JOURNAL OF TROPICAL LIFE SCIENCE
2024, Vol. 14, No. 3, 643 – 650
http://dx.doi.org/10.11594/jtls.14.03.19

Research Article

Phytochemical Profile and Antioxidant Activity of Hydro-distillated from *Gynostemma pentaphyllum* Makino with Hydrogen Gas

Min Young Kim *

Toxicology Laboratory, Department of Biotechnology (Biomaterials), College of Applied Life Science, Jeju National University, Jeju, 63243, Republic of Korea

Article history:
Submission April 2024
Revised September 2024
Accepted September 2024

**Corresponding author:*
E-mail: jeffmkim@jejunu.ac.kr

ABSTRACT

Hydrogen-rich *Gynostemma pentaphyllum* Makino distillate (HRGD) is made by distilling *G. pentaphyllum* Makino with hydrogen gas to produce the final product. The study evaluated the total phenolic and flavonoid contents of HRGD, as well as its antioxidant properties, including scavenging activities for various radicals such as 2,2-diphenyl-1-picryl-hydrazyl (DPPH), superoxide anion (O_2^-), hydrogen peroxide radical (H_2O_2), nitric oxide radical ($NO^\bullet$) and 2,2´-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$), along with a ferrous ion chelating assay. Total phenolic and flavonoid contents were 42330 mg gallic acid and 36300 mg rutin equivalents/100 g, respectively. HRGD exhibited significant antioxidant and free radical scavenging activities *in vitro*, which were dose-dependent and superior to those of standard antioxidants. HRGD exhibited higher scavenging activities for DPPH, O_2^-, H_2O_2, $NO^\bullet$ and $ABTS^{\bullet+}$ radicals, as well as ferrous ion chelating activity than standard antioxidants ($p < 0.05$). The results showed that HRGD could be an economical and readily accessible source of natural antioxidants and a possible food and pharmaceutical supplement.

Keywords: Antioxidant activity, Hydrogen-rich Gynostemma pentaphyllum Makino distillate, Phytochemical compounds

Introduction

Plants harbor compounds that can serve as active pharmacological products. *Gynostemma pentaphyllum* (Thunb.) Makino, a medicinal herb from the Cucurbitaceae family, is widely utilized in various Asian countries for its health benefits [1, 2]. It has been incorporated into foods and supplements. Recent pharmacological research has unveiled numerous advantageous properties of *G. pentaphyllum* Makino, including antimicrobial, anticancer, anti-aging, antifatigue, antiulcer, hypolipidemic, and immune-modulatory activities [1-5]. These benefits are attributed to saponins and flavonoids [1-5].

Until now, the majority of research on these species has concentrated on the chemical composition and pharmacological properties of *G. pentaphyllum* Makino, leaving the study of the plant's extraction technique for enhancing functional activity largely unexplored. Wang *et al.* (2017) have investigated the physicochemical characteristic and antioxidant capacity of *G. pentaphyllum* seed oil (GPSO) which was extracted by supercritical CO_2 fluid extraction (SFE) [6]. They reported that GPSO has direct and potent antioxidant activities; it may be due to several constituents with potential healthy biological properties, such as unsaturated fatty acids, tocopherols, phytosterols, and other lipid constituents [6]. SFE is a separation technology that uses supercritical fluid solvents (CO_2) for extraction [7]. In supercritical conditions, temperature does not significantly affect extraction yield; however, an increase in pressure enhances the extraction rate by improving oil solubility [7].

Steam distillation extraction, the method used in this study for *G. pentaphyllum* (Thunb.) Makino extraction is a technique used to extract compounds from a sample using steam [8,

How to cite:
Kim MY (2024) Phytochemical Profile and Antioxidant Activity of Hydro-distillated from *Gynostemma pentaphyllum* Makino with Hydrogen Gas. Journal of Tropical Life Science 14 (3): 643 – 650. doi: 10.11594/jtls.14.03.19.

9]. Steam distillation utilizes the volatility of a compound, allowing it to evaporate when heated with steam, and its hydrophobic properties facilitate separation into an oil phase during condensation [8, 9]. Previous study suggests that the highest recovery of saponins, which are the main functional ingredient of *G. pentaphyllum* Makino can be achieved with steam distillation extraction [10].

The potential for preventive and therapeutic applications of Hydrogen (H_2) has now been confirmed in various diseases. To date, multiple studies have shown that H_2 has beneficial effects in diverse animal models and human disease [11-13]. H_2 offers a safe and effective way of disease treatment and promotes researchers to reconsider the significance and benefits of medicinal gas in the human body, including its anti-oxidant, anti-inflammatory, and anti-allergic effects [11-13]. Moreover, it is classified as a food additive: The use of hydrogen as a reducing agent is proposed to conserve the quality of different foods such as fresh white cheese, dairy beverages enriched with polyunsaturated fatty acids, pasteurized orange juice, and strawberries [14]. The aim of this study was to determine the physicochemical composition of hydrogen-rich *G. pentaphyllum* (Thunb.) Makino distillate (HRGD), which can be used as sources of functional ingredients for food, nutraceutical and pharmaceutical applications, and also to qualify their total phenolics and anti-oxidant potentials.

Material and Methods

Preparation of HRGD

HRGD sample was provided by Youngmul company (Jeju, Korea). The whole plant of *G. pentaphyllum* (Thunb.) Makino was plucked in February 2019 from Mt. Halla on Jeju Island, South Korea. The whole fresh plant was washed using tap water, drained, and air-dried overnight. The dried *G. pentaphyllum* (Thunb.) Makino was soaked in water and extracted using a low-temperature vacuum distiller (Komachine Cosmos 660) (Kyunseo E&P, Incheon, Korea) [15, 16]. Steam distillation is a process in which a liquid is made to evaporate at a lower temperature (45-47℃) than normal [8, 9, 15]. The first distillate was filtered to exclude any precipitates, infused with dissolved hydrogen gas (400 ppb), and condensed using a rotary evap-orator (Buchi Rotavapor R-200, New Castle, DE, US). The concentrated extract was then lyophilized.

Determination of physicochemical constants

Physicochemical constants like loss on drying, extractive values, total ash value, and pH were determined as per methods described by WHO (1998).

Loss on drying

After precisely weighing it in a tarred evaporating dish, as much as 10 g of *G. pentaphyllum* (Thunb.) Makino was placed in (without first drying). This was weighed after five hours at 105℃. Drying and weighing were carried out at one-hour intervals until the weight remained constant. Two sequential weights, after drying for 30 min. and cooling for 30 min. in a desiccator, revealed not more than 0.1 g change, therefore reaching constant weight.

Extractive Values (Successive)

A certain amount of *G. pentaphyllum* (Thunb.) Makino was acquired, and the sugars were extracted by releasing it in cold water. The extracts were then dried in a desiccator until their weight was maintained, and it was quantitatively weighed. The percentage of the extracts that were extracted in proportion to the weight of the *G. pentaphyllum* (Thunb.) Makino was calculated.

Total Ash Value

Approximately 2-3 g of ground plant material was incinerated in a tarred platinum/silica crucible at a temperature not exceeding 450℃ until all carbon was removed. Subsequently, it was measured for weight after cooling. The percentage of ash relative to the air-dried plant material was determined.

pH Value at 1% and 10% Dilution

The drug was weighed exactly as much as 1 and 10 g. It was dissolved in precisely measured 100 mL of water and filtered, and the pH of the filtrate was assessed with a standardized glass electrode.

Total phenol and flavonoid contents

Total soluble phenolics were spectrophotometrically determined using Folin-Ciocalteu reagent (Sigma-Aldrich, MO) and gallic acid as the standard, as previously reported [17]. Total phenolic content was calculated as gallic acid

equivalents (GAE) per liter of sample, based on a standard curve of gallic acid.

The total flavonoid content was determined using an aluminum chloride colorimetric method [17]. Fifteen microliters of HRGD were mixed with 4.5 µL of 5% $NaNO_2$, 60 µL of distilled water and 4.5 µL of 10% $AlCl_3$. After incubation for 6 min, 60 µL of 4% NaOH solution was added to the mixture, and the final volume was made up to 150 µL with distilled water. The solution was thoroughly mixed, and the absorbance was immediately measured at 510 nm against the prepared blank, using standards with known rutin concentrations. The flavonoid content was calculated based on the calibration curves of rutin and expressed as milligrams of rutin equivalents (RE) per liter of the sample.

Free radicals scavenging activities

DPPH radical assay

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of the HRGD was measured using modified methods [17]. Briefly, 100 µL of freshly prepared 0.4 mM DPPH solution in methanol was added to the equal volume of each sample fraction. The reaction mixture was incubated for 10 min, and the absorbance was measured at 517 nm using a Spectra MR microplate reader (Dynex Technologies, Inc., Chantilly, VA, US). The commercial antioxidant butylated hydroxytoluene (BHT) was used as a positive control.

Hydrogen peroxide radical assay

Xanthine oxidase (0.25 units/mL) was added, and the mixture was left standing at room temperature for 25 min [17]. The absorbance was measured at 560 nm utilizing a microplate reader with reference to a suitable blank. The capacity of HRGD to scavenge hydrogen peroxide (H_2O_2) was assessed using a previously described method [17]. A 20 µL H_2O_2 (10 mM) was combined with 80 µL of HRGD and 100 µL of 100 mM phosphate buffer (pH 5.0). Following a 5-minute incubation at 37°C, 30 µL of freshly prepared 1.25 mM ABTS and 30 µL of 1 U/mL peroxidase were introduced to the reaction mixture. The absorbance of H_2O_2 at 405 nm was measured spectrophotometrically 10 min later at 37°C, relative to a phosphate buffer blank devoid of H_2O . All tests were conducted six times, with sodium pyruvate serving as the reference compound.

Superoxide radical assay

The superoxide anion scavenging activity of HRGD was measured by the reduction of NBT, following a previously reported method [10]. Superoxide radicals (O_2^-) were generated in a solution containing 50 mM sodium carbonate buffer (pH 10.5), 3 mM xanthine, 3 mM ethylenediamine tetraacetic acid (EDTA), 0.5 mM nitro blue tetrazolium (NBT) and 0.15% bovine serum albumin. HRGD or quercetin (positive control) was added to the solution.

Nitric oxide radical assay

The inhibition of nitric oxide radical ($NO^{\bullet}$) was measured using the Griess reduction [10]. Sodium nitroprusside in phosphate-buffered saline (10 mM, pH 7.0) was introduced to the extracts, and the mixtures (100 µL) were incubated at 25°C for a total during 3 hours. An equal volume of Greiss reagent was next added and allowed to stand for 5 minutes. The absorbance of these solutions was measured at 540 nm. BHT was utilized as a positive control.

$ABTS^{\bullet+}$ radical assay

The scavenging activity of HRGD against $ABTS^{\bullet+}$ radical was determined using previous protocols [17]. The ABTS reagent was prepared by mixing 5 mL of 7 mM ABTS with 88 µL of 140 mM potassium persulfate. The mixture was kept in darkness at the ambient temperature for 12-16 hours until the reaction reached finalization and the absorbance stabilized. The ABTS reagent was subsequently diluted with water in a ratio of 1:44 (v/v). To assess scavenging activity, 100 µL of ABTS reagent was combined with 100 µL of HRGD in a 96-well microplate, and the absorbance was recorded at 734 nm after 6 minutes. BHT served as the positive control.

Ferrous ion chelating activity

The chelating ability was determined according to the method of Kim *et al.* [17]. A volume of 5 µL of freshly prepared $FeCl_2$ (2 mM) was mixed with 250 µL of HRGD or EDTA (positive control). A 10 µL of 5 mM ferrozine was added to the mixture and absorbance readings were taken exactly 10 min later at 25ºC.

Statistical analysis

The results were expressed as mean ± standard deviation (SD) of triplicate values. Statistical

analysis was performed using SPSS version 12.0 using a nonparametric test (Mann-Whitney U test). A dose-response curve was plotted to determine IC_{50} values, which represent the concentration sufficient to achieve 50 % of the maximum scavenging capacity. Correlations among data obtained were analyzed using Pearson's correlation coefficient. A *p*-value less than 0.05 was considered statistically significant.

Results and Discussion

Physicochemical constants

The physicochemical properties of a given type of HRGD provide information regarding its structural stability and quality. The physicochemical properties of HRGD obtained by steam distillation are listed in Table 1. The successive extract value of HRGD was found to be 16.7%. The total ash value of HRGD indicated the amount of minerals and earthy material attached to the HRGD, and its value was calculated to be 9.521% w/w. The pH values of 1% and 10% solutions were 6.12 and 5.94, respectively. The value for loss on drying was found to be 1.507% w/w; less value of moisture content could prevent bacterial, fungal, and yeast growth (Table 1).

Contents of total phenolics and flavonoids

Plants possess high levels of polyphenols and flavonoids, which contribute to potent antioxidant activity, thereby imparting various defensive and disease-fighting properties [18]. The total phenolic content (TPC) of HRGD was calculated from the linear regression equation of the gallic acid standard calibration curve. The TPC was expressed in terms of milligram gallic acid equivalent per gram dry weight of HRGD (Table 2). The TPC of HRGD obtained in this study (42330 ± 322 mg GAE/100g) was higher than the TPC of *G. pentaphyllum* (Thunb.) Makino tea extract reported by Šamec *et al.* (2016) (1157 ± 35 and 811 ± 25 mg GAE/100g for 80% methanol and aqueous extracts, respectively) [19]. TPC is one of the important parameters of total antioxidant capacity and is widely used for the evaluation of the antioxidant properties of plants [20]. Phenolic compounds, including flavonoids, phenolic acids, stilbenes, coumarins, and tannins, are aromatic secondary plant metabolites that are considered non-harmful to human health [18, 21].

The total flavonoid content (TFC) of HRGD, calculated from the calibration curve (R^2 = 0.999), was 36,300 ± 189 rutin equivalents (RE) g/100 g (Table 2). In the previous study, the TFC of methanol and aqueous extracts of *G. pentaphyllum* (Thunb.) Makino tea were 539 ± 38 and 276 ± 19 mg RE/100 g, respectively [19], which is 67.3-131.5 folds lower than that of the HRGD (14.9 mg RE/100 g) achieved with steam distillation extraction in our study (Table 2). This implies that differences in secondary metabolites in *G. pentaphyllum* (Thunb.) Makino is greatly influenced by the extraction method. Flavonoids, in particular, play a role in modulating lipid peroxidation, which is implicated in conditions such as atherogenesis, thrombosis, and carcinogenesis [21]. The pharmacological effects of flavonoids have been shown to correlate with their antioxidant potential [21].

Table 1. General physicochemical parameters of HRGD

Test parameters	Results
Loss on drying at 105 ℃	1.507% w/w
Successive extract value	16.7%
Total ash	9.521% w/w
pH of 1.00% w/v soln.	6.12
pH of 10.00% w/v soln.	5.94

Note: HRGD: Hydrogen-rich *Gynostemma pentaphyllum* Makino distillate.

Table 2. Total phenolic and flavonoid contents of HRGD

Total phenolics (GAE[1)] mg/100 g)	Total flavonoids (RE[2)] mg/100 g)
42,330±322	36,300±189

Note: Each value is expressed as mean ± standard deviation (n = 3). HRGD: Hydrogen-rich *G. pentaphyllum* Makino distillate, [1)]GAE: Gallic acid equivalent, [2)]RE: Rutin equivalent.

Antioxidant activities

Free radicals are generated constantly in biological systems and have the potential to seriously harm tissues and biomolecules, resulting in a number of medical disorders [22-23]. Thus, the antioxidant activity of HRGD was evaluated using free radical scavenging and chelating methods. In this study, HRGD showed strong DPPH radical scavenging activity. The DPPH assay is frequently utilized to measure the capacity of antioxidants to eliminate free radicals, which are recognized as key contributors to biological damage resulting from oxidative stress [24]. Table 3 depicts a steady increase in the percentage inhibition of the DPPH radical by the HRGD up to a concentration of 10 µg/mL; after that, there was a leveling off with a much slower increase in inhibition. The maximum DPPH radical scavenging activity of HRGD was 62.5 ± 2.02% and the IC_{50} was 9.8 µg/mL (Table 3). The DPPH value of HRGD was higher than that of BHT (IC_{50} = 27 µg/mL).

Table 3. Antioxidant activity of HRGD

Activity (%)	Concentration (µg/mL)				IC_{50} value (µg/mL)
	10	20	40	80	
DPPH radical scavenging	50.8±0.45	55.0±4.91	59.7±2.11	62.5±2.02	9.8
Superoxide radical scavenging	35.7±1.96	57.0±9.11	61.8±1.54	73.1±0.18	18.8
Hydrogen peroxide radical scavenging	61.1±1.87	75.7±1.07	80.6±2.81	85.4±3.12	8.2
Nitric oxide radical scavenging	58.5±1.42	61.6±1.26	69.6±1.53	75.4±1.29	8.5
$ABTS^{\cdot+}$ radical scavenging	99.6±0.18	-	-	-	5.0
Ferrous ion chelating	53.4±0.43	64.3±0.76	69.3±0.75	75.9±0.62	9.4

Note: IC_{50} means the effective concentration at which the antioxidant activity was 50%, which was obtained by interpolation from linear regression analysis. Each value is expressed as mean ± standard deviation (n = 3). HRGD: Hydrogen-rich *Gynostemma pentaphyllum* Makino distillate.

Hydrogen peroxide functions as a weak oxidizing agent, inactivating certain enzymes mostly via the oxidation of essential thiol (-SH) groups. The compound can immediately traverse cell membranes; upon stepping into the cell, it likely connects with Fe^{2+} and potentially Cu^{2+} ions to generate hydroxyl radicals, which might explain many of its toxic effects. [24, 25]. As we can see from Table 3, HRGD showed a scavenging activity on the hydrogen peroxide radicals in a dose dependent manner. The maximum scavenging percentage of hydrogen peroxide radical was 85.4 ± 3.12% at the concentration of 80 µg/mL. Furthermore, the IC_{50} value of HRGD was 8.2 µg/mL. Nonetheless, when compared to sodium pyruvate (3.24 µg/mL), the hydrogen peroxide radical scavenging activity of HRGD was found to be low but comparable. The present study indicates that the HRGD has good scavenging activity for hydrogen peroxide but is not as efficient as the standard sodium pyruvate.

The superoxide anion is a relatively weak oxidant; nevertheless, it can contribute to the creation of more reactive species, such as singlet oxygen and hydroxyl radicals, which can result in tissue damage [26]. The effects of HRGD on superoxide anion radical scavenging activity were estimated by the nitro blue tetrazolium (NBT) assay, and the result is shown in Table 3. HRGD showed a scavenging activity on the superoxide radicals in a dose-dependent manner. The maximum scavenging percentage of superoxide radical was 73.1 ± 0.18% at the concentration of 80 µg/mL. Furthermore, the IC_{50} value of HRGD was 18.8 µg/mL, which was less than that of the quercetin standard (42.1 µg/mL). The results suggest that the plant extract is a more potent scavenger of superoxide radicals than the stand-

ard quercetin.

Nitric oxide's involvement in diverse disease states has garnered significant interest among scientists globally. Nitric oxide does not directly interact with bioorganic macromolecules, including DNA and proteins [27]. Under aerobic conditions, the nitric oxide molecule exhibits significant instability and reacts with oxygen to generate intermediates. such as $NO_2^{\bullet}$, $N_2O_4^{\bullet}$ and $N_3O_4^{\bullet}$ and stable products like nitrate, nitrite and peroxynitrite when reacted with superoxide, which is highly toxic to humans [27]. In this study, HRGD showed dose-dependent nitric oxide scavenging activity. The maximum scavenging percentage of nitric oxide radical was 72.12 ± 0.38% at the concentration of 80 µg/mL. Our results indicate that HRGD scavenged nitric oxide more efficiently (IC_{50} = 8.5 µg/mL) than BHT (IC_{50} = 75 µg/mL) (Table 3).

The ABTS assay is based on the inhibition of the absorbance of radical cation $ABTS^{\bullet+}$, which has a characteristic wavelength at 734 nm, by antioxidants [24]. Similar to DPPH, the decolorization of $ABTS^{\bullet+}$ radical reflects the capacity of an antioxidant species to donate electrons or hydrogen atoms to inactivate this radical cation [24]. The results obtained in this study indicated that HRGD exhibited a high $ABTS^{\bullet+}$ radical scavenging activity, and its percentage inhibition reached 99.6 ± 0.18% at a concentration of 10 µg/mL. The IC_{50} value of HRGD was 5 µg/mL, while the IC_{50} value of standard antioxidant BHT was 5.81 µg/mL (Table 3).

Ferrozine produces a violet complex with Fe^{2+} [24, 28]. In the presence of a chelating agent, complex formation is interrupted, and as a result, the violet color of the complex is decreased [24, 28]. The results demonstrated that the formation of the ferrozine-Fe^{2+} complex is inhibited in the presence of HRGD (Table 3). The IC_{50} values of HRGD and EDTA were 9.4 and 1.27 µg/mL, respectively.

In a previous study, the antioxidant potential of GPSO was determined by using DPPH and hydrogen peroxide radical scavenging activity and the ferric-reducing antioxidant power [6]. The overall antioxidant capacity of HRGD, which was extracted by steam distillation, was shown to be comparable to that of GPSO, which was extracted by SFE at a certain concentration (Table 3). The IC_{50} value of GPSO on scavenging DPPH was found to be 39.3 mg/mL, whereas the IC_{50} value of HRGD was 9.8 µg/mL; HRGD exhibited an IC_{50} value of 8.2 µg/mL towards scavenging hydrogen peroxide radical, while GPSO possessed a lower value of 73.52 mg/mL than HRGD (Table 2) [6]. As can be seen in Table 2, HRGD presents a greater degree of ferric-reducing antioxidant power with respect to dose than GPSO does. The antioxidant effects *in vitro* of HRGD were most likely due to the large content of total polyphenols and flavonoids (Table 1). These results strongly suggest that steam distillation extraction and hydrogen gas addition increase the contents of antioxidant capacity in *G. pentaphyllum* (Thunb.) Makino.

Correlation between antioxidant components and antioxidant activity

Knowledge about antioxidant properties and antioxidant components of HRGD is limited. Thus, linear correlation studies were carried out, and results were obtained from this study. Table 4 presents the Pearson's correlation coefficient between total phenols, total flavonoid contents and determined antioxidant capacity in HRGD. Our results show that there is a positive correlation between antioxidant capacity and composition. There are strong correlations between two antioxidant components (total polyphenols and flavonoids) and all free radicals (DPPH, superoxide, hydrogen peroxide, nitric oxide and $ABTS^{\bullet+}$)

Table 4. Coefficients of correlation between antioxidant compounds and antioxidant activities of HRGD

Antioxidant	DPPH	Superoxide	Hydrogen peroxide	Nitric oxide	$ABTS^{\bullet+}$	Chelating effect on ferrous ions
Total phenolics	0.913*	0.948*	0.920*	0.918*	0.985*	0.904*
Total flavonoids	0.890*	0.913*	0.898*	0.890*	0.930*	0.892*

Note: All values are absolute value of correlation coefficients. *$p < 0.05$ is considered statistically significant. HRGD: Hydrogen-rich *Gynostemma pentaphyllum* Makino distillate.

scavenging and chelating activities were found (r^2 = 0.890-0.985, $p < 0.05$) (Table 4). A high correlation between antioxidant capacities and their total phenolic contents indicated that phenolic compounds were a major contributor to the antioxidant activity of HRGD (Table 4). Similar results were obtained by Heo *et al*. (2009), which demonstrates that phenolics are the major contributors to the total antioxidant capacity of fruits, vegetables, and grains [30].

Conclusion

The study examined the phenolic content and antioxidant effect of HRGD to explore its potential as a source of bioactive compounds with various applications. Significant levels of phenolic compounds, including flavonoids, were detected. The findings also unveiled potent antioxidant properties, which were comparable to or even stronger than the standards used. These results imply that HRGD shows promise as a candidate for further exploration in the cosmetic, pharmaceutical, and food industries. Further molecular studies are currently underway to elucidate the mechanisms underlying the biological properties of HRGD.

Acknowledgment

This research was supported by the 2024 scientific promotion program funded by Jeju National University, Republic of Korea.

References

1. Huang D, Ming R, Xu S, et al. (2021) Chromosome-level genome assembly of *Gynostemma pentaphyllum* provides insights into gypenoside biosynthesis. DNA Research 28(5). doi: 10.1093/dnares/dsab018.
2. Nguyen NH, Ha TKQ, Yang JL, et al. (2021) Triterpenoids from the genus *Gynostemma*: Chemistry and pharmacological activities. Journal of Ethnopharmacology 268, 113574. doi: 10.1016/j.jep.2020.113574.
3. Liu H, Li X, Duan Y, et al. (2021) Mechanism of gypenosides of *Gynostemma pentaphyllum* inducing apoptosis of renal cell carcinoma by PI3K/AKT/mTOR pathway. Journal of Ethnopharmacology 271: 113907. doi: 10.1016/j.jep.2021.113907.
4. Wang Z, Zhao X, Liu Z, et al. (2019) Anti-diabetic activity evaluation of a polysaccharide extracted from *Gynostemma pentaphyllum*. International Journal of Biological Macromolecules 126: 209-214. doi: 10.1016/j.ijbiomac.2018.12.231.
5. Weng X, Lou YY, Wang YS, et al. (2021) New dammarane-type glycosides from *Gynostemma pentaphyllum* and their lipid-lowering activity. Bioorganic Chemistry 111: 104843. doi: 10.1016/j.bioorg.2021.104843.
6. Wang L, Pang M, Wang X, et al. (2017) Characteristics, composition, and antioxidant activities *in vitro* and *in vivo* of *Gynostemma pentaphyllum* (Thunb.) Makino seed oil. 97: 2084–2093. doi: 10.1002/jsfa.8013.
7. Yang Y, Hu B (2014) Bio-based chemicals from biorefining: lipid and wax conversion and utilization. Advances in Biorefineries 2014: 693-720. doi: 10.1533/9780857097385.2.693.
8. Gil KA, Jokic S, Cikos AM, Banozic M, et al. (2023) Comparison of different green extraction techniques used for the extraction of targeted flavonoids from edible *Feijoa* (*Acca sellowiana* (O.Berg) Burret) flowers. Plants (Basel) 12(7): 1461. doi:10.3390/plants12071461.
9. Russo D, Faraone I, Labanca F, et al. (2019) Comparison of different green-extraction techniques and determination of the phytochemical profile and antioxidant activity of *Echinacea angustifolia L.* extracts. Phytochemical Analysis 30 (5): 547-555. doi: 10.1002/pca.2847.
10. Chuyen HV (2003) Extraction of saponins, total soluble solids and antioxidant activity from Polyscias fruticosa roots. Food Research 7(3): 42-27. doi: 10.26656/fr.2017.7(3).019.
11. Ge L, Yang M, Yang NN, et al. (2017) Molecular hydrogen: a preventive and therapeutic medical gas for various diseases. Oncotarget 8(60): 102653-102673. doi: 10.18632/oncotarget.21130.
12. Ohsawa I, Ishikawa M, Takahashi K, et al. (2007) Hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals. Nature Medicine 13: 688-694. doi: 10.1038/nm1577.
13. Ohta S (2011) Recent Progress Toward Hydrogen Medicine: Potential of Molecular Hydrogen for Preventive and Therapeutic Applications. Current Pharmaceutical Design 17 (22): 2241–2252. doi: 10.2174/138161211797052664.
14. Sezer YC, Bulut M, Boran G, et al. (2022) The effects of hydrogen incorporation in modified atmosphere packaging on the formation of biogenic amines in cold stored rainbow trout and horse mackerel. Journal of Food Composition and Analysis 112: 104688. doi: 10.1016/j.jfca.2022.104688.
15. Roach WA (1958) A continuous low-temperature vacuum extractor. Annals of Botany 22(86): 293-295. doi: 10.1093/oxfordjournals.aob.a083613.
16. Tang L (2018) Reduced pressure distillation method for determining the acetic acid salt in sodium silicate sand. IOP Conference Series: Earth and Environmental Science 170: 022144. doi: 10.1088/1755-1315/170/2/022144.
17. Kim JH, Kim JH, Kim MY (2022) Effect of extraction solvents on antioxidant and skin-whitening potentials of defatted Camellia seed cakes. Journal of Applied and Natural Science 14 (2): 341-348. doi: 10.31018/jans.v14i2.3368.
18. Iqbal Y, Ponnampalam EN, Cottrell JJ, Suleria HAR, et al. (2022) Extraction and characterization of polyphenols from non-conventional edible plants and their antioxidant activities. Food Research International 157: 111205. doi: 10.1016/j.foodres.2022.111205.
19. Šamec D, Valek-Žulj L, Martinez S, et al. (2016) Phenolic acids significantly contribute to antioxidant potency of *Gynostemma pentaphyllum* aqueous and methanol extracts. Industrial Crops and Products 84: 104-107. doi: 10.1016/j.indcrop.2016.01.035.
20. Molole GJ, Gure A, Abdissa N (2022) Determination of total phenolic content and antioxidant activity of *Commiphora* mollis (Oliv.) Engl. Resin. BMC Chem-

istry 16: 48. doi: 10.1186/s13065-022-00841-x.
21. Shen N, Wang T, Gan Q, et al. (2022) Plant flavonoids: Classification, distribution, biosynthesis, and antioxidant activity. Food Chemistry 383: 132531. doi: 10.1016/j.foodchem.2022.132531.
22. Babu S, Manoharan S, Ottappilakkil H, et al. (2022) Role of oxidative stress-mediated cell death and signaling pathways in experimental fluorosis. Chemico-Boplogical Interactions 365: 110106. doi: 10.1016/j.cbi.2022.110106.
23. Halliwell B (2022) Commentary for "Oxygen free radicals and iron in relation to biology and medicine: Some problems and concepts" Archives of Biochemistry and Biophysics 718: 109151. doi: 10.1016/j.abb.2022.109151.
24. Magalhães LM, Segundo MA, Reis S, et al. (2008) Methodological aspects about *in vitro* evaluation of antioxidant properties. Analytica Chimica Acta 613(1): 1-19. doi: 10.1016/j.aca.2008.02.047.
25. Lee WJ, Lee SH (2022) Protocatechuic acid protects hepatocytes against hydrogen peroxide-induced oxidative stress. Current Research in Food Science 5: 222-227. doi: 10.1016/j.crfs.2022.01.006.
26. Fridovich I (2022) Reprint of: Biological effects of the superoxide radical. Archives of Biochemistry and Biophysics 726: 109228. doi: 10.1016/0003-9861(86)90526-6.
27. Lundberg JO, Weitzberg E (2022) Nitric oxide signaling in health and disease. Cell 185 (16): 2853-2878. doi: 10.1016/j.cell.2022.06.010.
28. Berker KI, Ozyurt D, Demirata B, et al. (2017) Solid-phase extraction spectrophotometric determination of total antioxidant capacity in antioxidant-poor samples by using the ferric-ferrozine method. Analytical Sciences 33 (6): 683-689. doi: 10.2116/analsci.33.683.
29. Jacobo-Velázquez DA, Cisneros-Zevallos L (2009) Correlations of Antioxidant Activity against Phenolic Content Revisited: A New Approach in Data Analysis for Food and Medicinal Plants. Journal of Food Science 74(9): R107-R113. doi: 10.1111/j.1750-3841.2009.01352.x.
30. Heo HJ, Kim YJ, Chung D, et al. (2007) Antioxidant capacities of individual and combined phenolics in a model system. Food Chemistry 104(1): 87-92. doi: 10.1016/j.foodchem.2006.11.002.