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ORIGINAL ARTICLE

Influence of Boiling Duration of GCSB-5 on Index Compound Content and Antioxidative and Anti-inflammatory Activity

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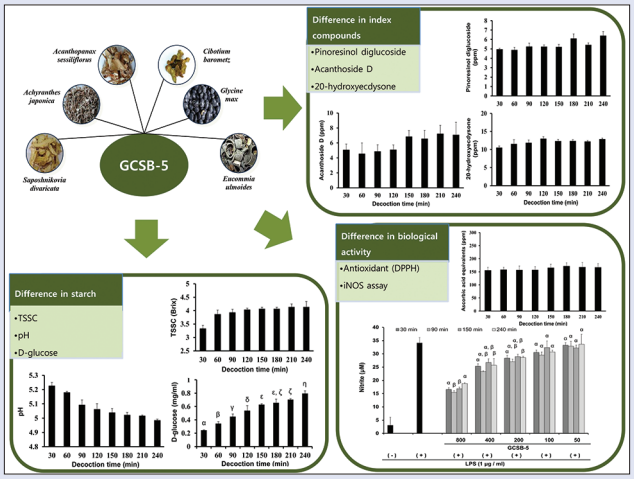
ABSTRACT

Background: GCSB-5, an herbal drug composition with an anti-inflammatory effect, is prepared by boiling, which is the most common herbal extraction method in traditional Korean medicine. Several parameters are involved in the process, i.e., extractant type, herb-to-extractant ratio, extraction temperature and pressure, and total boiling time. **Objectives:** The aim of this study was to examine the influence of boiling time on index compound amount and the antioxidative and anti-inflammatory activities of GCSB-5. **Materials and Methods:** Different samples of GCSB-5 were obtained by decocting for 30, 60, 90, 120, 150, and 240 min. Each sample was tested for hydrogen ion concentration (pH), total soluble solid content (TSSC), marker compound profiles, and antioxidative and anti-inflammatory activity. **Results:** pH was found to decrease while TSSC increased with extended decoction. Marker compound contents for GCSB-5 (acanthoside D for *Acanthopanax sessiliflorus* Seem, 20-hydroxyecdysone for *Achyranthes japonica* Nakai, and pinoresinol diglucoside for *Eucommia ulmoides* Oliver) remained relatively constant regardless of the length of boiling. Total D-glucose amount increased with longer boiling. The antioxidative and anti-inflammatory potentials of GCSB-5 were not substantially affected by decoction duration. **Conclusion:** Biological characteristics and marker compound content of GCSB-5 were not altered significantly in prolonged boiling.

Key words: Anti-inflammation, anti-oxidation, boiling duration, GCSB-5, traditional Korean medicine

SUMMARY

- Longer boiling duration of GCSB-5 did not increase yield in a time-dependent manner, but yields of 210 and 240 min samples were significantly higher
- Hydrogen ion concentration of GCSB-5 samples decreased while total soluble solid content and D-glucose concentration levels increased with boiling duration
- Although concentrations of some index compounds increased with extended boiling duration of GCSB-5, increase was small and not in a direct proportional relationship
- Antioxidative and anti-inflammatory properties of GCSB-5 were not substantially affected by decoction duration.



Abbreviations used: CAM: Complementary and alternative medicine; KIOM: Korea Institute of Oriental Medicine; KMD: Korean medicine doctor; TSSC: Total soluble solid content; pH: Hydrogen ion concentration; HPLC: High-performance liquid chromatography; NO: Nitric oxide; NO_2: Nitric dioxide; LPS: Lipopolysaccharide; DMSO: Dimethyl sulfoxide.

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INTRODUCTION

The use of complementary and alternative medicine (CAM) and the application of herbal drugs in treating various medical conditions are becoming more common worldwide.[1-4] The most frequently used form of herbal drug preparation in Korea is the hot boiling method. While various methods of extraction exist, boiling in hot water is the most widely used as it does not require additional preprocessing of raw materials, and the absorption rate of aqueous extracts or decoctions (*Tang*) is reported to be higher than that of tablet or pill forms (*Hwan*). However, it should be taken into consideration that the boiling method uses water as an extractant and heat is continuously applied to raw materials during extraction, rendering likely for physiochemical changes to take place during the process. A previous study on *Huanglianjiedu*, one of the most commonly used herbal prescriptions, revealed that duration of boiling and the herb-to-water ratio may affect extraction yield of active components.[5] Extraction of longan fruit pericarp under high pressure was positively correlated with the higher antioxidative activity of the extract compared to normal pressure.[6] Furthermore, the extraction yield of paeoniflorin, an active component used as index compound for *Paeonia lactiflora*,[7] was found to increase for up to 2 h of boiling and

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to decrease after 4 h, and the authors concluded that compounds may be degraded or denatured after 2 h of boiling at temperatures of 100°C or above.[8] Therefore, various factors concerning the extraction process such as temperature, time duration of extraction, extractant, and pressure should be taken into consideration as important factors as they may affect the biochemical nature and extraction yield of active constituents, leading to changes in drug potency.[9-12] Among the various plant, animal, mineral, or seashell materials used in CAM, *Chinemydis plastrum* and *Trionycis carapax* belong to a certain subset that is recommended "*Seon-jeon*," or "boil-before," implying that certain materials should be extracted longer than others in terms of boiling time. In addition, herbs such as *Pogostemon cablin*, *Aucklandia lappa*, *Amomum kravanh*, *Amomum villosum*, *Alpinia katsumadai*, *Santalum album*, and *Aquilaria agallocha* have been known to require "*Hu-ha*," or "boil-after," suggesting that these materials require extraction for shorter periods to achieve heightened effect. As demonstrated both historically and scientifically, various herbs or materials are decocted under specific conditions to reach maximum potency or to reduce adverse effects from potential toxicity. However, empirical knowledge of such techniques as "boil-before" or "boil-after" has yet to be standardized[13] and warrants further investigation.

GCSB-5 is a mixture of six different herbs, namely, *Saposhnikovia divaricata* Schischek, *Achyranthes japonica* Nakai, *Acanthopanax sessiliflorus* Seem, *Cibotium barometz* J. Smith, *Glycine max* Merrill, and *Eucommia ulmoides* Oliver. These herbs have a long history of use for musculoskeletal conditions such as osteoarthritis and herniated intervertebral disc. The Korean Medicine Clinical Practice Guideline for Lumbar Herniated Intervertebral Disc in Adults, recently published by the Korea Institute of Oriental Medicine, includes recommendations for *Chungpa-jun*, the main constituent of which is GCSB-5, for the treatment of intervertebral disc disorders.

A recent survey conducted in Korean medicine doctors within a hospital/clinic network that specializes in spinal disorders with the aim of investigating current practice patterns of Korean medicine treatment reported *Chungpa-jun* to be the most frequently prescribed herbal drug for lumbar disc herniation.[14] Furthermore, a retrospective cohort study on 6894 subjects from seven Korean medicine hospitals specializing in musculoskeletal disorders was conducted to assess possible hepatotoxicity of various herbal prescriptions, and results showed that *Chungpa-jun* was the most commonly used herbal medicine, accounting for over 40% of prescriptions.[15] The anti-inflammatory activity of GCSB-5 in acute and chronic inflammation models has been demonstrated both *in vitro* and *in vivo*,[16,17] with suppression of deteriorative change in a rat model of monosodium iodoacetate-induced osteoarthritis.[18] In addition, GCSB-5 was shown to exert neuroprotective effects in *in vitro* and *in vivo* peripheral nerve injury models,[19] and its clinical safety and efficacy have also recently been reported.[14]

In the current study, marker compound concentration and total starch content were analyzed in GCSB-5 samples extracted by various decoction time lengths. Anti-inflammatory and antioxidative properties were investigated using inducible nitric oxide synthase (iNOS) assay and 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assay, respectively, and the aim of this study was to establish optimal time length for boiling and standardize the preparation method of GCSB-5, an herbal mixture best known for its anti-inflammatory effect.

MATERIALS AND METHODS

Preparation and composition of GCSB-5

All raw constituents of GCSB-5 were purchased from Green Meong Pum Pharm Corp. (Namyangju, South Korea). *S. divaricata* Schischek (3.0 g), *A. japonica* Nakai (3.0 g), *A. sessiliflorus* Seem (3.0 g), *C. barometz* J. Smith (2.0 g), *G. max* Merrill (2.0 g), and *E. ulmoides* Oliver (1.0 g) in ground form was added to 140 ml of distilled water and boiled for either 30, 60, 90, 120, 150, 180, 210, or 240 min at 100°C. The decoction was passed through a 3 μm pore filter (Hyundai Micro, Seoul, South Korea) following cooling after the boiling process, and distilled water was added to the decoction to a final volume of 140 ml.

A total 70 ml of each sample was subjected to total soluble solid content (TSSC), hydrogen ion concentration (pH), total starch, DPPH assays, and high-performance liquid chromatography (HPLC) analysis. The remaining 70 ml of each sample was freeze-dried at −80°C for iNOS assay. All samples were extracted three times separately under the same conditions, with the exception of boiling duration, and then freeze-dried. Freeze-dried samples were stored at −20°C before being dissolved in distilled water immediately before assay.

Total soluble solid content and hydrogen ion concentration

Nonfreeze-dried samples were analyzed for TSSC and pH. TSSC was measured with PAL-1 (ATAGO Co., Tokyo, Japan). After calibration with distilled water, each sample was assessed for TSSC. The pH of each sample was measured with Orion Star A211 (Thermo Scientific, Waltham, USA). The pH meter was calibrated with premixed solutions of pH 4.0, 7.0, and 10.0 before assay. The mean was obtained from triplicate experiments.

High-performance liquid chromatography analysis

The marker contents of GCSB-5 were analyzed using HPLC (Shimadzu, Kyoto, Japan). Acanthoside D for *A. sessiliflorus* Seem, 20-hydroxyecdysone for *A. japonica* Nakai, and pinoresinol diglucoside for *E. ulmoides* Oliver were selected as index compounds. The standard markers were purchased from Sigma-Aldrich (St. Louis, USA), and HPLC grade organic solvents (J.T. Baker, Center Valley, USA) were used for analysis. The standards were dissolved in ethanol to achieve a concentration of 1 mg/ml, then diluted 2-fold and passed through a 0.2 μm pore filter. Each sample of different boiling durations was added with an equivalent volume of ethanol, ultrasonicated, and then passed through a 0.2 μm pore filter. A standard curve was obtained using the standard marker, and the index content of each sample was determined by calculating the area under the corresponding slope.

Acanthoside D

Analysis of acanthoside D, the marker compound of *A. sessiliflorus* Seem, was performed with a C18 column (4.6 mm × 250 mm, 5 μm; Agilent, Santa Clara, USA). The temperature of the column was set to 40°C and the UV wavelength to 210 nm. The mobile phase was composed of 10% acetonitrile (A) and 30% acetonitrile (B). Gradient profile was applied with 10% B at the start, 15% B from 5 to 15 min, and 10% B from 20 to 30 min. The flow velocity was maintained at 1 ml/min.[20-22]

20-hydroxyecdysone

Analysis of 20-hydroxyecdysone, the marker compound of *A. japonica* Nakai, was performed using a C18 column (4.6 mm × 250 mm, 5 μm; Agilent, Santa Clara, USA). Column temperature was set at 35°C and UV wavelength at 254 nm. Water (A) and acetonitrile (B) were selected as the mobile phase. Gradient profile was applied with 15% B at the start, 30% B from 8 to 15 min, 30% B from 15 to 30 min, and 15% B from 30 to 35 min. Flow velocity was maintained at 1 ml/min.[23-25]

Pinoresinol diglucoside

The analysis of pinoresinol diglucoside, the marker compound of *E. ulmoides* Oliver, was conducted according to the method given in Korean Pharmacopeia. C18 column (4.6 mm × 250 mm, 5 μm; Agilent,

Santa Clara, USA) was used for analysis. The temperature of the column was set to 35°C and UV wavelength to 230 nm. The mobile phase was composed of 0.1% formic acid (A) and acetonitrile (B). Gradient profile was applied with 5% B at the start, 20% B from 8 to 15 min, and 5% B from 30 to 35 min. The flow velocity was kept at 1 ml/min.

Total starch

The content level of starch in each sample was measured with a Megazyme kit (K-TSTA, Chicago, USA).[26,27] Measurement was conducted after the boiling and filtering process, before freeze-drying. A volume of 8 ml of 95% ethanol was added to 2 ml of each GCSB-5 extract. The samples were then vortexed, incubated at room temperature for 30 min, and centrifuged at 1800 ×*g*. Supernatants were removed and the pellet was resuspended in 1 ml of distilled water. After 3.9 ml of 1 mM acetate buffer and 0.1 ml of 66 unit amyloglucosidase were added to each sample, the mixture was incubated at 50°C for 30 min. The final samples were then subjected to a luminescence assay by adding glucose oxidase plus peroxidase and 4-aminoantipyrine freeze-dried powder dissolved in p-hydroxybenzoic acid and sodium azide buffer. Absorbance was read at 540 nm. A standard curve was obtained through multiple samples of D-glucose control at different concentrations (10, 50, 100, 500, and 1000 μg/ml), and the starch content in each GCSB-5 sample was quantified based on the curve.

1,1-diphenyl-2-picryl-hydrazyl

DPPH assay of nonfreeze-dried samples was performed as previously reported by Chang *et al.*[28] by monitoring absorbance at 540 nm with a microplate reader (TECAN, Chapel Hill, USA). The standard curve was plotted with multiple concentrations (1, 5, 10, 50, 100, and 500 μg/ml) of ascorbic acid (Sigma-Aldrich, St. Louis, USA) as standard. The scavenging activity of the GCSB-5 extract on DPPH radicals was measured by comparing absorbance values to the standard curve.

Nitric oxide

Murine macrophage RAW 264.7 cell line was purchased from the Korean Cell Line Bank (KCLB, Seoul, South Korea, No. 40071). Cells were cultured in DMEM (Gibco, Waltham, USA) supplemented with 10% fetal bovine serum (Gibco, Waltham, USA) and 1% antibiotic-antimycotic agent (Gibco, Waltham, USA) in a 5% CO_2 chamber maintained at 37°C. When RAW 264.7 cells are activated, NO is produced, generating nitric dioxide (NO_2) in the culture media. Concentration of the nitric product was measured by reaction with Griess reagent. RAW cells were first seeded into 96-well plates at a density of 5×10^5 cells/ml and then incubated in a 5% CO_2 chamber at 37°C for 24 h. The cells were stimulated using 1 μg/ml lipopolysaccharide (LPS; Sigma-Aldrich, St. Louis, USA) and then treated with different GCSB-5 extracts (boiled for 30, 90, 150, and 240 min) at various concentrations (50, 100, 200, 400, and 800 μg/ml). After incubation for 24 h, the supernatant was collected and an equal volume of Griess reagent (0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride and 1% sulfanilamide in 5% phosphoric acid) was added. Absorbance was read at 540 nm in a microplate reader (TECAN, Chapel Hill, USA) and the concentration of NO_2 in each supernatant sample was determined based on the standard curve obtained with sodium nitrite.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay for cell viability

After collection of supernatant, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (final concentration of 500 mg/ml) was added to each well and incubated at 37°C for 4 h. The remaining medium was then removed and dimethyl sulfoxide was added to dissolve the formazan. Cell viability (%) was determined as compared to the control group (LPS+) at an absorbance of 570 nm.

Statistical analysis

One-way ANOVA was performed to investigate influence of boiling time length on biological activity of GCSB-5; two-way ANOVA was carried out to assess time length and concentration. Duncan's multiple range test was conducted for *post hoc* analysis. All statistical analyses including computation of *P* values were executed with SPSS 18.0 statistical software packages (IBM, Armonk, USA).

RESULTS

Yield

First, 140 ml of distilled water was added to a total 14 g of combined raw materials. The mixture was then boiled with reflux extraction apparatus for different time lengths (30, 60, 90, 120, 150, 180, 210, and 240 min), cooled, and supplemented with distilled water to 140 ml. A total 70 ml of the extract was freeze-dried for additional experiments. Longer duration in boiling extraction did not increase yield in a time-dependent manner, but yields of 210 and 240 min samples were significantly higher than those of other samples [Figure 1a].

Total soluble solid content and hydrogen ion concentration

While TSSC values of various GCSB-5 samples stayed within 3.2–4.2 brix, TSSC of each sample was found to increase in proportion to boiling duration, and the difference in increase was especially significant between the 30 and 60 min time points. This finding suggests that longer boiling processes may result in increased extraction of total soluble solids. The pH of each sample ranged between 5.00 and 5.25 overall and showed tendency to decrease with longer boiling duration [Figure 1b and c].

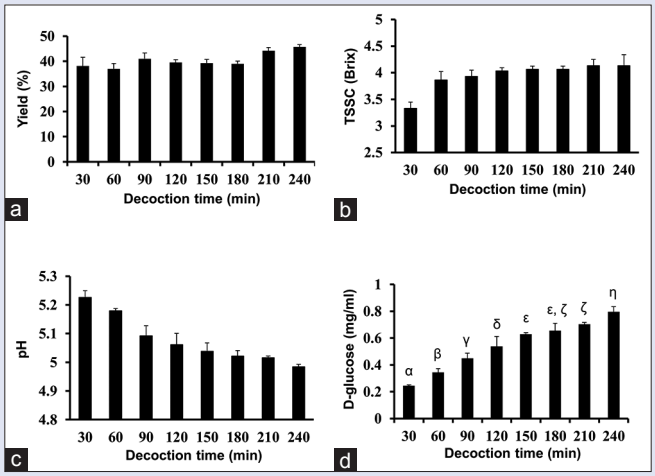

Figure 1: Difference in chemical properties of GCSB-5 by varying boiling time length. GCSB samples boiled for 30, 60, 90, 120, 150, or 240 min were analyzed for yield rate, total soluble solid content, hydrogen ion concentration, and D-glucose amount. Statistically significant differences between bars are indicated with different Greek alphabet characters; same alphabet characters imply no significant difference between bars ($P < 0.0001$) (a) yield rate, (b) total soluble solid content, (c) hydrogen ion concentration, (d) total starch

Total starch

Total starch in each decoction sample was hydrolyzed to D-glucose, and levels were measured. The concentration level of D-glucose in the 30 min sample was found to be 0.245 ± 0.005 mg/ml, whereas that in the 240 min sample was 0.796 ± 0.039 mg/ml. The concentration level of D-glucose was shown to significantly increase with extended decocting ($P < 0.0001$; Figure 1d).

Analyses of index compounds

The content level of various index compounds was investigated with different GCSB-5 samples. Concentration level of acanthoside D, the marker compound of *A. sessiliflorus* Seem, ranged from 4.55 ± 1.45 μg/ml to 7.22 ± 1.14 μg/ml among samples, and levels tended to increase after 150 min of exposure to boiling [Figure 2a]. Concentration of 20-hydroxyecdysone, the index compound of *A. japonica* Nakai, was found to be between 10.52 ± 0.45 μg/ml and 12.98 ± 0.58 μg/ml, and the current results suggest that boiling duration may not be relevant in terms of higher 20-hydroxyecdysone extraction [Figure 2b]. Pinoresinol diglucoside, the marker compound of *E. ulmoides* Oliver, was in the range of 4.88 ± 0.27 μg/ml to 6.40 ± 0.45 μg/ml. Concentration of the compound was shown to be highest at a decoction time of 180 and 240 min [Figure 2c]. Although concentrations of some index compounds increased with extended boiling duration, it was not in a direct proportional relationship and the increase was too small to be considered meaningful.

Antioxidant activity

The radical scavenging potential of GCSB-5 was investigated with DPPH assay. The standard curve was plotted, and serially diluted Trolox solutions (15.625, 31.25, 62.5, 125, and 250 μg/ml) were assayed for DPPH radical scavenging. The antioxidative activity of each GCSB-5 sample was quantified based on the curve. Results show that the antioxidative

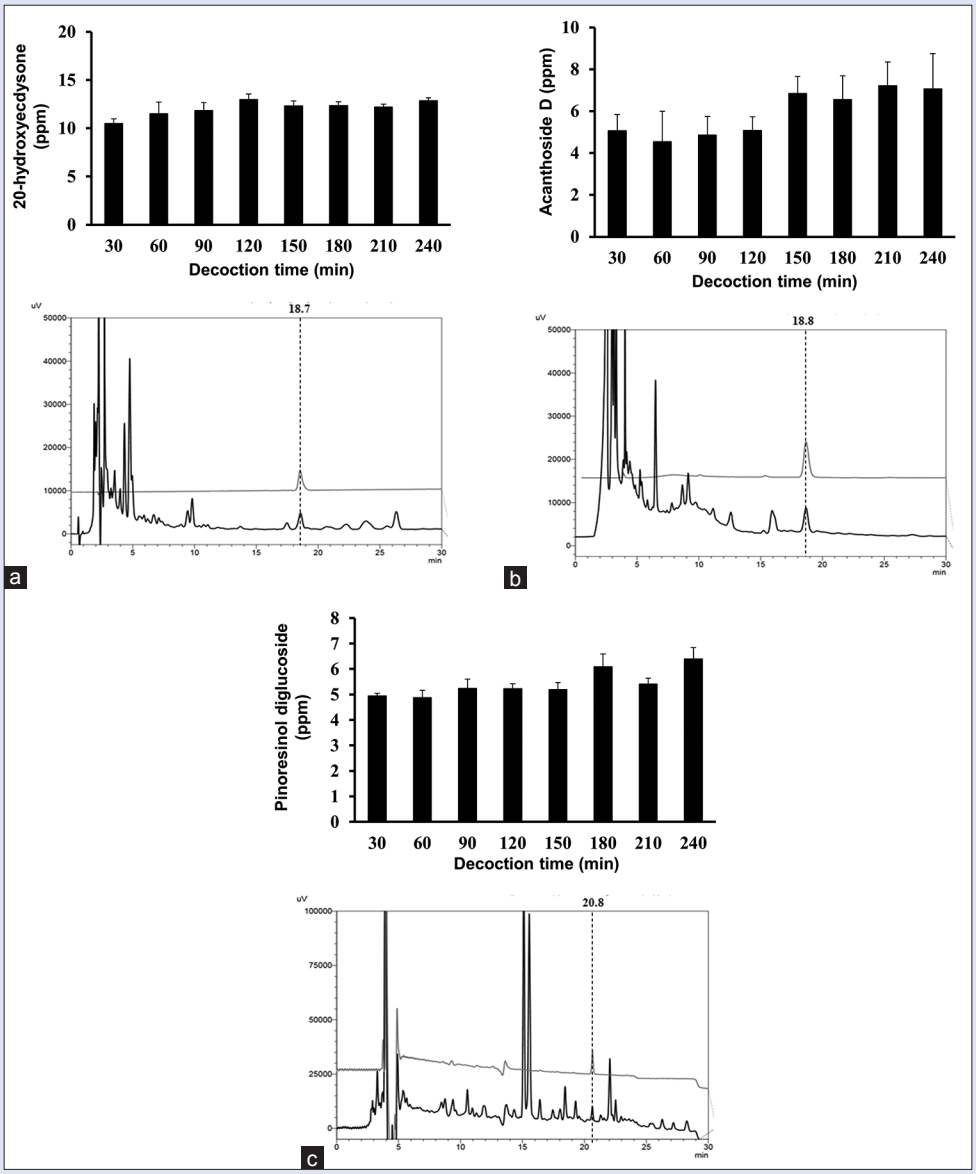

Figure 2: Difference in GCSB-5 index compounds by varying boiling time length. Amount of acanthoside D (*Acanthopanax sessiliflorus* Seem), 20-hydroxyecdysone (*Achyranthes japonica* Nakai), and pinoresinol diglucoside (*Eucommia ulmoides* Oliver) in samples were investigated using high-performance liquid chromatography. (a) acanthoside D, (b) 20-hydroxyecdysone, (c) pinoresinol diglucoside

characteristics of various GCSB-5 samples were similar, and differences were too small to be statistically significant [Figure 3a].

Anti-inflammatory activity

The anti-inflammatory properties of GCSB-5 (boiled for 30, 90, 150, and 240 min, respectively) were each investigated at multiple concentrations. GCSB-5 displayed anti-inflammatory activity in a dose-dependent manner expressed as decrease in generation of NO at higher concentrations of GCSB-5 ($P < 0.0001$). However, no association between concentration and boiling duration was found ($P = 0.437$). Thus, it is likely that boiling duration does not affect the anti-inflammatory effect of GCSB-5 [Figure 3b].

Cell viability was also investigated using MTT assay. The experimental conditions were identical to that of the NO assay, and results showed that viability (%) for all concentrations and time lengths was not <85% of controls. It can, therefore, be inferred that the current NO assay results are not a consequence of significant cytotoxicity [Figure 3c].

DISCUSSION

In this study, possible changes in index compound amount and antioxidative and anti-inflammatory activities of GCSB-5 by different extraction time lengths were investigated. GCSB-5 was chosen as subject for study in difference by extraction duration out of various herbal mixtures as its properties have been extensively reported in previous literature: clinical as well as *in vivo* studies on GCSB-5 for lumbar intervertebral disc herniation and osteoarthritis have been conducted,[18] and its *in vitro* anti-inflammatory activity with underlying mechanism[16,17] and nerve regenerative effects[19] have been reported. Previous studies on decocting methodology including boiling duration have mainly focused on yield difference in marker compounds and active constituents and chemical denaturation of these compounds.[9,29,30] The literature shows that marker compound and active constituent amount do not necessarily increase in proportion to boiling time length, and changes were observed in an herb-specific manner. The current study holds significance in that biological activities of the subject material GCSB-5 were directly compared among samples of different extraction durations. The aim of this study was to assess the role of boiling duration in chemical and biological properties of herbal extractions through determination of yield, TSSC, pH, and HPLC analysis of index compounds. DPPH assay and NO assay were also conducted to establish pharmacological relevance.

TSSC was found to increase with extended boiling time [Figure 1b]. It is natural for nonsoluble, high-molecular compounds to break down into soluble, low-molecular compounds through the boiling process. pH was shown to decrease with longer boiling [Figure 1c]. The current findings were consistent with previous literature,[31] and it may be conjectured that continuous physical stimulation, i.e., heat, may protonate decoction compounds. Such decoction factors as boiling duration and pressure may modify end product characteristics and should, therefore, be given due consideration. D-glucose amount following hydrolysis was investigated to quantify total starch amount. The results showed that extended boiling time length led to significant increase in total starch amount [Figure 1d]. As the raw materials constituting GCSB-5 are of herbal origin, it is probable that its aqueous extracts contain a certain amount of starch, and it should be noted that large amounts of starch are not always beneficial to the human body. Digestive rate negatively correlates with crystallization of starch,[32] and starch granules have a crystalline chemical structure imbedded in an amorphous matrix. A previous report linking naturally occurring

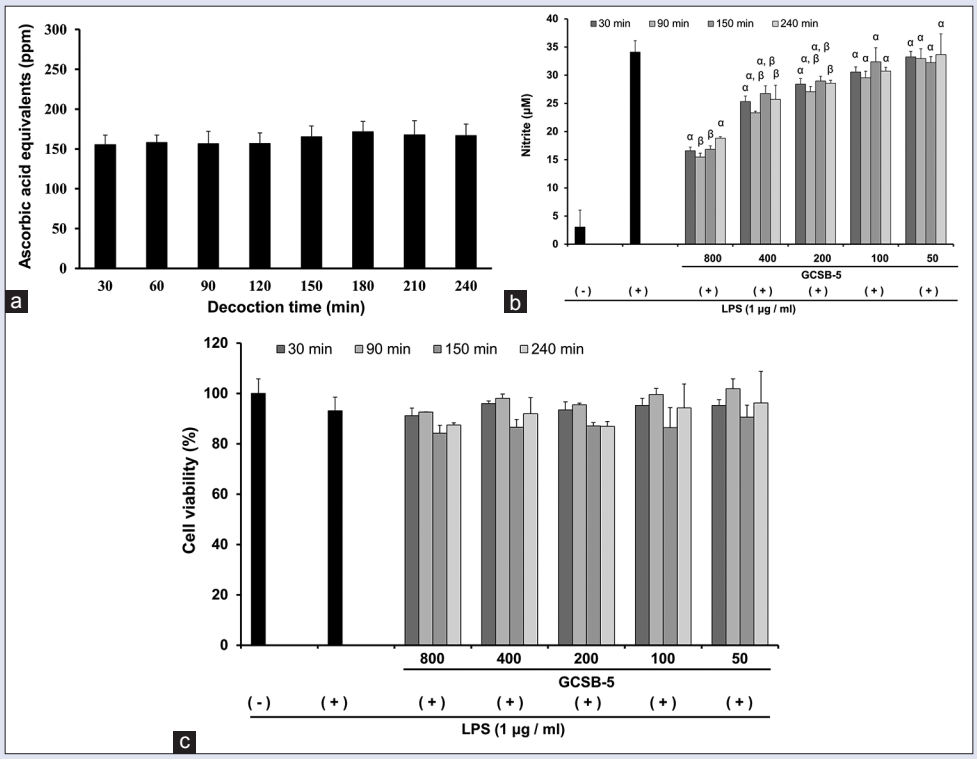

Figure 3: Difference in the biological activity of GCSB-5 by varying boiling time length. Antioxidative and anti-inflammatory activities of GCSB-5 samples were measured. Statistically significant differences between bars are indicated with different Greek alphabet characters; same alphabet characters imply no significant difference between bars (a) 1,1-diphenyl-2-picryl-hydrazyl assay ($P = 0.097$), (b) inducible nitric oxide synthase assay (by dose $P < 0.0001$, by time $P = 0.437$), (c) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

starch with slower absorption rate[31] further suggested that many individuals are only partially capable of digesting plant source starches, and it is possible that excessive starch in herbal decoctions may lead to adverse drug reactions such as bloating, indigestion, or acid reflux. In the current study, the total starch in each sample was broken down to D-glucose with amyloglucosidase, and the end product was quantified using a microplate reader.[33,34]

HPLC analyses of acanthoside D and pinoresinol di-glucose showed that compound amount tended to increase with extended boiling. Even so, increment did not occur in a time-dependent manner and was not directly proportional to boiling duration [Figure 2a and c]. Moreover, the increase was not significant and did not affect biological activities either way in antioxidative or anti-inflammatory effects. The radical scavenging potential of each GCSB-5 sample was evaluated with DPPH assay after standardization with ascorbic acid, a well-known antioxidant compound. Duration of decoction did not affect the antioxidative potential of GCSB-5 [Figure 3b]. In addition, iNOS assay was conducted to gauge whether length of boiling time affected the anti-inflammatory potential of GCSB-5. Results showed that while the anti-inflammatory effect of GCSB-5 increased in a dose-dependent manner, the difference between various GCSB-5 samples of different boiling duration failed to reach statistical significance. Based on these findings, it can be tentatively concluded that the length of the boiling process is irrelevant to the pharmacological activity of GCSB-5.

In summary, these results suggest that extraction of ineffectual materials such as starch increases with longer boiling duration, while pharmacological activities remain relatively constant. As such, 150 min was found to be preferable in terms of extraction yield of index compounds, and 30 min would suffice for pharmacological effect in GCSB-5 decoction. However, as appropriate extraction method or extraction time of herbal drugs varies by ingredient, further studies are needed to establish optimal methods of extraction for other commonly used prescriptions.

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Conflicts of interest

There are no conflicts of interest.

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