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# EVALUATION OF PHOSPHOMOLYBDENUM AND FERRIC REDUCING ANTIOXIDANT POWER ASSAYS IN EXTRACT COMBINATIONS OF *MANGIFERA INDICA* AND *EUPHORBIA HIRTA*

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***Abstract***

*Combining plant extracts at lower doses may enhance antioxidant effects through synergy. This study investigated the antioxidant capacity of combined Mangifera indica and Euphorbia hirta leaf extracts using metal ion-reducing power assays. The extracts were prepared via maceration with 70% ethanol. Phytochemical profiling was conducted through preliminary screening tests. Antioxidant capacity was assessed spectrophotometrically using the phosphomolybdenum and ferric reducing antioxidant power (FRAP) assays. The phosphomolybdenum assay involved incubation of the extracts with reagent at 95°C for 90 minutes, while the FRAP assay was conducted at 37°C for 15 minutes. Extract combinations were prepared in the ratios of 1:3, 1:1, and 3:1. Phytochemical screening revealed the presence of alkaloids, phenolics, flavonoids, steroids, and terpenoids in both extracts. In the phosphomolybdenum assay, the Mo(VI) reducing power of M. indica extract (29.154±0.664 mg AAE/g extract) was higher than that of E. hirta (27.948±0.667 mg AAE/g extract). Similarly, in the FRAP assay, the ferric reducing power of M. indica extract (55.304±1.284 mg AAE/g extract) exceeded that of E. hirta (48.009±1.873 mg AAE/g extract). The highest reducing powers were observed in Comb. 3, yielding 30.745±0.715 mg AAE/g extract (phosphomolybdenum) and 57.190±1.431 mg AAE/g extract (FRAP). Among the three combinations tested, only the 3:1 ratio demonstrated a synergistic antioxidant effect in both assays.*

***Keywords:*** *free radicals, medicinal plants, oxidative stress, phytochemicals, synergistic effect.*

## Introduction

Antioxidants play a crucial role in disease prevention by neutralizing harmful free radicals, thereby reducing oxidative stress and protecting cells from damage. They help maintain a strong immune system, which may prevent infections and chronic illnesses such as heart disease and cancer (Ayoka et al., 2022). Although the body naturally produces antioxidants to combat radical species, external sources of antioxidants become necessary when endogenous levels are insufficient (Kurutas, 2016; Vona et al., 2021).

Plants are rich sources of secondary metabolites, particularly phenolics and flavonoids, which are known for their antioxidant properties (Tungmunnithum et al., 2018). The antioxidant potential of phenolics and flavonoids arises from their ability to donate single electrons (single electron transfer, SET) and/or hydrogen atoms (hydrogen atom transfer, HAT) to free radical molecules (Miličević, 2024; Zeb, 2020).

The metal ion-reducing power assay is one of the antioxidant assays based on the SET mechanism. It evaluates an antioxidant's ability to donate an electron to a metal ion, thereby reducing it (Shahidi & Zhong, 2015).

*Mangifera indica* L., commonly known as mango, is traditionally recognized for its medicinal properties in Indonesia. In Bali, all parts of the *M. indica* plant are documented in the *Lontar Usada Taru Pramana* as treatments for stomachache, heartburn, and palpitations. Several studies have reported the strong antioxidant potential of *M. indica* extracts, particularly in metal ion-reducing assays. Duresa and Manaye (2017) evaluated the antioxidant capacity of three *M. indica* fruit extracts (50% ethanol) from different varieties (Nopha, Mettu, and Gore) using the phosphomolybdenum method. Their results revealed reducing powers of $0.372\pm3.45 \times 10^{-3}$, $0.360\pm3.55 \times 10^{-3}$, and $0.356\pm3.76 \times 10^{-3}$ mg AAE/25 g, respectively. Abdel-Aty et al. (2018) reported that *M. indica* seed kernel extract (80% methanol) exhibited Mo(VI) reducing power of 4.00±0.11 µg GAE/mL. Similarly, Tambunan and Sihotang (2024) found that ethanol extract of *M. indica* leaves demonstrated strong ferric reducing antioxidant power, with a FRAP value of 151.7 mg/mL. This capacity is attributed not only to phenolics and flavonoids but also to the presence of mangiferin (Mistry et al., 2023; Sekar et al., 2019).

In traditional medicine, high doses of herbal extracts are often administered to achieve pharmacological effects comparable to conventional drugs. However, prolonged use of high-dose extracts may lead to adverse side effects. Combining *M. indica* with other plant extracts may enhance efficacy while reducing negative outcomes. This approach offers a promising strategy for managing diseases related to oxidative stress (Ekor, 2014). Nevertheless, the interactions between herbal components in traditional formulations remain insufficiently explored.

*Euphorbia hirta* is a weed plant that has long been used in Indonesia as a traditional medicine for treating various ailments such as asthma, skin conditions, and digestive disorders (Sahertia et al., 2023). The extract of *E. hirta* contains phenolic, flavonoid, and terpenoid compounds, which contribute to its diverse pharmacological activities (Gupta et al., 2017; Wu et al., 2012). Numerous studies have indicated that *E. hirta* extract possesses antioxidant properties. For example, Caroline et al. (2018) reported that the methanol extract of *E. hirta* aerial parts (at 60 µg/mL) showed percentage reduction values of 53.68±3.75% and 54.06±3.78% in the phosphomolybdenum and FRAP assays, respectively. Similarly, Kain et al. (2022) demonstrated that the ethanol extract of *E. hirta* leaves exhibited ferric and Mo(VI) reducing power values of 595.99 and 525.84 mg AAE/g extract, respectively. Combining *Mangifera indica* with *E. hirta* may offer a novel approach to addressing health issues related to oxidative stress at lower doses.

Several in vitro studies have explored the combination of *M. indica* leaf extract with other plant extracts. Pamungkas and Retnaningtyas (2017) found that the combination of *M. indica* leaf extract with *Pandanus amaryllifolius* Roxb. leaf extract exhibited stronger antioxidant activity than either extract alone. Likewise, Kurniawati et al. (2021) reported that combining *M. indica* and *Annona muricata* leaf extracts produced a more potent DPPH radical scavenging effect than individual extracts. Nevertheless, no research has specifically examined the antioxidant capacity of the combination of *M. indica* and *E. hirta*. Therefore, this study is the first to report the antioxidant activity of this combination through the metal ion-reducing power mechanism. Additionally, a synergistic antioxidant effect was observed when the two extracts were combined.

## Methodology

### Tools and materials

The tools utilized in this study included an analytical balance (Ohaus), oven (Memmert), Buchner funnel (Iwaki), rotary evaporator (IKA), test tubes (Iwaki), hot plate stirrer (Thermo Scientific), and spectrophotometer (Genesys). *M. indica*

leaves were collected from residential plantations in Badung Regency, Bali Province, Indonesia, while the aerial parts of *E. hirta* were obtained from rice fields in the same region. All chemicals used were of analytical grade, including $FeCl_3$, Mg, HCl, chloroform, acetic anhydride, $H_2SO_4$, Mayer's reagent, Dragendorff's reagent, ammonium molybdate, sodium phosphate, methanol, 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), and acetate buffer (300 mM, pH 3.6).

**Extraction procedure**

Fresh *M. indica* leaves and the aerial parts of *E. hirta* were dried in an oven at 45°C for three days, milled, and filtered using a 200-mesh strainer. Ethanol (70% v/v) was used for maceration in a sample-to-solvent ratio of 1:10. Solvent removal was performed using a rotary evaporator (120 rpm, 45°C). The thick extracts obtained were stored at 4°C until use (Putra et al., 2020).

**Phytochemical screening**

A qualitative phytochemical screening was performed on *M. indica* and *E. hirta* extracts using standard tests. Alkaloids were detected using Mayer's and Dragendorff's reagents. Phenolics, flavonoids, steroids, and terpenoids were identified using 1% $FeCl_3$, Mg-conc. HCl, chloroform-acetic anhydride-conc. $H_2SO_4$, and chloroform-conc. $H_2SO_4$, respectively. The presence of phytochemicals was indicated by the formation of precipitates or color changes (Rajkumar et al., 2022).

**Phosphomolybdenum assay**

This assay determined the extracts' ability to reduce Mo(VI) to Mo(V). This method was chosen because it does not rely on organic solvents (commonly uses water) and employs reagents that are simple, sensitive, and cost-effective (Sadeer et al., 2020). In this assay, a specific volume of extract solution was mixed with 2 mL of phosphomolybdenum reagent to yield a final extract concentration of 100 µg/mL. After incubation at 95°C for 90 minutes, the absorbance was measured at 693 nm (Putra et al., 2022). The Mo(VI) reducing power was expressed as milligrams of ascorbic acid equivalent per gram of extract (mg AAE/g extract**).**

**FRAP assay**

The FRAP assay measures the ability of an extract to reduce Fe(III) to Fe(II). This method is known for its reproducibility, sensitivity, and compatibility with a wide range of biological samples, as well as its simple and affordable instrumentation (Sadeer et al., 2020). The assay was carried out by mixing a specific volume of extract solution with 0.5 mL of FRAP reagent, achieving a final extract concentration of 100 µg/mL. After incubation at 37°C for 15 minutes, the absorbance was recorded at 594 nm (Putra et al., 2022). The results were expressed as mg AAE/g extract.

**Determination of interaction type**

Extract combinations were prepared by mixing *M. indica* and *E. hirta* extracts in ratios of 1:3, 1:1, and 3:1. The interaction type was determined by comparing the theoretical value (TV) and experimental value (EV). The theoretical value was calculated based on the proportional contributions of the individual extracts. A synergistic interaction was defined when the EV was more than 5% higher than the TV. An antagonistic interaction was indicated when the EV was more than 5% lower than the TV. An additive interaction was observed when the difference between TV and EV was less than 5% (Nutmakul & Chewchinda, 2023).

**Data Analysis**

The results were presented as mean ± standard deviation (SD) based on triplicate experiments. Data analysis was conducted using GraphPad Prism 8. Differences between groups were evaluated using one-way ANOVA followed by Tukey's post hoc test.

**Results and Discussion**

**Phytochemical profile**

The presence of secondary metabolites in *M. indica* and *E. hirta* extracts was confirmed through a simple qualitative test, with the results presented in **Table 1**. The

findings demonstrated that *M. indica* and *E. hirta* extracts contained alkaloids, phenolics, flavonoids, steroids, and terpenoids. The presence of these secondary metabolites in plants is associated with a wide range of pharmacological properties. Phenolics and flavonoids, in particular, are classes of phytochemicals known for their potent antioxidant activity. The occurrence of phenolics and flavonoids in the ethanol extract of *M. indica* leaves was previously reported by Dhital (2017), who also noted its strong antioxidant effect. Similarly, previous studies have revealed that the ethanol extract of *E. hirta* contains phenolics, flavonoids, and terpenoids (Ahmad et al., 2017; Praveen et al., 2024). *E. hirta* extract has also exhibited strong antioxidant activity (Praveen et al., 2024).

**Table 1.** Phytochemical screening results

| Phytochemical | Reagent | Observed appearance | Result | |
|---|---|---|---|---|
| | | | ***M. indica*** | ***E. hirta*** |
| Alkaloids | Mayer | Yellowish precipitate | + | + |
| | Dragendorff | Purple precipitate | + | + |
| Phenolics | $FeCl_3$ 1% | Greenish black | + | + |
| Flavonoids | Mg-Conc. HCl | Orange | + | + |
| Steroids | Chloroform-acetic anhydride-conc. $H_2SO_4$ | Greenish-yellow fluorescence | + | + |
| Terpenoids | Chloroform-glacial acetic acid | Reddish-brown precipitate | + | + |

**Note:** "+" indicates the presence of phytochemicals.
**Abbreviations:** *Conc.* = concentrated; *M. indica* = *Mangifera indica*; *E. hirta* = *Euphorbia hirta*.

## Mo(VI) reducing power

The Mo(VI) reducing power was determined using the phosphomolybdenum method. This assay was based on the reduction of Mo(VI) to Mo(V) in the presence of antioxidants. A green complex was formed upon heating at 95°C for 90 minutes, which could be quantified by measuring absorbance at 695 nm (Jafri et al., 2017). Ascorbic acid was used as a standard to quantify the reducing power of the extracts. Figure 1 displays the linear calibration curve for ascorbic acid, where the absorbance increased proportionally with concentration. The linear regression equation obtained was $y = 0.0406x + 0.0359$, with an $R^2$ value of 0.9948.

Table 2 summarizes the Mo(VI) reducing power of *M. indica*, *E. hirta*, and their combined extracts. *M. indica* extract exhibited a slightly higher reducing power (29.154±0.664 mg AAE/g extract) compared to *E. hirta* extract (27.948±0.667 mg AAE/g extract); however, the difference was not statistically significant ($p > 0.05$). This suggests that both extracts had comparable reducing powers as measured by the phosphomolybdenum assay. When the two extracts were combined, the Mo(VI) reducing power increased proportionally with the concentration of *M. indica* extract.

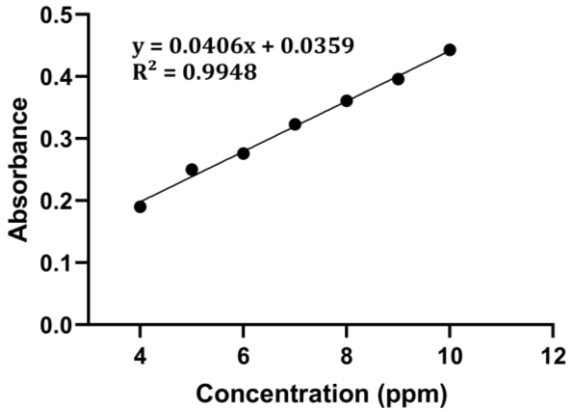


**Figure 1**. Linear curve of ascorbic acid for the phosphomolybdenum assay

**Table 2.** Interaction types of *M. indica* and *E. hirta* extract combinations in the phosphomolybdenum assay

| Extract | Combination ratio | Experimental value (mg AAE/g extract) | Theoretical value (mg AAE/g extract) | Interaction |
|---|---|---|---|---|
| *M. indica* | - | 29.154±0.664$^{bc}$ | - | - |
| *E. hirta* | - | 27.948±0.667$^{b}$ | - | - |
| Comb. 1 | 1:3 | 23.817±0.501$^{a}$ | 28.249 | Antagonistic |
| Comb. 2 | 1:1 | 29.693±0.464$^{c}$ | 28.551 | Additive |
| Comb. 3 | 3:1 | 30.745±0.715$^{c}$ | 28.852 | Synergistic |

**Note:** Superscript letters ($^{a}$, $^{b}$, $^{bc}$, $^{c}$) indicate statistically significant differences based on one-way ANOVA followed by Tukey's post hoc test ($p < 0.05$).
**Abbreviations:** AAE = Ascorbic Acid Equivalent.

The combination of antioxidant-containing extracts can result in three types of interactions: synergistic, antagonistic, or additive. Based on the interaction analysis, only Combination 3 (ratio 3:1) exhibited a synergistic effect, whereas Combination 1 (ratio 1:3) and Combination 2 (ratio 1:1) showed antagonistic and additive effects, respectively (see Table 2).

## Ferric reducing power

The ferric reducing power of the extracts was evaluated using the FRAP assay, which relies on the donation of electrons to the Fe(III) cation. Basically, the FRAP test measures the ability of an extract to reduce Fe(III) to Fe(II) under acidic conditions. This reduction leads to the formation of a blue Fe(II)-TPTZ complex in the presence of TPTZ (Munteanu & Apetrei, 2021).

In this study, ascorbic acid was used to generate a linear regression curve. The absorbance of the reaction solution with the FRAP reagent was measured at 593 nm. The resulting regression equation was y = 0.2099x + 0.2432, with a correlation coefficient (r) of 0.9992. The calibration curve for ascorbic acid is presented in Figure 2.

Table 3 summarizes the ferric reducing power of *M. indica*, *E. hirta*, and their combined extracts. The results indicate that the ferric reducing power of *M. indica* extract (55.304±1.284 mg AAE/g extract) was significantly higher ($p < 0.05$) than that of *E. hirta* extract (48.009±1.873 mg AAE/g extract). The ferric reducing power of the combined extracts increased with the proportion of *M. indica* in the mixture. The reducing power followed the order: Comb. 3 (3:1) > Comb. 2 (1:1) > Comb. 1 (1:3). Furthermore, combinations 1 and 2 exhibited antagonistic interactions, while only combination 3, which contained a higher proportion of *M. indica*, demonstrated a synergistic interaction.

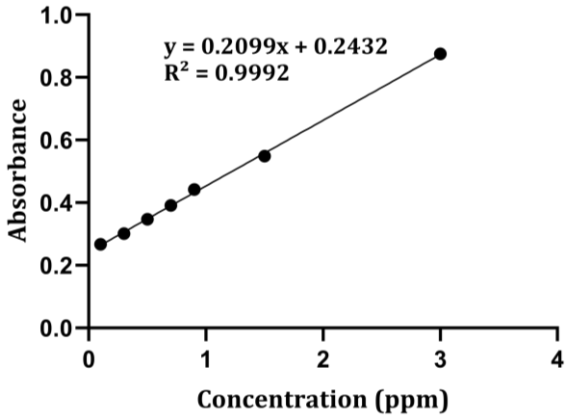


**Figure 2**. Linear curve of ascorbic acid for the FRAP assay

## Synergistic effect

Synergism is a central concept in traditional medicine, where formulations often consist of multiple herbal extracts. This synergy enhances efficacy, reduces the required dosage of each extract, and lowers potential toxicity (Yuan et al., 2017). A synergistic antioxidant effect occurs when the antioxidant capacity of two combined extracts exceeds the sum of their individual effects (Olszowy-Tomczyk, 2020). Moreover, synergy can emerge when a weaker antioxidant enhances the activity of a stronger one within the combination (Bayram & Decker, 2023). In this study, extract combinations were tested at three ratios: 1:3, 1:1, and 3:1. These ratios were

chosen based on the hypothesis that synergistic effects are more likely at higher concentration ratios. Lower ratios such as 1:2 or 2:1 may not demonstrate statistically significant synergy because the reducing power of the combined extracts is similar to, or even weaker than, the individual extracts.

Among the three combinations, only combination 3 (3:1) exhibited a synergistic interaction. This ratio indicates that the concentration of *E. hirta* was three times lower than that of *M. indica*. This suggests that *E. hirta* was the weaker antioxidant in the mixture. The synergistic antioxidant mechanism, in this case, might involve regeneration. For example, when *M. indica* reduced Mo(VI) to Mo(V) in the phosphomolybdenum assay, it became oxidized and lost antioxidant activity. The *E. hirta* extract might then regenerate *M. indica* to its active state, enabling it to continue reducing Mo(VI). A similar mechanism likely occurred in the FRAP assay. This process is commonly referred to as antioxidant regeneration (Putra et al., 2024).

**Table 3**. Interaction types of *M. indica* and *E. hirta* extract combinations in the FRAP assay

| Extract | Combination ratio | Experimental value (mg AAE/g extract) | Theoretical value (mg AAE/g extract) | Interaction |
|---|---|---|---|---|
| *M. indica* | - | 55.304±1.284$^{c}$ | - | - |
| *E. hirta* | - | 48.009±1.873$^{b}$ | - | - |
| Comb. 1 | 1:1 | 39.771±2.027$^{a}$ | 49.833 | Antagonistic |
| Comb. 2 | 1:3 | 48.654±1.182$^{b}$ | 51.657 | Antagonistic |
| Comb. 3 | 3:1 | 57.190±1.431$^{c}$ | 53.480 | Synergistic |

**Note:** Superscript letters ($^{a}$, $^{b}$, $^{c}$) indicate statistically significant differences based on one-way ANOVA followed by Tukey's post hoc test ($p < 0.05$).
**Abbreviations:** AAE = Ascorbic Acid Equivalent.

Several researchers have demonstrated the synergistic antioxidant effects of extract combinations. Crespo et al. (2019) employed a simplex-lattice design to investigate this phenomenon in a mixture of essential oil extracts from *Coriandrum sativum*, *Thymus vulgaris*, and *Anethum graveolens*. Based on FRAP and ABTS assays, the combination comprising 16.7% *C. sativum*, 66.7% *T. vulgaris*, and 16.7% *A. graveolens* exhibited the most significant synergistic effect. Similarly, Putra et al. (2022) examined the antioxidant synergy between *Citrus grandis* and *Blumea balsamifera* extracts using phosphomolybdenum, FRAP, DPPH, and ABTS assays. Their results indicated that the mixture demonstrated synergistic antioxidant activity at specific ratios. Benamar-Aissa et al. (2023) also reported synergistic effects from combining extracts of two *Artemisia* species, *A. campestris* and *A. herba-alba*, with *Citrus aurantium*. Using a Simplex Lattice Mixture Design and evaluating the combinations with FRAP, DPPH, and ABTS assays, they found that all mixtures exhibited synergistic antioxidant effects.

## Conclusion

This study demonstrated that *M. indica* and *E. hirta* extracts possess significant metal ion-reducing power. In the phosphomolybdenum assay, *M. indica* and *E. hirta* extracts exhibited Mo(VI) reducing power of 29.154±0.664 and 27.948±0.667 mg AAE/g extract, respectively. Meanwhile, in the FRAP assay, their ferric reducing powers were 55.304±1.284 and 48.009±1.873 mg AAE/g extract, respectively. When combined, the highest metal ion-reducing power was observed in Comb. 3 (3:1 ratio), with Mo(VI) reducing and ferric reducing powers of 30.745±0.715 and 57.190±1.431 mg AAE/g extract, respectively. A synergistic antioxidant effect was observed exclusively in Comb. 3, likely attributable to an antioxidant regeneration mechanism in which *E. hirta* extract regenerated the antioxidant potential of *M. indica*, thereby enhancing the overall

reducing power of the mixture. These findings suggest improved antioxidant efficacy, potential dose reduction, and decreased toxicity of the extract combination. However, since this study was conducted in an in vitro environment, the results might not fully translate to in vivo conditions. Therefore, further in vivo antioxidant evaluations using experimental animals are necessary to validate and extend these findings.

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