INTRODUCTION

Ha-Rak (Benchalokawichian) is one of the famous Thai herbal recipes, which has been included in the National List of Essential Herbal Medicines of Thailand. The recipe contains the five herbal roots including *Capparis micracantha*, *Clerodendrum indicum*, *Ficus racemosa*, *Harrisonia perforata* and *Tiliacora triandra* as its ingredients. There are some previous studies of the recipe that have been further investigated such as antiallergic, anti-inflammatory, and antioxidative activities, a study that elaborated on the cytotoxic constituents that responded to the cytotoxic activity of the recipe has been limited. The investigation of the extracts of Ha-Rak recipe on cytotoxicity toward SW620 cancer cell lines was performed in this study using MTT assay and the IC_{50} values of the tested extracts were obtained to evaluate their cytotoxic performance for the preliminary anticancer study. In addition to that, the amount of lupeol in the extracts and the herbal principles of the recipe was also determined. The contents of lupeol in the extracts of the recipe that obtained from different samples were also compared and sequentially analyzed with a one-way analysis of variance (ANOVA). Hence, the aim of the present work is to indicate the presence of a cytotoxic compound in Ha-Rak recipe and the results would be used as supportive data for preliminary assessment on the anticancer property of the recipe.
**MATERIALS AND METHODS**

**Materials and reagents**

The 12 marketed samples of Ha-Rak recipe, the herbal capsule preparations in Thailand were purchased from the traditional pharmacies in the month of October 2017. They were authenticated by comparing with genuine samples using the pharmacognostic methods that were described in a literature at the Department of Pharmacognosy, College of Pharmacy, Rangsit University, Thailand. The samples were numbered as 1-12. In addition, the raw plants that are the ingredients of the recipe were purchased and also authenticated in the same way. Lupeol (CAS no. 545-47-1) was supplied from Nanjing Spring & Autumn Biological Engineering Co., Ltd., Nanjing, China. All analytical solvents used in the extraction, sample preparation and chromatography were procured from Honeywell Burdick & Jackson®, Korea. The preparative process for analytical samples was performed by the solid-phase extraction (SPE) devices that containing the Bond Elut C18 cartridge (100 mg, 3.0 mL) and V AC Elut-20 (16×100 mm), which belong to Agilent Technologies, Inc. and they were purchased from Thai Unique Co., Ltd. All disposable accessories used for HPLC in the analytical method development were procured from S.N.P. Scientific Co., Ltd., Thailand.

**Preparation of the sample solutions**

The powdered herbs of Ha-Rak (20 capsules) were mixed in a mortar and weighed accurately (5.0 g) into a thimble of Soxhlet apparatus (C. Gerhardt GmbH & Co. KG, Germany). It was extracted in a Soxhlet extractor with 300 mL of methanol for 3 h. The extract was evaporated to dryness in a rotary evaporator (Rüedi Rotavapor R-114, Switzerland). The extract was reconstituted with small amounts of methanol and adjusted to 10 mL in a volumetric flask using methanol as a solvent. In addition, the powders of the five plants (5.0 g, each) that are the principles of the recipe were also extracted by the same method. They were prepared and adjusted to obtain 10 mL methanolic extracts as the sample solutions. Each sample solution (3.0 mL) was loaded onto a solid-phase extraction (SPE) cartridge (C18, 100 mg, 3.0 mL). The impurities in the sample were washed out with 3.0 mL of purified water and discarded. The analytes were subsequently eluted with the six cycles of 0.5 mL of methanol to collect 3.0 mL of the assay solution.

**Preparation of the standard solutions**

Lupeol (99% purity, 12.5 mg) was dissolved in methanol and adjusted into the 25.0 mL volumetric flask to furnish a stock standard solution (500 µg/mL). A stock standard solution was pipetted 0.2, 0.5, 1.0, 2.0, 3.0 and 4.0 mL individually, transferred to the 10 mL volumetric flasks, and further adjusted by methanol to afford 10, 25, 50, 100, 150 and 200 µg/mL of working standard solutions, as sequentially.

**Chromatographic conditions**

The HPLC system (Agilent Technologies, Inc., United States) was equipped with quaternary pump, degasser, autosampler, thermostat column chamber and photodiode array detector (DAD) belonging in 1260 Infinity series. All devices were connected with the Hewlett-Packard computer, which was installed with Agilent OpenLAB software (HPLC 1260 online) for integrating the results from instruments. The assay solutions and working standard solutions were filtered through 0.45 µm nylon membrane prior to analysis. Each filtered sample (20 µL) was injected by automatic injector to a system that comprised a COSMOSIL (Cholesterol Waters type) packed column (150 mm × 4.6 mm i.d., 5 µm) as a stationary phase and a mixture of acetonitrile and methanol (70:30) as a mobile phase. The flow rate of a system was conducted at 2.0 mL/min and the column chamber was set at ambient temperature. The chromatograms of each sample were recorded in the range of DAD wavelength (200-400 nm) and the wavelength at 210 nm was selected to evaluate the peak data of lupeol. The analytical method for the determination of lupeol was validated following the requirements of International Conference on Harmonisation (ICH) guidelines and it was demonstrated in our recent report. The quantification of lupeol in extracts

Lupeol in the extracts of Ha-Rak recipe and the five raw herbal materials was examined using the Soxhlet extraction with SPE and the validated HPLC-DAD methods. The content of lupeol in the extracts was calculated by a linear regression equation, which was obtained from a calibration curve of lupeol standard solutions and expressed as milligrams of lupeol per 100 grams of dry powdered recipe. The marker contents were examined in triplicate and their SD values were also displayed and further submitted in the statistical analysis.

**Cytotoxic assay**

An in vitro cytotoxic assay of the herbal extracts against human colon carcinoma cell lines (SW620) was performed using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) colorimetric method as described in a previous report. All extracts from the five plants of Ha-Rak recipe and its marketed products were dissolved in dimethylsulfoxide (DMSO) and diluted with culture medium to make a stock solution. A series of 2-fold dilutions of each stock solution was prepared for the assay and the final concentration of DMSO did not exceed 0.5% in each experiment. Dose response curves were plotted from 8 concentrations of a 2-fold serial solution of each sample (7.81, 15.62, 31.25, 62.50, 125, 250, 500 and 1000 µg/mL) against their percentage of cell survival in triplicate. The concentration of each extract that reduced the growth of a cancer cell line by 50% calculating from a curve was considered to report as the 50% inhibitory concentration (IC₅₀ value). Results of the MTT assay for tested samples were expressed in µg/mL. The cytotoxic activity of the herbal extracts was compared with that of lupeol, which was used as a positive control in this study.

**Statistical analysis**

A study of lupeol content in the herbal extracts was examined in triplicate and a mean ± SD of each sample was also demonstrated. The cytotoxic investigation of the extracts on SW620 cell lines was carried out in triplicate and their IC₅₀ values were also expressed as a mean ± SD. The statistical comparisons between the groups of samples were carried out using a one-way analysis of variance (ANOVA) with Tukey's multiple comparison posthoc test (SPSS, SPSS Inc., Chicago, United States). A value of P < 0.05 was determined for the statistical significance of all tested groups.

**RESULTS**

The investigation of lupeol in the extracts of Ha-Rak recipe, which were collected from 12 different sources (numbered as 1-12) along with the five herbal principles of the recipe was performed using the validated HPLC method and the lupeol content in each sample was calculated by using a linear regression equation that obtained from a calibration curve of lupeol standard solutions. The results of all analyzed extracts are summarized in Table 1. The HPLC chromatograms of 12 extracts that demonstrated a signal of lupeol were overlaid, as shown in Figure 1.

The content of lupeol in 12 different samples of the recipe was observed in the ranges between 20.12 ± 1.54 and 61.61 ± 6.47 mg/100g. These results demonstrate that there is no uniformity in regard to the amount of lupeol in these extracts. The highest yield of lupeol was found in sample 11, whereas the lowest content was observed in sample 1. One-way ANOVA showed that samples 1-12 had significant differences in the lupeol contents (P < 0.0001, F = 14.74, df = 11, 24). Tukey’s posthoc tests affirmed that the highest content of lupeol in sample 11 (61.61 | Pharmacognosy Journal, Vol 13, Issue 1, Jan-Feb, 2021 | 134
Table 1: Content of lupeol and cytotoxic activity of different Ha-Rak extracts.

| Sample | Content of lupeol (mg/100 g) | IC50 (µg/mL) |
|--------|-------------------------------|--------------|
| 1      | 20.12 ± 1.54                  | 47.10 ± 0.88 |
| 2      | 39.31 ± 6.19                  | ND           |
| 3      | 47.42 ± 6.30                  | ND           |
| 4      | 43.01 ± 7.18                  | ND           |
| 5      | 24.61 ± 3.47                  | ND           |
| 6      | 27.75 ± 4.48                  | ND           |
| 7      | 31.12 ± 5.79                  | ND           |
| 8      | 26.79 ± 2.39                  | ND           |
| 9      | 43.56 ± 2.51                  | 42.60 ± 0.91 |
| 10     | 34.02 ± 8.51                  | ND           |
| 11     | 61.61 ± 6.47                  | 37.30 ± 0.77 |
| 12     | 33.94 ± 1.61                  | ND           |
| C. indicum | 4.50 ± 0.21                  | 212.24 ± 2.28 |
| C. micracantha | ND          | ND           |
| F. racemosa | 250.62 ± 3.80                | 34.80 ± 0.69 |
| H. perforata | ND            | ND           |
| T. triandra | 7.94 ± 0.14                  | 30.10 ± 1.07 |
| Lupeol (positive control) | -                 | 30.50 ± 0.48 |

Values are shown as mean ± standard deviation in three independent experiments, ND is represented the non-determined data.

Figure 1: HPLC profile of lupeol in different Ha-Rak extracts, the signal of lupeol compound shows at 4.2 min in the overlay chromatograms of 12 marketed products (upper lines) and the standard chromatogram of authentic sample (bottom line).

± 6.47 mg/100g) differed from all samples (P < 0.01 to P < 0.001) except sample 3 (47.42 ± 6.30 mg/100g; P > 0.05). Conversely, sample 1 that showed the lowest yield of lupeol (20.12 ± 1.54 mg/100g) had a significant content (P < 0.01 to P < 0.001) as compared to samples 2, 3, 4, 9 and 11. Determination of the lupeol contents in the five raw plants of Ha-Rak recipe showed that F. racemosa possessed the highest yield of lupeol (250.62 ± 3.80 mg/100g). Its content was noted higher than the other plants and 12 extracted samples. On the other hand, C. indicum possessed the lowest lupeol content (4.50 ± 0.21 mg/100g), which was lower than any other contents that found in this study. Among five herbal principles, the amount of lupeol had not been observed in the extracts of C. micracantha and H. perforata, which were analyzed by the developed analytical method this time.

The extracts of 12 samples together with those of five herbal components of the recipe were selected to examine their effects on the inhibition of cancer cell growth. This study was carried out using the MTT assay against human colon adenocarcinoma (SW620) cell lines. Lupeol, a marker compound in the developed analytical method was used as a
positive control. The cytotoxicity of the selected extracts toward that cancer cell lines was expressed as the IC$_{50}$ values as displayed in Table 1 and the photomicrographs of cancer cells that were treated with the extracts at their active concentration values are shown in Figure 2. The extracts of samples 1, 9 and 11 that obtained from the different samples of the marketed recipe showed cytotoxic activity with the IC$_{50}$ values of 47.10 ± 0.88, 42.60 ± 0.91 and 37.30 ± 0.77 µg/mL, respectively. Thus, the order of cytotoxic strength among them was 11 > 9 > 1. Sample 11 had a large amount of lupeol, it also possessed a strong cytotoxic effect as compared to samples 1 and 9. On the other hand, a lower activity was observed in sample 1, which occupied a lupeol content lower than in the others. Observations indicated that the cytotoxicity of these extracts and their lupeol contents had a relationship. With respect to the five herbal principles, the extracts of C. indicum, F. racemosa and T. triandra demonstrated cytotoxicity with the IC$_{50}$ values of 212.24 ± 2.28, 34.80 ± 0.69 and 30.10 ± 1.07 µg/mL, respectively. F. racemosa extract owned the highest lupeol content, it had an inhibitory effect toward cancer cell proliferation better than samples 1, 9 and 11. Whereas, C. indicum possessed the lowest lupeol content, its cytotoxicity was observed to be lower than that of all extracts in this investigation. Among these extracts, T. triandra expressed the best IC$_{50}$ value as compared to the others, its activity was also observed stronger than a positive control. In addition, one-way ANOVA testing for the cytotoxic potency between the different extracted groups was performed. The difference on the cytotoxic effects among the extracted groups was noticed significantly ($P < 0.05, F = 3.96, df = 5, 12$). Posthoc tests indicated that the IC$_{50}$ value of sample 1 (47.10 ± 0.88 µg/mL) was significantly different from the IC$_{50}$ values of the extracts of T. triandra (30.10 ± 1.07 µg/mL) and a positive control (30.50 ± 0.48 µg/mL) ($P < 0.05$), whereas no significant difference was observed in the IC$_{50}$ value of sample 11 (37.30 ± 0.77 µg/mL) as compared to all tested extracts except for C. indicum.

**DISCUSSION**

The cytotoxicity against SW620 cancer cell lines of the extracts of Thai traditional Ha-Rak recipe and its herbal ingredients was examined to explore the potential of this herbal remedy for performing in-depth investigations on anticancer. Considering the chemical constituents of the herbal principles in the recipe, the cytotoxically active compounds such as pectolarigenin and perforatic acid were isolated and their activities against cancer cell lines were also investigated in a recent report.[19] However, the other promising cytotoxic agents have still been explored from the plants, which are the ingredients of the recipe. Lupeol, a cytotoxic compound that was determined as one of the active compounds in C. indicum[9] was selected as an analytical marker herein, in order to examine the content in the recipe and also determine whether it was responsible for the cytotoxic activity against SW620 cell line. Lupeol, a natural lupane-type triterpenoid derivative has been reported to function as a cytotoxic compound by enhancement of reactive oxidative species and induction of cell apoptosis in various cancer cell line tests.[20,21] In this study, the analyzed extracts demonstrated the lupeol contents varying from 20.12-61.61 mg/100g in 12 Ha-Rak marketed products and 4.50-250.62 mg/100g in the herbal principles of the recipe. A positive correlation between the lupeol contents of different extracts of the recipe and their cytotoxic

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**Figure 2:** Photomicrographs of SW620 cells treated with the extracts at their active concentrations after 72 h. (A) Culture medium; (B) normal control (DMSO); (C) lupeol; (D) sample 1; (E) sample 9; (F) sample 11; (G) C. indicum; (H) F. racemosa; (I) T. triandra.
Among the herbal ingredients, tested extracts is correlated with the presence of lupeol in the recipe. The lupeol contents, the results indicated that the cytotoxicity of the effect of extract and explain why the cytotoxic activity of that T. triandra harvest and the environment of crops. In this way, the significant substances such as the geography of plant habitats, the season of the are many regulating factors that would affect the yield of bioactive toward human colon carcinoma cell lines in our study. Referring to reports that showed bioactivities involving their lupeol profiles. Although a direct correlation between the cytotoxic performance and lupeol content was observed in the extracted samples of F. racemosa and C. indicum, it was inverse in the extract of T. triandra, which possessed the highest cytotoxic potency in this study. The capability on cytotoxicity of T. triandra might correspond with prominent cytotoxic alkaloids, i.e. tillicariancine, yanangcarine and ocanoalboline, which are found as its chemical constituents and displayed powerful cytotoxicity in various cancer cell tests. Thus, these alkaloids might be responsible for the effect of T. triandra extract and explain why the cytotoxic activity of that plant was stronger than a positive control in this study.

Based on the cytotoxic effect of the extracts of Ha-Rak recipe, concerning the lupeol contents, the results indicated that the cytotoxicity of the tested extracts is correlated with the presence of lupeol in the recipe. Among the herbal ingredients, F. racemosa and T. triandra are two considerable active principles, which exhibited remarkable cytotoxicity toward human colon carcinoma cell lines in our study. Referring to the investigation of chemical compounds in medicinal plants, there are many regulating factors that would affect the yield of bioactive substances such as the geography of plant habitats, the season of the harvest and the environment of crops. In this way, the significant varying contents of lupeol among 12 samples of Ha-Rak recipe were distinguished in our investigation.

CONCLUSION

The developed chromatographic method can be effectively used for quantification of lupeol, a cytotoxic compound in the extracts of Ha-Rak recipe and its herbal materials. Extracts from the recipe and its herbal principles including C. indicum, F. racemosa and T. triandra that contain significantly different contents of lupeol exhibited the cytotoxic activity toward SW620 cancer cell lines with varying degrees of IC₅₀ values. The direct proportion between the contents of lupeol and their cytotoxic degrees was disclosed in all tested samples excluding T. triandra. This study indicates the presence of lupeol in Ha-Rak recipe and elaborates the performance of its extract in order to perform further in-depth studies on an anticancer property.

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CONFLICTS OF INTEREST

No conflicts of interest.

REFERENCES

1. Singhachai C, Palanuwe C, Kyohara H, Yamada H, Ruangrungsi N. Pharmacognostic specification of five root species in Thai traditional medicine remedy; Ben-Cha-La-Ka-Wi-Chian. Pharmacoog J. 2011;3(22):1-11.
2. Jongchanapong A, Singhachai C, Palanuwe C, Ruangrungsi N, Toxiwat P. Antipyrretic and antinociceptive effects of Ben-Cha-La-Ka-Wi-Chian remedy. J Health Res. 2010;24(1):15-22.
3. Singhachai C, Palanuwe C, Kyohara C, Yamada H, Ruangrungsi N. Safety evaluation of Thai traditional medicine remedy: Ben-Cha-La-Ka-Wi-Chian. J Health Res. 2011;25(2):83-90.
4. Juckmeta T, Thongdeeong P, Itharath A. Inhibitory effect on β-hexosaminidase release from RBL-2H3 cells of extracts and some pure constituents of B. curcas. In vitro anticancer activity of Ficus racemosa. J. Ethnopharmacol. 2013;149:205-10.
5. Juckmeta T, Itharath A. Anti-inflammatory and antioxidant activities of Thai traditional remedy called “Ya-harakh”. J Health Res. 2012;26(4):205-10.
6. Nutmauk T, Pattananyapant K, Soonthornchareonun N, Shiomi K, Mori M, Prathantarakul S. Antiplasmodial activity, of a Thai traditional antipyretic formulation, Bencha-Loga-Wichan: A comparative study between the roots and their substitutes, the J Ethnopharmacol. 2016;193:125-32.
7. Nuaensara S, Kondo S, Itharath A. Antimicrobial activity of the extracts from Benchakloakwivian remedy and its components. J Med Assoc Thai. 2011;94(Suppl 7):S172-7.
8. Juckmeta T, Ruangnso P, Itharath A. Cytotoxic activities against two lung cancer cells of Thai anti-tumor drug. Planta Med. 2016;82(501):P976.
9. Somwong P, Suttsirs R. Cytotoxic activity of the chemical constituents of Clerodendrum indicum and Clerodendrum villusum roots. J Integr Med. 2018;16(11):57-61.
10. Jager S, Trojan H, Kopp T, Laszczyn MK, Scheffer A. Pentacyclic triterpene distribution in various plants-rich sources for a new group of multi-potent plant extracts. Molecules. 2009;14(6):2016-31.
11. Lansky EP, Paavilainen HM, Pavlus AD, Newman RA. Ficus spp. (fifl): Ethnobotany and potential as anticancer and anti-inflammatory agents. J Ethnopharmacol. 2008;119(2):195-213.
12. Das AK, Mandal V, Mandal SC. Design of experiment approach for the process optimisation of microwave assisted extraction of lupeol from Ficus racemosa leaves using response surface methodology. Phytochem Anal. 2013;24(3):230-47.
13. Somnill P, Itthipanuchtich P, Ruangnso N, Limpanasithikul W. Inhibitory effect of Harrisonia perforata root extract on macrophage activation. Thai J Pharmacol. 2010;32(1):168-71.
14. Saleem M, Lupeol, a novel anti-inflammatory and anti-cancer dietary triterpene. Cancer Lett. 2009;285(2):109-15.
15. Siven KS, Nguyen AH, Lee JH, Li F, Singh SS, Kumar AP, et al. Negative regulation of signal transducer and activator of transcription-3 signaling cascade by lupeol inhibits growth and induces apoptosis in hepatocellular carcinoma cells. Br J Cancer. 2014;111(7):1237-37.
16. Khatoon S, Irshad S, Pandey MM, Rastogi S, Rawat AK. A validated HPTLC method for quantification of lupeol, a cytotoxic compound against SW620 cells in the extracts of Ha-Rak recipe. Pharmacoog J. 2011;3(21):1-11.
17. Prathanturarug S. Antiplasmodial activities of a Thai traditional antipyretic remedy: Benchalokawichian. J Med Assoc Thai. 2012;85(1):1-6.
18. Bopage NS, Gunaherath GK, Jayawardena KH, Wijeyaratne SC, Abeysekera AM, et al. Cytotoxic activity of the bioactive principles from T. astringens and its components. J Integr Med. 2019;16(1):57-61.
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**GRAPHICAL ABSTRACT**

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