa, Asia, Europe). *L. mexicana complex* and the *viannia* subgenus cause the disease in the New World (South America) [2,4]. In this disease, the patient generally presents with one or several ulcer (s) or nodule(s) in the skin (Fig.1a).

b. Diffuse Cutaneous Leishmaniasis (DCL)

The diffuse cutaneous leishmaniasis cause disseminated nodules, plates or lumps on the face arms and legs (Fig.1b). DCL due to *L. aethiopica* in Ethiopia, Kenya and Yemen never heal spontaneously and relapses after treatment [5]. *L. mexicana* and *L. amazonensis* cause DCL in Caribbean's and Brazil [2].

c. Mucocutaneous Leishmaniasis (MCL)

Mucocutaneous leishmaniasis is a cutaneous condition which occurs at the site of a fly bite, and is characterized by an ulceration of the skin [6]. MCL produce extensive disfiguring destruction of mucosa and cartilage of the mouth (Fig. 1c), nose, ear, and pharynx leading to a severe disfigurement of the face [2]. MCL occur due to *L. aethiopica* in the Old World and *L. Brasiliensis* complex in the New World [5].

d. Visceral Leishmaniasis (VL)

Visceral leishmaniasis (VL), also known as kala-azar is the most severe form of leishmaniasis. The sponge migrates to the internal organs analogous as liver, spleen (hence 'visceral') and bone gist and if left undressed will nearly always results in the death of the host.

The symptoms include fever, weight loss, mucosal ulcers, fatigue, anemia and substantial lump of the liver and spleen (Fig. 1d). Of particular concern, according to the World Health Organization (WHO), is the arising problem of HIV/ VL co-infection (8). Several species of *leishmania* are known to give rise to the visceral form of the illness. The Old-World species are *L. donovani* and *L. infantum* and the New World species are *L. chagasi*.

e. Post kala-azar Dermal Leishmaniasis (PKDL)

Post kala-azar dermal leishmaniasis (PKDL) is sequel of kala-azar that may appear on the skin of affected individualities up to 20 years after being partially treated, untreated or indeed in those considered adequately treated [9,10]. It's generally associated with *L. donovani* which gives different complaint patterns in India and Sudan. In the Indian variant, nodes enlarge with time and form pillars but infrequently ulcerate (Fig. 1e), but nodes from the African variety frequently ulcerate as they progress. Nerve involvement is common in African variety but rare in Indian key [11].

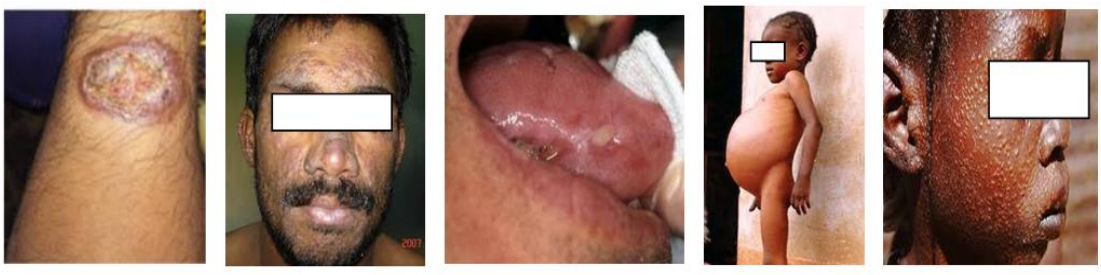


Fig. 1. Clinical manifestation of leishmaniasis (a) Cutaneous leishmaniasis (b) Diffuse Cutaneous leishmaniasis (c) Mucocutaneous leishmaniasis (d) Visceral leishmaniasis and (e) Nodular post-kala-azar dermal leishmaniasis (PKDL) [7]

1.3 Epidemiology and Geographical Distribution of Leishmaniasis

1.3.1 Global Situation

Human leishmaniasis is endemic in 88 counties of the world in which 350 million people are considered to live at risk of infection. There are 2 million new cases of leishmaniasis annually and 14 million infected people worldwide [12]. Leishmaniasis accounts for ~2 million disability adjusted life years in ~90 countries, most of which are in the developing world [13].

1.3.2 Ethiopian Situation

In Ethiopian cutaneous leishmaniasis is dominantly caused by endemic *L. aethiopica* species and rarely by *L. tropica* and *L. major*. It is widely distributed at altitude between 1500-2700m almost everywhere in the country [14,15].

DCL and MCL are found in areas where LCL occurs [16]. Visceral leishmaniasis is distributed throughout the lowlands and semi-desert areas (below altitude 1500m) with varying degree of endemicity. So far VL cases have been reported from at least 40 localities with annual burden estimated to be 4,500-5,000 [17].

1.3.3 Life Cycle of Leishmaniasis

Leishmania is protozoan parasite founds as a motile flagellated promastigote in the sand fly vector and as a round non-flagellated amastigote inside the phagolysosome of the macrophage [18], Fig. 2. The disease is transmitted through the bite of female phlebotomine sand flies [19].

1.4 Treatment of leishmaniasis

1.4.1 Drugs approved for treatment of leishmaniasis

Proven therapies against human leishmaniasis include pentavalent antimonials (sodium stibogluconate and meglumine antimonate) amphotericin B, pentamidine**,** miltefosine and paromomycin [21].

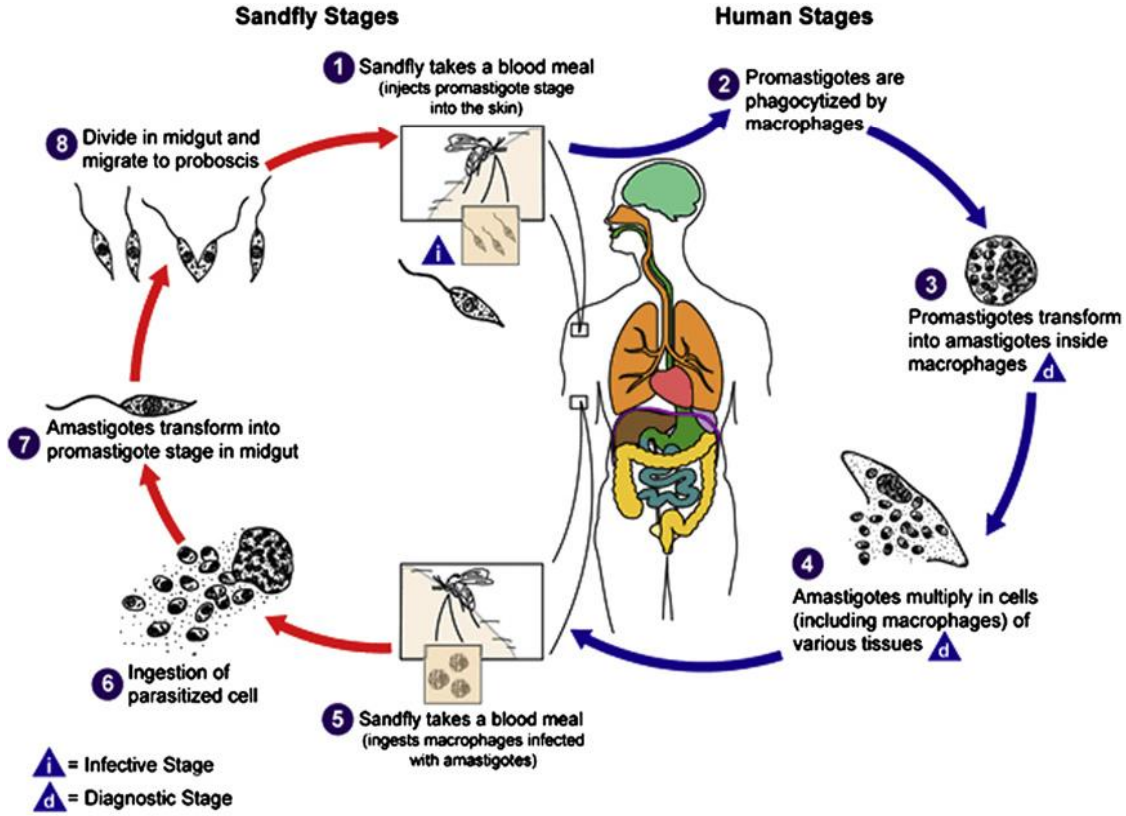


Fig. 2. Life cycle of leishmania parasite [20]
(Source: http://aapredbook.aappublications.org/content/images/large/2006/1/071_07.jpeg)

These drugs are unsatisfactory because of their frequent side effect and increasing drug resistance, therefore, new, safer and more efficacious drugs are urgently required [22].

In this regard, 4(3H)-quinazolinone offer prospects for discovering new compounds with therapeutics properties.

1.4.2 Biological Activities of Quinazolinones

4(3H)-quinazolinone are a frequently encountered heterocyclic in medicinal chemistry literature with applications including antibacterial and antifungal [23], anti-inflammatory and analgesic [24], antimalarial [25], antihypertensive [26] and antiviral [27].

1.4.3 Quinazolinones as antileishmanial agents

Several indolo [2,1-b] quinazolin-6,12-dione (tryptanthrin) derivatives (8-methylindolol [2,1-b] quinazolin-6,12 dione, 8-fluoroindolo [2,1-b] quinazolin-6 ,12-dione and 8-chloro indolo [2,1-b] quinazolin-6,12-dione had displayed remarkable *in vitro* antileishmanial activity against *Leishmania donovani* amastigotes [28].

Rational design from the above-mentioned results that quinazolinone ring system could be promising scaffold for design of antimalarial and antileishmanial agents. Some of our reported quinazolinone derivatives showed pronounced antimalarial activity encouraged by this result it was decided to synthesize novel compounds having essentially in their structure the quinazolinone counterpart linked to different aryl derivatives to investigate the effect of such molecular variation on the antimalarial and antileishmanial activities.

1.5 Synthesis of Quinazolinone Derivatives

4(3H)-quinazolinone derivatives can be synthesized in many ways. Some of the methods include

a. Niementowski quinazoline synthesis

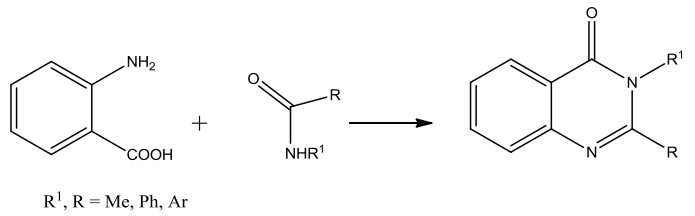
This method is the formation of 4-oxo-3,4-dihydroquinazolines by cyclization of anthranilic acid by amides [29], scheme 1.

b. One-pot synthesis of 2,3 disubstituted -4(3H)-quinazolinones

A one-pot, three-component condensation of anthranilic acid with aromatic amines and ortho-esters on the surface of KAl $(SO_4)_2$.12 H_2O (Alum) under microwave irradiation as a new efficient method to produce 2,3 – disubstituted -4(3H) –quinazolinones in good yields. 2,3- distributed-4(3H)-quinazolinones formation can be rationalized as shown in scheme 2 [30].

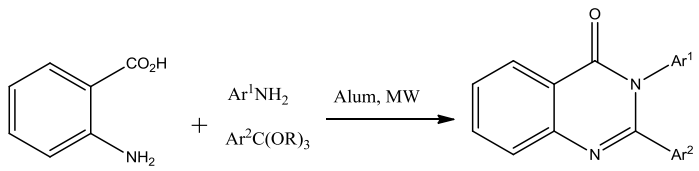
c. One-pot synthesis of 2-(alkylamino) or 2-(arylamino) -4(3H)- quinazolinones

A convenient one-pot preparation of 2-(alkylamino) and 2-(arylamino)-4(3H)-quinazolinones in synthetically useful yields is reported, scheme 3. The approach is based on the reaction of isothiocyanates with 2- aminobenzamide. The reaction could be either heated under reflux for 1- 4.5 hours in toluene (15ml), or exposed to microwave irradiation for 3-4 minutes. The solid material was crystallized from ethanol [31].



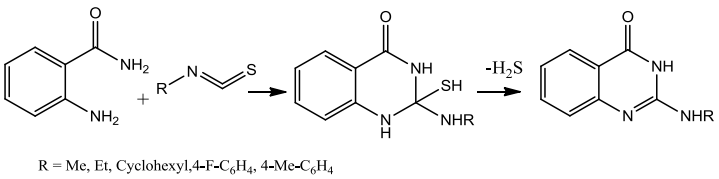
R^1, R = Me, Ph, Ar

Scheme 1. Synthesis of 2,3-disubstituted 4(3H)- quinazolinones



R = Me, Et, Ph

Scheme 2. One-pot synthesis of 2,3-distributed 4(3H)-quinazolinones



Scheme 3. One pot synthesis of 2-substituted 4(3H)-quinazolinones

1.6 Experimental

1.6.1 Test Animals

Swiss albino male mice of weight 20-30g and age 6-8 weeks were obtained from Leishmaniasis diagnosis and research laboratory (LDRL), School of Medicine, Addis Ababa University. The mice had been acclimatized to the laboratory condition before use for a period of 7 days. The animals were housed in standard cages and maintained on standard pelleted diet and water [32].

1.6.2 Culture medium for antileishmanial activity

RPMI-1640 (Gibco, Invitrogen Co.,UK) 10% heat-inactivated fetal calf serum (HIFCS), 1% penicillin-streptomycin and 1% L-glutamine all from Sigma Chem. Co., St. Louis, USA were supplied to make complete culture mediums.

1.6.3 Culture conditions

The *L. aethiopica* isolate was grown first on Novy-MacNeal-Nicolle (NNN) medium and then in tissue-cultured flasks containing RPMI 1640 medium supplemented with 10% HIFCS and 1% 100 IU penicillin/ml streptomycin solution at 22^0C for promastigotes [33,34].

1.6.4 Reference Drugs

For the in vitro Miltefosine/ hexadecyl phosphocholine (AG Scientific, San Diego, CA, USA), and amphotericin B deoxycholate (Fungizone®, ER Squibb, UK) were included as reference drugs (positive control) in the *in vitro* antileishmanial activity testing of the synthesized compounds.

2. METHODS

2.1 Synthesis of 4(3H)-Quinazolinone Derivatives

a. Synthesis of 2-methyl-3,1-benzoxazin-4-one II (Acetanthranil) [35]

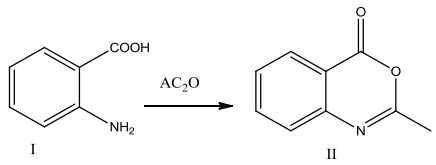
A solution of anthracitic acid **I** (20g, 0.145 moles) in acetic anhydride (23 ml) was heated under reflux for 1 hr. The excess acetic anhydride was then washed with anhydrous petroleum ether, where upon a solid mass was obtained which, without purification was suitable for the subsequent reaction, scheme 4. Reported melting point is 79-80^{0}C [36].

b. Synthesis of 3-aryl-2-methyl-4(3H)-Quinazolinone; III a-c [37] and 3-arylamino-2-methyl-4(3H)-Quinazolinone; d [38]

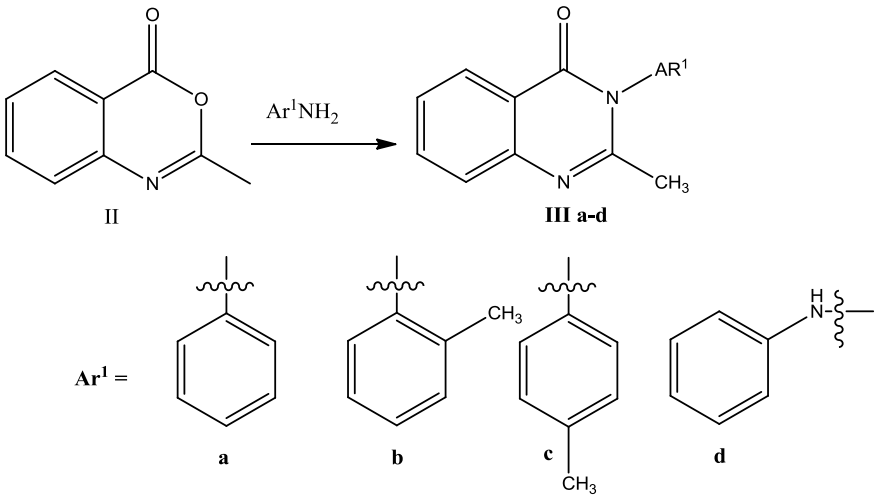
A mixture of Acetanthranil: II (1.175gm,7.5 mmole) and equimolar amounts of the appropriate aromatic amine or phenyl hydrazine was heated under reflux at 190^0C for 5 hrs. The dark sticky mass formed was cooled and recrystallized from ethanol, scheme 5.

c. Synthesis of 3-aryl-2-(substituted styryl)-4(3H)-Quinazolinones; IV a-f [39].

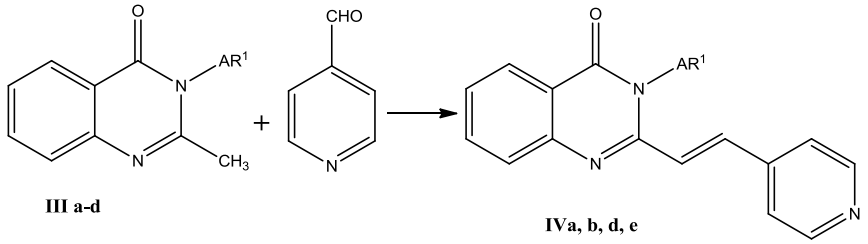
To a solution of 3-aryl-2-(substituted styryl)-4(3H)-Quinazolinones; **III a-c** (10mmole) and 3-arylamino-2-methyl-4(3H)-quinazolinones; **III d** (10 mmole) in acetic anhydride (10ml), an equimolar amount of appropriate aldehyde was added and 10mg of anhydrous zinc chloride as catalyst. The reaction mixture was heated under reflux for 10 hrs. and set aside at room temperature. The reaction mixture was poured into ice-cooled water (50ml). The separated solid product was filtered, dried and recrystallized from ethanol, scheme 6, 7 and 8.



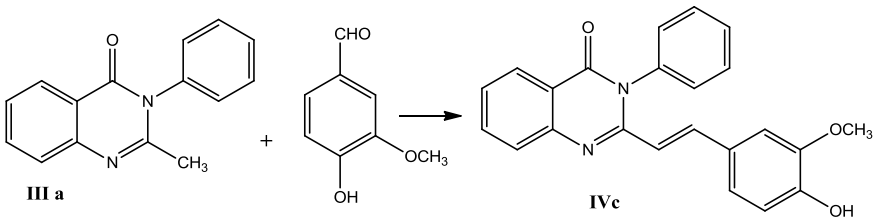
Scheme 4. Synthesis of 2-methyl-3,1-benzoxazin-4-one



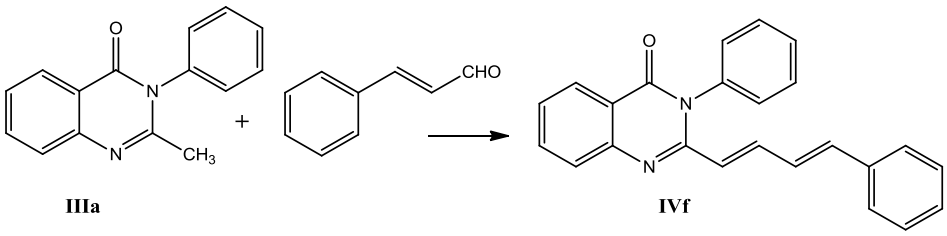
Scheme 5. Synthesis of 3-aryl-2-methyl-4(3H)-Quinazolinone



Scheme 6. Synthesis of (*E*)-3-aryl-2-(2-(pyridine-4-yl) vinyl)-4(3H)-quinazolinones



Scheme 7. Synthesis of 2-(4-hydroxy-3-methoxystyryl)-3-phenyl-4(3H)-quinazolinone



Scheme 8. Synthesis of (*1E, 3E*)-3- phenyl-2-(4-phenylbuta-1,3-dienyl)-4(3H)- quinazolinone

2.2 Preparation of Stock and Working Concentration for Antileishmanial Activity

Stock concentration of 10mg/ml were prepared by dissolving 10mg of each of the six compounds in dimethyl sulfoxide (AMSO) up to 1ml Eppendorf tube mark. Then stocks were diluted using complete RPMI (440 ml RPMI + 50 ml HIFCS + 5 ml penicillin-streptomycin solution (100 IU penicillin/ml-100 µgm/ml streptomycin solution) and 5 ml L-glutamine) to obtain aliquots of 10ugm/ml.

Aliquots of the synthesized compounds were then diluted to 0.004329 µmol/ml (4 µg/ml of amphotericin B). Three-fold serially diluted with complete RPMI gave the final eight working concentrations of each of the synthesized compounds. In addition, various working concentrations of the standard drugs (2, 1, 0.4, 0.1, 0.04, 0.02,0.004, and 0.002µg/ml for amphotericin B [40] and three-fold serial dilution from 40 to 0.01829µg/ml ranging from highest to lowest for miltefosine) were prepared and used as positive controls for antileishmanial activity screening. All the prepared drugs were stored at -20^{0}C and retrieved only during use.

2.3 Biological Activity Assay

2.3.1 *In vitro* antileishmanial assay

All biological assays were performed in LDRL, Faculty of Medicine, Addis Ababa University. Throughout the assay, the highest concentration of DMSO was adjusted not to exceed 1.0% of the synthesized compound preparation, a concentration that showed no growth inhibitory effect. The *L. aethiopica* isolate studied was also treated with standard antileishmanial drugs to compare their susceptibility with that of the synthesized compounds. Assays were done based on reported methods [33, 34, 41].

2.4 Antipromastigote Assay

2.4.2 Determination of percent inhibitory concentration

In a 96- well microtiter plate, 100µl of each of the eight three-fold serial dilutions of synthesized compounds were added in triplicate wells. Then 100 µl of suppression of parasites (3.0 x 10^6 promastigotes/ml of *L. aethiopica*) were added in duplicate (the third well left with only the target compounds of each dilution) to each well and contents of the plates were then maintained in humidified atmosphere at 22^{0}C under at 5% CO_2.

After 68 hrs. of incubation, 10µlof fluorochrome resazurin solution (12.5 mg dissolved in 100 ml of distilled water) was added into each well, and the fluorescence intensity was measured after a total incubation time of 72 hrs. using Victor3 Multilabel Counter (Perkin-Elmer), at an excitation wavelength of 530 nm and emission wavelength of 590 nm [34]. The IC_{50} values were evaluated from sigmoidal dose-response curves using computer software Graph pad prism 5.0 (Graph Pad Software, Inc., San Diego, CA).

The Alamar blue® (Resazurin) (7-hydroxy-3H-phenoxazin-3-one-10-oxide) assay permitted a simple, rapid, reliable, sensitive, cost-effective method for continuous monitoring of cell cultures. It helps to accurately and quantitatively measure proliferation of *Leishmania* [42].

The dye (Resazurin) employed in this method is soluble and stable in culture medium and non-toxic to cells. The dye also does not affect the secretory abilities of cells [43].

2.4.3 *In vivo* Acute Toxicity Test

The oral acute toxicity of compounds **IVa, IVb, IVc, IVd, IVe and IVf** was investigated using male Swiss Albino mice (20g each) according to reported methods [44, 45]. The mice were divided into groups of six mice each and fasted overnight. The compounds were given orally, suspended in 1% gum acacia, in dose of 10, 50, 100, 200 and 300mg/kg. After oral administration to the target compounds, the mice were observed closely during 24 hrs. with special attention to the first four hours. Additionally, the test compounds were investigated for their parenteral acute toxicity in groups of six mice per cage. The compounds or their vehicle, propylene glycol (control) was given by intraperitoneal injection in doses of 20, 40, 80, 120, 140mg/kg. The percentage survival was followed up to 7 days [46]. Acute toxicity signs like sedation, lacrimation, hair erection, blinking, urination, muscle weakness, convulsion in motor, diarrhea, sleep, coma and death were checked in the test mice.

2.5 Statistical Analysis

The results of the study were expressed as mean ± standard deviation and statistical significance for suppressive test was determined by one-way ANOVA using Origin 6.0 software. Data on survival time, %parasitemia and % suppression was analyzed using Microsoft Office Excel 2010. All data was analyzed at 95% confidence interval (P = 0.05).

The IC_{50} values for invitro promastigote assay of target compounds were evaluated from sigmoidal dose-response curves using computer software Graph Pad Prism 5.0.
Percentage parasitemia and percentage suppression fore antimalarial activity were calculated using the following formulae:

$$\% \text{ Parasitemia} = \frac{\text{Number of infected RBC}}{\text{Number of total RBC}} \times 100$$

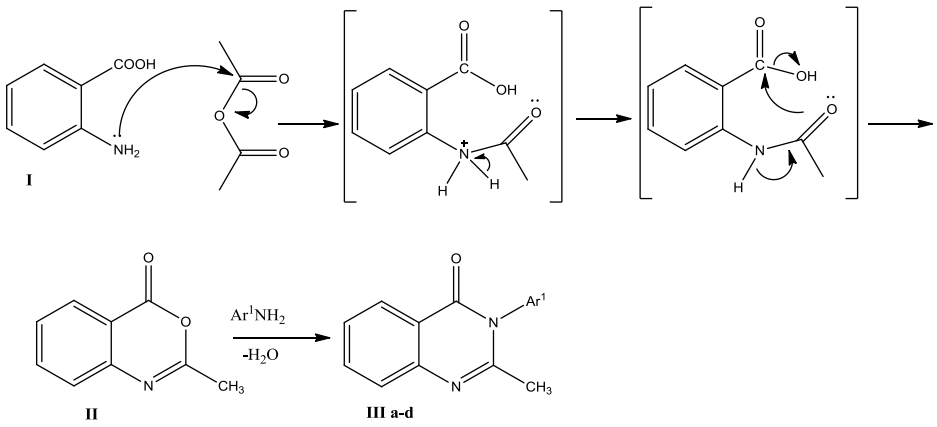
$$\% \text{ Suppression} = \frac{\text{Parasitemia in untreated group - Parasitemia in treated group}}{\text{Parasitemia in untreated group}} \times 100$$

3. RESULT AND DISCUSSION

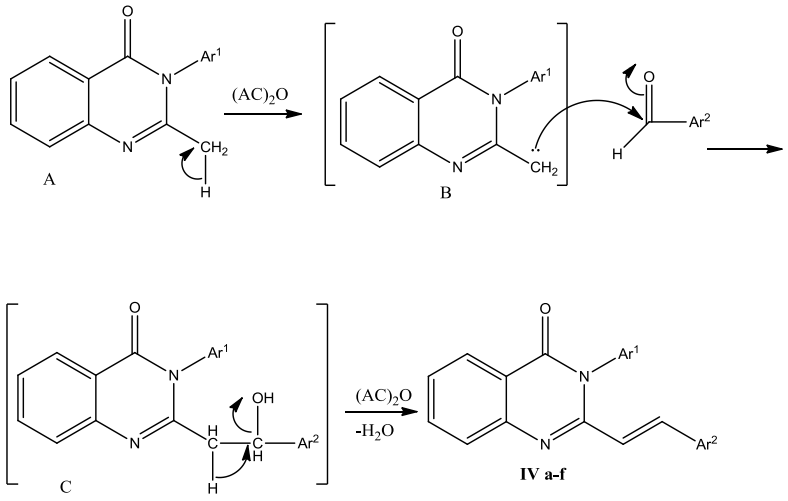
3.1 Synthesis of the Target Compound

The largest compounds were synthesized according to schemes 6, 7 and 8. The intermediates 2-methyl-3,1-benzoxazin-4-one (Acetanthranil) **II** was prepared by heating under reflex anthranilic acid with acetic anhydride, scheme 4. The mechanism of synthesis of Acetanthranil starts with acetylating amino group of anthranilic acid followed by dehydration by the effect of acetic anhydride, scheme 9.

Condensation of acetanthranil **II** with appropriate aromatic amines or phenyl hydrazine afforded compounds **III a-d** schemes 5. Compounds **IVa-f** were synthesized by condensations of 2-methyl-3-substituted 4 (3H)-quinazolinone with acetic anhydride in the presence of anhydrous zinc chloride. Acetic anhydride will abstract hydrogen from the methyl group to form carbanion which in turn attacked the carbonyl group of the selected aldehydes to offered the hydroxyl intermediate **C**. Finally, dehydration by acetic anhydride and zinc chloride afforded the target compounds (**IV a-f**) scheme 10.



Scheme 9. Mechanism of formation of intermediate compounds (II and III a-d)



Scheme 10. Mechanism of formation of target compounds (IVa-f)

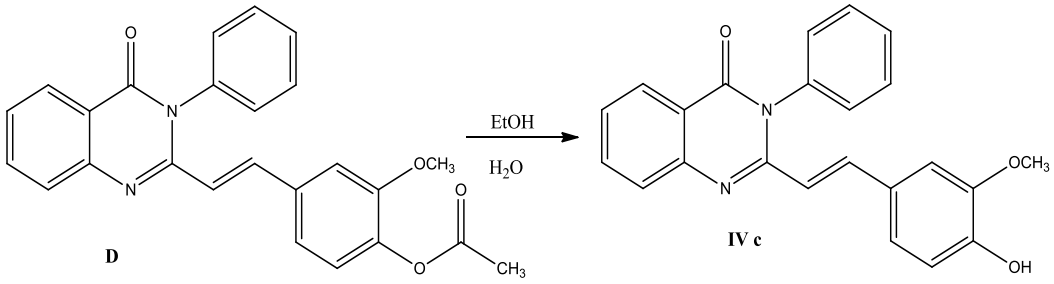
The crude compounds **D,** scheme 11 crystallized from reaction mixture was subjected to IR spectral analysis and showed two characteristic peaks at 1673 and 1763 cm^{-1} assigned for acetyl carbonyl group and quinazolinone carbonyl group. After crystallization from aqueous ethanol (95%), the obtained ^1H NMR spectrum devoid from characteristic peak of the acetyl methyl group confirming hydrolysis of the weak phenolic ester.

Physical constants and yield percent of the synthesized compounds are listed in table 1. The progress of the reactions in the synthesis part of the work was monitored by thin-layer chromatography (TLC). The R_f values for each synthesized compound were calculated, Table 1. The spots on the TLC plate were visualized using iodine vapor.

The Compounds were synthesized in a good yield (59.5-85%). All the synthesized compounds were readily soluble in dimethyl sulphoxide and partially soluble in methanol and chloroform. The structures of the final compounds were verified based on data from elemental microanalysis, IR and ^1H NMR spectral studies.

3.2 Elemental Microanalysis

Elemental microanalyses were performed on Perkin Elmer 2400 elemental analyzer and were founds with ±0.4% of the theoretical values, Table 2.



Scheme 11. Hydrolysis of weak phenolic ester to (E)-2-(4-hydroxy-3-methoxystyryl)-3-phenyl-4(3H)-quinazolinone IVc

Table 1. Physical constants and yield % of the synthesized compounds

Cpd No.	Mol. Formula	Mol. Wt (g/mol)	Yield (%)	M.P (^{0}C)	R_f [Chloroform: Benzene (9:1)]
IVa	$C_{21}H_{15}N_3O$	325.36	71.2	250-252	0.718
IVb	$C_{22}H_{17}N_3O$	339.39	78.0	223-225	0.744
IVc	$C_{23}H_{18}N_2O_3$	370.40	63.4	260-262	0.795
IVd	$C_{22}H_{17}N_3O$	339.39	59.5	224-226	0.690
IVe	$C_{21}H_{16}N_4O$	340.38	67.5	264-266	0.738
IVf	$C_{24}H_{18}N_2O$	350.41	85.0	228-230	0.643

Table 2. Results of the elemental microanalysis

	% Composition					
Cpd No.	**Estimated**			**Found**		
	C	**H**	**N**	**C**	**H**	**N**
IVa	77.52	4.65	12.91	**77.21**	**4.82**	**13.13**
IVb	77.86	5.05	12.38	**78.10**	**4.85**	**12.11**
IVc	74.58	4.90	7.56	**74.27**	**5.22**	**7.81**
IVd	77.86	5.05	12.38	**78.08**	**4.79**	**12.54**
IVe	74.10	4.74	16.26	**74.36**	**4.48**	**16.24**
IVf	82.26	5.18	7.99	**82.46**	**4.88**	**8.08**

3.3 Spectroscopic Analysis of the Synthesized Compounds

Infrared (IR) spectra were recorded on a SHIMADZU 8400 SP FT-IR spectrophotometer using Nujol disc technique. ^{1}H NMR spectrum was recorded on Bruker Avance DMX400-FT-NMR spectrometer and the chemical shifts are given in δ (ppm) downfield from tertramethylsilane (TMS) which served as an internal standard. Splitting patterns were designed as follows: *s*: singlet; *d*: doublet; *m*: multiplet. The summarized characteristic stretching and bending IR vibration frequencies and the ^{1}H NMR chemical shifts for each compound are discussed below.

^{1}H NMR ($CDCl_3/CCl_4$) ppm: 6.55 (*d*, 1H, J = 15.53 Hz, vinyl-C_2 H), 7.16 (d, 2H, J = 5.29 Hz, pyridine-$C_{2,6}$ H), 7.35 (*d*, 2H, J = 6.38 Hz, phenyl-$C_{3,5}$ H), 7.55 (*t*,1H, J = 8.08 Hz, quinazolin-C_7 H), 7.58-7.67 (*m*, 3H, phenyl-$C_{2,4,6}$ H), 7.78-7.86 (*m*, 2H, quinazolin-$C_{6,8}$ H), 7.90 (*d*, 1H, J = 15.53 Hz, vinyl-C_1 H), 8.32 (*dd*,1H, J_1 = 0.490Hz, J_2 = 1.407 Hz quinazolin-C_5 H) and 8.57 (*d*,2H,J= 5.29 Hz, pyridine-$C_{3,5}$ H).

The IR spectrum compound **Iva** (Fig 3), showed strong characteristic band at 1680 cm^{-1}attributed to 4 (3H)-quinazolinone carbonyl group stretching. The peak appeared at low frequency as it is amide carbonyl group. Moreover, a medium characteristic band for C=N appeared at 1593 cm^{-1}.

^{1}H NMR spectrum of compound **Iva** (Fig 3), showed two doublets at 6.55 ppm and 7.90 ppm attributed to vinyl protons. They have the same coupling constant, J = 15.53 Hz confirming the existence in (*E*) configuration [47]. in addition, two doublets appeared at 7 .16 ppm and 8.57 ppm assigned to protons of pyridine protons which assure the formation of the regret's compounds.

^{1}H NMR (DMSO-d_6) ppm: 2.4 (*s*, 3H, p-tolyl CH_3), 6.60 (*d*, 1H, j = 15.70 Hz, vinyl C_2 H), 7.30-7.45 (*m*, 6H *p*-tolyl $C_{2,35,6}$ H and pyridine-$C_{2,6}$ H), 7.60 (t, 1H, J=7.84 Hz, quinazolin-C_7 H), 7.75-7.85 (*m* ,2H, vinyl-C_1 H and quinazolin-C_8 H), 7.90 (*t*, 1H, J =7.10 Hz, quinazolin-C_6 H), 8.15 (*dd*, 1H, J_1 = 0.460 Hz, J_2 = 1.391 Hz, quinazolin-C_5H) and 8.55 (*d*, 2H, j = 5.28 Hz, pyridin-$C_{3,5}$ H).

The IR spectrum of compound **IVb** (Fig 4), showed strong characteristic absorption band at 1682 cm^{-1} that indicated the presence of a 4(3H)-quinazolinone carbonyl group. The band appeared at low frequency as it is amidic carbonyl group. Strong absorption at 1614 cm^{-1} assigned to the C=N stretch.

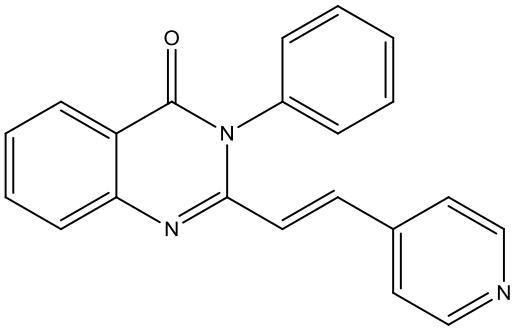


Fig. 3. (*E*)-3-phenyl-2-[2-(pyridine-4-yl) vinyl]-4(3H)-quinazolinone IVa:
IR (Nujol) (cm^{-1}): 1680 (C=O) and 1593 (C=N)

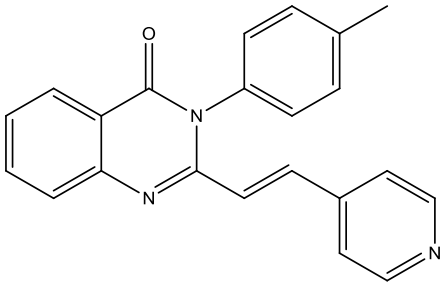


Fig. 4. (*E*)-2-[2-(pyridine-4-yl) vinyl]-3-p-tolyl-4(3H)-quinazolinone IVb
IR (Nujol) (cm^{-1}): 1682 (C=O) and 1614 (C=N)

^{1}H NMR spectrum of compound **IVb** (Fig 4), showed two doublet peaks at 6.60 and 7.80 ppm that appeared as overlapping multiplet with quinazolin-C_8H attributed to vinylic protons. The coupling constant, J = 15.70 Hz confirm that the compounds is in (*E*) configuration [47]. In addition, the overlapping multiplet peaks at 7.30-7.45 ppm afford to four aromatic protons of *p*-tolyl and pyridine-$C_{2,6}$ H. Similarly, doublet peak at 8.55 ppm due to pyridine-$C_{3,5}$ protons. The presence of these peaks confirmed the formation of the target compound.

^{1}H NMR ($CDCl_3$/CCL_4) ppm:3.8 (*s*,3H, -O-CH_3), 6.25 (*d*,1H, J=15.69 Hz, vinyl-C_2H), 6.85-7.0 (*m*,3H, 4-hydroxy-3-methoxphenyl-$C_{2,5,6}$ H), 7.35 (*d*, 2H, J=7.84 Hz, phenyl-$C_{3,5}$ H),7.50 (*t*,1H, J = 7.79 Hz, quinazolin-C_7 H), 7.55-7.65 (*m*,3H, phenyl-$C_{2,4,6}$ H) ,7.80 (*m*, 2H, quinazolin-$C_{6,8}$ H), 7.92 (*d*,1H, J = 15.69 Hz, vinyl-C_1 H), 8.30 (*dd*,1H, J_1= 0.510 Hz, J_2 =1.430 Hz quinazolin-C_5H).

The IR spectrum of compound **IVc** (Fig 5), revealed characteristic medium band at 1673 cm^{-1} attributed to the quinazolinone carbonyl group. The appeared at low frequently as it is amidic carbonyl group. The other characteristic medium band at 1637 cm^{-1} assigned to C=N stretching. The bands at 1206 and 1120 cm_{-1} are characteristic to ether symmetric and asymmetric stretching.

The^{1}HNMR spectrum of compound **IVc** (Fig 5), showed two doublet peaks at 6.25 and 7.92 ppm attributed to vinyl protons. The coupling constant of these protons (J= 15.69 Hz) confirm that the compounds is in (*E*) configuration [47]. In addition, the peaks at 6.8 -7.0 ppm (*m*,3H) afforded to aromatic protons of 4-hydroxy-3-methoxyphenyl group and the up-field singlet peak at 3.8 ppm conforms the formation of the target compound.

^{1}HNMR (DMSO-d_6) ppm:2.4 (*s*,3H, CH_3), 6.60 (*d*,1H, J=15.63Hz, vinyl-C_2 H),7.30-7.45 (*m*, 6H, tolyl-$C_{3,4,5,6}$ H and pyridin-$C_{2,6}$ H),7.60 (*t*,1H, J=7.7.3 Hz, quinazolin-C_7 H),7.75 -7.85 (*m*,2H, vinyl-C_1 H and quinazolin-C_8 H),7.90 (t, 1H, J=7.34 Hz, quinazolin-C_6 H), 8.15 (*dd*, 1H, J_1 =0.415 Hz, J_2 =1.390Hz quinazolin-C_5 H) and 8.55 (*d*,1H, J=5.32 Hz pyridine-$C_{3,5}$ H).

The IR spectrum of compound **IVd** (Fig 6), exhibited a characteristic absorption band at 1652 cm^{-1} attributed to quinazolinone carbonyl group. The peak appeared at low frequency as it is amidic carbonyl group. The absorption band appeared at 1634 cm^{-1} is due to C=N stretching.

The ^{1}HNMR spectrum of compound **IVd** (Fig 6), displayed two doublets at 6.60 ppm and in overlapping multiplet at 7.75-7.85 ppm. The coupling constant of these protons (J=15.63 Hz) confirm that the compound is in (*E*) configuration [47]. The overlapping multiplet peaks at 7.30-7.45 ppm attributed to two protons of pyridine-$C_{2,6}$ H. The highly de-shielded peak at 8.55 ppm afforded to pyridine-$C_{3,5}$ H confirm the foundation of the trade compounds.

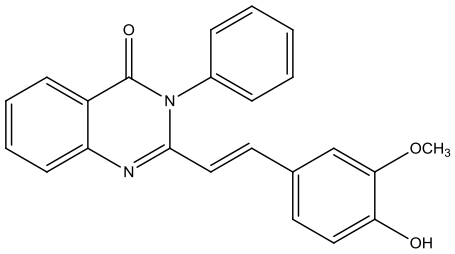


Fig. 5. (*E*)-2-(4-hydrox-3-methoxystystyryl)-3-phenyl-4(3H)-quinazolinone IVc:
IR (Nujol) (cm^{-1}): 1673 (C=O);1637 (C=N); 1120 and 1206 (C-O-C)

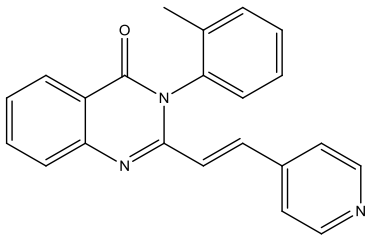


Fig. 6. (*E*)-2-[2-(Pyridin-4-yl)-vinyl]-3-o-tolyl-4(3H)-quinazolinone IVd:
IR (Nujol) (cm^{-1}):1652 (C=O) and 1634 (C=N)

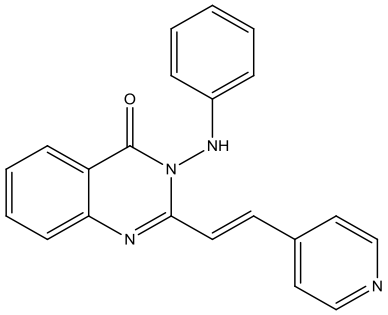


Fig. 7. (*E*)-2-[2-(pyridin-4-yl)-vinyl]-3-phenylamine-4(3H)-quinazolinone IVe:
IR (Nujol) (cm^{-1}): 3250 (N-H); 1700 (C=O) and 1625 (C=N)

^{1}HNMR (DMSO-d_6) ppm: 6.70 (*d*,2H,J=8.39Hz, phenyl-$C_{2,6}$ H), 6.85 (*t*,1H, J=7.36Hz, 3-phenylamine-C4 H), 7.23 (*t*,2H, J=8.34Hz, 3-phenylamne-$C_{3,5}$ H), 7.58 (*t*,1H, J=7.61Hz, quinazolin-C_7 H),7.62 (*d*,2H, J=5.31Hz, pyridine-$C_{2,6}$ H), 7.67 (*d*,1H, J=15.90Hz,vinyl-C_2 H),7.82 (*d*,1H, j=8.27Hz, quinazolin-C_8 H), 7.90 (*t*,1H, J=8.30Hz, quinazolin-C_6 H), 8.00 (*d*,1H, J=15.90Hz, vinyl-C_1 H), 8.15 (*dd*,1H, J_1=0.450Hz, J_2=0.976Hz, quinazolin-C_5 H), 8.60 (*d*,2H, pyridine-$C_{3,5}$ H) and 9.25 (*s*,1H, 3-phenylamine N-H).

The IR spectrum of compound **IVe** (Fig 7), revealed a sharp absorption at 3250 cm^{-1} which is characteristic for the presence of N-H group. Another characteristic medium band at 1700 cm^{-1} assigned to the 4(3H)-quinazolinone carbonyl group. The peak appeared at high frequency as it forms five membered rigs by hydrogen bond with the N-H. In addition, the band at 1652 cm^{-1} were attributed to the C=N stretching.

The ^{1}HNMR spectrum of compound **IVe** (Fig 7), displayed two doublets at 7.67 ppm and 8.00 ppm assigned to vinylic protons. They have the same coupling constants, J= 15.90Hz confirming that the compound is in (*E*) configuration [47]. In addition, two doublets at 7.62 & 8.60 ppm appeared due to pyridine protons which assure the formation of the target compound.

^{1}HNMR ($CDCl_3$ /CCL_4) ppm: 5.95 (*d*,1H, J=14.69 Hz, butdiene-C_4 H), 6.75 (*t*, 1H, J = 15.43 Hz, butdiene-C_2 H), 6.95 (d, 1H, J= 15.43Hz, butdien-C_1 H), 7.25-7.35 (*m*, 5H, butadiene-C_3 H), quinazolin-$C_{6,8}$ H and phenyl-$C_{3,5}$ H),7.73 -7.88 (*m*,3H, quinazolin-C_7H and phenyl $C_{2,6}$ H), 7.40 -7.70 (*m*,6H, phenyl-C_4 H and but-phenyl-$C_{2,3,4,5,6}$ H) and 8.3 (*dd*,1H, J_1 =0.897Hz, J_2=1.380Hz quinazolin-C_5H)

The IR spectrum of compound **IVf** (Fig 8), exhibited strong characteristic bands at 1679 cm^{-1} for carbonyl group and the peak appeared at low frequency as it is amidic carbonyl group. Medium band at 1625 cm^{-1} was assigned for C=N stretching.

In the ^{1}HNMR spectrum of compound **IVf** (Fig 8), the protons of the conjugating double bonds of 2-(4-phenylbuta-1,3-dienyl) appeared at 5.95 ppm, 6.75 ppm and 6.95 ppm for carbon number 4,2 and 1 proton respectively. The coupling constants (J= 14.69 Hz and J= 15.43 Hz) of these three protons confirming that the compound is in (*E, E*) configuration. In addition, coupling constants of protons on carbon number 1 and 2 (J=15.43 Hz) show the they are adjacent protons [47].

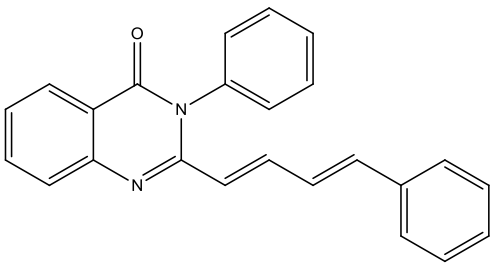


Fig. 8. (*1E,3E*)-3-phenyl-2-[4 phenylbut-1,3-dienyl]-4(3H)-quinazoline IVf:
IR (Nujol) (cm^{-1}): 1679 (C=O) and 1625 (C=N)

Table 3. Anti-promastigote activity (IC_{50}) of IVa-f and standard drugs against *L. aethiopica* strain (CL/039/09)

Test compound	IC_{50} values (µg/ml)	IC_{50} values (ng/ml)
IVa	0.03766	37.66
IVb	0.00538	5.38
IVc	0.00412	4.12
IVd	0.00110	1.10
IVe	0.03017	30.17
IVf	0.03894	38.94
Amphotericin B	0.04359	43.59
Miltefosine	3.17	3170.00

The protons of butadiene-C_3H appeared as over applying multiplet at 7.25-7.35 ppm with two protons of quinazolin-$C_{6,8}$ H and phenyl-$C_{3,5}$ H. The five protons on but-phenyl showed overlapping multiplet peak with one proton of phenyl-C_4 H at 7.40-7.70 ppm confirming the formation of the target compound.

3.4 Biological Activity Results

3.4.1 *In vitro* antileishmanial activity of the synthesized compounds

The *in vitro* antileishmanial activity of the synthesized compounds **IVa-f** was performed against the isolated of *L. aethiopica* (CL039/09) that causes cutaneous leishmaniasis in Africa especially in Ethiopia. The isolate was also treated with standard antileishmanial drugs for comparison.

3.4.2 Anti-promastigote assay

The inhibitory Activity of the synthesized compounds on promastigotes was assessed on clinical isolate of *Leishmania aethiopica* strain (CL/039/09) as described in the experimental section. Moreover, the 50% inhibitory concentration (IC_{50}) of the synthesized compounds and reference drugs were evaluated from fluorescence characteristic of resazurin, Table 3.

The tested compounds exhibited better inhibitory activity as indicated by their lower IC_{50} than amphotericin B and miltefosine, drug that have clinically applied to the treatment of leishmaniasis. This high potency of the synthesized quinazolinone derivatives is also in agreement with that of tryptanthrin that constrains a quinazolinone moiety and displayed remarkable *in vitro* antileishmanial activity [28].

Among the test compounds, **IVd** is the potent followed by **IVc** and **IVb** respectively. Compound **IVe** had the next lower IC_{50} while **IVa** and **IVf** displayed comparable IC_{50} which is slightly lower than amphotericin B.

In general, all compounds showed better antileishmanial activity as indicated by their lower IC_{50} values than amphotericin B and miltefosine, the positive controls used, Table 3. Moreover, the observed IC_{50} values of amphotericin B (0.04359 µg/ml) was in a good agreement with the reported IC_{50} values amphotericin B (0.047 ± 0.008 µg/ml) on the same strain [34].

3.4.3 *In vivo* acute toxicity test

Oral and parental acute toxicity test was performed for all compound **IVa-f**. The test compounds were administrated through oral route in doses of 10, 50, 100, 200 and 300 mg /kg. The results indicated that test compounds proved to be non-toxic and well tolerated by the experimental animal up to 300 mg/kg. Moreover, these compounds were tested for their toxicity through parenteral route in doses of 20, 40, 80, 120, 140 mg/kg [46]. The results revealed absence of any acute toxicity signs like lacrimation, hair erection, blinking, urination, muscle weakness, sedation and convulsion, reduction in motion, diarrhea, sleep, coma and death in both oral and parental studies for all test compounds.

4. CONCLUSION

Novel 2-aryl,3-substituted-4(3H)-quinazolinone derivatives, **IVa-f** were synthesized to investigate their antileishmanial activities. The target compounds were obtained in a good yield (59.5- 85%) by applying different chemical reactions like cyclization and condensation reaction. The chemical structure of the final compounds was verified by using elemental microanalysis, IR and ^{1}HNMR.

The *in vitro* antileishmanial activity was performed against the isolate of *L. aethiopica* (CL039/09) that causes cutaneous leishmaniasis in Africa especially in Ethiopia. All the tested compound exhibited better IC_{50} (0.00110µg/ml - 0.03894 µg/ml) than amphotericin B (0.04359 µg/ml) and miltefosine (3.17 µg/ml). Among the tested compounds, **IVd** (IC_{50} =

0.00110 µg/ml) is the most potent followed by **IVc** (IC_{50} =0.00412 µg/ml) and **IVb** (IC_{50} = 0.00538) respectively. The results of acute toxicity indicated that all test compounds (**IVa-f)** proved to be non-toxic and well tolerated by the exper imental animals up to 300 mg/kg in oral and 140 mg/kg in parenteral studies. All the test compounds showed antileishmanial activities higher than the reference drugs- amphotericin B and miltefosine. Meanwhile acute toxicity test showed that all test compounds have good safety margin. Therefore, the test compounds **IVa-f** would represent a fruitful matrix for the development of new class for antileishmanial agents that would deserve further investigation and derivatization.

AVAILABILITY OF DATA AND MATERIALS

All the data that support the findings of our study are available at Addis Ababa University, School of Pharmacy. Data are however available from the authors upon reasonable request and with permission.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

ETHICAL CONSIDERATION

A letter of ethical approval was obtained from the Ethical Review Board of the School of Pharmacy, Addis Ababa University [Ref. No. ERB/SOP/187/04/2020]. Guidelines outlined in the National Committee for Research Ethics in Science and Technology (NENT) for the use of animals in research were met.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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