Insight into the secret of how indigo naturalis works: in vitro and in vivo validation of the critical role of bile

xiaorong Xu

Chengdu University of Traditional Chinese Medicine

Fei Ran

Chengdu University of Traditional Chinese Medicine

Yanan He

Chengdu University of Traditional Chinese Medicine

Huamei Gou

Chengdu University of Traditional Chinese Medicine

Wei Liao

Chengdu University of Traditional Chinese Medicine

Fang Wang

Jiangxi University of Traditional Chinese Medicine

Ming Yang

Jiangxi University of Traditional Chinese Medicine

Li Han (✉ hanliyx@163.com)

Chengdu University of Traditional Chinese Medicine

Dingkun Zhang

Chengdu University of Traditional Chinese Medicine

Research

Keywords: indigo naturalis, bile, antipyretic, bile duct ligation, in vitro and in vivo

Posted Date: July 10th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-40856/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background: Indigo Naturalis (IN) has positive therapeutic effects on cancer, leukemia, colitis, psoriasis, fever and other diseases that seriously endanger human health. It contains 10% organic matter and 90% inorganic matter with extremely hydrophobic. However, the clinical usage of IN is mainly by oral administration. And how IN exerts its effect after oral administration is still a secret remained to be discovered.

Methods: In this study, *in vitro* and *in vivo* experiments were performed. The *in vitro* experiments simulate the *in vivo* process of IN by different digestive fluid and the effects of a small amount and normal amount of bile on the organic and inorganic matter release of IN were compared. And the surface morphology and elements of IN in different solutions were also observed. In addition, 2, 4-dinitrophenol-induced fever rat model was also used to investigate the intervention effect of IN on the fever of normal rats and bile duct ligation rats.

Results: It was found that bile plays an important role in promoting the carrier dissolution and release of inorganic and organic matters from IN. And IN had better antipyretic effect on normal rats than on bile duct ligation rats, which further proved that bile is important to the curative effect of IN.

Conclusions: The results indicate that the calcium carbonate carrier translates into a slightly soluble state in the acidic simulated gastric fluid. And a small amount of organic matter was released from the calcium carbonate carrier. When IN entering the simulated intestinal fluid which mixed with bile, a large amount of carrier dissolved, and organic substances such as indirubin dissolved out in large quantities, thereby being absorbed into the blood and exerting therapeutic effect. The above findings have certain guiding significance for the clinical application of IN. For patients with insufficient bile secretion such as cholangiotomy, oral IN may have poor curative effect and even doesn't work.

Background

IN is a Traditional Chinese Medicine with a long history of application. It is widely used in internal medicine, surgery, pediatrics, gynecology, infectious and other diseases [1]. The earliest medicinal efficacy of IN is recorded in Theory of Medicine which published in the Tang Dynasty. It can be taken internally or externally and used alone or in combination with gypsum and bupleurum [2]. IN is a dry powder, blocks or granules derived from dried leaves and stems of *Baphicacanthus cusia* (Nees) Bremek., *Polygonum tinctorium* Ait., or *Isatis indigotica* Fort [3]. The traditional manufacturing method is to harvest the stems and leaves in autumn, place them in the soaking pool and introduce the river water till they are submerged completely. Then immerse and ferment for several days. When the pool liquid turn green [4] remove the residue, add lime, stir fully, stand for a 1 to 2 days to make $\text{Ca}(\text{OH})_2$ react with CO_3^{2-} in the immersion liquid to generate CaCO_3 crystal nucleus, providing a carrier for sedimentation and attachment for indigo and indirubin. Then discard the supernatant and collect the sediment at the bottom and move it to the small pool, let it stand, and then discard the supernatant to get crude indigo. The crude indigo is

purified through water fly removal and other impurities, and after drying, the finished product is obtained [5].

Due to its manufacturing process, the material composition of IN is about 10% organic and about 90% inorganic matter [6]. Its organic components are mainly indole alkaloids, such as indigo, indirubin [7], etc. The inorganic components contain more than 70% calcium carbonate and about 10% silica. IN's traditional efficacy are clearing heat and detoxifying, diarrhea, convulsions, cooling blood and removing spots. Modern pharmacological studies have shown that IN has anti-tumor, anti-bacterial, fungal and anti-inflammatory effect. It also has encouraging results in the treatment of leukemia, psoriasis, ulcerative colitis [8-10], etc.

Because hydrophobic indigo, indirubin and other organic substances are deposited on the surface of CaCO_3 , it prevents the growth of CaCO_3 crystal nuclei and forms a fine IN microstructure [11]. It makes IN exhibit the physical characteristics of light and loose body and strong hydrophobicity, difficult to enter the water. Thus, the traditional clinical usage of IN is mainly pills and powder. However, it is unclear how IN changes and exerts curative effects in *vivo* after oral administration. This is of great significance for the research of IN's clinical usage, the development of related preparations and further research.

In this paper, the in *vivo* process of IN was studied through both *vitro* and *vivo* experiments. In *vitro* experiments, dissolution apparatus was used to simulate IN's in *vivo* process through simulated gastric fluid (SGF), bile and simulated intestinal fluid (SIF) and to explore the secrets of its effectiveness by observing the changes of its morphological structure and elements. The content of inorganic and organic matters in different solutions was also measured. 2,4-dinitrophenol-induced rat fever model was used to compare the antipyretic effect of IN on normal rats and bile duct ligation rats. And the antipyretic experiment was performed to verify the inference in *vitro*.

Materials And Methods

Reagents

Indigo Naturalis (IN) was purchased by Sichuan Jiangyou Hengyuan Pharmaceutical Group Co., Ltd. It was identified by Professor Xu Run-chun, and deposited at the Chengdu University of Traditional Chinese Medicine, Chengdu, 611137, China. Indigo Naturalis contained 2.1% indigo and 0.15% indirubin by HPLC. Standards of indigo and indirubin were purchased from Chroma-Biotechnology Co., Ltd (Chengdu, China). Their purity is more than 98 %. Calconcarboxylic acid was purchased from Shanghai Maclean Biochemical Technology Co., Ltd (Shanghai, China). Medical suture needle was purchased from Shanghai Hankang Medical Devices Co., Ltd (Shanghai, China). Ethyl acetate, N-N-dimethylformamide (DMF) and glycerin were purchased from Chengdu KeLong Chemical Factory (Chengdu, China). Liquid chromatography (LC)-grade methanol and acetonitrile was purchased from Fisher Scientific (Loughborough, United Kingdom). Ultra-high purity water was prepared using a Millipore-Q system (Millipore Corporation, Billerica, MA, USA).

Preparation of simulated gastric fluid (SGF) [12] 16.4 mL of dilute hydrochloric acid was added to 800 mL of distilled water, shaken and diluted to 1000 mL.

Preparation of simulated intestinal fluid (SIF) [12] 250 mL of 0.2 mol·L⁻¹ potassium dihydrogen phosphate solution mix up with 118 mL of 0.2 mol·L⁻¹ sodium hydroxide solution, shaken and diluted to 1000 mL.

Animals

Male Sprague-Dawley rats (200± 20 g) were obtained from Chengdu Dashuo Laboratory Animal Co., Ltd. (Chengdu, China). Animals were housed under a 12-hour light/dark cycle at a temperature of 22 ± 2°C and relative humidity of 55 ± 5%. The animals had free access to food and water.

Inorganic matter dissolve from IN

Preparation of samples

25g IN was added to different solutions which water bath to 37°C. Measure 200mL of each solution and stir for 1 h, set the rotation speed as 100 r·min⁻¹. After stirring, filter, collect the filtrate, and dry the IN powder. It was divided into SGF group, SGF+SIF+SSF group, SGF+SIF+Bile 1.335 (SSBL) group and SGF+SIF+Bile 5.34 (SSBH) group.

Take 30 mL of the filtrate of each group, adjust the PH to 13, add moderate calconcarboxylic acid into the filtrate, titrate with EDTA titrant three times in parallel respectively, record the consumed EDTA volume when the solution changes from purplish red to pure blue, calculate the calcium ion concentration of each group.

Organic matter dissolve from and residue in IN

HPLC methods

The UPLC separations were performed using a Thermo Scientific™ Dionex™ UltiMate™ 3000 (Waltham, Massachusetts). The chromatographic column is Welchrom C18 column (250 × 4.6 mm, 5 × μm). The mobile phase is methanol-water with equal elution (70% methanol). The detection wavelength is 289 nm. The volume flow rate is 1 mL·min⁻¹. Column temperature was 25°C. The injection amount is 10 μL.

Standard solution preparation

Preparation of the mixed reference substance solution: indigo and indirubin reference substance were accurately weighed, produced solution containing indigo 10.046 μg·mL⁻¹, indirubin 71.509 μg·mL⁻¹.

Preparation of dissolution samples from IN

Collect the filtrates obtained in Figure 1 under item 3.1.1 respectively, accurately measure 10 mL, extract with 30 mL ethyl acetate for 30 min each time, and extract 5 times. The upper liquid was placed in an evaporation dish and evaporated in a water bath, 10 mL DMF was added to dissolve. After cooling and passing 0.22 microporous membrane, take the continuous filtrate.

Preparation of residue samples of IN

Collect and dry the IN powder obtained by filtering different solutions under item 3.1.1 in Figure 1. Accurately weigh 50 mg of dried IN powder. Ultrasonically extract with 100 mL DMF for 30 min, cool, and then filtered with 0.22 μm microporous membrane to obtain HPLC sample solution.

Preparation of standard curve

The reference solutions of indigo and indirubin have a mass concentration of $10.046 \mu\text{g}\cdot\text{mL}^{-1}$ and $71.509 \mu\text{g}\cdot\text{mL}^{-1}$, respectively. It was diluted to 7 different concentrations, and the samples were injected under the above chromatographic conditions for measurement. The standard curve was obtained by setting the injection amount (X, mg) as the abscissa and the peak area (Y) as the ordinate. The regression equation is as follows.

indigo: $y = 1135.3x + 0.3353 (R^2 = 0.9999)$, indirubin: $y = 113526x + 0.3353 (R^2 = 0.9999)$.

Surface and structural properties of residue IN

Appearance observation

IN medicinal materials and IN powder separated from SGF, SSF, SSBH were each taken a little in an ion sputtering device and the appearance and morphological characteristics of the powders were observed by JSM-7500F SEM (Japan Electronics Corporation) after gold plating.

Surface element analysis

The Mapping mode of xflash Energy spectrometer (German, Brooke) was used to conduct surface scanning on the selected areas of IN medicinal material and IN powder separated from SGF, SSF, SSBH (the effective area was 80 mm^2 , the collection count was $> 200\,000 \text{ CPS}$, and the typical resolution was $\text{MnKa}125 \text{ eV}$), to obtain the surface distribution images of N, Ca and Si elements, and to characterize nitrogen organic matter, calcium carbonate and silicon dioxide respectively.

Effect of bile on antipyretic effect of IN

Preparation and experimental grouping

The rats were subjected to adaptive measurement of anal temperature every morning and evening (at 9:00 a.m. and 17:00 p.m.) for 3d before the experiment [13]. Before each measurement, apply a small amount of glycerin on the probe of the electronic thermometer, insert the rat's rectum 3 cm, (make sure

that the depth of each insertion is consistent), record the temperature after the reading is stable. And rectal temperatures with fluctuations beyond 0.5°C between the morning and evening measurements were excluded. A total of 60 rats were randomly divided into six groups (n = 10 per group) according to weight, including normal control group, model group, IN group, bile duct ligation normal (BLN) group, bile duct ligation model group (BLM), bile duct ligation IN (BLIN) group.

Bile duct ligation

Rats of BLN group, BLM and BLIN group were anesthetized with 20% urethane (1g·kg⁻¹). The belly of the rat is shaved, and the abdomen was disinfected 3 times with 75% alcohol. The abdominal cavity was opened along the midline of the abdomen, the common bile duct was ligated, sutured, and the wound was disinfected with iodophor. Incubate until awake, and feed in a single cage for 3 days [14].

Model preparation and drug intervention

Prior to the modeling, the rats were fasted for 12 h (free access to water). The rectal temperature of all rats was measured twice prior to modeling (original temperature). Subsequently, 2,4-dinitrophenol dissolved in physiological saline (3 mg·mL⁻¹) was injected in the back of rats (10 mL·kg⁻¹) to induce fever in rats except normal group and the BLN group. Fever in rats (both body temperature increased by 0.3°C), accompanied by tremors, shortness of breath and atrophy, indicate that the model was successfully created. Immediately after the successful modeling, the IN group and the BLIN group were intragastric administrated with the IN solution (0.9 g·kg⁻¹), and others groups were gavaged distilled water according to body weight.

After drug intervention treatment, the anal temperature was measured once every 30 min for each group and recorded continuously for 5 h. And HT-19 Infrared thermal imager is used to take pictures of different groups of rats to observe the body temperature status. The change curve of anal temperature was plotted and processed by SPSS 21.0 statistical software.

Statistical analysis

All data were reported as $\bar{x}(\pm s)$. Significance of each group was analyzed with one-way analysis of variance. The values of various groups were evaluated by one-way ANOVA and difference test. $*P < 0.05$ and $**P < 0.01$, calculated using SPSS software (version 21), were considered statistically significant.

Results

Inorganic matter dissolve from IN

The concentration of calcium ion in different solutions was calculated respectively. It was discovered that the SSBH group has higher concentration than other groups. And it was bile rather than stir that play an important role in the solubility of calcium ion. It was showed in figure 2.

Organic matter dissolve from IN

According to the above chromatographic conditions, the contents of indigo and indirubin in different IN solutions were determined by HPLC. The results are showed in figure 3. It could be found that the content of indigo and indirubin in the SSBH group are much higher than SSBL group. Compared with indigo, bile has a better solubilizing effect on indirubin and the effect of bile to promote the dissolution of effective substance in IN is related to the quantity of bile.

Organic matter residue in IN

The contents of indigo and indirubin in IN that filtered and dry from different solutions were also determined by HPLC. The results show that SSBH group contains less indigo and indirubin compared to the other groups. It indicated that IN dissolves more indigo and indirubin under the influence of bile. It was also showed in figure 3.

Appearance observation

The appearance shapes of IN powder in different solutions are showed in figure 4. In the IN original powder, the calcium carbonate carrier is closely arranged, and it can be seen that a large amount of IN is attached to the carrier. In the IN powder isolated from SGF, the arrangement of the carrier is loose, the structure is generally complete, and it is slightly soluble. And from the SSF group, it can be seen that the carrier has a small amount of dissolution, which may be caused by long-term stirring. In IN powder separated from the SSBH, it can also be seen that the carrier dissolves a lot, and the IN attached to the surface of the carrier decreases. The above results reveal the changing process of IN in the digestive tract. After oral administration, IN enters acidic gastric fluid, and the calcium carbonate carrier forms a slightly soluble state. Then it moves through the stomach and enters the intestine along with the bile secreted by the gallbladder. Next, intestinal peristalsis, the surface actives agent or other physiology of bile make the carrier dissolve in a large amount and releases a lot of IN, promoting the dissolution of the chemical components in IN.

Surface element analysis

The surface element analysis diagram of four kinds of IN powder is showed in figure 5. It can be seen that the content of calcium element is: IN original powder > IN from SGF > IN from SSF > IN from SSBH. Moreover, the N element in IN from SSBH is significantly reduced compared with other groups. However, the content of Si does not change much. It confirmed the dissolution state of the carrier in the digestive tract after oral administration of IN and indicates that bile promotes the release of nitrogen compounds in IN. In summary, the slight dissolution of the carrier does not release a large amount of active ingredients, and bile rather than stirring plays an important role in the process of releasing the active ingredients and acting.

Bile on antipyretic effect of IN

It can be observed that the temperature of the model group is significantly higher than that of the other groups in Figure 6. In the fever rats, the tails are red, and the temperature of the control group does not change much, the perianal is redder, while the tail is bluish compared to other groups with lower temperature. Of course, the perianal temperature measured by the infrared instrument is far less accurate than the rectal temperature measured by the electronic thermometer, but you can visually see the fever rats in different group.

The rectal temperatures of the rats at different time points were recorded to assess the pyrexia rat model versus control rats. The temperature rise value of each group of rats at the temperature monitoring point ($\Delta T/^{\circ}\text{C}$ = measured body Temperature-basic body temperature) was calculated. The average anal temperature change curve was drawn in figure 7. The rectal temperatures of model rats were significantly increased after injection of 2, 4-dinitrophenol. However, the rectal temperatures of the IN and BLIN group were significantly suppressed on the 30 min after treating with IN at the dose of $0.9\text{g}\cdot\text{kg}^{-1}$. And the antipyretic effect of IN group was better than BLIN group.

Discussion

In this experiment, *in vivo* and *in vitro* studies have confirmed that bile is the key to the release of active ingredients by IN. In *in vitro* simulation experiments, it can be seen that the group of SSBH contains the most calcium ions which illustrates calcium carbonate carrier dissolved mostly in intestines. And the dissolution of organics in different solutions is $\text{SSBH} > \text{SSBL} > \text{SGF} > \text{SSF}$. The morphological structure and surface element analysis experiments show that the N element is reduced partly due to the addition of bile.

And the above results suggest that IN undergoes the following steps after oral administration. First, IN enters acidic SGF, and the calcium carbonate carrier translates into a slightly soluble state. Then, it moves through the stomach and enters the intestine along with the bile secreted by the gallbladder. Finally, the carrier dissolve in a large amount and releases a lot of IN because of the intestinal peristalsis, surface active agent or other physiology of bile causing the dissolution of the chemical components in IN. In addition, the bile is the key to the dissolution of indigo and indirubin in IN and the effect of bile on the release of indirubin in IN is better than indigo according to HPLC results. The release of organics explains the phenomenon that the main medicinal ingredient indirubin is low in IN, but has good oral effects [15-17]. It also indicates that the normality of bile secretion has a significant influence on the effectiveness of some drugs when used in clinically. For patients with insufficient bile secretion, oral IN may not be effective as expected or even has no effect. Because bile is secreted into the intestine, it can be inferred that the main absorption site of IN after oral administration is in the intestine.

The commonly used classic fever models are lipopolysaccharide, dry yeast and 2,4-dinitrophenol [18-20]. The lipopolysaccharide model requires intraperitoneal injection, and it is a bacterial fever model. The use of lipopolysaccharide model may cause abdominal sutures in bile duct ligation rats, such as dehiscence and low immunity. Moreover, the model of endotoxin may lead to lower survival rate of surgery rats, so it

should not be used. The dry yeast fever model is due to local skin ulceration after injection, which is more harmful to rats after surgery. However, 2,4-dinitrophenol is a strong metabolic stimulant which can stimulate aseptic inflammation in animals by injecting subcutaneously and is a non-infectious fever model. It induced fever quickly and the harm is small. Thus, 2,4- dinitrophenol rats fever model was used to induce fever. The results showed that the normal rats and the bile duct ligation rat model group had stable fever, and there was no significant difference which means the selected model is reasonable. And normal group has better antipyretic effect than bile duct ligation group at the same dose of IN. It is also proved that bile is the key to the antipyretic effect of IN. Of course, according to literature reports, bile duct ligation will affect the change of intestinal flora, such as *Bifidobacteria*, etc., which may also be one of the influencing factors of IN's antipyretic effect [21, 22].

In this experiment, a simple method was used to explore the oral absorption mechanism of IN. It was found that bile can obviously promote the release of indigo and indirubin. And the solubilizing effect on indirubin is better than indigo. The significant effect of bile on the dissolution of indirubin may be one of the mechanisms of the curative effect of indirubin. Undoubtedly, the kind of extraction solvent has a certain influence on the measured content of indigo and indirubin. However, the dissolution trend of indigo and indirubin under the influence of bile is consistent. And making clear the oral absorption site of IN has a certain reference value for the development of related preparations. But this experiment simulates the oral process of IN in *vitro*, without considering the effects of enzymes, intestinal flora and other biological environments, and the mechanism of bile enhancing the dissolution of organic matter from IN needs further study.

Conclusion

The in *vivo* and in *vitro* experiments results suggest that bile is of great significance for the release and absorption of organic matter in IN to play a therapeutic effect. Therefore, when IN is used orally in clinic, the patient's bile secretion should be considered.

Abbreviations

IN: Indigo Naturalis; SGF: simulated gastric fluid; SIF: simulated intestinal fluid ;SSF: simulated gastric fluid+simulated intestinal fluid , SSBL: simulated gastric fluid+simulated intestinal fluid + bile 1.335g. SSBH: simulated gastric fluid+simulated intestinal fluid+ bile 5.34g. BLN: bile duct ligation normal, bile duct ligation model group (BLM), bile duct ligation IN (BLIN) group.

Declarations

Acknowledgements

Not applicable.

Author Contributions

Ming Yang, Dingkun Zhang, Li Han and Xiaorong Xu conceived and designed the paper; Xiaorong Xu, Fei Ran and Huamei Gou performed the experiment. Yanan He and Fang Wang gave some advice for improving the paper; other people gave some advice for improving the picture; Xiaorong Xu wrote the paper.

Funding

This project was financially supported by the National Natural Science Foundation of China [NO. 81773918]; the Innovative research team of traditional Chinese medicine of Chengdu University of traditional Chinese medicine [CXTD2018006].

Availability of data and materials

The datasets used during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This study was conducted in strict accordance with the recommendations of the Guidelines for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of China. The protocol and experimental designs were approved by the Ethical Committee of Chengdu University of Traditional Chinese Medicine (Approval ID: 2017BL-003). All possible steps were taken to avoid the animals' suffering at any stage of the experiments. At the end of the study, the animals were sacrificed following anesthesia with excessive urethane.

Consent for publication

The manuscript is approved by all authors for publication

Competing interests

The authors declare no competing financial interests.

References

- [1] Lu JF, Yan XH. Clinical application of Qingdai. Chinese and Foreign Health Digest.2010;7:385-6.
- [2] David J. Chinese drugs of plant origin—chemistry, pharmacology and use in traditional and modern medicine: By W. Tang and G. Eisenbrand, Springer, New York, Heidelberg, 1993;32: 1081.
- [3] Zhang ZG, Yang R, Kang, LI. Study on water grind processing method of Indigo Naturals. Chin .Pat. Med.2002;24:202-4.
- [4] Keiichi Aino, Kikue. Microbial Communities Associated With Indigo Fermentation That Thrive in Anaerobic Alkaline Environments. Frontiers in microbiology.2018;9:2196.

- [5] Liu J, Wang YH, Guo DH. The Processing Technique of Traditional Indigo Dyes. *silk monthly*. 2009; 42-50.
- [6] Chen TQ, Wu JZ. Wenxiong. Jianqingdai inorganic element composition SEM/EDS, ICP. AES and IR analysis. *Nat. Prod. Res. Dev.* 2007;19: 837-40.
- [7] Wu XX. Chen XF, Dan J. Characterization of anti-leukemia components from Indigo naturalis using comprehensive two-dimensional K562/cell membrane chromatography and in silico target identification. *Scientific Reports*. 2016;6: 30
- [8] Naganuma M, Sugimoto S, Mitsuyama K. Efficacy of Indigo Naturalis in a Multicenter Randomized Controlled Trial of Patients With Ulcerative Colitis. *Gastroenterology*. 2018;154: 935-47.
- [9] Cheng YC, Wu Q. Clinical efficacy and IL-17 targeting mechanism of Indigo naturalis as a topical agent in moderate psoriasis. *BMC complementary and alternative medicine*. 2017;17:439.
- [10] Xie Q, Yu L, Wang XQ. A novel realgar-indigo naturalis formula more effectively induces apoptosis in NB4 cells. *Pakistan journal of pharmaceutical sciences*. 2019;32: 957-62.
- [11] Wei Q, Yang M, Xu RC. New conception of Qingdai's Processing Principle. *Chin. Tradit. Pat. Med.* 2004;26: 116-18.
- [12] Chinese Pharmacopoeia C, 2015. *Pharmacopoeia of the People's Republic of China*. People's Medical Publishing House, Beijing, 199.
- [13] Zhang X, Wang Y, Li SJ. The Potential Antipyretic Mechanism of Gardeniae Fructus and Its Heat-Processed Products With Plasma Metabolomics Using Rats With Yeast-Induced Fever. *Frontiers in Pharmacology*. 2019; 10: 491.
- [14] Inja Cho, Bon NK, Eun HK. Bile duct ligation of C57BL/6 mice as a model of hepatic encephalopathy. *Anesthesia and Pain Medicine*. 2020;15:19.
- [15] Li Z, Zhu C, An B. Li, Indirubin inhibits cell proliferation, migration, invasion and angiogenesis in tumor-derived endothelial cells. *OncoTargets and therapy*. 2018;11: 2937-44.
- [16] Zhao Y, Han P, Liu L. Indirubin modulates CD4(+) T-cell homeostasis via PD1/PTEN/AKT signalling pathway in immune thrombocytopenia. *Journal of cellular and molecular medicine*. 2019;23: 1885-98. 10.
- [17] Xiao Z, Hao Y, Liu B. Indirubin and meisoindigo in the treatment of chronic myelogenous leukemia in China. *Leukemia & lymphoma*. 2002; 43:1763-68.
- [18] Kalambaheti T, Cooper GN, Jackson, GD. Role of CINC-1 and CXCR2 receptors on LPS-induced fever in rats. *Pflugers Archiv : European journal of physiology*. 2019;471: 301-11.

[19] Zhang F, Wang D, Li X. Metabolomic study of the fever model induced by baker's yeast and the antipyretic effects of aspirin in rats using nuclear magnetic resonance and gas chromatography-mass spectrometry. *Journal of pharmaceutical and biomedical analysis*. 2013;81-82:168-177.

[20] Liu S, Wang N, Chen PS. Effect of huanglianjiedu tang on fever in rats induced by 2, 4-dinitrophenol. *Journal of traditional Chinese medicine*. 2013;33:80-87.

[21] Wei DD, Wang JS, Duan JA. Duan. Metabolomic Assessment of Acute Cholestatic Injuries Induced by Thioacetamide and by Bile Duct Ligation, and the Protective Effects of Huang-Lian-Jie-Du-Decoction. *Frontiers in pharmacology*.2018;9:458.

[22] Kalambaheti T, Cooper GN, Jackson GD. Role of bile in non-specific defence mechanisms of the gut. *Gut*.1994;35: 1047-51.

Figures

Figure 1

Preparation of samples. SGF*: simulated gastric fluid without pepsin; SIF#: simulated intestinal fluid without pancreatin.

Figure 2

Vs SGF group, * $P < 0.05$; Vs SSF group, ** $P < 0.01$; Vs SSBL group, ** $P < 0.01$. Data are expressed as $\bar{x}(\pm s)$ (n = 3).

Figure 3

The content of indigo and indirubin. (A: HPLC chart of IN dissolution, B: HPLC chart of IN residue, C: content of indigo dissolve from IN, D:content of indigo residue in IN, E:content of indirubin dissolve from IN, F:content of indirubin residue in IN.)

Figure 4

Scanning electron micrograph of IN isolated from different solutions. A: original IN, B: SGF, C: SSF, D, SSBH

Figure 5

Photographs of surface elements of IN powder isolated from different solutions. A: original IN, B: SGF, C: SSF, D, SSBH

Figure 6

Infrared images of rats. A: normal rats, B: bile duct ligation rats.

Figure 7

The average anal temperature change curve in rats ($n = 10$, $\bar{x}(_) \pm s$).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [GraphicalAbstract.jpg](#)