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Research Article
Bioactive γ-butyrolactones from Endophytic Fungus *Aspergillus versicolor*

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Abstract
Background and Objective: The γ-butyrolactones is a popular class of fungal metabolites, possessing variable biological activities. In this study, the anti-microbial, anti-leishmanial, anti-malarial and cytotoxic activities of γ-butyrolactone derivatives: Aspernolides L (1) and M (2) and butyrolactones I (3) and VI (4) separated from *Aspergillus versicolor* isolated from *Pulicaria crispa* (Asteraceae) roots were assessed. Moreover, their radioligand displacement affinity on human cannabinoid and opioid receptors was estimated. **Materials and Methods:** The anti-microbial effect of compounds 1-4 was assessed against *Aspergillus fumigates* (*A. fumigates*), *Cryptococcus neoformans* (*C. neoformans*), methicillin-resistant *Candida albicans* (*C. albicans*), *Staphylococcus aureus* (*S. aureus*), *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Escherichia coli* (*E. coli*), using modified CLSI/NCCLS methods. Their anti-leishmanial capacity towards *Leishmania donovani* (*L. donovani*) as well as anti-malarial potential towards *Plasmodium falciparum* (*P. falciparum*) [(W2, Indo-China) and (D6, Sierraleon)], using plasmodial lactate dehydrogenase (pLDH) assay were evaluated. Moreover, the binding affinity towards opioid (subtypes δ, κ and μ) and cannabinoid (CB1 and CB2) receptors were tested using *in vitro* radioligand displacement assay. The cytotoxic effect was evaluated towards epidermoid (KB), malignant melanoma (SK-MEL), ovarian (SK-OV-3) and ductal (BT-549) carcinomas. **Results:** Compounds 1 and 2 had moderate anti-microbial effect towards *C. albicans*, *A. fumigates*, *E. coli* and *P. aeruginosa* with IC_{50} ranged from 2.60-6.04 mM. Moreover, compounds possessed anti-leishmanial potential towards *L. donovani* with IC_{50} 2.31 and 3.47 mM, respectively and IC_{90} 5.67 and 3.89 mM, respectively compared to pentamidine. While, compound 3 displayed moderate activities towards *C. neoformans* and *A. fumigates* with IC_{50} 7.90 and 9.75 mM, respectively. On the other hand, compound 2 revealed activity towards D6 and D6 S1 clones (chloroquine-sensitive strains of *P. falciparum*) with IC_{50} 2.16 and 1.43 mM, respectively. Compounds 1-4 exhibited good binding affinity towards the CB1 receptor with 71.2, 80.5, 69.8 and 66.1%, respectively displacement values. Moreover, compound 2 showed affinity to d-, k- and m-receptors with displacement values 61.2, 73.5 and 61.3%, respectively. Furthermore, compound 1 displayed cytotoxic potential towards SK-MEL with IC_{50} 0.70 mM and 2 exhibited the highest activity towards KB, SK-MEL, BT-549 and SKOV-3 cell lines with IC_{50} 1.2, 0.9, 0.1 and 0.8 mM, respectively. **Conclusion:** Compounds 1-2 could be drug leads for anti-microbial, anti-leishmanial, cytotoxic and anti-malarial agents. The good binding affinity of 1-4 towards the CB1 receptor may also provide additional information on the possible analgesic and anti-inflammatory properties of the butyrolactones which are not reported earlier.

Key words: *Aspergillus versicolor*, γ-butyrolactones, anti-microbial, anti-leishmanial, anti-malarial, cannabinoid, opioid receptors, cytotoxicity

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Infectious diseases caused by fungi, bacteria, parasites and viruses are important factors of morbidity and mortality in all regions of the world particularly in developing countries[1]. The resistance of parasites, fungi and bacteria to anti-parasitic and anti-microbial agents, respectively has grown in the last decades[2]. The prevalence of their resistant has increased due to extensive misuse of anti-microbial and anti-parasitic agents in treatment of infectious diseases[3]. This has made the current available anti-microbial and anti-parasitic drugs insufficient to control these infections and lead to major health problems[4,5]. Thus, many researchers have focused on the investigation of natural products as a potential source of anti-microbial and anti-parasitic agents[6]. Recent years have witnessed a significant progress in the investigation of endophytic fungi. Diverse structural types of bioactive metabolites isolated from endophytes have attracted the scientists' attention as new drug leads for various infectious diseases[7-11]. *Aspergillus* genus (family Aspergillaceae) a diverse and large genus, contains ≈180 filamentous species of prominent pharmaceutical and commercial potentials[10,12]. It is popular as a wealthy source of γ-butyrolactone derivatives, which are a class of fungal metabolites, composing of a 5-membered lactone ring to which two benzene rings are attached. They show a wide variety of activities as anti-malarial, cytotoxic, anti-H1N1, anti-microbial, anti-cholinesterase, antioxidant and cyclin-dependent kinase and lipoxygenase inhibitor[7,10]. Surveying the current available literatures revealed the existence of minor reports regarding the anti-leishmanial and anti-malarial activities of this class of metabolites[7]. Moreover, their binding affinity to human cannabinoid and opioid receptors has not been previously evaluated. The γ-butyrolactones: Aspernolides L (1) and M (2), butyrolactone I (3) and VI (4) (Fig. 1) separated from *Aspergillus versicolor* isolated from *Pulicaria crispa* (*Asteraceae*) roots, which was collected from Gabal Al-aquiq, Al Madinah Al Munawwarah, KSA were previously characterized on basis of their MS, 1D and 2D NMR data[13]. The purpose of this study was to assess their anti-leishmanial, anti-malarial and human cannabinoid and opioid receptors displacement affinity. In addition, their anti-microbial and cytotoxic capacities were tested. Also, this study aimed to highlight new bioactivities for butyrolactones, especially for those isolated from the endophytic fungus *Aspergillus versicolor*.

MATERIALS AND METHODS

This study was conducted from November, 2016 to March, 2017 in the Department of Medical Microbiology and Parasitology, Faculty of Medicine, Princess Al-Jawhara Center of Excellence in Research of Hereditary Disorders, King Abdulaziz University, Saudi Arabia.

Anti-microbial assay: Compounds 1-4 were assessed for their anti-microbial effect against *Aspergillus fumigates* ATCC 90906, *Cryptococcus neoformans* ATCC 90113, methicillin-resistant *Candida albicans* ATCC 90028, *Staphylococcus aureus* ATCC 33591, *Pseudomonas*

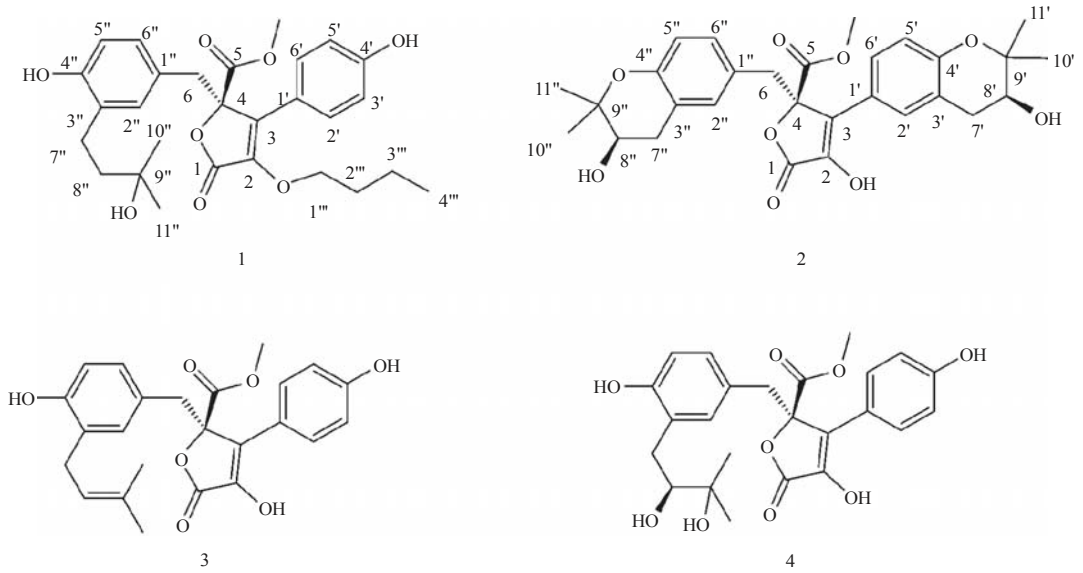

Fig. 1: Chemical structures of tested compounds 1-4

aeruginosa ATCC 27853 and *Escherichia coli* ATCC 35218, using modified CLSI/NCCLS methods. Twenty percent DMSO/saline was added to dilute the samples, then were transported to 96 well microplates in duplicate. Ciprofloxacin and amphotericin B were utilized as anti-bacterial and anti-fungal, respectively[7,14,15].

Anti-leishmanial assay: The *in vitro* anti-leishmanial capacity of compounds 1-4 was evaluated towards *Leishmania donovani* promastigotes as previously stated. The experiment was undertaken in triplicate. Pentamidine (positive standard) was used[7,14].

Anti-malarial assay: Compounds 1-4 were assessed on *Plasmodium falciparum* [chloroquine resistant (W2, Indo-China) and sensitive (D6, Sierraleon)]. The assay was based on the estimation of plasmodial LDH potential as mentioned previously[10,16]. Both artemisinin and chloroquine were utilized as positive control and DMSO as negative one.

Cytotoxicity assay: The cytotoxic effect was evaluated towards epidermoid (KB), malignant melanoma (SK-MEL), ovarian (SK-OV-3) and ductal (BT-549) carcinomas *in vitro* as previously published. DMSO and doxorubicin were utilized as negative and positive controls, respectively[7,16].

Cannabinoid receptor radioligand displacement: This evaluation was carried out from November, 2016 to March, 2017. National Center for Natural Products Research, Department of Pharmacognosy, School of Pharmacy, The University of Mississippi, MS 38677, USA. Metabolites of compounds 1-4 were estimated in binding affinity with subtypes of cannabinoid receptors, CB1 and CB2[17,18]. Each compound (10 μM) was incubated with 1.9165 (nM, CB2) or 1.6975 (nM, CB1). Cannabinoid receptors agonist [^{3}H]-CP-55,940 and CB1 (7 μg) or CB2 (1 μg) membrane were incubated at 37°C for 90 min in a 96-well plate with gentle agitation in a 0.2 mL Tris-HCl (50 mM), EDTA (20 mM), NaCl (154 mM) and 0.2% fatty-acid BSA radio immuno assay grade at pH 7.4. The reaction was ended by vacuum filtration through a 0.5% polyethylenimine pre-soaked UniFilter (96 GF/C filter), followed by washing with Tris-HCl (50 mM) at pH 7.4. 25 μL MicroScint20 was added to the dried filters. Micro-plate scintillation counter (TopCount NXT) was utilized to read the plates. The binding in the existence of vehicle (1.0% DMSO) and 10.0 μM CP-55,940 were known as total and nonspecific bindings, respectively. Each experiment was repeated in triplicate. The difference between nonspecific and total binding was the specific binding.

Opioid receptors radioligand displacement: All metabolites were assessed for binding competition assays towards the subtypes of opioid receptors (μ, δ and κ). Ten micrometers of tested compound were incubated for 60 min in a 96-well plate with DPDPE [0.65835 nM, tyrosyl-3,5-^{3}H(N), δ], DAMGO [0.9479 nM, tyrosyl-3,5-^{3}H(N), μ] or 0.62295 nM [phenyl-3,4-^{3}H]-U-69,593 (κ) in a 0.2 mL final volume of Tris-HCl (50 mM) at pH 7.4 with 20 μg (δ and μ) or 15 μg (κ) of membrane. The reaction was ended via vacuum filtration through a UniFilter 96 GF/B filter presoaked with 0.3% BSA, followed by washing with 4°C Tris-HCl (50 mM) at pH 7.4. MicroScint20 (50 μL) was added to the dried filters. Each experiment was repeated in triplicate. Specific binding, total binding and percent displacement were determined using the following equation[17,19]:

$$\text{Percent displacement} = \frac{100\text{-(Compounds binding-nonspecific binding)}}{\text{Specific binding}} \times 100$$

The binding 10 μM U-69,593 (κ), DAMGO (μ), or DPDPE (δ) was known as a nonspecific binding. The competitive binding assay was carried out by estimating 12 triplicate 3-fold serial dilutions of compound (300 μM) and naloxone hydrochloride (3 μM, positive control) with 15 μg of δ membrane and 1.87 nM [^{3}H]-PDPE.

Statistical analysis: The obtained data were expressed as mean±standard error of mean using the student t-test. The statistical significance was evaluated by one-way analysis of variance (ANOVA). The values were considered to be significantly different when $p<0.05$.

RESULTS AND DISCUSSION

It is commonly known that it is more economical and easier to produce valuable products from a microbial source. Plant derived endophytic fungi have to cope with both plant defense and competing microorganisms and have, therefore, developed a diverse and rich secondary metabolites as part of their survival strategy. The finding out of new metabolites further widened the structural diversity of the bioactive compounds generated by the plant endophytes. Natural butyrolactones have been separated from different *Aspergillus* species as *A. versicolor*, *A. terreus*, *A. flavipes*, *A. insuetus* and in addition *Allantophomopsis lycopodina* and bacterium *Xenorhabdus szentirmaii*[7,20]. They possess variable bioactivities.

Table 1: Anti-microbial activity results of tested butyrolactones

	IC_{50} (mM)					
Compound number	*C. albicans*	*A. fumigates*	*C. neoformans*	MRS	*E. coli*	*P. aeruginosa*
1	4.31±0.17*	3.09±0.13*	>20	>20	6.04±0.31*	2.78±0.11*
2	5.41±0.25*	3.20±0.20*	>20	>20	2.60±0.09*	4.60±0.18*
3	>20	9.75±0.26*	7.90±0.21*	>20	>20	>20
4	>20	>20	>20	>20	>20	>20
Ciprofloxacin	-	-	-	0.11	0.07	0.05
Amphotericin B	0.35	0.41	0.08	-	-	-

Each value represents the Mean±SEM, n = 3, *Significant different from control at p<0.05

Table 2: *In vitro* anti-leishmanial activity results of tested butyrolactones

	Leishmania donovani	
Compound number	IC_{50} (mM)	IC_{90} (mM)
1	2.31±0.11*	5.67±0.28*
2	3.47±0.23*	3.89±0.18*
3	24.18±1.67*	32.91±1.24*
4	40.00	40.00
Pentamidine	0.85	1.04

Each value represents the Mean±SEM, n = 3, *Significant different from control at p<0.05

Table 3: Anti-malarial activity results of tested butyrolactones

	IC_{50} (mM)			
Compound number	*P. falciparum* (D6 clone)	*P. falciparum* (D6 S1 clone)	*P. falciparum* (W2 clone)	*P. falciparum* (W2 S1 clone)
1	89.9±4.76*	65.20±3.07*	59.1±2.11*	68.7±1.99*
2	2.16±0.07*	1.43±0.09*	200	200
3	101.3±6.49*	200	119.5±8.23*	200
4	200	200	200	200
Chloroquine	0.52	0.011	-	-
Artemisinin	-	-	0.91	0.008

Each value represents the Mean±SEM, n = 3, *Significant different from control at p<0.05

The butyrolactones 1-4 were evaluated for their anti-microbial capacity towards *C. albicans*, *A. fumigates*, *C. neoformans*, *E. coli*, methicillin-resistant *S. aureus* and *P. aeruginosa*. Compounds 1 and 2 showed moderate activity against *C. albicans*, *A. fumigates*, *E. coli* and *P. aeruginosa* with IC_{50} ranged from 2.60-6.04 mM, respectively. While, 3 displayed moderate activity towards *A. fumigates* and *C. neoformans* with IC_{50} 9.75 and 7.90 mM, respectively (Table 1).

Leishmaniasis causes high morbidity and mortality levels and is famed as a main tropical public health problem by the WHO[21]. The available drugs for treating it are expensive, toxic and sometimes ineffectual[22]. The insistent demand for substitutional treatments led to natural products screening for their potential use in leishmaniasis therapy. The anti-leishmanial activity of compounds 1-4 was assessed against *L. donovani* promastigotes. It is noteworthy that 1 and 2 compounds exhibited anti-leishmanial potential towards *L. donovani* with IC_{50} 2.31 and 3.47 mM, respectively and IC_{90} 5.67 and 3.89 mM, respectively compared to pentamidine (IC_{50} 0.85 mM and IC_{90} 1.04 mM). However, compound 3 showed weak activity (Table 2). These findings suggested that both 1 and 2 compounds could be potential anti-leishmanial compounds. Malaria is an infectious illness caused by four *Plasmodium* species and is considered the world most devastating human parasitic infection[23]. The multidrug resistance to the existed anti-malarial agent demands the discovery of novel drugs and treatment efforts to eliminate this lethal disease. Endophytic fungal metabolites possess a great diversity of chemical constituents and have been assessed for anti-plasmodial effect as potential provenances of new anti-malarial agents[7,10,24]. The anti-malarial activity towards chloroquine resistant (W2, Indo-China) and sensitive (D6, Sierraleon) strains of *Plasmodium falciparum* was tested. The results showed that only compound 2 exhibited activity against both chloroquine-sensitive strain of *P. falciparum* (D6 S1 clone and D6 clone) with IC_{50} 2.16 and 1.43 mM, compared to chloroquine (IC_{50} 0.52 and 0.011 mM, respectively). However, the rest of compounds showed weak activity (Table 3). Thus, compound 2 could be a promising anti-malarial drug lead.

Table 4: Results of binding affinity assay of tested butyrolactones for human cannabinoid (subtypes CB1 and CB2) and opioid receptors (subtypes d, k and m)

Compound numbers	CB1% displacement	CB2% displacement	δ% displacement	κ% displacement	μ% displacement
1	71.2±0.87*	48.7±0.33*	56.4±0.29*	0	35.8±0.15*
2	80.5±0.99*	0	61.2±0.51*	73.5±0.67*	61.3±0.91*
3	69.8±0.76*	0	40.1±0.35*	0	43.5±0.76*
4	66.1±0.59*	0	20.4±0.08*	0	33.8±0.21*
CP-55,940	91.56	102.98	-	-	-
Naloxone	-	-	99.7	101.5	101.9

Each value represents the Mean±SEM, n = 3, *Significant different from control at p<0.05

Table 5: Results of cytotoxic activity of tested butyrolactones

	IC_{50} (mM)			
Compound numbers	SK-MEL	KB	BT-549	SKOV-3
1	0.70±0.06*	14.0±0.90*	4.6±0.22*	3.4±0.10*
2	0.90±0.11*	1.2±0.09*	0.1±0.07*	0.8±0.01*
3	9.20±0.19*	18.0±1.10*	6.0±0.11*	12.1±0.09*
4	11.30±0.86*	14.7±0.95*	15.1±0.82*	10.9±0.34*
Doxorubicin	0.121	0.028	0.041	0.319

Each value represents the Mean±SEM, n = 3, *Significant different from control at p<0.05

In addition, they were assessed using *in vitro* radioligand binding affinity assays of opioid (subtypes δ, κ and μ) and cannabinoid (CB1 and CB2) receptors. Compounds 1-4 (Conc. 10 μM) selectively inhibited 71.2, 80.5, 69.8 and 66.1%, respectively of the specific binding of [^{3}H]-CP-55,940 to HEK cell membranes representing CB1.

Moreover, only compound 1 showed moderate inhibition with affinity 48.7% of [^{3}H]-CP-55,940 specific binding to HEK (human embryonic kidney) cell membranes representing CB2. Interestingly, compounds 1 and 2 inhibited 56.4 and 61.2% of [^{3}H]-DPDPE specific binding to HEKhDOR cell membranes expressing d-opioid receptors at 10 μM, respectively. On the other hand, compounds 3 and 4 showed moderate affinity. Only, compound 2 displaced 73.5% of the specific binding of U-69,593 radioligand to HEKhKOR cell membranes expressing k-receptors. Moreover, compound 2 inhibited 61.3% of [^{3}H]-DAMGO specific binding to HEKhMOR cell membranes expressing m-receptors. While, compounds 1, 3 and 4 showed moderate inhibition with affinity values 35.8, 43.5 and 33.8%, respectively (Table 4). This is the first account of the estimation of the affinity of this kind of compounds towards human opioid and cannabinoid receptors. These receptors are G-protein coupled receptors and have been long recognized to control pain[32]. Several subtypes of cannabinoid and opioid G-protein receptor systems have been identified, the opioid receptor system involves mainly μ (mu), κ (kappa) and δ (delta) receptors, while the cannabinoid receptor system includes CB1 and CB2 receptors. Agonists of cannabinoid and opioid receptors are known to produce powerful analgesia and have been explored pharmacologically for the treatment of various neuropathic pains[25,26]. Particular interest has been concentrate in CB2 as a goal for treating neuropathic pain, various inflammations and various pathologies[27]. The CB1 is found primarily in the central nervous system (CNS) in areas involved in reward, regulation of appetite and nociception. It has been implicated in cancer, Alzheimer's disease, pain and obesity. CB1 receptor agonists are able to impair cognition and memory, to alter the control of motor function and to produce anti-nociception[28-30]. It is noteworthy that tested compounds showed good selective cannabinoid and opioid receptors receptor binding affinity. Thus, these compounds could be potential anti-inflammatory. Hence, this study may also provide as additional information on the possible analgesic and anti-inflammatory properties of the butyrolactones which are not reported earlier. Furthermore, compounds 1-4 were assessed for their *in vitro* cytotoxic effect towards KB, SK-MEL, BT-549 and SKOV-3 cell lines. Compounds 3 and 4 showed weak activity towards tested cancer cells compared to doxorubicin. However, compound 1 showed highest activity against SK-MEL with IC_{50} values of 0.70 mM compared to doxorubicin (IC_{50} 0.121 mM) and moderate effect towards SKOV-3 and BT-549 with IC_{50} 3.4 and 4.6 mM, respectively compared to doxorubicin (IC_{50} 0.041 and 0.319 mM, respectively). Compound 2 exhibited highest activity towards KB, SK-MEL, BT-549 and SKOV-3 cell lines with IC_{50} values of 1.2, 0.9, 0.1 and 0.8 mM, respectively (Table 5).

CONCLUSION

Among the tested butyrolactones, compounds 1-2 had anti-microbial, anti-leishmanial, cytotoxic and anti-malarial capacities. Compounds 1-4 exhibited good binding affinity towards the CB1 receptor. Moreover, compound 2 showed affinity to d-, k- and m-receptors. The *in vitro* binding affinity of butyrolactones on human cannabinoid and opioid receptors is revealed for the first time in this study. These

results confirmed that these compounds are considered as lead structures for novel CB1 selective ligand. This could be one of the possible mechanisms of the anti-inflammatory effect of butyrolactones. However, both intensive *in vitro* and *in vivo* investigations are required to confirm these effects. Moreover, further *in vitro* examinations are necessary to explore the possible mechanisms of these metabolites.

SIGNIFICANCE STATEMENTS

This study will help the researcher to know more about the pharmacological effects of butyrolactones. The study would aid the researcher to discover the significance of γ-butyrolactones in the development of potent, safe and effective anti-inflammatory, anti-microbial, anti-malarial, anti-leishmanial and cytotoxic agents. Furthermore, from the viewpoint of synthetic organic chemistry, the γ-butyrolactones are challenging and interesting targets to test novel synthetic strategies.

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