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Original Article
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Toxicity Evaluation of a Non-Pain Pharmacopuncture Extract Using a Bacterial Reverse Mutation Test

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Objectives: The objective of this study was to assess the genotoxicity of a no-pain pharmacopuncture (NPP) extract developed in 2022 using a bacterial reverse mutation assay, aiming to further substantiate the safety profile of NPP.
Methods: The genotoxicity evaluation involved a bacterial reverse mutation assay to assess the mutagenic potential of NPP extracts with and without metabolic activation. Histidine-requiring *Salmonella typhimurium* strains (TA98, TA100, TA1535, and TA1537) and tryptophan-requiring *Escherichia coli* strains (WP2uvrA) were used in the assay.
Results: The NPP extract did not induce a revertant colony count exceeding two times that of the negative control at any dose level in any of the tested strains, both with and without metabolic activation. Additionally, no growth inhibition or precipitation was observed in the presence of NPP.
Conclusion: Based on the findings, it can be concluded that the NPP extract exhibited no mutagenic potential in the *in vitro* genotoxicity tests conducted.

Keywords: bacterial reverse mutation test, genotoxicity test, Mutong, no-pain pharmacopuncture, safety

INTRODUCTION

Assessing genotoxicity is paramount for natural product medicines, including herbal medicines, as they have the potential to induce lethal mutations and damage genetic material [1]. Herbal prescriptions in Korean medicine (KM) and traditional Chinese medicine often comprise multiple herbs to harness their synergistic effects. Evidence suggests that this enhances the therapeutic benefits and mitigates toxicity [2]. Comprehensive toxicity and therapeutic efficacy validation studies ensure the safe utilization of single and compound formulations of natural products and herbal medicines [3].

Pharmacopuncture, a modern acupuncture treatment, involves the extraction and purification of pure herbal medicines with diluted solutions, which are then injected into treatment areas or acupuncture points [4, 5]. Given that pharmacopuncture is administered directly into the treatment site, bypassing the gastrointestinal tract, the toxicity of the herbal extracts used in pharmacopuncture therapy is of critical importance. To date, pharmacopuncture solutions have been developed and used in the form of compound extracts of herbal medicines with diverse effects. In KM pharmacopuncture, these extracts are dispensed by external herbal dispensaries that adhere to standardized protocols in line with domestic pharmaceutical practices certified by the Ministry of Health and Welfare. Clinicians recognize the significance of toxicity testing and quality control in pharmacopuncture [6, 7]. However, only a limited number of pharmacopuncture fluids have undergone toxicity evaluation [7].

No-pain pharmacopuncture (NPP), or Mutong pharmacopuncture, is a compound extract comprising four medicinal herbs: Corydalis Radix (CT), Paeoniae radix (PR), Glycyrrhizae Radix et Rhizoma (GR), and Chaenomelis Fructus (CF). Developed in 2022, NPP is used to alleviate pain in KM clinical practice [5, 7, 8]. To date, only one clinical study has reported its efficacy in plantar fasciitis [9]. A safety validation in rats

involved a single intramuscular dose toxicity assessment [8], an *in vivo* micronucleus assay [7], and an *in vitro* chromosomal aberration assay [10]. However, the bacterial revertant mutation test, one of the three most common battery assays, has not been conducted. Therefore, this study aimed to validate the genotoxicity of NPP through a bacterial revertant mutation test, contributing to its safety profile for clinical use.

MATERIALS AND METHODS

1. Preparation of the NPP extract

The NPP extract used in this study was comprised of CT (2 mg/mL), CF (0.3 mg/mL), PR (2 mg/mL), and GR (2 mg/mL). The extract was manufactured at Namsangcheon External Herbal Dispensary (Yongin, Korea). The extraction method has been detailed in a previous study [7, 8].

2. Test strains

We assessed NPP's mutagenic potential using histidine-requiring *Salmonella typhimurium* strains (TA98, TA100, TA1535, and TA1537) and tryptophan-requiring *Escherichia coli* strains (WP2uvrA) with and without metabolic activation. All test strains were provided by MOLTOXTM, Inc. (Boone, NC, USA). We employed several positive controls, including sodium azide (SA), 2-nitrofluorene (2-NF), 2-aminoanthracene (2-AA), 9-aminoacridine (9-AA), and 4-nitroquinoline oxide (4-NQO). We dissolved these compounds in distilled water or dimethyl sulfoxide, froze them in a cryogenic freezer (OPR-DFU-657CEV, Operon, Korea) at temperatures ranging from –80°C to –60°C, and thawed them on the day of treatment.

3. Test methods

We conducted a dose range exploration study to determine the highest dose level, with the high dose set at 100% NPP and sequentially diluted using a geometric ratio of two to generate the lower dose levels (50%, 25%, 12.5%, 6.25%, and 3.13%). We then performed the main study in duplicate with dose levels of 100%, 50%, 25%, 12.5%, and 6.25% of NPP, using three plates for each dose. We incubated various concentrations of NPP with the test strains at 37°C for 48 h with or without metabolic activation using the S9 mixture. The combinations of substances and doses included: 2-NF at 5.0 μg/plate versus TA98 without the S9 mixture; SA at 1.5 μg/plate versus TA100 and TA1535 without the S9 mixture; 9-AA at 80 μg/plate versus TA1537 without the S9 mixture; 4-NQO at 0.3 μg/plate versus WP2uvrA without the S9 mixture; 2-AA at 1.0 μg/plate versus TA98, at 2.0 μg/plate versus TA100, at 3.0 μg/plate versus TA100 and TA1537, and 10.0 μg/plate versus WP2uvrA with the S9 mixture.

We visually observed the test substance's sedimentation and counted and recorded the number of revertant colonies post-treatment. Following the culture test, we performed automatic colony counting using an automatic colony counter (Proto-COL3, Synbiosis, London, UK), with manual counting carried out if necessary.

To confirm growth inhibition, we used a stereomicroscope (45× magnification; SZ61, Olympus, Tokyo, Japan) to assess the presence or absence of a background lawn while enumerating the number of revertant colonies. We determined growth inhibition if there was a significant decrease in the number of revertant colonies compared to the negative control group or a notable reduction in the background lawn due to colony thinning or disappearance. We determined a positive test result if the number of revertant colonies of more than one strain more than doubled compared with that of the negative control group, there was a dose-dependent increase, or the outcome was reproducible.

4. High-performance liquid chromatography analysis

We employed a high-performance liquid chromatography (HPLC) analysis to identify and quantify five main compounds in NPP: tetrahydropalmatine and coptisine in CT, quercetin in CT, CF, and GR, paeoniflorin in PR, and glycyrrhizin in GR. We performed the liquid chromatography using an Agilent VWD UV detector (280 nm) (Santa Clara, CA, USA) coupled with a Watchers 120 ODS-BP column (4.6 × 150 nm, 5 μm) at a flow rate of 1 mL/min and an injection volume of 10 μL. The mobile-phase solvents consisted of distilled water in A and 0.1% phosphoric acid in acetonitrile in B. We used a stock solution of NPP for this analysis, which had a concentration 100 times higher than NPP used as a finished product (6.3 mg/mL) in KM clinical practice.

5. Statistical analysis

We directly enumerated the number of revertant colonies

and determined the mean and standard deviation. However, we did not conduct any formal statistical analyses.

RESULTS

1. Investigation findings on dose ranges

We conducted an initial exploratory study to determine the upper dosage limit for the main investigation. The highest dose was set at NPP 100%, which was then serially diluted using a geometric ratio of two to generate lower dose levels (50%, 25%, 12.5%, 6.25%, and 3.13%). We observed no growth inhibition or precipitation attributable to NPP at any dosage level across all strains, regardless of the presence (with the S9 mixture) or absence (without the S9 mixture) of metabolic activation (Fig. 1).

2. Main bacterial reverse mutation test results for NPP

Following the preliminary dose-ranging study, the main investigation revealed that NPP administered at varying doses did not significantly increase in the average revertant colony count across all tested bacterial strains (TA100, TA1535, TA98, TA1537, and WP2uvrA) compared to the negative control group. In the NPP treatment group, the revertant colony count for each strain at all dosage levels remained below two times that observed in the negative control group, regardless of the presence or absence of metabolic activation. We observed no indications of growth inhibition or precipitation induced by NPP across all dose levels for each bacterial strain, regardless of the presence or absence of metabolic activation. Additionally, the validity of the analysis was confirmed as the mean count of revertant colonies per strain in the positive control group exceeded twice that of the negative control group (Table 1, Fig. 2).

3. HPLC analysis results

The HPLC analysis revealed retention times of 29.57 min for tetrahydropalmatine, 12.42 min for quercetin, 32.42 min for coptisine, 14.28 min for paeoniflorin, and 32.33 min for glycyrrhizin. The NPP extract contained 0.80 μg/mL of tetrahydropalmatine and 0.25 mg/mL of quercetin, while coptisine, paeoniflorin, and glycyrrhizin were not detected (Fig. 3).

DISCUSSION

Herbal medicine has been a cornerstone of healthcare for millennia, valued for its therapeutic properties [11]. However,

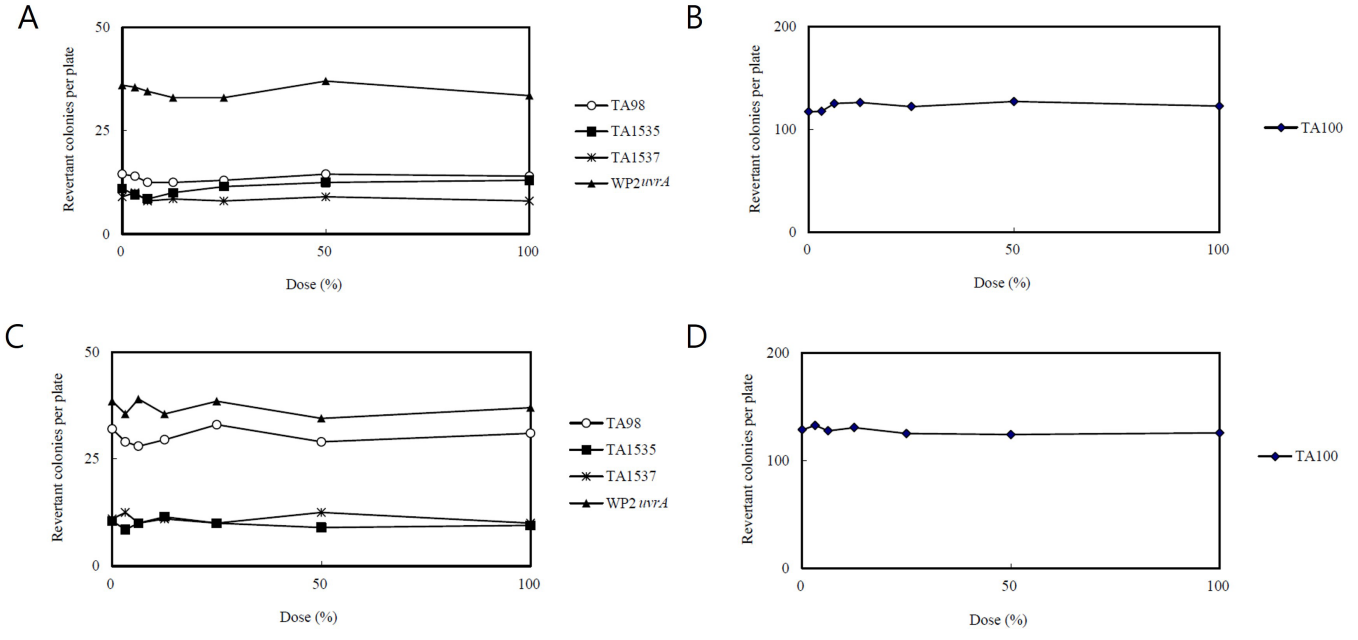

Figure 1. Dose-response curve in the dose-range exploratory study of the bacterial reverse mutation test of NPP. In the absence of metabolic activation in the first and second main studies (TA98, TA1535, TA1537, and WP2uvrA (A), and TA100 (B)). In the presence of metabolic activation in the first and second main studies (TA98, TA1535, TA1537, and WP2uvrA (C), and TA100 (D)).

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Table 1. Results of the bacterial reverse mutation test for the no-pain pharmacopuncture extract in *Salmonella typhimurium* (TA100, TA1535, TA98, and TA1537) and *Escherichia coli* (WP2uvrA) with or without metabolic activation (S9 mixture)

Strain	With S9 mixture				Without S9 mixture			
	Test substance	Dose (%)	First main study Mean ± SD	Second main study Mean ± SD	Test substance	Dose (%)	First main study Mean ± SD	Second main study Mean ± SD
TA98	Saline	0	16 ± 1	28 ± 1	Saline	0	16 ± 1	16 ± 1
	NPP	6.25	18 ± 1	16 ± 1	NPP	6.25	16 ± 1	16 ± 1
		12	17 ± 1	16 ± 1		12	16 ± 1	16 ± 1
		25	17 ± 2	16 ± 1		25	16 ± 1	16 ± 1
		50	16 ± 1	16 ± 1		50	16 ± 1	16 ± 1
		100	15 ± 1	16 ± 1		100	16 ± 1	16 ± 1
	2-NF	5.0 μg/plate	592 ± 11	16 ± 1	2-AA	1.0 μg/plate	16 ± 1	16 ± 1
TA100	Saline	0	116 ± 3	16 ± 1	Saline	0	16 ± 1	16 ± 1
	NPP	6.25	119 ± 3	16 ± 1	NPP	6.25	16 ± 1	16 ± 1
		12	125 ± 3	16 ± 1		12	16 ± 1	16 ± 1
		25	125 ± 4	16 ± 1		25	16 ± 1	16 ± 1
		50	123 ± 3	16 ± 1		50	16 ± 1	16 ± 1
		100	122 ± 4	16 ± 1		100	16 ± 1	16 ± 1
	SA	1.5 μg/plate	638 ± 21	16 ± 1	2-AA	2.0 μg/plate	16 ± 1	16 ± 1
TA1535	Saline	0	13 ± 1	16 ± 1	Saline	0	16 ± 1	16 ± 1
	NPP	6.25	16 ± 1	16 ± 1	NPP	6.25	16 ± 1	16 ± 1
		12	16 ± 1	16 ± 1		12	16 ± 1	16 ± 1
		25	16 ± 1	16 ± 1		25	16 ± 1	16 ± 1
		50	16 ± 1	16 ± 1		50	16 ± 1	16 ± 1
		100	16 ± 1	16 ± 1		100	16 ± 1	16 ± 1
	SA	1.5 μg/plate	16 ± 1	16 ± 1	2-AA	3.0 μg/plate	16 ± 1	16 ± 1
TA1537	Saline	0	16 ± 1	16 ± 1	Saline	0	16 ± 1	16 ± 1
	NPP	6.25	16 ± 1	16 ± 1	NPP	6.25	16 ± 1	16 ± 1
		12	16 ± 1	16 ± 1		12	16 ± 1	16 ± 1
		25	16 ± 1	16 ± 1		25	16 ± 1	16 ± 1
		50	16 ± 1	16 ± 1		50	16 ± 1	16 ± 1
		100	16 ± 1	16 ± 1		100	16 ± 1	16 ± 1
	9-AA	80.0 μg/plate	16 ± 1	16 ± 1	2-AA	3.0 μg/plate	16 ± 1	16 ± 1
WP2uvrA	Saline	0	16 ± 1	16 ± 1	Saline	0	16 ± 1	16 ± 1
	NPP	6.25	16 ± 1	16 ± 1	NPP	6.25	16 ± 1	16 ± 1
		12	16 ± 1	16 ± 1		12	16 ± 1	16 ± 1
		25	16 ± 1	16 ± 1		25	16 ± 1	16 ± 1
		50	16 ± 1	16 ± 1		50	16 ± 1	16 ± 1
		100	16 ± 1	16 ± 1		100	16 ± 1	16 ± 1
	4-NQO	0.3 μg/plate	16 ± 1	16 ± 1	2-AA	10 μg/plate	16 ± 1	16 ± 1

2-AA, 2-aminoanthracene; 2-NF, 2-nitrofluorene; 4NQO, 4-nitroquinoline N-oxide; B(a)P, benzo(a)pyrene; NPP, no-pain pharmacopuncture; SA, sodium azide; SD, standard deviation.

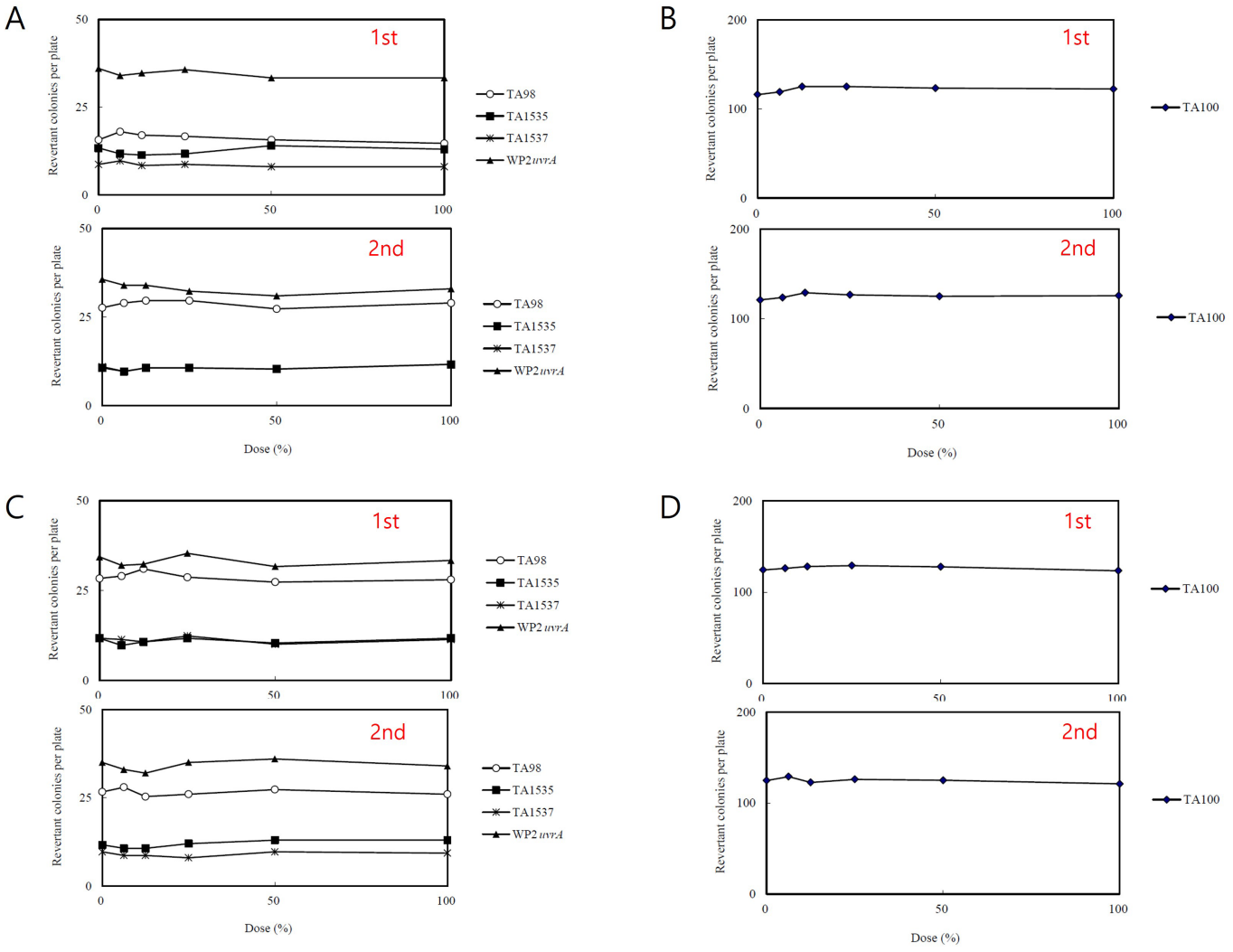

Figure 2. Dose-response curve in the bacterial reverse mutation test of NPP. In the absence of metabolic activation in the first and second main studies (TA98, TA1535, TA1537, and WP2uvrA (A), and TA100 (B)). In the presence of metabolic activation in the first and second main studies (TA98, TA1535, TA1537, and WP2uvrA (C), and TA100 (D)).

amidst increasing awareness of food and drug safety, improvements in global living standards, rising environmental pollution, the influx of foreign medicinal materials, uncertain plant sourcing, and the indiscriminate use of health products and traditional remedies containing herbs, concerns regarding the safety of herbal medicines have escalated [12, 13]. Documentation regarding the quality, safety, and efficacy of common herbal formulas remains limited [14].

The NPP extract comprises a blend of four herbs—CT, CF, PR, and GR—long used in Asian traditional medicine for their analgesic, anti-inflammatory, and muscle-relaxant properties. Numerous studies have validated the effectiveness and safety of each herb [7-10, 15]. Nonetheless, comprehensive evidence for NPP, a novel combination of these herbs, is lacking. Currently, research reports NPP's therapeutic efficacy in a singular case of plantar fasciitis patient [9], while its safety has been demonstrated in a single-dose toxicity study in rat thigh muscle [8]. The absence of genotoxicity is indicated by a rat micronucleus assay [7] and an *in vitro* chromosome aberration assay [10], forming a battery of three assays to assess genotoxicity.

Genotoxicity testing assesses direct DNA or chromosomal damage by a test substance, resulting in morphological changes or functional abnormalities, and serves as a primary screening tool for drug carcinogenicity [16]. Test methods are categorized based on indicators such as gene mutations, chromosomal aberrations, and effects on DNA damage or repair [17, 18].

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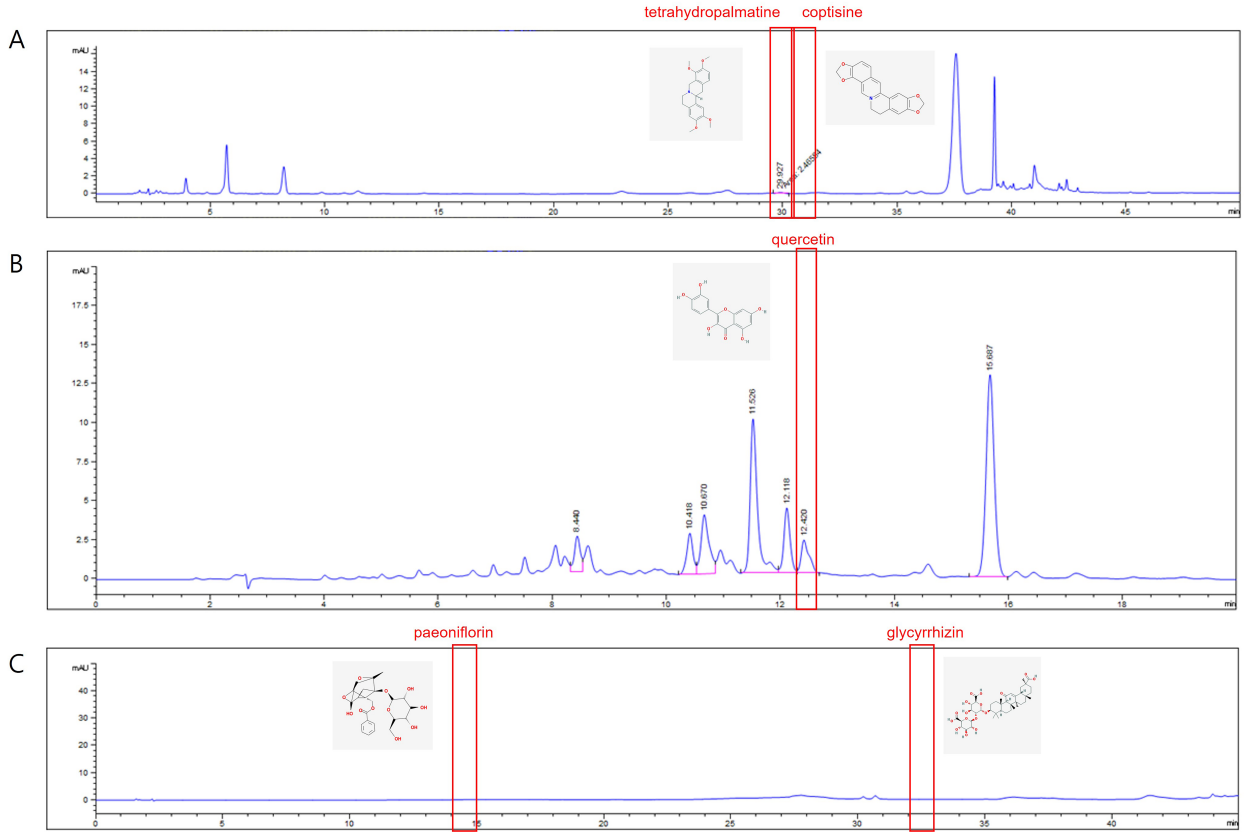

Figure 3. HPLC chromatogram of standard and NPP-derived tetrahydropalmatine, quercetin, coptisine, paeoniflorin, and glycyrrhizin. Identification and quantification of five major compounds of NPP were performed using HPLC analysis. (A) Tetrahydropalmatine and coptisine. (B) Quercetin. (C) Paeoniflorin and glycyrrhizin. HPLC, high-performance liquid chromatography; NPP, no-pain pharmacopuncture.

Given the diverse mechanisms of toxicity, a battery of multiple genotoxicity tests is required rather than relying on a single assessment. The three most common assays include the bacterial revertant mutation test, the *in vitro* chromosomal aberration test, and the *in vivo* micronucleus test [19], which all follow ICH and OECD guidelines [17, 18]. To assess the potential genotoxicity of NPP extracts, we conducted a bacterial reverse mutagenesis test, which was not included in the three-assay battery.

The bacterial reverse mutation test, or the Ames test, is a pivotal screening method using *Salmonella* and *E. coli* bacterial strains to identify potential genetic damage and gene mutations induced by test substances [20-22]. This method was endorsed by the National Toxicology Program following extensive chemical evaluations. A positive outcome in the bacterial reverse mutation test closely correlates with findings from rodent carcinogenicity tests [23]. Among the three standard genetic toxicity tests mandated by the Korea Food and Drug Administration, the revertant mutation test necessitates evaluation across a minimum of five strains, including *S. typhimurium* TA98, TA100, TA1535, TA1537, or TA97, TA97a, TA102, or *E. coli* (WP2uvrA pKM101). Additionally, dose steps must be set at five or more levels, with a minimum requirement of three plates per dose [24, 25]. In this study, to assess the safety profile of the NPP solution, we established five doses of NPP treatment groups: 100%, 50%, 25%, 12.5%, and 6.25%, employing histidine-requiring *S. typhimurium* TA98, TA100, TA1535, and TA1537, and tryptophan-requiring *E. coli* WP2uvrA pKM101. We incorporated commonly used positive controls, such as SA, 2-NF, 2-AA, 9-AA, and 4-NQO, in this study [25].

This study revealed no evidence of mutagenicity induced by the NPP extract across all tester strains compared to concurrent negative controls under both metabolic activation and non-activation conditions. The study ensured the reproducibility of dose-setting and test results, obtaining more than four doses of the test substance with no discernible growth inhibition. Moreover, the mean values of returning colonies for both negative and positive controls fell within the historical control data

range. The number of returning colonies for each strain's positive control significantly exceeded twice that of the negative control. Additionally, no adventitious bacterial contamination was identified, underscoring the study's proper execution and ensuring reliability.

In traditional medical practice, CT is commonly incorporated into medicinal compounds, known for its alkaloid content comprising tertiary amines, quaternary alkaloids, and non-alkaloids [26]. Over 80 alkaloids have been isolated and identified from CT, with corydaline and tetrahydropalmatine distinguished for their potent analgesic effects [27-30]. Tetrahydropalmatine exhibits anti-inflammatory and analgesic properties, stimulates adrenocorticotropic hormone secretion in rat pituitary glands, and possesses analgesic potency comparable to opium at a fraction of the dose [29-32]. Another constituent, quercetin, present in CT, CF, and GR, is a flavonoid with reported analgesic, anti-inflammatory, and antioxidant effects [32]. Additionally, PR contains paeoniflorin and GR contains glycyrrhizin, which both impact pain and inflammation [31, 33-35]. For this study, the literature corroborated the analgesic and anti-inflammatory effects of these five principal compounds in NPP constituent herbs, guiding the subsequent HPLC analysis to confirm their presence. However, we only detected tetrahydropalmatine and quercetin and did not identify coptisine, paeoniflorin, and glycyrrhizin. These compounds may have been lost during the extraction, purification, or dilution processes or were present in trace amounts below the detection threshold. Future studies could use tetrahydropalmatine and quercetin data for NPP quality control and explore a broader spectrum of compounds from each herb to establish their pharmacological efficacy.

CONCLUSION

Our genotoxicity assessments reveal that the NPP extract does not induce mutations in bacterial models. While initial findings from single-dose toxicity evaluations and three genotoxicity assays appear promising, NPP's comprehensive safety profile warrants further investigation. Additional scrutiny through diverse toxicity assessments, human safety trials, and extensive clinical studies are warranted to substantiate NPP's safety and efficacy.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest in this work.

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