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1 **Gastroprotective potential against indomethacin and safety assessment of the homology of**
2 **medicine and food formula cuttlebone complex**

3

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1 **ABSTRACT**

2 Cuttlebone complex (CBC), the homology of medicine and food formula, comprised of five
3 herbal medicines (*Endoconcha Sepiae*, *Radix Paeoniae Rubra*, Fresh Ginger, *Fructus Amomi*, and
4 *Radix Glycyrrhizae*) and two food ingredients (*Zingiber zerumbet* and chitosan). Herein, the
5 gastroprotective potential against indomethacin and safety assessment of CBC were investigated. In
6 gastroprotective model, CBC effectively decreased indomethacin-increased gastric ulcerous lesions
7 and increased indomethacin-reduced prostaglandin E2 levels in gastric mucosa. In genotoxicity,
8 CBC treatment did not increase revertant colonies in five *Salmonella typhimurium* strains and
9 chromosome aberrations in Chinese hamster ovary CHO-K1 cells with or without S9 metabolic
10 activation. The oral supplementation of CBC did not increase micronucleus formation in peripheral
11 blood of mice. In subacute toxicity study, body weight and blood biochemical parameters were
12 normal observed in CBC-treated rats. In conclusion, CBC was considered as a non-toxic formula
13 and could be used to remedy indomethacin-induced gastric damage.

14

15 **Keywords:** Gastroprotective potential; Genotoxicity; Homology of medicine and food; Cuttlebone
16 complex; Subacute toxicity

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1 **Introduction**

2 Homology of medicine and food (HMF) is referred as combination the function of food and
3 medicine scientifically with nutritional value, healthcare activities, and prevention and treatment of
4 diseases.[1] Cuttlebone complex (CBC), the HMF formula, comprised of five herbal medicines,
5 including *Endoconcha Sepiae*, *Radix Paeoniae Rubra*, fresh ginger root, *Fructus Amomi* and *Radix*
6 *Glycyrrhizae*, and two food ingredients, including chitosan and *Zingiber zerumber*. *Endoconcha*
7 *Sepiae*, also called cuttlebone and Hai Piao Xiao in Chinese, is the internal structure from *Sepiella*
8 *maindroni* de Rochebrune or *Sepia esculenta* Hoyle. *Radix Paeoniae Rubra*, also called white
9 peony and Bai Shao Yao in Chinese, is the dried root of *Paeonia lactiflora* Pall. Fresh ginger root,
10 also called Sheng Jiang in Chinese, is the fresh rhizome of *Zingiber officinale* Rosc. *Fructus Amomi*
11 also called Sha Ren in Chinese, is the dried ripe fruits of *Amomum villosum* Lour., A. *longiligulare*
12 T. L.Wu and *A. xanthioides* Wall. *Radix Glycyrrhizae*, also called Gan Cao in Chinese, is the roots
13 and rhizomes of *Glycyrrhiza uralensis* Fisch., *G. inflata* Bat., and *G.glabra* L. Chitosan is the
14 shrimp shell of *Pandalus borealis* through deacetylation. *Zingiber zerumbet*, also called pinecone
15 ginger, is the fresh root of *Zingiber zerumbet (L.)* Smith.

16 Gastric ulcer is one of the most common diagnosed gastrointestinal disorders in clinical
17 practice.[2] Many factors could be responsible for gastric ulcer, including inadequate dietary habits,
18 cigarette smoking, stress, infection by *Helicobacter pylori*, and excessive consumption of
19 non-steroidal anti-inflammatory drugs (NSAIDs).[3] Indomethacin, one of the NSAIDs, is commonly
20 used as a prescription medication to cure fever, pain, stiffness and swelling and was shown to have
21 the abilities to induce gastric ulcer in rats.[4] There are plenty of studies have indicated that several
22 possible mechanisms are involved in indomethacin-induced gastric ulcer, including production of
23 reactive oxygen species-mediated mitochondrial damage, induction of apoptosis in gastric mucosa,
24 and inhibition of prostaglandin synthesis.[5,6] The use of natural plant extracts and herbal medicines
25 have been regarded as an alternative therapy for the treatment of gastric ulcer.[7,8] Several studies
26 have indicated that the components in CBC possessed gastroprotective potential. For example,



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1 cuttlebone, Sheng Jiang, and chitosan were shown to exhibit anti-gastric ulcer activity.[9-11] Sheng
2 Jiang and Gan Cao were shown to exhibit anti-Helicobacter pylori activity.[12,13] White peony and
3 Sha Ren were shown to promote gastrointestinal movement.[14,15] Pinecone ginger was shown to
4 improve stomachache and fever.[16] A main therapeutic trait of HMF is that crude extractions used in
5 combination exhibited synergistic or additive effects in disease prevention and treatment. However,
6 few scientific studies have supported the claim in terms of modern pharmacology. Herein, we
7 explored the effects of CBC against indomethacin-induced gastric damages in rats.
8 Analysis of the genotoxicity of the HMF formulas is important because the materials with
9 genotoxicity may cause gene mutations and increase the risk of some chronic diseases including
10 cancer.[17] Based on the Enforcement Rules of the Health Food Control Act established by the
11 Ministry of Health and Welfare, health food products in Taiwan should be evaluated for their
12 genotoxicity and pharmacological effects. In the present study, we further investigated the
13 genotoxicity of CBC using bacterial reverse mutation in *Salmonella typhimurium strains*,
14 chromosome aberrations in Chinese hamster ovary CHO-K1cells, and micronucleus formation in
15 mice and subacute toxicity of CBC using 28 days repeated feeding study in male and female rats as
16 recommended by Taiwan Food and Drug Administration (TFDA). The results obtained from this
17 study will provide the safety information of CBC before its commercialization as a health food.
18
19 **Materials and methods**
20 **Preparation of CBC and analysis of calcium content in CBC**
21 The formulation of CBC was developed by Ko Da Pharmaceutical Co. Ltd., Taoyuan, Taiwan.
22 The herbal samples such as Gan Cao, Sha Ren, Bai Shao Yao and Sheng Jiang were identified in the
23 R&D center of our company, where voucher specimens have been kept. The raw materials,
24 including Gan Cao, Sha Ren and Sheng Jiang, were extracted in boiled water for 1 h. The filtrates
25 were collected and the residues were further extracted in boil water for 1 h. All collected filtrates
26 were subjected to vacuum and reduced-pressure concentration to obtain extracts. The fresh



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1 Pinecone Ginger and Bai Shao Yao were sliced and allowed to dry in 60°C oven. The fresh
2 cuttlebone was blanched in boil water containing 4% acetic acid for 15 min followed by dried in
3 60°C oven. After dried process, the Pinecone Ginger, Bai Shao Yao and cuttlebone were grinded
4 using pulverizer to obtain excipients. The extracts, excipients and chitosan were mixed and stirred
5 followed by dried in 60°C oven. The mixtures were grinded using pulverizer, and then passed
6 through 60-sieve mesh followed by granulation using 75% ethanol. The granulated mixtures were
7 passed through 30-sieve mesh to obtain CBC. The proportion of cuttlebone, white peony, pinecone
8 ginger, chitosan, Sheng Jiang, Sha Ren, and Gan Cao in CBC were 66.66% (w/w), 9.33% (w/w),
9 5.0% (w/w), 5.0% (w/w), 4.67% (w/w), 4.67% (w/w), and 4.67% (w/w), respectively.
10 The calcium content in CBC was measured using inductively coupled plasma-optical emission
11 spectrophotometer (ICP-OES) (PerkinElmer Inc., Massachusetts, USA). Briefly, 0.2 g of CBC was
12 mixed with 6 mL of nitric acid (70%, v/v) and 1.5 mL of $H_2O_2$ (30%, v/v) for 20 min at room
13 temperature. The mixtures were digested on the MARSXpress Microwave Digestion System (CEM
14 World Headquarters, North Carolina, USA) at 205°C with 800W for 20 min. The levels of calcium
15 in the digested samples were determined by ICP-OES analysis. The ICP conditions were set as
16 follows: the power was 1400W; plasma flow was 15 L/min, auxillary flow was kept at 0.2 L/min,
17 nebulizer flow was 0.7 L/min, pump rate was kept at 1.5 ml/min, calcium was monitored at
18 wavelength of 317.933 nm and plasma view was in radial mode. The calcium content in CBC was
19 calculated by comparing the calcium standard and represented as mg $g^{-1}$ or %.
20
21 **Indomethacin-induced gastric ulceration in rats**
22 Male Sprague-Dawley (SD) rats of 6-wk-old were purchased from BioLASCO Co., Ltd (Yilan,
23 Taiwan). This study protocol was approved by the Institutional Animal Care and Use Committee of
24 Chung Shan Medical University (approval No: CSMU-1178). Rats were housed in cages with
25 controlled temperature (25 ± 2°C) and humidity (65 ± 5%) with 12 h-light/dark cycles. During
26 accommodation for 1 wk and experimental periods, rats were supplied with a standard rodent diet



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1 (Lab 5001, Purina Mills) and water ad libitum. Rats were randomly divided into five groups (n = 8
2 for each group) as follows: group 1, control (oral supplementation with double distilled water);
3 group 2, indomethacin (35 mg $kg^{-1}$); group 3, indomethacin + low dose of CBC (310 mg $kg^{-1}$);
4 group 4, indomethacin + medium dose of CBC (620 mg $kg^{-1}$); group 5, indomethacin + high dose of
5 CBC (930 mg $kg^{-1}$). Rats were orally received once with indomethacin for 1 h, and then orally
6 treated once with CBC for an additional 4 h. After scarification, the stomach were removed
7 carefully and opened along the greater curvature, and then washed with ice-cold saline solution. The
8 gastric lesion in rats was observed in thread-like pattern and the length of ulcerous lesion was
9 measured immediately using caliper at the end of experiments. The gastric mucosal damages were
10 expressed as ulcer index in which the calculation of ulcer index using a 0-3 scoring system as the
11 severity of each damage.[18] The severity was defined based on the length of the damage as follows: 0
12 = no ulcerous lesion; 1 = ulcerous lesion < 1 mm length; 2 = ulcerous lesion 2-4 mm length and 3 =
13 ulcerous lesion > 4 mm. The ulcer index was calculated as the sum of the total scores divided by the
14 number of animals. The prostaglandin $E_2$ ($PGE_2$) content in gastric mucosa was determined by
15 ELISA. Briefly, the stomach was homogenized in 50 mM potassium phosphate buffer, and then
16 centrifuged (18000 × g) at 4°C for 10 min to obtain supernatant. The supernatant was analyzed
17 using commercial $PGE_2$ ELISA kit (R&D Systems, Inc., USA) according to supplier's instructions.
18 The $PGE_2$ content was expressed as ng $mg^{-1}$ protein. For histopathological analysis, gastric tissues
19 were fixed in 10% formalin and embedded in paraffin followed by sectioned into 2 μm using
20 microtome. The paraffin-embedded tissues were dried at 60ºC for 1 h and deparaffinized using a dip
21 of xylene and gradient concentration of ethanol. The microslide was stained with hematoxylin and
22 eosin stain, and observed under microscope (BX51, Olympus, Tokyo, Japan).
23

24 **Preparation of rat S9 fraction**

25 The rat liver S9 microsomal was prepared based on the published method.[19] Male SD rats
26 (6-wk-old; 200 g) were purchased from BioLASCO Co., Ltd (Yilan, Taiwan) and this study



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1 protocol was approved by the Institutional Animal Care and Use Committee of National Taiwan
2 University (approval No: NTU- 20130324). Rats were treated with enzyme-inducing agents,
3 including β-naphthoflavone (80 mg $kg^{-1}$) and phenobarbital (80 mg $kg^{-1}$), for consecutive three days
4 via intraperitoneal injection. Rat livers were removed under a sterile condition, rinsed with ice-cold
5 1.15% of KCl (pH 7.4) buffer and homogenized in ice-cold KCl buffer. The homogenate was
6 subjected to a centrifugation of 9,000 × g for 20 min to obtain S9 microsomal fraction. The
7 composition in the 1 mL of S9 mix are 8 mM $MgCl_2$, 33 mM KCl, 5 mM glucose 6-phosphate, 4
8 mM NADP, 100 mM sodium phosphate (pH 7.4) and 0.1 mL of microsomal S9 fraction. The S9
9 microsomal pellets were suspended in $KH_2PO_4$ buffer (pH = 7.4) and stored at -80°C until used.
10
11 **Ames test**
12 The *Salmonella typhimurium* strains, including TA98 (△uvrB/rfa/ pKM101), TA100
13 (△uvrB/rfa/ pKM101), TA102 (rfa/ pKM101), TA1535 (△uvrB/rfa) and TA1537
14 (△uvrB/pkM101), were purchased from the Food Industry Research and Development Institute
15 (Hsinchu, Taiwan). The Ames test was used to determine the mutagenicity of CBC using plate
16 incorporation method.[20] Briefly, 0.1 mL of CBC solution with different concentrations, 0.1 mL of
17 fresh bacterial broth, and 0.5 mL of sterile buffer (for the assay without S9 fractions) or 0.5 mL of
18 S9 fractions (for the assay with S9 fractions) were mixed with 2.0 mL of overlay agar. The mixtures
19 were poured over the minimal glucose plate and allowed to solidify. The plate was incubated for 48
20 h at 37°C and the number of revertant colonies per plate was counted. As positive controls without
21 S9 fractions, 0.5 µg per plate of 4-nitro-o-phenylenediamine for TA98, 4 µg per plate of sodium
22 azide for TA100 and TA1535, 0.5 µg per plate of mitomycin C for TA102, and 5 µg per plate of
23 9-aminoacridine for TA1537 were used. As positive controls with S9 fractions, 1 µg per plate of
24 benzo[a]pyrene for TA98, TA102 and TA1535, and 4 µg per plate of 2-aminoanthracene for TA100
25 and TA1537 were used. Based on the rule reported previously,[21] the value of the positive control



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1 group should be significantly higher than that of the control group. To confirm the validity, the
2 values in control group for TA98, TA100, TA102, TA1535 and TA1537 should be 30-60, 70-240,
3 240-320, 15-35 and 2-15 CFU, respectively. Mutagenicity was judged to be positive when the
4 revertants in CBC treatment increased more than 2-fold, as compared to that in the control. We also
5 calculated the mutagenic index to reflect the mutagenicity of CBC. The mutagenic index was
6 calculated as the average number of revertants per plate with the test group divided by the average
7 number of revertants per plate with the negative control.[22] All the experiments were performed in
8 three independent assays.
9
10 **Chromosomal aberration assay in CHO-K1 cells**
11 The Chinese hamster ovary fibroblast cell line (CHO-K1, BCRC Number 60006) was
12 purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells
13 were growth in McCoy's 5A medium containing 10% (v/v) fetal bovine serum (FBS), 0.22% (w/v)
14 sodium bicarbonate, 2 mM of glutamine, 100 μg $mL^{-1}$ of streptomycin, and 100 units $mL^{-1}$ of
15 penicillin-G (GIBCO/BRL, Rockville, MD, USA) in a humidified incubator under 5% $CO_2$ and
16 95% air at 37°C. The S9 fractions isolated from the rat livers was prepared as described above. For
17 determination of the chromosomal aberration,[23] cells were treated with CBC for 3 h containing S9
18 fractions and for 3 h or 20 h containing no S9 fractions. Cells were harvested at 20 h of incubation
19 for metaphase observation, and then exposed to Colcemid (0.1 μg $mL^{-1}$). 10 μg $mL^{-1}$ of
20 cyclophophamide monohydrate for S9 fractions and 0.07 μg $mL^{-1}$ of mitomycin C for no S9
21 fractions were used as positive controls. Cells were then incubated with 75 mM KCl hypotonic
22 solution for 20 min, fixed in the mixture of acetic acid and methanol (1:3, v/v), and stained with 5%
23 Giemsa for 5 min on the glass slide. A total of 100 metaphases of chromosomal aberrations were
24 analyzed and summed up by morphological changes, including chromosome gap, chromosome
25 break, dicentric, ring, chromatid gap, chromatid break, acentrin fragment, and multiple aberrations.
26



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1 **Micronucleus assay in peripheral blood of mice**

2 Male ICR mice of 8-wk-old were purchased from BioLASCO Co., Ltd (Yilan, Taiwan). This
3 study protocol was approved by the Institutional Animal Care and Use Committee of National
4 Taiwan University (approval No: NTU- 20130324). Mice were housed in cages with controlled
5 temperature (25 ± 2°C) and humidity (65 ± 5%) with 12 h-light/dark cycles. After accommodation
6 for 1 wk, mice were randomly divided into five groups (n = 10 for each group) as follows: group 1,
7 control (oral supplementation with double distilled water); group 2, low dose of CBC (oral
8 supplementation with 3 g $kg^{-1}$); group 3, medium dose of CBC (oral supplementation with 6 g $kg^{-1}$);
9 group 4, high dose of CBC (oral supplementation with 10 g $kg^{-1}$); group 5, positive control
10 (intraperitoneal injection with 2 mg $kg^{-1}$ of mitomycin C). After 24 and 48 h of administration, the
11 peripheral blood samples were collected from the retro-orbital plexus in a 5-mL vacutainer tube
12 containing $K_3EDTA$. For slide preparation, 1 mg of acridine orange (AO)(Sigma Chemical Co., St.
13 Louis, MO) dissolved in 1 mL of double distilled water, and then the AO solution was spread out
14 evenly on the pre-heated (70°C) slide. Blood samples (5 µL) dropped onto AO-slides, stained and
15 fixed at 4°C. Slides were examined under a fluorescent microscope. A total of 1000 blood cells
16 were counted and analyzed the number of AO-stained micronucleus.

17

18 **Subacute 28 days repeated feeding toxicity assay in rats**

19 Male and female Wistar rats of 6 to 8-wk-old were purchased from BioLASCO Co., Ltd (Yilan,
20 Taiwan). This study protocol was approved by the Institutional Animal Care and Use Committee of
21 National Taiwan University (approval No: NTU- 20130324). Rats were housed in cages with
22 controlled temperature (25 ± 2°C) and humidity (65 ± 5%) with 12 h-light/dark cycles. After
23 accommodation for 1 wk, rats were randomly divided into eight groups (n = 10 for each group) as
24 follows: group 1, male control (oral supplementation with double distilled water); group 2, male
25 low dose of CBC (oral supplementation with 3 g $kg^{-1}$); group 3, male medium dose of CBC (oral
26 supplementation with 6 g $kg^{-1}$); group 4, male high dose of CBC (oral supplementation with 10 g

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1 kg$^{-1}$); group 5, female control (oral supplementation with double distilled water); group 6, female
2 low dose of CBC (oral supplementation with 3 g kg$^{-1}$); group 7, female medium dose of CBC (oral
3 supplementation with 6 g kg$^{-1}$); group 8, female high dose of CBC (oral supplementation with 10 g
4 kg$^{-1}$). Rats were treated with CBC daily for 28 consecutive days. During the accommodation and
5 experimental periods, rats were supplied a standard rodent diet (Lab 5001, Purina Mills) and water
6 ad libitum. The body weights of rats were measured weekly. At the end of experiment, rats were
7 sacrificed with $CO_2$ asphyxiation and blood samples were collected with cardiac puncture. All
8 organs, including heart, liver, spleen, lung, kidney, adrenal, testes, epididymis and ovary, were
9 isolated and weighed. Biochemical analysis was performed on rats serum for the examination of
10 parameters, including high density lipoprotein (HDL), low density lipoprotein (LDL), glutamic
11 oxaloacetic transaminease (GOT), glutamine pyruvic transaminase (GPT), blood urea nitrogen
12 (BUN), creatinine (CRE), total cholesterol (T-Cho), triglyceride (TG), globulin, albumin, total
13 protein, glucose, $Na^+$, $Cl^-$, $Ca^{2+}$, $Mg^{2+}$, and phosphorus. All analyses were carried out using Express
14 Plus Automatic Clinical Chemistry Analyzer (Beijing, China). For hematological analysis, the blood
15 samples collected in $K_3EDTA$ to obtain plasma. The parameters, including white blood cells (WBC),
16 red blood cells (RBC), hemoglobin (Hb), hematocrit (HCT), mean corpuscular volume (MCV),
17 mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC),
18 platelets (PLT), and lymphocytes, were conducted by System-450 Automated Hematology Analyzer
19 (Tokyo, Japan).
20
21 **Statistics analysis**
22 Values are expressed as mean ± SD and analyzed using one way ANOVA followed by
23 Fisher's protected least significant difference test for comparisons of group mean, when the F value
24 was significant ($P < 0.05$). Chi-square test was used to analyze the statistical differences of ulcer
25 incidences in the stomach between the indomethacin-treated rats and those treated with
26 indomethacin and supplemented with CBC. All statistical analyses were performed using SPSS for



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1 Windows, version 10 (SPSS, Inc.); a $P$ value < 0.05 is considered statistically significant.

2

3 **Results**

4 **The content of calcium and calcium carbonate in CBC**

5 The content of calcium in CBC accounted for 236 ± 12 mg/g (23.6 ± 1.2%). Calcium

6 carbonate levels were calculated as calcium content multiplied by 2.5 (the ratio of molecular weight

7 in calcium carbonate with 100 and calcium with 40). By calculation, the content of calcium

8 carbonate in CBC was 590 ± 31 mg $g^{-1}$ or 59.0 ± 3.1%.

9

10 **Gastroprotective effects of CBC against indomethacin-induced gastric damages in rats**

11 Rats were orally pre-treated once with indomethacin for 1 h, and then orally received once

12 with CBC for 4 h to determine the therapeutic effects of CBC against gastric ulceration induced by

13 indomethacin. All of the 8 mice in the indomethacin-treated group developed gastric ulcer, whereas

14 6 and 3 of the 8 mice in the medium- and high-CBC group developed gastric ulcer, respectively

15 (Table 1). Indomethacin treatment significantly increased ulcer index (Table 1), ulcerous lesions

16 (Fig. 1, upper panel), and erosion and blood congestion (Fig. 1, lower panel), but decreased $PGE_2$

17 content (Table 1). CBC treatment effectively ameliorated indomethacin-induced ulcerous lesions

18 (Fig. 1, upper panel), erosion and blood congestion (Fig. 1, lower panel) and ulcer index (Table 1)

19 in a dose-dependent manner. In addition, CBC treatment significantly and dose-dependently

20 increased indomethacin-reduced $PGE_2$ content in gastric mucosa (Table 1), and that this effect of

21 high dose of CBC group returned to the control levels (Table 1).

22

23 **Ames test**

24 The mutagenicity of CBC was examined using five *Salmonella typhimurium* strains of TA98,

25 TA100, TA102, TA1535 and TA1537 with or without S9 metabolic activation. Results revealed that

26 CBC (0.3-5 mg per plate) did not increase the number of revertant colonies and mutagenic index in



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1 any bacterial strains with or without S9 metabolic activation, as compared to control group (Table
2 2). The positive controls significantly increased the number of revertant colonies and mutagenic
3 index in comparison with control group ($P < 0.01$)(Table 2).
4
5 **Chromosomal aberration *assay***
6 CBC (0.3-5.0 mg $mL^{-1}$) treatment did not induce cytotoxicity in CHO-K1 cells at 24 h of
7 incubation (data not shown), indicating that CHO-K1 cells has abilities to produce a sufficient
8 number of metaphases for the chromosomal aberration assay. The total aberrant cells in 100
9 metaphases with chromosomal aberrations were summed up by morphological changes, including
10 chromosome gap, chromosome break, dicentric, ring, chromatid gap, chromatid break, acentrin
11 fragment, and multiple aberrations. Results revealed that CBC treatment did not significantly
12 increase the total aberrant cells in S9 metabolic activation for 3 h and in no S9 metabolic activation
13 for 3 h or 20 h, as compared to control group (Fig. 2). The positive controls, including
14 cyclophophamide monohydrate for S9 metabolic activation and mitomycin C for no S9 metabolic
15 activation, significantly increased the number of total aberrant cells in comparison with control
16 group ($P < 0.01$) (Fig. 2).
17
18 **Micronucleus assay**
19 During experimental periods, no abnormal changes were observed in the general appearance of
20 mice in CBC and mitomycin C treatment. A total of 1000 blood cells were counted in peripheral
21 blood of mice, and we found that CBC treatment did not significantly increase the number of
22 micronucleus, as compared to control group (Fig. 3). In contrast, mitomycin C treatment
23 significantly increased the number of micronucleus at 24 h (4.4 ± 1.6) and 48 h (8.8 ± 1.9) after
24 supplementation ($P < 0.01$) (Fig. 3), indicating that this study was considered as an acceptable
25 experimental condition.
26



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1 **Subacute 28 days repeated toxicity assessment**

2 No toxicity signs were observed throughout the study following orally supplementation of
3 CBC (3, 6 and 10 g $kg^{-1}$) for 28 consecutive days. CBC treatment did not cause any significantly
4 changes in body weights and relative organ weights at the end of experiment (Table 3). All
5 hematological values did not show any statistics differences among control and CBC-treated groups
6 (Table 4). For blood biochemical parameters, high dose of CBC treatment significantly increased
7 LDL levels (Table 4) and this value was still within the normal ranges. However, in other
8 parameters, CBC treatment did not exhibit significant differences in comparison with control group
9 (Table 4).

10

11 **Discussion**

12 The main question addressed by this study was to examine the effect of CBC on
13 indomethacin-induced gastric damages in rats. The ulcerogenic activity of indomethacin is
14 associated with inhibition of prostaglandins synthesis in the stomach tissues.[5,24] In accordance with
15 this concept, we herein found that indomethacin treatment significantly increased ulcerous lesions,
16 erosion and blood congestion, and ulcer index, but decreased $PGE_2$ content in stomach mucosa of
17 rats. Calcium carbonate is the main chemical composition in cuttlebone and accounted for 59.0% in
18 CBC. Calcium carbonate has been shown to prevent the gastric lesions induced by acidified aspirin
19 through acid-neutralization.[10] Herein, our findings indicated that pre- and post-supplementation
20 with CBC significantly increased indomethacin-reduced $PGE_2$ content leading to inhibition of
21 indomethacin-induced gastric ulcer in rats. The main gastroprotective potential may be attributed to
22 the high content of calcium carbonate in CBC. Synergistic actions are of vital importance in herbal
23 medicines for prevention and treatment of chronic diseases.[25] The possible explanations for the
24 synergistic actions are that different herbal medicines may mediate either the same or different
25 target in a synergistic way as well as may decrease the adverse effects or increase pharmacological
26 activity by herbal-herbal interaction.[26] Although more study is needed to clarify the role of calcium



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1 carbonate and synergistic/additive effects of CBC in gastroprotective potential, these findings
2 indicate a potential clinical application for CBC.
3 Another question addressed by this study was to determine the genotoxicity and subacute
4 toxicity of CBC intended for human consumption. Therefore, the systematic toxicological
5 researches using different experimental methods must be performed to predict the toxicity and to
6 build the criteria for selecting a non-toxic dose of herbal medicine for humans. *In vitro* toxicological
7 studies, including Ames test and chromosomal aberrations analysis, have accepted as safety
8 assessment for food, cosmetics, and clinical medication.[27] Ames test (*Salmonella*/microsome
9 reversion assay or mutagenicity test), a rapid bacterial reverse mutation assay, were designed to
10 detect any substances, including herbal medicines, food ingredients and environmental pollutants,
11 that can produce genetic damage leading to gene mutations such as frame-shift mutations and base
12 pair substitution.[28] CBC treatment up to 5 mg/plate did not significantly increase the number of
13 revertant colonies and mutagenic index with or without S9 metabolic activation in five *Salmonella*
14 strains. In addition, significant increases in revertant number of colonies and mutagenic index were
15 observed in positive controls, indicating that this assay was valid.
16 The chromosomal aberrations analysis plays an important role in the determination of health
17 foods prior to selling. Chromosomal aberrations, one of the critical predictors in substances with
18 genotoxicity and mutagenicity, are the classical genotoxic response in tumor initiation and
19 development processes.[29] Mutagenicity can be measured by examining chromosomal or chromatid
20 structural changes, including gap, break, dicentric, ring, acentrin fragment, and multiple aberrations,
21 in cultured mammalian cells.[29] The Chinese hamster ovary fibroblast CHO-K1 cell line with
22 characteristics of mutagen sensitive and low chromosome number is commonly used to determine
23 chromosomal aberrations.[30] Herein, we found that there were no statistical differences in the
24 number of metaphases with structural aberrations between control and CBC-treated groups at any
25 concentrations with or without S9 metabolic activation in CHO-K1 cells. In addition, the positive

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1 controls treatment significantly increased the number of total aberrant cells in the presence or
2 absence of S9 fractions, indicating that this assay was valid.
3 Analysis of micronucleus in peripheral blood is commonly applied to evaluate the effects of
4 mutagens on chromosomal damage in humans and animals and has been used to identify the dietary
5 factors have a significance impact on genotoxicity *in vitro* and *in vivo*.[31,32] Based on the OECD
6 TG474 mammalian erythrocyte micronucleus test guideline,[33] mice or rat is appropriate mammalian
7 species in micronucleus assay. Previous study has showed that micronucleus formation appeared in
8 a similar incidence in mouse and rat, there are no major inter-specific differences in the total
9 percentages of nuclear anomalies.[34] Our findings revealed no significance in the number of
10 micronucleus from peripheral blood at any doses of CBC (3, 6 and 10 g $kg^{-1}$) in comparison with
11 control mice. These results demonstrated that CBC has no mutagenicity in Ames test, chromosomal
12 aberration assay and micronucleus assay.
13 The 28 days repeated feeding study has been used to determine the subacute oral toxicity that
14 provided safety information of health foods prior to the commercialization.[35] No toxicological signs,
15 including body weights changes and relative organ weights, were found during entire experimental
16 period. Hematological system has been considered to use as a critical marker to monitor the
17 physiological changes in humans and animals because it is sensitive to toxic substances.[36] Our
18 findings revealed that rats were treated with CBC for 28 consecutive days resulted in no significant
19 changes in hematological values. Similarly, no statistics significances were observed in all blood
20 biochemical parameters between control and CBC-treated rats, indicating that CBC did not cause
21 abnormalities in liver and renal functions, blood lipids, nutritional status, and electrolyte balance in
22 rats. These results indicated that CBC has no subacute toxicity in male and female rats and the no
23 observed adverse effect level (NOAEL) is 10 g $kg^{-1}$ in rats.
24 It should be noted that just a batch-specific formulation of CBC was used to investigate
25 gastroprotective potential and safety assessment in this study. As registered/certificated as a health
26 food in Taiwan, the specification was established based on the proportions of each component in



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1 CBC from three independent batches of formulation. The main variation of CBC formula was
2 attributed to the yield of dried-extracts, including Gan Cao, Sha Ren and Sheng Jiang. The
3 acceptability criterion for each dried-extract weight was ±10% during manufacture. Therefore, the
4 specification of cuttlebone, white peony, pinecone ginger, chitosan, Sheng Jiang, Sha Ren, and Gan
5 Cao in CBC was 65.61-67.76% (w/w), 9.2-9.5% (w/w), 4.90-5.07% (w/w), 4.90-5.07% (w/w),
6 4.20-5.13% (w/w), 4.20-5.13% (w/w), and 4.20-5.13% (w/w), respectively. In addition, the calcium
7 content in CBC was obtained from three independent batches of formulation.

8 We stipulated that the recommended dietary allowance (RDA) of CBC in humans is 6 g per
9 day. In gastroprotective model, a formula is available for converting human equivalent dose (HED)
10 to animal dose in mg/kg, i.e., multiply the human dose in mg $kg^{-1}$ per day by 6.2.[37] The medium
11 dose (620 mg $kg^{-1}$) of CBC in rats was obtained by the equation: 100 mg $kg^{-1}$ (6 g per 60 kg person)
12 × 6.2. The low (310 mg $kg^{-1}$) and high dose (930 mg $kg^{-1}$) of CBC were selected based on two-fold
13 and a half fold difference from the medium dose, respectively. In micronucleus assay and subacute
14 toxicity assessment, the doses of CBC were 30, 60, and 100-fold relative to RDA of product (6 g
15 per 60 kg person translated into 100 mg $kg^{-1}$) according to the health food safety assessment
16 guideline as recommended by TFDA.

17 Several added values are involved in this study. One is that CBC could be used as the
18 substitute of stomach drugs and as dietary supplement without toxicological effects for relieving
19 gastric damage induced by indomethacin. According to the statistical data from TFDA, more than
20 22 billion of stomach drugs were taken during 2013. Another is the marine waste reuse and
21 increased income for fisherman. Cuttlebone, the major component in CBC, is the internal structure
22 of cuttlefish and usually regarded as marine waste and thrown it away.

23 In conclusion, we demonstrated that CBC exhibited gastroprotective potential against
24 indomethacin-induced gastric ulcer in rats, and the in-depth studies for identifying the molecular
25 mechanism underlying such actions is needed in the future. We also demonstrated that CBC had no
26 genotoxicity in assays of Ames test, chromosomal aberration assay, and micronucleus assay as well



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1 as no subacute toxicity in 28 days repeated feeding study. Therefore, CBC could be considered as
2 safe and non-toxic HMF formula. These results provided useful information on CBC for
3 development of complementary and alternative medicine.
4
5 **Funding sources**
6 This research was supported by E10200100-527 from the Conventional Industry Technology
7 Development (CITD) of Industrial Development Bureau, Ministry of Economic Affairs, Taiwan
8 (R.O.C.).
9
10 **Conflict of interest statement**
11 We declare no conflict of interest involved in this study.
12
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1 **Table 1. Effects of cuttlebone complex (CBC) on indomethacin-induced gastric ulcer index,**
2 **ulcerous incidence and gastric $PGE_2$ content in rats**[1]

3

| | Incidence (%) | Ulcer index[2] | $PGE_2$ content (ng $mg^{-1}$ of protein) |
|---|---|---|---|
| Control | 0/8 (0) | $0.0 \pm 0.0^a$ | $0.29 \pm 0.03^a$ |
| Indomethacin 35 mg $kg^{-1}$ | 8/8 (100) | $6.52 \pm 0.23^b$ | $0.06 \pm 0.02^b$ |
| Indomethacin + CBC 310 mg $kg^{-1}$ | 8/8 (100) | $3.66 \pm 0.37^c$ | $0.12 \pm 0.02^c$ |
| Indomethacin + CBC 620 mg $kg^{-1}$ | 6/8 (75) | $1.71 \pm 0.45^d$ | $0.26 \pm 0.01^d$ |
| Indomethacin + CBC 930 mg $kg^{-1}$ | 3/8 (37.5)* | $0.45 \pm 0.12^a$ | $0.28 \pm 0.01^a$ |

4

5 [1] Rats were orally treated once with indomethacin (35 mg $kg^{-1}$) for 1 h, and then orally treated once
6 with different doses of CBC (310, 620 and 930 mg $kg^{-1}$) for an additional 4 h. Values in ulcer index
7 and $PGE_2$ content are expressed as mean ± SD, n = 8; mean in each column not sharing a letter
8 differ statistically, $P < 0.05$.

9 [2] The ulcer index was calculated as the sum of the total scores divided by the number of animals.

10 * $P < 0.05$ compared with indomethacin-treated group using Chi-square test.

11

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**Table 2. Effects of cuttlebone complex (CBC) on mutagenic activity in five *Salmonella typhimurium* strains without and with S9 metabolic activation.**

| | Number of revertant colonies per plate expressed as mean ± SD and (mutagenic index[#]): without S9 metabolic activation | | | | |
|---|---|---|---|---|---|
| | TA98 | TA100 | TA102 | TA1535 | TA1537 |
| NC[1] | 39 ± 2 | 184 ± 5 | 279 ± 5 | 15 ± 1 | 5 ± 1 |
| CBC 0.3 mg per plate | 38 ± 3 (0.97) | 179 ± 5 (0.97) | 280 ± 7 (1.00) | 16 ± 2 (1.07) | 4 ± 1 (0.80) |
| CBC 0.6 mg per plate | 39 ± 2 (1.00) | 179 ± 6 (0.97) | 282 ± 4 (1.01) | 16 ± 1 (1.07) | 4 ± 2 (0.80) |
| CBC 1.2 mg per plate | 38 ± 1 (0.98) | 178 ± 6 (0.97) | 277 ± 3 (0.99) | 15 ± 1 (1.00) | 5 ± 2 (1.00) |
| CBC 2.5 mg per plate | 38 ± 3 (0.97) | 182 ± 4 (0.99) | 283 ± 3 (1.01) | 14 ± 1 (0.93) | 5 ± 1 (1.00) |
| CBC 5 mg per plate | 40 ± 2 (1.01) | 176 ± 7 (0.96) | 274 ± 7 (0.98) | 16 ± 1 (1.00) | 4 ± 1 (0.80) |
| PC[2] | 142 ± 4 (3.62)[3,*] | 501 ± 14 (2.72)[4,*] | 940 ± 18 (3.37)[5,*] | 156 ± 6 (10.4)[4,*] | 22 ± 5 (4.4)[6,*] |
| | Number of revertant colonies per plate expressed as mean ± SD and (mutagenic index): with S9 metabolic activation | | | | |
| | TA98 | TA100 | TA102 | TA1535 | TA1537 |
| NC[1] | 32 ± 1 | 166 ± 6 | 306 ± 8 | 16 ± 1 | 11 ± 1 |
| CBC 0.3 mg per plate | 32 ± 2 (1.00) | 154 ± 5 (0.93) | 303 ± 3 (0.99) | 15 ± 1 (0.94) | 12 ± 2 (1.09) |
| CBC 0.6 mg per plate | 31 ± 3 (0.97) | 166 ± 6 (1.00) | 302 ± 3 (0.99) | 17 ± 1 (1.06) | 12 ± 1 (1.09) |
| CBC 1.2 mg per plate | 31 ± 3 (0.99) | 157 ± 3 (0.95) | 303 ± 8 (0.99) | 16 ± 1 (1.00) | 11 ± 1 (1.00) |



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| | | | | | |
|---|---|---|---|---|---|
| CBC 2.5 mg per plate | 30 ± 4 (0.96) | 164 ± 4 (0.99) | 311 ± 5 (1.02) | 17 ± 1 (1.06) | 11 ± 1 (1.00) |
| CBC 5 mg per plate | 32 ± 2 (1.01) | 168 ± 4 (1.01) | 306 ± 7 (1.00) | 18 ± 2 (1.13) | 11 ± 2 (1.00) |
| PC[2] | 145 ± 5 (4.57)[7,*] | 575 ± 5 (3.46)[8,*] | 941 ± 19 (3.08)[7,*] | 88 ± 2 (5.5)[7,*] | 45 ± 3 (4.09)[8,*] |

[#]The mutagenic index was calculated as the average number of revertants per plate with the test group divided by the average number of revertants per plate with the negative control.

[1] Negative control; [2] positive control; [3] 0.5 µg per plate of 4-nitro-o-phenylenediamine; [4] 4 µg per plate of sodium azide; [5] 0.5 µg per plate of mitomycin C; [6] 5 µg per plate of 9-aminoacridine; [7] 1 µg per plate of benzo[a]pyrene; [8] 4 µg per plate of 2-aminoanthracene; [*] $P < 0.05$ compared with the negative control group.



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**Table 3. Effects of CBC on body weight and relative organ weight in rats at the end of experiment[1].**

| | Male | | | | Female | | | |
|---|---|---|---|---|---|---|---|---|
| | | CBC (g kg$^{-1}$) | | | | CBC (g kg$^{-1}$) | | |
| | Control | 3 | 6 | 10 | Control | 3 | 6 | 10 |
| Body weight (g) | 378 ± 17 | 371 ± 24 | 379 ± 16 | 373 ± 16 | 248 ± 18 | 239 ± 13 | 243 ± 10 | 247 ± 11 |
| Heart (%) | 1.14 ± 0.04 | 1.16 ± 0.07 | 1.17 ± 0.10 | 1.12 ± 0.06 | 0.82 ± 0.07 | 0.79 ± 0.07 | 0.81 ± 0.05 | 0.74 ± 0.20 |
| Liver (%) | 11.03 ± 1.00 | 10.76 ± 1.10 | 11.10 ± 0.81 | 10.89 ± 1.11 | 7.32 ± 0.82 | 6.59 ± 0.49 | 6.82 ± 0.59 | 7.00 ± 0.58 |
| Spleen (%) | 0.95 ± 0.12 | 0.98 ± 0.17 | 0.93 ± 0.09 | 0.94 ± 0.06 | 0.67 ± 0.07 | 0.58 ± 0.08 | 0.66 ± 0.11 | 0.67 ± 0.04 |
| Lung (%) | 1.45 ± 0.09 | 1.47 ± 0.17 | 1.46 ± 0.08 | 1.49 ± 0.12 | 1.15 ± 0.08 | 1.10 ± 0.04 | 1.11 ± 0.08 | 1.19 ± 0.07 |
| Kidney (%) | 2.62 ± 0.20 | 2.61 ± 0.26 | 2.78 ± 0.20 | 2.65 ± 0.18 | 1.77 ± 0.22 | 1.64 ± 0.12 | 1.68 ± 0.12 | 1.73 ± 0.17 |
| Adrenal gland (%) | 0.06 ± 0.01 | 0.06 ± 0.01 | 0.07 ± 0.01 | 0.06 ± 0.01 | 0.08 ± 0.01 | 0.07 ± 0.01 | 0.07 ± 0.01 | 0.08 ± 0.00 |
| Testes (%) | 3.38 ± 0.33 | 3.42 ± 0.29 | 3.44 ± 0.27 | 3.33 ± 0.34 | - | - | - | - |
| Epididymis (%) | 0.87 ± 0.05 | 0.90 ± 0.05 | 0.86 ± 0.07 | 0.83 ± 0.06 | - | - | - | - |
| Ovary (%) | - | - | - | - | 0.69 ± 0.13 | 0.81 ± 0.26 | 0.67 ± 0.19 | 0.56 ± 0.07 |

[1] Rats were orally treated with CBC (3, 6 and 10 g kg$^{-1}$) daily for 28 consecutive days and relative organ weights were calculated as % of individual body weights. Data are expressed as mean ± SD (n = 10 for each group).



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**Table 4. Effects of CBC on hematological and blood biochemical parameters in rats at the end of experiment[1].**

| | Male | | | | Female | | | |
|---|---|---|---|---|---|---|---|---|
| | | CBC (g $kg^{-1}$) | | | | CBC (g $kg^{-1}$) | | |
| | Control | 3 | 6 | 10 | Control | 3 | 6 | 10 |
| | | | | hematological parameters | | | | |
| WBC ($10^3$ cells $\mu L^{-1}$) | 4.8 ± 0.7 | 5.8 ± 1.5 | 5.7 ± 1.3 | 5.3 ± 0.8 | 2.5 ± 0.6 | 2.1 ± 0.7 | 3.1 ± 0.7 | 3.6 ± 1.0 |
| RBC ($10^6$ cells $\mu L^{-1}$) | 8.0 ± 0.3 | 8.0 ± 0.3 | 8.2 ± 0.5 | 8.2 ± 0.4 | 8.0 ± 0.4 | 8.3 ± 0.3 | 8.2 ± 0.4 | 8.4 ± 0.4 |
| Hb (g/dL) | 15.4 ±0.5 | 15.2 ± 0.4 | 15.9 ± 0.9 | 16.1 ± 0.7 | 15.1 ± 0.6 | 15.4 ± 0.7 | 15.3 ± 0.5 | 16.0 ± 0.6 |
| HCT (%) | 47.2 ±1.7 | 46.5 ± 1.2 | 48.7 ± 2.4 | 48.6 ± 1.8 | 46.5 ± 1.8 | 47.1 ± 1.8 | 47.0 ± 1.2 | 47.8 ± 1.5 |
| MCV (fl) | 58.7 ± 1.4 | 58.4 ± 2.0 | 59.3 ± 1.6 | 59.5 ± 1.9 | 58.0 ± 1.5 | 57.1 ± 2.2 | 57.4 ± 1.9 | 57.0± 1.6 |
| MCH (pg) | 19.2 ± 0.6 | 19.1 ± 0.6 | 19.5 ± 0.6 | 19.7 ± 0.5 | 18.9 ± 0.4 | 18.6 ± 0.6 | 18.7 ± 0.5 | 19.0 ± 0.6 |
| MCHC (g $dL^{-1}$) | 32.7 ± 0.5 | 32.7 ± 0.3 | 32.7 ±0.5 | 33.2 ±0.5 | 32.5 ±0.5 | 32.6 ± 0.5 | 32.6 ± 0.4 | 33.3 ± 0.5 |
| PLT ($10^3$ cells $\mu L^{-1}$) | 770 ± 101 | 799 ± 102 | 777 ± 66 | 792 ± 75 | 723 ± 91 | 711 ± 88 | 723 ± 83 | 716 ± 92 |
| Lymphocytes (%) | 77.8 ± 6 | 79.7 ± 5 | 81.2 ± 5 | 80.6 ± 3 | 80.1 ± 7 | 79.2 ± 6 | 80.0 ± 6 | 81.5 ± 4 |
| | | | | Blood biochemical parameters | | | | |
| GOT (U $L^{-1}$) | 117 ±16 | 112 ± 17 | 113 ± 15 | 104 ± 15 | 130 ± 19 | 119 ± 13 | 118 ± 15 | 107 ± 16 |
| GPT (U $L^{-1}$) | 39.3 ± 5 | 39.9 ± 7 | 39.5 ± 6 | 34.8 ± 4 | 38.7 ± 5 | 37.1 ± 6 | 35.2 ± 6 | 36.5 ± 7 |
| BUN (mg $dL^{-1}$) | 19.3 ± 3 | 19.5 ± 3 | 20.7 ± 3 | 20.3 ± 3 | 23.3 ± 2 | 22.1 ± 3 | 24.3 ± 4 | 26.3 ± 5 |
| CRE (mg $dL^{-1}$) | 0.43 ± 0.05 | 0.37 ± 0.08 | 0.40 ± 0.06 | 0.39 ± 0.07 | 0.47 ± 0.04 | 0.46 ± 0.04 | 0.46 ± 0.08 | 0.46 ± 0.10 |
| Glucose (mg $dL^{-1}$) | 93.1 ± 15 | 88.9 ± 23 | 107.2 ± 53 | 78.7 ± 17 | 70.2 ± 16 | 71.8 ± 15 | 74.1 ± 16 | 74.2 ± 12 |
| T-CHO (mg $dL^{-1}$) | 62.0 ± 14 | 65.2 ±12 | 59.4 ± 10 | 65.3 ± 11 | 71.8 ± 13 | 67.7 ± 13 | 75.1 ± 12 | 67.3 ± 10 |



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| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| TG (mg $dL^{-1}$) | 47.1 ± 16 | 48.7 ±12 | 32.5 ± 11 | 53.1 ± 25 | 37.4 ± 8 | 34.1 ± 6 | 33.6 ± 9 | 29.2 ± 13 |
| HDL (mg $dL^{-1}$) | 14.3 ± 3 | 15.5 ± 3 | 17.3 ± 3 | 18.0 ± 3 | 22.1 ± 3 | 22.3 ± 4 | 21.9 ± 3 | 20.1 ± 3 |
| LDL (mg $dL^{-1}$) | 6.9 ± 1.8 | 7.4 ± 1.9 | 5.9 ± 1.2 | 6.7 ± 0.9 | 4.2 ± 0.3 | 3.9 ± 0.3 | 3.8 ± 0.2 | 5.4 ± 0.3* |
| Globulin (g $dL^{-1}$) | 2.2 ± 0.1 | 2.2 ± 0.1 | 2.3 ± 0.1 | 2.2 ± 0.1 | 2.2 ± 0.1 | 2.2 ± 0.2 | 2.2 ± 0.2 | 2.2 ± 0.2 |
| Albumin (g $dL^{-1}$) | 4.0 ± 0.2 | 3.9 ± 0.1 | 4.0 ± 0.2 | 4.0 ± 0.2 | 4.1 ± 0.2 | 4.3 ± 0.2 | 4.1 ± 0.2 | 4.1 ± 0.2 |
| Total protein (g $dL^{-1}$) | 6.2 ± 0.2 | 6.1 ± 0.1 | 6.3 ± 0.3 | 6.1 ± 0.3 | 6.3 ± 0.3 | 6.5 ± 0.3 | 6.2 ± 0.4 | 6.2 ± 0.5 |
| $Na^+$ (meq $L^{-1}$) | 142 ± 1 | 143 ± 1 | 143 ± 1 | 142 ± 1 | 142 ± 4 | 144 ± 1 | 142 ± 2 | 141 ± 2 |
| $Cl^-$ (meq $L^{-1}$) | 99 ± 1 | 99 ± 1 | 101 ± 2 | 101 ± 1 | 100 ± 1 | 101 ± 1 | 100 ± 1 | 99 ± 2 |
| $Mg^{2+}$ (mg $dL^{-1}$) | 4.0 ± 0.2 | 3.6 ± 0.3 | 3.7 ± 0.4 | 3.5 ± 0.3 | 4.2 ± 0.3 | 3.9 ± 0.3 | 3.8 ± 0.2 | 3.7 ± 0.3 |
| $Ca^{2+}$ (mg $dL^{-1}$) | 10.9 ± 0.4 | 11.0 ± 0.3 | 11.1 ± 0.6 | 11.0 ± 0.5 | 10.3 ± 0.3 | 10.3 ± 0.2 | 10.6 ± 0.4 | 10.9 ± 0.6 |
| Phosphorus (meq $L^{-1}$) | 9.6 ± 0.7 | 10.1 ± 0.8 | 10.3 ± 0.6 | 10.0 ±0.7 | 7.6 ± 0.7 | 7.6 ± 0.9 | 8.8 ± 1.4 | 8.9 ± 1.0 |

[1]Rats were orally treated with CBC (3, 6 and 10 g $kg^{-1}$) daily for 28 consecutive days. Data are expressed as mean ± SD (n = 10 for each group). *represented as statistical significance in comparison with the control group ($P < 0.05$). Abbreviations: white blood cells (WBC); red blood cells (RBC); hemoglobin (Hb); hematocrit (HCT); mean corpuscular volume (MCV); mean corpuscular hemoglobin (MCH); mean corpuscular hemoglobin concentration (MCHC); platelets (PLT); glutamic oxaloacetic transaminease (GOT); glutamine pyruvic transaminase (GPT), blood urea nitrogen (BUN); creatinine (CRE); total cholesterol (T-Cho); triglyceride (TG); high density lipoprotein (HDL), low density lipoprotein (LDL).



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**Figure captions**

**Figure 1.** Effects of cuttlebone complex (CBC) on indomethacin-induced gastric damages in rats. Macroscopic (upper panel) and histopathological (lower panel) observation of gastric mucosa. The solid arrows indicated the ulcerations (upper panel); the solid and dotted arrows indicated erosion and blood congestion, respectively (lower panel).

**Figure 2.** Effects of CBC on chromosome aberration in Chinese hamster ovary cells. The percentage of total aberrant cells was expressed as mean ± SD; *represented as statistical significance in comparison with the control group ($P < 0.01$). 10 $\mu g\ mL^{-1}$ of cyclophophamide monohydrate for S9 metabolic activation and 0.07 $\mu g\ mL^{-1}$ of mitomycin C for no S9 metabolic activation were used as positive controls.

**Figure 3.** Effects of CBC on micronucleus formation in ICR mice. Data are expressed as mean ± SD from 10 mice. *represented as statistical significance in comparison with the control group ($P < 0.01$).

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## Figure 1

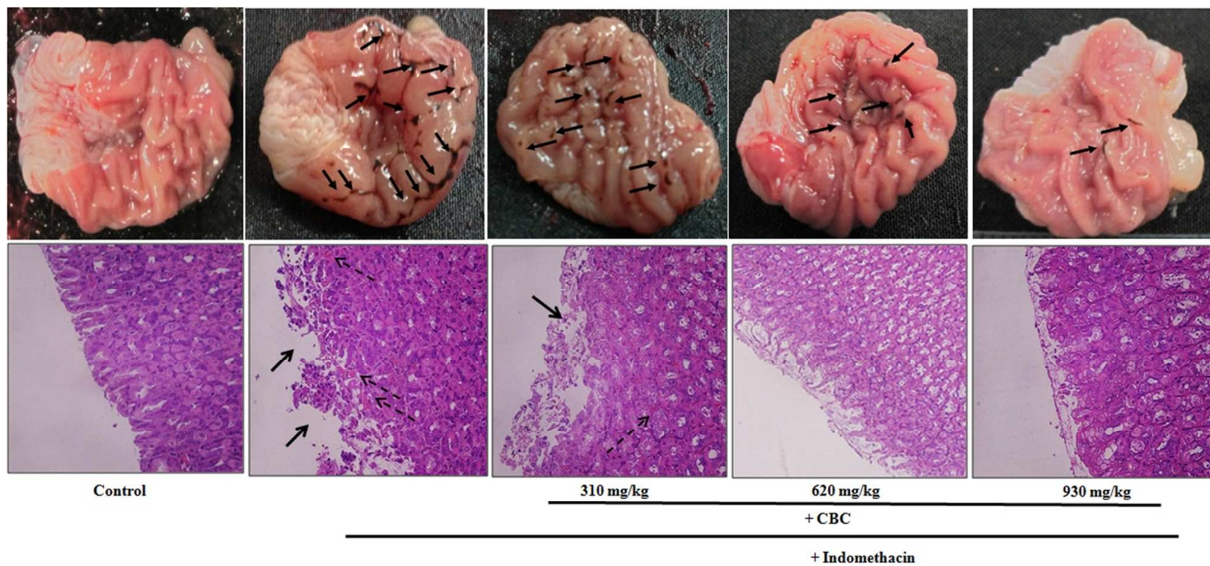


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Figure 2

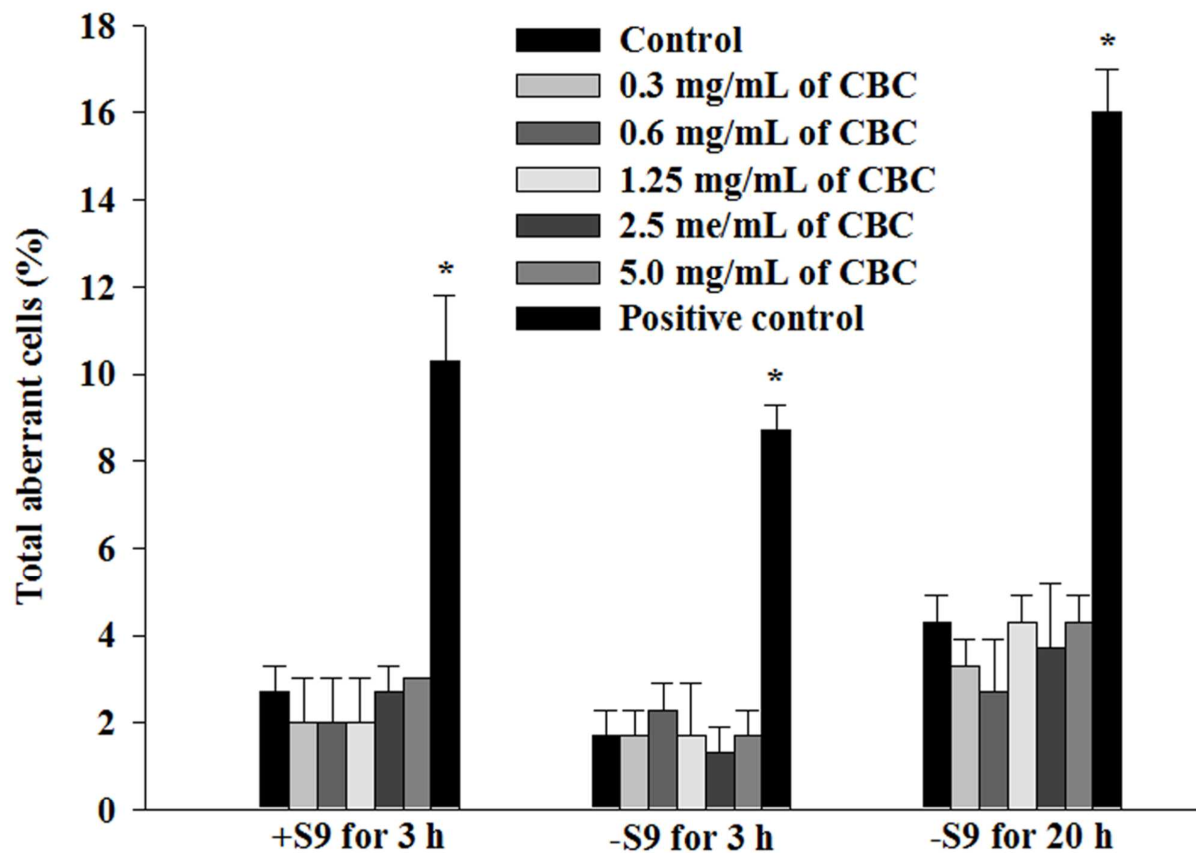


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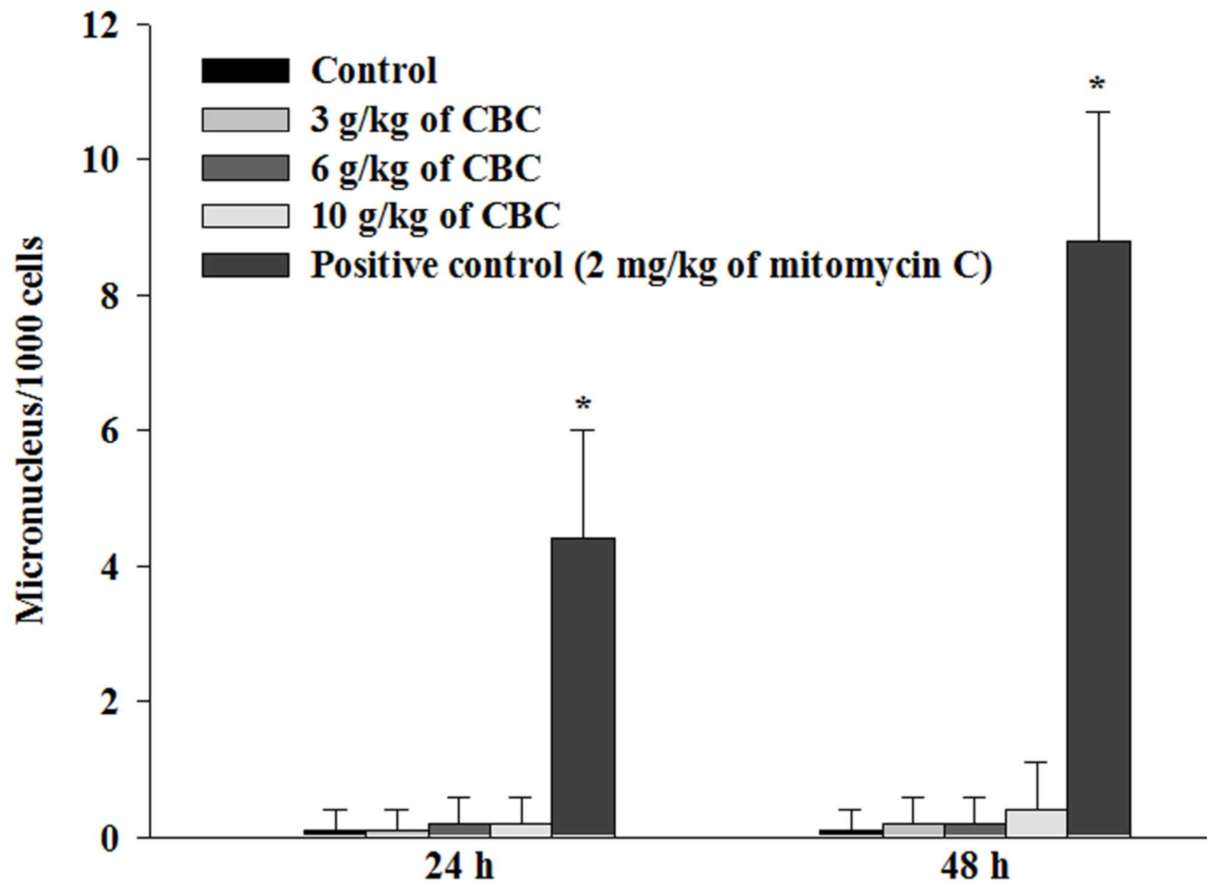
Figure 3

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