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Metabolomic analysis of Thai Herbal Analgesic Formula based on ultra-high-performance liquid chromatography-quadrupole time-of-flight mass spectrometry

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ABSTRACT

Sahatsatara formula (STF), a Thai herbal medicine formula which has been commonly used as analgesic drugs to relieve musculoskeletal pain and numbness in Thai traditional medicine. The pharmacological studies of its ingredients have represented that have anti-inflammatory and antioxidant properties. However, the quality markers (Q-markers) for STF are still unknown and require further investigation. The primary goal of this study was to establish the chemical profile of STF though metabolomic analysis. Untargeted metabolomics were used to analyze global components and accurately qualify compounds. Multivariate analysis (MVA) was used to classify STF extract at three different concentrations and a quality control sample. Furthermore, samples' characteristics and identification-related markers were observed and compounds matched to the Traditional Chinese medicine library in UNIFI software. According to the results, chemical analysis revealed 63 compounds in positive mode and 33 compounds in negative mode within STF. Notably, 19 potential Q-markers were tentative identified in all three concentrations of STF, including alkaloids, terpenes, phenols, organic acids, disaccharides, fatty acids, glycosides, quinonoids, and steroids. The compounds exhibited pharmacological effects such as anti-inflammatory activity, anti-oxidant activity, and analgesic properties, which correlated to traditional properties of STF. Consequently, this study provides insights into the chemical profiles of the STF and identifies potential markers that can be utilized for qualitative and quantitative quality control of STF. Additionally, the findings can also be useful for further research into STF's anti-inflammatory properties through in vitro assays, as well as exploring its clinical efficacy to support evidence-based medicine for STF.

1. Introduction

The Thai herbal Sahatsatara formula (STF) has been used for more than three decades by Thai Traditional medicine doctors to relieve musculoskeletal pain, numbness, and anti-flatulence. It has been registered in the National List of Essential Medicines (NLEM)

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Abbreviations

HM	Herbal medicine
HPTLC	High Performance Thin Layer Liquid Chromatography
GMP	Good Manufacturing Practice
OPLS-DA	Orthogonal Partial Least Square Discriminal Analysis
PCA	Principal Component Analysis
PIC/S	Pharmaceutical Inspection Co-operation Scheme
RSD	Relative standard deviation
TLC	Thin Layer Chromatography
TTM	Thai Traditional Medicine
STF	Sahatsatara formula
WHO	World Health Organization
VIP	Variable Importance in the Projection
UPLC-QTOF-MS	Ultra-Performance Liquid Chromatography coupled with Quadrupole Time-of-Flight Tandem Mass Spectrometry
QTOF	Quadrupole Time-of-Flight

by the Ministry of Public Health since 2011. STF is a polyherbal formula consisting of twenty-one ingredients composed of *Piper nigrum* L., *Plumbago indica* L., *Piper retrofractum* Vahl, *Kleinhovia hospita* L., *Terminalia chebula* Retz., *Baliospermum solanifolium* (Burm.) Suresh, *Acorus calamus* L., 1, 7, 7 – trimethylbicyclo (2.2.1) heptan -2- one, *Myristica fragrans* Houtt. [Mace], *Myristica fragrans* Houtt. [Fruits], *Lepidium sativum* L., *Anethum graveolens* L., *Ferula assa-foetida* L., *Pimpinella anisum* L., *Cuminum cyminum* L., *Merremia vitifolia* (Burm.f.) Hallier.f., *Nigella sativa* L., *Anacyclus pyrethrum* (L.) Lag., *Atractylodes lancea* (Thunb.) DC., *Picrorhiza kurroa* Royle ex Benth., and Retz. Most of ingredients exhibit various pharmacological activities (Supplementary Material, Table S1) [1]. Ensuring quality control of herbal medicines (HMs) is crucial to ensuring consistency, and aligning with the World Health Organization's (WHO) traditional medicine strategy [2]. However, the complex nature of HMs compose of over 2,000 compounds within a single herb [3] presents challenges in identifying specific compound markers in polyherbal formulas. Previous study have focused on the fingerprint patterns of standard piperine compared to piperine in STF using thin layer chromatography (TLC) and quadrupole time-of-flight mass spectrometry (QTOF) [1]. Furthermore, piperine was used for a quantitative marker by using high performance thin layer liquid chromatography (HPTLC) and ultra-performance liquid chromatography (UPLC) approaches [4]. However, relying on a single compound for clinical efficacy and mechanism of action of STF is insufficient.

Metabolomics, a research approach that identifies and quantifies the unique chemical fingerprints of small molecules involved in cellular reactions, referred to as metabolite profiling, hold promise. There are two approaches to metabolomics: targeted and untargeted.; The targeted approach focuses on analyze a specific set of metabolites, while the untargeted approach aims to analyze all metabolites [5–7]. Presently, researchers face challenges in establishing good quality control assessments standardizations for HM worldwide. As a result, untargeted metabolomic analysis is becoming more popular as a technique for discovering new biomarkers and understanding biochemical changes in plant metabolites [8]. Therefore, the goal of this research is to investigate metabolomic profiling of STF using UPLC-QTOF-MSE, UNIFI platform, and multivariate statistical analyses (MVA) such as Principal Component Analysis (PCA) and Orthogonal Partial Least Square Discriminant Analysis (OPLS-DA) to identify potential new biomarkers for STF with pharmacological activities.

2. Materials and methods

2.1. Materials and reagents

The STF powder utilized in this study was produced by the Ayurved Siriraj Manufacturing Unit of Herbal Medicine and Products, Center of Applied Thai Traditional Medicine (CATTM)., The manufacturing process adhered to good manufacturing practice guidelines set by the Pharmaceutical Inspection Cooperation Scheme (PIC/S GMP). Furthermore, all components of the STF powder were authenticated by experienced TTM practitioners from the Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand.

The reference standards used in the analysis included caffeine (3-Methyl-C13,99%), L-Phenylalanine- 1–13C, sodium formate and sodium hydroxide, were purchased from Sigma - Aldrich (Missouri, USA). Cholic Acid-[2H4] was purchased from Isosciences and chemical company (Pennsylvania, USA). Ethanol (AR grade) and 2-Propanol (LC-MS grade) were purchased from Scharlau (Barcelona, Spain). Formic acid, methanol and acetonitrile (Optima™ LC-MS grade) were purchased from Fisher chemical (Loughborough, UK). Leucine Enkephalin was purchased from Waters Corporation (Milford, Massachusetts, USA). Ultrapure water using a Milli-Q water purification system with a resistivity of 18.2 MΩ-cm. (Millipore, Billerica, MA, USA).

2.2. Sample preparation and extraction

The STF powder was subjected to extraction using ethanol 80% at ratio of 1:10 (w/v). The extraction process involved sonication for 1 h, followed by filtration through GF/A filter paper particle filtration size of 1.6 μm (Whatman, England), respectively. The resulting solution was freeze-dried and stored in a desiccator until further use. For untargeted metabolomics analysis, 1 mg of STF powder extract was dispersed in 1 mL of absolute methanol and vortexed for 10 min. The mixture was then centrifuged at 12,000 rpm for 5 min at 4 °C and serial dilution at 25, 50, 100 μg/mL with absolute methanol were prepared. The supernatant was filtered through 0.2 μm polyvinylidene difluoride (PVDF) hydrophilic syringe filter, resulting in a final volume of was 1 mL, including the internal standards (IS) solution. The prepares samples were injected into the ultra-performance liquid chromatography coupled with quadrupole time-of-flight tandem mass spectrometry (UPLC-Q-TOF-MS^E) system. To ensure analysis stability and suitability, a quality control (QC) sample was prepared by pooling and adding 100 μL of the QC sample to each sample. The QC sample contained all of the components present in the analysis. All samples were stored at 4 °C, and the injection volumes for all samples were all 5 μL, respectively.

2.3. Ultra-high-performance liquid-chromatography with quadrupole time-of-flight tandem mass spectrometry conditions

Chromatographic analysis was conducted using a Waters Acquity UPLC® system (Waters Corporation, Milford, USA). The protocol for LCMS-QTOF analysis was adopted from a previous study [9]. Chromatographic separation was carried out at 40 °C, using a Waters ACQUITY HSS T3 (100 mm × 2.1 mm, 1.8 μm) column with a guard column. The mobile phases consisted of eluent A (0.1% formic acid aqueous solution) and eluent B (0.1% formic acid in absolute methanol). The flow rate was set at 0.4 mL/min. The gradient profiles were as follows: 0–1 min, 0%–0% B; 1–16 min, 0%–100% B; 16–20 min, 100%–100% B; 20–22 min, 100%–0% B; 22–24 min, 0%–0% B.

Mass spectrometric detection was performed by Waters® SYNAPT G2-Si mass spectrometer (Waters Corporation, Milford, USA) with positive and negative ion detections with electrospray ionization (ESI) source for acquisition in MS^E mode to investigate untargeted metabolomic analysis. The MS^E mode set up as full scan mode which allowed both precursors and fragmentation data to be simultaneously acquired during a single run. This method consisted of three functions; low energy (LE) applied collision energy of 4 eV, high energy (HE) acquired spectra through ramp trap collision energy of 15–40 eV and the lock mass data for internal on-the-fly mass calibration. The MS mass scanned range 50–1,200 m/z with a scan time of 0.5 s in continuum mode, preserving the peak shape of the exact-mass precursor and product ions. The source conditions in resolution mode: capillary voltage 3 kV, sample cone 40 V, source offset 80 V, source temperature 150 °C, desolvation temperature 500 °C, cone gas flow rate 50 L/h, and desolvation gas (N2) flow rate at 1,000 L/h. During data acquisition, the mass to charge ratio (m/z) was 200 pg/mL solution of leucine enkephalin (Waters, USA) infused continuously at 5 μL/min.via a lock spray interface. This generated were reference in positive mode at m/z 556.2771 $[M+H]^+$ and negative mode at m/z 554.2615 $[M-H]^-$ to ensure mass accuracy and reproducibility.

2.4. Data analysis of STF

Data acquisition and processing were performed using MassLynx™ V4.2 software and UNIFI 1.8.0 platforms (Waters, Manchester, UK) to investigated untargeted metabolomics profiles of STF. Initially, the raw data was compared with Waters Traditional Chinese medicine library in both modes. Tentative identification of major compounds was conducting using the following parameters: retention time (RT) range of 0–24 min, target match tolerance ±10 ppm. high energy intensity threshold 1,000 counts, low energy intensity threshold 50,000 counts to reduce background noise and adducts form were selected H+, Na+, NH4+ for positive ionization mode and H-, CHOO-, CH3COO- for negative ionization mode. Subsequently, a workflow was implemented to filters the data using following parameters: RT range of 0.5–20 min, mass error ±5 ppm. and a response more than 50,000 counts. Furthermore, the potential marker from UNIFI software were identified and elucidated using online databases including ChEBI, ChEBML, NIST, KEGG, ChemSpider, Pubchem, and Pubmed. All identify herbal components were reported to have related pharmacological activities in STF.

2.5. Metabolomic analysis

To investigate the untargeted metabolomics based on UPLC-QTOF-MS^E combined with multivariate analysis (MVA). It was performed by UNIFI software transfers the data to EZinfo software (Waters Corp., MA, USA). It was used the MS^E raw data. The parameters were set as follows: mass range 100–1,500 Da, RT range 0–24 min, mass tolerance 0.10 mDa, minimum intensity 5%, marker intensity threshold 1,000 counts, RT window 0.20 min, RT and m/z were set similarly. Multiple injections and samples were compared. This study used multivariate statistical analysis, including principal component analysis (PCA) and orthogonal projections to latent structures discriminant analysis (OPLS-DA). The PCA score plots revealed distinct differentiation among the three concentrations of STF. Subsequently, an OPLS-DA predictive component and S-plot were generated to visualize the differences between STF concentration markers. To identify significant metabolites, markers were filtered based on their variable importance in the projection (VIP) values. Metabolites with VIP value > 2 and p-value <0.05 were considered statistically significant.

3. Results

3.1. Metabolomic profiles and tentative identification compounds of STF

In this study, the metabolic profiling of STF was represented the base peak intensity (BPI) chromatograms of STF at concentration of 25, 50, and 100 μg/mL as shown in Fig. 1 (A-G). To ensure quality in the metabolomics data, the percentage relative standard deviation %RSD was calculated to assess the repeatability of procedures in this experiment. The %RSD was calculate on the area of each identification compound obtained from five injections. The RSD values (X) were classified into three categories: $0 < X \leq 10\%$, $10 < X \leq 20\%$, or $20 < X \leq 30\%$. The compounds showing %RSD of most identification compounds below 10% and less than 30% (Supplementary Material, Fig. S1).

A comprehensive analysis of chemical compounds that matched TCMs library in The UNIFI software was performed based on retention time. The highest representation of matched compounds was observed in the STF sample at a concentration of at STF extracts 100 μg/mL in both ESI^+ and ESI^- modes (Supplementary Material, Figs. S2–S5). The total number of tentative identification compounds that matched the TCM library of each mode was 63 compounds in positive ionization mode (Supplementary Material, Table S2) and 33 compounds in negative ionization mode (Supplementary Material, Table S3). Nineteen compounds were represented or tentatively characterized in all three STF concentrations, as shown in Table 1. These compounds enriched the STF with phytochemical components including five alkaloids, five terpenes, two phenols, two organic acids and disaccharides, fatty acids, glycosides, quinonoids and steroids. All compounds were identified based on mass fragmentation patterns, which were confirmed by comparing them with patterns reported in literature.

3.2. Biomarker discovery of STF

PCA is an efficient unsupervised clustering tool to observe the clustering trends between individual samples for finding the similarities or differences among STF samples at concentration 25, 50, and 100 μg/mL. The PCA scores plot showed STF extracts concentrations at 25, 50, 100 μg/mL and pool QCs sample (Fig. 2). The QCs sample were clustered in PCA which performed stability of the system. In addition, The PCA also classified three concentration of STF extracts were distinctly separated to the clusters in both ionizations mode. The numbers of sample injections were described as follows: STF extracts 25 μg/mL (n = 5), STF extracts 50 μg/mL (n = 5), and STF extracts 100 μg/mL (n = 5). A total of 557 variables (positive mode) and 313 variables (negative mode) were analyzed. The PCA scores plots of data were shown in Fig. 2. Two parameters, R^2X [1] and R^2X [2] are used to which provided R^2X [1] = 0.4365 and R^2X [2] = 0.2628 in positive mode and R^2X [1] = 0.3019 and R^2X [2] = 0.1645 in negative mode. These parameters suggested the model performed a relatively more excellent prediction in positive mode than negative mode.

In order to distinguish different concentrations of STF, OPLS-DA models were established in both modes. OPLS-DA analysis, S-plot and VIP value were employed for compound separation. A comparison between the three STF concentrations groups were showed better differentiation in positive and than negative mode. The variables to distinguish the data composing of t [1] (x-axis) represented the group differences for each STF concentration, while to Ref. [1] (y-axis) represented score differences within the class. Each point in the score plot according to an observation and variations can occur due to systematic changes in the experimental plan, as depicted in Fig. 3 (A-F).

Supplementary Material, Fig. S6, demonstrates the coefficients versus VIP values. X-variables with positive VIP values and large positive/negative coefficient values represent compounds that were similarly present in STF concentrations of 25, 50, 100 μg/mL in both positive and negative modes. Additionally, Fig. 3 (A-F) the covariance p [1] and correlation p(corr) [1] loadings from a two class OPLS-DA model, visualized in the in S-Plot. The points in the S-plot represent Exact Mass/Retention Time pairs. In both modes, the upper right quadrant of each S-plot, when comparing the STF concentrations showed components that were more abundant in higher

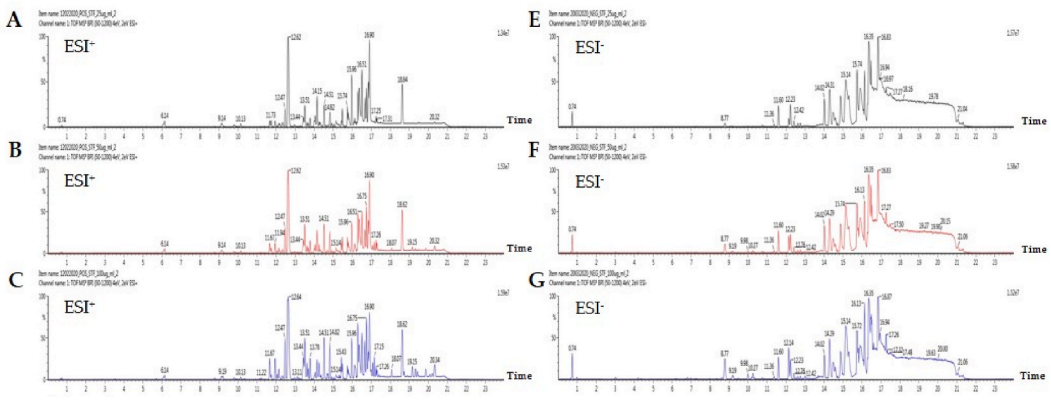

Fig. 1. Base peak intensity (BPI) chromatograms of STF at 25 μg/mL (A), 50 μg/mL (B) and 100 μg/mL (C) in positive mode (ESI^+); at 25 μg/mL (E), 50 μg/mL (F) and 100 μg/mL (G) in negative mode (ESI^-).

Table 1
Identified STF compounds and components source by UPLC-QTOF-MS^E.

No.	RT (min.)	Formula	Adduct	Calculate Mass (Da)	Theoretical Mass (Da)	Mass Error (ppm)	MS^E Fragmentation	Identification	Phytochemical group
1	0.74	$C_{12}H_{22}O_{11}$	$[M-H]^-$	342.1158	342.1162	−1.24	173.0447,191.0552	β-Gentiobiose	Disaccharides
2	8.78	$C_{14}H_6O_8$	$[M-H]^-$	302.0060	302.0063	−0.96	174.9523	Ellagic acid	Phenols
3	11.96	$C_{16}H_{17}NO_3$	$[M+H]^+$	271.1199	271.1208	−3.56	201.0529,135.0428	Piperyline	Alkaloids
4	12.14	$C_{30}H_{48}O_6$	$[M+Na]^+$	504.3456	504.3451	0.97	201.0525, 135.0428	Esculentagenic acid	Terpene
5	12.14	$C_{16}H_{16}O_3$	$[M+NH_4]^+$	256.1090	256.1099	−3.37	201.0525, 135.0428, 115.0530	Orchinol	Phenols
6	12.47	$C_{14}H_{16}N_2O_5$	$[M+NH_4]^+$	292.1068	292.1059	2.85	135.0428, 288.1588	Astragaline E	Alkaloids
7	12.47	$C_{17}H_{21}NO_3$	$[M+Na]^+$	287.1513	287.1521	−2.96	135.0428, 138.0901	Piperanine	Alkaloids
8	12.64	$C_{17}H_{19}NO_3$	$[M+Na]^+$	285.1358	285.1365	−2.26	201.0536, 115.0527, 135.0428,171.0430	Piperine	Alkaloids
9	13.51	$C_{19}H_{18}O_3$	$[M+NH_4]^+$	294.1248	294.1256	−2.40	141.0690, 169.0634, 227.0690	IsotanshinoneIIA	Quinonoids
10	13.63	$C_{14}H_{22}O$	$[M+NH_4]^+$	206.1661	206.1671	−4.12	–	Longicamphenylone	Terpene
11	13.79	$C_{19}H_{22}O_3$	$[M+NH_4]^+$	298.1565	298.1569	−1.26	135.0429, 211.0994	Ostruthin	Terpene
12	14.52	$C_{21}H_{27}NO_3$	$[M+Na]^+$	341.1986	341.1991	−1.49	135.0426, 293.1790	Pipernonaline	Alkaloids
13	15.81	$C_{16}H_{26}$	$[M+CH3COO]^-$	218.2034	218.2035	−0.34	–	Patchoulene	Terpene
14	16.15	$C_{21}H_{40}O_4$	$[M+Na]^+$	356.2921	356.2927	−1.59	319.1940	2,3-Dihydroxypropyl oleate	Organic acid
15	16.69	$C_{20}H_{34}O_2$	$[M+Na]^+$	306.2556	306.2559	−0.96	295.1941, 279.2162	ent-Kauran-16β,17-diol	Terpene
16	16.95	$C_{20}H_{36}O_2$	$[M+Na]^+$	308.2708	308.2715	−2.35	319.1939, 270.2419	Ethyl linoleate	Fatty acid
17	17.32	$C_{22}H_{42}O_2$	$[M-H]^-$	338.3183	338.3185	−0.57	183.0121	Erucic acid	Organic acid
18	17.41	$C_{36}H_{62}O_8$	$[M-H]^-$	622.4428	622.4445	−2.74	385.1906, 242.9431, 130.9660	Ginsenoside Rh2	Glycosides
19	17.41	$C_{35}H_{60}O_6$	$[M+Na]^+$	576.4404	576.4390	2.29	469.3297, 397.3824	β-Daucosterol	Steroids

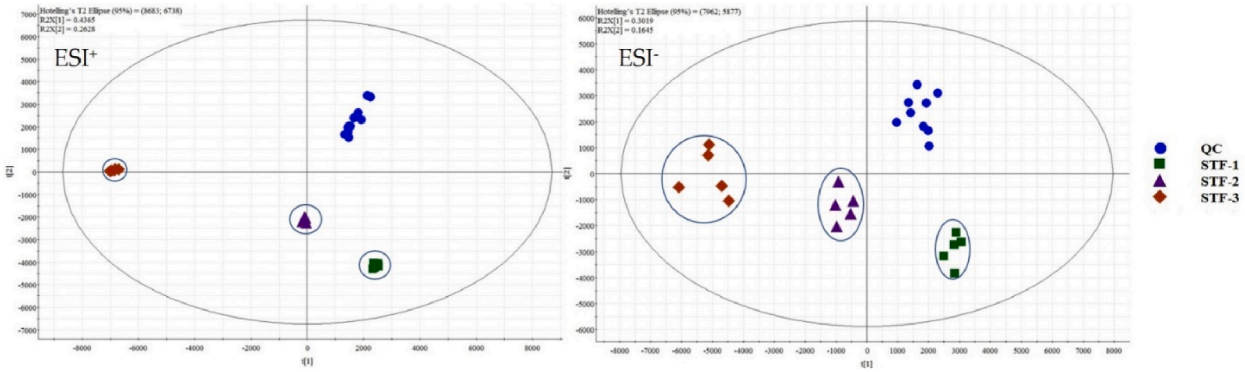

Fig. 2. Principle component analysis scores plot (PCA) of STF at 25 μg/mL (STF-1), 50 μg/mL (STF-2) and 100 μg/mL (STF-3) in positive (ESI^+) and negative mode (ESI^-). QC: Quality Control.

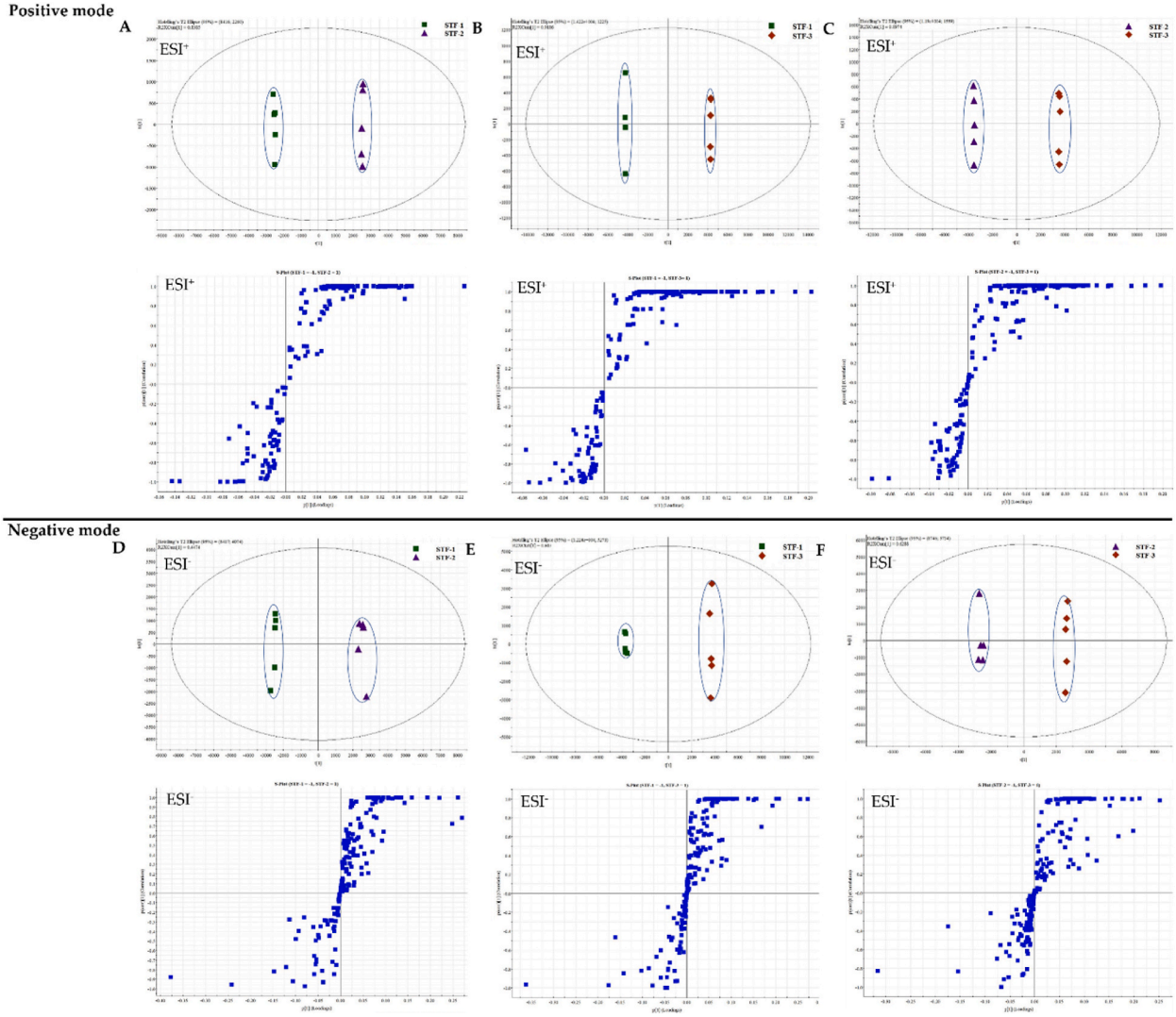

Fig. 3. The OPLS-DA/S-plot of STF at 25 μg/mL (STF-1) and 50 μg/mL(STF-2) (A), 25 μg/mL (STF-1) and 100 μg/mL (STF-3) (B), 50 μg/mL (STF-2) and 100 μg/mL (STF-3) (C) in positive mode (ESI^+); at 25 μg/mL (STF-1) and 50 μg/mL(STF-2) (D), 25 μg/mL (STF-1) and 100 μg/mL (STF-3) (E), 50 μg/mL (STF-2) and 100 μg/mL (STF-3) (F) in negative mode (ESI^-).

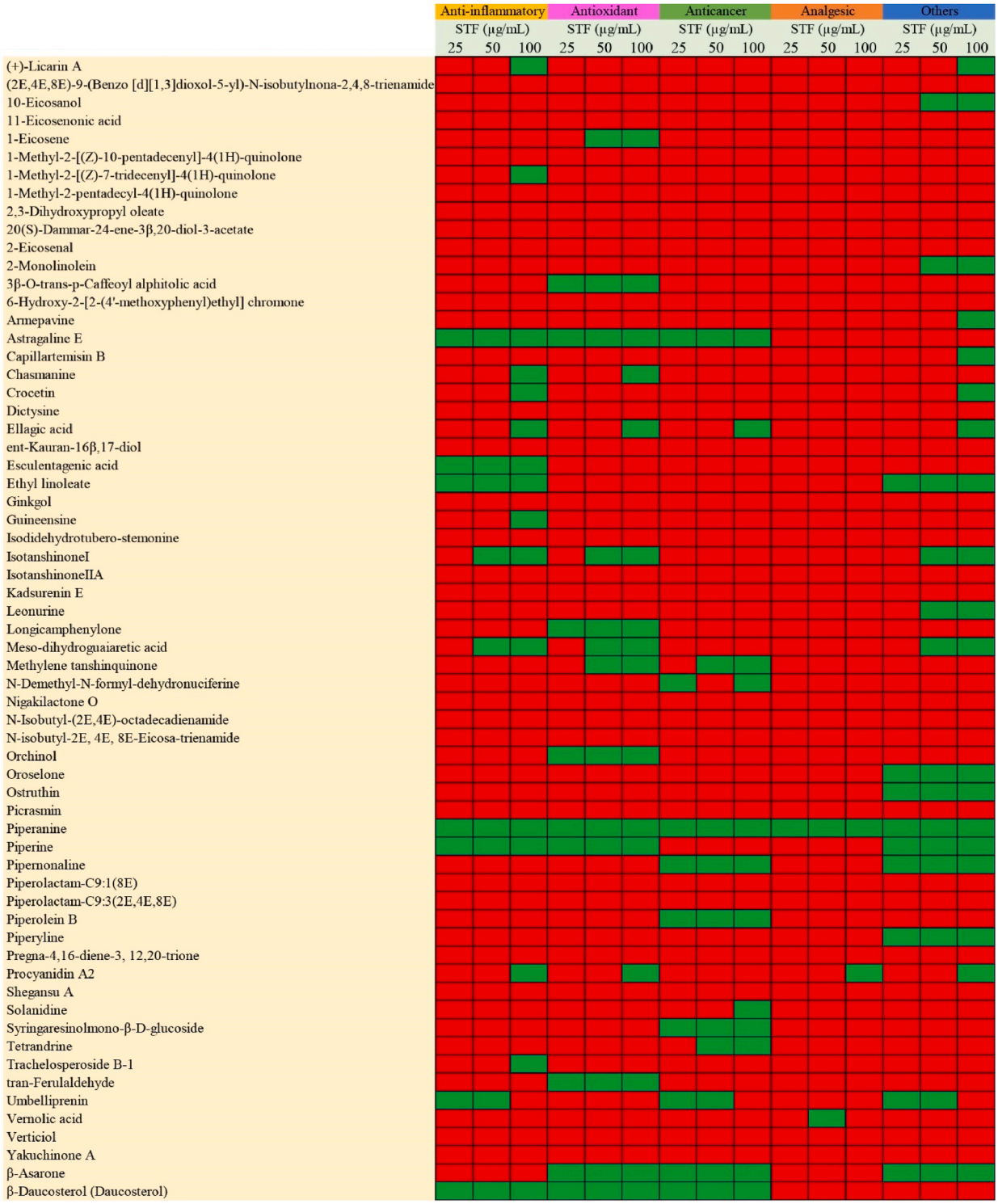

Fig. 4. a. The heat map represents the pharmacological activities of tentatively identified compounds that match the TCM library related to concentration of STF extracts at concentrations 25, 50, 100 μg/mL in positive ionization. b. The heat map represents pharmacological activities of tentatively identified compounds that match TCM library related to concentration of STF extracts at concentrations 25, 50, 100 μg/mL in negative ionization.

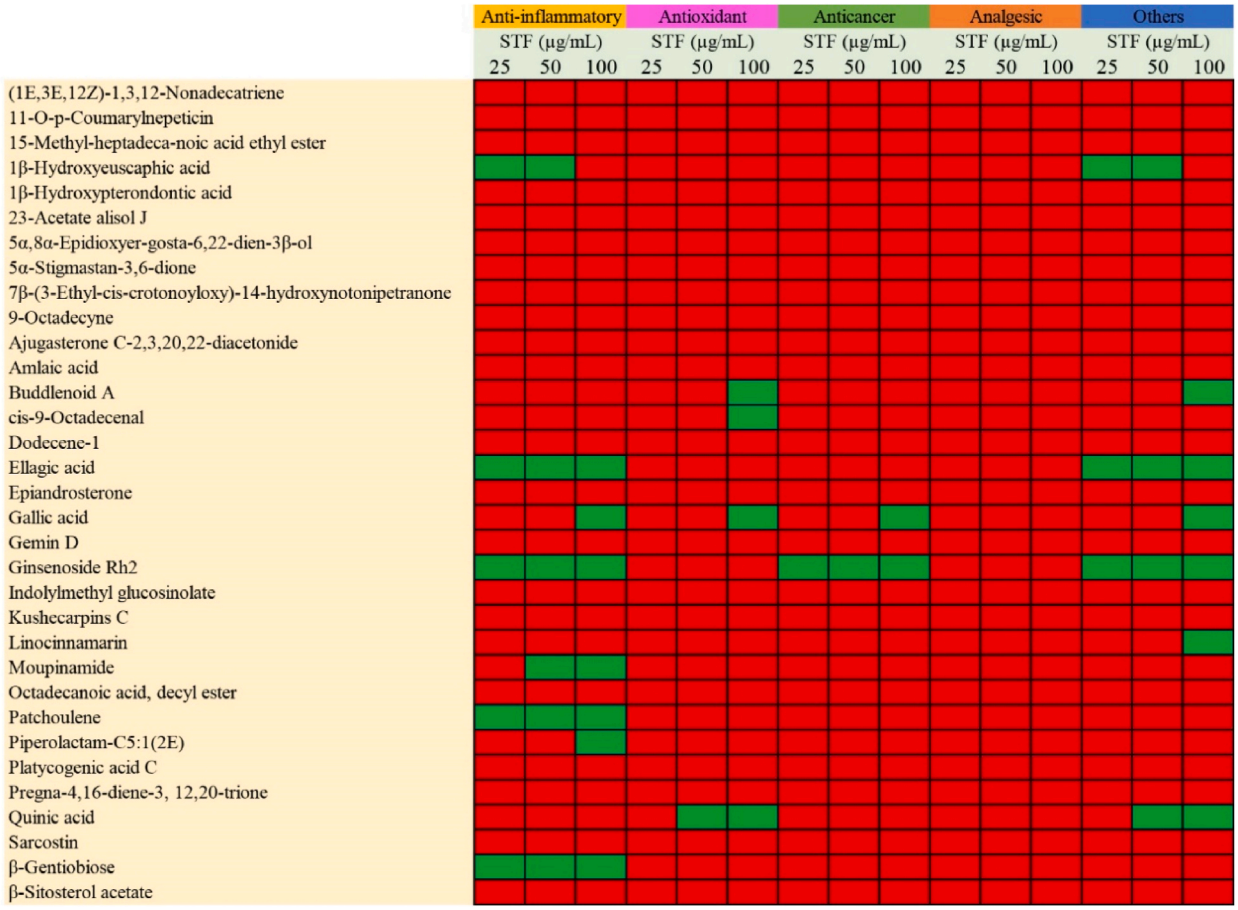

Fig. 4. (*continued*).

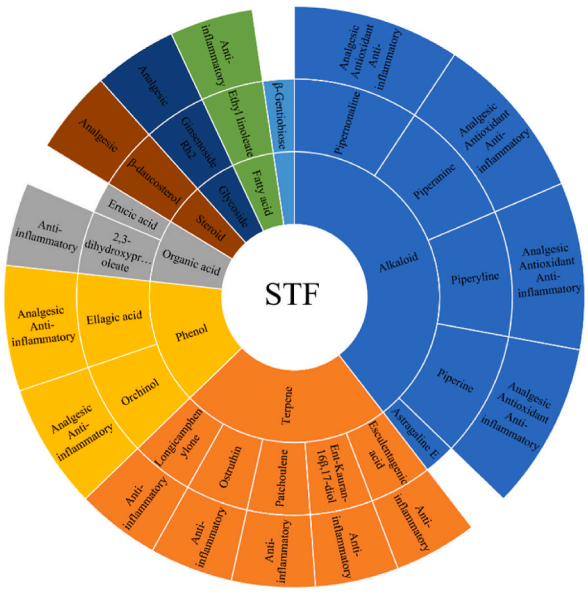

Fig. 5. A summary diagram of chemical compounds identification of STF at all three concentrations related to pharmacological effects based on literature.

STF concentrations. Conversely, the positive mode revealed more pronounced compound differences, while the lower left quadrant shows lower concentration results. Moreover, the x-axis represented the contribution between the groups, while the y-axis represented the reliability based on VIP values greater than >2 and p-values less than 0.05. Compound identification varied among the three concentrations of STF extract (Supplementary Material, Tables S2–S3).

3.3. Chemical profile markers

Nineteen identified compounds were represented in all three concentrations, making them suitable Q-markers for STF. The mass spectra of these identified compounds in STF at the three concentrations are depicted in Supplementary Material, Figs. S7–S25. The results were further supported by the presence of six major ingredients in STF *Piper nigrum* L. (PN); piperine, piperanine, piperyline and pipernonaline, *Plumbago indica* L. (PI), *Piper retrofractum* Vahl (PR); piperine, piperanine and pipernonaline, *Terminalia chebula* Retz. [Fruit] [10]; ellagic acid, *Acorus calamus* L. (AC) and *Baliospermum solanifolium* (Burm.) Suresh [10]. These ingredients accounted for more than 80% of total weights. Futhermore, the pharmacological effects of compounds in STF at each concentrations were reviewed on previous literature, as shown in Figuer 4a-4b and summarize, as shown in Fig. 5.

4. Discussion

The method development of untargeted metabolomics approach for discover quality marker of STF in this study was controlled and prevented experimental bias by monitoring several parameters to ensure accuracy and reproducibility of method and instrument during analysis. The procedure was controlled since study design until post-analytical data. To begin with pre-analytical stage; we kept sample extraction in container protective from light besides desiccator was used to preserved sample stability which were effect from environment temperature and humidity. Next, in analytical stage, we selected the internal standard which was not presented in nature or in the herbal samples that is radioactive reference standards including caffeine-C13 for positive ionization mode and cholic-[2H4] for negative ionization mode to observed intensity and peak area along the experiment by injected at the beginning and the end of analysis then spiked in all samples and QCs. During analysis lock mass was injected then the data was observed until finish acquisition [11]. The final step was post-analytical stage were controlled data processing method for tentative compound identification by creating method subjected to data acquisition that was MSe in both ionization modes in the UNIFI platform then set parameters were matched with TCMs library. However, no quantification was provided. As a result, the study preferred untargeted metabolomics screening to suggest new quality marker for STF by matching and identifying compounds in the TCMs library and confirm by public online databases and explore relative pharmacological activities in previous literature studied [12]. Interestingly, based on the result targeted metabolomics research should be done for further.

A potential quality marker throughput UPLC-QTOF/MS for untargeted metabolomics profiles in STF presence of nineteen compounds in three concentration which were consistent. Among these compounds, 14 were identified in positive ionization mode and five in negative ionization mode. The primary classification of phytochemicals in STF was alkaloids for five tentative identification compounds. There were determined in seed of *Piper nigrum* L. and fruit of *Piper retrofractum* Vahl. in STF about 33.6% of total formula weight; piperyline, piperanine, piperine, pipernonaline. These compounds have presented several pharmacological effects such as anti-inflammatory, antioxidant, anti-mutagenic, anti-cancer effects, antidepressant-like effects, anti-diarrheal properties, protective to gastric ulcer, antifungal activity, and analgesic [13,14].Notably, astragaline E was not previously study reported associated with several pharmacological effects. Another, phytochemical group identified was five terpenes including monoterpenoid and compound such as; ostruthin, patchoulene. diterpenoids and sesterterpenoids; ent-Kauran-16β,17-diol, longicamphenylone. triterpeniods; esculentagenic acid. The pharmacological effects of terpenes include antifungal activity, antibacterial activity and anti-inflammation properties [11,12,15]. Polyphenols, represented by compounds like ellagic acid [16] in fruit of *Terminalia chebula* Retz. contained 10.4% was also identified in STF and orchinol was not identified previously studied in STF ingredients. This compound associated with anti-inflammatory and analgesic properties [16]. One quinonoid derivatives; isotanshinoneIIA was not previously reported in literature review as a component of STF ingredients. IsotanshinoneIIA have demonstrated anti-inflammatory properties and are commonly used in Chinese herbal medicine as well as to remove blood stasis and [17]. One organic ester; dihydroxypropyl oleate was shown anti-inflammatory effect. One glycoside; ginsenoside Rh2, one steroid, β-daucosterol or β-Sitosterol β-D-glucoside found in root of *Plumbago indica* L. contained 22.4%.The pharmacological effect was the pain relief [18].Otherwise, three tentative identification compounds still imprecise on its related pharmacological activities including disaccharide; β-Gentiobiose, fatty acid; ethyl linoleate and organic ester; erucic acid that suggest to studied therapeutic properties in further. The majority of the phytochemical properties of the identified STF compound were associated with traditional uses such as anti-inflammatory, antioxidant, and analgesic that related to traditional usage which described by hot tasted of recipe can increase fire and stimulate wind element for pain relieving. However, previous studied was reported isolate piperine 10 mg/kg bw/day effect on maternal reproductive and embryonic development in mice. Besides piperine was reported inhibit cytochrome P-450 (CYP3A4, CYP2E1, CYP2C9) it may lead to increase drug bioavailability [19]. NLEM was described contraindication of STF used with pregnant women and be aware herb-drug interaction including phenytoin, propranolol, theophylline and rifampicin. Additionally, Ellagic acid has safety when used as additives for food [20]. Thus, no data reported about potential toxicity in others compounds. As the TTM theory, the structure of HMs formula play the critical role for TTM aspects, it compose of main, secondary and seasoning ingredients that will increase or decrease efficacy in the formula when used the herb combination. Therefore, the herb-drug interactions study about piperine in STF still imprecise need more investigate and report for further.

Different STF concentrations of STF 25, 50, and 100 g/mL was more pronounced in the positive mode than negative mode in PCA

analysis. However, plumbagin a principal component of *Plumbago indica* L. (Supplementary Material, Table S1), was not detected in our analysis. In addition, *Plumbago indica* L. was found 22.4% of STF samples, or as a secondary ingredient in the formula. This result suggests that the presence of a main constituent in the formula does not make it a reliable biomarker. Many external factors, such as quality of raw material, and the part of the plant used can have an impact on metabolomics profile data. Environmental condition until harvest time are also concern in HMs quality control.

Furthermore, the processing parameters used in the UNIFI software had limited ranges of mass error, retention time, and response, which might have resulted in low intensity and higher mass error in our results. Other studies have used wider ranges of response including values $\geq$ 200 counts to capture a greater number of compounds [21].

To summarize, our study identified or tentatively characterized nineteen chemical substances in STF beneficial for national herbal standardization and traditional indications of STF based on previous pharmacological studies on its components. These untargeted methods also be applied to other TTM formulas. Furthermore, our results can serve as a foundation of valuable evidence-based medicine for future research, including the development of a method for quantifying STF using certified reference standards, and in *vitro* studies employing anti-inflammatory assays. Furthermore, this work could provide information for clinical research on STF treatment and investigation into the metabolic pathways and mechanism of action of STF.

5. Conclusion

This study provides evidence of STF chemical profiles which we found were not only piperine as a Q-marker, but also the nineteen chemical components in STF. These include alkaloids (piperyline,astragaline E, piperanine, pipernonaline), terpene (long-icamphenylone, ostruthin, patchoulene, ent-Kauran-16β,17-diol, esculentagenic acid). phenols (ellagic acid, orchinol), steroids (β-daucosterol), quinonoids (IsotanshinoneIIA), disaccharides (β-Gentiobiose), glycosides (ginsenoside Rh2), fatty acid (ethyl linoleate) and organic acids (Erucic acid, 2,3-dihydroxypropyl oleate) with related pharmacological effect to TTMs aspects. These findings fill a data gap in terms of quantitative quality control determinations that can be used in the future. Additionally, this study identified potential STF markers that could be used to standardize and control herbal quality in terms of therapeutics and contraindications. It is also useful for future research of STF in *vitro* anti-inflammatory assays and human metabolite analysis to reveal metabolic pathways.

Declarations

Author contribution statement

Pravit Akarasereenont: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Manmas Vannabhum: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Natchaya Ziangchin: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Puthida Thepnorarat: Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e18296.

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