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Pharmacological investigations of natural β_2-adrenoceptors agonists on rat uterus *in vitro* and *in silico* studies

Ph.D. Thesis

By

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Szeged, Hungary

2012

~~xX♥@ DEDICATION @♥Xx~~

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I dedicate this work

To my lovely parents,

To my wife and kids

To my brothers and sisters

To all whom I love

With my deepest love and

Respect.

~~xX♥@ Aimun @♥Xx~~

Publications list

Publications related to the PhD thesis

1. **Aimun Abdelgaffar Elhassan Ahmed**, Robert Gaspar, Arpad Marki, Andrea Vasas, Mahmoud Mudawi Eltahir Mudawi, Judit Hohmann and George Falkay.
   Uterus-Relaxing Study of a Sudanese Herb (El-Hazha).
   American J. of Biochemistry and Biotechnology 6 (3): (2010) 231-238, …….. **IF: 1.493**
2. **Aimun AE. Ahmed**, Arpad Marki, Robert Gaspar, Andrea Vasas, Mahmoud M.E. Mudawi, Balázs Jójárt, Judit Verli, Judit Hohmann, and George Falkay.
   β_2-Adrenergic activity of 6-methoxykaempferol-3-O-glucoside on rat uterus: *in vitro* and *in silico* studies.
   European Journal of Pharmacology 667 (2011) 348–354……..... **IF: 2.737**
3. **Aimun AE. Ahmed**, Arpad Marki, Robert Gaspar, Andrea Vasas, Mahmoud M.E. Mudawi, Balázs Jójárt, Renáta Minorics, Judit Hohmann, and George Falkay.
   In vitro and *in silico* pharmacological investigations of a natural alkaloid.
   Medicinal Chemistry Research, DOI:10.1007/s00044-011-9946-0,……..... **IF: 1.058**

Other publication

Ahmed A EE, Eltyeb I B, Mohamed A H.
Pharmacological activities of Mangifera indica Fruit Seed Methanolic Extract.
Omdurman Journal of Pharmaceutical Sciences (2006), **1**(2): 216-231, (Local Sudanese).

Abstract and presentation related to the thesis

In vitro and *in silico* pharmacological investigations of natural β_2-Adrenoceptors agonists. The 3rd PharmSciFair Conference, June 13-17, 2011, Prague, Czech Republic.

Contents

List of abbreviations

- AH2: Methanolic extract of the El-hazha herb (*Haplophylum tuberculatum)*.
- β_2-ARs: β_2-Adrenoceptors
- BA: The estimation equilibrium binding affinity of drug candidate calculation as the experimental free energy of binding (ΔG_E) from the experimental data.
- cAMP: Cyclic *adenosine monophosphate*.
- EC_{50}: The concentration of the drug producing 50% of their maximal relaxing effect.
- E_{max}: The maximal relaxing effect of the drug.
- GPCRs: G protein-coupled receptors
- HAP: haplopine-3,3'-dimethylallylether.
- HMM: Homology molecular modeling
- ICI118,551: 3-(isopropylamino)-1-[(7-methyl-4-indanyl)oxy]butan-2-ol (β_2-ARs antagonist).
- LE: Ligand efficiency.
- 6-MKG: 6-methoxykaempferol-3-*O*-glucoside.
- MOE: Molecular Operating Environment software.
- TBM: template-based protein modelling.
- RMSD: Root mean square deviation
- pA_2: Negative logarithm of the competitive antagonist concentration at which the agonist concentration should be doubled to reach the same effect that without the antagonist.
- pdb: Protein Data Bank file format.
- pD_2: Negative logarithm of the non-competitive antagonist concentration that reduces an agonist effect to its $E_{max}/2$.

1. INTRODUCTION

1.1 Premature labour as medical challenge

A normal pregnancy should last about 40 weeks. Occasionally, labour may begin prematurely before the 37th week of pregnancy because uterine contractions cause the cervix to open earlier than normal. When this happens, the baby is born premature and can be at risk for health problems [1]. The specific causes of premature labour are not known and is still medical challenge [2] due to its incidence has not decreased recent several decades [3,4].and no effective primary means of its prevention [5]. It was estimated that in 2005, 12.9 million births, or 9.6% of all births worldwide, were preterm. Approximately 11 million (85%) of these preterm births were concentrated in Africa and Asia, while about 0.5 million occurred in each of Europe and North America (excluding Mexico) and 0.9 million in Latin America and the Caribbean. The highest rates of preterm birth were in Africa and North America (11.9% and 10.6% of all births, respectively), and the lowest were in Europe (6.2%) [6,7].

1.1.1 High risk factors for premature labour

Self ability: Women are at greatest risk for premature labour if:

- They are pregnant with multiples [8].
- They have had a previous premature birth.
- They have certain uterine or cervical abnormalities [9,10].

Medicals [11]*:*

- Recurring bladder and/or kidney infections.
- Urinary tract infections, vaginal infections, and sexually transmitted infections
- Infection with fever (greater than 101 degrees F) during pregnancy.
- Unexplained vaginal bleeding after 20 weeks of pregnancy.
- Chronic illness such as high blood pressure, kidney disease or diabetes.
- Multiple first trimester abortions or one or more second trimester abortions.
- Underweight or overweight before pregnancy.
- Clotting disorder (thrombophilia).
- Being pregnant with a single fetus after *in vitro* fertilization.

- Short time between pregnancies (less than 6-9 months between birth and beginning of the next pregnancy).

Life style [12,13]*:*

- Little or no prenatal care.
- Smoking.
- Drinking alcohol.
- Using illegal drugs [14].
- Domestic violence, including physical, sexual or emotional abuse.
- Lack of social support.
- High levels of stress.
- Low income.
- Long working hours with long periods of standing [15].

1.1.2 Tocolysis as medical solution for premature labour

Tocolytic agents are drugs designed to inhibit the contractions of myometrial smooth muscle cells. The aim of tocolysis is not only to stop uterine contractions and to prevent preterm delivery, but also to decrease the prenatal morbidity and mortality associated with preterm birth [16]. The main drugs used as tocolytics are indomethacin and other prostaglandins inhibitors [17], calcium channel blockers such as nifedipine [18,19], β-adrenergic agonists and oxytocin receptors antagonist, while the medical prevention consists of antibiotic or progesterone administration [20].

One of the most well-known mechanism of action through which a tocolytic agent acts was to relax the uterus, so generally any agent has ability to relax the uterus can be considered as a tocolytic agent. Clouse [2] demonstrates that relaxation of rat myometrium is mediated by β_2-adrenoceptors, also α_1/β-adrenoceptor ratio determines not only the spontaneous motor activity of the rat uterus, but also the potency of the agents with tocolytic effect [21].

In clinical practice many drugs were used to inhibit the preterm labour incidence such as, magnesium sulfate is a medication given through an iv. A large dose is given initially and then a smaller continuous dose is given for 12-24 hours or more. Whilst corticosteroids are medication given 24 hours before birth to help accelerate the baby's lung and brain maturity.

Tocolytics are sometimes used to decrease the frequency of contractions, and may make women feel better. Among these β_2-ARs agonists were of therapeutic potential due to their use for asthma [22] and to inhibit preterm labour [19] as tocolytic agent.

Numerous pharmacological agents have been utilized experimentally to inhibit preterm labour, but none has proven to be ideal. β_2-ARs agonists provide the best combination of safety and efficacy [23], also a combination of nifedipine and β_2-adrenoceptors agonists should be considered for the treatment or prevention of preterm birth [24,25].

A few β_2-ARs agonists were from natural origin. This information encourages us to attempt to isolate and test the pharmacological features of the isolated compound on β_2-adrenergic system.

1.2 Ethno-pharmacology of El-hazha

During search for new β_2-adrenoceptors agonists from natural origin to solve main medical challenge pre-term labour, a Sudanese herb El-hazha (*Haplophyllum tuberculatum*) (Forssk.) A. Juss. (Rutaceae); was selected as a target source due to its extensive traditional uses in this area [26]. The herb is used in Sudan as an antispasmodic, to treat allergic rhinitis [27] and gynaecological disorders, asthma and breathing difficulties.

This plant was also well-known among herbalists and widely used traditionally in other counties such as Saudi Arabia [28] and Oman to treat skin discoloration, infections and parasitic diseases [29] due to its importance, new species were discovered recently in Spain [30].

Its essential oils were investigated for antimicrobial activity by Alburtamani *et al*. [31] and were found to cause partial inhibition of the growth of *Escherichia coli*, *Salmonella choleraesuis* and *Bacillus subtilis* to the same extent as gentamycin sulfate.

Its cardiovascular effect were studied by Mohamed e*t al.* [27], who reported that its aqueous extract significantly decreased the contractility and the heart rate, but did not affect the flow rate of the isolated perfused rabbit heart. This action was not blocked by atropine, but the muscarinic antagonist blocked the fall in blood pressure was seen when the extract was administered to anaesthetized cats. The extract also stimulated rabbit aortic strip, rat vas

deferens and rat anococcygeus muscles. These adrenergic effects were largely reduced by phentolamine.

Its hepatoprotective activity was investigated by Ali *et al.* [31] on the liver damage induced by paracetamol in mice and proved to be relatively ineffective protecting only 16% of the animals against the lethal effect of paracetamol (1 g/kg) in comparable to that of the standard hepatoprotective agent silymarin.

When its cytotoxic activity was checked against 11 tumor cell lines, strong cytotoxic activity was observed [33].

Both its uses to relax the uterus and to treat asthma and inspiration difficulties catalyzed us to carry out this study to evaluate their effects in order to find a new therapeutic agent(s) to aid in solving of two major medical challenges (preterm labour inhibition and asthma control). Literature survey revealed that the aqueous extract of this plant obviously possess contracting activity, whilst traditional uses suggested contradictory applications such as muscle relaxant and contracting effect in the same time.

On the other hands and from phytochemical's point of view, many compounds were isolated from this plant, including alkaloids [31,34,35]. Among these compounds, 6-methoxykaempferol-3-*O*-glucoside (6-MKG) [36] and haplopine-3,3'dimethylallylether (HAP) [37] were isolated and indentified early.

Although literature revealed their isolation and identification their pharmacological profile remains undetermined or controversial. In this study we attempt to proof and determine the pharmacological profile of both 6-MKG and HAP based on their uterus-relaxing activity using β_2-adrenergic receptors as a main target.

1.3 β_2-Adrenoceptors

β-Family of adrenergic receptors have three subtypes: β_1, β_2, and β_3 that share high sequence similarity among these subtypes, whilst differences in the mechanisms of ligand entry and exit may play a role in receptor subtype selectivity of ligand binding [38].

β_2-ARs are belong to the G protein-coupled receptors (GPCRs) which are the largest family of cell-surface receptors involved in signal transduction and are encoded by the largest gene family in most animal genomes.

β_2-ARs represent one of the most important drug discovery targets [39,40]. Among the GPCRs, β_2-ARs serve as the targets of 50% of drugs in the market [38].

The active binding site of β_2-ARs was determined from the crystal structure of the β_2-AR–carazolol complex structure, where residues Asp^{192} and Phe^{193} of ECL2 and Lys^{305} and Tyr^{308} of TM7, whilst there is a salt bridge between Asp^{192} and Lys^{305}. Carazolol formed close interactions with several polar transmembrane residues Asp^{113} (TM3), Asn^{312} (TM7), Tyr^{316} (TM7), Ser^{203} (TM5), and Ser^{207} (TM5) were considered as finger print for optimum activity, whilst the ECL2 was found to be critical to ligand-binding kinetics due to its conformational flexibility [41].

β_2-ARs acts through a 2^{nd} messenger cAMP. GPCRs are in a conformational equilibrium between inactive and activating states [42] and binding of and activating ligand enables the receptor to catalyze the exchange of GTP for GDP in a heterotrimeric G protein (Fig. 1). The conformation of the TM5–ICL3–TM6 region is linked to the equilibrium between inactive and active forms of the receptor [43], and stabilized by the third intracellular loop (ICL3) that links TM5 and TM6, which is a major site of interaction with G proteins [44].

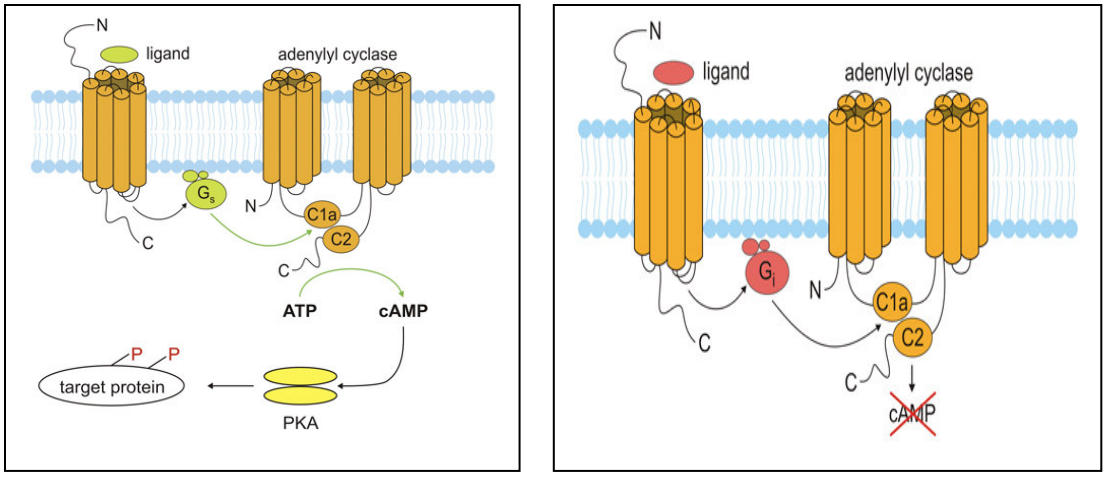


Fig. 1. Activation and inhibition of the β-ARs by a ligand and the release of the (cAMP).

The main physiological functions for β_2-ARs are bronchial and vascular smooth muscle relaxation [45,46].

It is believed that G-protein-coupled receptors are the most promising target proteins for drug research, thus β_2-ARs are an important pharmaceutical target for pulmonary and

cardiovascular diseases [47]. They are also useful in the treatment of nervous system injury and premature labour. Major pharmaceutical industries are investing enormous amounts of time and money to achieve this object. This study is a bird's eye view on the some aspects β_2-adrenoceptors in drug discovery.

Useful examples of some β_2-ARs agonists and antagonists and their efficacy from Yao *et al.* [48], were isopretronol (full agonist); epinepherine (full agonist); norepinepherine (strong partial agonist); salbutamol (partial agonist); dopamine (weak partial agonist); halostachine (very weak partial agonist); catechol (very weak partial agonist); alprenolol (natural antagonist) and ICI118,551 (inverse agonist). Whilst fenoterol, formoterol, salmeterol, clenbuterol, bambuterol, indacaterol (β_2-selective agonists).

1.4 Molecular homology modelling and molecular docking

In the drug discovery field different techniques were used [49], the common direct one is once the target was identified, drug search starts in which a mixture of experimental and computer-based methods were involved [50,51].

Recently, *in silico* modeling of receptor-ligand interactions has become a common reference to biological studies carried out in the computer, joining the traditional terms *in vivo* and *in vitro* to describe the location of experimental studies [52,53]. These methods act as complementary to the classical pharmacology and ligand screening ones [54,55], aimed to saving time and money in order to speed-up the research signals [56].

Nowadays*, in silico* homology modeling was used extensively to prepare the drug targets [56]. The crystal structure of β_2- ARs was determined in 2007 by Kobilka and his group [57] while important modification was made by de Graaf and Rognan [58], thus used in this study, as a template for homology modeling process [59] with more realistic resembling results.

Also Hetenyi *et al.* [60] reported that we can use the *in silico* data using the same useful equation (Eq. I) for the estimation equilibrium binding affinity (BA) of drug candidate calculation as the experimental free energy of binding (ΔG_E) from the experimental data to calculate the various Ligand efficiency (LE) values on the basis of a set of biologically relevant structural and thermodynamic experimental data.

Protein modelling was a challenge in drug discovery, because predicting the accurate 3D structure of proteins has always been and remains a complicated assignment [61]. In template-based protein modelling (TBM), the accuracy of protein structures, particularly their binding sites, is essential for the success of modeling protein complexes. Overall, approximately 50% of complexes with their interfaces modeled by high-throughput techniques had accuracy suitable for meaningful docking experiments. This percentage will grow with the increasing availability of co-crystallized protein-protein complexes [62].

TBM structure techniques rely on the study of principles that dictate the 3D structure of natural proteins from the theory of evolution viewpoint [64], recently, this modeling type becomes a popular modeling.

TBM involves several steps; identification of homologues (templates), alignment of target to template, structure building, refinement and validation.

Target protein can be aligned to the template using sequence-sequence, sequence-profile or profile-profile alignments. This is called pair-wise or single or multiple sequence alignment.

Standard homology molecular modeling (HMM) which always selects the sequence with the best score led to a lower root mean square deviation (RMSD) relative to the available crystal structure compared to optimized sequence alignment generated using HMM.

TBM has been widely used in the process of drug design and discovery [64]. Various recent applications of homology modeling in the drug discovery process include lead identification [65,66], lead optimization, understanding of selectivity [67], explanation of resistance development [68], binding site analysis and mutation studies [69].

After building the target, molecular docking of ligands were performed to determine the interaction between the ligand and receptor molecule (Fig. 2), because docking technique was a good predictor of molecule pharmacologic chaperoning capability [70]. Discovery of novel DNA gyrase and HIV protease inhibitors were developed by using docking simulations [71,72].

Computational approaches that dock small molecules into the structures of macromolecular targets and score their potential complementarily to binding sites are widely

used in hit identification and lead optimization based on structure-based design and screening strategies [72].

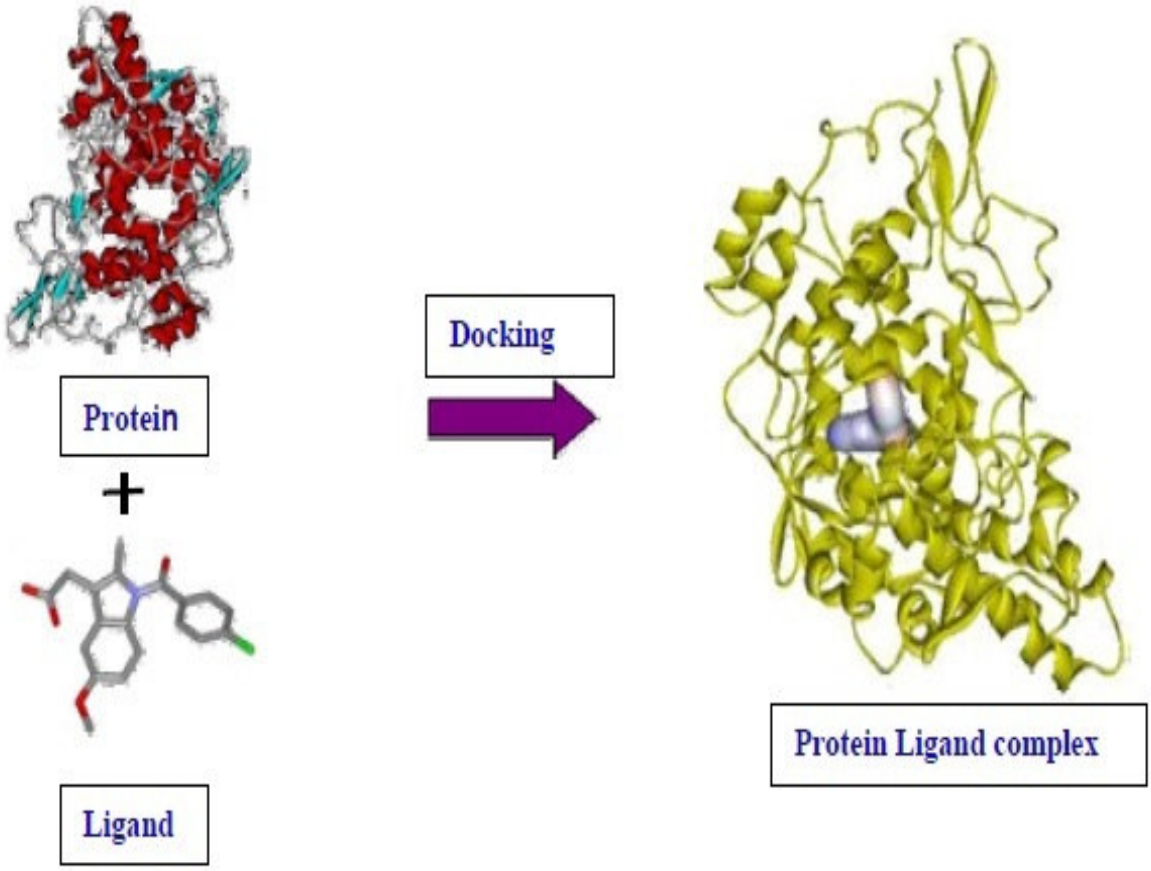


Fig. 2. Schematic diagram of docking approach after determination of the target and the ligand.

2. AIMS

Plants still a rich source for drugs, and a recent trend in medicine to return to the nature in treating disease and to discover new therapeutic agents.

This study was an attempt to proof and evaluate some traditional uses of El-hazha herb in gynaecological area as uterus-relaxing agent, to isolate and characterized its active agents based on the above mentioned activity using β_2-adrenergic receptors as a main target.

General aim:

- To find a new potential therapeutic natural tocolytic agent(s) or substantial lead substance from plant origin to aid in solving the medical challenge premature labour.

Specific aims:

- To investigate the relaxing activity of El-hazha herb regarding to its traditional uses on non-pregnant and late-pregnant rat uteri *in vitro*.
- Attempt to isolate pure active compound(s) based on this relaxing activity by biological-guided fractionation and by using β_2-adrenoceptors as a main target.
- To determine the pharmacological profiles of isolated compounds using *in vitro* and *in silico* techniques.

3. MATERIALS AND METHODS

3.1 Chemicals

Plant material

The aerial parts of *Haplophylum tuberculatum* (El-haza) were collected in the North part of Sudan (Abu hamad, Nahr El-Neel State) in November 2008 and identified by Dr. Wai'l E abdalla and Yahia S. Mohamed [Medicinal and Aromatic Plants Research Institute (MAPRI), Khartoum, Sudan]. A voucher specimen (No. M23/08) has been deposited at the Herbarium of MAPRI.

Standard drugs

Terbutaline, propranolol, progesterone, prazosin, non-specific phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) (1mM) and forskoline were purchased from Sigma-Aldrich (Hungary), whilst ICI118,551 was purchased from TOCRIS (Hungary).

Radioligand

The radioligands [^{3}H] dihydroalprenolol (DHA), β-adrenergic antagonist and [^{3}H]ICI118,551, selective β_2-adrenergic antagonist were purchased from Amersham International plc (UK).

Assay Kits

The commercial competitive cAMP Enzyme Immunoassay (EIA) Kit was purchased from (Sigma-Aldrich Ltd, Hungary).

3.2 Animals

Ethical considerations for housing and handling the animals

The animals were treated in accordance with the European Communities Council Directives (86/609/ECC) and the Hungarian Act for the Protection of Animals in Research (XXVIII.tv.32. §). All experiments involving animal subjects were carried out with the approval of the Hungarian Ethical Committee for Animal Research (registration number: IV/1758-2/2008). Sprague-Dawley rats (Charles-River Laboratories, Hungary) were kept at 22 ± 3 o C, the relative humidity was 30 – 70% and the light/dark cycle was 12/12 h. They

were maintained on a standard rodent pellet diet (Charles-River Laboratories, Hungary) with tap water available *ad libitum*. The animals were sacrificed by CO_2 inhalation.

Mating of the animals

Mature female (180-200 g) and male (240-260 g) rats were mated in a special mating cage. A metal door, which was movable by a small electric engine, separated the rooms for the male and female animals. A timer controlled the function of the engine. Since rats are usually active at night, the separating door was opened before dawn. Within 4-5 h after the possibility of mating, vaginal smears were taken from the female rats, and a sperm search was performed under a microscope at a magnification of 1200 times. If the search proved positive, or if smear taking was impossible because of an existing vaginal sperm plug, the female rats were separated and were regarded as first day-pregnant animals.

3.3 Softwares

The main softwares that used during our study were:

1- The AutoDock4 with AutoDockTools 1.5 were obtained freely via their official internet site [73].

2- Molecular Operating Environment (MOE) was purchased from Chemical Computing Group Inc., Canada [74].

3- The scientific graphing, curve fitting and statistics package, Prism 4.0 was purchased from GraphPad software Inc., USA.

3.4 Extraction, Fractionation, Isolation and identification of the active compounds

The methanolic-maceration of the plant produced a yield's percentage of (5.5%), while the steps of fractionations were highlighted in a summary diagram (Fig.3) emphasizing on that mentioned as most active pharmacologically and used throughout the study. At the end tow active compounds were isolated from the dried aerial parts of El-hazha by biologically guided fractionation and their structures were determined by NMR spectral data analysis. The produced amount was reconstituted by distilled water or DMSO and diluted by distilled water to get the desired concentration for all pharmacological tests.

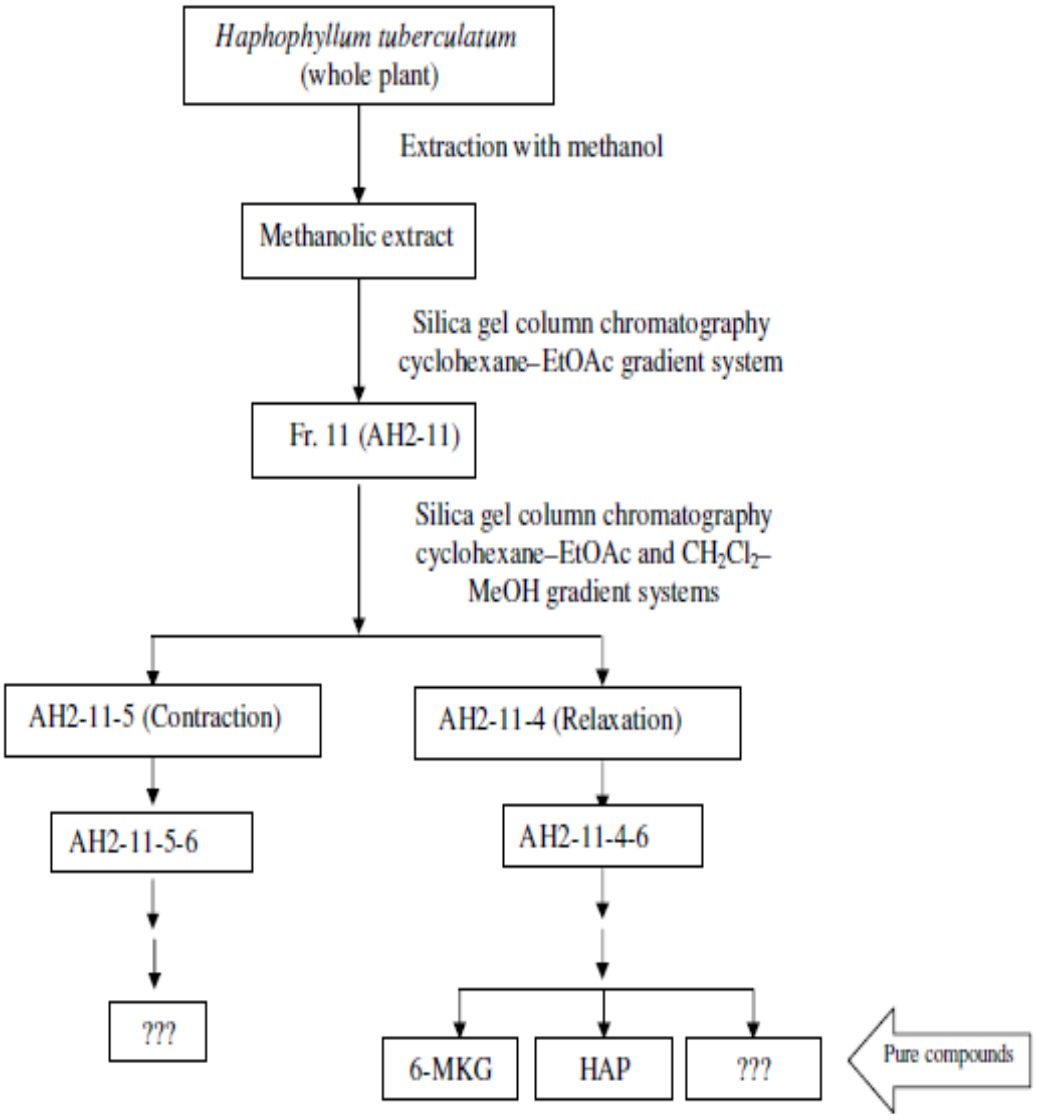


Fig. 3. Diagram illustrated the main steps of Bioactivity-guided fractionation of the methanolic-extract of El-hazha emphasized on the most active fractions resulted and used throughout the study. 6-MKG: 6-Methoxykaempferol-3-*O*-glucoside [75], HAP: haplopine-3,3'-dimethylallylether [76].

3.5 Pharmacological methods

3.5.1 In vitro studies

Isolated organ bath studies

Uteri were removed from rats (250–350 g) on non-pregnant and day 22 of pregnancy (270–350 g). Muscle rings 5 mm long were sliced from the uterine horns and mounted vertically in an organ bath containing 10 ml de Jongh solution (composition: 137 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 4 mM NaH_2PO_4, 6 mM glucose, pH=7.4). The organ bath was maintained at 37 °C and carbogen (95% O_2+5% CO_2) was bubbled through it. After mounting, the rings were equilibrated for about 1 h before the experiments were undertaken; with a solution change every 15 min. The initial tension of the preparation was set to about 1.5 g, which was relaxed to about 0.5 g at the end of equilibration. The tension of the myometrial rings was measured with a gauge transducer (SG-02; Experimetria Ltd., Budapest, Hungary) and recorded with a SPEL Advanced ISOSYS Data Acquisition System (Experimetria Ltd., Budapest, Hungary). Uteri were pre-contracted by 25 mM KCl and cumulative concentration-response curves were constructed in each experiment for 6-MKG, HAP or terbutaline in different concentrations (range from 10^{-9} to 10^{-4} M) in the presence and absence of ICI 118,551 (10^{-5} M). Following the addition of each concentration of the tested material, recording was performed for 5 minutes. Concentration-response curves were fitted; E_{max}, EC_{50} and pA_2 values were determined and compared statistically using computer program Prism 4.

Progesterone treatment of pregnant rats

The progesterone treatment of the pregnant rats was started on day 15 of pregnancy. Progesterone was dissolved in corn oil and injected subcutaneously every day up to day 21 at a concentration of 0.5 mg/0.1 ml/kg. On day 22, the uteri were collected and the organ bath studies were performed as described above. The experimental data on the non-treated and the progesterone-treated animals were analysed statistically.

Measurement of uterine cAMP accumulation

Uterine tissue samples from 22-day (intact term pregnant) pregnant rats were incubated in de Jongh solution as mentioned above. Cyclic AMP generation was stimulated with 6-MKG for 10 min, in the presence of the non-specific phosphodiesterase inhibitor3-

isobutyl-1-methylxanthine (IBMX) (1 mM), and Forskoline (10^{-5}M), then the samples were immediately frozen in liquid nitrogen and stored there until the extraction of cAMP [77]. Frozen tissue samples were ground, weighed, homogenized in ten volumes of ice-cold 5% trichloroacetic acid and centrifuged at 600×g for 10 min. The supernatants were extracted with three volumes of water-saturated diethyl ether. After drying, the extracts were stored at −70°C until the cAMP assay. Uterine cAMP accumulation was measured with a commercial competitive cAMP Enzyme Immunoassay (EIA) Kit (Sigma-Aldrich Ltd, Budapest, Hungary), and tissue cAMP levels were expressed in pmol/mg tissue.

Radioligand binding assay

Membrane preparation

The 22-day pregnant rats were killed as mentioned above. Tissues (brain or uterine) were cut and homogenized in buffer (0.01 M Tris–HCl, 0.25 M sucrose, pH 8.0) with an Ultra-Turrax T25 homogenizer, and centrifuged (20,000×g, 10 min, 4°C). The supernatants were stored at 4°C, and the pellets were re-suspended and re-centrifuged. After mixing, the supernatants were centrifuged (50,000×g, 60 min 4°C). The pellets were re-suspended, aliquoted and stored at −70°C for used in radioligand binding assays [78].

Displacement assay

The affinities of the tested compound 6-MKG and standard drugs (terbutaline, ICI118,551) for β_2-adrenergic receptors were measured on above mentioned membrane preparation using [^{3}H] ICI118,551 [79] as radioligand (concentration: 2 nM, specific activity: 18.8 Ci/mM). Under standard assay conditions, the final reaction mixture volume was 300 µl consisted of diluted membrane preparation (protein content approximately 0.5–1 mg/ml), 100 µl [^{3}H]ICI118,551 (specific activity: 18.8 Ci/mM) and 100 µl unlabeled ligand (10^{-5} M) for nonspecific binding or 100 µl incubation buffer (consisting of 0.05 M Tris–HCl, 0.01 M $MgCl_2$ and 2.5% ethanol, pH 7.42) for total binding. Following the incubation period, the membranes were collected on a Whatman GF/C filter, using a Brandel M24 Cell Harvester and washed with 3×10 ml ice-cold buffer (50 mM Tris–HCl, pH 7.42). The bound radioactivity was determined in a HighSafe scintillation cocktail using Wallac1409 liquid scintillation counter. Specific binding was determined by subtracting the nonspecific binding from the total binding values. All assays were carried out at least three times in duplicate,

and values are given as means ± SEM. Displacement experiments were individually analyzed, the affinity was determine by calculating the inhibition constants (K_i) using computer program Prism 4.

3.5.2 *In silico* studies

Preparation of the ligands

The structure of 6-MKG and HAP were drawn using Symyx Draw Editor software [80], and then converted to pdb file format and the structure was minimized by Molecular Operating Environment (MOE) software developed by Chemical Computing Group Inc. [74]. The pdb files of the standard ligands (epinephrine, norepinephrine, propranolol, isoproterenol and ICI118,551) were obtained from the PubChem except terbutaline, which was downloaded from DrugBank as a pdb file [81,82].

Homology model of rat β_2-adrenergic receptor

The homology model of rat β_2-adrenerg receptor was built using 'Homology modeling modul' of MOE 2009.10 with the ligand supported option. The template was the modified human β_2-adrenoceptors published by de Graaf and Rognan. [58], which is able to select full or partial agonists in virtual screening. After the alignment, certain residues were deleted from the sequence (1-29, 231-263 and 343-418, due to the absence of an appropriate template), which resulted in 91.5% homology identity (Fig. 10). During the modelling the MMFF94x force field was applied. After the model building the complex obtained was subjected to further refined using a modified method, as described in the de Graaf article: (1) distance restraints were applied between the appropriate heavy and hydrogen atoms; (2) minimization of the ligand position was performed with flexible side chain atoms of the interacting residues; (3) minimization with flexible residues, which were closer than 4.5 Å from any ligand atom and restraints applied remained intact; and (4) restraints were removed and final minimization was performed. We have applied these steps because without these restraints, the Ser^{203} and Ser^{204} side chains projected out from the active site. During the minimization steps the same force field was applied and the gradient was set to 0.05 kcal/mol $Å^2$.

Docking studies

In this study, the set of target compounds including 6-MKG were docked on rat β_2-adrenergic receptor binding site using AutoDock 4.2 software [73]. The initial structure of

the compounds was prepared in MOE and was minimized (grad <0.001) using the MMFF94 force field [83,87].

Before docking analysis, the docking files were prepared regarding to the AutoDock4 requirements using the AutoDock Tools 1.5.4, [88] in which all possible flexible bonds of the ligands, the partial atomic charges (Gasteiger–Marsili formalism), and the Kollman charges for all atoms in the β_2-AR were assigned. Finally, polar hydrogens were added on the receptor. All other parameters were kept at their default values. The grid box (60, 60, 60 Å) was centred (3.767, 14.309, 4.648) on the isoproterenol docked by [89] and the lattice point distance was set to 0.375 Å. This grid centre was used for all other ligands. All simulations used the hybrid Lamarckian Genetic Algorithm, with an initial population of 100 randomly placed individuals and 10 million maximum number of energy evaluations. The lowest energy cluster returned for each compound was used for further analysis. The interactions of the ligands on β_2-AR were visualized and the figures were created using AutoDock Tools v1.5.4, VMD 1.8.5 Software [90] and MOE.

3.5.3 Comparative *in silico* - *in vitro* ligand efficiency estimation

A set of standard β_2-adrenoceptors and the isolated compound HAP were explored in MOE to calculate different parameters and evaluated regarding their efficiencies in order to see the drug candidate properties. Although the scoring function of AutoDock easily can overestimate the ΔG_{bind} value because of its additive nature, even in case of larger ligands, AutoDock4 was used to obtain the ΔG values for our ligand *in silico* after docking. However, the ligand efficiency (LE) value was introduced to normalize the free binding energy values [91], ligand efficiency indices ($\Delta G/MW$ and $\Delta G/NHA$, respectively) were calculated for our ligands regarding the molecular weight (MW) and number of heavy atoms (NHA), where ΔG is the free energy of binding.

The following equation was used to calculate the LE from *in vitro* data:

$$LE = \frac{-RT \ln EC_{50}}{NHA} \quad \text{..... (Eq. I)}$$

Where, R: is the gas constant (1.9876 Kcal/mol); T: is the temperature (298.15 K); EC_{50}: is the concentration of the drug producing 50% of their maximal relaxing effect and NHA: is the number of heavy atoms in the ligand structure.

On the other hands, Bemebenek *et al.* [92] rules for identifying a drug candidate [The ΔG/NHA must be deeper than -0.24 kcal/mol, molecular weight lower than 500 g/mol and the number of heavy atoms between 20 and 70] were applied for our set.

3.6 Data Analysis

pA_2-value calculation

The pA_2-value for the antagonist ICI118,551 with all other agonists was calculated using the following equation:

$$pA_2 = pA_x + \log (x\text{-}1) \quad \text{..... (Eq. II)}$$

Where, pA_x = -log value of antagonist concentration. The pD_2 was calculated from the *in vitro* EC_{50} results then applied to the above mentioned equation (Eq. II).

Ligand efficiency calculation in silico and in vitro

The estimation equilibrium binding affinity (BA) of drug candidate calculation as the experimental free energy of binding (ΔG_E) from the experimental data was used to calculate the various LE's on the basis of a set of biologically relevant structural and thermodynamic experimental data using the above mentioned equation (Eq. I).

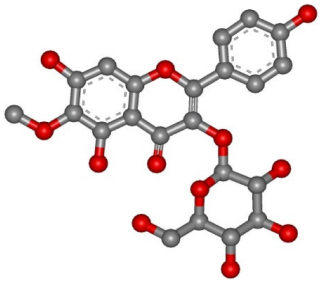
Statistical Analysis

The statistics was done by using Prism 4.0 (GraphPad Software, USA) computer program. For the statistical evaluations, data were analyzed by performing two-tailed unpaired t-test to compare the significance mean differences for various results together. The differences were considered to be significant at levels of $p \leq 0.05$.

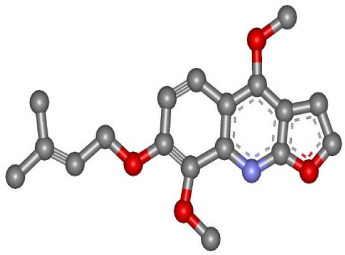
4. RESULTS

4.1 Isolated compounds

Two active compounds were isolated from the most active fraction. and their structure were determined by NMR spectral data analysis. Their structures were identical with those published by Wei *et al.* 2004 [10] and Al-Rehaily *et al.* 2003 [3], respectively. The chemical formulas, and the molecular weights are:



6-Methoxykaempferol-3-*O*-glucoside (6-MKG).
Ch. Formula: $C_{22}H_{22}O_{12}$
M. wt g/mol: 478.40



Haplopine-3,3'-dimethylallylether (HAP).
Ch. Formula: $C_{18}H_{19}NO_4$
M. wt g/mol: 313.13

4.2 *In vitro* results

4.2.1 Contractility studies on non-pregnant and late-pregnant rat uteri

Methanolic extract (AH2) produces basically reasonable relaxant effect on uterine contraction elicit by (25 mM KCl) in a dose dependent manner for both (NP) and (LP). This effect was changed and flocculated reasonably after several fractionations processes (Fig. 3). Also the two isolated compounds 6-MKG and HAP appears similar relaxant activity in both uteri, Fig. 4

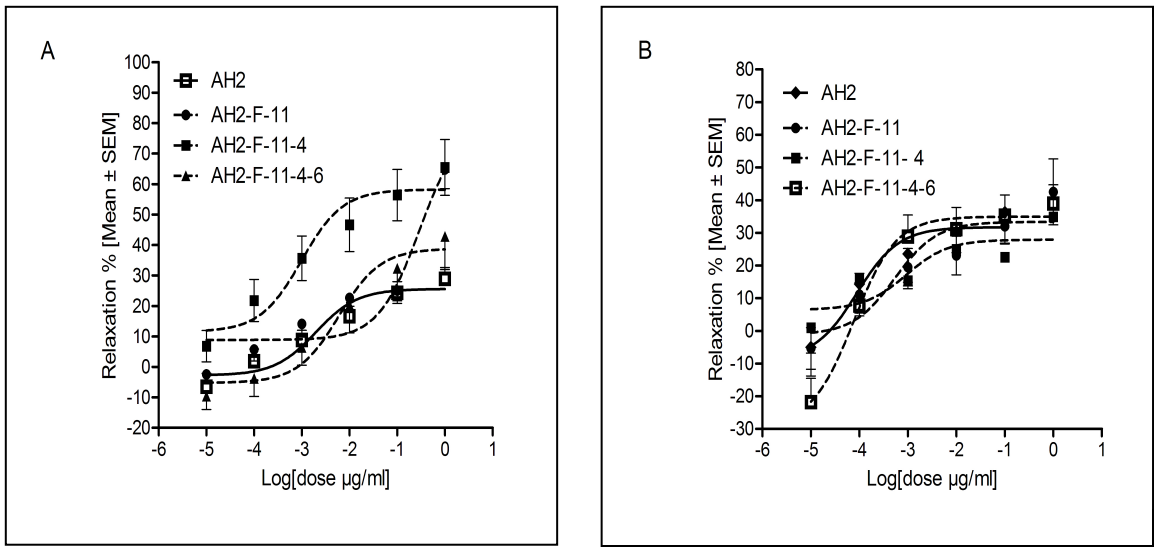


Fig. 4: Showed the (AH2) and its best fractions relaxant effects on (**A**) non-pregnant (NP) and (B) late-pregnant (LP) isolated rat uterus *in vitro*.

When the two compounds alone or in combination were compared to terbutaline (β_2-ARs agonist), *In vitro* results revealed that both compounds were able to relax both non-pregnant (Fig. 5A) and late-pregnant (Fig. 5B) uterine contractility with 50% and 80% of the E_{max} of terbutaline, whilst the EC_{50} was lower than that of terbutaline.

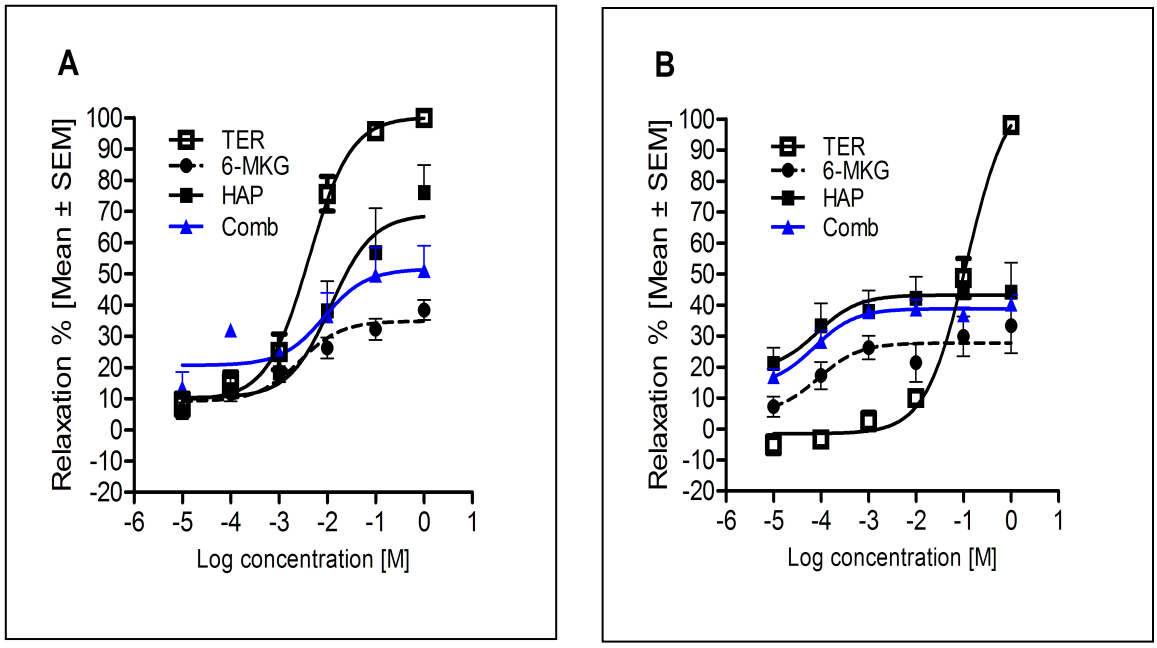


Fig. 5: Comparison of the relaxant effect of 6-MKG, HAP and their combination to the standard terbutaline on (**A**) non-pregnant (NP) and (**B**) late-pregnant (LP) isolated rat uterus *in vitro*.

Our study found this relaxant effect was mediated by β-ARs. The non-selective β-ARs blocker propranolol blocks competitively the effect of the most active extract fraction at preliminary steps of our study, while progesterone pre treatment of the (LP) rat uterus did not alter significantly the effect of this fraction.

The β_2-ARs antagonist ICI118,551 competitively antagonized the relaxing effect of both 6-MKG and HAP comparable to terbutaline (Table 1) and their combination Fig. 6. Besides similar effect was observed for their combination in both uteri, Fig. 5.

Table 1: Effect of ICI118,551 on the relaxing activities of terbutaline, 6-MKG, HAP and their combination on non-pregnant and late-pregnant rat uteri *in vitro*

Ligand	pA_2 [mean ± SEM], N=6-8		Emax [mean ± SEM]%, N=6-8			
			NP		LP	
	NP	LP	without ICI	with ICI	without ICI	with ICI
TER	**4.8 ± 0.3**	**2.9 ± 0.1**	**101.8 ± 0.5**	**74.6 ± 8.2**	**112.1 ± 1.7**	**25.2 ± 4.4**
6-KMG	4.5 ± 0.2	7.7 ± 0.4***	35.9 ± 2.3***	27.2 ± 3.9**	40.1 ± 1.3***	15.1 ± 3.6
HAP	4.7 ± 0.1	3.3 ± 0.02	80.8 ± 11.8*	16.5 ± 4.5**	51.8 ± 8.4***	35.2 ± 4.3
Combination	2.8 ± 0.6***	1.6 ±0.2***	52.1 ± 7.8***	40.9 ± 1.5*	39.0 ± 2.6***	33.4 ± 4.5

Terbutaline was used as positive control to compare and relate other to it throughout the table. TER: terbutaline, HAP: isolated compound; pA_2: negative logarithm of the antagonist concentration that reduces an agonist effect to its $E_{max}/2$; N: total number of observations; ICI: ICI18,551 *: p>0.05, **: p>0.01.

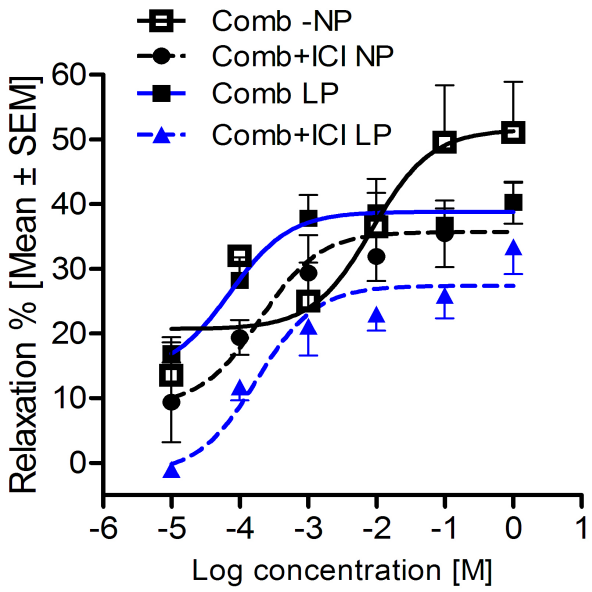


Fig. 6: The β_2-ARs antagonist ICI118,551 competitively antagonized the relaxing effect of 6-MKG and HAP combination in both uteri.

When the 6-MKG, HAP were added together, 6-MKG reduced significantly the activity of HAP in NP rat uterus Fig. 7A, whilst the addition of two compounds in the same time with terbutaline, both decreased the terbutaline effect in both uteri, Fig. 7B

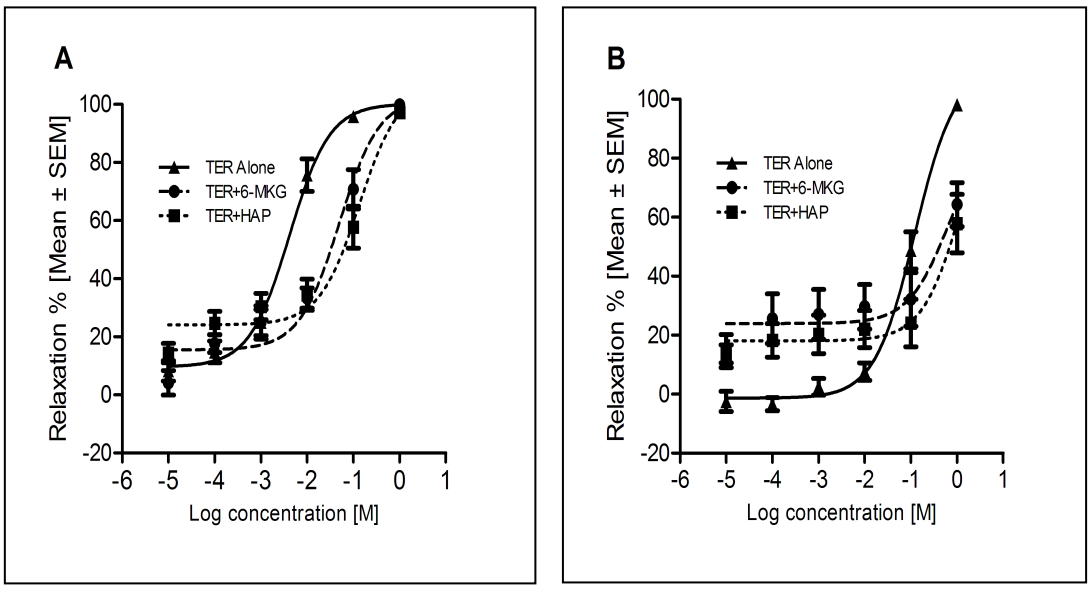


Fig. 7: Effect of 6-MKG, HAP on to the standard terbutaline relaxant effect on (**A**) non-pregnant (NP) and (**B**) late-pregnant (LP) isolated rat uterus *in vitro*.

4.2.3 Effect of 6-MKG on cAMP level

6-MKG induced the level for uterine cAMP on late-pregnant rat uterus, Fig.8

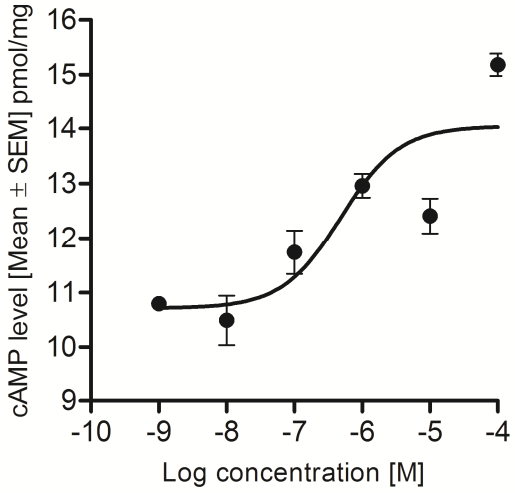


Fig. 8: Dose-response curves of the effect of 6-MKG on the cAMP induction level of the late-pregnant rat uterus in vitro. Values are means of 6-8 observations; vertical bars denote standard errors of the mean (S.E.M.).

4.2.4 Radioligand binding assay

The affinity of the methanolic-extract (AH2) and its most active sub fractions from different fractionation steps for β-adrenergic receptors were tested on rat brain membrane preparation, using tritiated dihydroalprenolol ([^{3}H] DHA, 2 nM) as a radioligand. All of the ligands displace the radioligand from the target receptor.

The AH2 displace the radiligand only in very high concentration (10^4 µg/ml) with un-estimated Ki ,while its other sub fractions showed better displacement affinities and the K_i values of AH2-11-4 and AH2-11-4-6 were (1.2 ±1.0) x10^{-2} , (1.1 ±1.1) x10^{-3} and 3.8 ± 3.8 µg/ml respectively (Fig. 9A).

The affinity of the terbutaline, ICI 118,551 and 6-MKG for β_2-adrenergic receptors were tested on 22-day pregnant rat uterine membrane preparation, using [^{3}H]ICI 118,551 as radioligand (2 nM). All of the ligands displace the radioligand from the target receptor, the K_i values of 6-MKG, terbutaline and ICI118,551 were 35.37±1.9, 479.9±2.0 and 181.9±1.6 nM respectively (Fig. 9B).

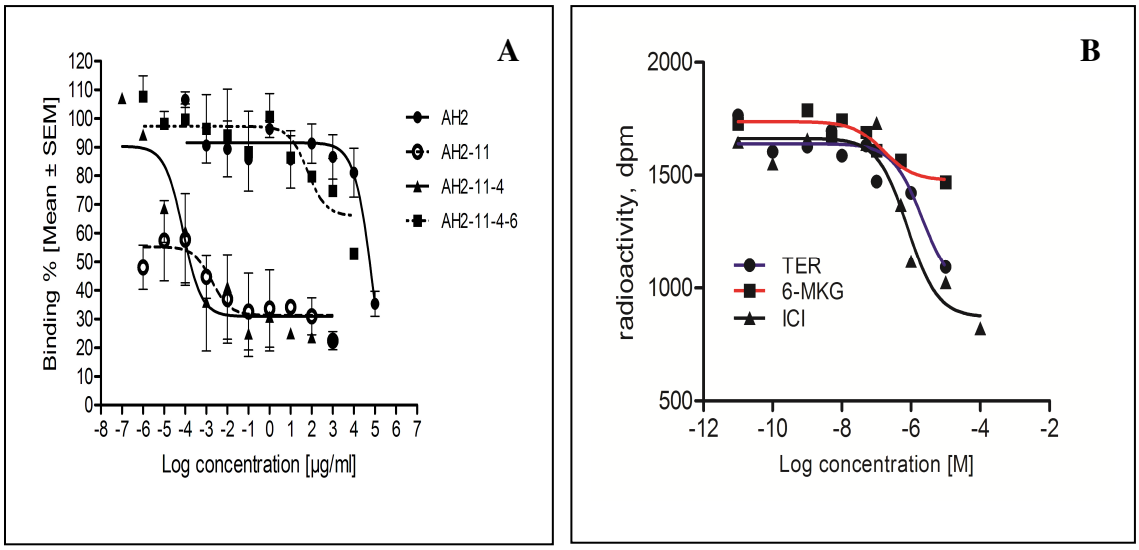


Fig. 9. (**A**) The affinity of the methanolic-extract and its most active sub fractions for β-ARs on rat brain membrane preparation, using Dihydroalprenolol [^{3}H] DHA (2 nM) as an isotopes radio ligand, (**B**) The affinity of the terbutaline, ICI 118,551 and 6-MKG for β_2-ARs on 22-day pregnant rat uterine membrane preparation, using [^{3}H]ICI 118,551 as radio ligand (2 nM).

4.3 *In silico* results

4.3.1 Homology modeling

The Customized rat homology modeling for the β_2-AR that modeled from the template of the modified human β_2-AR. The N-, and C-terminal parts of the receptor together with the 3rd intracellular loop was not included in our homology modelling routine, which resulted in 91.5 % homology identity (Fig. 10).

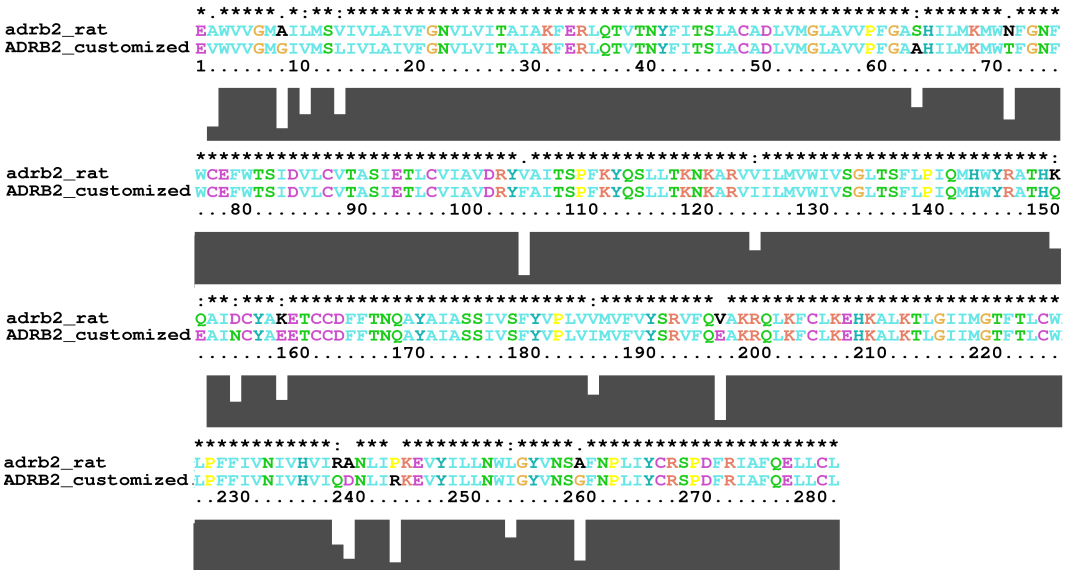


Fig. 10: Alignment between rat and human customized β_2-AR after deletion of the 1-29, 231-263 and 343-418 residues.

The αC RMSD (all residues) value between the template and the modelled structure was 1.04 Å, and the RMSD value for the binding site forming anchor points was 0.84 Å, oriented towards the receptor interior. The applied restraints kept the orientation of the Ser^{203} and Ser^{204} side chains toward the active site. The homology model was validated by docking of noradrenaline that detected all of the important interactions (Asp^{113}, Phe^{193}, Ser^{203}, Ser^{204}, Asn^{312}) therefore this receptor model was suitable for molecular docking calculations.

4.3.2 Molecular Docking

The results of docking studies of the six standard ligands and our compounds 6-MKG and HAP summarized in the Table 2.

All standard compounds showed strong interactions with the Asp^{113}, Phe^{193} and Asn^{312} residues, while adrenaline, noradrenaline and isoproterenol, which contains catechol ring, forms interactions with Ser^{203} and Ser^{204} too. The 6-MKG also interacts with common interaction points (Asp^{113} and Asn^{312}) residues which stabilize the receptor – ligand complexes, but it has a different interaction pattern. The glycopyranoside side chain forms an H-bond with $Cys^{191}$and Tyr^{316} but the benzopyrane ring did not interact with the Ser^{203} and Ser^{204} residues (Fig. 8). The 6-MKG displayed the lowest ΔG_{bind} and calculated K_d values, followed by ICI 118,551, propranolol, isoproterenol, terbutaline, adrenaline and

noradrenaline. In the radioligand binding assay, the same order of potencies was determined for 6-MKG, ICI 118,551 and terbutaline.

The HAP possesses the similar ΔG_{bind} and K_i values to terbutaline. There are three common interaction points (Thr^{118}, Phe^{193}, Asn^{312}) which stabilize the receptor-ligand complexes that present for terbutaline, while for the HAP Thr^{118} was replaced by Thr^{110} , Phe^{193} was absent and an unique basic bond (His^{93}) was present instead of Asn^{312}.

Table 2. Interaction points, estimated free energy of binding (ΔG_{bind}) and calculated K_d of docked ligands for the rat β_2-adrenoceptor. All of the mentioned interactions were H-bonds, except for that of Phe^{193}, which was a benzyl-benzyl interaction. ICI: ICI 118,551; PROP: propranolol; TER: terbutaline; ISO: isoproterenol; nADR: noradrenaline; ADR: adrenaline.

Interactions		Tested Ligands		Standard ligands					
Residue	Type	6-MKG	HAP	ICI	PROP	TER	ISO	nADR	ADR
Asp^{113}	acidic	+		+	+	+	+	+	+
Thr110	polar		++						
Thr^{118}	polar					+			
Cys^{191}	polar	+							
Phe^{193}	polar			+	+	+	+	+	+
Ser^{203}	polar						+	+	+
Ser^{204}	polar						+	+	+
Asn^{293}	polar						+	+	+
Asn^{312}	polar	+		+	+	+	+	+	+
Tyr^{316}	polar	+							
His93	basic		+						
$-\Delta G_{bind}$, kcal/mol		-11.53±0.06	-8.12 ± 0.12	-9.10±0.05	-10.30±0.07	-8.18±0.05	-8.45±0.04	-5.66±0.12	-6.65±0.02
calculated K_d, nM		3.55±4.5	1.12±0.5 µM	214.28±57.2	28.28±4.4	1.01±0.3 µM	641.71±75.6	19.7±3.5 µM	13.38±2.5 µM

4.4 Comparative *in vitro* - *in silico* ligand efficiency estimation results

As shown in Table 3, Our isolated compounds 6-KMG and HAP satisfy the characteria published by Bemebenek *et al.* [92] for identifying a drug candidate by exhibited ΔG/NHA value deeper than -0.24 kcal/mol, molecular weight lower than 500 g/mol and with the number of heavy atoms of 34 and 23 respectively. In addition, standard ligands set values were presented in a tabular manor.

When we calculate ligand efficiency for 6-KMG, HAP and terbutaline for both *in vitro* and *in silico* data regarding the number of heavy atoms Table 4, we got same value which similar to the exact E_{max} value that calculated by Prism software from the *in vitro* results as shown in Fig. 5.

Table 3: Number of heavy atoms (NHA), molecular weight (MW, g/mol), estimated free energy of binding (ΔG_{bind}, kcal/mol) and calculated ligand efficiencies (ΔG/NHA, kcal/mol per non-hydrogen atom; ΔG/MW, kcal/g) of docked ligands for the rat β_2-AR. ICI: ICI 118,551; PROP: propranolol; TER: terbutaline; ISO: isoproterenol; nADR: noradrenaline; ADR: adrenaline ; and HAP: isolated compound.

compound	**NHA**	MW	ΔG	**ΔG/NHA**	ΔG/MW
ICI	20	278.416	-9.10	-0.455	-0.033
nADR	12	170.188	-5.66	-0.472	-0.033
ADR	13	184.215	-6.65	-0.512	-0.036
ISO	15	212.269	-8.45	-0.563	-0.040
PROP	19	260.357	-10.30	-0.542	-0.040
TER	**16**	226.296	**-8.18**	**-0.511**	-0.036
6-MKG	**34**	480.422	**-11.53**	**-0.339**	-0.024
HAP	**23**	313.353	**-8.12**	**-0.353**	-0.026

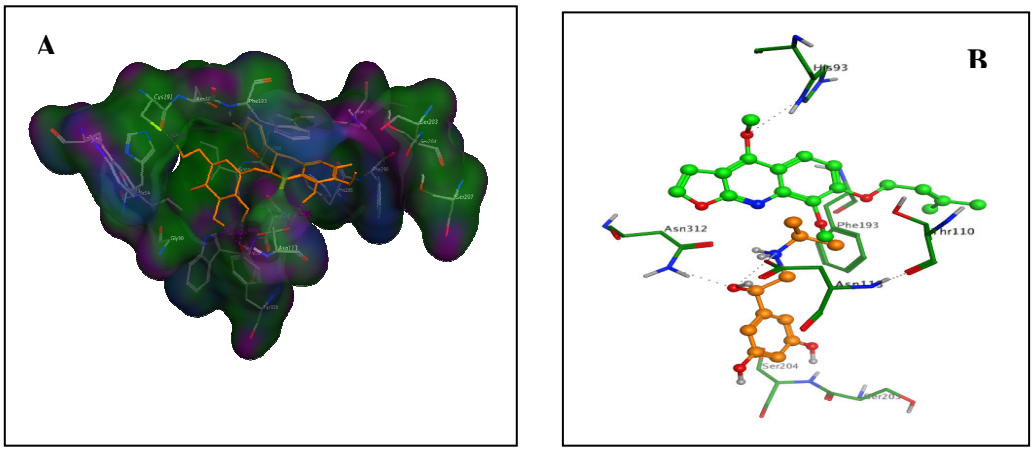


Fig. 11. (**A**) Illustration of the lowest energy conformation of 6-MKG (orange-large molecule with thick grey carbon) at the rat β_2-ARs binding site. The residues are the determined interaction points within the active pocket of the receptor. The coloured surface shows the van der Waals interaction surface (purple: H-bonding, green: hydrophobic, blue: mild polar). (**B**) Illustration of the lowest energy conformation of HAP (green) and the standard terbutaline (orange) docked into the active site of customized rat β_2-AR. Pictures made by MOE.

Table 4. comparison of calculated Ligand efficiency (LE) value based on number of heavy atoms *in vitro* and *in silico* for terbutaline and isolated compound HAP, *In vitro* E_{max} value.

compound	NHA	*In vitro* (NP) N=6-8		*In silico*		E_{max}±SEM
		EC_{50}	LE[a]	ΔG, kcal/mol	LE[b]	*In vitro*
TER	**16**	**5.94 ± 3.2 E-07**	**-5.31E+02**	**-8.18 ± 0.05**	**-0.511**	**101.8 ± 0.5**
6-MKG	34	6.11± 2.4 E-07	-2.41E+02	-11.53 ± 0.06	-0.339	35.93 ± 2.9
HAP	23	6.34 ± 3.9 E-07	-3.68E+02	-8.12 ± 0.12	-0.353	80.8 ± 11.8
Ratio of 6 -MKG/TER			**45.39%**		**66.34%**	**35.29%**
Ratio of HAP/TER			**69.30%**		**69.08%**	**79.37%**

TER: terbutaline, HAP: isolated compound; NP: non-pregnant rat uterus; NHA: Number of heavy atoms; MW: Molecular weight; ΔG_{bind}: estimated free energy of binding; E_{max}: the maximal relaxing effect of TER or HAP against KCl-induced contraction, EC_{50}: the concentration of the TER or HAP producing 50% of their maximal relaxing effect against (KCl-induced contraction) in the system; S.E.M.: standard errors of the mean; N: total number of observations.

[a] $LE=-RT\ln(IC_{50})/NHA$, [b] $LE=\Delta G/NHA$.

5. DISCUSSION

Finding therapeutics to act on potential drug targets is a challenging and often very expensive process [93], different important therapeutic agents were developed from plant and recently many research work was emphasized on plants to find natural new drugs.

Although the Human genome project was completed in 2003 [94,95], besides huge work and efforts were done on developing and producing biotechnology drugs from protein origin [96,97], these drugs have many disadvantages [98], which redirected the researchers back to the nature looking for small molecules specially from medicinal herbs. On the other hand, biotechnology drugs cover only little area of drugs unlike the classical drugs.

Natural alkaloids such as atropine and others [99] were considered among the most active classical drugs. African medicinal plants are a rich drug source and biologically-active compounds can be isolated from it by fractionation [100] depending on the ethno-pharmacological information.

Premature labour still a health challenge world-wide [2], thus huge efforts were done and required to found a solution for it, this work was an attempt to find a natural solution.

However, the efficacy and safety of tocolytics are not adequate, new agents are therefore required including substances from natural sources. Many plants have been recently investigated world-wide in the search for tocolytic or uterus-relaxing agent such as *Curcuma aeruginosa* Roxb. rhizome [101], *Scutellaria baicalensis* root [102] and *Ficus capensis* Thunb. [103].

El-Hazah, is named locally in Sudan as "a plant of all disease". Due to its extensive use traditionally this plant subjected to different studies in different directions, but this study was the first novel complete work, deep pharmacological study with detailed assay guided fractionation and isolation and investigations of its two main active agents.

5.1 Fractionation and activity investigations

Although El-hazha is used traditionally as an aqueous infusion, in our investigation we use the methanolic-extract (AH2) from the plant, because the aqueous extract of the plant was investigated by Mohamed *et al*. [27] and gives potent contracting activity.

In general pilot screening, AH2 exerts relaxant effect in both non-pregnant and late-pregnant uteri, whilst the radioligand binding assay revealed that the extract exerts binding affinity to β-AR only in relatively very high concentration, these findings necessitates its fractionation to clarify this affinity.

Fractionation gives different sub-fractions with different efficacies on both isolated uteri, but we deal only with the most active ones, the fraction that showed best pharmacological activity was selected for the next steps.

Fractionation affect significantly the biological extract activity, in non-pregnant uterus, the extract activity was increased significantly by fractionation then decreases after further fractionation steps. In late-pregnant uterus the activity did not affected significantly by fractionation. These findings were supported with radioligand binding assay experiments results, so we continue the study using the most active sub fraction (AH2-11), because we thought that this relaxant effect may be attributed to synergistic effects of the extract compounds.

AH2-11 was used to perform experiments to verify the role of β-adrenergic receptor in mediating this relaxant activity, in non-pregnant, propranolol has no effect on the fraction's relaxant activity which may be taken as an evidence of a role of other mechanism(s) such as direct muscle effect [104] or Ca+-channel blocking activity. But in the late-pregnant the β-AR was clearly identified by the significant propranolol antagonistic effect on the fraction activity.

Further conformity test for the β-AR role in this relaxation was done by pre-treatment of pregnant rats by progesterone because the gestagen-induced increases in the myometrial β_2-AR density and the amount of activated G proteins coupled to β-ARs. Progesterone pre-treatment increases the expression of the β_2-ARs during pregnancy and alters the effects of β_2-AR agonists on the pregnant myometrium [25], progesterone treatment did not potentiate the β- receptors sensitivity to this fraction, these findings can be explained by, the β-AR was only partially participated in this relaxation.

β-AR activity of the semi-purified fraction was not strong, even after the isolation of its two main compounds which were found to be a partial agonist after comprehensive further investigations.

5.2 Pharmacodynamics of the isolated compounds

6-MKG and HAP were isolated from El-hazha by fractionation [100] based on its β-adrenergic activity, and then subjected to *in vitro* and *in silico* pharmacological investigations on rat uterus.

Both 6-MKG and HAP evidently have agonistic features, because they produced approximately 50% and 80% of the maximum activity of terbutaline on the isolated rat uterus, with a higher binding affinity for the β_2-adrenoceptors in both *in vitro* radioligand for 6-MKG and *in silico* docking experiments.

On the other hands, 6-MKG exhibited a lower EC_{50} on pregnant rat uterus than that of terbutaline (half), but bound to the β_2-ARs with higher affinity and lower efficacy, and was therefore a weak agonist.

ICI 118,551 in the late-pregnant uteri completely blocked the effect of 6-MKG unlike terbutaline (partially), suggesting that 6-MKG is only a weak agonist.

Since the density of β-ARs increases in late pregnancy relative to that in the non-pregnant rat [106], we investigated 6-MKG activity in the late-pregnant isolated rat uterus, where only slightly different results were obtained from those on the non-pregnant rat uterus. The E_{max} of 6-MKG was 25% of that for terbutaline, while EC_{50} was higher for 6-MKG than for non-pregnant, where it was 1000x that for terbutaline.

The β_2-adrenoceptor are GPCR that acts through cAMP [42]. 6-MKG induced a cAMP level enhanced significantly in a dose-dependent manner in the late-pregnant uteri, and thus 6-MKG is an agonist, because Klukovits *et al.* [78] revealed that terbutaline as agonist induced a cAMP level in similar mannor. Besides, the radioligand binding assay revealed that 6-MKG has better affinity than terbutaline and ICI118,551 for the β_2-adrenoceptors.

HAP exerts uterus relaxant activity less than that of terbutaline, which was in line with *in silico* results. HAP possibly has β_2-AR agonistic activity showing similar binding affinity than that of terbutaline. This hypothesis has been supported by the action of β_2-antagonist ICI118,551 which blocked the relaxant effect of both HAP and terbutaline with same pA_2 value. However, the E_{max} of HAP was more depressed by the antagonist, which suggests that HAP has a lower efficacy feature compared to terbutaline.

The comparison of the isolated compound 6-MKG and HAP to the original herb methanolic extract revealed that 6-MKG and HAP should play a major role in the relaxant effect of the extract. However, we can only compare the E_{max} values, because 6-MKG and HAP were used in molar concentration, while the methanolic extract was used in µg/ml, so the EC_{50} values cannot be compared to each other.

β_2-ARs has only one binding site, so addition of two agonist at the same time compete with each other and interferes with others leading to synergistic additive or partial agonistic pharmacological effects. To check this our isolated compounds were added alone or in combination to the standard terbutaline, where they decreased significantly the terbutaline relaxing effect on rat uteri, thus serves as partial agonists.

On other hands 6-KMG significantly decreased the maximal effect of HAP from 80% to 60 % and it considered as partial agonist. Besides, the ICI118,551 competitively blocked the combination effect in both uteri.

5.3 *In silico* studies

The human β_2-adrenoceptor was the first non-rhodopsin GPCR to be cloned, but the X-ray structure of this receptor was solved only in 2007 [107], (PDB ID: 2RH1). To build the homology model of the rat β_2-adrenoceptors, we have used the customized model of 2RH1 developed by de Graaf and Rognan. [58]. The receptor structure obtained by homology modelling with its RMSD values and the interaction points (Asp^{113}, Phe^{193}, Ser^{203}, Ser^{204}, Asn^{312}) with noradrenalin proved that it is a good starting point for molecular docking calculations. Homology modeling was required for rat receptor from human one because our study was carried in *in vitro* rat uterus as main target and to compare the results logically.

In case of the reference molecules we have identified 3 common interaction points (Asp^{113}, Phe^{193} and Asn^{312}), and other two residues (Ser^{203} and Ser^{204}) represented by ligands containing the catechol ring (adrenaline, noradrenaline, isoprenaline). These findings confirm the efficiency of our homology model.

Using the scoring function of AutoDock4 the estimated free binding energy values were calculated for each ligand. These estimated values can't be compared against the experimental ΔG_{bind} and K_d values, because the standard error of the scoring function is around 2.5 kcal/mol.

The 6-MKG binds to the rat β_2-ARs with low ΔG_{bind} and K_d values and has different interaction points than that of terbutaline, adrenaline, noradrenaline and ICI 118,551. The position of glycopyranoside ring is stabilized by Asn^{312} (electrostatic interaction), Cys^{191} (H-bond) and the Tyr^{316} (two strong H-bonds), while the Asp^{113} forms an H-bond with the 5-hydroxy part of the flavone ring. Therefore the benzopyrane ring of 6-MKG anchors far from the Ser^{203} (3.4 – 5.2 Å), Ser^{204} (3.8 – 4.7 Å) and Ser^{207} (4.6 – 6.0 Å) and it was not able to showed these typical catechol interactions.

The *in silico* findings revealed that HAP resembles the standard terbutaline in ΔG_{bind} and K_i, and differ from it on the orientation within the active pocket and the replacement of the acidic interaction at Asp^{113} by the basic interaction at His^{93}.This slight difference leads to observe slight activity difference consequently, because activity similarly feature is matter of binding energy and affinity constant (K_d) property.

Although partial-agonist is a compound that can activate receptors but is unable to elicit the maximal response of the receptor system, both 6-KMG and HAP were partial β_2-ARs agonists, because partial agonists may have 50% response doses lower or higher than full agonists [108]. Information regarding the binding sites of partial agonists is still not sufficient to explain why they cannot fully activate the receptor

Partial agonists were of therapeutic essentials, nicotine receptor partial-agonists may help people to stop smoking such as cytisine, a drug widely used in central and eastern Europe for smoking cessation [109], while partial agonists of dopamine receptors was used in schizophrenia [110] and psychosis [111,112]. Lastly the β-adrenergic partial agonist alifedrine implicated in the management of cardiac performance with acute ischemic left ventricular failure [113].

5.4 Comparative *in silico* - *in vitro* functional studies

Ligand efficiency (LE) was a useful metric for measuring the impact on activity of the addition of more molecular bulk where molecules that achieve a given potency with fewer heavy atoms are by definition more efficient [114]. It allows the combination of pharmacodynamic (IC_{50}, ΔG_{bind}) and pharmacokinetic (MW, NHA, NoC, etc) properties into unique measures Bemebenek *et al.* [92]. Identifying a drug candidate the ΔG/NHA value must be deeper than -0.24 kcal/mol, the molecular weight lower than 500 g/mol and the

number of heavy atoms between 20 and 70. In our case, both isolated compounds 6-KMG and HAP satisfies this feature.

Although in this study we only emphasizing on the pharmacodynamic property of the isolated compound so only three parameters (MW, ΔG_{bind} and NHA) were used and did not discuss other factors related to pharmacokinetics features.

For *in silico* LE calculation we use experimental free energy of binding (ΔG_{bind}), while for *in vitro* we use inhibitor concentration at 50 % inhibition (IC_{50}) as BA pharmacodynamics representing parameter.

The correlation of our calculated LE and the E_{max} revealed that there was a direct strong relation between the efficiency and *in vitro* efficacy, because we got similar value in both cases when comparing our compounds 6-KMG and HAP to the standard terbutaline.

6. SUMMARY

Our work can be summarized in a brief informative way in which, El-hazha was used many years in Sudan to treat different aliments, by these findings its ethno-pharmacology in the field of gynaecology has been proofed to some extent and two new natural tocolytic agents were isolated from it in order to aid in solving the problem of premature labour.

Our results revealed that the fractionation affect significantly the relaxant activity of the plant methanolic extract and there were strong evidences that showed the presence of a partial role for β-AR on mediating this relaxation activity or may be its complete, but inhibited by the existence of other contracting substances that needs further separation and isolation.

The purification leads to a discovery of a new novel natural therapeutic agent(s), considered as a useful tocolytic.

The isolated compound, 6-MKG exerts weak β_2-adrenoceptor agonistic activity, whilst HAP exhibited strong activity. 6-MKG and HAP serve as a starting point in future drug development aimed at the production of a new safe, effective and bio-accessible therapeutic agent, and can be considered a natural compounds of potential significance for the treatment of premature labour.

Both 6-KMG and HAP were categorized as partial β_2-adrenoceptors agonist due to they cannot fully activate the receptor., but still of therapeutic essentials that implicated in the management of several medicinal disorder such as premature labour, bronchial asthma and even cardiac performance with acute ischemic left ventricular failure.

Future work is recommended to investigate the pharmacokinetics and toxicological features for the isolated compounds in order to be available for evidence-based clinical therapy.

7. CONCLUSSION

Finally we can concluded that, plants still a rich source for drugs, and a recent trend in medicine to return to the nature in treating disease and to discover new therapeutic agents.

The traditional use for El-hazha as relaxing agent may be due to the presence of active compounds that act on β_2-Adrenergic receptor.

We investigated the plant methanolic extract and found it showed significant relaxant activity in which β_2-adrenergic receptors participate substantially in mediating it. These findings stimulate the isolation and the pharmacological characterization of its active compounds.

Confirmation and scientifically proof of its mentioned traditional use, even it seems contradictory from the first point of view.

The fractionations significantly affect the activity of its methanolic-extract, existence of partial role for β-AR on mediating the plant relaxant activity

Both isolated compounds, 6-KMG and HAP were β_2-AR agonists with potential therapeutic value in preventing premature labour.

6-KMG and HAP can be categorized as partial β_2-ARs agonists.

Both pure compounds can serve as a starting point in future drug development aimed at the production of a new safe, effective and bio-accessible therapeutic agent.

Future work was recommended to investigate the pharmacokinetics and toxicological features for the isolated compounds in order to be available for evidence-based clinical therapy.

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ACKNOWLEDGMENT

I would like to express my warmest thanks to my supervisor, **Prof. Dr. Goerge Falkay**, for offering me the possibility to work in his group under his direct supervision that allow me subjected to his experience guidance throughout the study. Also my thanks to **Dr. István Zupkó,** the heads of the Department of Pharmacodynamics and Biopharmacy for his kind care during the whole period I spend for Ph.D. studies.

I am also thankful **to Dr. Robert Gaspar** my kind co-supervisor and **Dr. Arpad Marki** for their valuable help and support.

I am grateful to my big family represented by **all members** of the Pharmacodynamics & Biopharmacy at faculty of pharmacy, Szeged University for support and awareness during my stay between them.

I also wish to thanks **my co-authors** and **colleagues** for the pleasant co-operation, inspiriting discussion and stimulating me to complete this thesis.

Finally, I am likewise to thank the **government of Sudan** represented by Minister of Higher education and scientific research for providing me the full scholarship.

BIOGRAPHICAL SKETCH

Aimun AE. Ahmed was born on Dec. 22, 1975 in Shandi, Sudan. In July 2001, he obtained his B.Pharm degree in Pharmacy, Khartoum University. In July 2006, he obtained his M.Pharm degree from Faculty of Pharmacy University of Khartoum at the department of Pharmacology. He joined Department of Pharmacodynamics and Biopharmacy at faculty of Pharmacy Szeged University as a graduate student in Sept. 2008. He will defend his Ph.D. thesis in Pharmacology in February 2012.

APPENDIX

I.

American Journal of Biochemistry and Biotechnology 6 (3): 231-238, 2010
ISSN 1553-3468

Uterus-Relaxing Study of a Sudanese Herb (El-Hazha)

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Abstract: Problem statement: The aim of this study is to investigate the pharmacological effects of the Methanolic-extract (AH2) of El-Hazha and its sub-fractions. **Approach:** These investigations were carried out on *in vitro* isolated uterus preparations from Non-Pregnant (NP) and Late-Pregnant rats (LP). In parallel displacement radio-ligand binding assay was performed for β-Adrenergic Receptors (β-ADR). **Results:** Showed that the herb and its different fractions produced dose-dependent relaxant effect ($p<0.05$, t-test, $n = 6$) on uterine contraction elicit by 25 mM KCl in both NP and LP uteri that affected significantly by fractionation, however, the effect of the most active fraction (AH2-11) was reversed by prior addition of propranolol (non-specific β-antagonist), but not affected by progesterone pre treatment of the LP rats. In addition, AH2 only in high concentration displaced isotopes from β-ADR, this affinity changed markedly by fractionation. **Conclusion:** We validate the fractionation effect on its relaxant activity and found partial role for β-ADR on mediating this activity. Future study was recommended to isolate and investigate its active components to enhance this activity or to discover a new novel natural therapeutic agent(s).

Key words: *Haplophylium tuberculatum*, radio-ligand binding assay, β-Adrenergic Receptors (β-ADR), Non-Pregnant (NP), Late-Pregnant rats (LP), Sudanese plant, charles-river laboratories, medicinal and aromatic plants, tocolytic agent, pharmacological effects, progesterone treatment, uterus-relaxing study

INTRODUCTION

Despite extensive efforts, the incidence of preterm birth has not decreased recent several decades (Monga and Creasy, 1995; Hannah, 2000). Preterm labor is still a health challenge, because there are as yet no effective primary means of its prevention (Koucky *et al*., 2009).

Tocolytic agents are drugs designed to inhibit the contractions of myometrial smooth muscle cells. The aim of tocolysis is not only to stop uterine contractions and to prevent preterm delivery, but also to decrease the prenatal morbidity and mortality associated with preterm birth (Tsatsaris *et al*., 2004). The main drugs used as tocolytics are indomethacin and other prostaglandins inhibitors (Vermillion and Landen, 2001), calcium channel blockers such as nifedipine (Pryde *et al*., 2001; Giles and Bisits, 2007), β-adrenergic agonists and oxytocin receptors antagonist, while the Medical prevention consists of antibiotic or progesterone administration (Tara and Thornton, 2004).

One of the most well-known mechanism of action through which a tocolytic agent acts was to relax the uterus, so generally any agent has ability to relax the uterus can be considered as a tocolytic agent (Clouse *et al*., 2007) demonstrates that relaxation of rat myometrium is mediated by β_2-adrenoceptors. also α1/β-adrenoceptor ratio determines not only the spontaneous motor activity of the rat uterus, but also the potency of the agents with tocolytic effect (Zupko *et al*., 1998).

However, the efficacy and safety of tocolytics are not adequate, new agents are therefore required including substances from natural sources. Many plants have been recently investigated world-wide in the search for tocolytic or uterus-relaxing agent such as *Curcuma aeruginosa* Roxb. Rhizome (Thaina, 2009), *Scutellaria baicalensis* root (Shih *et al*., 2009) and *Ficus capensis* Thunb (Owolab *et al*., 2009).

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El-Hazha (*Haplophyllum tuberculatum*) (Forssk.) A. Juss. (*Rutaceae*); is an herb indigenous to the northern part of Sudan, North Africa and other areas of the Middle East (Boulus, 1983). Named locally in Sudan as "a plant of all disease", it is to be found in every Sudanese home as an emergency drug that used extensively by old Sudanese in the rural areas. The herb is utilized in Sudan as an antispasmodic, as an antiflatulant, to relieve toothache and to treat allergic rhinitis (Mohamed AH *et al*., 1996), malaria, gynaecological disorders, asthma, inspiration difficulties, renal disorders and others.

This plant is also well known among herbalists and widely used traditionally in other counties such as Saudi Arabia (Mohammed *et al*., 1991) and Oman (Mossa *et al*., 1987).

Its essential oils were investigated for antimicrobial activity by (Al-Burtamani *et al*., 2005) and were found to cause partial inhibition of the growth of *Escherichia coli, Salmonella choleraesuis* and *Bacillus subtilis* to the same extent as gentamycin sulfate.

Its cardiovascular effect were studied by (Mohamed AH *et al*., 1996), who reported that its aqueous extract significantly decreased the contractility and the heart rate, but did not affect the flow rate of the isolated perfused rabbit heart. This action was not blocked by atropine, but the muscarinic antagonist blocked the fall in blood pressure seen when the extract was administered to anaesthetized cats. The extract also stimulated rabbit aortic strip, rat vas deferens and rat *anococcygeus* muscles. These adrenergic effects were largely reduced by phentolamine.

Its hepatoprotective activity was investigated on the liver damage induced by paracetamol in mice by (Ali *et al*., 2001) and proved to be relatively ineffective protecting only 16% of the animals against the lethal effect of paracetamol (1 g kg^{-1}) in comparable to that of the standard hepatoprotective agent silymarin.

When its cytotoxic activity was checked against 11 tumor cell lines, where strong cytotoxic activity was observed (Varamini *et al*., 1992).

On the other hands and from phytochemical's point of view, some compounds of this Sudanese plant were isolated by (Khalid and Waterman, 1981).

Both its uses to relax the uterus and to treat asthma and inspiration difficulties catalyzed us to carry out this study to evaluate their effects in order to find a new therapeutic agent(s) to aid in solving of two major medical challenges (preterm labor inhibition and asthma control).

Literature survey revealed that the aqueous extract of this plant obviously possess contracting activity, while traditional uses suggested contradictory applications such as muscle relaxant and contracting effect in the same time.

This study was an attempt to proof and evaluate some of its traditional uses in gynaecological area as uterus-relaxing agent and to see the influence of fractionation on its activity.

MATERIALS AND METHODS

Plant material:

Crude plant collection and identification: The aerial parts of *Haplophylum tuberculatum* (El-Hazha) were collected freshly during the 1rst week of November 2008 from their natural habitats in the North part of Sudan (Abu-hamad, Nahr El-Neel State). The voucher specimens (No. M23/08) were identified by Dr. Wai'l E. Abdalla and Yahia S. Mohamed of Herbarium of Medicinal and Aromatic Plants Research Institute (MAPRI), Khartoum, Sudan, where the specimens were also deposited for future references.

Extraction, fractionation and preparation of the plant material for pharmacological tests: The air-dried powdered aerial parts of *Haplophylum tuberculatum* (AH) (2 kg) were extracted with Methanol for 1h. The MeOH extract (AH2) was concentrated and completely dried under vacuum to yields 315 g of the crude extract which reconstituted in distilled water to get the desired concentration and tested pharmacologically (Ganguly *et al*., 2007), then fractionated and tested. The most active fraction in each step was selected based on comparing the *in vitro* pharmacological results, then fractionated and tested. The most active fractions are (AH2-11) 88.31 g, (AH2-11-4) 4.31 g and (AH2-11-4-6) 161mg (Fig. 1).

Pharmacological studies:

Ethical considerations for housing and handling the animals: The animals were treated in accordance with the European Communities Council Directives (86/609/ECC) and the Hungarian Act for the Protection of Animals in Research (XXVIII.tv.32. §). All experiments involving animal subjects were carried out with the approval of the Hungarian Ethical Committee for Animal Research (registration number: IV/1758-2/2008). Sprague-Dawley rats (Charles-River Laboratories, Hungary) were kept at 22 ± 3°C, the relative humidity was 30-70% and the light/dark cycle was 12/12 h. They were maintained on a standard rodent pellet diet (Charles-River Laboratories, Hungary) with tap water available *ad libitum*. The animals were sacrificed by CO_2 inhalation.

Mating of the animals: Mature female (180-200 g) and male (240-260 g) rats were mated in a special mating

cage. A metal door, which was movable by a small electric engine, separated the rooms for the male and female animals. A timer controlled the function of the engine. Since rats are usually active at night, the separating door was opened before dawn. Within 4-5 h after the possibility of mating, vaginal smears were taken from the female rats and a sperm search was performed under a microscope at a magnification of 1200 times. If the search proved positive, or if smear taking was impossible because of an existing vaginal sperm plug, the female rats were separated and were regarded as first day- pregnant animals.

Isolated organ bath studies: Uteri were removed from non-pregnant (180-200 g), 22-day-pregnant (270-350 g) rats. Muscle rings 5 mm long were sliced from the uterine horns and mounted in an organ bath (8 parallels) containing 10 ml de Jongh solution (in mM: 137 NaCl, 3 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 12 $NaHCO_3$, 4 NaH_2PO_4, 6 glucose, pH 7.4). The organ bath was maintained at 37 o C and carbogen (95% O_2+5% CO_2) was bubbled through it. After mounting, the rings were equilibrated for about 1 h before experiments were undertaken; with a solution change every 15 min. The initial tension was set to about 1.5 g, which was relaxed to about 0.5 g at the end of equilibration. The tension of the myometrial rings was measured and recorded with a gauge transducer and an S.P.E.L. Advanced ISOSYS Data Acquisition System (Experimetria Ltd., Hungary), respectively. Contractions were elicited with KCl (25 mM) and cumulative dose-response curves were constructed in each experiment by addition of plant extract or its fraction(s) in different concentrations (range from 10^{-5}-100 µg ml^{-1}) alone or in the presence of propranolol (β-blocker) (10^{-6} mM). Recording was performed for 5 minutes. Dose-response curves were fitted from areas under curves (AUCs) using Prism 4.0 computer program. E_{max} and EC_{50} values were calculated (E_{max}: the maximum relaxant effect of extract or its fractions, EC_{50}: the concentration of extract or its fraction (s) alone which elicits half of the maximum relaxant effect of extract or its fraction (s) or one of its fraction (s).

Progesterone treatment of pregnant rats: The progesterone treatment of the pregnant rats was started on day 15 of pregnancy. Progesterone was dissolved in corn oil and injected subcutaneously every day up to day 21 at a concentration of 0.5 mg 0.1^{-1} ml kg^{-1}. On day 22, the uteri were collected and the organ bath studies were performed as described above. The experimental data on the non-treated and the progesterone-treated animals were analyzed statistically.

Radio-ligand binding assay:

Membrane preparation: The selected tissue of abundant receptors of interest was rat brain membrane. Animals were sacrificed by rapid cervical dislocation, both side of the skull were cut from back to forward. The intact brain was expose and removed carefully using forceps. The brain were freed from other tissues and homogenized in ice-cold homogenizing buffer (20 mM $NaHCO_3$) in a ratio of 1:5. The homogenate was centrifuged at 15500 rpm speed for 40 min, the re-suspended pellets were centrifuged under the same conditions. Finally the pellet were re-suspended in binding buffer (50 mM tris + 0.5 mM EDTA with pH = 7.5) and divided into small stock aliquots 2.6 mL each and frozen at -70°C which diluted and used in radio-ligand binding displacement assays.

Displacement assay: The affinities of the tested extract and its fractions for β-adrenergic receptors were measured on above membrane preparation using [3H] Dihydroalprenolol (DHA) (β-adrenergic antagonist) as radioligand (~1.5 nM).Radioligand were purchased from Amersham International plc (UK). Under standard assay conditions, the final incubation system volume was 300 µl consisted of diluted membrane preparation (protein content approximately 0.5-1 mg ml^{-1}), radio-ligand and incubation buffer or with the tested extract or its fractions (its concentration ranging from 10^{-7}-10^{4} µg mL^{-1}), following the incubation period, the membranes were collected on a Whatman GF/C filter, using a Brandel M24 Cell Harvester. Filters were collected in liquid scintillation vials and the radioactivity was measured with LKB Wallac liquid scintillation counter. The experiment were performed at 25°C for 45 min, the nonspecific binding were determined using 10^{-5}M Alprenolol. Displacement experiments were analyzed individually with the computer program Prism 4.0 to determine the inhibition constants (Ki) of the investigated agents.

Statistical analysis: The statistics was done by using Prism 4.0 (GraphPad Software, USA) computer program. For the statistical evaluations, data were collected from at least 6 animals and analyzed by performing two-tailed unpaired t-test to compare the significance mean differences for various results. The differences were considered to be significant at levels of $p \leq 0.05$.

RESULTS

Phytochemical extraction and fractionation: The Methanolic-maceration of the plant produced a yield's percentage of (5.5%), while the steps of fractionations were highlighted in a summary diagram (Fig.1) emphasizing on that mentioned as most active pharmacologically and used throughout the study.

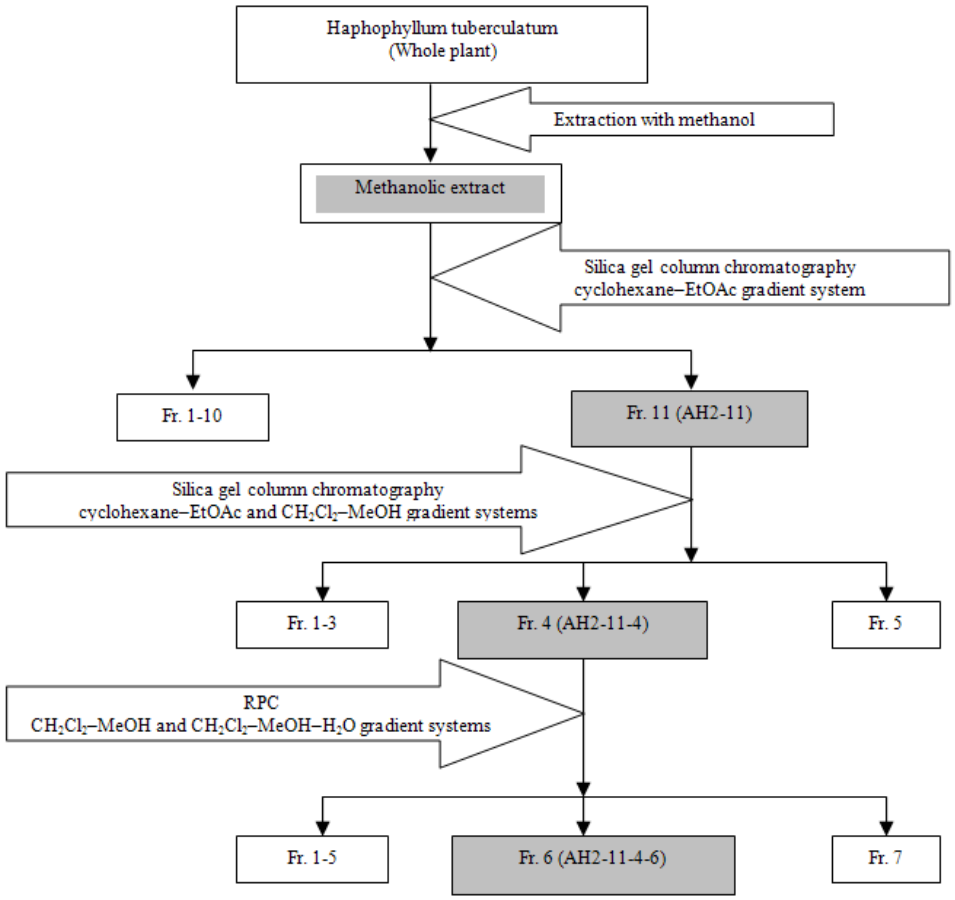

Fig. 1: Diagram illustrated the main steps of Bioactivity-guided fractionation of the Methanolic-extract of El-Hazha emphasized on the most active fractions resulted and used throughout the study.

Table1: The basic effects of the Methanolic-extract on non-pregnant and late-pregnant rat uterus *in vitro* results, general pilot screening for the direct plant relaxant activity

Gestation period	E_{max} ± SEM %	EC_{50} ± SEM (µg mL^{-1})
Non-pregnant	25.7 ± 3.6	(2.2 ± 0.6) $\times 10^{-3}$
Late-pregnant	39.9 ± 5.8	(0.4 ± 0.3) $\times 10^{-3}$
P-value	0.094	0.027 *

*: $p<0.05$; E_{max}: The maximal relaxing effect of the Methanolic-extract against (KCl-induced contraction); EC_{50}: The concentration of the Methanolic-extract producing 50% of their maximal relaxing effect of Methanolic-extract against (KCl-induced contraction) in the system

***In vitro* pharmacological studies:**

Rat uterus results:

Effect of the Methanolic-extract on rat uteri: Basically, the extract showed relaxant activity in NP and LP rat uterus without significant difference in E_{max} ($p = 0.094$), but with significant difference in EC_{50} ($p = 0.027$), Table 1.

Effect of the different AH2 fractions after the 2 nd and 3rd fractionation on rat uteri: AH2 and its fractions showed different activity level on both uteri type (data not shown), while the most active fraction was selected for the next step.

Effect of the most active fractions of AH2 on rat uteri: In non-pregnant (Fig. 2A and Table 2), the basic AH2 relaxant activity was increased significantly after fractionation while it decreased by further fractionation.

In late-pregnant unlike non-pregnant results, (Fig. 2B and Table 2), the extract activity was not significantly affected by different fractionations steps.

After this step the fraction AH2-11 was considered as the most active one and selected for the advanced β- adrenergic study using propranolol as standard blocker (Fig. 3 and Table 3) and progesterone treatment (Fig. 4).

Table 2: Relaxant effect of the Methanolic-extract AH2 and its most active fractions on non-pregnant and late-pregnant rat uterus *in vitro* results

	Non-pregnant		Pregnant D 22	
Fraction	E_{max} ± SEM (%)	EC50 ± SEM (µg mL^{-1})	E_{max} ± SEM (%)	EC50 ± SEM (µg mL^{-1})
AH2	25.6 ± 2.6	(2.2 ± 0.6) ×10^{-3}	31.7 ± 5.4	(0.4± 0.3) ×10^{-3}
AH2-11	81.0 ± 12.6 *	1.3 ± 0.6	33.4 ± 4.5	(4.5 ± 3.8) ×10^{-2}
AH2-11-4	58.2 ± 5.0	(7.8 ±5.9) ×10^{-3}	27.9 ± 5.2	(4.4 ± 2.1) ×10^{-5}
AH2-11-4-6	38.8 ± 6.3	(6.8 ±3.2) ×10^{-3}	34.9 ± 3.5	(1.7 ± 0.7) ×10^{-4}

*p<0.05, AH2 was used as control for its different fractions, E_{max}: the maximal relaxing effect of the Methanolic-extract AH2 or its sub fractions (AH2-11, AH2-11-4 and AH2-11-4-6) against KCl-induced contraction; EC_{50}: the concentration of the Methanolic-extract AH2 or its sub fractions (AH2-11, AH2-11-4 and AH2-11-4-6) producing 50% of their maximal relaxing effect of Methanolic-extract against KCl-induced contraction in the system

Table 3: Propranolol antagonistic effect on the relaxant effect of the most active fraction AH2-11 on isolated non-pregnant and late-pregnant rat uterus in vitro

	Non-pregnant		Pregnant D 22	
Most active Fraction AH2-11	E_{max} ± SEM (%)	EC50 ± SEM (µg mL^{-1})	E_{max} ± SEM (%)	EC50 ± SEM (µg mL^{-1})
Alone	81.0 ± 12.6	1.3 ± 0.6	49.5 ± 3.9	(3.9 ± 3.2) ×10^{-2}
With Propranolol [10^{-6} M]	124.1 ± 16.9	(34.6 ± 8.8) ×10^{-2}	29.5 ± 3.4 **	(0.5 ± 0.4) ×10^{-3}

*: p<0.05, E_{max}: the maximal relaxing effect of the most active fraction AH2-11 against KCl-induced contraction; EC_{50}: The concentration of the most active fraction AH2-11 producing 50% of their maximal relaxing effect against KCl-induced contraction in the system

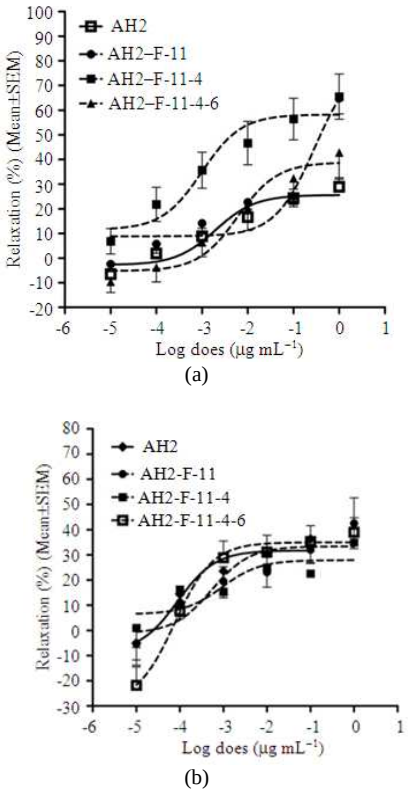

Fig. 2: Dose-response curves of the relaxing effect of the Methanolic-extract (AH2) (continuous-line) and its most active fractions (dotted-lines) on non-pregnant (a) and late-pregnant (b) rat uterus in vitro against KCl-induced control contractions. Values presented are means of 6-8 observations, while vertical bars denote Standard Errors of the Mean (SEM)

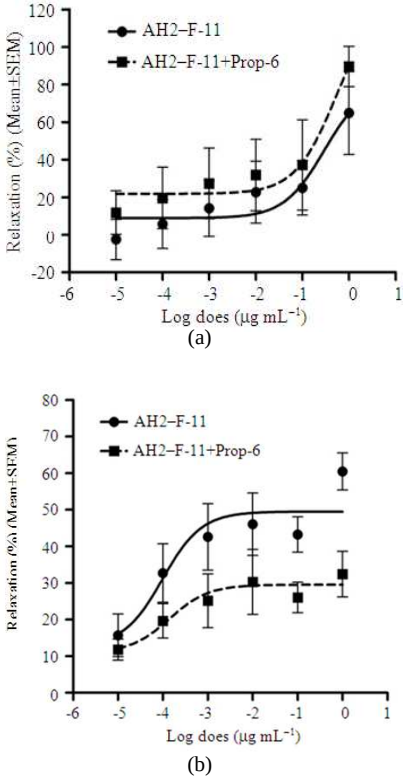

Fig. 3: Dose-response curves of the most active fraction AH2-11 alone (continuous-lines) and with propranolol 10^{-6} (dotted-line) on non--pregnant (a) and late-pregnant (b) on isolated rat uterus in vitro against KCl-induced control contractions. Values presented are means of 6-8 observations, while vertical bars denote Standard Errors of the Mean (SEM)

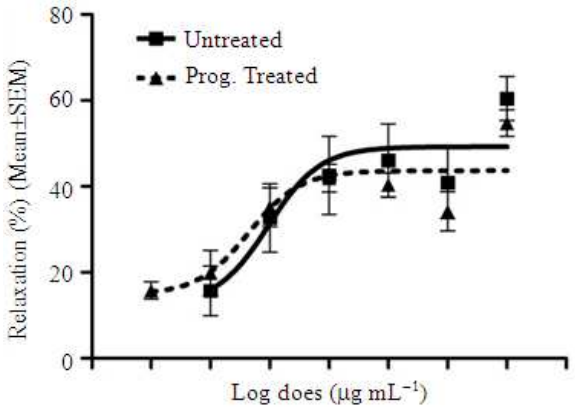

Fig. 4: Dose-response curves of the most active fraction AH2-11, untreated (continuous-lines) and progesterone-treated (dotted-line) on isolated late-pregnant rat uterus (Day22) in vitro against KCl-induced control contractions. Values presented are means of 6-8 observations, while vertical bars denote Standard Errors of the Mean (SEM)

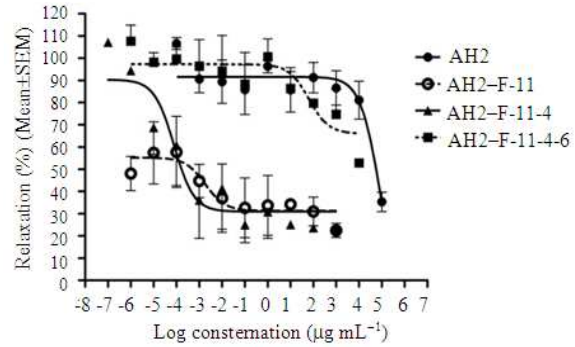

Fig. 5: The displacement curves of AH2 and its most active fractions on β-adrenergic receptors using Dihydroalprenolol [3H] DHA as a radioligand and isotopes on rat brain membrane preparation. Values presented are means of 6-8 observations, while vertical bars denote Standard Errors of the Mean (SEM)

Propranolol effect on the relaxant activity of the most active fraction on rat uteri: Propranolol in concentration of 10^{-6} M had no significant effect on neither the curve nor its parameters of the most active fraction AH2-11 on non-pregnant rat uterus (Fig. 3A and Table 3).

On late-pregnant rat uterus (Fig. 3B and Table 3), propranolol in concentration of 10^{-6}M exerts significant effect on the E_{max}% of the curve ($p = 0.0049$) but with insignificant ($p=0.184$) effect on the EC_{50} of the most active fraction AH2-11 under investigation.

Effect of AH2-11 on late-pregnant progesterone-treated rat uterus results: The progesterone treatment for late-pregnant rat uterus did not alter significantly neither the E_{max}% nor the EC_{50} of the curve of the most active fraction AH2-11 (Fig. 4).

Radio-ligand binding assay: The affinity of the Methanolic-extract and its most active sub fractions from different fractionation steps for β-adrenergic receptors were tested on rat brain membrane preparation, using Dihydroalprenolol [^{3}H] DHA (2 nM) as an isotopes radioligand. All of the ligands displace the radioligand from the target receptor.

The AH2 showed the displacement of the isotopes only in very high concentration (10^4 µg mL^{-1}) with un-estimated Ki ,while its other sub fractions showed better displacement affinities and the K_i-values of AH2-11-4 and AH2-11-4-6 were $(1.2 \pm 1.0) \times 10^{-2}$, $(1.1 \pm 1.1) \times 10^{-3}$ and 3.8 ± 3.8 µg mL^{-1} respectively (Fig. 5).

DISCUSSION

Plants still a rich drug source (Wahab *et al.*, 2008), El-Hazha, is named locally in Sudan as “a plant of all disease”. Due to its extensive use traditionally this plant subjected to different studies in different directions, but the novelty of this study arises from many factors which can be summarized as; deep pharmacological study with detailed assay guided fractionation, while other studies either general or used only the crude extract without fractionation, It involve the use of a pharmacological methods like RLB-assay and It done on both non-pregnant and late-pregnant (D22) rat uterus.

Traditionally El-Hazha is used as an aqueous infusion. In our investigation we made a Methanolic-extract (AH2) from the plant, because the polarity of the two solvents are quite similar, the extract contains probably the same components. Previously, the aqueous extract of the plant was investigated by (Mohamed AH *et al*., 1996) and gives potent contracting activity. The chloroformic fraction contains non-polar compounds and it was never used traditionally.

In general pilot screening, AH2 exerts relaxant effect in both uteri with insignificant difference in E_{max}%, but the EC_{50} was significantly differ, while the RLB assay revealed that the extract exerts binding affinity to β-ADR only in relatively very high concentration, these findings necessitates its fractionation to clarify this affinity.

Fractionation gives different sub-fractions with different efficacies on both isolated uteri, but we deal only with the most active ones. Fractions that produce relaxation ≤35% on NP and ≤25% on LP after first

fractionation and that produce relaxation activity ≤25% on NP and 20% on LP after the 2nd fractionation process which calculated as E_{max} ±SEM% were mentioned and considered as active. Moreover, the fraction that showed best pharmacological activity was selected for the next steps.

The fractionation effect on the biological extract activity was achieved by relating AH2 to its most active fractions. In NP uterus, the extract activity was increased significantly ($p = 0.026$) by fractionation then markedly decreases, but still at a level (2X) greater.

In LP uterus the activity did not affected significantly by fractionation, but the original extract relaxant activity still exist. In spite that on this stage of pregnancy the sensitivity of the uterus to β-adrenergic activity was weaker than the non pregnant (Gaspar *et al*., 2005).

These findings were supported with and confirmed by radio-ligand binding assay experiments results. In which at the beginning fractionation improved AH2 affinity to β-ADR, but after the 3rd fractionation process the affinity was deteriorated to a level similar to the AH2 before fractionation.

We continue the study using the most active sub fraction AH2-11, because we thought that this relaxant effect may be attributed to synergistic effects of the extract compounds that can be explain by the huge number of compounds that appears in the TLC plate (not shown here).

The selected Fraction AH2-11 as most active one when used to perform further experiments to verify the role of β-adrenergic receptor in mediating the above mentioned relaxant activity using propranolol as a blocker, both rat uteri revealed that; in NP, propranolol has no effect on the fraction's relaxant activity which may be taken as an evidence of a role of other mechanism(s) involves rather than β-ADR such as direct muscle effect (Ali *et al*., 1992) or Ca+-channel blocking activity. But in the LP data the β-ADR was clearly identified by the propranolol antagonistic effect on the fraction activity in which its dose -response curve was significantly ($p = 0.0049$) shifted to the right.

Further conformity test for the β-ADR role in this relaxation was done by pre-treatment of pregnant rats by progesterone because (Gálik *et al*., 2008) reported that, Progesterone pre-treatment increases the expression of the β2-ADR during pregnancy and alters the effects of β_2-ADR agonists on the pregnant myometrium. In addition, gestagen-induction increases in the myometrial β_2-ADR density and the amount of activated G proteins coupled to β-ADRs.

Although (Gaspar *et al*., 2005) found on the late stage of pregnancy the sensitivity of the uterus to β-adrenergic activity was weaker than the non-pregnant, unfortunately the progesterone treatment did not potentiate the β-receptors sensitivity to this fraction, these findings can be explained by that, the β-ADR only partially participated in this relaxation besides other possible mechanism(s) or may be hide by the presence of other compounds in this semi-purified fraction.

TLC chromatogram showed the complexity of the active fractions. Even the sub-sub-sub fraction contains large number of structurally related compounds exists in a very small amount, thus their isolation is a very difficult process. These compounds may contribute the various traditional uses and the local name (plant of all diseases) of the plant. Finally, the separation may affect markedly its biological activity due to the well-known plant synergism phenomenon.

CONCLUSION

Finally we can concluded that, by these findings and demonstration we confirm and proof its mentioned traditional use, even it seems contradictory from the first point of view, The fractionation significantly affect the activity of its Methanolic-extract.

There is a partial role for β-ADR on mediating this relaxation activity or may be its complete, but inhibited by the existence of other contracting substances that needs further separation and isolation.

The suggested purification may lead to a discovery of a new novel natural therapeutic agent(s) useful to aid solving two major medical problems pre term labour and asthma.

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European Journal of Pharmacology 667 (2011) 348–354

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Pulmonary, Gastrointestinal and Urogenital Pharmacology

β_2-Adrenergic activity of 6-methoxykaempferol-3-*O*-glucoside on rat uterus: *In vitro* and *in silico* studies

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ARTICLE INFO

Article history:
Received 26 November 2010
Received in revised form 12 May 2011
Accepted 22 May 2011
Available online 2 June 2011

Keywords:
β_2-Adrenoceptor
Radioligand binding assay
cAMP
Homology modelling
Docking
El-hazha

ABSTRACT

6-Methoxykaempferol-3-*O*-glucoside (6-MKG) was isolated from a Sudanese herb (El-hazha). The pharmacological effects of 6-MKG were tested on isolated non-pregnant or late-pregnant rat uteri *in vitro*, whilst docking studies were carried out modelling of the binding of 6-MKG to the rat β_2-adrenoceptor *in silico*. *In vitro* studies revealed that 6-MKG was able to relax both the non-pregnant and the late-pregnant uterine contractility with 50% of the E_{max} of terbutaline, whilst the EC_{50} for 6-MKG was at least half than that of terbutaline. The β_2-adrenoceptors antagonist 3-(isopropylamino)-1-[(7-methyl-4-indanyl)oxy]butan-2-ol (ICI118,551) competitively antagonised the relaxing effect of 6-MKG. Radioligand binding and cAMP studies confirmed the β_2-adrenoceptors agonistic property of the compound. In *in silico* docking studies, 6-MKG bound to rat β_2-adrenoceptors with low ΔG_{bind} value (-11.53 ± 0.06 kcal/mol) and it interacted with four residues of the active site (Asp^{113}, Asn^{312}, $Cys^{191}$and Tyr^{316}). It is concluded that 6-MKG exerts weak β_2-adrenoceptor agonistic activity and can be considered a natural compound with potential therapeutic significance in the field of premature pregnant uterine contractions and asthmatic problems.

1. Introduction

El-hazha (*Haplophyllum tuberculatum*) (Forssk.) A. Juss. (Rutaceae) is an herb indigenous to the northern part of Sudan, North Africa and other areas of the Middle East (Boulus, 1983). Named locally in Sudan "a plant of all disease", it is to be found in every Sudanese home as an emergency drug and is used extensively by the old Sudanese in the rural areas. The herb is utilised in Sudan as an antispasmodic, an antiflatulant, to relieve toothache, and to treat allergic rhinitis (Mohamed et al., 1996), malaria, gynaecological disorders, asthma, inspiration difficulties, renal disorders and others.

This plant is well-known amongst herbalists and is widely used traditionally in other countries, such as Saudi Arabia (Al-Yahya et al., 1991) and Oman, to treat skin discolouration, infections and parasitic diseases (Mossa et al., 1987). Due to its importance, new species were reported recently from Spain (Navarro et al., 2004).

Our previous investigation results revealed that the Methanolic-extract of the plant had significant relaxant activity (Ahmed et al., 2010). This finding stimulated the isolation and pharmacological characterization of its active compounds.

β_2-Adrenoceptor agonists are of therapeutic potential due to their use for asthma (D'Urzo et al., 2010) and to inhibit pre-term labour (Giles and Bisits, 2007), which is still medical challenge (Clouse et al., 2007). A few β_2-adrenoceptor drugs are of a natural origin. This encouraged us to attempt to isolate and test the pharmacological features of isolated compounds on the β_2-adrenergic system.

Although the literature revealed the isolation and identification of 6-methoxykaempferol-3-*O*-glucoside (6-MKG) (Wei et al., 2004), its pharmacological profile remains undetermined.

In this study we set out to determine the pharmacological profile of 6-MKG on the basis of its uterus-relaxing activity, using β_2-adrenoceptors as main target.

2. Materials and methods

2.1. Phytochemical aspects

2.1.1. Plant material

The aerial parts of *H. tuberculatum* (El-hazha) were collected in the north of Sudan (Abu-hamad, Nahr El-Neel State) in November 2008 and identified by Dr. Wai'l E. abdalla and Yahia S. Mohamed from MAPRI (Medicinal and Aromatic Plants Research Institute, Khartoum, Sudan). A voucher specimen (No. M23/08) has been deposited at the Herbarium of MAPRI.

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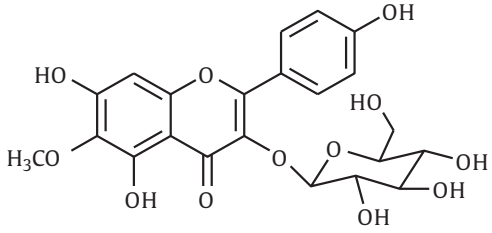
doi:10.1016/j.ejphar.2011.05.066

2.1.2. Isolation and identification of 6-MKG

6-MKG, 3,4′,5,7-tetrahydroxyflavone-3-*O*-β-D-glucopyranoside, was isolated from the dried aerial parts of El-hazha, and its structure was determined by NMR spectral data analysis. The structure was identical with that published by Wei et al. (2004). The chemical formula of this compound is $C_{22}H_{22}O_{12}$, with a molecular weight of 478.40 and the following structure:



A total of 10.8 mg was isolated, dissolved and diluted with distilled water to obtain the desired concentration for all pharmacological tests (Ganguly et al., 2007).

2.2. In vitro studies

2.2.1. Isolated organ bath studies

2.2.1.1. Ethical considerations for housing and handling the animals. The animals were treated in accordance with the European Communities Council Directives (86/609/ECC) and the Hungarian Act for the Protection of Animals in Research (XXVIII.tv.32. §). All experiments involving animal subjects were carried out with the approval of the Hungarian Ethical Committee for Animal Research (registration number: IV/1758-2/2008). Sprague–Dawley rats (Charles-River Laboratories, Hungary) were kept at 22 ± 3 °C; the relative humidity was 30–70% and the light/dark cycle was 12/12 h. They were maintained on a standard rodent pellet diet (Charles-River Laboratories, Hungary), with tap water available *ad libitum*. The animals were sacrificed by CO_2 inhalation.

2.2.1.2. Mating of the animals. Mature female (180–200 g) and male (240–260 g) rats were mated in a special mating cage. A metal door, which was movable by a small electric engine, separated the rooms for the male and female animals. A timer controlled the function of the engine. Since rats are usually active at night, the separating door was opened before dawn. Within 4–5 h after the possibility of mating, vaginal smears were taken from the female rats, and a sperm search was performed under a microscope at a magnification of 1200 times. If the search proved positive, or if smear taking was impossible because of an existing vaginal sperm plug, the female rats were separated and were regarded as first day-pregnant animals.

2.2.1.3. Uterus preparation. Uteri were removed from non-pregnant rats (180–200 g) and on pregnancy day 22 from pregnant rats (270–350 g). Muscle rings 5 mm long were sliced from the uterine horns and mounted vertically in an organ bath containing 10 ml de Jongh solution (composition: 137 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 4 mM NaH_2PO_4, 6 mM glucose, pH = 7.4). The organ bath was maintained at 37 °C and carbogen (95% O_2 + 5% CO_2) was bubbled through it. After mounting, the rings were equilibrated for about 1 h before the experiments were undertaken; with a solution change every 15 min. The initial tension of the preparation was set to about 1.5 g, which was relaxed to about 0.5 g at the end of equilibration. The tension of the myometrial rings was measured with a gauge transducer (SG-02; Experimetria Ltd., Budapest, Hungary) and recorded with a SPEL Advanced ISOSYS Data Acquisition System (Experimetria Ltd., Budapest, Hungary).

Uteri were pre-contracted with 25 mM KCl and cumulative concentration–response curves were constructed in each experiment for 6-MKG or terbutaline in different concentrations (range from 10^{-9} to 10^{-4} M) in the presence or absence of 3-(isopropylamino)-1-[(7-methyl-4-indanyl)oxy]butan-2-ol (ICI 118,551) (10^{-5} M). Following the addition of each concentration of the tested material, recording was performed for 5 min. Area under the curve were evaluated and concentration–response curves were fitted; E_{max}, EC_{50} and pA_2 values were determined and compared statistically by using the computer program Prism 4.0. (GraphPad Software, USA).

2.2.2. Measurement of uterine cAMP accumulation

Uterine tissue samples from 22-day pregnant rats were incubated in de Jongh solution as mentioned above. Cyclic AMP (cAMP) generation was stimulated with 6-MKG for 10 min, in the presence of the non-specific phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) (1 mM), and forskoline (10^{-5} M), and the samples were then immediately frozen in liquid nitrogen and stored until the extraction of cAMP (Gaspar et al., 2007). Frozen tissue samples were ground, weighed, homogenised in ten volumes of ice-cold 5% trichloroacetic acid and centrifuged at 600 × *g* for 10 min. The supernatants were extracted with three volumes of water-saturated diethyl ether. After drying, the extracts were stored at −70 °C until the cAMP assay. Uterine cAMP accumulation was measured with a commercial competitive cAMP Enzyme Immunoassay (EIA) Kit (Sigma-Aldrich Ltd, Budapest, Hungary), and tissue cAMP levels were expressed in pmol/mg tissue.

2.2.3. Radioligand binding assay

2.2.3.1. Membrane preparation. The 22-day-pregnant rats were killed as mentioned above. The uterine tissues were cut and homogenised in buffer (0.01 M Tris·HCl, 0.25 M sucrose, pH 8.0) with an Ultra-Turrax T25 homogeniser, and centrifuged (20,000 × *g*, 10 min, 4 °C). The supernatants were stored at 4 °C, and the pellets were re-suspended and re-centrifuged. After mixing, the supernatants were centrifuged (50,000 × *g*, 60 min, 4 °C). The pellets were re-suspended, aliquoted and stored at −70 °C until use in radioligand binding assays (Klukovits et al., 2009).

2.2.3.2. Displacement assay. The affinities of the tested compound 6-MKG and a standard drug (terbutaline or ICI118,551) for the β_2-adrenoceptors were measured on the above-mentioned membrane preparation by using [^{3}H]ICI118,551 (Bilski et al., 1983) as radioligand (concentration: 2 nM, specific activity: 18.8 Ci/mmole). Under standard assay conditions, the final reaction mixture volume was 300 µl containing the diluted membrane preparation (protein content approximately 0.5–1 mg/ml), 100 µl [^{3}H]ICI118,551 and 100 µl unlabelled ligand (10^{-5} M) for non-specific binding, or 100 µl incubation buffer (consisting of 0.05 M Tris·HCl, 0.01 M $MgCl_2$ and 2.5% ethanol, pH 7.42) for total binding. Following the incubation period, the membranes were collected on a Whatman GF/C filter, using a Brandel M24 Cell Harvester, and washed with 3 × 10 ml ice-cold buffer (50 mM Tris·HCl, pH 7.42). The bound radioactivity was determined in a HighSafe scintillation cocktail by using a Wallac1409 liquid scintillation counter. Specific binding was determined by subtracting the non-specific binding from the total binding. All assays were carried out at least three times in duplicate, and values are given as means ± S.E.M. Displacement experiments were analysed individually; the affinity was determined by calculating the inhibition constants (K_i) using the computer program Prism 4.0.

2.3. In silico studies

2.3.1. Preparation of ligands

The structure of 6-MKG was drawn by using Symyx Draw Editor software (Symyx, 2010) and then converted to pdb file format, and the structure was minimised with the Molecular Operating Environment (MOE) software developed by Chemical Computing Group Inc. (CCG, 2009), The pdb files of the standard ligands (epinephrine, norepinephrine,

propranolol, isoproterenol and ICI118,551) were obtained from the PubChem database whilst that of terbutaline, was downloaded from DrugBank as a pdb file (DrugBank, 2006, 2008).

2.3.2. Homology model of rat β_2-adrenoceptor

The homology model of rat β_2-adrenergic receptor was built using 'Homology modelling modul' of MOE 2009.10 with the ligand supported option. The template was the modified human β_2-adrenoceptors published by De Graaf and Rognan (2008), which is able to select full or partial agonists in virtual screening. After the alignment, certain residues were deleted from the sequence (1–29, 231–263 and 343–418, due to the absence of an appropriate template), which resulted in 91.5% homology identity (Fig. 5). During the modelling the MMFF94x force field was applied. After the model building the complex obtained was subjected to further refined using a modified method, as described in the De Graaf article: (1) distance restraints were applied between the appropriate heavy and hydrogen atoms; (2) minimization of the ligand position was performed with flexible side chain atoms of the interacting residues; (3) minimization with flexible residues, which were closer than 4.5 Å from any ligand atom and restraints applied remained intact; and (4) restraints were removed and final minimization was performed. We have applied these steps because without these restraints, the Ser^{203} and Ser^{204} side chains projected out from the active site. During the minimization steps the same force field was applied and the gradient was set to 0.05 kcal/mol $Å^2$.

2.3.3. Docking studies

In this study, the set of target compounds including 6-MKG was docked on the rat β_2-adrenoceptor binding site by using AutoDock 4.2 software (Morris et al., 2009). The initial structure of the compounds was prepared in MOE and was minimised (grad <0.001) by using the MMFF94 force field (Halgren, 1996a,b,c,d; Halgren and Nachbar, 1996).

Before the docking analysis, the docking files were prepared with regard to the AutoDock4 requirements, using AutoDock Tools 1.5.6 (Sanner, 1999), in which all possible flexible bonds of the ligands, the partial atomic charges (Gasteiger-Marsili formalism), and the Kollman charges for all atoms in the β_2-adrenoceptors were assigned. Finally, polar hydrogens were added to the receptor. All other parameters were kept at their default values. The grid box (20.25, 18, 16.5 Å) was centred (5.2, 14.3, 4.2) on the isoproterenol (Soriano-Ursu' et al., 2009; Soriano-Ursúa et al., 2009) and the lattice point distance was set to 0.375 Å. This grid centre was used for all other ligands. All simulations used the hybrid Lamarckian Genetic Algorithm, with an initial population of 100 randomly placed individuals and a maximum of 10 million energy evaluations. The lowest energy cluster returned for each compound was used for further analysis. The energy of this final receptor–ligand complex was minimised in MOE (MMFF94 force field, gradient 0.001 kcal/mol $Å^2$) by applying the following steps(1) minimisation of all hydrogens, (2) minimisation with the flexible residues (fixed position of ligand and receptor backbone) and (3) minimisation with flexible residues and ligand. The interactions of the ligands on the β_2-adrenoceptors were visualised and the figures were created by using AutoDock Tools v1.5.4 and MOE.

2.4. Statistical analysis

Statistical evaluations were performed with the Prism 4.0 computer program. Data were analysed by means of the two-tailed

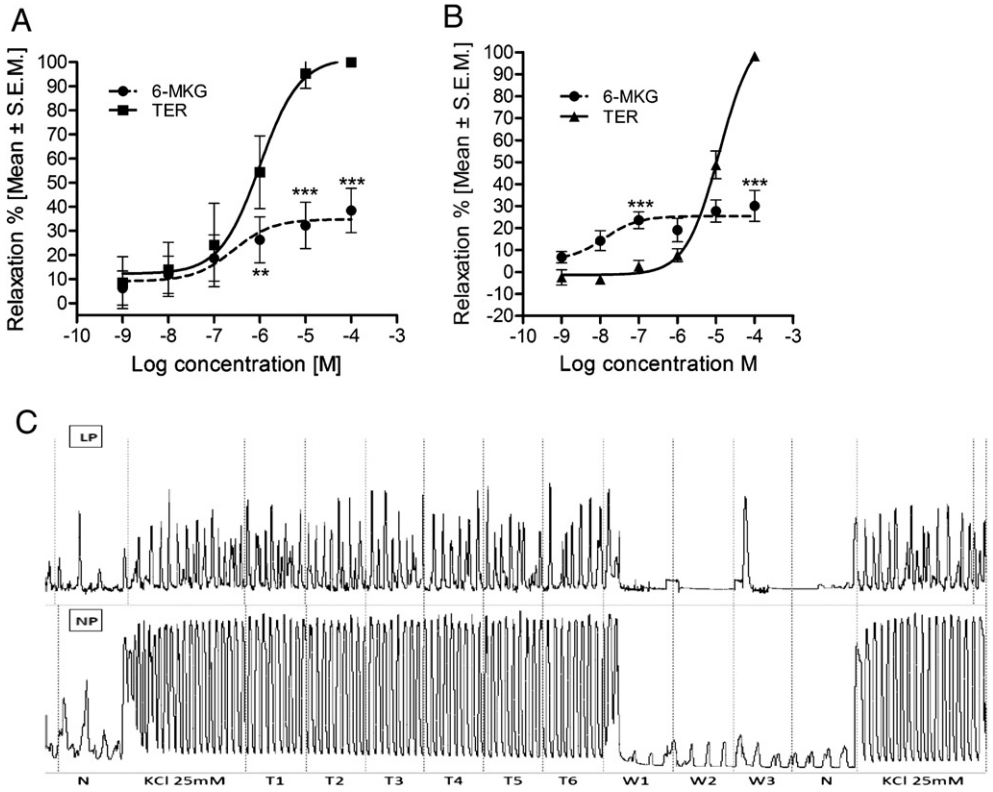


Fig. 1. Dose–response curves of the relaxing effect of 6-MKG (dotted-line) and terbutaline (continuous-line) on the non-pregnant (A) and the late-pregnant (B) rat uterus *in vitro*, pre-contracted with 25 mM KCl. Values presented are means of 6–8 observations; vertical bars denote standard errors of the mean (S.E.M.), **, P<0.01, ***, P<0.001 for 6-MKG against. The E_{max} values of 6-MKG and terbutaline in A are 34.9 ± 2.3% and 100.2 ± 2.8%, respectively, with P<0.001, whilst the EC50 values are $2.7\times10^{-7}\pm1.9$ and $4.2\times10^{-7}\pm1.2$ M, respectively, whilst E_{max} values of 6-MKG and terbutaline in B are [25.4 ± 2.7]% and [110.1 ± 5.1]%, respectively, with P<0.0001, whilst the EC_{50} values are $1.3\times10^{-8}\pm3.6$ and $1.2\times10^{-5}\pm1.2$ M, respectively, with P<0.001. Chart graph (C) representing the contraction of untreated tissues during the whole period of our experiments for both non-pregnant (NP) and late-pregnant (LP) rat uteri. N: normal tissue activity, T: time interval (5 min), W: washing out.

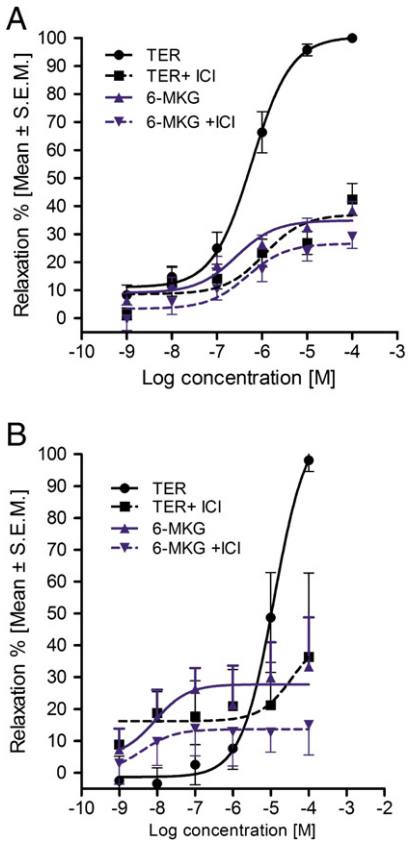


Fig. 2. Effects of terbutaline (black) and 6-MKG (blue) on the non-pregnant (A) and the late-pregnant (B) uterine contractions in the presence (dotted line) or the absence (continuous line) of ICI118,551. Values are means of 6–8 observations; whilst vertical bars denote standard errors of the mean (S.E.M.).

unpaired *t*-test to compare the mean differences for various results. The differences were considered to be significant $P \leq 0.05$.

3. Results

3.1. In vitro studies

3.1.1. Isolated rat uterus

6-MKG and terbutaline were both found to induce a concentration-dependent relaxation on the isolated non-pregnant and the late-pregnant rat uterus pre-contracted with 25 mM KCl (Fig. 1).

On the non-pregnant uterus (Fig. 1A), the E_{max} of 6-MKG decreased and differed significantly from that of terbutaline, whereas the EC_{50} values did not differ significantly.

On the late-pregnant uterus (Fig. 1B), both E_{max} and EC_{50} of 6-MKG had low difference from the terbutaline values.

Table 1
pA_2 values of the action of ICI118,551 on the relaxing effect of terbutaline and 6-MKG in non-pregnant and late-pregnant rat uterus *in vitro*.

Ligand	pA_2 [mean ± S.E.M.], N = 6–8	
	Non-pregnant	Late-pregnant (day 22)
TER	4.7 ± 0.1	2.9 ± 0.1
6-MKG	4.5 ± 0.2	7.7 ± 0.4
P-value	0.52	<0.001

TER: terbutaline, 6-MKG: isolated compound, $P<0.05$: statistically significant, pA_2: negative logarithm of the antagonist concentration that reduces an agonist effect to $E_{max}/2$, N: total number of observations.

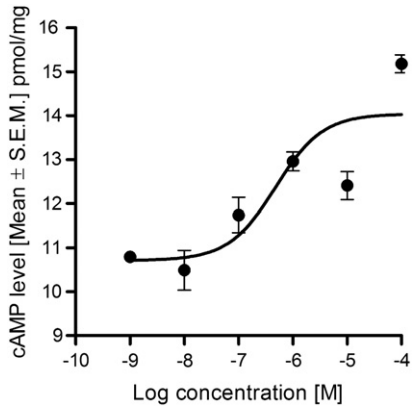


Fig. 3. Dose–response curves of the effect of 6-MKG on the cAMP induction level of the late-pregnant rat uterus *in vitro*. Values are means of 6–8 observations; vertical bars denote standard errors of the mean (S.E.M.).

There were no signs of fatigue or decreased contractions in both non-pregnant (NP) and late-pregnant (LP) untreated rat uteri samples during the whole period of our experiments (Fig. 1C).

The selective β_2-adrenoceptor antagonist ICI118,551 competitively antagonised the relaxing effect of 6-MKG and terbutaline and shifted the curves to the right to a significant extent in the late-pregnant (Fig. 2B and Table 1) and to an insignificant extent in the non-pregnant (Fig. 2A and Table 1) rats.

Also, 6-MKG induced an elevation in the level of uterine cAMP in the late-pregnant rat uterus (Fig. 3).

In comparison to Methanolic extract (ME), the 6-MKG produced higher relaxant effect and similar effect (E_{max}) in non-pregnant and late-pregnant rat uterus, respectively (Table 2).

3.1.2. Radioligand binding assay

The affinities of terbutaline, ICI 118,551 and 6-MKG for the β_2-adrenoceptors were tested on 22-day-pregnant rat uterine membrane preparations, with [^{3}H]ICI118,551 as radioligand (2 nM). All of the ligands displaced the radioligand from the target receptor in a monophasic manner; the K_i values of 6-MKG, terbutaline and ICI118,551 were 35.37 ± 1.9, 479.9 ± 2.0 and 181.9 ± 1.6 nM, respectively (Fig. 4).

3.2. In silico studies

3.2.1. Homology model of the rat β_2-adrenoceptor

The N- and C-terminal parts of the receptor, together with the 3rd intracellular loop, were not included in our homology modelling routine, which resulted in 91.5% homology identity (Fig. 5).

The αC RMSD (all residues) value between the template and the modelled structure was 1.04 Å, and the RMSD value for the binding site forming anchor points was 0.84 Å, oriented towards the receptor interior. The applied restraints kept the orientation of the Ser^{203} and Ser^{204} side chains toward the active site. The homology model was validated by docking of noradrenaline that detected all of the important

Table 2
Comparison of the herb methanolic extract (ME) and pure compound (6-MKG) on isolated non-pregnant and late-pregnant rat uteri *in vitro*.

Ligand	E_{max} ± S.E.M. %, N = 6–8	
	Non-pregnant	Late-pregnant (day 22)
ME	25.6 ± 2.6	31.7 ± 4.7
6-MKG	34.9 ± 2.3[a]	27.6 ± 3.2

ME: Methanolic extract of the herb, 6-MKG: isolated compound, E_{max}: the maximal relaxing effect of ME or 6-MKG against KCl-induced contraction, N: total number of observations.

[a] $P<0.01$.

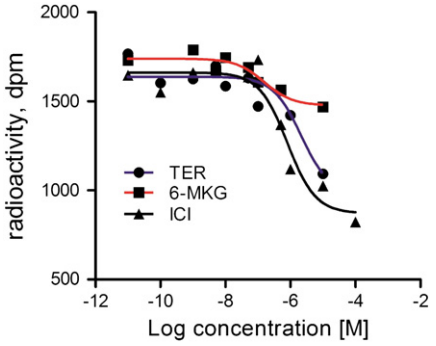


Fig. 4. Displacement curves of 6-MKG, terbutaline (TER) and ICI118,551 (ICI) on β_2-adrenoceptors with [^{3}H]ICI118,551 as radioligand on rat uterus membrane preparations.

interactions (Asp^{113}, Phe^{193}, Ser^{203}, Ser^{204}, Asn^{312}, Fig. 6A) therefore this receptor model was suitable for molecular docking calculations.

3.2.2. Docking studies

The results of docking studies on the six standard ligands and 6-MKG were presented in Table 3. All standard compounds showed strong interactions with the Asp^{113}, Phe^{193} and Asn^{312}, whilst adrenaline (Fig. 6B), noradrenaline (Fig. 6A) and isoproterenol, which contains catechol ring, forms interactions with Ser^{203} and Ser^{204}. The 6-MKG also interacts with common interaction points (Asp^{113} and Asn^{312}) residues which stabilise the receptor–ligand complexes, but it has a different interaction pattern. The glycopyranoside side chain forms an H-bond with Cys^{191}and Tyr^{316} but the benzopyrane ring did not interact with the Ser^{203} and Ser^{204} residues (Fig. 7). The 6-MKG displayed the lowest ΔG_{bind} and calculated K_d values, followed by ICI118,551, propranolol, isoproterenol, terbutaline, adrenaline and noradrenaline. In the radioligand binding assay, the same order of potencies was determined for 6-MKG, ICI118,551 and terbutaline.

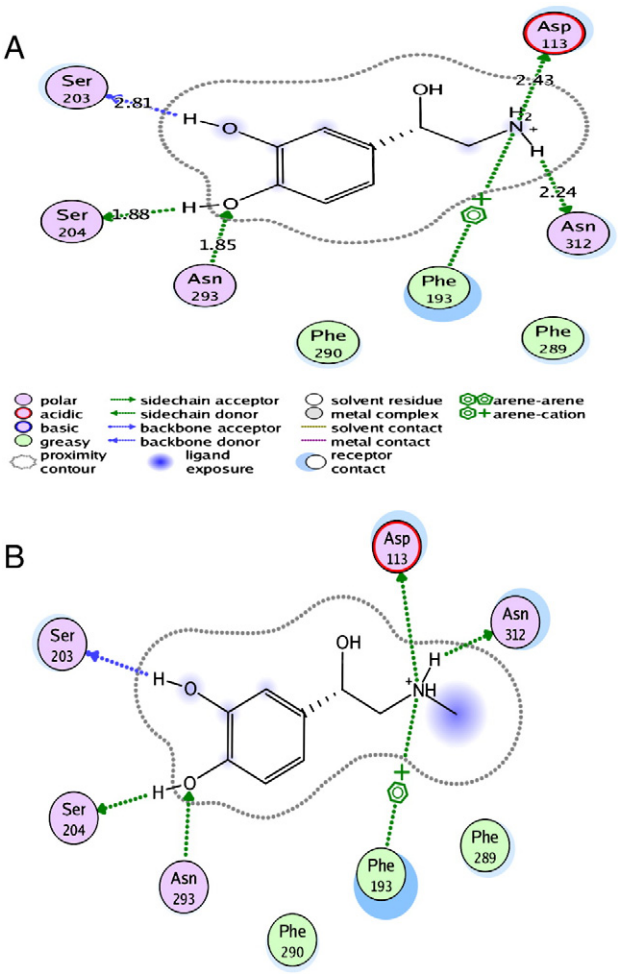


Fig. 6. Schematic representation of noradrenalin (A) and adrenaline (B) key interactions. Schemes were made by MOE facility.

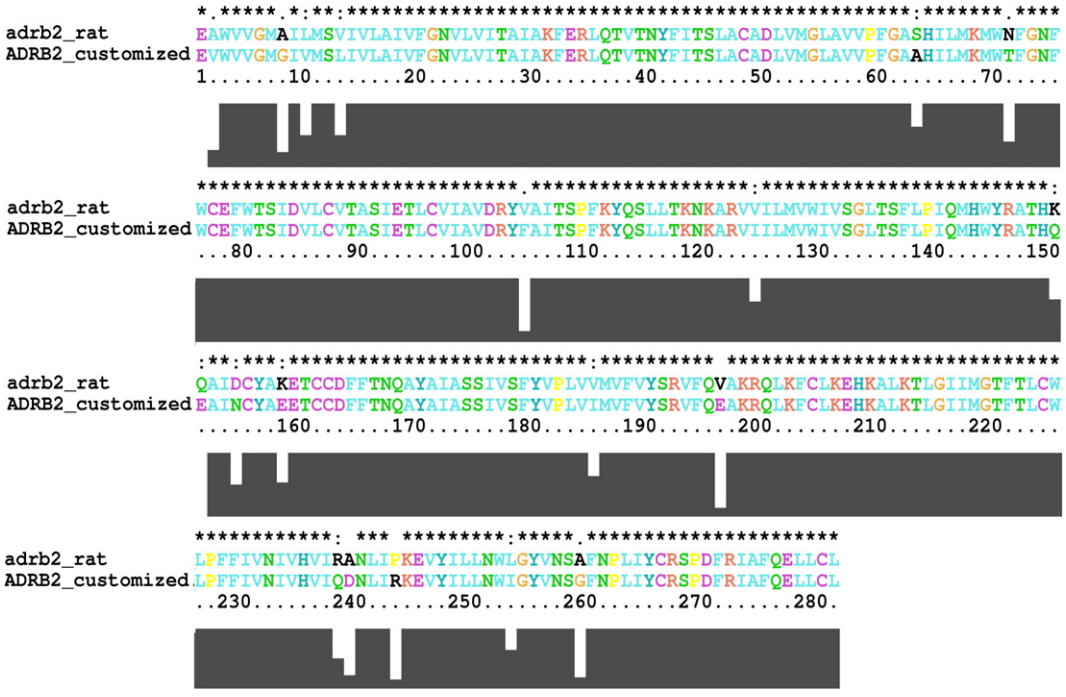


Fig. 5. Alignment between rat and human customised β_2-adrenoceptor after deletion of the 1–29, 231–263 and 343–418 residues.

Table 3
Interaction points, estimated free energy of binding (ΔG_{bind}) and calculated K_d of docked ligands for the rat β_2-adrenoceptor. All of the mentioned interactions were H-bonds, except for that of Phe^{193}, which was a benzyl-benzyl interaction. ICI: ICI118,551; PROP: propranolol; TER: terbutaline; ISO: isoproterenol; nADR: noradrenaline; ADR: adrenaline.

Residues	ICI	PROP	TER	ISO	nADR	ADR	6-MKG
Asp^{113}	+	+	+	+	+	+	+
Thr^{118}			+				
Cys^{191}							+
Phe^{193}	+	+	+	+	+	+	
Ser^{203}				+	+	+	
Ser^{204}				+	+	+	
Asn^{293}				+	+	+	
Asn^{312}	+	+	+	+	+	+	+
Tyr^{316}							+
$\pm\Delta G_{bind}$, kcal/mol	-9.10 ± 0.05	-10.30 ± 0.07	-8.18 ± 0.05	-8.45 ± 0.04	-5.66 ± 0.12	-6.65 ± 0.02	-11.53 ± 0.06
Calculated K_d, nM	214.28 ± 57.2	28.28 ± 4.4	1.01 ± 0.3 μM	641.71 ± 75.6	19.7 ± 3.5 μM	13.38 ± 2.5 μM	3.55 ± 4.5

4. Discussion

El-hazha is a plant that possesses uterus-relaxant activity (Ahmed et al., 2010). We therefore fractionated and isolated 6-MKG from it by bioassay-guided fractionation procedures (Hostettmann et al., 2000) and subjected it to post-isolation *in vitro* pharmacological investigations and *in silico* molecular modelling with docking studies.

6-MKG evidently has agonistic features, because it produced approximately 50% of the maximum activity of terbutaline on the isolated rat uterus, with a higher binding affinity for the β_2-adrenoceptors in both *in vitro* radioligand and *in silico* docking experiments.

On the other hands, it exhibited a lower EC_{50} on pregnant rat uterine than that of terbutaline (half), but bound to the β_2-adrenoceptors with higher affinity and lower efficacy, and was therefore a weak agonist.

In the organ bath studies we proved that the contractions of both non-pregnant and late pregnant rat uteri were not changed by time, so all the changes we recorded related to the effects of our extract or compound.

ICI118,551 shifted the 6-MKG and terbutaline dose–response curves somewhat (insignificant) to the right in the non-pregnant rat uterus, illustrating the weak competition for the β_2-adrenoceptors (Jankovic et al., 1999).

ICI118,551 in the late-pregnant uteri completely blocked the effect of 6-MKG unlike terbutaline (partially), suggesting that 6-MKG is only a weak agonist.

Since the density of β-adrenoceptors increases in late pregnancy relative to that in the non-pregnant rat (Gaspar et al., 2005), we investigated 6-MKG activity in the late-pregnant isolated rat uterus, where only slightly different results were obtained from those on the non-pregnant rat uterus. The E_{max} of 6-MKG was 25% of that for terbutaline, whilst EC_{50} was higher for 6-MKG than for non-pregnant, where it was 1000× that for terbutaline.

The β_2-adrenoceptor acts on a GPCR through cAMP (Weis and Kobilka, 2008). 6-MKG induced a cAMP level enhanced significantly in a dose-dependent manner in the late-pregnant uteri, and thus 6-MKG is an agonist, because (Klukovits et al., 2009) revealed that terbutaline as agonist induced a cAMP level in similar mannor. Besides, the radioligand binding assay revealed that 6-MKG has better affinity than terbutaline and ICI118,551 for the β_2-adrenoceptors.

The comparison of the isolated compound 6-MKG to the original herb methanolic extract revealed that 6-MKG should play a major role in the relaxant effect of the extract. However, we can only compare the E_{max} values, because 6-MKG was used in molar concentration,

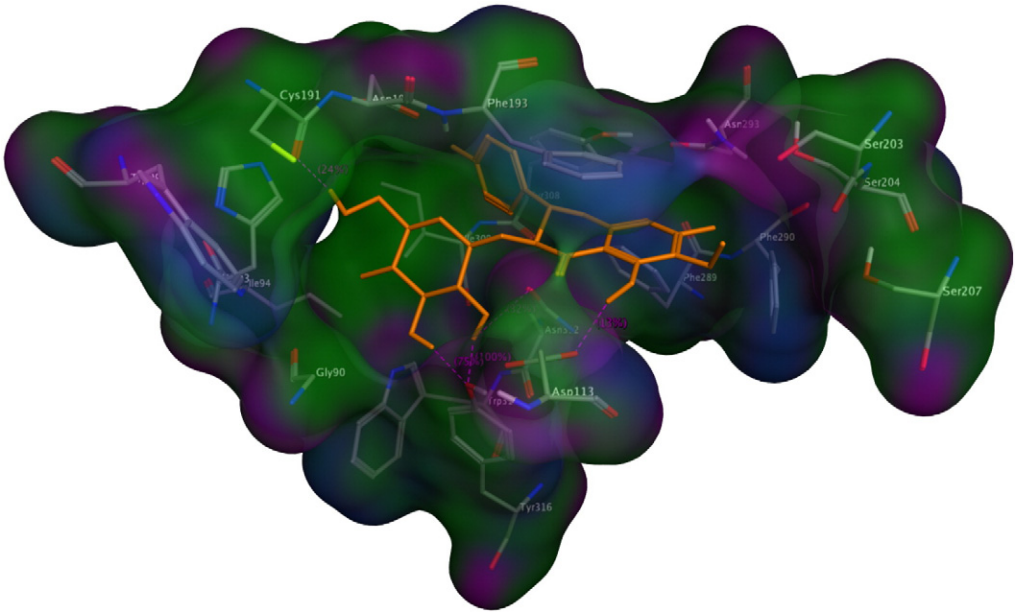


Fig. 7. Illustration of the lowest energy conformation of 6-MKG (orange-large molecule with thick grey carbon) at the rat β_2-adrenoceptors binding site. The residues are the determined interaction points within the active pocket of the receptor. The coloured surface shows the van der Waals interaction surface (purple: H-bonding, green: hydrophobic, blue: mild polar). Picture was made by MOE.

whilst the methanolic extract was used in μg/ml, so the EC_{50} values cannot be compared to each other.

The human β_2-adrenoceptor was the first non-rhodopsin GPCR to be cloned, but the X-ray structure of this receptor was solved only in 2007 (Rasmussen et al., 2007, PDB ID: 2RH1). To build the homology model of the rat β_2-adrenoceptors, we have used the customised model of 2RH1 developed by De Graaf and Rognan (2008) which prefers the binding of agonists. The receptor structure obtained by homology modelling with its RMSD values and the interaction points (Asp^{113}, Phe^{193}, Ser^{203}, Ser^{204}, Asn^{312}) with noradrenalin proved that it is a good starting point for molecular docking calculations.

In case of the reference molecules we have identified 3 common interaction points (Asp^{113}, Phe^{193} and Asn^{312}), and other two residues (Ser^{203} and Ser^{204}) represented by ligands containing the catechol ring (adrenaline, noradrenaline, isoprenaline). These findings confirm the efficiency of our homology model.

Using the scoring function of AutoDock4 the estimated free binding energy values were calculated for each ligand. These estimated values can't be compared against the experimental ΔG_{bind} and K_d values, because the standard error of the scoring function is around 2.5 kcal/mol. The 6-MKG binds to the rat β_2-adrenoceptors with low ΔG_{bind} and K_d values and has different interaction points than that of terbutaline, adrenaline, noradrenaline and ICI118,551. The position of glycopyranoside ring is stabilised by Asn^{312} (electrostatic interaction), Cys^{191} (H-bond) and the Tyr^{316} (two strong H-bonds), whilst the Asp^{113} forms an H-bond with the 5-hydroxy part of the flavone ring. Therefore the benzopyrane ring of 6-MKG anchors far from the Ser^{203} (3.4–5.2 Å), Ser^{204} (3.8–4.7 Å) and Ser^{207} (4.6–6.0 Å) and it was not able to showed these typical catechol interactions.

Finally, 6-MKG exerts weak β_2-adrenoceptor agonistic activity and can be considered a natural compound of potential significance for the treatment of premature labour and relaxation of the lung smooth muscle, and thus use for asthma treatment. 6-MKG can serve as a starting point in future drug development aimed at the production of a new safe, effective and bio-accessible therapeutic agent.

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Med Chem Res
DOI 10.1007/s00044-011-9946-0

MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

In silico and in vitro pharmacological investigations of a natural alkaloid

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Received: 29 June 2011 / Accepted: 10 December 2011

Abstract Haplopine-3,3′-dimethylallyl ether (HAP) was isolated from a Sudanese herb (El-hazha), characterized and tested on isolated rat uterus rings pre-contracted with KCl by means of in vitro and *in silico* docking studies on a customized rat β_2-adrenoceptor (β_2-AR). The in vitro results revealed that HAP produces uterus-relaxant effects similar to those of terbutaline. Its E_{max} (the maximum relaxing effect against KCl-induced contraction) was approximately 80% that of terbutaline, whilst its EC_{50} (The concentration producing 50% of E_{max}) was ten times lower than that for terbutaline, showing greater potency and less efficacy. ICI-118,551 (a selective β_2-AR antagonist) exhibited similar antagonism as concerns the relaxing effects of HAP and terbutaline, with $pD_2' = 6.3 \pm 0.2$ and $pA_2 = 6.7 \pm 0.2$, respectively. The *in silico* results of β_2-AR showed that HAP behaves similarly terbutaline except in the orientation within the active pocket. However, HAP has unique basic bond (His^{93}) instead of the acidic bond at the most important site (Asp^{113}) of β_2-AR. This study suggests that the affinity is closely related to the interactions of the oxygen groups, whilst the difference in efficacy may be due to the lack of the Asp^{113} interaction. The correlation of the calculated ligand efficiency and E_{max} revealed that there was a direct strong relation between the efficiency and in vitro efficacy. Finally, it was concluded that HAP can be categorized as a β_2-AR agonist; further pharmacokinetic and toxicological studies are required to complete its profile.

Keywords *In silico* · β_2-Adrenergic receptor · Rat uterus · Homology modelling · Docking · El-hazha · Ligand efficiency

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Introduction

Researchers have recently turned back to nature in the search for molecules from medicinal herbs after huge efforts have been devoted to the development and production of biotechnological drugs of protein origin (Piascik, 1996; Joe, 2003), because these drugs have many disadvantages (Sethuraman and Stadheim, 2006), such as rapid hydrolysis, they elicit an immune response, their effects are of short duration and they are expensive. Moreover, biotechnological drugs cover only a relatively small area of drugs, unlike the classical drugs.

Natural alkaloids such as atropine and others are still considered among the most active classical drugs. African medicinal plants are a rich drug source and biologically active compounds can be isolated from them by fractionation, depending on the ethno-pharmacological information.

El-hazha (*Haplophyllum tuberculatum*) (Forssk.) A. Juss. (Rutaceae) is a herb indigenous to the northern part of Sudan, North Africa and other areas of the Middle East (Boulus,

Published online: 30 December 2011

1983). Named locally in Sudan 'a plant of all disease', this herb was selected as a target source to search for new β_2-adrenergic receptor drugs of natural origin with which to solve the two main medical challenges of pre-term labour and asthma, due to its extensive traditional uses in this area. The herb is also used as an antispasmodic, to treat allergic rhinitis (Mohamed *et al*., 1996), gynecological disorders, asthma and breathing difficulties in Sudan.

The traditional use of El-hazha as a relaxing agent was found to be due to the presence of active compounds that act on the β_2-adrenoceptors (β_2-ARs), because this herb possesses putative β_2-adrenergic agonistic activity (Ahmed *et al*., 2010).

β_2-ARs agonists are of clinical importance for both asthma treatment (D'Urzo *et al*., 2010) and premature labour inhibition (Giles and Bisits, 2007) as tocolytic agents.

In silico homology modelling has been used extensively to prepare drug targets that save time and money by speeding-up research signals (Klebe, 2006). The crystal structure of β_2-ARs was determined in 2007 (Cherezov *et al*., 2007), and an important modification was made by de Graaf and Rognan (2008). This model can distinguish between agonist and antagonists, and is therefore used as a template for homology modelling processes (Sabio *et al*., 2008). We have performed *in silico* docking study to find the interaction points of haplopine-3,3'-dimethylallyl ether (HAP) and terbutaline within the β_2-AR binding site and to determine the energy relations of ligand–receptor interaction.

It has been reported that *in silico* data can be applied in the same useful equation for estimation of the equilibrium binding affinity (BA) of drug candidates as for the experimental free energy of binding (ΔG_{bind}) from the experimental data to calculate the various ligand efficiencies (LEs) on the basis of a set of biologically relevant structural and thermodynamic experimental data (Hetenyi *et al*., 2007).

Many compounds have been isolated from El-hazha, including alkaloids (Khalid and Waterman, 1981; Al-Rehaily *et al*., 2001; Al-Burtamani *et al*., 2005), but few of them have been tested pharmacologically. Among them, HAP was isolated and identified early in 2003 (Al-Rehaily *et al*., 2003), but its pharmacological profile remains uncertain. This study set out to investigate the pharmacological activity of this natural alkaloid HAP.

Materials and methods

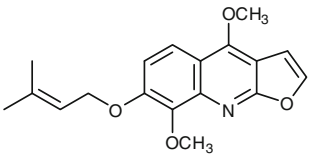
Phytochemical aspects

Plant material

The aerial parts of *H. tuberculatum* (El-hazha) were collected in the north of Sudan (Abu hamad, Nahr El-Neel State) in November 2008 and identified by Dr. Wai'l E Abdalla and Yahia S. Mohamed [Medicinal and Aromatic Plants Research Institute (MAPRI), Khartoum, Sudan]. A voucher specimen (No. M23/08) has been deposited at the Herbarium of MAPRI.

Isolation and identification of HAP

HAP or furoquinoline alkaloid 7-isopentenyloxy-γ-fagarin was isolated from the dried aerial parts of El-hazha and its structure was determined by NMR spectral data analysis. The structure was identical with that published by Al-Rehaily *et al*. (2003). The chemical formula of this compound is $C_{18}H_{19}NO_4$ and the molecular weight is 313.13 g/mol, with the following structure:



A total of 10 mg was produced and reconstituted by DMSO, then diluted with distilled water to give the desired concentration for all pharmacological tests (Ganguly *et al*., 2007).

In silico studies

Preparation of ligands

The structure of the isolated compound was drawn by using Symyx Draw Editor 3.2.1.60 software (Symyx Technologies, 2010), then converted to pdb file format, and minimized with the Molecular Operating Environment (MOE) software developed by Chemical Computing Group Inc. (2009). The pdb file of standard ligand terbutaline (accession number DB00871) was downloaded from DrugBank (Wishart *et al*., 2006) as a pdb file.

Docking studies

In this study, terbutaline and HAP were docked on the rat β_2-AR binding site by using AutoDock 4.2 software (Morris *et al*., 2009). The homology model of β_2-ARs was built during previous study (Ahmed *et al*., 2011). The initial structures of the compounds were prepared in MOE, and minimized (grad < 0.001) by using the MMFF94 force field (Halgren and Nachbar 1996; Halgren 1996a, b, c, d).

Before docking analysis, the docking files were prepared with regard to the AutoDock4 requirements, using the AutoDock Tools 1.5.4 (Sanner, 1999), in which all possible flexible bonds of the ligands, the partial atomic charges

(Gasteiger–Marsili formalism), and the Kollman charges for all atoms in the β_2-ARs were assigned. Finally, polar hydrogens were added. All other parameters were kept at their default values. The grid box (60, 60, 60 Å) was centred (3.767, 14.309, 4.648) on the isoproterenol (Soriano-Ursúa *et al*., 2009a, b) and the lattice point distance was set to 0.375 Å. This grid centre was used for all other ligands. All simulations used the hybrid Lamarckian Genetic Algorithm (Morris *et al*., 1998), with an initial population of 100 randomly placed individuals and a maximum number of energy evaluations of 10 million. The lowest energy cluster returned for each compound was used for further analysis. The interactions of the ligands on the β_2-ARs were visualized and the figures were created by using AutoDock Tools v1.5.4 and MOE.

In vitro studies

Housing and handling of the animals

The animals were treated in accordance with the European Communities Council Directives (86/609/ECC) and the Hungarian Act for the Protection of Animals in Research (XXVIII.tv.32. §). All experiments involving animal subjects were carried out with the approval of the Hungarian Ethical Committee for Animal Research (registration number: IV/1758-2/2008). Sprague–Dawley rats (Charles-River Laboratories, Hungary) were kept at 22 ± 3°C; the relative humidity was 30–70% and the light/dark cycle was 12/12 h. They were maintained on a standard rodent pellet diet (Charles-River Laboratories, Hungary) with tap water available ad libitum. The animals were killed by CO_2 inhalation.

Isolated organ studies

Uteri were removed from non-pregnant rats (250–350 g). Muscle rings 5 mm long were sliced from the uterine horns and mounted vertically in an organ bath containing 10-ml de Jongh solution (composition: 137 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 4 mM NaH_2PO_4, 6 mM glucose, pH: 7.4). The organ bath was maintained at 37°C and carbogen (95% O_2 + 5% CO_2) was bubbled through it. After mounting, the rings were equilibrated for about 1 h before the experiments were undertaken with a solution change every 15 min. The initial tension of the preparation was set to ~1.5 g, which was relaxed to ~0.5 g at the end of equilibration. The tension of the myometrial rings was measured with a gauge transducer (SG-02; Experimetria Ltd., Budapest, Hungary) and recorded with a SPEL Advanced ISOSYS Data Acquisition System (Experimetria Ltd., Budapest, Hungary).

Normal spontaneous contractions were recorded, contractions were then elicited with KCl (25 mM) and cumulative dose–response curves were constructed in each experiment for HAP or terbutaline in different concentrations (range from 10^{-9} to 10^{-4} M) in the presence and absence of 10^{-5} M ICI-118,551 (a selective β_2-AR antagonist). Following the addition of each concentration of the tested material, recording was performed for 5 min. Dose–response curves were fitted from the evaluated areas under curves (AUCs). E_{max}, EC_{50}, and pA_2 values were determined and compared statistically by using the computer program Prism 4 (GraphPad Software Inc. San Diego, CA, USA).

In silico and in vitro LE estimation

A set of standard β_2-ARs and the isolated compound HAP were explored in MOE to calculate various parameters and evaluated with regard to their efficiencies in order to determine the drug candidate properties. Although the scoring function of AutoDock can easily overestimate ΔG_{bind} because of its additive nature, even in the case of larger ligands, AutoDock4 was used to obtain ΔG_{bind} for the ligand after *in silico* docking. However, the LE value was introduced to normalize the free binding energy values (Garcia-Sosa *et al*., 2008); LE indices (ΔG/molecular weight (MW) and ΔG/number of heavy atoms (NHA), respectively) were calculated for ligands relative to the MW and NHA, where ΔG_{bind} is the free energy of binding.

The following equation was used to calculate the LE from the in vitro data: $LE = -RT \ln(EC_{50})/NHA$.

On the other hands, rules for identifying a drug candidate ΔG/NHA must be less than −0.24 kcal/mol, MW less than 500 g/mol and NHA between 20 and 70 (Bembenek *et al*., 2009) were applied for our set.

Statistical analysis

The statistical analyses were performed by using the Prism 4.0 (GraphPad Software, USA) computer program. For the statistical evaluations, data were analysed by means of two-tailed unpaired *t* tests to compare the significance of mean differences for various results. All data were obtained from at least six animals. The differences were considered to be significant at $P \leq 0.05$.

Results

In silico docking studies

There are four common ligand–side chain interactions (Asp^{113}, Thr^{118}, Phe^{193} and Asn^{312}), which stabilize the receptor–ligand complex for terbutaline, whilst for HAP the Thr^{118} was replaced by the Thr^{110}, Asp^{113}, Phe^{193} and Asn^{312} were absent and unique basic interaction (His^{93}) was

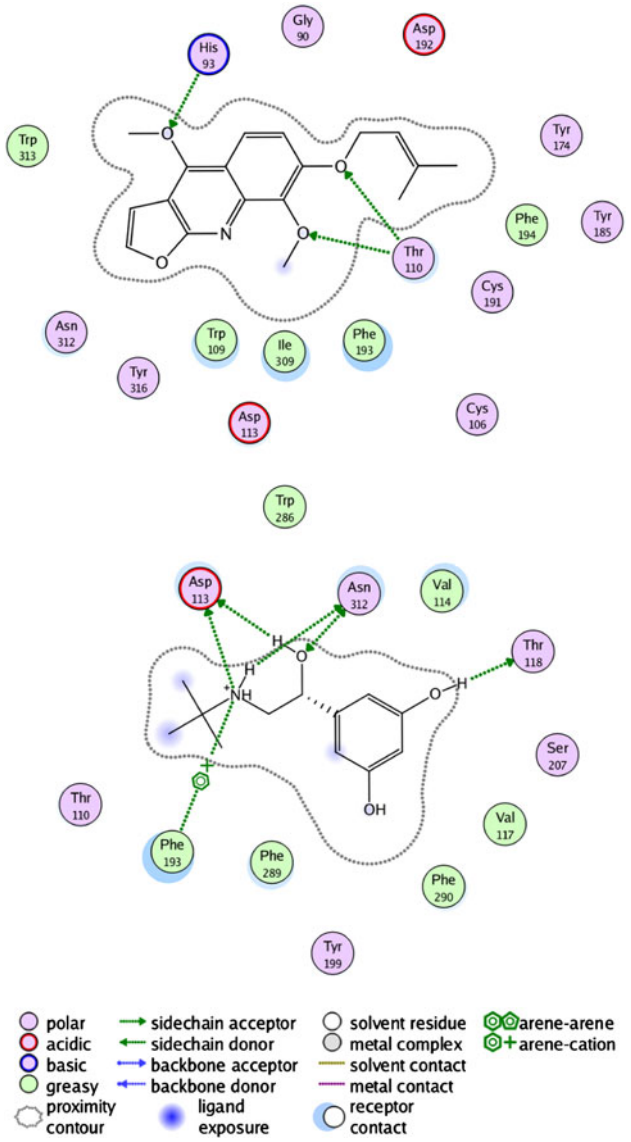


Fig. 1 Illustration of the interaction points of the lowest energy conformation of HAP (*up*) and terbutaline (*down*) docked into the active site of a customized rat β_2-adrenoceptor

determined (Fig. 1). The calculated ligand–receptor interaction parameters of the classical standard terbutaline and HAP are presented in Table 1. Whilst the binding energy (ΔG_{bind}), van der Waals energy and calculated K_d values are very similar, the intermolecular electrostatic and H-bond energy values of HAP are ten times higher than that of terbutaline. The protonated δ-nitrogen of His^{93} forms one and the γ-OH of the Thr^{110} forms two H-bonds with three different oxygen of HAP (H-bond energy: −0.40 kcal/mol). In the case of terbutaline, the Thr^{118} builds one, the Asp^{113} participates in two and the Asn^{312} forms three H-bonds (H-bond energy: −3.07 kcal/mol). There is an ionic (γ-OH of Asp^{113} with protonated N of terbutaline) and a cation–π ligand–receptor interaction (Phe^{193} with protonated N of terbutaline) too. The final intermolecular energy values—calculated by Autodock 4—are very similar (HAP: −10.09 kcal/mol, terbutaline: −10.77 kcal/mol).

The position of the lowest energy conformation of HAP and the standard terbutaline docked into the active site of customized rat β_2-adrenoceptor is different. Figure 2 illustrates the orientation of the ligands in the binding

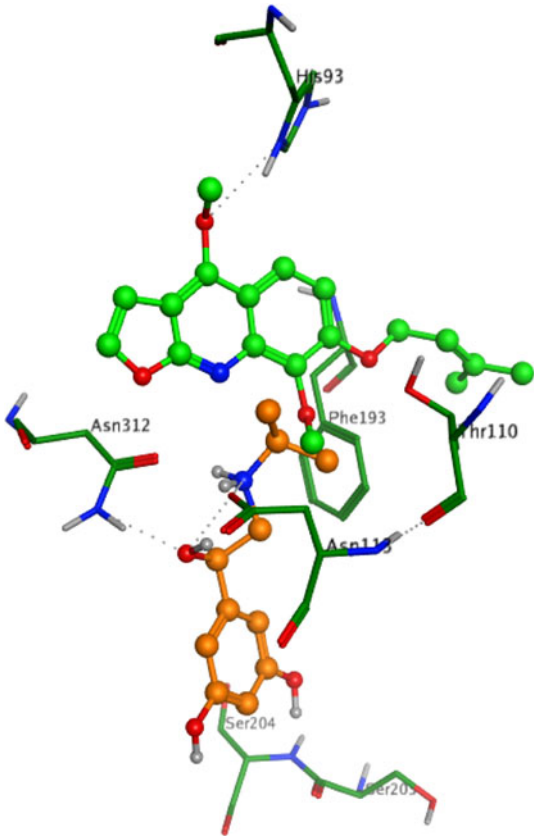


Fig. 2 Different binding orientation of HAP (*ball and stick, upper horizontal*) and the standard terbutaline (*ball and stick, down vertical*) docked into the active site of a customized rat β_2-adrenoceptor

Table 1 Ligand–receptor interaction energies of HAP and terbutaline with β_2-adrenergic receptor

Ligand	ΔG	Ligand–receptor electrostatic energy	Intermolecular H-bond energy	Ligand–receptor vander Waals energy	K_d
Terbutaline	−8.68	−1.95	−3.07	−8.82	1.35
HAP	−8.59	0.11	−0.40	−11.97	1.12

The unit is kcal/mol for all energy values and μmol for K_d

pocket: terbutaline occupy the binding cavity of adrenergic ligand determined by others (Rasmussen *et al*., 2007), whilst the HAP binds into different position.

In vitro isolated organ studies

Effects of HAP and terbutaline on isolated rat uterus

Both HAP and terbutaline induced concentration-dependent relaxant activity on isolated non-pregnant rat uterus pre-contracted with KCl (Fig. 3). E_{max} for HAP differed significantly from that for terbutaline, whilst the EC_{50} value was not different as compared with terbutaline. DMSO as vehicle was ineffective on the basal tissue activity.

Effect of ICI-118,551 on relaxant activity of HAP and terbutaline on rat uterus

The selective β_2-adrenoceptor antagonist ICI-118,551 at 10^{-5} M was without any significant effect on the non-pregnant tissue activity in the presence or absence of KCl. Results obtained by AUC comparison are presented in Fig. 4.

ICI-118,551 antagonized the relaxing effects of HAP and terbutaline and shifted the terbutaline curve to the right, and HAP curve downward similarly with insignificant differences in pA_2 and pD_2' (Fig. 5; Table 2).

LE estimation

As shown in Table 3, HAP satisfies the criteria (Bembenek *et al*., 2009) for identifying a drug candidate by exhibiting ΔG/NHA value less than −0.24 kcal/mol, a MW less than 500 g/mol and 23 NHA.

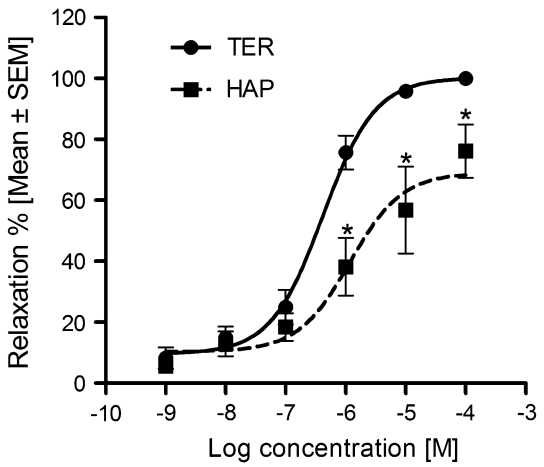


Fig. 3 Dose–response curves of the relaxing activity of HAP (*dashed line*) and terbutaline (*continuous line*) on non-pregnant rat uterus in vitro against KCl-induced control contractions. Values presented are means of 6–8 observations, whilst *vertical bars* denote standard errors of the mean (SEM), $*P < 0.05$, for HAP against terbutaline

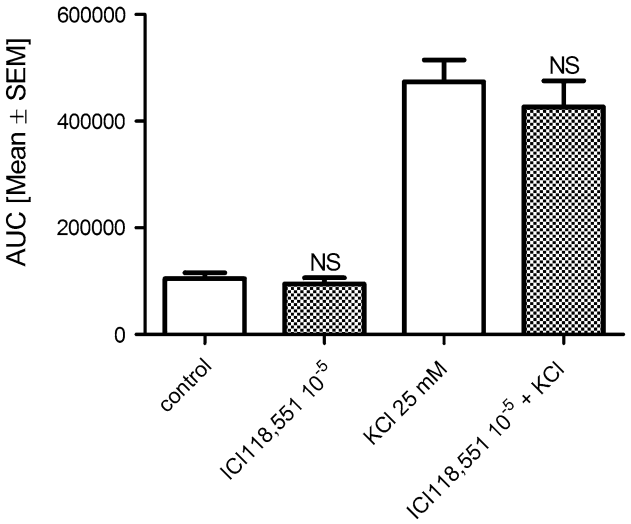


Fig. 4 Effects of the selective β_2-adrenoceptor antagonist ICI-118,551 on tissue activity, illustrated as AUC columns, on non-pregnant rat uterus in vitro against KCl-induced control contractions in the presence (*full column*) and absence (*empty column*) of ICI-118,551. Values presented are means of 6–8 observations, whilst *vertical bars* denote standard errors of the mean (SEM); *NS* not significant statistically

Calculations LE of HAP and terbutaline for both the in vitro and the *in silico* data regarding NHA (Table 4) yielded a similar value as for E_{max} calculated with the Prism software from the in vitro results, as shown in Fig. 3.

Discussion

HAP isolated from El-hazha by fractionation was subjected to in vitro and *in silico* pharmacological investigations on the rat uterus.

The *in silico* findings revealed that HAP resembles the standard terbutaline in ΔG_{bind} and calculated K_d, but differs from it as concerns the orientation within the active pocket, and the H-bond, electrostatic and van der Waals energy values. The electrostatic energy approximately describes the recognition of the small molecule by the protein (Purohit *et al*., 2011). The terbutaline possesses negative electrostatic energy (−1.95 kcal/mol) meaning better interaction with the receptor, whilst HAP has a positive value (0.11 kcal/mol) representing weak recognition. The formation of H-bonds and van der Waals contacts require appropriate orientation and distance between interacting atoms. We have determined only three H-bonds between HAP and β_2-AR side chains whilst the terbutaline forms five H-bonds and possesses about ten times lower H-bond energy (−0.40 and −3.07 kcal/mol, respectively). The van der Waals interaction energy of HAP is much lower (−11.97 kcal/mol) than that of terbutaline (−8.56 kcal/mol). Figure 1 shows that there are 16 residues, which interact

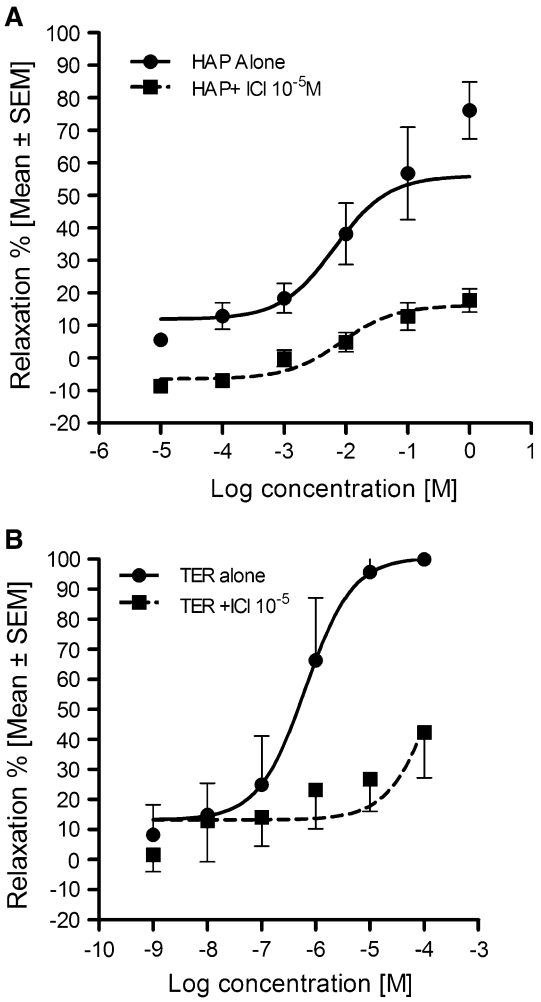


Fig. 5 Effects of the selective β_2-adrenoceptor antagonist ICI-118,551 on the relaxing activities of HAP (**a**) and terbutaline (**b**), illustrated as concentration–response curves of the relaxing effect of HAP and terbutaline on the non-pregnant rat uterus in vitro against KCl-induced control contractions in the presence (*dotted line*) and absence (*continuous line*) of ICI-118,551. Values presented are means of 6–8 observations, whilst *vertical bars* denote standard errors of the mean (SEM)

Table 2 Effects of ICI-118,551 on the relaxing activities of terbutaline and HAP on non-pregnant rat uteri in vitro

Ligand	pA_2[a] and pD_2'[b] [mean ± SEM], $N = 8$	E_{max} [mean ± SEM]%, $N = 8$	
		Without ICI	With ICI
TER	6.7 ± 0.2	101.8 ± 0.5	39.0 ± 3.8
HAP	6.3 ± 0.2	80.8 ± 11.8	16.5 ± 4.5
P value	NS	$P > 0.05$	$P > 0.01$

TER terbutaline, *HAP* isolated compound; pA_2 for terbutaline; pD_2' for HAP, *N* total number of observations, *ICI* ICI-118,551

[a] $pA_2 = pA_x + \log(x - 1)$, where $x = EC_{50}^{ANT}/EC_{50}$

[b] $pD_2' = pA_x + \log(x - 1)$, where $x = E_{max}/E_{max}^{ANT}$

Table 3 Number of heavy atoms (NHA), molecular weight (MW, g/mol), estimated free energy of binding (ΔG_{bind}, kcal/mol) and calculated LEs (ΔG/NHA, kcal/mol per non-hydrogen atom; ΔG/MW, kcal/g) of docked ligands for the rat β_2-adrenoceptor

Compounds	NHA	MW	ΔG	ΔG/NHA	ΔG/MW
ICI	20	278.416	−9.10	−0.455	−0.033
nADR	12	170.188	−5.66	−0.472	−0.033
ADR	13	184.215	−6.65	−0.512	−0.036
ISO	15	212.269	−8.45	−0.563	−0.040
PROP	19	260.357	−10.30	−0.542	−0.040
TER	16	226.296	−8.18	−0.511	−0.036
HAP	23	313.353	−8.12	−0.353	−0.026

ICI ICI-118,551, *PROP* propranolol, *TER* terbutaline, *ISO* isoproterenol, *nADR* noradrenaline, *ADR* adrenaline, *HAP* isolated compound

with HAP whilst terbutaline binding position is stabilized by only 12 residues. These findings suggest that the recognition of HAP by the β_2-AR is little bit poorer than that of terbutaline, but HAP forms slightly more stable complex with the receptor.

HAP exerts a lower uterus-relaxant activity than that of terbutaline, which is in line with the *in silico* a result. HAP possibly has β_2-AR agonistic activity, displaying a BA similar to that of terbutaline. This hypothesis is supported by the results with the β_2-adrenoceptor antagonist ICI118,551, which decrease the maximal effect of HAP and the potency (EC_{50}) of terbutaline. However, the E_{max} of HAP was more depressed by the antagonist, which suggests that HAP has a lower efficacy as compared with that of terbutaline.

LE has proved to be a useful measure of the impact of the addition of more molecular bulk on activity, where molecules that achieve a given potency with fewer heavy atoms are by definition more efficient (Kuntz *et al*., 1999). It allows the combination of pharmacodynamic (EC_{50} and ΔG_{bind}) and pharmacokinetic (MW, NHA, etc.) properties into unique measures (Bembenek *et al*., 2009). For identification of a drug candidate, the ΔG/NHA must be below −0.24 kcal/mol, MW less than 500 g/mol and NHA between 20 and 70. The isolated compound HAP satisfied these criteria.

In this study, we only concentrated on the pharmacodynamic property of our compound. We therefore used only three parameters (MW, ΔG_{bind} and NHA) and did not discuss other factors related to pharmacokinetic features.

For *in silico* LE calculation, we used the experimental free energy of binding (ΔG_{bind}), whilst in vitro we used the concentration at 50% inhibition (EC_{50}) as the parameter representing the BA pharmacodynamics.

The correlation of the calculated LE and E_{max} revealed that there was a direct strong relation between the efficiency and in vitro efficacy, because we observed similar

Table 4 Comparison of calculated LE based on NHA in vitro and *in silico* for terbutaline and the isolated HAP, in vitro E_{max} value

Compounds	NHA	In vitro (NP), $N = 6–8$		*In silico*		$E_{max} \pm$ SEM
		EC_{50}	LE[a]	ΔG (kcal/mol)	LE[b]	In vitro
TER	16	5.94 ± 3.2E−07	−5.31E+02	−8.18 ± 0.05	−0.511	101.8 ± 0.5
HAP	23	6.34 ± 3.9E−07	−3.68E±02	−8.12 ± 0.12	−0.353	80.8 ± 11.8
Ratio HAP/TER			69.30%		69.08%	79.37%

TER terbutaline, *HAP* isolated compound, *NP* non-pregnant rat uterus, *NHA* number of heavy atoms, *MW* molecular weight, ΔG_{bind} estimated free energy of binding, E_{max} maximum relaxing effect of TER or HAP against KCl-induced contraction, EC_{50} concentration of the TER or HAP producing 50% of the maximum relaxing effect against KCl-induced contractions in the system, *SEM* standard error of the mean, *N* total number of observations

[a] $LE = -RT \ln(EC_{50})/NHA$, [b] $LE = \Delta G/NHA$

values in both cases when comparing HAP with the standard terbutaline.

Finally, HAP is of potential therapeutic value as a β_2-adrenergic agonist and may be of value in solving the medical problems of asthma and preterm labour. Its value in folk medicine has been confirmed by this experiment.

Future study is recommended to investigate the pharmacokinetics and toxicological features of HAP so that these are available as concerns evidence-based clinical therapy.

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