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Isolation and characterization of an anti-microbial biflavonoid from the chloroform-soluble fraction of methanolic root extract of *Ochna schweinfurthiana* (Ochnaceae)

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The chromatographic investigation of the chloroform soluble fraction of methanolic root extract of *Ochna schweinfurthiana* using a combination of silica and sephadex LH-20 led to the isolation of tri-methoxy derivative of lophirone A. The structure of this new compound was determined using both 1 and 2D nuclear magnetic resonance (NMR). The antimicrobial activity of the isolated compound was also investigated using agar diffusion and broth dilution techniques. Clinical isolates obtained from the Department of Medical Microbiology, Ahmadu Bello University Teaching Hospital, Zaria, Nigeria were used for the studies. The compound was found to show activity against *Staphylococcus aureus*, *Streptococcus pyrogenes*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia* and *Samonella typhi*, but was not active at the tested dose on methicillin resistant *S. aureus, Bacillus subtilis, Corynobacterium ulcerans, Escherichia coli* and the only fungi tested *Candida albicans.* The low concentration for minimum inhibitory concentration (MIC, 5 µg/ml) and minimum bactericidal concentration (MBC, 20 µg/ml) suggests the compound has a good antimicrobial activity against the susceptible organisms and validates the ethno medicinal use of the plant in the treatment of various bacterial infections, including infected wounds and typhoid fever.

Key words: *Ochna schweinfurthiana* extract, antimicrobial, tri-methoxy, lophirone A, nuclear magnetic resonance (NMR).

INTRODUCTION

Antimicrobial resistance (AMR) is not a recent phenomenon, but it is a critical health issue today that has evolved to become a worldwide public health threat (World Health Organization (WHO), 2012). AMR is driven by both appropriate and inappropriate use of anti-infective medicines for human, animal health and food production,

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production, together with inadequate measures to control the spread of infections (WHO, 2002; Goosens et al., 2005; Mathew et al., 2007; Orzech and Nichter, 2008).

At least 2 million people become infected with bacteria that are resistant to antibiotics each year in the United States out of which about 23,000 die as a direct result of these infections, while many more people die from other complications related to antibiotic resistance (Centers for Disease Control and Prevention (CDC), 2013). Most European countries similarly witness a seemingly unimpeded increase of antimicrobial resistance in the major Gram-negative pathogens which could unavoidably lead to loss of therapeutic treatment options (European Centre for Disease Prevention and Control (ECDC), 2012).

Natural products have been the most significant source of drugs and drug leads in history (Cragg and Newmann, 2005). The emergence of multidrug resistance in human and animal pathogenic bacteria as well as undesirable side-effects of certain antibiotics has triggered immense interest in the search for new antimicrobial drugs of plant origin (Ahmed and Beg, 2001). Theoretically, bacteria will continue to develop resistance once exposed to any antimicrobial agent, thereby imposing the need for a permanent search and development of new drugs (Silver and Bostian, 1993). Amongst the priority actions required to hold back the spread of AMR and curtail its potential catastrophic effect is the continuing research and development of newer effective antimicrobial agents (European Commission (EC), 2011) and higher plants represent a potential source of novel antibiotic prototypes (Meurer–Grimes et al., 1996).

Ochna schweinfurthiana F Hoffm is a shrub or small tree up to 4 m tall that belongs to the family Ochnaceae. The family has been reported to be a rich source of complex dimmers of biflavonoids and chalcones (Anuradha et al., 2006; Ichino et al., 2006; Jayaprakasam et al., 2000; Kaewamatawong et al., 2002). We have previously reported the anti-microbial effect of the methanol and acetone extracts of the stem bark and leaves extract of the *O. schweinfurthiana* (Abdullahi et al., 2010) and also isolated and investigated the anti-microbial activity of a flavonoid di-glycoside from the butanol soluble fraction of methanolic leaves extract of *O. schweinfurthiana* (Abdullahi et al., 2011). In continuation of our search for bio-active compounds from the plant, we hereby report the isolation and antimicrobial effect of a new biflavonoid from the chloroform soluble fraction of the methanol extract of the root of O. *schweinfurthiana.*

MATERIALS AND METHODS

Collection, identification and preparation of plant

The whole plant material of *O. schweinfurthiana* was collected in Samaru-Zaria, Nigeria in June 2007. It was authenticated by Mr. Musa Muhammad of the herbarium section of Biological Sciences Department, Ahmadu Bello University, Zaria, and a voucher specimen (number 900229) was deposited. The leaves were removed, air-dried and powdered.

Extraction

The powdered root of *O. schweinfurthiana* (2.7 kg) was extracted three times each for 3 days with methanol (3.5 L) by cold maceration. The solvent was removed *in vacuo* to afford a reddish-brown product (336 g) referred to as *O. schweinfurthiana* root methanol extract coded (OSR). 100 g of the extract was suspended in distilled water and filtered. The filtrate was then successively partitioned with hexane, chloroform, ethylacetate and n-butanol to afford chloroform, ethylacetate, butanol and residual aqueous fractions.

Thin layer chromatography (TLC)

The chloroform-soluble portion of the methanol extract of *O. schweinfurthiana* (SRC) was subjected to TLC using pre-coated aluminium plate. The solvent systems used were chloroform and methanol (9:1 and 15:1). The spots on TLC were visualized under ultraviolet (UV), spraying with Gibbs reagent followed by exposure to ammonia solution or spraying with 10% sulphuric acid followed by heating at a temperature of 110°C for 5 to 10 min.

Chromatographic separation

Separation of chloroform-soluble fraction of O. schweinfurthiana root extract

Column chromatography, silica gel: Chloroform soluble fraction (5 g) was mounted over a glass column (75 × 3.5 cm) packed with silica gel (60 to 230 mesh). The column was eluted continuously using n-hexane, dichloromethane and methanol mixture by gradient elution technique; 50 ml each was collected as eluates and the progress of elution was monitored using TLC. A total of 10 fractions (A1-A10) from 110 collections were made; fractions A7 and A8 (coded SR-8A) were combined and further purified by Sephadex LH-20 gel filtration column chromatography. In both cases, the progress of separation was monitored using TLC.

Gel filtration: Further purification of SR-8A was undertaken using sephadex LH-20 eluted with methanol. The progress of separation was monitored using TLC. Repeated gel filtration led to the isolation of a compound coded 8A.

Phytochemical analysis

The isolated compound was subjected to shinoda and ferric chloride tests (Silva et al., 1998).

Spectral analysis

UV spectra were obtained in methanol on a Helios-zeta, UV-VIS Spectrophotometer. IR spectra was recorded (KBr) on Shimadzu FTIR8 400S Fourier Transform Infrared Spectrophotometer. NMR spectra (both 1D and 2D) were obtained on a Bruker AVANCE (600 MHz for ^{1}H and 125 MHz) for ^{13}C spectrometer, using the residual solvent peaks as internal standard. Chemical shift values (δ) were reported in parts per million (ppm) relative to internal solvent standard and coupling constants (*J* values) were given in Hertz. Heteronuclear multiple bond correlation (HMBC) spectroscopy spectra were optimized for a long range $J_{H\text{-}C}$ of 7Hz ($d_6 = 0.07s$).

The solvent used was deutrated chloroform (CDCl$_3$)

Antimicrobial assay

The microorganisms tested include *Staphylococcus aureus,* methicillin resistant *Staphylococcus aureus, Streptococcus pyogenes, Bacillus subtilis, Corynobacterium ulcerans, Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumonia, Salmonella typhi* and *Candida albicans.* All the organisms were clinical isolates obtained from the Department of Medical Microbiology, Ahmadu Bello University Teaching Hospital, Zaria, Nigeria.

Reference drug

Sperfloxacin 5 mcg/disc made by Himedia Laboratories Pvt. Ltd. Mumbai, India was used as the reference drug.

Susceptibility studies

Preliminary antimicrobial activity of the compound was carried out using stock concentration of 20 µg/ml. The microorganisms were maintained on agar slant. The inocula were prepared by inoculating the test organisms in nutrient broth and incubating them for 24 h at 37°C for the bacteria, while for *C. albicans*, Sabouraud Dextrose broth was used and was incubated for 48 h. After incubation, the broth cultures were diluted to 1:1000 for the Gram-positive bacteria and 1:5000 for the Gram-negative bacteria. One milliliter of the diluted cultures was inoculated into a sterile molten nutrient agar at 45°C and poured into sterile petri-dish. Similarly, 1 ml of the diluted fungal suspension was poured into sterile Sabouraud dextrose agar plates and the excess sucked up with Pasteur pipette. These were swirled gently and allowed to solidify. Wells were bored into the solidified inoculated nutrient agar plates using cork borer of 6 mm diameter. The wells were filled with 0.1 ml of the compound. Sparfloxacin standard disc was also placed on the agar plate. 1 h was allowed for the compound to diffuse into the agar after which the plates were incubated overnight at 37 and 25°C for bacteria and fungi, respectively. At the end of incubation period, diameter of inhibition zone was measured using transparent ruler and recorded. The compound and standard antibiotic were tested in duplicate and mean zones of inhibition were calculated.

Minimum inhibitory concentration (MIC)

MIC was determined using broth dilution method (Volleková et al., 2001). Two fold serial dilutions of the compound were made to obtain concentrations of 20, 10, 5, 2.5 and 1.25 µg/ml. 0.2 ml suspension of standard inoculum of each organism was inoculated to the different concentrations of the compound. The test tubes were then incubated at 37°C for 24 h after which they were observed for growth. Inhibition of growth was indicated by a clear solution. The MIC was defined as the lowest concentration of the compound inhibiting the visible growth of each microorganism.

Minimum bactericidal concentration (MBC)

The contents of the MIC tubes and the preceding tubes in the serial dilution were sub-cultured into appropriately labeled nutrient agar plates by dipping a sterile wire loop into each test tube and streaking the surface of the labeled nutrient agar plates. The plates were then incubated at 37°C for 24 h after which they were ob-served for colony growth. The lowest concentration of the subculture with no growth was considered as minimum bactericidal concentration (Volleková et al., 2001).

RESULTS AND DISCUSSION

Compound 8A was obtained as a colorless amorphous powder (15 mg) from the chloroform-soluble fraction of *O. schweinfurthiana* root. It gave pink-reddish colour upon spraying with 10% H$_2$SO$_4$ in methanol and heating over hot plate at 110°C plate for 5 to 10 min. It also gave a purple colour on spraying with GIBBS reagent signifying the presence of phenolic ring.

The UV in methanol showed absorptions at 243, 247 and 277nm suggestive of an isoflavonoid nucleus (Enas et al., 2012). The IR spectrum (KBr), Vmax cm^{-1} comprised of absorption bands at 3061 (C-H aromatic stretching), 1574 and (aromatic C=C). Absence of absorption band at 1620 to 1670 suggests compound as an isoflavonoid or a chalconoid (Mabry et al., 1970; Peng et al., 2006).

The ^{1}H-NMR spectrum exhibited a characteristic proton signal downfield at δ_H 12.62 corresponding to a free H-bonded OH group. It also displayed signals typical of a 1, 2, 4-trisubstituted benzene ring indicated by a set of meta-coupled proton at (δ_H 6.70 (d, *J*=2.2 Hz, H-3B$_1$), δ_H 6.77 (dd*, J*=2.2 Hz, 8.58 Hz, H-5B$_1$) and an ortho-coupled proton downfield at δ_H 7.93, (d, *J*=8.94), representing H-6B$_1$ (Mabry et al., 1970). The proton resonance which appeared as a singlet further downfield at δ_H 8.06 presumably due to the influence of a keto group suggests a H-β$_1$ proton in an isoflavone system (Mabry et al., 1970; Pegnyemb et al., 2003).

Another set of 1, 2, 4-trisubstituted benzene ring system is represented by ^{1}H NMR resonances at (δ_H 6.24 (d, *J*=2.5 Hz, H-3'), δ_H 6.39 (dd*, J*=2.5 Hz, 9.1 Hz, H-5') and an ortho-coupled proton downfield at δ_H 8.17 (d, *J*=9.1, H-6')) (Pegnyemb et al., 2003). This ring sub structure is in close proximity with a carbonyl group as confirmed by the HMBC spectroscopy.

The ^{1}H NMR spectrum also exhibited two closely overlapping 1, 4-disubstituted benzene rings which was adequately visualized with Bruker Topsins Software. The protons integrated for 8 hydrogens and these 2 AA'BB' systems comprise of ortho-coupled protons at δ_H7.22, (d, *J*=8.7, 2H) assignable to H-2''/6'' and δ_H6.64 (d, *J*=8.7, 2H) assignable to H-3''/5'' in Ring A$_1$ as well as δ_H7.19 (d, *J*=8.52, 2H) assignable to H-2'''/6''' and δ_H6.68 (d, *J*=8.52, 2H) assignable to H-3'''/5''' of Ring A$_2$. The above assignment was corroborated by the Homonuclear Correlation Spectroscopy (^{1}H-^{1}H COSY) and the Heteronuclear Correlation Spectroscopy (HSQC).

The ^{13}C-NMR spectrum exhibited signals for 33 carbons, including the intensely overlapping (double) signals at δ_c129.12 (C-2''/6''), 128.43 (C-2'''/6'''), 113.87 (C-3''/5'') and 113.92 (C-3'''/5''') (Markham et al., 1978). The carbon chemical shift values showed 8 of the carbons are oxygenated. The distortion-less enhancement
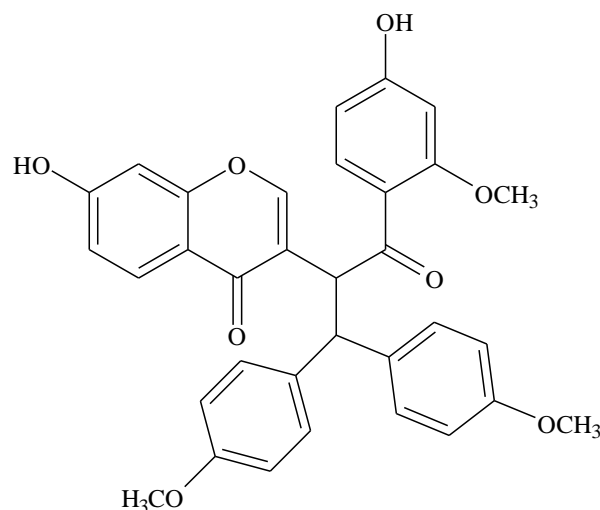
Figure 1. Tri-methoxy lophirone A.

by polarization transfer (DEPT) experiments established the nature and chemical environments of the carbon atoms and allowed the identification of 13 quaternary carbon atoms including 2 carbonyl groups at δ_c 203.47 and 175.28, and 15 aromatic sp^2 hybridized carbon atoms suggesting the presence of about 4 benzene rings in the molecule. The 3 singlet signals at δ_c 55.53, 55.13 and 55.06 are due to methoxy (OCH$_3$) group attached to aromatic ring system. The ^{13}C-NMR spectrum and the DEPT experiments also showed 2 aliphatic sp^3 carbon resonances at δ_c 52.97 and 43.04 corresponding to ^{1}H NMR aliphatic resonances at δ_H 4.69 (d, J=12.3) and δ_H 6.07 (d, J=12.3) due to α- and β protons, respectively. The cross peaks observed in the ^{1}H-^{1}H COSY further confirmed these aliphatic resonances as an ethinyl chain.

Heteronuclear multiple bond correlations between protons and carbons in the molecule (up to 3 bonds) facilitated the assignments of the H- and C-signals not captured in the HSQC, the quaternary carbons, the three methoxy groups as well as established the connectivity between the ethinyl chain to the three aforementioned substructures.

The summary of the 1 and 2D results of compound 8A established and characterized the structure of 8A as tri-methoxy lophirone A (Figure 1), which is, to our know-ledge, a new compound. Tri-methoxy lophirone A is a derivative of known compounds: lophirone A (Ghogomu et al., 1987; Messanga et al., 2001; Pegnyemb, 2003; Anuradha et al., 2006), calodenone (Messanga et al., 1992; Pegnyemb et al., 2003; Likhitwiyawuid et al., 2005; Anuradha et al., 2006) and afzelone D (Pegnyemb et al., 2003). The difference between Compound 8A (tri-methoxy lophirone A) and the three known compounds, being the presence of 3-methoxy groups in 8A instead of none, one and two methoxy groups in lophirone A, calodenone and afzelone D, respectively (Table 1).

The results of antimicrobial investigations suggest the compound has activity against *S. aureus*, *S. pyrogenes*, *P. aeruginosa*, *K. pneumonia* and *S. typhi*, but were not active, at the tested dose, on methicillin resistant *S. aureus, B. subtilis, C. ulcerans, E. coli* and the only fungi tested, *C. albicans* (Table 2).

The low concentration for the MIC (5 µg/ml) and the MBC (20 µg/ml) as reflected in Table 3 suggests the compound has a good antimicrobial activity against the susceptible organisms considering that compounds with MICs of less than 100 µg/ml are regarded as having strong antimicrobial potential (Tang et al., 2003).

S. pyogenes has been implicated in many important human diseases including skin infections, pharyngitis and rheumatic fever, and certain strains have already developed resistance to macrolides, tetracyclines and clindamycins (Malhotra-Kumar et al., 2009).

P. aeruginosa and *K. pneumonia* have also been implicated along with *E. coli* amongst the causative agents in urinary tract infection (UTI) which is the most common infectious diseases at the community level (Linhares et al., 2013).

S. typhii bacteria is the causative agent for typhoid fever and this work has further confirmed the ethno-medicinal use of various parts of *O. schweinfurthiana* in treating typhoid fever, skin and other bacterial infections.

Conclusion

Conclusively, the results of the study showed the root of *O. schweinfurthiana* contains an antimicrobial biflavonoid (tri-methoxy lophirone A) as one of its constituents: its antimicrobial activity is significant and may serve as a lead towards the development of more potent, safe and cost effective antimicrobial agents. The result also validates

Table 1. ^{1}H NMR, ^{13}C NMR, DEPT, HSQC and HMBC Summary on 8A in (CDCl$_3$).

Position	DEPT	δC	δH, J = Hz	HMBC
B$_1$-1	C	117.1	-	-
2	C	157.7	-	-
3	CH	114.02	6.70 (1H, d, J = 2.22 Hz)	C-1, 5
4	C	161.1	-	-
5	CH	115.06	6.77 (1H, dd, J = 2.22, 8.6 Hz)	C-1, 3
6	CH	127.94	7.93 (1H, d, J = 8.94 Hz)	C-2, 4, c-$_1$
c-$_1$	C	175.28	-	-
α$_1$	C	121.19	-	-
β$_1$	CH	155.26	8.06 (1H, s)	C-2, c-$_1$, α$_1$, α$_2$
B$_2$-1'	C	113.7	-	-
2'	C	166.8		
3'	CH	100.83	6.24 (1H, d, J = 2.34 Hz	C-1', 2', 4', 5'
4'	C	165.8	-	-
5'	CH	107.96	6.39 (1H, dd, J = 2.34, 9.1 Hz)	C-1', 3'
6'	CH	132.67	8.17 (1H, d, J = 9.1 Hz)	C-, 2', 4' and c-$_2$
c-$_2$	C	203.49	-	-
α$_2$	C	43.04	6.07 (H, d, J = 12.12 Hz)	C- β$_1$, β$_2$, α$_1$, c-$_1$, c-$_2$,1", 1'''
β$_2$	C	52.97	4.69 (H, d, J = 12.12 Hz)	C- 1", 2", 1''', 2''' c-$_2$
A$_1$- 1"	C	135.28	-	-
2"	CH	129.12	7.22 (2H, d, J = 8.7 Hz)	C- 2", 3", 4", β$_2$
3"	CH	113.87	6.64 (2H, d, J = 8.7 Hz)	C-1" 3", 4"
4"	C	157.99		
5"	CH	113.87	6.64 (2H, d, J = 8.7 Hz)	C-1" 3", 4"
6"	CH	129.12	7.22 (2H, d, J = 8.7 Hz)	C- 2", 3", 4", β$_2$
A$_2$- 1'''	C	134.16	-	-
2'''	CH	128.43	7.19 (2H, d, J = 8.52 Hz)	C- 2''' 3''', 4''', β$_2$
3'''	CH	113.92	6.68 (2H, d, J = 8.52 Hz)	C-1''' 3''', 4'''
4'''	C	157.91	-	-
5'''	CH	113.92	6.68 (2H, d, J = 8.52 Hz)	C-1''' 3''', 4'''
6'''	CH	128.43	7.19 (2H, d, J = 8.52 Hz)	C- 2''' 3''', 4''', β$_2$,
OH	-	-	12.62 s	-
2-OCH$_3$	CH$_3$	55.53	3.72 s	-
4"-OCH$_3$	CH$_3$	55.13	3.66 s	-
4"-OCH$_3$	CH$_3$	55.06	3.60 s	-

Table 2. Susceptibility of 8A to various pathogenic test organisms.

Test organism	Mean zone of inhibition (mm)	
	8A (20 µg/ml)	Sparfloxacin (5 µg/ml)
Staphylococcus aureus	19	22
Methicillin resistant *Staph. aureus*	0	19
Streptococcus pyogenes	17	24
Bacillus subtilis	0	27
Corynbacterium ulcerans	0	19
Escherichia coli	0	14
Klebsiella pneumoniae	19	14
Salmonella typhi	17	27
Pseudomonas aeruginosa	16	17
Candida albicans	0	0

0: No activity.

Table 3. Minimum inhibitory and minimum bactericidal concentrations of 8A against the test organisms.

Test organism	Concentration (µg/ml)	
	MIC	MBC
Staphylococcus aureus	5	20
Methicillin resistant Staphylococcus aureus	R	R
Streptococcus pyogenes	5	20
Bacillus subtilis	R	R
Corynbacterium ulcerans	R	R
Escherichia coli	R	R
Klebsiella pneumoniae	5	20
Salmonella typhi	5	20
Pseudomonas aeruginosa	5	20
Candida albicans	R	R

R: Resistant, no activity against organisms.

the ethno medicinal use of the plant in the treatment of various bacterial infections, including infected wounds and typhoid fever. This is the first report of isolation of this compound, to the best of our search.

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Full Length Research Paper

Latent natural product and their potential application as anti-infective agents

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Extract of *Acorus calamus* (AC) and *Ferula asafetida* (FA) were tested against different bacterial pathogens by well-cut agar diffusion method. To analyzed the *in vitro* activities of *A. calamus* and *F. asafetida*, four different bacteria (*Escherichia coli*, *Staphylococcus epidermidis*, *Staphylococcus aureus* and *Bacillus subtilis*) were used. *A. calamus* had a broad spectrum antimicrobial effect against different bacterial pathogens. Streptomycin was used as standard drug with significant activity values, that is, 34 mm against *S. aureus*, 36 mm against *S. epidermidis*, 30 mm against *B. subtilis* and 24 mm against *E. coli*. Analysis of the data showing that, the crude extract of *A. calamus* in n-hexane exhibited superior activity against *S. epidermidis*. The dichloromethane extract of *F. asafetida* was found low against *E. coli* and *B. subtilis*. Results were compared concomitantly to standard drugs: streptomycin. Phytochemical screening of *A. calamus* and *F. asafetida* showed the presence of terpenoids, saponins, flavonoids, alkaloids, tannins, glycosides and reducing sugar components. The high potency of *A. calamus* and *F. asafetida* against these microbes could provide an example of prospecting for new compounds. Based on the current conclusion, it can be accomplished that these plants have antimicrobial activity, which is as potent as standard antimicrobial drugs against specific microorganisms.

Key words: *Acorus calamus*, *Ferula asafetida,* infection, inhibition, phytochemical screening, medicinal plants.

INTRODUCTION

The phytochemical research, in which ethno pharmacological properties of plants are evaluated, leads to the discovery of new anti-infective agents from higher plants. Owing to the development of drug resistive strains in human pathogens against commonly used antibiotics, it is necessary to discover new antimicrobial substances from plants and other sources. Popular European books on medicinal plants touted *calamus* as a "wonder drug." It was commonly used in folk medicine as a "nervine," most likely linked to the tranquilizing effect of cis-isoasarone. In Exodus 30: 23, 24, 34, it has been written that God ordered Moses to make the Holy Oil, one of its constituents was an aromatic reed which some authorities have suggested might have been *Acorus calamus*. Some

people strung together its dried root pieces, steam it throughout the home and is thought to "kill" sickness. *A. calamus* Linn, commonly known as sweet flag, is an aromatic medicinal plant belonging to the Araceae family. It has been long known for its medicinal value, it is wild or cultivated throughout the Himalayas at an altitude of 6000 ft. The rhizomes of *A. calamus* contain an aromatic oil that has been used medicinally since ancient times and has been harvested commercially (Meena et al., 2010). The most common way of ingesting *A. calamus* is by chewing it. It can be peeled and washed to remove the bitterness and then eaten raw like a fruit. The dried and powdered rhizome has a spicy flavor and is used as a substitute for ginger, cinnamon and nutmeg. A pinch of

the powdered rhizome is used as a flavoring in tea. The inner portion of young stems makes a very palatable salad. Sweet flag has a very long history of medicinal use in Chinese and Indo-Pak herbal traditions (Khan, 2012).

It is widely employed in modern herbal medicine as its sedative, laxative, diuretic, and carminative properties (Marcy at el., 2005). It is used in Ayurveda to counter the side effects of all hallucinogens. Both roots and leaves of *A. calamus* have shown strong antioxidant (Devi et al., 2011). It is used as antimicrobial against various bacteria, filamentious fungi, and yeast (Balakumbahan et al., 2010). *A. calamus* is effective against cattle tick, *Rhipicephalus (Boophilus) microplus* (Gosh et al., 2011). A recent study showed that beta-asarone isolated from *Acorus calamus* oil inhibits adipogenesis in 3T3-L1 cells and thus reduces lipid accumulation in fat cells (Johnson et al., 1995). Protective effect against acrylamide induced neurotoxicity (Lee, 2011). Chewing the root of *calamus* helps fight tobacco addiction, that is, it kills the taste for tobacco over time (Shukla et al., 2006). The plant is externally used to treat skin eruptions, rheumatic pains and neuralgia. It is used in incense sticks and is widely used as insecticide for lice, bedbugs, worms, etc. Sweet Flag is used as an antihistamine (Brown et al., 1989). *A. calamus* uses are abortifacient, anodyne, aphrodisiac, aromatic, carminative, diaphoretic, emmenagogue, febrifuge, hallucinogenic, homeopathy, odontalgic, sedative, stimulant, stomachic, tonic, and vermifuge.

Ferula is a genus of about 170 species of flowering plants in the family Apiaceae. It is native to the Mediterranean region East to Central Asia, mostly growing in arid climates. It is strictly distributed in Pakistan, India, Iran, and Afghanistan. *Ferula asafetida* has a pungent, unpleasant smell when raw, but in cooked dishes, it delivers a smooth flavor, reminiscent of leeks. Plant prefers full sun and dry soil (Morningstar and Desai, 1991). *F. asafetida* has shown remarkable antioxidant and antihemolytic activities. It reduces the growth of indigenous microflora in the gut, reducing flatulence. It is effective against Swine flu virus. Used for treating chronic bronchitis and whooping cough, as well as reducing flatulence.

F. assafoetida has also been reported to have contraceptive/abortifacient activity, *F. assafoetida* oleo-gum-resin has been reported to be antiepileptic. Roots yield an oleo-gum-resin used as an expectorant, antispasmodic and spice inhaling this gum prevents hysterical attacks. *F. asafetida* is useful in alleviating toothache. This spice is used as a digestive aid, in food as a condiment and in pickles (Wichtl, 2004).

MATERIALS AND METHODS

Plant

Rhizome of *A. calamus* and gum of *F. asafetida* were collected from Malakand, Pakistan. The taxonomic identification of both plants was carried out by Dr. Farrukh Hussain at the Department of Botany

(DOB), University of Peshawar, Pakistan. A voucher specimen under the scientific name of the plant (bot. 20012 (pup) and bot. 20013 (pup)) was deposited at the herbarium of Department of Botany, University of Peshawar, Pakistan.

Preparation of crude extract

Plant materials were dried under shade and powered. From these powered materials, weighed amount was taken in separate thimbles. This was suspended above the flask containing the solvent n-Hexane fitted with reflux condenser. The flask was heated (60 to 65°C for 5 to 6 h) on heating mantle; the evaporated solvent upon condensation trickled into the extraction chamber containing the plant material. At the end of the extraction process, the flask containing the n-Hexane extract was removed. After the removal of n-Hexane extract, the plant material remains in thimble and the next solvent of high polarity was passed like dichloromethane and extract was obtained. Ethyl acetate at the end of highly polar solvent ethanol was passed and thus different extract were obtained according to increasing polarities of solvents. The solvents obtained were evaporated under vacuum using rotary evaporator. The plants extracts were collected in labeled vials. The plant materials taken for extraction were *A. calamus* (70 g) and *F. assafoetida* (65 g). The crude extracts were subjected for anti-bacterial activity and their phytochemical screening.

Qualitative analysis of chemical constituents

Chemical analysis for the presence of major classes of secondary metabolites (alkaloids, tannins, anthraquinones, glycosides, reducing sugars, saponins, flavonoids, phlobatanins, steroids and terpenoids) in the crude extracts was carried out according to the method described by Pearson (1976).

Antimicrobial assay

Preparation of medium

Two types of media were used, solid and liquid media. Nutrient agar 16.8 g was dissolved in distilled water and volume was made up to 0.6 L. It was autoclaved at 120°C for 15 min. Media was cooled and allowed to solidify in Petri dishes. Nutrient broth 1.9 g was dissolved in distilled water and volume was made up to 150 ml. 8 ml of this broth was added to screw capped test tubes, which were placed in autoclave at 120°C for 15 min, then refrigerated at 37°C.

Preparation of tested materials

The test sample were prepared by dissolving 21 mg of crude extract in 1 ml of DMSO in Eppendorff tube and kept for 1 h. To another Eppendorff tube, 1 mg of streptomycin was dissolved in 1 ml DMSO and kept for 1 h.

Antibacterial assay

Cup-plate diffusion method

Antibacterial activity of plant extracts was carried using cup-plate agar diffusion method (14) with some small modifications. One milliliter from each standard bacterial stock suspension was mixed thoroughly with 40 to 45 ml of sterile Molten Mueller-Hinton agar 40°C, poured into sterile Petri-dishes and left to solidify. Then, four

cup-shape wells (120 mm diameter) were made in each plate using sterile cork-borer. The agar disks were removed and four alternate cups were filled with extract using sterile adjustable pipettes. Four Petri-dishes with two alternate cups were used with the respective solvent instead of the extracts as control. The plates were then incubated in upright position for 22 to 24 h at room temperature. Two replicates were carried out for each extract. After incubation period, the inhibition zones diameters were measured.

Disc diffusion method

The antibacterial assay for plant extract was also conducted using disc diffusion method as illustrated by Abdel-Wahab et al. (2009). The nutrient agar solution (16 ml) was poured and kept overnight in a refrigerator. Whatman filter paper discs of 6 mm diameter were impregnated with 10 µl of the solution of crude extract (at 4 mg/ml) dissolved in dimethyl sulfoxide (DMSO). Standard disc of streptomycin sulphate (10 µg/disc) was used as positive control, while DMSO was used as a negative control. The Petri dishes were inverted and incubated for 24 h at 37°C. Clear inhibition zones around the discs indicated the presence of antimicrobial activity.

RESULTS

Phytochemical screening

Phytochemical screening of chemical constituents of crude extract in different solvents showed the presence of alkaloids, tannins, anthraquinones, glycosides, reducing sugars, saponins, flavonoids, phlobatanins, steroids and terpenoids constituents as shown in Tables 1 and 2, according to Syed et al. (2013).

Antimicrobial screening

The results were summarized in Table 3. However, results were interpreted in terms of commonly used terms: sensitive, intermediate and resistant. Findings of cup-plate diffusion method for ethanolic extracts of *A. calamus* and *F. asafetida* are exposed as shown in Figures 1 and 2.

Antibacterial activities of *A. calamus* and *F. asafetida*

The antibacterial activity of crude extracts, that is, n-Hexane, dichloromethane, ethyl acetate and ethanol of the selected plants. Traditionally, *A. calamus* and *F. asafetida* were used to treat bacterial infections. The results from the current study were screened for their microbial activity against four standard bacteria, namely, *Staphylococcus aureus, Staphylococcus epidermidis, Bacillus subtilis* and *Escherichia coli.* The pattern of inhibition varied with the plant's extract, the solvent used for extraction and the organism tested. DMSO was used as negative control and no inhibition was shown by it against all the bacteria. Streptomycin, an antibiotic was used as a positive control. Streptomycin was used as

standard drug with significant activity values (Figure 3).

Results of the antibacterial activities of the plants extracts are summarized in Table 3. For all the crude extracts, the highest antibacterial activities were shown by n-hexane and ethyl acetate crude extract of *A. calamus* followed by dichloromethane fraction of *F. asafetida*. The n-hexane extract of *A. calamus* showed good activity against *S. epidermidis*. Non-significant activity of n-hexane extract of *A. calamus* was seen against *E. coli* and *B. subtilis*, while no activity was shown against *S. aureus*. Non-significant activity was seen for dichloromethane extract against *E. coli* and *B. subtilis*, while no activity was seen against *S. aureus* and *S. epidermidis*. The ethyl acetate of *A. calamus* showed low activity against *S. epidermidis*, non significant against *B. subtilis* and *S. aureus*, while no activity was seen against *E. coli*. *A. calamus*, ethanolic extract showed no activity against *B. subtilis, S. aureus* and *E. coli,* while non significant against *S. epidermidis*.

The dichloromethane extract of *F.asafetida* was found low against *E. coli* and *B. subtilis*. While exhibited non-significant activity against *S. epidermidis* and no activity against *S. aureus*. The n-Hexane extract of *F. asafetida* showed no activity against *S. epidermidis* and *E. coli* while non-significant against *S. aureus* and *B. subtilis*. The ethyl acetate extract was found non-significant against *B. subtilis*, *S. aureus* and *E. coli*, while no activity was seen against *S. epidermidis*. In addition, *F. asafetida* ethanolic extract showed non-significant activity against *S. epidermidis* and *E. coli* and *B. subtilis* while no activity was seen against *S. aureus.*

DISCUSSION

In the present study, the n-Hexane extract of *A. calamus* (2 mg/ml) showed that it is potent against bacterial strains as compared to its other extracts as shown in Figure 1. The zone of inhibition was 16 mm against *S. epidermidis*. So this plant can be further processed for isolation of active components, inhibiting the growth of this pathogen which may lead to the discovery of potent antibiotic. The potency of the other extracts of *A. calamus* against bacterial strains was not good enough. This showed that the active components of *A. calamus* are more soluble in n-hexane. The presence of saponins in *A. calamus,* shown by its dichloromethane and ethanolic extract is the cause of hyperlipdemia in rats, while diarrhea does not occur with the large dose of the extract with ethanol. Alcoholic extract is responsible for anti-inflammatory effect, while n-hexane for spasmolytic effect of the plant. The presence of flavonoids in dichloromethane extract is responsible for showing antibacterial activity, particularly, against *S. aureus.* Ethyl acetate extract showed 14 mm zone of inhibition against *S. epidermidis*, whose phytochemical screening showed the presence of alkaloids, tannins and terpenoids. This showed that out of these three constituents, one, two or all are responsible for its

Table 1. Secondary metabolites detected in different extracts of *Acorus calamus*.

Chemical component	n-Hexane	DCM	Ethyl acetate	Ethanol
Alkaloids	+	++	++	+++
Tannins	-	-	++	++
Anthraquinones	-	-	-	-
Glycosides	-	-	-	++
Reducing sugar	-	-	-	++
Saponins	-	+	-	+++
Flavonoids	-	+	-	+
Phlobatanins	-	-	-	-
Terpenoids	++	+++	++	++

+++ = High concentration; ++ = Moderate concentration; + = Low concentration, - = Absent

Table 2. Secondary metabolites detected in different extracts of *Ferula asafetida.*

Chemical component	n-Hexane	DCM	Ethyl acetate	Ethanol
Alkoloids	++	++	+++	+++
Tannins	-	-	-	+
Anthraquinones	-	-	-	-
Glycosides	-	-	-	++
Reducing sugar	-	-	-	+
Saponins	-	++	-	+++
Flavonoids	-	-	-	++
Phlobatanins	-	-	-	-
Terpenoids	++	+++	++	++

+++ = High concentration; ++ = Moderate concentration; + = Low concentration, - = absent

Table 3. Antibacterial activity of *A. calamus and F. asafetida.*

Extraction solvent	Inhibition zone (mm)*					Inhibition zone (mm)*				
	A. calamus	E.C	S.E	S.A	B.S	*F. asafetida*	E.C	S.E	S.A	B.S
Hexane	Extract	0	0	12	10	Extract	12	16	0	12
Dichloromethane	Extract	14	12	0	15	Extract	12	0	8	10
Ethyl acetate	Extract	10	0	12	12	Extract	0	14	10	12
Ethanol	Extract	12	12	0	10	Extract	0	10	0	0
Streptomycin	1 mg/ml	24	36	34	30	1 mg/ml	24	36	34	30

E.C.: *Escherichia coli*; S.E.: *Staphylococcus epidermidis*; S.A.: *Staphylococcus aureus*; B.S.: *Bacilis subtilis.*

antibacterial activity.

Conclusion

The antimicrobial effects of *A. calamus* and *F. asafetida* extracts against the studied bacteria suggest that, different parts of *A. calamus* and *F. asafetida* possess remarkable therapeutic action that can support the traditional usage of this plant in the treatment of bacterial diseases such as gastrointestinal infection, diarrhea, respiratory and skin diseases. These antimicrobial activities are likely due to the presence of secondary metabolites like tannins, flavonoids, alkaloids, saponins, terpenes and glycosides in *A. calamus* and *F. asafetida*. The high potency of *A. calamus* and *F. asafetida* against
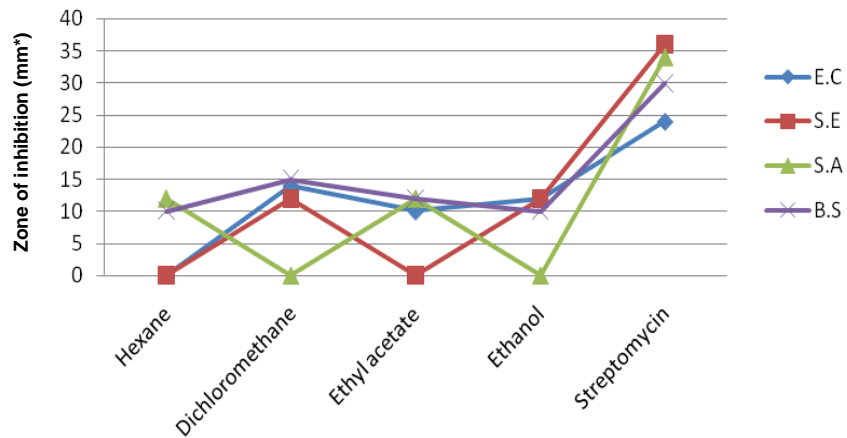
Figure 1. Antibacterial activities of *Acorus calamus* in different solvent.
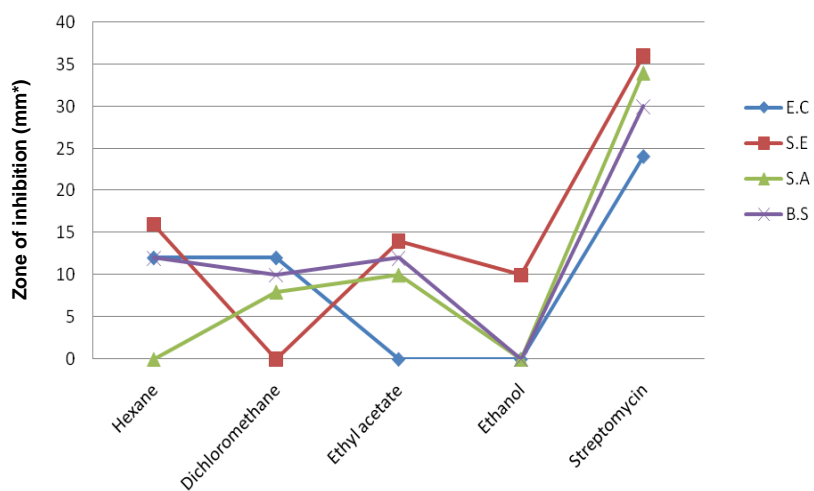

Figure 2. Antibacterial activities of *Ferula assafoetida* in different solvents.
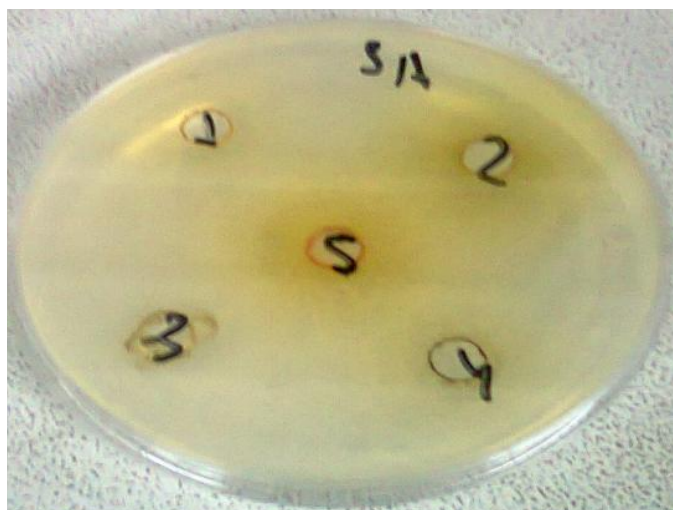

Figure 3. Cup-plate diffusion method for ethanolic extracts of the fruits and seeds against *Staphylococcus aureus.*

these microbes could provide an example of prospecting for new compounds.

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Full Length Research Paper

The effect of medium chain saturated fatty acid (monolaurin) on levels of the cytokines on experimental animal in *Entamoeba histolytica* and *Giardia lamblia* infection

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The aim of this study was to demonstrate the effect of medium chain saturated fatty acid (monolaurin) on experimentally infected *Giardia lamblia* and *Entamoeba histolytica*, and measurement of IFN-γ, TNF-α, IL-4, IL-10, TGF-β levels of cytokines. To study the effect of monolaurin on the duodenal mucosa of the studied infected hamsters, a group of sixty golden Syrian hamsters were used, which were further divided into two subgroups: Subgroup I, in which hamsters were infected by oral administration of 10,000 *G. lamblia* cysts and Subgroup II, in which hamsters were infected by 10,000 *E. histotytica* cysts. Each subgroup was divided into (6) groups. Subgroup I included the group from (1 to 6), although subgroup II included from 7 to 12 groups. In *G. lamblia* infected subgroup I, best results were observed by the reduction in both vegetative and cystic forms, respectively shown in group (6) treated with combination of metronidazole and monolaurin post infection 94.68 and 96.55%, respectively. In the Subgroup II infected with *E. histolytica*, the high reduction in trophozoite and cystic forms in intestinal contents were in the group (12) which was treated with a combination of metronidazole and monolaurin post infection (90.12 and 92.56%, respectively). Cytokines levels IFN-γ, TNF-α, IL-4, IL-10 and TGF-β were measured in serum using sandwich enzyme-linked immunosorbent assay (ELISA). The best result was shown in the group (6) treated with a combination of metronidazole and mololaurin post infection 130,129, 35, 165 and 240 Pg/ml. Also histopathological examination gave the best healing in the groups (6) infected with *G. lamblia* than thos infected by *E. histolytica*.

Key words: *Giardia lamblia*, *Entamoeba histolytica,* lauric acid, monolaurin, IFN-γ, TNF-α, IL-4, IL-10, TGF-β, histopathological examination.

INTRODUCTION

Entamoeba histolytica (*E. histolytica*) and *Giardia lamblia* (*G. lamblia*) are common causes of diarrhea and malabsorption in humans (Rauch et al., 1990). The infection may produce severe acute diarrhea in children less than five years of age with chronic infections resulting in weight loss and growth retardation (Fraser, 1994; Fraser et al., 1997; Newman et al., 2001). A parasitological assessment of drinking water in Egypt demonstrated a high prevalence of *G. lamblia*, an intestinal parasite of humans and various animals (Stauffer et al., 2006). *E. histolytica* is a protozoan causing amebic dysentery. Invasive amoebiasis is manifested by amebic colitis which

can be complicated by intestinal perforation and peritonitis or hepatic abscess which may be fatal (Petri et al., 1987). There are little data on the true prevalence and incidence of *E. histolytica* infection in Africa; however, Egypt has high rates of asymptomatic infection as detected by the stool examination (Stauffer et al., 2006).

Therapeutic strategy has included diverse pharmaceutical agents of traditional use such as metronidazole, quinacrine and furazolidone (Gardner and Hill, 2001; Harris et al., 2001). However, evidence points to an increasing frequency of cases refractory to treatment with these drugs (Mendelson, 1980; Brasseur and Favennec, 1995; Sarker et al., 2010). Of these, metronidazole and albendazole may be the most representatives of anti-*E. histolytica* and *G. lamblia* of traditionally and recently used. Lemee et al. (2000) pointed to an increased frequency of cases refractory to treatment with these drugs and emergence of the drug-resistance species. Metronidazole has proved to acquire potential carcinogenicity and mutagenic effect in rats (Hill, 2000).

Cytokines may have a role in *G. lamblia* and *E. histolytica* infection. Cytokines or Interleukins are secreted by lymphocytes, monocytes or macrophages. They act on other cells of the immune system to regulate their function. Interleukins cause inflammatory response in parasitic diseases. (Beutler and Cerami, 1989). Tumor necrosis factors (TNF), a mediator of inflammatory response also plays an important role in parasitic diseases (Beutler and Cerami, 1986). Furthermore, this cytokine promotes the proliferation and recruitment of monocytes and neutrophils to inflammatory sites being the dominant cytokine for *E. histolytica* and *G. lamblia* parasite (Singer and Solaymani, 2010).

Singer and Nash (2000) illustrated the importance of T-cells in the control of giardiasis. Neither Th1 nor Th2 cells were absolutely necessary for the clearance of Giardia infection. This suggests that in the absence of Th1 cells, Th2 cells are sufficient for clearance of the parasite, or that in the absence of Th2 cells, Th1 cells are sufficient. Alternatively, Th3 cells (mucosal T cells) may play the major role in *E. histolytica* and *G. lamblia* infection. However, in interferon-gamma deficient animals, parasite clearance was delayed when compared to controls, suggesting the Th1 response may be more substantial in controlling Giardia infections. T-cell cytokines may also induce the production and release of antigiardial defensins into the intestinal lumen (Singer and Nash, 2000).

The drugs of choice in amebiasis are nitroimidazoles (Freeman et al., 1997; Iamp et al., 1999). It is effective and available at low cost. Trophozites of *E. histolytica* are able to adapt to therapeutically relevant levels of the drug (Samarawickerma et al., 1997; Wassmann et al., 1999). Lauric acid is a naturally derived fatty acid belonging to the medium chain saturated fatty acids (MCSFAs) which is suggested to have antimicrobial and antiparasitic properties. MCSFAs are natural fats that are easily digested, quickly absorbed and readily utilized for energy production

in mammals. Due to their relative ease of absorption by the body, these compounds are ideal for individuals with digestive tract problems such as diarrhea and malabsorption of long chain fatty acids containing foods (Petschow et al., 1998).

Lauric acid (dodecanoic acid, C: 12) is a medium chain saturated fatty acid which is reported to have anti-giardial effect with lethal dose 50 (LD_{50}) concentration comparable to that of metronidazole which is the drug of choice in treatment of giardiasis. Dodecanoic acid appears to induce *in vitro* trophozoite death by accumulating within the parasite cytoplasm resulting in rupture of cell membrane (Rayan et al., 2005). Lauric acid is transformed into a substance called "monolaurin" in the human body. Monolaurin is a glyceride ester derivative of lauric acid (Hegde, 2006). Also, it was reported that monolaurin can destroy various pathogenic bacteria and protozoa such as *G. lamblia.* This is why lauric acid is the fundamental building block of the most effective anti-pathogenic of all the medium chain saturated fatty acids (MCSFAs) (Rayan et al., 2005).

The present study was carried out to evaluate lauric acid, as a treatment of plant origin on *G. lamblia* or *E. histolytica* infection. This work also studies the histopathological changes occurring in the small intestine of infected animals and following treatment.

MATERIALS AND METHODS

G. lamblia and *E. histolytica* cysts were obtained from diarrheic patients attending parasitology laboratory in outpatient clinic of Theodore Bilharz Reasearch Institute (TBRI). Each hamster was infected orally by 10,000 of either *G. lamblia* or *E. histolytica* cysts. The excreta of hamsters were examined daily to evaluate the time of maximal cyst excretion. A group of sixty golden Syrian hamsters were used, which were further subdivided into two subgroups: Subgroup I: in which hamsters were infected by oral administration of 10,000 *G. lamblia* cysts and Subgroup II: in which hamsters were infected by 10,000 *E. histotytica* cysts through an esophageal tube. Each subgroup divided into (6) group: Subgroup I included the group from (1 to 6) although subgroup II included 7 to 12 groups. Group (1) constituted infected animals added as control (5 animals), group (2) infected with Giardia cysts (5 animals) and treated with metronidazole in a dose of 120 µg/kg receiving twice daily for 5 successive days. Group (3) infected with Giardia cysts (5 animals) received dose of 500 mg/kg monolaurin for 7 consecutive days pre infection, group (4) infected with Giardia cysts (5 animals) received 500 mg/kg monolaurin for 7 consecutive days post infection. Group (5) infected with Giardia cysts (5 animals) received dose (2/3) 300 mg/kg monolaurin for 7 days consecutive days pre infection, then treated with metronidazole 1/3 dose twice daily for 5 successive days. Group (6) infected with Giardia cysts (5 animals) received a dose of 300 mg/kg monolaurin for 7 consecutive days post infection in a combination treated with metronidazole 1/3 dose twice daily for 5 successive days. Group (7) infected with *E. histolytica* cysts. Group (8) infected with *E. histolytica* cysts and treated with metronidazole120 µg/kg twice daily for 5 successive days. Group (9) infected with *E. histolytica* cysts (5 animals) and treated dose 500 mg/kg monolaurin for 7 consecutive days pre infection. Group (10) infected with *E. histolytica* cysts (5 animals) three consecutive days post infection. Group (11) infected with *E. histolytica* cysts (5 animals) received a dose of 300 mg/kg monolaurin

for 7 consecutive days pre infection, then treated with metronidazole 1/3 dose twice daily for 5 successive days and group (12) infected with *E. histolytic*a cysts (5 animals) received a dose of 300 mg/kg monolaurin for 7 consecutive days post infection in combination with metronidazole 1/3 dose twice daily for 5 successive days. The number of trophozoites were investigated in the duodenal part of intestine of hamsters.

Drugs

*Metronidazole (flagyle) was supplied by Rhone Opulence Rorer Company, as suspension. The dose given to each hamster was 120 µg/kg twice daily for 5 successive days. The dose for hamsters was calculated according to the chart for drug doses in experimental animals Paget and Barnes (1964). *Monolaurin (lauric acid) was supplied by Manufactured for Ecological formulas CONCORD, CA 9456 (Rayan et al., 2005). The dose was 500 mg/kg given for 7days, pre and post infection.

Parasitological study

In the group infected with *G. lamblia* and *E. histolytica* one week following infection, stool analysis was performed for all (12) groups to verify infection. Analysis was repeated every other day till end of second week post-infection. Treatment to all groups were given one week following infection. One week later stool analysis was done by direct examination of fresh stool for trophozoites and merthiolate iodine formaldhyde concentration (MIFC) technique (Blagg et al., 1955). Hamsters infected with *G. lamblia* and *E. histolytica* were sacrificed ten days after treatment and small bowel was removed and the duodenal contents were analyzed and the number of vegetative forms was counted. In hamsters infected with *E. histolytica,* large intestine was excised and colonic contents were examined. In the group treated with monolaurin pre-infection, hamsters were administered monolaurin daily for one week pre infection and the hamsters were sacrificed after three weeks post infection.

Histopathological examination

After sacrifice of the animals, part of the small intestine were fixed by formaline then pieces of tissues were processed for paraffin embedding stained with hematoxylin-eosin and masson trichrome stain (Bancroft and Stevens, 1975).

Serum cytokine and chemokine measurement

Levels of the cytokines IFN-γ, TNF-α, IL-4, IL-10, TGF-β (R & D systems Inc., Minneapolis) were measured in serum using sandwich enzyme-linked immunosorbent assay (ELISA) (eBioscience). The results were expressed as pg/ml, based on standard curves (Baqai, 1996). In brief, ELISA plates were coated with 50 µl (Iµg/ml) of capture antibody (IFN-γ, TNF-α, IL-4, IL-10, TGF-β) (Beckton Dickenson & Co.) and allowed to incubate at 4°C overnight. Plates were washed six times with Phosphate buffered saline (PBS)/Tween 20. Excess protein binding sites were blocked with 200 µl of skimmed milk. The wash step was repeated and 50 µl of serum samples were added and incubated 1 h in water bath at 37°C. The washing step was repeated and the biotin labeled anti-(IFN-γ, TNF-α, IL-4, IL-10, TGF-β) monoclonal detector antibody (Iµg/ml) (Beckton & Dickenson & Co) was added to each well and the plates incubated at room temperature for an hour. After a further washing step 100 µl of avidin-alkaline phosphatase was added to each well and the plates incubated at room temperature for 30 min. The washing step was repeated and 100 µl of p-nitrophenyl phosphate (pNpp) (Sigma Aldnch) (Img/ml) in 0.2 M tris

buffer was added to each well to detect bound antibody. The re-action was visualized by the addition of 100 µl/well of p-nitrophenyl phosphate (pNpp) (Sigma) substrate solution for 30 min in the dark at room temperature. The reaction was stopped by adding 50 µl/well of 8 N H_2SO_4 and plates were read at 405 nm using ELISA microplate reader (Bio Rad).

Ethical considerations

The experimental animal studies were conducted in accordance with international valid guidelines and they were maintained under convenient conditions at the Schistosom Biological Supply Progrm (SBSP) animal house of Theodor Bilharz Research Institute (TBRI), Cairo, Egypt.

Statistical analysis

The statistical package for social sciences (SPSS) for Windows (version 11) computer program was used for statistical analysis. Means of different groups were compared using unpaired 2-tailed students t-test. Data were considered significant if "p" values were less than 0.05.

RESULTS

Parasitological parameters

Table 1 showed that the reduction in trophozoite forms of *G. lamblia* in intestinal contents was 67.0% in group (3) treated with monolaurin pre-infection and 87.34% in the group (4) treated with monolaurin post-infection compared to infected control group (1). The reduction in the cysts of *G. lamblia* treated with monolaurin pre and post infection (group 3 and 4) was 73.96 and 91.15%, respectively (Table 2). Tables 1 and 2 showed that there was a reduction in trophozoite and cystic forms of *G. lamblia* in intestine (92.15 and 93.23, respectively), when treated with metronidazole (group 2). The best results in a percentage reduction rate in both vegetative and cystic forms, respectively was shown in the group (6) treated with a combination of metronidazole and monolaurin post infection 94.68 and 96.55%, respectively (Tables 1 and 2). In Tables 3 and 4, in the group infected with *E. histolytica,* the high reduction in trophozoite and cystic forms in intestinal contents were in the in group (12) treated with a combination of metronidazole, and monolaurin post infection were 93.08 and 92.56%, respectively. The reduction in trophozoite forms of *E. histolytic* in the groups (9 and 10) treated with monolaurin pre and post infection were 75.32 and 88.15%, respectively (Table 3). The difference for both treated groups was statistically significant from respective untreated control hamsters at (P < 0.001). The reduction in number of the cysts of *E. histolytic* pre and post infection (group 9 and 10) were 76.66 and 89.59%, respectively (Table 4). When treated with metronidazole in group (8) there were reduction in trophozoite and cysts forms of *E. histolytic* in intestinal contents were 89.14 and 90.03%, respectively (Tables 3 and 4).

Table 1. Effect of monolaurin on vegetative forms (trophozoite) in the small intestine infected with *Giardia lamblia*.

Group	Vegetative forms in small intestine (trophozoite) mean±SE	% reduction
Control infected groups	39.50±0.65	0
Infected treated with Metronidazole	3.1±0.95*	92.15
Treated with monolaurin pre-infection .	13.0±0.20**	67.0
Treated with monolaurin post-infection .	5.1±0.23***	87.34
Treated with monolaurin pre-infection + metronidazole.	9.9±0.12****	74.93
Treated with metronidazole + monolaurin post-infection	2.1±0.11****	94.68

Date were as mean ± SE (mean ± standard devination). *Significant difference compared to the infected control group (P > 0.001).

Table 2. Effect of monolaurin on the number of cysts excreted in stool infected with *Giardia lamblia*.

Group	Cysts/gm stool	% reduction
Control groups	7250.00±35.35	-
Infected treated with Metronidazole	490.2±25.22*	93.23
Treated with monolaurin pre infection	1887.5±21.34**	73.96
Treated with monolaurin post-infection	610.2±15.11***	91.15
Treated with monolaurin pre-infection + metronidazole	400.9±0.22****	94.48
Treated with metronidazole + monolaurin post-infection	250.0±0.012*****	96.55

Date were as mean±SE (mean ± standard deviation). *Significant difference compared to the infected control group (P > 0.001).

Table 3. Effect of monolaurin on vegetative forms (Trophozoite) in the small intestine infected with *E. histolytic.*

Group	Vegetative forms in small intestine (trophozoite) mean±SE	% reduction
Control infected groups	50.65±1.35	
Infected treated with Metronidazole	5.5±0.35*	89.14
Treated with monolaurin pre- infection	12.5±1.13**	75.32
Treated with monolaurin post- infection	6.0±2.11***	88.15
Treated with monolaurin pre-infection + metronidazole	5.0±0.02****	90.12
Treated with metronidazole + monolaurin post-infection	3.5±0.22*****	93.08

Date were as mean ± SE (mean ± standard devination). *Significant difference compared to the infected control group (P > 0.001).

Serum cytokine and chemokine measurement

Levels of the cytokines IFN-γ, TNF-α, IL-4, IL-10, TGF-β (R&D systems Inc., Minneapolis) were measured in serum using sandwich ELISA. There is a significant reduction observed in all groups, the greatest reduction in different cytokines was observed in group 3 and 6 while there is no significant reduction in the level of TGF-β (Table 5).

Table 4. Effect of monolaurin on the number of cysts excreted in stool infected with *E. histolytic.*

Group	Cysts/gm stool Mean± SE	% reduction
Control groups	6725.00±55.55	-
Infected treated with metronidazole	670.0±23.44*	90.03
Treated with monolaurin pre-infected	1569.2±11.24**	76.66
Treated with monolaurin post-infected	700.0±12.34***	89.59
Treated with monolaurin pre-infection + Metronidazole .	600.3±21.20****	91.07
Treated with metronidazole + monolaurin post-infection .	500.0±11.22*****	92.56

Date were as mean ± SE (mean ± standard devination). *Singnificant difference compared to the infected control group (P > 0.001)

Table 5. Effect of monolaurin on cytokines infected with *Giardia lamblia*.

Parameter	IFN – γ	TGF –b	IL – 4	IL – 10	IL – 6
	Pg/ml ± SEM				
Normal	166±5.11	256±29.2	17.9±1.7	99 ±12.4	140±5.1
Infected	612±19.6	131.2±31.1	69.6±12	510±29.1	587±94
Infected treated with Metronidazole	155.17±6.90	144.21±12.14	43.11±4.32	188.32±50.20	255.17±6.90
Treated with monolaurin pre- infection	311.23±2.72	219±33.7	27±1.3	107±20.2	164±32.6
Treated with monolaurin post-infection	267.81±5.32	142.62±23.90	61.31±14.11	121.22±12.13	171.81±4.32
Treated with monolaurin pre-infection + metronidazole	145.22±4.88	140.01±33.23	40.00±2.11	175.22±22.32	250.12±2.30
Treated with metronidazole + monolaurin post-infection	130.02±3.38	129.11±11.11	35.05±2.22	165.22±22.11	240.22±3.33

The Levels of the cytokines IFN- γ, TNF-α, IL-4, IL-10, TGF-β were measured in serum using sandwich ELISA.

In sub-groups infected with *E. histolytica*. There is a significant reduction observed in all groups, the greatest reduction in different cytokines was observed in group 9 and 12 while there is no significant reduction in the level of TGF-β (Table 6).

Histopathological examination

In the control infected non treated hamsters, atrophic degeneration of the intestinal villi was ob-served (Figure 1). Again, the group given monolaurin post-infection revealed partial villi atrophy (Figure 2), but exhibited complete healing in the group treated post-infection (Figure 3 and 4). While the group given combination of metronidazole and monolaurin post-infection with *E. histolytic* showed degeneration and healing in intestinal villi (Figure 5).

DISCUSSION

Millions of people are annually infected with *E. histolytica and G. lamblia,* making the diseases a major cause of morbidity worldwide (Teles et al., 2011). Metronidazole is known to be the drug of choice for treatment of trichomoniasis and giardiasis. However, some adverse reaction appears to be related to the high dosage and duration of treatment, resistance to the drug had led to the search for other suitable treatment (Martinez and Caumes, 2000). In human giardiasis, therapeutic failure that was recorded recently is up to 5 to 20% of cases (Fallah et al., 2007) occurs due to low compliance of drug the-rapy, frequent re-infection or emergence of parasite resistance to metronidazole and/or

Table 6. Effect of monolaurin on cytokines infection with *E. histolytica.*

Parameter	IFN – γ	TGF –β	IL – 4	IL – 10	IL – 6
	Pg/ml±SEM				
Normal	166±5.11	256±29.2	17.9±1.7	99±12.4	140±5.1
Infected	612±19.6	131.2±31.1	69.6±12	510±29.1	587±94
Infected treated with metronidazole	170.22±5.50	150.21±11.24	43.11±4.32	192.22±40.50	255.17±6.90
Treated with monolaurin pre-infection	315.27±2.81	233.0±12.6	28.02±2.3	117.6±20.2	170±22.5
Treated with monolaurin post-infection	277.81±5.32	152.73±13.80	64.31±12.12	125.02±12.13	176.52±4.44
Treated with monolaurin pre-infection+metronidazole	168.11±4.4	148.22±22.3	40.22±2.12	169.33±34.22	245.13±5.72
Treated with metronidazole + Monol post-infection	150.01±4.4	135.73±13.70	30.21±13.14	150.13±22.11	234.22±6.22

The Levels of the cytokines IFN- γ, TNF-α, IL-4, IL-10, TGF-β were measured in serum using sandwich ELISA.
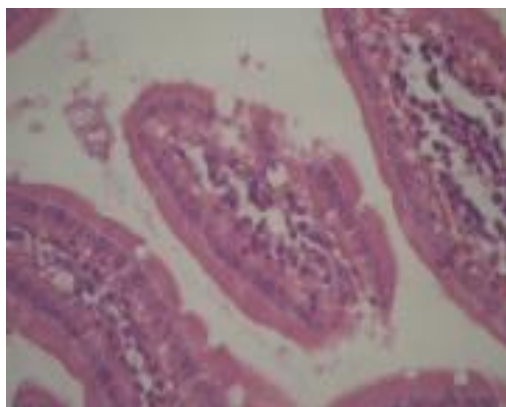

Figure 1. Infected control of *E. histotytica* degeneration of the intestinal mucosa (40×).
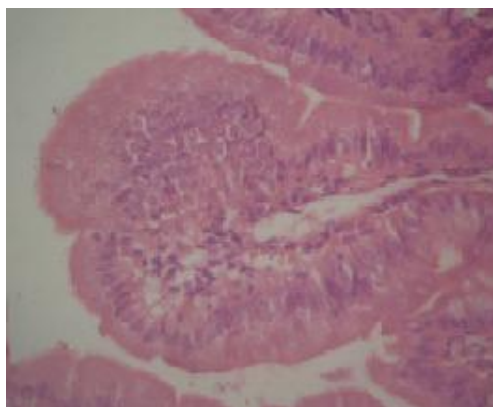

Figure 2. Healing of the intestinal villi after metronidazole treatment of *E. histotytica* (Hx &E ×40).
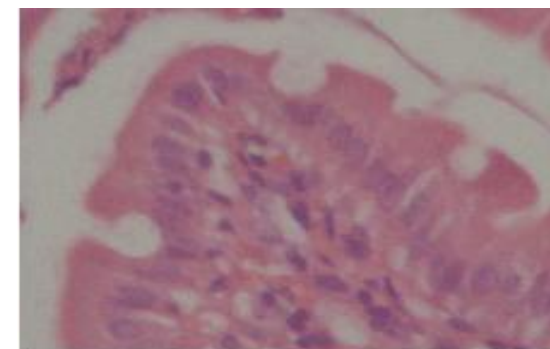

Figure 3. Treated with monolaurin post-infection with *E. histotytic* showing-degeneration and heal in intestinal villi (40×) E.

nitromidazole (Lemee et al., 2000).

In this study regarding the effect of metronidazole on *G. lamblia* infection, it was found that there was a high significant difference between control and all treated *G. lamblia* infected groups. Metronidazole administration resulted in a percentage reduction rate of 92.15 and 93.23% in both vegetative and cystic forms, respectively

(Tables 1 and 2) group (2), compared to 67 and 73.96% when monolaurin was given pre-infection in group (3). The best results were in a percentage reduction rate in both vegetative and cystic forms obtained in group (6) which was treated with a combination of metronidazole and monolaurin post infection 94.68 and 96.55%, respectively. These results are in agreement

with Amer et al. (2007) who made a trial to increase the effectiveness of metronidazole on *G. lamblia*. The study revealed a highest cure rate in vegetative forms (99.32%) and cystic forms (98.6%) in hamsters infected with *Giardia* and treated by a combination of metronidazole and lactospore. The authors added that, when metronidazole or lactospore were given separately, the reduction rates of trophozoites
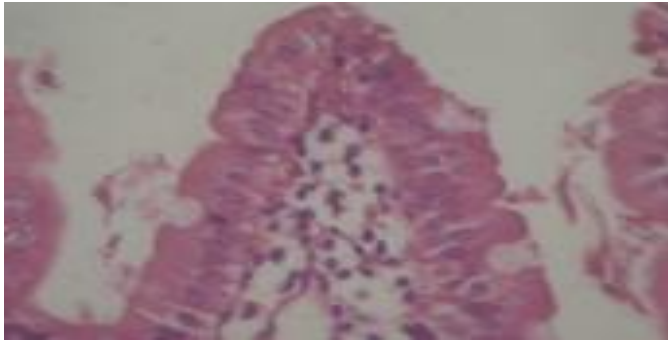
Figure 4. Treated with monolaurin Post-infection with *E. histotytic*a – reagenration and helling in instinal filli (40×) E.H.
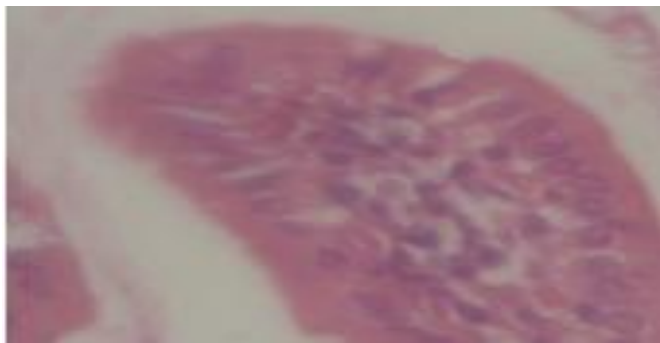

Figure 5. Treated by combination of metronidazole and monolaurin for week post infection with *E. histotytic*a showing complet healing in intestine.

were 92.22 and 63.4%, respectively and of cystic forms were 93.8 and 79.5%, respectively. Another study reported 96.4 to 99.14% cure from Giardia cysts and 86.5 to 78.1% reduction in the vegetative forms in hamsters treated with metronidazole. Moreover, it was found that a cure rate over 90% in cystic forms *Giardia* when treated with metronidazole in other reports (Fawzy et al., 2003).

Monolaurin, the monoglyceride of lauric acid is the most powerful antiviral, antibacterial and antifungal fatty acid found in coconut oil. It has the greatest overall antimicrobial effect. It also had antiprasitic effect on blastocysts *in vitro* (Hassan et al., 2010). Monolaurin which is a natural compound derived from coconut was evaluated for its antigiardial effects on giardia as stated by Rayan et al. (2005).

In the present study in subgroup infected by *G. lamblia,* the best results in a percentage reduction rate in both vegetative and cystic forms was respectively observed in group (6) which was treated with combination of metronidazole and monolaurin post infection 94.68 and 96.55%, respectively. In the group (4) receiving mono-laurin post infection, the number of trophozoites and cysts forms of *G. lamblia* in intestinal contents were 87.34 and 91.15%, respectively compared to infected control group.

This agree with Helmy (2010) who evaluated the effect of monolaurin, against *G. lamblia* in infected hamsters which the highest percentages of reduction in the number of *Giardia* cysts and trophozoites were in the group that received combined treatment (98.83 to 96.95%) followed by the group that received the metronidazole as treatment (93.77 to 95.5%) and the lowest percentage of reduction in the group that received the lauric acid as treatment (82.03 to 78.76%).

Similarly, in study of Hassan et al. (2010) reported the administration of monolaurin (lauric acid) was effective in the group infected with blastocystis. Dodecanoic acid (monolaurin ML) at 500 and 700 µg/ml induced highly significant reduction of concentration of blastocystis cells in culture after 2 h incubation (p < 0.01). Higher concentration (1000 µg/ml) caused rapid death of the parasite with viable cells were detected after 30 min incubation. These results agree with those found by Rayan et al. (2005) who confirmed that the dodecanoic acid has an anti-giardia effect with an LD_{50} concentration comparable to that of metronidazole.

Our result agrees with Fahmy et al. (2008) whose results showed that combined treatment of metronidazole together with arthemer and rosemary gave best results in highest percent reduction of cyst count in stool analysis and vegetative forms in small intestine caused by *G. lamblia*. Mixed treatment of metronidazole plus artemisia and rosemary showed complete regeneration of intestinal cells and sings of recovery was noticed.

El-Shennawy et al. (2009) studied the effect of pomegranate on the intestinal *G. lamblia* and concluded that the highest trophozoite reduction 98.7% was obtained in the group receiving metronidazole with pomegtanate (leaves). By histology, healing of mucosal ulcerations, preserved villi and reduced chronic inflammatory infiltrate of the lamina propria were detected with combined therapy.

Role of cytokines in *G. lamblia* infection is not clear. *G. lamblia* normally does not penetrate the epithelial barrier, therefore the spontaneous elimination of the parasite depends largely on immune mechanism. Cytokines or other inflammatory mediators may play a role in *G. lamblia* infection. IL4 appears to have some relationship in patients with *G. lamblia* infection as reported previously (Baqai, 1996).

Chronic diarrhoea and malabsorption produces mucosal inflammation associated with T cell activation and cytokine release (Farthing, 1993). Cytokines were not altered after infection of colonic cell with *G. lamblia* (Jung et al., 1995). As *G. lamblia* is a non invasive parasite, TNF alpha did not appear to have any role in giardiasis. This is in contrast with patients suffering from amoebiasis where TNF alpha was found because *E. histolytica* is an invasive parasite (Wang et al., 1992). TNF appears to act synergistically with other cytokines (Neta et al., 1988) and may be of therapeutic benefit in *G. lamblia* infection (Belosevic and Daniels, 1992).

In this study, the Levels of the cytokines IFN-γ, TNF-α,

IL-4, IL-10, TGF-β were measured in serum using sandwich ELISA. The best result was shown in group (6) which was treated with a combination of metronidazole and mololaurin post infection 130,129, 35, 165 and 240 Pg/ml in the subgroup I, infected with *G. lamblia*. In the present study, there is no significant reduction observed in all groups, the greatest reduction in different cytokines was observed in group 3, 6, 9 and 12, while there is no significant reduction in the level of TGF-β.

Our results agreed with Huma and Rakhshanda (2000) whose results indicate that IL-4 being an inflammatory regulator appears to have some relationship with it, probably because *G. lamblia* is a non invasive parasite giardiasis, while TNF alpha was not detected in patients. In our study, histopathological examination revealed complete healing of intestinal mucosa and regeneration and healing in intestinal villi after treatment with combination of metronidazole and monolaurin post-infection in group infected with *G. lamblia*, while partial healing of the lining epithelium of the intestine was noticed after treatment with monolaurin post infection or metronidazole treatment. This agrees with Helmy (2010) who showed that histopathological examination and electron microscopic examination revealed complete healing of intestinal mucosa after the combined treatment, while partial healing of the lining epithelium of the intestine was noticed after metronidazole or lauric acid treatment. Amoebiasis is a significant cause of morbidity worldwide and is the third leading cause of death from parasitic diseases. Although, metronidazole is the drug of choice for treatment of amoebiasis and has been used in clinical practice for many years, inappropriate usage could lead to drug resistance (Bansal et al., 2006). Drug resistance in *E. histolytica* is uncommon but differences in drug susceptibility between different isolates have been reported and resistance of metronidazole may be develop in future (Bansal et al., 2006).

In subgroup II, infected with *E. histolytica*, results showed that the high reduction in trophozoite and cystic forms in intestinal contents in group (12) treated with combination of metronidazole and monolaurin post infection were 93.08 and 92.56%, respectively. Then, the group (10) treated with monolaurin post-infection gave the best result in percent of reduction of cyst count in stool analysis and vegetative form 88.15 and 89.59% when infected with *E. histolytica*.

In our study, in the reduction in cytokines when infected *with E. histolytica*, the best result was observed in group (12) which was treated with combination of metronidazole and mololaurin post infection; the level of different cytokines was 150,135, 30, 150 and 234 Pg/ml (IFN-γ, IL-4, IL-6, IL-10). In the group (8) which treated with metronidazole, the reduction in cytokines levels show in group (2) which was 170, 150, 43.1, 192 and 255 Pg/ml (IFN-γ, IL-4, IL-6, IL-10).

Histopathological examination revealed complete healing in the group treated with combination of metronidazole and monolaurin post-infection. While the group given the treatment post-infection with *E. histolytic* showed degeneration and healing in intestinal villi. Our work showed promising results with monolaurin, which can be used as a complementary food product in combination with metronidazole in treatment for *G. lamblia* or *E. histolytica* infection.

Conclusion

In *G. lamblia* infected subgroup I, best results were observed by the reduction in both vegetative and cystic forms, respectively shown in group (6) treated with combination of metronidazole and monolaurin post infection 94.68 and 96.55%, respectively. In the subgroup II infected with *E. histolytica*, the high reduction in trophozoite and cystic forms in intestinal contents were in group (12) which was treated with a combination of metronidazole and monolaurin post infection (90.12 and 92.56%, respectively). Cytokines levels IFN-γ, TNF-α, IL-4, IL-10, TGF-β were measured in serum using sandwich ELISA. The best result was shown in the group (6) treated with a combination of metronidazole and monolaurin post infection 130,129, 35, 165 and 240 Pg/ml. Also histopathological examination gave best healing in groups (6) infected with *G. lamblia* than those infected by *E. histolytica*.

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Full Length Research Paper

Puerarin inhibits acute nociceptive responses via the P2X$_3$ receptor in rat dorsal root ganglia

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The effects of puerarin on acute nociception mediated by the P2X$_3$ receptor in rat dorsal root ganglia (DRG) were studied. Intrathecal injection of adenosine triphosphate (ATP) or α,β-methylene-ATP (α,β-meATP) could potentiate acute nociceptive responses induced by intraplantar injection of ATP or α,β-meATP in rat hindpaw. Intraplantar injection of formalin increased the expression of P2X$_3$ in rat DRG and induced acute nociceptive responses. These induced nociceptive responses were inhibited by intrathecal injection of puerarin, which also decreased the up-regulated expression of P2X$_3$ mRNA and protein in DRG induced by intraplantar injection of formalin. Molecular docking studies revealed that puerarin could interact perfectly with homology-modeled rat P2X$_3$ receptor (rP2X$_3$). rP2X$_3$ can be restricted to binding ATP because of its combination with puerarin (increasing the concentration of free ATP), therefore blocking the rP2X$_3$ channel. Whole cell patch clamp recording showed that puerarin inhibited the potentiation of P2X$_3$ receptor-mediated currents induced by lipopolysaccharide. These results demonstrated that puerarin inhibited nociceptive responses induced by ATP, α,β-meATP, or formalin and its inhibitory effect was mediated by reduction of inflammatory pain-induced up-regulation of P2X$_3$ receptor expression and blockade of ATP binding sites on the P2X$_3$ receptor. Our results suggest that puerarin could decrease acute pain mediated by the P2X$_3$ receptor in the DRG.

Key words: Puerarin, acute nociceptive response, P2X$_3$ receptor, dorsal root ganglia, whole cell patch clamp.

INTRODUCTION

Peripheral administration of purine 2X (P2X) agonists rapidly causes nociceptive behaviors in experimental animals and pain sensation in humans (Andó and Sperlágh, 2013; Burnstock, 2013; Sperlágh et al., 2006). Most of the nociceptive responses to peripheral adenosine triphosphate (ATP) are mediated by the P2X$_3$ receptor, and the P2X$_3$ receptor plays a crucial role in facilitating pain transmission. Animal experiments have demonstrated that a dose-dependent nociceptive response could be observed after the intraplantar injection of α,β-meATP in conscious rats, such as paw lifting, withdrawing, licking, and other pain defensive

behaviors (Andó et al., 2010; Cherkas et al., 2012; Ford and Undem, 2013). The number of pain defensive behaviors was significantly reduced after formalin injection into the paws of P2X$_3$-deficient mice (Cockayne et al., 2005; Souslova et al., 2000). It has been shown that P2X$_3$ mRNA and protein expression in the dorsal root ganglia (DRG) were increased, and that ATP-gated currents mediated by the P2X$_3$ receptor from primary sensory neurons were significantly enhanced after neuropathic pain and inflammatory pain stimuli (Borsani et al., 2010; Calvert et al., 2008; Honore et al., 2002; Krimon et al., 2013; Okubo et al., 2010; Tsuda et al.,

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2007; Xu et al., 2012; Yu et al., 2013). These results suggest that ATP and inflammatory substances (such as formalin) are involved in the transmission of pain via activation of the P2X$_3$ nociceptor (Borsani et al., 2010; Fountain, 2013; Li et al., 2013; Nones et al., 2013; Pan et al., 2009).

Radix puerariae (*R. puerariae*) is the dried root of *Pueraria lobata* (Willd.) Ohwi and *Pueraria thomsonii* benth. In China, *R. puerariae* is known as 'Ge Gen', and has been used as a traditional medicine for the management of various diseases including cardiovascular disorders. *R. puerariae* is also known as Kadzu root in the West and contains significant amounts of the isoflavonoid puerarin (PUE) [4H-1-benzopyran-4-one, 8-b-D-glucopyranosyl-7-hydroxy-3-(4-hydroxyphenyl), $C_{21}H_{20}C_9$], which is a major active ingredient extracted from the traditional Chinese medicine Ge-gen (*Radix Puerariae*; Rong et al., 1998). The uses of Ge Gen described in pharmacopoeias and in traditional systems of medicine are for the treatment of fever, pain, diabetes mellitus, measles, acute dysentery, or diarrhea, and PUE is widely used in the treatment of cardiovascular diseases in China (Gao et al., 2007; Rong et al., 1998; Zhang et al., 2013). PUE has been shown to possess antioxidant properties such as scavenging reactive oxygen species, increasing superoxide dismutase activity and inhibiting protein nonenzymatic glycation (Guo et al., 2003; Xu, 2003). Both Ge Gen and PUE soup exhibit very similar effects on the inhibition of inflammatory responses and oxidative damage (Peng et al., 2013), suggesting that PUE is the functional active ingredient in anti-nociceptive responses and analgesia. Thus, to understand basic mechanisms underlying PUE inhibition of nociceptive responses, we studied the effects of PUE on acute nociceptive responses in rats. Nociception was induced by both intrathecal and intraplantar injection of ATP/α,β-meATP or intraplantar injection of formalin. The expression levels of P2X$_3$ mRNA and protein after intraplantar injection of formalin were assessed.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats (180 to 230 g) were provided by the Center of Laboratory Animal Science of Nanchang University. The animals were housed in plastic cages (five per cage) with room temperatures between 21 and 25°C. Animal use was inspected and approved by the Animal Care and Use Committee of Medical College of Nanchang University. The IASP's ethical guidelines for pain research in animals were followed. All animals were treated in accordance with ARVO Statement for the use of Animals in Ophthalmic and Vision Research in China.

Antibodies and reagents

P2X$_3$ antibody was purchased from Chemicon International Inc. (Temecula, CA). Array slides were obtained from Qiagen (Valencia,

CA, USA). β-Actin was from Advanced Immunochemicals (Long Beach, CA). PUE was the product of Kangenbei Pharmaceutical Limited Corporation, China. ATP, α,β-methylen-ATP (α,β-meATP), and LPS were obtained from Sigma-Aldrich (St. Louis, MO). Formalin was obtained from Shanghai Reagent Company (20100300, GB685-79, Shanghai, China). All drugs were dissolved and diluted in 0.9% saline (NS). Other antibodies and reagents are further described in the text.

Animal groups

Animals were randomly divided into three groups and each group was further divided into subgroups as follows and the PUE/NS, NS/NS group for sharing:

Groups of nociceptive behavior induced by α,β-meATP were: NS/α,β-meATP (meaning is "intrathecal injection of NS/intraplantar injection of α,β-meATP", and the following description as the same), PUE/α,β-meATP, α,β-meATP/α,β-meATP, and α,β-meATP+PUE/α,β-meATP. Groups of nociceptive behavior induced by ATP were: NS/ATP, PUE/ATP, ATP/ATP, and ATP+PUE/ATP. Groups of nociceptive behavior induced by formalin were: NS/formalin, PUE/formalin, PUE/NS, and NS/NS.

Observation of rat pain defensive behaviors

Experimental rats were raised in the laboratory for 1 week before being tested. The laboratory temperature was maintained between 21 and 25°C. Rats were placed inside a transparent organic glass box (20×30×30 cm^3) on a stainless steel mesh floor and allowed to acclimate for behavioral experiments. For intrathecal injections, ATP (10 μmol/L), α,β-meATP (1 μmol/L), or different concentrations of PUE were injected into the L5 and L6 space with a trace syringe (18-G needle) after rats were anesthetized with ethylether. The total volume of intrathecal injection was 15 μl for each experiment. A successful injection was indicated by movement (swing) of the animal tail and hind limbs. Rats awoke after 2 to 6 min. For intraplantar injections, ATP (10 μmol/L), α,β-meATP (1 μmol/L), or 2.5% formalin was injected into the left foot with a trace syringe (26G needle). The drugs used for intraplantar injections were diluted to 100 μl of NS and injected at one time. After injection, animals were monitored for 60 min to observe nociceptive responses, such as paw lifting, withdrawing and licking, the occurrence of nociceptive responses from the paw of the injected side were counted. The frequency of lifting, withdrawing and licking the foot/5 min (times/5 min) were measured for estimating the effects of intrathecally applied PUE on nociception induced by α,β-meATP, ATP or formalin injected into rat hindpaw. Each rat was tested only once.

Reverse transcription-polymerase chain reaction (RT-PCR) analyses

Two hours after the intraplantar injection of NS or formalin (2.5%), animals were anesthetized with penthiobarbital sodium (Shanghai Xingya Medical Company, Batch No: 050101), and then ipsilateral L4-L6 DRG were dissected and harvested. The expression of P2X$_3$ mRNA in DRG was detected by RT-PCR. Total RNA was isolated from DRG by the TRIZOL Reagent (Invitrogen) with the guanidinium isothiocyanate method and subjected to DNase I digestion (Pharmacia; 0.1 U/ml, 15 min, 37°C) to eliminate genomic contamination. P2X$_3$ forward and reverse primer sequence genes were 5′-CAACTTCAGGTTTGCCAAA-3′ and 5′-TGAACAGTGAGGGCCTAGAT-3′, respectively, and the size of the product was 519 bp. β-actin, as an internal control, was also amplified using specific primers (forward and reverse sequences were 5′-TAAAGACCTCTATGCCAACACAGT-3′ and 5′-

CACGATGGAGGGGCCGG ACTCATC-3′), with the size of the product being 240 bp. Band densities were measured using the Gel Imaging System software (Junyi Shanghai) and normalized to each β-actin internal control.

Immunohistochemistry for detecting immunoreactivity

Ipsilateral L4-L6 DRG dissection and harvest were the same as described earlier. The isolated DRGs were washed using phosphate-buffered saline (PBS). After fixing with 4% paraformaldehyde (PFA) for 24 h, the ganglia were dehydrated by 20% sucrose for overnight at 4°C, and then ganglia were cut 20 μm in thickness via a cryostat. Immunohistochemical staining was performed using a SP-9001 kit (Beijing Zhongshan Biotech Co). Rabbit anti-P2X$_3$ was obtained from Chemicon International, Inc. (1:2500 dilution in PBS), biotinylated goat anti-rabbit secondary antibody and streptavidin-horseradish peroxidase were obtained from Beijing Zhongshan Biotech Company. The average optical density of P2X$_3$ receptor expression in ganglia was analyzed using an image scanning analysis system (HMIV-2000, Wuhan). Background was determined by average optical density (OD) of ten random areas (from positive cell). Negative control experiments were also conducted to confirm P2X$_3$ receptor expression in the ganglia (figure not shown).

Western blotting analysis

Animals were sacrificed and the collected tissues were quick frozen in tubes on dry ice. DRG was then isolated immediately and rinsed in ice-cold PBS. Ganglia were homogenized by mechanical disruption in lysis buffer and incubated on ice for 50 min. Homogenate was then pelleted at 12000 rpm for 10 min and the supernatant was collected. The quantity of total protein was determined in the supernatant using the Lowry method. After being diluted with sample buffer and being heated to 95°C for 10 min, samples containing equal amounts of protein (20 μg) were separated by SDS-polyacrylamide gel (10%) electrophoresis using a Bio-Rad electrophoresis device, and subsequently transferred onto nitrocellulose (NC) membrane under the same system. The labeled proteins were visualized with enhanced chemiluminescence on high-performance film (Shanghai Pufei Biotech Co). Chemiluminescent signals were collected on autoradiography film, and the band intensity was quantified using AlphaImager 2200 software. The antibodies and their dilutions were: rabbit polyclonal anti-P2X$_3$ (1:1000; Chemicon International Co.), monoclonal β-actin (1:10,000; Advanced Immunochemicals, Long Beach, CA), and secondary antibody (goat anti-rabbit IgG (1:3000, Beijing Zhongshan Biotech Co.). Band densities were normalized to each β-actin internal control.

Homology modeling and molecular docking

The MOE 2012.10 Docking program was used for protein ligand docking calculations (Naim et al., 2007; Sanner, 1999). Docking models the interaction between a ligand and a receptor active site by computer simulation. The technique of docking is to position the ligand in different orientations and conformations within the binding site to calculate optimal binding geometries and energies. MOE's Dock application searches for favorable binding configurations between small- to medium-sized ligands and a not-too-flexible macromolecular target, usually a protein. For each ligand, a number of configurations called poses are generated and scored. The score can be calculated as either a free energy of binding, which takes into account solvation and entropy, or the enthalpic term of the free energy of binding, or a qualitative shaped-based numerical measure. The final top-scoring poses, along with their scores and conformation energies, are written to a database where they are ready for further analysis.

Based on the published crystal structure of the zfP2X$_4$ channel in its closed state (Kawate et al., 2009), the extracellular loop and transmembrane portion of the rat P2X$_3$ receptor (rP2X$_3$) was modeled. The standard modeling techniques implemented in SPDBV4.1.0 (Swiss-PdbViewer) generate a homology model of the rP2X$_3$ (Guex and Peitsch, 1997). Homology modeling was performed with the SWISS-MODEL online server for automated protein homology modeling (Kiefer et al., 2009). Protein Data Bank entry 3I5D, which is believed to represent the closed state of the channel, was used as a template. The sequence of rP2X$_3$ was retrieved from accession number P49654 of the UniProtKB database. Sequence alignment between the template and the model sequence was performed using a modified version of the alignment algorithm. In this approach, alignments are computed by optimizing a function based on residue similarity scores. Structure obtained from homology modeling was verified by PROCHECK (Pontius et al., 1996). PUE (CID 5281807) was downloaded from Pubchem, and prepared by ChemBioDraw Ultra 11.0 and Chimera1.6.1.

Whole cell patch clamp recording

Full details of the electrophysiological methods have been previously reported (Kong et al., 2013). In brief, rats were anesthetized with urethane (1.2 g/kg, i.p.) and DRG neurons were superfused continuously with external solution containing (in millimolar): NaCl 150, KCl 5, CaCl$_2$ 2.5, MgCl$_2$ 1, 4-(2-hydroxyethyl)- 1-piperazineethanesulfonic acid (HEPES) 10, and D-glucose 10 (osmolarity adjusted to 340 mM with sucrose, pH adjusted to 7.4 with NaOH). Cells were patch-clamped in the whole-cell configuration using pipettes with a resistance of 3 to 5 MΩ when filled with the following solution (in millimolar): KCl 140, MgCl$_2$ 2, HEPES 10, EGTA 11, and ATP 5 (pH adjusted to 7.2 with KOH). Cells were held at −60 mV, data were filtered at 1 kHz, and acquired by means of a DigiData 132XInterface and pClamp 10.0 software (Molecular Devices, Sunnyvale, CA, USA). To obtain stable and reproducible P2X$_3$ receptor currents, its synthetic and specific agonist α,β-methylene-ATP (α,β-meATP) was applied with a fast superfusion system, and current peak amplitudes were measured. The drugs were dissolved in external solution and delivered by gravity flow from an array of tubules (500 μm OD, 200 μm ID) connected to a series of independent reservoirs. The distance from the tubule mouth to the cell examined was approximately 100 μm.

Statistical analysis

Statistical analyses of the data were performed using SPSS 17.0. All results were expressed as mean ± standard errorSE. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by the Fisher post hoc test for multiple comparisons and the unpaired t test for between two groups comparisons, $p < 0.05$ was considered significant. Graphs were prepared using Sigmaplot 11.0 software.

RESULTS

A successful injection was indicated by movement (swing) of the animal tail and hind limbs. No animal deaths in the experimental process.
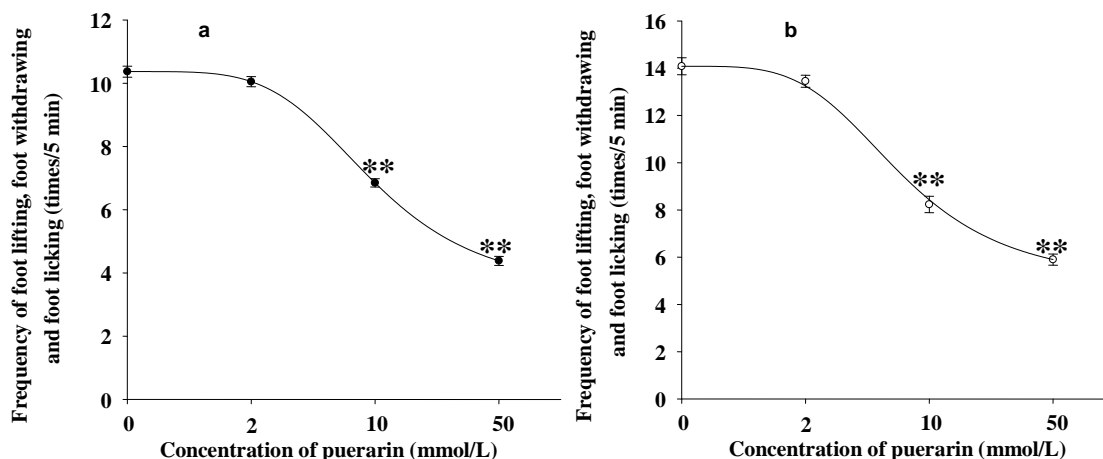
Figure 1. Effects of intrathecal administration of puerarin on acute nociception induced by both intrathecal and intraplantar injection of P2X$_3$ receptor agonist α,β-meATP or ATP. (a) Acute nociception induced by both intrathecal and intraplantar injection of α,β-meATP. (b) Acute nociception induced by both intrathecal and intraplantar injection of ATP. **p < 0.01 compared with no puerarin, n = 5 rats in each group, data are expressed as mean ± SE.

Table 1. Effects of intrathecally applied PUE on nociception induced by α,β-meATP injected into rat hindpaw

Group	Frequency of lifting, withdrawing and licking the foot/5 min
NS/NS	1.25±0.11
PUE/NS	1.37±0.03
NS/α,β-meATP	4.38±0.14***
PUE/α,β-meATP	2.13±0.09
α,β-meATP/α,β-meATP	10.37±0.17***###
α,β-meATP+PUE/α,β-meATP	6.85±0.13@@

The significant difference was denoted as ***p<0.001 compared with the data in NS/NS group or PUE/NS group, ###p<0.001 compared with the data in NS/α,β-meATP group or PUE/α,β-meATP, and @@p<0.01 compared with the data in α,β-meATP/α,β-meATP group; n=5 rats in each group, data shows with mean±SEM.

PUE reduces nociceptive behavior mediated by α,β-meATP

The P2X$_3$ agonist α,β-meATP can induce nociceptive responses in animalsb(Andó and Sperlágh, 2013; Burnstock, 2013). Intrathecal co-administration of PUE (2, 10, 50 mmol/L) and α,β-meATP (1 μmol/L) produced significant and dose-dependent reduction of nociceptive paw lifting, withdrawing and licking behavior in rats injected with α,β-meATP into the hindpaw and potentiated by intrathecal injection of α,β-meATP (1 μmol/L) (Figure 1a).

There was no obvious pain response in NS/NS rats. When NS (15 μl) was intrathecally injected, acute nociception in the rat hindpaw induced by intraplantar injection of α,β-meATP (1 μmol/L; NS/α,β-meATP) was higher than that in the NS/NS group (n = 5 per group, unpaired t test, t_8=17.234, p < 0.001) (Table 1). Both

intrathecal injection of PUE (10 mmol/L) and intraplantar injection of α,β-meATP (1 μmol/L) produced less nociceptive paw lifting, withdrawing, and licking behaviors in comparison with rats treated with intrathecal injection of NS and intraplantar injection of α,β-meATP (1 μmol/L; one-way ANOVA, $F_{3,16}$ = 348.479, p < 0.01, n = 5 per group).

Intrathecal injection of α,β-meATP (1 μmol/L) could potentiate the acute nociceptive responses in rat hindpaw induced by intraplantar injection of α,β-meATP (1 μmol/L). The acute nociceptive responses in α,β-meATP/α,β-meATP rats were higher than those in rats treated with intrathecal injection of NS and intraplantar injection of α,β-meATP (n = 5 per group, unpaired t test, t_8 = −42.052, p < 0.001) (Table 1). Nociceptive behaviors in rats treated with intrathecal co-administration of PUE (10 mmol/L) and α,β-meATP (1 μmol/L)/intraplantar injection of α,β-meATP were reduced in comparison with rats

Table 2. Effects of intrathecally applied PUE on nociception induced by ATP injected into rat hindpaw.

Group	Frequency of lifting, withdrawing and licking the foot/5 min
NS/NS	1.25±0.11
PUE/NS	1.37±0.03
NS/ATP	8.22±0.31***
PUE/ATP	5.27±0.16
ATP/ATP	14.08±0.36***###
ATP+PUE/ATP	8.43±0.25@@

The significant difference was denoted as ***p<0.001 compared with the data in NS/NS group or PUE/NS group, ###p<0.001 compared with the data in NS/ATP group or PUE/ATP group, and @@p<0.01 compared with the data of ATP/ATP group; n=5 rats in each group, data shows with mean±SEM.

Table 3. Effects of intrathecally applied PUE on nociception induced by formalin injected into rat hindpaw.

Group	Frequency of lifting, withdrawing and licking the foot/5 min
NS/NS	1.25±0.11
PUE/NS	1.37±0.03
NS/formalin	53.93±0.99**
PUE/formalin	40.8±0.23###

The significant difference was denoted as **p<0.01 compared with the data in NS/NS group, ###p<0.001 compared with the data in NS/formalin group, no difference between NS/NS group and PUE/NS group; n=5 rats in each group, data shows with mean±SEM.

injected with α,β-meATP (α,β-meATP/α,β-meATP; one-way ANOVA, $F_{5,24}$ = 883.653, p < 0.01, n = 5 per group) (Table 1). Our results showed that PUE inhibited nociceptive responses induced by the intrathecal injection of α,β-meATP in rat hindpaw (Table 1).

PUE reduces nociceptive behavior mediated by ATP

ATP can induce the nociceptive behaviors of animals. Intrathecal co-administration of PUE (2, 10, 50 mmol/L) and ATP (10 µmol/L) produced significant and dose-dependent reduction of nociceptive paw lifting, withdrawing and licking behaviors in ATP injected into rat hindpaw potentiated by the intrathecal injection of ATP (10 µmol/L) (Figure 1b).

There was no obvious pain response in rats both treated with intrathecal injection of NS and intraplantar injection of NS. Acute nociception of rats both treated with intrathecal injection of 15 µl NS and intraplantar injection of 100 µl ATP (10 µmol/L) was increased compared with rats in the NS/NS group (n = 5 per group, unpaired t test, t_8 = 20.861, p < 0.001) (Table 2). There was no significant difference between rats treated with intrathecal injection

of PUE and intraplantar injection of NS and rats treated with NS/NS (n = 5 per group, unpaired t test, t_8= −1.000, p = 0.347 > 0.05; Table 2).

Intrathecal injection of ATP could potentiate the acute nociceptive responses induced by intraplantar injection of ATP in rat hindpaw. In the intrathecal injection of ATP (10 µmol/L) and intraplantar injection of ATP (10 µmol/L) group, acute nociception was significantly increased in comparison with that in the NS/ATP group (n = 5 per group, unpaired t test, t_8 = −12.281, p < 0.001) (Table 2). Intrathecal co-administration of PUE (10 mmol/L) and ATP (10 µmol/L)/intraplantar injection of ATP, decreased nociceptive behaviors compared with rats intrathecally injected with ATP and intraplantar injected ATP (one-way ANOVA, $F_{5,24}$ = 435.099, p < 0.01, n = 5 per group) (Table 2). No significant difference between the NS/ATP group and ATP+PUE/ATP group was found (one-way ANOVA, $F_{5,24}$ = 435.099, p = 0.52, n = 5 per group) (Table 2). These results indicate that the intrathecal injection of PUE decreased nociceptive behaviors induced by the intrathecal injection of ATP (Table 2).

PUE reduces nociceptive behavior mediated by formalin

After the knockout of the $rP2X_3$, no receptor expression is seen in the DRG, and no spontaneous pain behavior is caused by formalin (Cockayne et al., 2005; Souslova et al., 2000), suggesting that inflammatory substances formalin could produce acute nociceptive responses via the $rP2X_3$. To identify the effect of PUE on acute nociception caused by formalin (2.5%), we compared the acute nociceptive responses of rats in the NS/NS, PUE/NS, NS/formalin, and PUE/formalin groups.

Results showed that acute nociception in rats treated with intrathecal injection NS and intraplantar injection of formalin (NS/formalin) was increased compared with that in rats of other groups (one-way ANOVA, $F_{3,16}$ = 2801.646, p < 0.01, n=5 per group) (Table 3). After both intrathecal injection of PUE (10 mmol/L) and intraplantar injection of formalin, nociceptive paw flinching, licking, and guarding behaviors in rat hindpaw were obviously reduced compared with those in rats treated with intrathecal injection NS and intraplantar injection of formalin (n=5 per group, unpaired t test, t_8 = 13.674, p < 0.001) (Table 3). These aforementioned results indicated that PUE decreased the nociceptive behaviors induced by formalin, which might be mediated via the $rP2X_3$ in rat DRG (Table 3).

RT-PCR demonstrates that PUE decreases the up-regulation of $P2X_3$ mRNA induced by formalin in the DRG

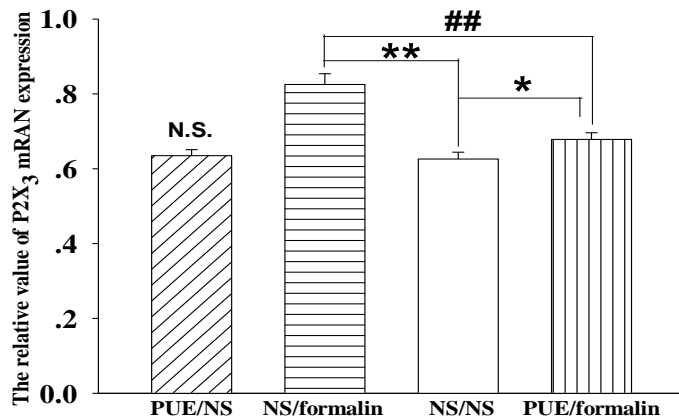To identify the mechanisms underlying formalin

Figure 2. Puerarin reduction of formalin-induced up-regulation of P2X$_3$ mRNA expression in DRG. RT-PCR analysis of DRG showed the up-regulated expression of P2X$_3$ mRNA in the NS/formalin group (p < 0.01). Upper panels: representative gels showing RT-PCR products for P2X$_3$ mRNA and β-actin internal control in the same sample. Lower panels: each band density (in arbitrary units) was normalized to its β-actin internal control, and the mean ± SE was shown for the number of independent samples indicated. The expression of P2X$_3$ mRNA in the PUE/formalin group was decreased in comparison with the NS/formalin group (p < 0.01). All experiments were conducted in triplicate.
*p < 0.05; **p < 0.01 compared with the NS/NS group. ##p < 0.01 compared with the NS/formalin group. N.S. denotes no significant difference between the NS/NS and PUS/NS groups.

involvement in acute nociception, the expression of P2X$_3$ mRNA in rat DRG was further studied using RT-PCR. We studied the effects of PUE on the expression of P2X$_3$ mRNA induced by formalin. On the basis of image analysis, the stain values (average optical density) of P2X$_3$ mRNA expression in NS/NS, PUE/NS, NS/formalin, and PUE/formalin groups were 0.6259 ± 0.0185, 0.6350 ± 0.0161, 0.8253 ± 0.0286, and 0.6783 ± 0.0180, respectively (n = 10 for each group). The stain values of P2X$_3$ mRNA expression in the NS/formalin group were significantly larger than those in the NS/NS, PUE/NS, and PUE/formalin groups (one-way ANOVA, $F_{3,36}$ = 195.924, p< 0.01, n = 10 per group) (Figure 2). The stain values of P2X$_3$ mRNA expression in the PUE/formalin group were larger than those in the NS/NS group (n = 10 per group, unpaired t test, t_{18} = -2.296, p = 0.034) (Figure 2). The expression of P2X$_3$ mRNA in the PUE/formalin group was smaller than that in the NS/formalin group (n = 10 per group, unpaired t test, t_{18} = 13.777, p<0.01) (Figure 2). These results suggest that PUE decreased the up-regulated expression of P2X$_3$ mRNA induced by formalin in rat DRG.

PUE decreases the up-regulation of P2X$_3$ immunoreactivity induced by formalin in the DRG

Immunohistochemistry was used to explore whether PUE

affected the increased expression of P2X$_3$ immunoreactivity in DRG after the intraplantar injection of formalin. On the basis of image analysis, the stain values (average optical density) of P2X$_3$ expression in NS/NS, PUE/NS, NS/formalin, and PUE/formalin groups were 0.6337 ± 0.0382, 0.6178 ± 0.0589, 0.8240 ± 0.0639 and 0.7078 ± 0.0381, respectively (n = 10 for each group). The stain values of P2X$_3$ expression in the NS/formalin group were significantly larger than those in NS/NS, PUE/NS, and PUE/formalin groups (one-way ANOVA, $F_{3,36}$ = 72.332, p < 0.01, n = 10 per group) (Figure 3). The stain values of P2X$_3$ expression in the PUE/formalin group were smaller than those in NS/formalin group (n = 10 per group, unpaired t test, t_{18} = 5.892, p < 0.01) (Figure 3). These findings further confirmed that the nociception induced by the intraplantar injection of formalin involved the rP2X$_3$ and that PUE inhibited the up-regulated expression of the rP2X$_3$ in the DRG.

Puerarin decreases the up-regulatioin of P2X$_3$ protein in DRG

To identify whether PUE affected the expression of P2X$_3$ protein in rat DRG after the intraplantar injection of formalin, the expression of P2X$_3$ protein in the DRG was further studied using western blotting. Image analysis showed that average optical density of P2X$_3$ protein expression (normalized to β-actin) in NS/NS, PUE/NS, NS/formalin, and PUE/formalin groups was 0.5556 ± 0.0378, 0.5486 ± 0.0371, 0.9727 ± 0.0541, and 0.6854 ± 0.0374, respectively (n = 10 for each group). Optical density of P2X$_3$ protein expression in the NS/formalin group was significantly larger than the NS/NS, PUE/NS, and PUE/formalin groups (one-way ANOVA, $F_{3,36}$ = 220.533, p < 0.01, n = 10 per group) (Figure 4). No difference in the intensity of P2X$_3$ protein expression was found between the NS/NS and PUE/NS groups (n = 10 per group, unpaired t test, t_{18} = -0.416, p = 0.682) (Figure 4). The expression of P2X$_3$ protein in the PUE/formalin group was smaller than that in the NS/formalin group (n =10 per group, unpaired t test, t_{18} = 13.799, p < 0.01) (Figure 4). Consistent with the immunohistochemistry findings, PUE could inhibit the up-regulated expression of P2X$_3$ protein in rat DRG induced by the intraplantar injection of formalin.

Molecular docking of PUE in a homology-modeled rP2X$_3$

The docking experiments revealed that the hydrophilic cavity formed between two adjacent subunits of the homotrimer presumably represented the ATP-binding site. Of the already-studied conserved residues, many were oriented toward the groove of the pocket, indicating that they may bind ATP directly (Lys63, Lys65, Phe171, Thr172, Asn279, Arg281, Lys299) (Kawate et al., 2009).
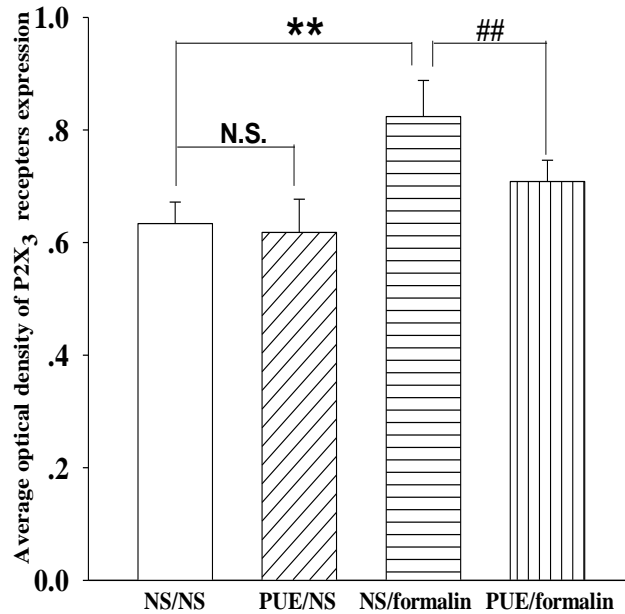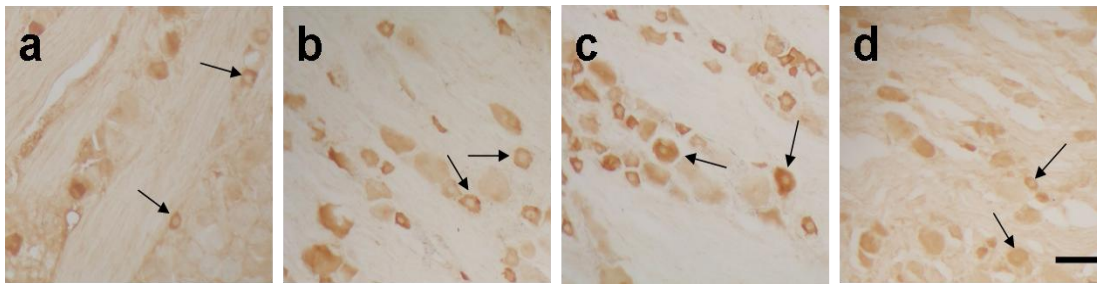
Figure 3. Reduction of P2X$_3$ immunoreactivity by puerarin in rat DRG neurons treated with formalin. P2X$_3$ immunoreactivity in the DRG was quantified (n = 10 each group). Representative results of P2X$_3$ immunoreactivity expression are shown in the upper panel for the NS/NS group (a), PUE/NS group (b), NS/formalin group (c), PUE/formalin group (d). Arrows indicate immunostained neurons; scale bar = 50 μm. Lower panels illustrate the average optical density of P2X$_3$ immunoreactivity expression in each group. The expression level of P2X$_3$ immunoreactivity in PUE/formalin group (d) was lower than the NS/formalin group (c; p<0.01). **p < 0.01 compared with the NS/NS group; [##]p < 0.01 compared with the NS/formalin group; N.S. = no significant difference.

PUE was shown to be involved in agonist (ATP) binding, which was situated on the opposite sites of the same subunit and was therefore able to form a binding pocket only at the interface of two adjacent subunits (Figure 5a and b). PUE could interact with the protein near the ATP-binding pocket and form hydrogen bonding with Gly66, Gly130, and Arg281 (Figure 5b and c). Interaction energies for the docked-complexes were calculated by MOE 2012.10 as shown in Table 4. The final score of docking between P2X$_3$ and PUE (Kcal/mol) showed that PUE could match and interact perfectly with the rP2X$_3$ (Table 4). The perfect match enabled PUE to interact with residues both deep in the ATP-binding pocket and in the outer sphere.

PUE inhibited the potentiation of P2X$_3$ receptor-mediated currents induced by LPS

The P2X$_3$ receptor is expressed in neurons of the DRG (Burnstock, 2013; Cheng et al., 2013; Noma et al., 2013). P2X$_3$ receptor-mediated currents can be potentiated by LPS (0.5 μg/ml; Franceschini et al., 2013). In this work, the effect of PUE on the potentiation of P2X$_3$ receptor-mediated currents induced by LPS (0.5 μg/ml) was investigated. Figure 6a shows the examples of membrane currents induced by selective P2X$_3$ receptor agonist α,β-meATP (100 μM) to DRG neurons under control conditions, 5 h after LPS (0.5 μg/ml), 5 h after PUE (10 mM) + LPS (0.5 μg/ml), and 5 h after PUE (10 mM). In
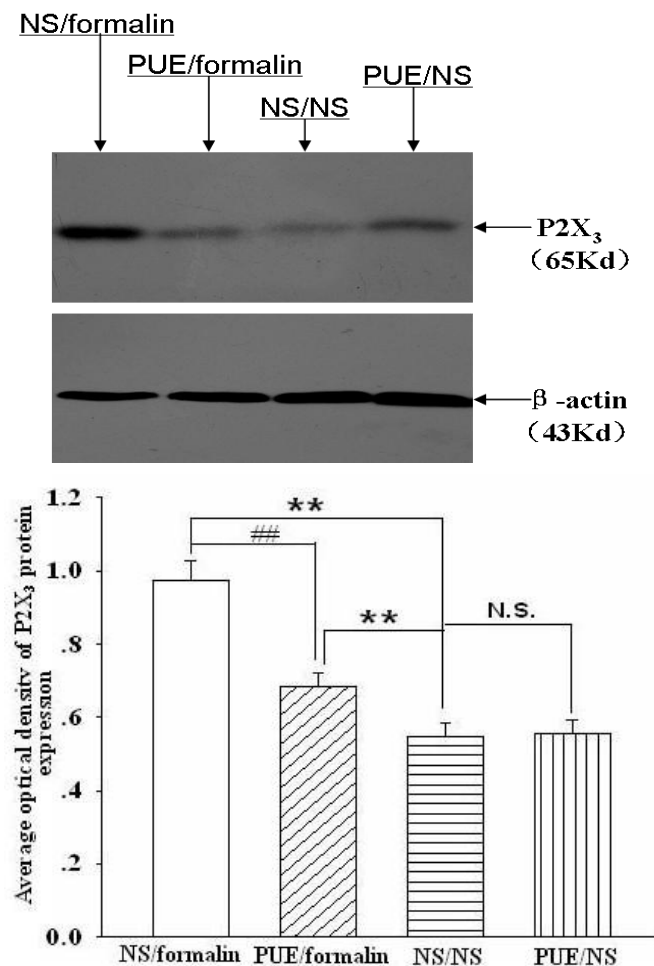
Figure 4. Reduction of P2X$_3$ protein expression by puerarin in rat DRG treated with formalin. The expression levels of P2X$_3$ protein in DRG were measured using western blotting. Representative results are shown in the upper panel. Equal amounts of lysates generated from DRG of each group were electrophoresed under denaturing conditions. The anti-P2X$_3$ antibody recognized a strong band of the expected size (65 kDa). The blot was simultaneously probed for the smaller housekeeping protein β-actin (43 kDa). Lower panel: each band density (in arbitrary units) was normalized to its β-actin internal control. All experiments were conducted in triplicate. **p < 0.01 compared with the NS/NS group; ##p < 0.01 compared with the NS/formalin group. N.S. = no significant difference between the NS/NS vs PUS/NS groups.

In all cases, the agonist application elicited a fast-developing inward current (Figure 6a) that rapidly decayed, because of receptor desensitization, a characteristic typical of currents mediated by P2X$_3$ receptors. When DRG neurons were treated for 5 h with LPS or PUE, a significant potentiation or inhibition of P2X$_3$ receptor-mediated currents was observed (p < 0.01) (Figure 6a and b). Nevertheless, when DRG neurons were treated for 5 h with PUE and LPS, the potentiation of α,β-meATP-mediated currents were inhibited as compared with DRG neurons treated for 5 h with LPS (p > 0.05).
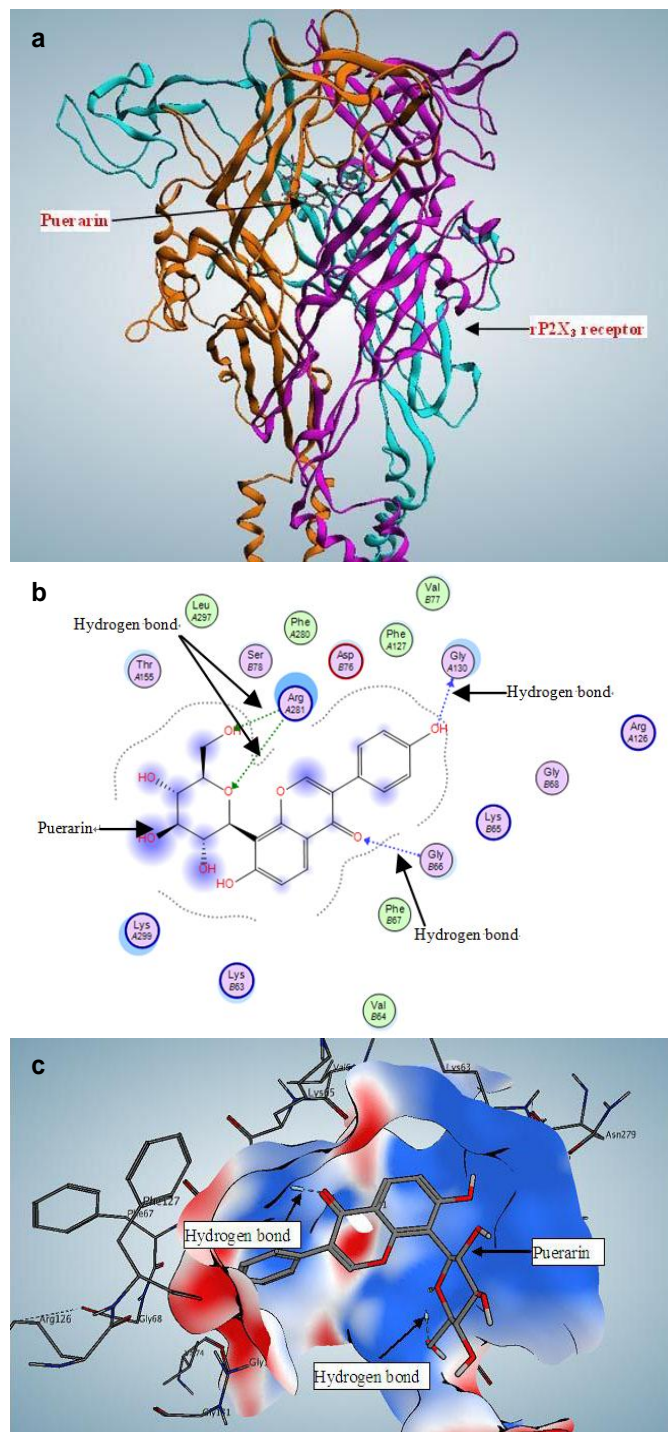

Figure 5. Computer simulation modeling of puerarin docking with the rP2X$_3$ receptor. Molecular docking of puerarin on a homology-modeled rP2X$_3$ receptor was simulated. The groove of the pocket may bind ATP directly (Lys63, Lys65, Phe171, Thr172, Asn279, Arg281, Lys299). ATP-binding sites were located at opposite sites of the same subunit and were therefore able to form a binding pocket only at the interface of two adjacent subunits (Figure 5a, b). Puerarin could interact with rP2X$_3$ receptor protein at the site close to the ATP-binding pocket and form hydrogen bonds with Gly66, Gly130, and Arg281 (Figure 5b, c). The perfect fit enabled the puerarin to interact with residues both deep in the ATP-binding pocket and in the outer sphere.
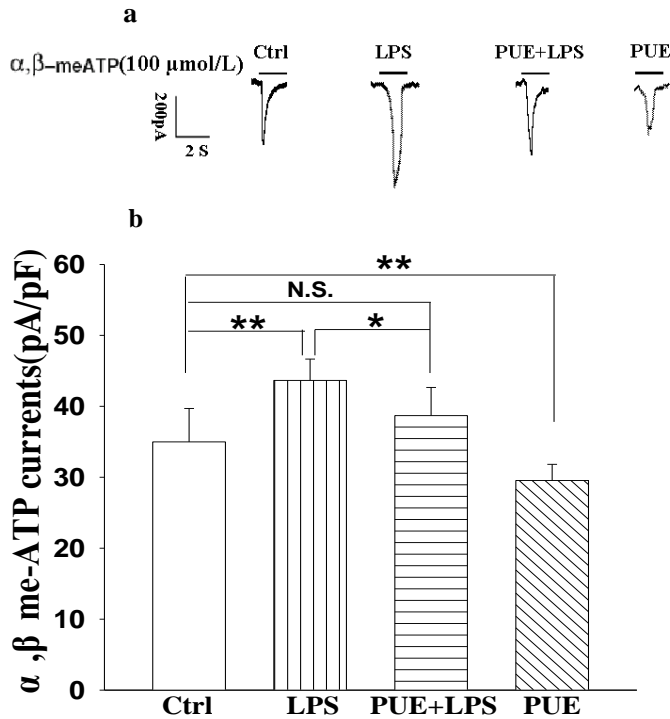
a

b

Figure 6. Inhibition of puerarin on the potentiation of P2X$_3$ receptor-mediated currents induced by LPS. Neuronal P2X$_3$ receptor-mediated responses in control conditions and after treatment with LPS or PUE+LPS were observed. (A) Representative examples of currents induced by application of α,β-meATP (100 μM) to DRG neurons in control condition, 5 h after LPS (0.5 μg/ml) or 5 h after PUE (10 mM)+LPS (0.5 μg/ml) application. (B) Histograms show average peak amplitudes of P2X$_3$-mediated currents (Ctrl, n = 32; LPS, n = 36; PUE+LPS, n = 32). *$p < 0.05$, **$p < 0.01$, N.S. denotes no significant difference.

Table 4. MOE score of P2X$_3$ and puerarin (Kcal/mol).

S/N	mol	mseq	S
1	puerarin	1	-6.1829
2	puerarin	1	-6.1699
3	puerarin	1	-6.1657
4	puerarin	1	-6.0725
5	puerarin	1	-6.0383
6	puerarin	1	-6.0048
7	puerarin	1	-5.9896
8	puerarin	1	-5.9528
9	puerarin	1	-5.9207
10	puerarin	1	-5.9158
11	puerarin	1	-5.8375
12	puerarin	1	-5.8337
13	puerarin	1	-5.8220
14	puerarin	1	-5.7890
15	puerarin	1	-5.7775
16	puerarin	1	-5.7738
17	puerarin	1	-5.7658
18	puerarin	1	-5.7526
19	puerarin	1	-5.7411
20	puerarin	1	-5.7391
21	puerarin	1	-5.7053

Mol is the research object. How many research objects are shown in MSEQ. S represents the final score.

DISCUSSION

Studies have shown that intraplantar injection of α,β-meATP or ATP produced nociceptive behaviors (such as paw lifting, withdrawing, and licking) and other pain defensive behaviors in conscious rats (Andó et al., 2010; Cherkas et al., 2012; Ford and Undem, 2013). The frequency of these pain defensive behaviors was significantly reduced after formalin injection into the claw of P2X$_3$-deficient mice (Cockayne et al., 2005; Souslova et al., 2000). Activation of the P2X$_3$ receptor was suggested to be involved in signal transmission of pain induced by ATP and inflammatory substances, such as formalin (Borsani et al., 2010; Fountain, 2013; Li et al., 2013; Nones et al., 2013; Pan et al., 2009). Our results showed that nociceptive responses could be induced by the intraplantar injection of ATP or α,β-meATP (P2X$_3$ receptor agonist) in conscious rats and such nociceptive responses were potentiated by intrathecal injection of ATP or α,β-meATP, indicating the nociceptive responses were activated by the P2X$_3$ receptor.

The effective ingredients of *Pueraria lobata* include a variety of flavonoids, such as daidzein, daidzin, PUE, and puerarin-7-xyloside (Peng et al., 2013; Rong et al., 1998), which have been clinically used for cardiovascular and cerebrovascular diseases (Gao et al., 2007; Rong et al., 1998). PUE (a major active ingredient extracted from the traditional Chinese drug called Ge Gen) is widely used for myocardial infarction, coronary heart disease, angina, and other cardiovascular diseases (Gao et al., 2007; Rong et al., 1998; Zhang et al., 2013). As both *Pueraria lobata* and puerarin soup have anti-inflammatory effects (Rong et al., 1998; Zhang et al., 2013), it is highly possible that PUE may have anti-nociceptive effects as well, especially for those nociceptive responses associated with inflammation. In the present study, we have observed that intrathecal injection of PUE inhibited the acute nociception induced by intraplantar injection of α,β-meATP or ATP and strengthened by intrathecal injection of ATP or α,β-meATP in rats. These results suggest that PUE inhibits the nociceptive responses via P2X$_3$ receptors.

It has been reported that neuropathic and inflammatory pain stimuli up-regulate the expression of P2X$_3$ mRNA, and protein in DRG and enhance ATP-gated currents mediated by P2X$_3$ receptor in primary sensory neurons (Borsani et al., 2010; Burnstock, 2013; Cheng et al., 2013; Joseph and Levine, 2012; Krimon et al., 2013; Noma et al., 2013; Prado et al., 2013). It has also been shown that the spontaneous pain behaviors induced by

formalin are significantly reduced in P2X$_3$ receptor knockout mice (Cockayne et al., 2005; Pan et al., 2009; Souslova et al., 2000), and that a variety of pain allergic reactions induced by formalin can be significantly reduced by a specific P2X$_3$ antagonist, A-317491 (Jarvis et al., 2002; Pan et al., 2009). These studies suggest that formalin may injure cells and sensory nerve endings, resulting in release of a large amount of ATP, leading to the up-regulation of P2X$_3$ receptor expression in DRG and ultimately producing nociceptive behavior responses (Calvert et al., 2008; Honore et al., 2002; McGaraughty et al., 2003; Nalepa et al., 2010; Okubo et al., 2010; Xu et al., 2012; Yu et al., 2013). In this study, it was observed that nociceptive behaviors in the NS/formalin group were significantly enhanced compared with those in the NS/NS group. In addition, the expression levels of P2X$_3$ mRNA, and protein in DRG were significantly increased. These results suggested that formalin increases P2X$_3$ expression and strengthens ATP or α,β-meATP-induced paw lifting, withdrawing, and licking and other acute nociceptive responses. When PUE was intrathecally injected, the up-regulated expression of P2X$_3$ mRNA and protein in DRG induced by the intraplantar injection of formalin was significantly reduced compared with the intrathecal injection of NS and intraplantar injection of formalin. Additionally, the nociceptive responses induced by formalin were also significantly decreased. These results indicate that PUE plays a role in the inhibition of formalin-induced acute nociceptive responses by acting on the P2X$_3$ receptor.

Homology modeling of other P2X receptor family members can be generated using the X-ray structure of the closed-state zebrafish (zf) P2X$_4$ receptor as a template (Kawate et al., 2009). Our result for molecular docking of PUE on a homology-modeled rP2X$_3$ indicated that puerarin could block ATP binding sites. As shown in Figure 5, PUE could interact with the rP2X$_3$ protein at the site proximal to the ATP-binding pocket and form hydrogen bonds with Gly66, Gly130, and Arg281. Interaction energies for the docked-complexes were calculated by MOE 2012.10 and are shown in Table 4. A higher value of negative interaction energy was an indicator of more efficient interaction between the rP2X$_3$ and PUE. The rP2X$_3$ can be restricted to binding ATP (increasing the concentration of ATP), because of its combination with PUE and therefore the channel of the rP2X$_3$ is blocked. Taken together, our results indicate that PUE may inhibit transmission of nociceptive information caused by inflammatory substances (ATP or formalin) via down-regulation of the expression levels of P2X$_3$ receptor and blockade of ATP-binding sites of the P2X$_3$ receptor in the DRG.

The P2X$_3$ receptor plays a crucial role in pain transduction (Burnstock et al., 2011). Inflammatory substances, such as LPS, may facilitate the release of ATP, which activated hyper-responsive P2X3 receptor, and then could amplify nociceptive signaling (Franceschini et al., 2013, Leung and Cahill, 2010). Our

studies showed that LPS enhanced the P2X$_3$ agonist α,β-meATP-mediated currents, and PUE repressed α,β-meATP -mediated currents. When DRG neurons were treated with PUE and LPS, the up-regulated α,β-meATP currents were decreased. Therefore, these results suggest that PUE can inhibit the α,β-meATP-mediated currents or the potentiation of P2X$_3$ receptor-mediated currents induced by LPS in acute pain.

Conclusions

Our results showed that PUE inhibited P2X$_3$ receptor-mediated acute nociceptive responses by reduction of formalin-induced up-regulation of P2X$_3$ receptor expression and blockade of ATP binding sites of P2X$_3$ receptor in the DRG. Thus, PUE could decrease acute pain mediated by the P2X$_3$ receptor in the DRG.

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