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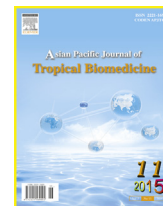
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Anti-inflammatory and antipyretic properties of Kang 601 heji, a traditional Chinese oral liquid dosage form



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ABSTRACT

Objective: To evaluate the scientific basis for the use of Kang 601 heji (K-601) as an anti-inflammatory and antipyretic agent using appropriate animal models.

Methods: Carrageenan-induced rat paw and xylene-induced ear oedemas were models used to investigate anti-inflammatory actions of K-601. Lipopolysaccharide-induced pyrexia model was used to evaluate antipyretic activity in Wistar rats. The anti-inflammatory and antipyretic mechanisms were evaluated by detecting prostaglandins E₂, nitric oxide, interleukin-1 β and tumour necrosis factor- α levels using appropriate reagents and ELISA kits.

Results: The results revealed that K-601 reduced the level of inflammations in both anti-inflammatory models in a dose-dependent manner. The same was true for the antipyretic model. The possible mechanisms of actions were through the inhibition of prostaglandins E₂, interleukin-1 β , tumour necrosis factor- α and nitric oxide.

Conclusions: K-601 has proven anti-inflammatory and antipyretic actions. The findings provide a scientific basis for the use of K-601 as anti-inflammatory and antipyretic agent in traditional Chinese medicinal practice.

1. Introduction

Inflammation is defined as the biological response of the body to local injury and infection, characterized by redness, oedema, fever, pain, and loss of function. It involves a cascade of events elicited by numerous stimuli that include infectious agents, ischaemia, thermal and physical injury, and antigen–antibody interaction [1]. Even though inflammation is the defence mechanism of the body to eliminate or limit the spread of injurious stimuli and heal the wounds, excessive inflammation

is associated with onset of diseases like rheumatoid arthritis, asthma, periodontitis, inflammatory bowel disease, atherosclerosis, Alzheimer's disease and even cancer such as gall bladder carcinoma [2]. Non-steroidal anti-inflammatory drugs are widely used in the treatment of acute and chronic inflammation, pain, and fever. Their use is associated with adverse effects like severe gastritis, peptic ulcer, nausea, vomiting, salt and water retention, worsening of renal function in renal or cardiac and cirrhotic patients, hypersensitivity, *etc.* [3]. Therefore, there is a need for screening and development of anti-inflammatory drugs which are devoid of these unwanted effects. As herbal medicines lack these side effects, the current trend is to conduct investigations on plant-based drugs. According to the World Health Organization, about 80% of the world population still relies mainly on traditional herbal remedies [4].

Fever is the regulated rise in body temperature above the normal range which is usually considered as a sign of an infection. Though a defence mechanism, sustained fever has

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potentially harmful effects such as dehydration, delirium, convulsions, cardiopulmonary strain, localized lesions and other teratological consequences [5]. The most common cause of fever is bacterial infections, and mimicked experimentally by the systemic injection of lipopolysaccharide (LPS). LPS is a major component of Gram-negative bacterial cell wall. The induction of fever involves an array of cytokines [interleukin-1 β (IL-1 β), interleukin-6 (IL-6), tumour necrosis factor- α (TNF- α), etc.] and other mediators leading to the production of cyclooxygenase-2-dependent prostaglandin E₂ (PGE₂) [6].

Kang 601 heji (K-601) is a traditional Chinese remedy for common cold comprising five herbs, *Lonicera japonica* Thunb. (*L. japonica*), *Isatis indigotica* Fort. (*I. indigotica*), *Rheum palmatum* L., *Phellodendron chinense* Schneid. (*P. chinense*), and *Scutellaria baicalensis* Georgi (*S. baicalensis*). It is usually used due to its ability to alleviate the symptoms of common cold such as headache, fever, inflammation and general bodily pain, etc. Though data from scientific literature provide information on individual herbs in the formulation, there is no scientific evidence available of this unique formulation to justify its use. The aim of this work was to investigate the anti-inflammatory and antipyretic properties of this formulation using appropriate animal models. This work also aimed at identifying the chemical make-up of this formulation which could help explain its possible mechanisms of action.

2. Materials and methods

2.1. Drug formulation, chemicals and reagents

K-601 was purchased from Nanjing Children Hospital Indomethacin (Shaanxi Yuupeng Pharm. Co. Ltd) and dexamethasone (Jinan Limin Pharmaceutical Co., Ltd.) were used in this study. Sterile normal saline solution was purchased from Anhui Shuanghe Pharmaceutical Co., Ltd. LPS (*Escherichia coli* 055:B5) was purchased from Sigma–Aldrich. Carrageenan was bought from Aladdin Chemistry Co. Ltd. High performance liquid chromatography grade acetonitrile was purchased from Merck (Darmstadt, Germany). Distilled water was purified by Milli-Q system (Millipore, USA). High performance liquid chromatography grade methanol was gotten from Jiangsu Han-bon Sci. & Tech. Co. Ltd. (Jiangsu, China). Analytical grade formic acid was purchased from Nanjing Chemical Factory (Nanjing, China). Kits for ELISA were purchased from Cusabio Biotech., Co., Ltd. (Wuhan, China).

2.2. Identification of chemical constituents of K-601

About 1 mL of K-601 was dissolved in 1 mL of distilled water. The resultant solution was centrifuged at 13000 r/min for 10 min and the supernatant was transferred to a sample vial for ultra performance liquid chromatography-mass spectrometer (UPLC-MS) analysis.

Chromatographic analysis was performed on an Agilent 1290 Series (Agilent Corp., Santa Clara, CA, USA). UPLC system equipped with a binary pump, micro degasser, an auto sampler and a thermostatically controlled column compartment. Chromatographic separation was carried out at 25 °C on a Zorbax RRHD eclipse plus C₁₈ column (2.1 mm × 50 mm, 1.8 μ m). The mobile phase consisted of 0.1% formic acid solution (A) and acetonitrile (B) using a gradient elution of 0%–5% B at 0–6 min,

5%–8% B at 6–15 min, 8%–15% B at 15–20 min, 15%–20% B at 20–30 min, 20%–30% at 30–35 min, 30%–35% at 35–45 min, 35%–40% at 45–60 min. The flow rate was kept at 0.2 mL/min, and the sample volume injected was set at 5 μ L. Detections were carried out by Agilent 6530 Q/TOF mass spectrometer (Agilent Corp., Santa Clara, CA, USA) equipped with an electrospray ionization interface. The parameters of operation were as follows: drying gas N₂ flow rate, 10.0 L/min; temperature, 330 °C; nebulizer, 35 psig; capillary, 3000 V; skimmer, 60 V; Oct RFV, 250 V. Each sample was analyzed in both the positive and negative modes due to the selective sensitivities to different components of the formulation-providing better information for molecular formulae and structural identification. Mass spectra were recorded across the range *m/z* 100–1000 with accurate mass measurements.

2.3. Animals

Male Wistar albino rats from Yangzhou University Cooperative Medicine Center weighing 180–220 g were used for the study. They were housed in acryl fibre cages at (24 \pm 2) °C, humidity (50 \pm 1)% and were kept on a 12 h light/dark cycle. They were fed with standard pellet diet from Jiangsu Provincial Synergistic Engineering Co. Ltd. and water *ad libitum*. Animals were made to acclimatize with the new environment for 7 days before experimentation. Then they were fasted 12 h before each test. The animal care and use complied with the Provisions and General Recommendations of the Chinese Experimental Animals Administration Legislation. The experiments were performed with the approval from the Animal Ethics Committee of China Pharmaceutical University.

2.4. Evaluation of anti-inflammatory property in Wistar rats

2.4.1. Carrageenan-induced paw oedema

Carrageenan-induced paw oedema was one of the models used for evaluation of anti-inflammatory effect as described by Winter *et al.* with some modifications [7]. The baseline values of the paw volume were taken at 0 h using a plethysmometer. A total of 48 rats were divided into six groups, with eight rats in each group. The drugs were administered in the following manner:

Group 1 received 4 mL of distilled water as the negative control;

Group 2 received 10 mg/kg of indomethacin as the positive control;

Groups 3, 4, 5 received 100 mg/kg, 200 mg/kg and 400 mg/kg of K-601, respectively;

Group 6 received normal saline.

After 1 h, 0.1 mL of carrageenan (1% in normal saline) was injected into the subcutaneous tissue of the right hind paws of each animal and the paw volumes were measured at 1, 2, 3, 4 and 5 h. Oedema was expressed as the mean increase in paw volume relative to control animals. The percentage inhibition of oedema was calculated using the following equation:

$$\text{Inhibition (\%)} = \frac{[(V_t - V_0)_{\text{control}} - (V_t - V_0)_{\text{treated}}]}{(V_t - V_0)_{\text{control}}} \times 100$$

where V₀ is paw volume at 0 h, V_t is paw volume at 1, 2, 3, 4, 5 h.

2.4.2. Xylene-induced ear oedema

Six groups ($n = 8$) of the rats were treated as follows:

Groups 1, 2, 3 received 100 mg/kg, 200 mg/kg, and 400 mg/kg body weight of K-601;

Group 4 received 10 mg/kg of indomethacin;

Group 5 received 4 mg/kg of dexamethasone *i.p.*;

Group 6 received normal saline as negative control.

After the administration of the drugs for 60 min, each animal received 20 μ L of xylene on the anterior and posterior surfaces of the right ear. The left ear was considered as normal control. After another 60 min, the animals were sacrificed and both ears were sampled with a punch (5 mm diameter) and weighed. The extent of oedema was evaluated by the weight difference between the right and left ear biopsies of the same animal.

2.5. Evaluation of antipyretic activity

2.5.1. LPS-induced fever in Wistar rats

The animals were randomly divided into six groups ($n = 8$). Animals in Groups 1, 2, 3 received 100, 200 and 400 mg/kg body weight of the drug, K-601 by gavage. In Group 4, each animal received 10 mg/kg body weight of indomethacin. Animals in Group 5 received 4 mg/kg body weight of dexamethasone via intraperitoneal injection. Animals in Group 6 received the vehicle, pyrogen-free water. After 30 min the animals in Group 6 received pyrogen-free sterile normal saline solution while the other groups were injected intraperitoneally with LPS (500 μ g/kg) dissolved in pyrogen-free sterile normal saline solution. Body temperature (Tb) was taken every h for 5 h after LPS administration.

These experiments were performed during the light phase of the circadian cycle. Tb was measured by gently inserting a small thermoprobe about 4 cm into the rectum until a beep sound heard. During the temperature measurements, each animal was held gently and performed at least twice 2 days prior to the experiment to avoid changes in rectal temperature secondary to handling. Only animals with stable body temperature and in the range 32.4–37.0 °C were used in the investigation of the influence of the drug on the Tb of the animals upon induction of pyrexia with LPS.

2.6. Mechanisms of activity

2.6.1. Test for cytokines (IL-1 β , TNF- α) and PGE₂

The IL-1 β , TNF- α and PGE₂ levels in the supernatants were measured using ELISA kits (Cusabio Biotech., Co. Ltd., Wuhan, China) according to the manufacturer's instructions. Briefly, diluted standards or samples were added to 96-well plates pre-coated with affinity purified polyclonal antibody specific rat IL-1 β , TNF- α and PGE₂. Into these wells, enzyme-linked polyclonal antibodies were added and incubated at 37 °C for 60 min, and washed 5 times. After the addition of the substrate solutions, their intensities were detected at 450 nm, which were proportional to the productions of IL-1 β , TNF- α and PGE₂.

2.6.2. Nitric oxide (NO) assay

The amount of nitrite as an indicator of NO production was measured using Griess reagent. In brief, supernatants from the plasma of each animal in each model were mixed with same volume of Griess reagents A & B. After incubation at room temperature for 10 min in a dark place, the absorbance was measured at 540 nm using Biotek Synergy 2 spectrophotometer.

2.7. Histopathology of the inflamed paw of Wistar rats

Biopsies of right hind paws of the rats were collected after the induction of inflammation with carrageenan for 5 h. Tissues slices were fixed in formalin-acetic acid fixative (100% formalin & 1% acetic acid) at room temperature, dehydrated, embedded in paraffin and sectioned into 4 μ m. Tissue sections were stained with haematoxylin and eosin and examined with Olympus IX51, Japan microscope. The tissue slices were randomly taken from the paws of rats from the following groups: indomethacin (positive control) group, group that received the highest dose of K-601 (400 mg/kg body weight), the model group as well as from rats not given the carrageenan injection (negative control).

2.8. Statistical analysis

The results were expressed as mean \pm SEM. Differences between control and treatment groups were analyzed using One-way analysis of variance (ANOVA) with GraphPad Prism 5.0 software as described in Dunnett's test. $P < 0.05$ was considered significant; $P < 0.01$ was considered very significant while $P < 0.001$ was considered highly significant.

3. Results

3.1. Identification of constituents of K-601

Using a strategy developed in our lab [8], 50 compounds were tentatively identified by UPLC-QTOF/MS. The retention times and MS data of the characterized compounds are summarized in Table 1.

3.2. Evaluation of anti-inflammatory activity in Wistar rats

3.2.1. Effects on carrageenan-induced paw oedema

The paw size increased steadily until it peaked at 3 h after injection with carrageenan (seen clearly in the negative control group). The various doses of K-601 reduced the paw size at the time points in a dose-dependent manner. The highest dose of 400 mg/kg even proved more effective in reducing inflammation after 5 h than the control drug, indomethacin. This is shown in Figure 1.

3.2.2. Effects on xylene-induced ear oedema

The effect of the drug K-601 on the reduction of the ear size of the rats upon induction of oedema with xylene was investigated. In this model varying doses (100, 200, and 400 mg/kg body weight) of the formulation were given and their effects were compared to two positive controls (indomethacin and dexamethasone) and a negative control. Figure 2 illustrates the outcome in bar chart. The effect of the formulation was dose-dependent, 400 mg/kg body weight giving the highest level of inhibition (thus with the lowest weight of the inflamed ears). The two positive controls exhibited comparable effects with dexamethasone giving higher inhibition rate. The inhibition rates compared to the control group gave P -values as follows: $P < 0.05$, $P < 0.01$, $P < 0.001$ respectively for 200 mg/kg and 400 mg/kg of K-601 and the positive controls respectively.

Table 1

Compounds identified in K-601 in positive and negative ion modes.

No.	t_R (min)	ESI mode	Fragment ions (m/z)	Formula	Identity	Abund. ($\times 10^6$)	Peak abundance	Source
1	4.265	–	169.0134, 125.0246	C ₇ H ₆ O ₅	Gallic acid ^a	16.06	High	RP
2	6.174	–	331.0670, 271.0433, 211.0235, 169.0139	C ₁₃ H ₁₆ O ₁₀	Gallic acid 3-O-β-D-glucopyranoside	2.93	Medium	RP
3	11.765	–	389.1051, 345.1085, 183.0641, 165.0548	C ₁₆ H ₂₂ O ₁₁	Secologanoside	16.70	High	LJ
4	17.136	–	353.0871, 191.0540	C ₁₆ H ₁₈ O ₉	Chlorogenic acid ^a	4.78	Medium	LJ
5	17.360	–	179.0331, 135.0430	C ₉ H ₈ O ₄	Caffeic acid ^a	3.17	Medium	LJ
6	22.593	–	353.0869, 191.0533, 179.0316, 173.0424, 161.0187, 135.0421	C ₁₆ H ₁₈ O ₉	4-O-caffeoylquinic acid	22.43	High	LJ
7	22.999	–	353.0863, 191.0531, 179.0316, 173.0324	C ₁₆ H ₁₈ O ₉	5-O-caffeoylquinic acid	21.06	High	LJ
8	24.536	–	357.1197, 195.0671, 151.0761, 125.0251	C ₁₆ H ₂₂ O ₉	Sweroside	9.72	Medium	LJ
9	26.053	–	367.1028, 191.0534	C ₁₇ H ₂₀ O ₉	Caffeoyl-CH ₂ -O-quinic acid	8.36	Medium	LJ
10	32.450	–	269.0439, 240.0408, 211.0400	C ₁₅ H ₁₀ O ₅	Aloe-emodin ^a	0.21	Low	RP
11	33.286	–	609.1475, 301.0325	C ₂₇ H ₃₀ O ₁₆	Rutin ^a	0.53	Low	SC
12	33.455	–	445.0756, 283.0241, 239.0326,	C ₂₁ H ₁₈ O ₁₁	Rhein-8-O-β-D-glucopyranoside	0.04	Low	RP
13	33.658	–	447.0897, 285.0396	C ₂₁ H ₂₀ O ₁₁	Luteolin-7-O-β-D-glucoside	0.75	Low	RP
14	33.692	–	463.0895, 301.0340, 271.0291	C ₂₁ H ₂₀ O ₁₂	Hyperoside	0.42	Low	LJ
15	33.861	–	461.0737, 285.0339	C ₂₁ H ₁₈ O ₁₂	Scutellarin	0.47	Low	SB
16	33.937	–	593.1519, 447.0962, 285.0401	C ₂₇ H ₃₀ O ₁₅	Lonicerin ^a	0.52	Low	LJ
17	34.756	–	515.1189, 353.0873, 191.0551, 179.0346	C ₂₅ H ₂₄ O ₁₂	1,5-O-dicaffeoylquinic acid or 4,5-O-dicaffeoylquinic acid or 1,4-O-dicaffeoylquinic acid	0.05	Low	LJ
18	36.809	–	269.0476, 167.0507	C ₁₅ H ₁₀ O ₅	Baicalein ^a	0.12	Low	SB
19	37.817	–	269.0456, 240.0408, 211.0400, 167.0518	C ₁₅ H ₁₀ O ₅	Emodin ^a	12.33	High	RP
20	38.152	–	269.0452, 225.0539, 197.0564, 183.0448	C ₁₅ H ₁₀ O ₅	Apeginin	0.24	Low	LJ
21	38.701	–	253.0488, 225.0556	C ₁₅ H ₁₀ O ₄	Chrysophanol	0.22	Low	RP
22	39.326	–	253.0504, 225.0557	C ₁₅ H ₁₀ O ₄	Chrysin	0.31	Low	RP
23	39.410	–	431.0987, 395.1032, 311.0588, 269.0468	C ₂₁ H ₂₀ O ₁₀	Aloe-emodin-8-O-glucoside	0.40	Low	RP
24	40.027	–	459.0952, 283.0612, 268.0376	C ₂₂ H ₂₀ O ₁₁	Wogonoside ^a	16.97	High	SB
25	40.263	–	283.0605, 268.0363, 240.0372	C ₁₆ H ₁₂ O ₅	Physcion	0.72	Low	RP
26	43.895	–	269.0463, 241.0521, 225.0543	C ₁₅ H ₁₀ O ₅	Norwogonin	0.26	Low	SB
27	45.416	–	313.7373, 229.2099, 211.1348, 171.1012	C ₁₈ H ₃₄ O ₅	Sanleng acid	2.90	Medium	RP
28	47.544	–	297.0409, 253.0501, 225.0517	C ₁₆ H ₁₀ O ₆	6-Methyl-rhein	0.17	Low	RP
29	52.055	–	283.0260, 239.0343, 211.0411, 183.0413	C ₁₅ H ₈ O ₆	Rhein ^a	0.28	Low	RP
30	4.076	+	180.1286, 121.0535	C ₁₁ H ₁₈ NO	Candicine	4.02	Medium	PC
31	20.496	+	314.1716, 269.1167, 237.0903, 192.1009, 143.0486	C ₁₉ H ₂₃ NO ₃	Lotusine	24.34	High	PC
32	22.430	+	342.1687, 192.1007	C ₂₀ H ₂₄ NO ₄	Phellodendrine ^a	25.40	High	SB
33	23.461	+	344.1854, 299.1283, 207.0766, 175.0748, 137.0590	C ₂₀ H ₂₆ NO ₄	Tembetarine	20.21	High	PC
34	23.498	+	342.1730, 297.1121, 265.0859	C ₂₀ H ₂₃ NO ₄	Magnoflorine ^a	25.39	High	PC
35	24.120	+	342.1684, 297.1102, 265.0852	C ₂₀ H ₂₃ NO ₄	Tetrahydrojatrorrhizine	25.32	High	PC
36	28.613	+	356.1853, 311.1271, 279.1016	C ₂₁ H ₂₅ NO ₄	Menisperine	22.31	High	PC
37	30.260	+	312.1591, 297.1361, 267.1191, 252.1016	C ₁₉ H ₂₁ NO ₃	Veticuline	0.32	Low	PC
38	31.966	+	356.1854, 192.1017	C ₂₁ H ₂₅ NO ₄	Tetrahydropalmatine	24.19	High	PC
39	31.975	+	356.1855, 297.0821, 192.1017, 177.0780, 148.0752	C ₂₁ H ₂₆ NO ₄	N-Methyltetrahydrocolumbamine	23.19	High	PC
40	35.193	+	338.1377, 322.1072, 308.0908, 294.1118, 280.0951	C ₂₀ H ₂₀ NO ₄	Jatrorrhizine ^a	2.03	Medium	PC
41	37.544	+	352.1534, 336.1228, 322.1074, 308.1277, 294.1121	C ₂₁ H ₂₂ NO ₄	Palmatine ^a	25.38	High	PC
42	37.722	+	336.1233, 334.1071, 306.0764, 292.0968	C ₂₀ H ₁₈ NO ₄	Berberine ^a	23.26	High	PC
43	37.783	+	445.0772, 271.0608	C ₂₁ H ₁₈ O ₁₁	Baicalin ^a	24.40	High	SB
44	38.119	+	320.0911, 292.0962	C ₂₀ H ₁₈ NO ₄	Epiberberine	0.06	Low	PC
45	38.600	+	263.0820	C ₁₆ H ₁₀ N ₂ O ₂	Indigotin	0.25	Low	II
46	42.955	+	437.3389, 409.3480, 366.0660	C ₂₆ H ₃₀ O ₇	Obacunone	0.12	Low	PC
47	44.121	+	329.2210, 316.0589, 301.0377, 287.0624, 273.0374	C ₁₇ H ₁₄ O ₇	Iristectorigenin A or Iristectorigenin B	1.73	Medium	LJ
48	44.121	+	329.2210, 316.0589, 301.0377, 287.0624, 273.0374	C ₁₇ H ₁₄ O ₇	Iristectorigenin A or Iristectorigenin B	1.73	Medium	LJ
49	50.202	+	453.1858, 425.1962, 367.1909	C ₂₆ H ₃₀ O ₈	Obaculactone	2.33	Medium	PC
50	51.520	+	285.0760, 270.0522	C ₁₆ H ₁₂ O ₅	Wogonin ^a	2.80	Medium	SB

^a Confirmed with reference compounds. The relative abundance of the compounds measured by the peak height in the extracted ion chromatogram $>10.00 \times 10^6$, defined as major constituent, thus high level; $(1.00 - 10.00) \times 10^6$, defined as minor constituent, meaning moderate level; $<1.00 \times 10^6$ defined as trace constituent, hence present in low levels. PC: *P. chinense*; LJ: *L. japonica*; II: *I. indigotica*; SC: *S. baicalensis*; RP: *Rheum palmatum*.

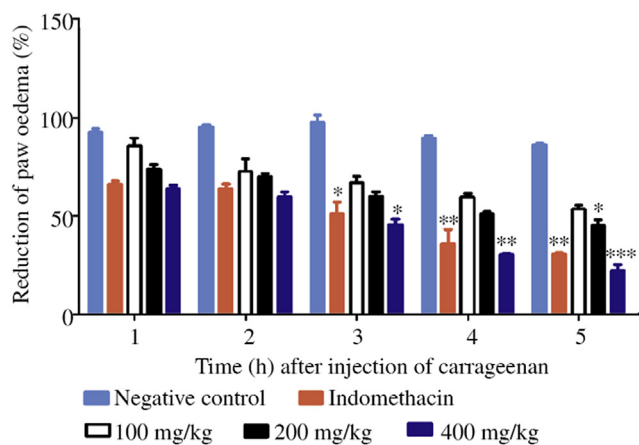


Figure 1. The effects of varying doses of K-601 and indomethacin on the paw size of rats with time. The asterisk denotes the significance levels in comparison with the negative control: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

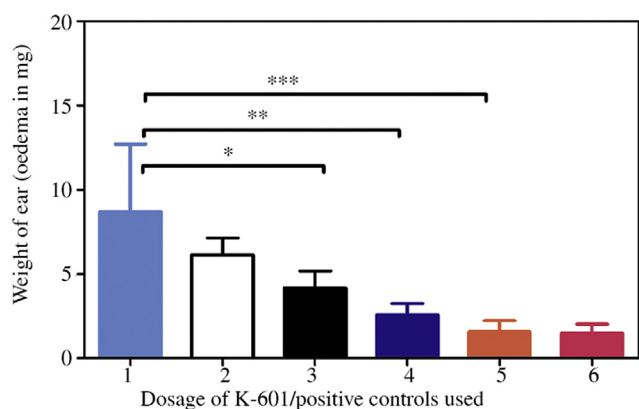


Figure 2. The effects of varying doses of K-601, indomethacin and dexamethasone on the xylene-induced ear oedema of rats. 1: Control; 2: 100 mg/kg; 3: 200 mg/kg; 4: 400 mg/kg; 5: Indomethacin; 6: Dexamethasone. The asterisks indicate the levels of significance in comparison with the control: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

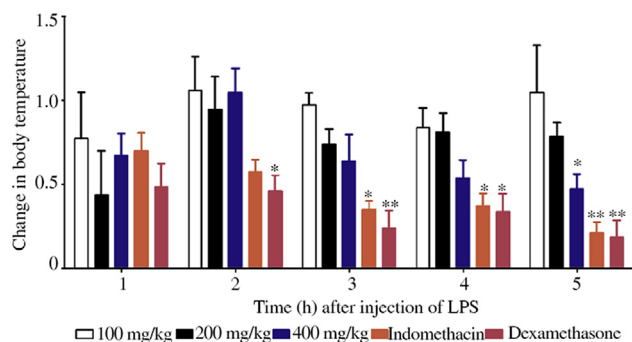


Figure 3. The effects of different doses of K-601, indomethacin and dexamethasone on LPS-induced fever with time. The asterisks denote the levels of significance in comparison with the negative control group: * $P < 0.05$, ** $P < 0.01$.

3.3. Evaluation of antipyretic activity in Wistar rats

The LPS-induced fever peaked 2 h after injection. The reduction in body temperature was also dose-dependent using K-601 with 400 mg/kg body weight giving the maximum effect. This high dose however was not as effective as the positive controls, indomethacin and dexamethasone. Dexamethasone (4 mg/kg) proved a little more effective than indomethacin at reducing the body temperature 5 h after experimentation probably due to the fact that it was given *i.p.* as shown in Figure 3.

3.4. Histopathological analysis of rat paw

The histopathological results shown in Figure 4 showed no cellular infiltration or oedema in the control group (Figure 4A). In contrast, polymorphonuclear infiltration and swelling followed carrageenan injection (Figure 4B). After treatment with 400 mg/kg of K-601, there were significant reductions in the polymorphonuclear infiltrations and oedema as shown in Figure 4C which compared with the standard control drug of indomethacin (Figure 4D).

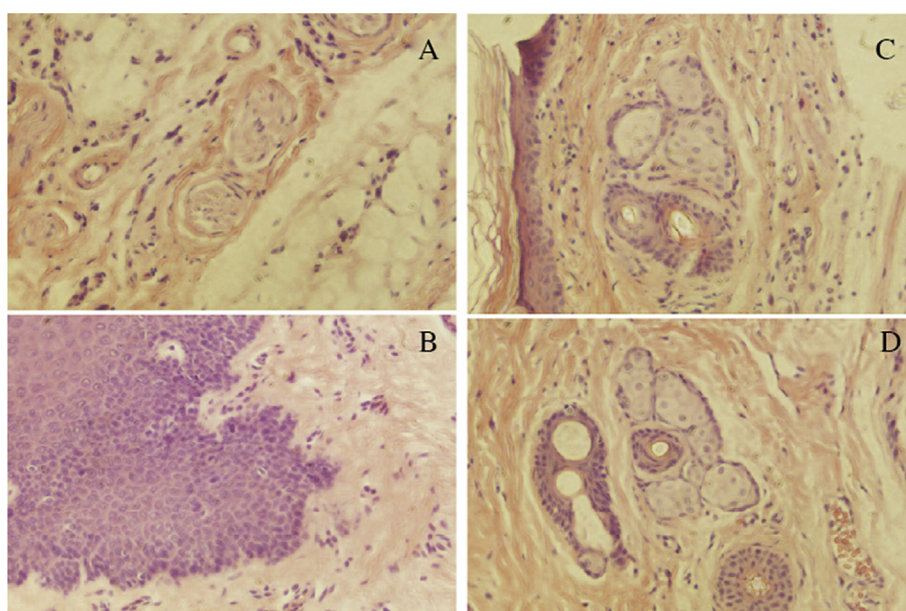


Figure 4. Histological changes in oedema paws 5 h after injection of carrageenan. Paws were cut 5 h after injection of carrageenan and subjected to histochemical staining of paw tissues. A: Saline control group; B: Carrageenan model group; C: 400 mg/kg body weight of K-601; D: indomethacin as positive control drug. Magnification $\times 20$.

4. Discussion

Tentative identification of compounds could be an arduous task especially when reference compounds are not available. With reference compounds, similar compounds could be identified based on their retention times, fragmentation patterns and other data in literature. In the absence of reference compounds, a lot of strategies have been employed for tentative identification and structural confirmation. The strategy employed in the identification was a method called diagnostic ion filtering strategy [8]. Briefly the diagnostic ions corresponding to a mother skeleton obtained from reference compounds are used to screen the same type of compounds. Then, the molecular ions of screened peaks are calculated using both negative and positive ion modes. Next, the accurate molecular formula of each peak obtained was applied to screening for a hit against various chemical databases. A most possible structure that contains such a substructure and substituent groups can be determined from these candidates by comparison of characteristic product ions and fragmentation pathways.

The outcome of the identification of the constituents of the formulation K-601 revealed it contains a variety of compounds ranging from flavonoids, isoflavonoids, to alkaloids, *etc.* In total, 50 compounds were tentatively identified in both the positive and negative ion modes. The identification of the chemical make-up of this formulation proved useful in explaining its possible mechanisms of actions.

Carrageenan-induced paw oedema is a good, reliable and repeatable *in vivo* experimental model employed to investigate anti-inflammatory agents. Following carrageenan injection, the development of oedema is believed to be biphasic [9,10] that eventually leads to signs of inflammation such as oedema, hyperalgesia and erythema. The early phase involves the release of inflammatory mediators such as histamine, serotonin and bradykinins while the late phase is associated with the release of prostaglandins [10]. It is also known that platelet activating factor and arachidonic acid metabolites play a vital role in the phase 1 of inflammation [11]. The resultant effect leads to increased vascular permeability, which enhances the infiltration of neutrophils and the accumulation of plasma fluid into the interstitial space which leads to oedema [9]. The results of this study reveals that inflammation was inhibited at both phases though the latter phase showed more significance. It was also revealed that the levels of inhibition of inflammation were dose-dependent. The dose of 400 mg/kg body weight gave better results in terms of the reduction of inflammation compared with the control drug of indomethacin at times 3 h, 4 h and 5 h.

The xylene-induced ear oedema is another inflammation model that was used. Xylene, a well-known inflammatory agent, provokes acute inflammatory response in the ear of the rat leading to serious oedematous changes of skin when applied to the ear. The acute inflammatory response, severe vasodilation and oedematous changes of the skin is partially associated with phospholipase A2 as reported by Luber-Narod *et al.* [12], Kim *et al.* [13] and Wang *et al.* [14]. The formulation K-601 also reduced inflammation in the ears of the rats in a dose-dependent manner, with 400 mg/kg giving the highest outcome.

Fever is commonly studied in the laboratory by injecting animals with bacterial LPS or mediators of its action such as pro-inflammatory cytokines, IL-1 β , IL-6 and TNF- α . It has been

reported that LPS-induced fever in rats is polyphasic with different underlying mechanisms [15]. The response of the animals to the fever depends on the serotype and dose of LPS, injection route and stress, ambient temperature, animal strain, gender differences [16–18]. Tb of the animals increased 1 h after injection of LPS and peaked at 2 h and 5 h. Though the underlying process of fever initiation and sustenance are multiple, varied and complex, it is believed that LPS causes fever by stimulating peripheral macrophages to synthesize and release pyrogenic cytokines such as IL-1 β , TNF- α and IL-6. These cytokines are transported to the thermoregulatory centre of the anterior hypothalamus and consequently stimulate the production of PGE₂. PGE₂ ultimately leads to the elevation of temperature, hence fever. PGE₂ is a major mediator of fever and present in all phases of the polyphasic LPS-induced fever [16,19]. NO is also found to be a part of several systems that are involved in Tb regulation and NO acting peripherally has been found to be pyretic [20].

In the investigation of K-601 as a potential antipyretic agent, it was found that it possesses the ability to reduce fever but also in a dose-dependent manner as shown in Figure 3. Its effect however even with the high dose of 400 mg/kg body weight though significant after 5 h could not compare with the positive controls of dexamethasone and indomethacin.

The possible mechanisms of actions of this formulation could be due to the inhibition of NO production, inhibition of cytokines release as well as the inhibition of PGE₂ release in inflammation and at the various phases of LPS-induced pyrexia.

The probable reasons for the activity of K-601 as an anti-inflammatory and antipyretic agent could be traced back to its composition. As earlier mentioned, this formulation contains flavonoids, isoflavonoids, alkaloids, *etc.* Flavonoids have been found to have anti-oxidative and free radical scavenging activities. They could regulate cellular activities of inflammation-related cells, thus, mast cells, macrophages, lymphocytes and neutrophils. Certain flavonoids modulate the enzyme activities of arachidonic acid, metabolizing enzymes such as phospholipase A2, cyclooxygenase, lipoxygenase and the NO producing enzyme, NO synthase. Therefore inhibition of these enzymes by flavonoids leads to reduced production of arachidonic acid, prostaglandins, leukotrienes and NO, which are crucial mediators of inflammation [21]. Another anti-inflammatory property of flavonoids is their ability to inhibit nuclear factor kappa B transcription activation [22,23].

Alkaloids have also been found to be good anti-inflammatory agents. For instance indole alkaloids from the roots of *I. indigotica* has been found to have inhibitory effects on NO production [24], alkaloidal fraction from *Alstonia scholaris* including picrinine, vallesamine and scholaricine was also found to inhibit NO and prostaglandin, PGE₂ production. Various alkaloids therefore exhibit their activities via different mechanisms.

The histopathological analyses also showed the extent to which K-601 could ameliorate inflammation. K-601 (400 mg/kg body weight) proved effective in preventing polymorphonuclear infiltration into the site of inflammation (paw) which compared favourably with the control drug, indomethacin.

Herbal formulations have been found to contain bioactive compounds that usually act in synergy to give the desired pharmacological effects. Thus the effects of the formulation, K-601 could be due to different compounds acting in consonance and not necessarily one specific compound.

Our findings however, are in agreement with other researchers who worked on the individual components of this formulation. *L. japonica* is commonly used for the treatment of sores, carbuncles, furuncles, swelling and infections caused by exopathogenic wind-heat or epidemic febrile diseases at the early stage of life. It is also found to have hepatoprotective, cytoprotective, antimicrobial, antioxidative, antiviral and anti-inflammatory effects [25]. *I. indigotica* has been found useful in preventing and treating influenza, tonsillitis, and malignant infectious diseases [26] especially severe acute respiratory syndrome and H1N1-influenza [27] because of its anti-viral, anti-bacterial, anti-inflammatory, anti-tumour, and immune regulatory functions. *P. chinense* is a well-known oriental folk medicine as an effective anti-inflammatory, antiphlogistic and antipyretic agent in clinical usage [28]. *S. baicalensis* is traditionally used for the treatment of inflammation, asthma as well as throat disorders, e.g. cough, tonsillitis and pharyngitis [29]. The combined effects of all these components were thus investigated.

In conclusion, the formulation K-601 was found to possess anti-inflammatory and antipyretic activities. It was found to inhibit the production of NO, the release of cytokines (IL-1 β , TNF- α) and PGE₂. The outcome of this research provides a solid foundation for further pharmacological studies on this unique formulation which is actually ongoing.

Conflict of interest statement

We declare that we have no conflict of interest.

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