

## Phytochemical Analysis and Evaluation of Antioxidant and Antibacterial Activities of Kratom-Containing Formulations of *Ya-Gae-Bid-Na-Ron*

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### Abstract:

**Objective:** This study aimed to evaluate the antioxidant and antibacterial activities of *Ya-Gae-Bid-Na-Ron* (YGBNR).

**Material and Methods:** YGBNR formulations were prepared using kratom collected from different provinces in Thailand. Mitragynine content was determined using high-performance liquid chromatography (HPLC), while total phenolic content (TPC), total tannin content (TTC), and total flavonoid content (TFC) were determined using colorimetric methods. Antioxidant power was estimated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric-reducing antioxidant power (FRAP) assays. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of YGBNR were determined using a broth dilution technique and plating of the bacterial solution, respectively.

**Results:** Mitragynine content, TPC, TTC, and TFC varied among different sources of kratom. DPPH assay indicated that YGBNR had an average  $IC_{50}$  of  $0.44 \pm 0.04$  mg/mL, and the FRAP assay ranged from  $61.48 \pm 0.55$  to  $102.98 \pm 0.94$  mg gallic acid equivalent (GAE), per gram of dry extract. YGBNR extracts did not exhibit any antibacterial activity. However, the volatile oil fraction showed MIC against *Escherichia coli* and *Pseudomonas aeruginosa* at a concentration of 3 mg/mL.

**Conclusion:** Phytochemical analysis revealed the abundance of phenolic compounds in YGBNR. YGBNR exhibits promising antioxidant potential, but does not exert any antibacterial activity.

**Keywords:** antibacterial, antioxidant, kratom, *Ya-Gae-Bid-Na-Ron*

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## Introduction

Kratom (*Mitragyna speciosa* (Korth.) Havil.; Rubiaceae) is a well-known psychoactive plant. While some countries have banned kratom and classified it as a poisonous narcotic, its legal status is maintained in other jurisdictions<sup>1</sup>. More than 50 alkaloids have been identified in this plant, the majority of which are indole and oxindole alkaloids; such as mitragynine, paynantheine, speciogynine, speciociliatine, and mitraphylline<sup>2</sup>. Kratom also produces other secondary metabolites; including tannins, flavonoids, and triterpenoids.

In Thai folkloric medicine, the leaf and bark of kratom are used in the management of diarrhea. The Ministry of Public Health of Thailand, Department of Thai Traditional and Complementary Medicine published the recipes containing kratom for treating diarrhea and for substituting opioids in pain management. Fifty-six remedies containing kratom have been documented in the National Materia Medica, with included: *Pra-Sa-Kra-Tom*, *Ya-Gae-Bid-Hua-Luk*, *Ya-Gae-Bid-Na-Ron* (YGBNR).

The pharmacological activities of kratom have been recently explored. Mitragynine and 7-OH mitragynine act in the central nervous system (CNS) to produce analgesic effects. Mitragynine and 7-OH mitragynine are partial  $\mu$ -opioid receptor agonists<sup>3,4</sup> and competitive  $k$ - and  $d$ - opioid receptor antagonists<sup>5</sup>. The binding affinity of mitragynine to the opioid receptor is lower than morphine, suggesting that it has a lower addictive potential<sup>4</sup>. Via its opioid action, kratom extract and mitragynine can reduce gastric secretion and appetite in rat models<sup>6,7</sup> in addition to decreasing stool frequency and amount<sup>8</sup>.

YGBNR is composed of herbs; including nine leaves of kratom (*Mitragyna speciosa* (Korth.) Havil.), one slice of ginger (*Zingiber officinale* L.), one fruit of Java long pepper (*Piper retrofractum* Vahl), and one ripe fruit of chili (*Capsicum frutescens* L.). It has been prescribed for the treatment of diarrhea with flatulence. In this present study,

other potential medical applications of kratom were explored, by determining the antioxidant and antibacterial activities of kratom-containing YGBNR formulations.

## Material and Methods

### Plant materials and formulation

Mature kratom leaves were purchased from Surat Thani, Songkhla, and Chumphon provinces of Thailand. Ginger rhizome was purchased from Phitsanulok, Thailand. Java long pepper fruit was purchased from Kanchanaburi, Thailand. Ripen chili fruit was from Songkhla, Thailand. All plant samples were dried in an oven at 50°C. Samples were ground to powder in a Cyclone Mill Twister and sieved (No. 60; particle size 344  $\mu$ m). YGBNR was prepared by mixing 16 g kratom, 1 g ginger, 2.8 g Java long pepper, and 2.4 g chili in a plastic bag to achieve a homogeneous mixture. Three different YGBNR formulations (No. 1–3) were prepared from each of the three sources of kratom.

### Preparation of the extracts

Ten grams of plant powder was submerged in 200 mL of the appropriate solvent: 80% v/v ethanol for kratom and YGBNR and 95% v/v ethanol for Java long pepper and chili. The mixtures were shaken for 8 hr and macerated at room temperature for 16 hr. The filtrate was then collected and evaporated to dryness: extracts were kept in a freezer. The volatile oil of YGBNR was obtained through the hydro-distillation method using a Clevenger apparatus. Fifty grams of YGBNR powder and 200 mL of distilled water were mixed in a round-bottomed flask. The water was distilled for 5 hr, upon which the volatile fraction was then collected.

### Determination of phytochemical compounds

#### Mitragynine content

Mitragynine content in the dry extracts of kratom leaves and YGBNR were determined using HPLC, as previously described<sup>9</sup>. Sample solutions were prepared at a

concentration of 1 mg/mL in methanol and filtered through a nylon membrane (0.22  $\mu$ m). The HPLC system consisted of a Shimadzu Prominence *i* equipped with a photodiode array (Shimadzu, Kyoto, Japan). The C18 column was a VertiSep™ ultra-performance silica (UPS) column (250x4.6 mm internal diameter, 5  $\mu$ m; Vertical, Bangkok, Thailand). The mobile phase consisted of a mixture of 20 mM ammonium acetate (pH 6) and acetonitrile, at a ratio of 35:65. The column was eluted isocratically at a flow rate of 1.0 mL/min with an injection volume of 20  $\mu$ L. The wavelength for the detection of the analyte was 225 nm. The area under the peak was used to calculate the mitragynine content from the standard curve. The amount of mitragynine was expressed as % w/w of the dry extract.

#### **Total phenolic content (TPC)**

The TPC was estimated using Folin Ciocalteu's method<sup>10</sup>. Samples were diluted with methanol, as appropriate, before the addition of 10% v/v Folin Ciocalteu's reagent in distilled water. After 5 min, 7.5% w/v of Na<sub>2</sub>CO<sub>3</sub> was added. The mixture was incubated at room temperature for 60 min in the dark. The absorbance at 760 nm was measured. The amount of total phenolic was expressed as gallic acid equivalents (mg) per gram extract. The experiment was performed in triplicate.

#### **Total tannin content (TTC)**

The total condensed tannin content was determined using the acidified vanillin method<sup>11</sup>. Samples were analyzed in a 96-well plate in the presence of 4% w/v vanillin-hydrochloric reagent. The absorbance of each solution was measured at 500 nm. The TTC was expressed as catechin equivalents (mg) per gram extract: the experiment was performed in triplicate.

#### **Total flavonoid content (TFC)**

The TFC was determined using the aluminum chloride colorimetric method; as described previously with modifications<sup>12</sup>. In a 96-well plate, each diluted sample was mixed with 5% w/v NaNO<sub>2</sub> and incubated for 6 min. After incubation, 10% w/v AlCl<sub>3</sub> was added and the reaction was again incubated for 6 min. To neutralize the reaction, 4% w/v NaOH was added and the absorbance was immediately measured at 510 nm. For the blank sample, the samples were mixed with distilled water. The amount of total flavonoid was expressed as rutin equivalents (mg) per gram extract: the experiment was performed in triplicate.

#### **Determination of antioxidant power**

##### **2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay**

The hydroxyl radical scavenging activity was determined using the DPPH assay<sup>13</sup>. The DPPH reagent was freshly prepared in methanol and added to the sample. After 30 min incubation at room temperature, the absorbance of the solution was measured at 517 nm. The sample blank was the sample mixed with methanol. The control sample was methanol without DPPH. Gallic acid was used as standard. The percent of inhibition was calculated using the following formula:

$$\% \text{ inhibition} = \frac{[(\text{OD}_{\text{control}}) - (\text{OD}_{\text{sample}} - \text{OD}_{\text{blank, sample}})]}{\text{OD}_{\text{control}}}$$

The IC<sub>50</sub>, expressed in mg/mL, was computed from the plot of the % inhibition vs. concentration of standard or sample.

##### **Ferric-reducing antioxidant power (FRAP) assay**

The total antioxidant power was determined using the FRAP assay<sup>14</sup>. The FRAP reagent was a mixture of 28 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tris (2-pyridyl)-s-triazine (TPTZ) solution in 40 mM HCl and 20 mM ferric chloride hexahydrate (FeCl<sub>3</sub>.6H<sub>2</sub>O) in a ratio 10:1:1 v/v. The

FRAP solution was kept in the dark. The sample was mixed with FRAP reagent and the absorbance of the reaction mixture was measured at 593 nm. The antioxidant power was calculated as gallic acid (mg) equivalents per gram extract. The sample was analyzed in triplicate.

#### Determination of antibacterial activity

Antibacterial activity was determined using broth microdilution technique, as described previously, with modifications<sup>15</sup>. The tested microbes were *Staphylococcus aureus* ATCC 25933, *Escherichia coli* ATCC 25922, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Enterococcus faecalis*. *K. pneumoniae*, *P. aeruginosa*, and *E. faecalis* were collected and isolated from patients at the Maharaj Nakhon Si Thammarat Hospital, Thailand. Prior to testing, bacteria were cultured on tryptic soy agar at 37°C for 24 hr. A single colony was inoculated into 2x Mueller–Hinton broth (MHB) and incubated at 37°C for 3 hr. The bacterial solutions were diluted to achieve a turbidity equal to 0.5 McFarland standard and further diluted with 0.85% w/v NaCl solution by 100-fold. For sample preparation, 30 mg of the extract was weighed and dissolved in dimethyl sulfoxide (0.5 mL), and adjust the volume with distilled water to 5 mL. The stock solution was diluted with 2x MHB using serial dilution. The test bacteria (10 µL) were added to each sample and mixed. The mixtures were incubated at 37°C for 24 hr. To detect viable bacteria, resazurin solution (10 µL) was added and the solutions were incubated for 3 hr. A change of color from blue to pink indicated viable bacterial cells. The lowest concentration of each sample that inhibited bacterial viability was the minimum inhibitory concentration (MIC). The solutions in the wells whose color did not change were plated on Mueller–Hinton agar (MHA) and incubated for 3 hr. The lowest concentration where there was no growth of bacteria was the minimum bactericidal concentration (MBC).

Tetracycline was used as a positive control. The samples were tested in triplicate.

#### Statistical analysis

The data were expressed as mean±standard deviation. Differences among groups were analyzed using one-way analysis of variance (ANOVA), followed by the Scheffe test using Statistical Package for the Social Science software (SPSS software V26). A significant difference was considered for p-values<0.05.

## Results

Three YGBNR formulations were prepared (No. 1, 2, and 3) using kratom collected from Surat Thani, Songkhla, and Chumphon, respectively. Their phytochemical profiles were determined, which included mitragynine content, TPC, TTC, and TFC; as summarized in Table 1. The results show that the kratom extract, the principal component in YGBNR, contributed markedly to the phytochemical characteristics of the formula. This correlation between kratom and YGBNR phytochemical profiles suggests that the kratom extract contributes greatly to the biological effects of YGBNR.

Next, the antioxidant potential of the formulations was determined using the DPPH and FRAP assays. Kratom (Surat Thani) extract exhibited the lowest scavenging activity  $IC_{50}$  of  $0.35\pm 0.01$  mg/mL, followed by the extracts from ginger, chili, and Java long pepper. Interestingly, YGBNR extracts showed similar free radical scavenging potency to kratom and ginger extracts (p-value>0.05). The results of the FRAP assay suggest that the highest gallic acid equivalent was in kratom, followed by ginger. Conversely, ferric reducing power was much lower in the Java long pepper and chili. The antioxidant activities of each extract vary with the TPC, TTC, and TFC, suggesting that these phytochemicals are responsible for the antioxidant powers of the formulation (Table 1).

The antibacterial activities of YGBNRs were evaluated and compared for bacterial viability and growth in the presence of the plant extracts and volatile oil fraction. As shown in Table 2, the extracts did not exhibit antibacterial activity against *S. aureus*, *K. pneumoniae*, and *E. faecalis*. Only the ginger extract inhibited the growth of

*E. coli* and *A. baumannii*, with MICs of 3 mg/mL and 1.5 mg/mL, respectively. Distillation of YGBNR formula No. 3 resulted in a volatile oil fraction that exhibited bacteriostatic effects against *E. coli* and *P. aeruginosa*, with an MIC of 3 mg/mL. These results indicate that the extracts have low antibacterial activities against the tested bacteria.

**Table 1** Phytochemical contents and the antioxidant power of the formulation

| Sample           | Mitragynine content (%w/w dry extract) | TPC mg GAE/g dry extract  | TTC mg CE/g dry extract   | TFC mg RE/g dry extract   | Antioxidant power             |                            |
|------------------|--|---------------------------|---------------------------|---------------------------|-------------------------------|----------------------------|
|                  |  |                           |                           |                           | DPPH IC <sub>50</sub> (mg/mL) | FRAP mg GAE /g dry extract |
| YGBNR No.1       | 5.32 <sup>b</sup> ±0.13                | 123.74 <sup>c</sup> ±1.96 | 143.41 <sup>b</sup> ±2.60 | 538.69 <sup>c</sup> ±3.05 | 0.39 <sup>a</sup> ±0.00       | 90.92 <sup>d</sup> ±1.12   |
| YGBNR No.2       | 3.66 <sup>c</sup> ±0.37                | 141.01 <sup>b</sup> ±1.44 | 123.81 <sup>c</sup> ±2.14 | 596.50 <sup>b</sup> ±1.48 | 0.41 <sup>a</sup> ±0.00       | 102.46 <sup>c</sup> ±1.61  |
| YGBNR No.3       | 4.49 <sup>d</sup> ±0.09                | 104.75 <sup>d</sup> ±2.35 | 81.30 <sup>d</sup> ±0.83  | 478.56 <sup>d</sup> ±1.30 | 0.48 <sup>a</sup> ±0.00       | 64.53 <sup>e</sup> ±2.33   |
| Kratom           | 6.41 <sup>a</sup> ±0.16                | 186.98 <sup>a</sup> ±0.30 | 201.27 <sup>a</sup> ±0.99 | 834.27 <sup>a</sup> ±3.24 | 0.35 <sup>a</sup> ±0.01       | 115.46 <sup>a</sup> ±1.51  |
| Ginger           |  | 126.20 <sup>c</sup> ±1.84 | 8.56 <sup>e</sup> ±0.82   | 319.92 <sup>e</sup> ±2.21 | 0.38 <sup>a</sup> ±0.01       | 107.48 <sup>b</sup> ±0.71  |
| Java long pepper |  | 18.91 <sup>f</sup> ±0.93  | 1.66 <sup>f</sup> ±0.03   | 108.13 <sup>f</sup> ±2.76 | 6.67 <sup>c</sup> ±0.29       | 10.88 <sup>f</sup> ±2.04   |
| Chili            |  | 32.76 <sup>e</sup> ±1.09  | 1.46 <sup>f</sup> ±0.10   | 74.01 <sup>g</sup> ±4.27  | 4.04 <sup>b</sup> ±0.12       | 13.56 <sup>e</sup> ±0.42   |
| Gallic acid      |  |                           |                           |                           | 0.02±0.00                     |                            |

Statistical analysis (One-way ANOVA; the Scheffe test) was performed. Means with the same letter <sup>(a-f)</sup> are not significantly different. YGBNR No.1, 2, and 3 contained kratom powder from Surat Thani, Songkhla, and Chumphon, respectively. GAE=gallic acid equivalent, YGBNR=Ya-Gae-Bid-Na-Ron, TPC=total phenolic content, CE=catechin equivalent, TTC=total tannin content, TFC=total flavonoid content, RE=rutin equivalent, DPPH=2,2-diphenyl-1-picrylhydrazyl, IC<sub>50</sub>=the concentration of antioxidant at 50% inhibition, FRAP=ferric-reducing antioxidant power

**Table 2** The antibacterial activities of the sample (MIC/MBC; µg/mL)

| Sample           | <i>S. aureus</i> |      | <i>E. coli</i> |      | <i>A. baumannii</i> |       | <i>K. pneumoniae</i> |     | <i>P. aeruginosa</i> |       | <i>E. faecalis</i> |      |
|------------------|------------------|------|----------------|------|---------------------|-------|----------------------|-----|----------------------|-------|--------------------|------|
|                  | MIC              | MBC  | MIC            | MBC  | MIC                 | MBC   | MIC                  | MBC | MIC                  | MBC   | MIC                | MBC  |
| YGBNR No.1       | ND               | ND   | ND             | ND   | ND                  | ND    | ND                   | ND  | 3,010                | NO    | ND                 | ND   |
| YGBNR No.2       | ND               | ND   | ND             | ND   | ND                  | ND    | ND                   | ND  | ND                   | ND    | ND                 | ND   |
| YGBNR No.3       | ND               | ND   | ND             | ND   | ND                  | ND    | ND                   | ND  | ND                   | ND    | ND                 | ND   |
| Ginger           | ND               | ND   | 3,010          | NO   | 1,505               | NO    | ND                   | ND  | ND                   | ND    | ND                 | ND   |
| Chili            | ND               | ND   | ND             | ND   | ND                  | ND    | ND                   | ND  | ND                   | ND    | ND                 | ND   |
| Java long pepper | ND               | ND   | ND             | ND   | ND                  | ND    | ND                   | ND  | ND                   | ND    | ND                 | ND   |
| Kratom           | ND               | ND   | ND             | ND   | ND                  | ND    | ND                   | ND  | ND                   | ND    | ND                 | ND   |
| Volatile oil     | ND               | ND   | 3,010          | NO   | ND                  | ND    | ND                   | ND  | 3,010                | NO    | ND                 | ND   |
| Tetracycline HCl | 0.12             | 1.95 | 0.01           | 0.01 | 31.25               | 62.50 | 125                  | 125 | 1.95                 | 15.63 | 0.12               | 0.49 |

YGBNR No.1, 2, and 3 contained kratom powder from Surat Thani, Songkhla, and Chumphon, respectively. Volatile oil was prepared by hydro-distillation of YGBNR formula No.3.

YGBNR=Ya-Gae-Bid-Na-Ron, MIC=minimum inhibitory concentration, MBC=minimum bactericidal concentration, µg/mL=microgram per milliliter, ND=not detected, No=no bactericidal effect

## Discussion

The use of kratom in Thai Traditional medicine has been documented and studied<sup>16</sup>. Folk medicine prescribes kratom to relieve pain, cough, and diarrhea. In the preparation of the traditional formulation, kratom has either been used as a single ingredient or combined with other herbs. YGBNR is a formulation composed of four plants of kratom leaves, ginger rhizome, Java long pepper fruit, and chili fruit, which are all easily accessible. YGBNR is also indicated for the management of diarrhea, which may be caused by infection from food poisoning. In Thai, the name YGBNR literally means “recommended for use in the summer”. Therefore, we found it interesting to establish the antioxidant and antibacterial activities of kratom-containing YGBNR to support other medical purposes it may serve.

We prepared the YGBNR extracts using the maceration method in ethanol and performed phytochemical analysis (Table 1). Mitragynine content varied from 3% – 6% (w/w), which varied depending on the source of kratom. Kratom leaves collected from Chumphon and Surat Thani contained high levels of mitragynine. Kratom contained more phenols, tannins, and flavonoids, compared to ginger, Java long pepper, and chili. This could explain the antioxidant activity of kratom in the DPPH assay. In contrast, the phenolic content was a more important determinant of activity in the FRAP assay. Thus, formulas No. 1 and 2 had the highest antioxidant power, which was probably due to the kratom and ginger in the formulas. Recently, it was reported that ethanolic kratom extract contained the TPC of  $252.92 \pm 1.15$  mg GAE/g extract and TFC of  $26.07 \pm 0.01$  mg quercetin equivalent (QE)/g extract<sup>17</sup>. The methanol extract had a TPC of  $105.58 \pm 5.43$  mg GAE/g extract and TFC of  $91.12 \pm 17.27$  mg CE/g extract<sup>18</sup>. This present study showed lower TPC, but higher TFC and TTC. These results suggest that YGBNR can be used as an antioxidant to protect against oxidative stress. Future studies on the mechanism of action are recommended. Interestingly, no antibacterial

activities were observed in the YGBNR formulations for the bacteria tested.

The efficacy of the active pharmaceutical ingredient in the recipe depends on the quality of the kratom leaves. Alkaloid content; especially mitragynine, varies based on seasonal changes, altitude, and geographical origin<sup>19</sup>. Kratom is distributed primarily in the southern regions of Thailand. This present study reveals that kratom leaves from Surat Thani and Chumphon have outstanding quality kratom in terms of mitragynine content.

YGBNR is a formula containing kratom leaves as a significant ingredient and has been used to treat diarrhea with flatulence. Antidiarrheal activity of the YGBNR is expected from mitragynine in kratom due to its opioid effect that causes a reduction in gastrointestinal mobility<sup>8</sup>. Long-term use of YGBNR can increase the risk of constipation. Adding ginger, Java long pepper, and chili may reduce the undesired impact of flatulence. The antioxidant activity of YGBNR may be due to its phenolic-related contents. Preclinical and clinical tests are warranted to confirm the efficacy and safety of this formulation.

## Conclusion

YGNBR contains kratom as its principal ingredient and has been previously shown to reduce gastrointestinal mobility in diarrhea. This study's phytochemical analysis showed the abundance of phenolic compounds in YGBNR, which corresponded to the antioxidant power of the formulation. However, YGBNR has no antibacterial activity.

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## Conflict of interest

The authors declare no conflict of interest.



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