TYPE Original Research
DOI 10.53905/igim.v1i03.17

INSPIRETECH GLOBAL INSIGHT
INSPIRE GLOBAL INSIGHT MULTIDISCIPLINARY JOURNAL

OPEN ACCESS

EDITED BY
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RECEIVED: June 11, 2026
ACCEPTED: July 19, 2026
PUBLISHED: July 27, 2026

CITATION APA STYLE 7
Leo, H. B., Regaletha, T. A. L., Purnasari, C. ., & Pua Upa, N. F. . (2026). Antagonistic Interaction Between Piper betle and Peperomia pellucida Ethanolic Extracts Reduces Antibacterial Activity Against Cutibacterium acnes. *Inspire Global Insight Journal*, 1(03), 88-92. https://doi.org/10.53905/igim.v1i03.17

Antagonistic Interaction Between *Piper betle L* and *Peperomia pellucida L* Ethanolic Extracts Reduces Antibacterial Activity Against *Cutibacterium acnes*

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ABSTRACT

Acne vulgaris is a chronic inflammatory disorder of the pilosebaceous unit in which Cutibacterium acnes plays a central pathogenic role. Rising resistance of C. acnes to conventional antibiotics such as clindamycin has intensified interest in plant-derived alternatives. This study aimed to evaluate the antibacterial activity of combined ethanolic extracts of green betel leaf (Piper betle L) and pepper elder leaf (Peperomia pellucida L) against C. acnes and to determine the combination ratio with the strongest antibacterial effect. A true experimental, post-test-only control group design was used. Leaves were macerated in 95% ethanol (1:10, w/v) for three days. Seven treatment groups were tested by the Kirby-Bauer disc diffusion method: single extract of P. betle 15%, single extract of P. pellucida 15%, combinations at ratios of 1:1, 1:2, and 2:1 at a fixed total concentration of 15%, a positive control (standardized clindamycin disc), and a negative control (sterile distilled water). Each group was replicated four times, determined using the Federer formula. Zones of inhibition were measured with a caliper after 24 h of incubation at 37 °C. Data were analyzed with Shapiro-Wilk, Levene's, one-way ANOVA, and Tukey's HSD post-hoc tests ($\alpha = 0.05$). All extract treatments inhibited C. acnes growth. The single P. betle 15% extract produced the largest inhibition zone among plant treatments (12.19 ± 0.44 mm, strong category), whereas single P. pellucida 15% extract produced 8.96 ± 0.30 mm (moderate category). Combination ratios of 1:1, 1:2, and 2:1 produced inhibition zones of 9.57 ± 0.77 mm, 8.76 ± 0.39 mm, and 8.56 ± 0.73 mm, respectively, all within the moderate category. The positive control produced 27.19 ± 0.95 mm (very strong), while the negative control produced no inhibition zone. One-way ANOVA revealed a statistically significant difference among treatment groups ($p < 0.001$), and Tukey's HSD indicated that the combinations did not significantly exceed the single P. pellucida extract, while the single P. betle extract significantly outperformed all combination ratios. Combined ethanolic extracts of P. betle and P. pellucida exhibit antibacterial activity against C. acnes, but combination did not enhance efficacy beyond that of the single P. betle extract, suggesting an antagonistic rather than synergistic interaction. The 1:1 ratio was the most effective among the combinations tested.

Keywords
acne vulgaris; cutibacterium acnes; piper betle l; peperomia pellucida l; antibacterial activity; extract combination.

INTRODUCTION

Acne vulgaris is a chronic inflammatory condition of the pilosebaceous unit characterized by comedones, papules, and pustules, and it remains one of the most common dermatological complaints worldwide. In Southeast Asia, reported prevalence ranges from 40% to 80%, with the highest burden among adolescents aged 14–19 years. Beyond its visible manifestations, acne carries a substantial psychosocial burden, including anxiety, depression, and diminished self-esteem associated with residual scarring, underscoring the clinical and public-health relevance of effective, accessible treatment options (Cui et al., 2022; Hay et al., 2016).

Among the pathogenic contributors to acne, *Cutibacterium acnes* (formerly *Propionibacterium acnes*) is a key anaerobic, Gram-positive commensal that proliferates within occluded follicles, promotes lipase-mediated hydrolysis of sebum triglycerides into pro-inflammatory free fatty acids, and activates innate immune pathways via Toll-like receptors, driving comedogenesis and inflammation(Huang et al., 2024; B. Zhang et al., 2019; Y. Zhang et al., 2023). Topical and oral antibiotics such as clindamycin, erythromycin, and tetracycline remain first-line adjuncts to acne therapy, but their prolonged and often unsupervised use has been paralleled by a global rise in antimicrobial resistance(Karadağ et al., 2020).

Regional surveillance data illustrate the scale of the problem. In Japan and South Korea, resistance rates of C. acnes to macrolide-lincosamide antibiotics are comparatively low (around 3–4%), whereas in Indonesia, resistance to tetracycline, erythromycin, and clindamycin has been reported at 12.9%, 45.2%, and 61.3%, respectively. Such disparities highlight both the urgency of the resistance problem in Indonesia specifically and the need for alternative antibacterial agents that are less prone to selecting resistant strains, motivating renewed interest in plant-derived antimicrobials as adjunct or alternative therapies(Angelini,

2024; Subramani et al., 2017).

Piper betle L (green betel) is a tropical plant long used in traditional Indonesian medicine; its leaves contain flavonoids, tannins, saponins, terpenoids, and phenolic compounds, including chavicol and eugenol derivatives, that confer bactericidal activity against both Gram-positive and Gram-negative organisms(Asan et al., 2022; Boripun et al., 2022). Previous work combining green, red, and black betel leaf extracts has demonstrated antibacterial activity against C. acnes, supporting the plant's relevance to acne-related pathogens. Similarly, *Peperomia pellucida L* (pepper elder, locally known as suruhan) has traditionally been applied to skin infections and bruises, and its extracts contain alkaloids and polyphenolic compounds with demonstrated antimicrobial capacity, including inhibitory effects on P. acnes reported in agar-well diffusion assays(Abere et al., 2012; Nelson et al., 2016)

Although the individual antibacterial potentials of P. betle L and P. pellucida L have each been documented, evidence regarding their combined or synergistic effect against C. acnes remains scarce. Combining two botanicals with distinct phytochemical profiles is a common strategy in phytopharmacology, motivated by the hypothesis that complementary mechanisms of action may produce additive or synergistic potentiation exceeding the effect of either agent alone(Abdelmalek et al., 2025; Guo et al., 2025). However, such combinations can equally result in additive, indifferent, or antagonistic interactions, depending on the specific compounds and their relative proportions an outcome that can only be established empirically(Caesar et al., 2019; Pirrone et al., 2011).

This gap the absence of empirical data on the combined antibacterial effect of P. betle L and P. pellucida L ethanolic extracts against C. acnes, and the lack of a defined optimal mixing ratio provided the rationale for the present study. Establishing whether combination confers any advantage over single-plant extracts is a necessary step before such combinations can be rationally considered for development into herbal anti-acne formulations that avoid the resistance liabilities of conventional antibiotics.

Accordingly, this study aimed to evaluate the antibacterial activity of ethanolic extracts of *P. betle L* and *P. pellucida L*, administered singly and in three combination ratios (1:1, 1:2, and 2:1) at a fixed total concentration of 15%, against *C. acnes* in vitro, and to identify the ratio yielding the greatest zone of growth inhibition.

MATERIALS AND METHODS

Study Design

This study employed a true experimental, post-test-only control group design conducted entirely in vitro, allowing full control over extraneous variables (temperature, sterilization, incubation time, and media nutrient content) that could otherwise influence bacterial growth.

Test Organism and Plant Material

The test organism was a laboratory isolate of Cutibacterium acnes, maintained on Nutrient Agar and standardized to a 0.5 McFarland turbidity suspension prior to inoculation. Fresh green betel (Piper betle L) leaves (4 kg) were collected from Oepura, and pepper elder (Peperomia pellucida L) leaves (3 kg) from Bakunase, both in Kupang, East Nusa Tenggara, Indonesia. Plant identity was confirmed by the UPT Materia Medica Herbal Laboratory, Batu, East Java Provincial Health Office. Leaves were washed, air-dried, and pulverized to yield 200 g of P. betle L and 137 g of P. pellucida L simplicia.

Extraction and Phytochemical Screening

Each simplicia was macerated in 95% ethanol at a 1:10 (w/v) ratio for three days at room temperature with daily agitation, filtered, and concentrated under reduced pressure using a vacuum rotary evaporator to obtain a thick extract. Extraction of P. betle L yielded 8 g of concentrated extract (4.0% yield), and extraction of P. pellucida L yielded 5 g (3.65% yield). Both extracts were confirmed ethanol-free before use and screened qualitatively for alkaloids (Mayer and Wagner reagents), tannins ($FeCl_3$), flavonoids (Mg/HCl), saponins (foam test), and terpenoids (Liebermann–Burchard-type color reaction).

Experimental Groups and Sample Size

Seven treatment groups were tested: (1) single P. betle L extract 15%, (2) single P. pellucida L extract 15%, (3) combination ratio 1:1, (4) combination ratio 1:2, (5) combination ratio 2:1 — all combinations prepared at a fixed total concentration of 15% — (6) positive control (standardized clindamycin disc), and (7) negative control (sterile distilled water). The number of replicates per group was determined using the Federer formula, $(t - 1)(r - 1) \geq 15$, where $t = 7$ treatment groups; solving for r yielded $r \geq 3.5$, rounded up to four replicates per group.

Antibacterial Activity Assay

Antibacterial activity was assessed using the Kirby-Bauer disc diffusion method. One milliliter of standardized bacterial suspension was spread evenly onto Mueller-Hinton Agar using a sterile cotton swab and allowed to absorb for 10 minutes. Blank paper discs (6 mm diameter) pre-soaked for 30 minutes in each extract concentration were placed aseptically onto the inoculated agar, alongside clindamycin discs (positive control) and sterile distilled water-soaked discs (negative control). Plates were incubated at 37 °C for 24 hours, after which the diameter of the clear zone surrounding each disc was measured with a caliper and classified according to standard inhibition-zone categories (weak, moderate, strong, very strong).

Statistical Analysis

Data normality was assessed using the Shapiro-Wilk test, and homogeneity of variance using Levene's test. As the data were normally distributed and variances homogeneous, group means were compared using one-way ANOVA, followed by Tukey's HSD post-hoc test to identify pairwise differences between groups, with statistical significance set at $p < 0.05$. All analyses were performed using standard statistical software.

Ethical Considerations

This study involved only in vitro microbiological testing of plant extracts and a standard bacterial isolate, with no human or animal subjects. Nonetheless, the study protocol received ethical clearance from the Health Research Ethics Committee of the

affiliated institution prior to commencement, in accordance with institutional and national research-ethics requirements.

RESULTS

Extraction Yield and Phytochemical Screening

Maceration of 200 g P. betle L simplicial yielded 8 g of concentrated extract (4.0% w/w), while 137 g of P. pellucida L simplicia yielded 5 g (3.65% w/w). Both extracts tested negative for residual ethanol and positive for alkaloids, tannins, flavonoids, saponins, and terpenoids (Table 1), confirming the presence of phytochemical classes previously associated with antibacterial activity.

Table 1. Phytochemical Screening of P. betle L and P. pellucida L Ethanolic Extracts

Compound	P. betle Extract	P. pellucida Extract
Alkaloids	Positive (+)	Positive (+)
Tannins	Positive (+)	Positive (+)
Flavonoids	Positive (+)	Positive (+)
Saponins	Positive (+)	Positive (+)
Terpenoids	Positive (+)	Positive (+)

Antibacterial Activity

All extract treatments produced measurable zones of inhibition against *C. acnes* (Table 2). The single P. betle 15% extract produced the largest mean zone of inhibition among plant-derived treatments (12.19 ± 0.44 mm; strong category), followed by the single P. pellucida 15% extract (8.96 ± 0.30 mm; moderate category). Combination ratios of 1:1, 1:2, and 2:1 produced mean inhibition zones of 9.57 ± 0.77 mm, 8.76 ± 0.39 mm, and 8.56 ± 0.73 mm, respectively — all classified as moderate. The positive control (clindamycin disc) produced the largest zone overall (27.19 ± 0.95 mm; very strong), while the negative control produced no inhibition (0 mm).

Table 2. Mean Inhibition Zone Diameters Against C. acnes (n = 4 per group)

Treatment	Mean Diameter ± SD (mm)	Category
P. betle L 15% (single)	12.19 ± 0.44	Strong
P. pellucida L 15% (single)	8.96 ± 0.30	Moderate
Combination 1:1	9.57 ± 0.77	Moderate
Combination 1:2	8.76 ± 0.39	Moderate
Combination 2:1	8.56 ± 0.73	Moderate
Positive control (clindamycin)	27.19 ± 0.95	Very strong
Negative control (sterile water)	0	None

Statistical Analysis

Shapiro-Wilk testing confirmed normal distribution of inhibition-zone data in all groups ($p > 0.05$ for each; negative control excluded owing to zero variance), and Levene's test confirmed homogeneity of variance ($p = 0.192$). One-way ANOVA showed a statistically significant difference in mean inhibition-zone diameter among treatment groups ($p < 0.001$), leading to rejection of the null hypothesis. Tukey's HSD post-hoc comparisons (Table 3) showed that the single P. betle L 15% extract differed significantly from every other group, including all three combination ratios and the positive control (all $p < 0.001$), confirming its distinctly superior activity among the plant-derived treatments. In contrast, the single P. pellucida L extract and the three combination ratios (1:1, 1:2, 2:1) formed a statistically homogeneous subset, with no significant pairwise differences among them ($p > 0.05$ in all comparisons). Notably, the 1:1 combination did not differ significantly from the positive control ($p = 0.227$), the smallest p-value among all combination-versus-positive-control comparisons, although its absolute mean diameter (9.57 mm) remained far below that of clindamycin (27.19 mm).

Table 3. Tukey's HSD Post-Hoc Pairwise Comparisons (p-values)

Group	P. betle 15%	P. pellucida 15%	1:1	1:2	2:1	Positive control
P. betle L 15%	-	0.001*	0.001*	0.001*	0.001*	0.001*
P. pellucida L 15%	0.001*	-	0.768	0.998	0.944	0.001*
1:1	0.001*	0.768	-	0.515	0.279	0.227
1:2	0.001*	0.998	0.515	-	0.997	0.001*
2:1	0.001*	0.944	0.279	0.997	-	0.001*
Positive control	0.001*	0.001*	0.227	0.001*	0.001*	-

*Statistically significant difference ($p < 0.05$).

Overall, these findings indicate that combining *P. betle L* and *P. pellucida L* extracts at the ratios and concentration tested did not enhance antibacterial activity relative to the single P. betle L extract; instead, all combination ratios performed at a level statistically indistinguishable from the weaker single P. pellucida L extract.

DISCUSSION

This study confirms that ethanolic extracts of both *P. betle L* and *P. pellucida* L possess antibacterial activity against *C. acnes* when applied singly, consistent with prior reports attributing this activity to bioactive compounds such as flavonoids, tannins, saponins, and terpenoids. These phytochemicals exert their antibacterial effects through multifaceted mechanisms, including the disruption of bacterial membrane integrity, denaturation of structural and transport proteins, and interference with cell wall/peptidoglycan synthesis (Wahyuni et al., 2023). The markedly stronger activity of the single *P. betle* L extract relative to *P. pellucida* L aligns with earlier findings that betel leaf extracts exert comparatively potent activity against both Gram-positive and Gram-negative bacteria (Aditya, 2023). This superiority likely reflects a higher concentration or greater potency of phenolic constituents—specifically chavicol and eugenol—in *Piper betle L*, which damage bacterial membranes and induce protein leakage, whereas *P. pellucida L* exhibits an alkaloid- and polyphenol-dominant profile with potentially differing potencies (Asan et al., 2022; Marchese et al., 2017; Putrajaya et al., 2019).

Contrary to the initial hypothesis that combining two phytochemically distinct extracts would potentiate antibacterial activity, none of the three combination ratios exceeded—or even matched—the activity of the single *P. betle L* extract; instead, each combination statistically resembled the weaker single *P. pellucida L* extract. This pattern indicates an antagonistic interaction rather than additive or synergistic effects. Mechanisms of antagonism in botanical combinations are complex, involving competitive binding of different phytochemicals to overlapping bacterial targets, chemical interactions between disparate compound classes reducing overall bioavailability, or simple dilution of the highly potent *P. betle L* constituents when a fixed total concentration is shared with the less active *P. pellucida L* fraction (Caesar et al., 2019; Caesar & Cech, 2019).

These results diverge from studies on other betel-leaf combinations—such as the combination of green, red, and black betel leaf extracts against *P. acnes*—which reported measurable antibacterial effects for combined formulations (Herdiana et al., 2023). However, such studies often lack rigorous benchmarking against single-extract potency, making direct comparisons difficult. Our findings resonate with the broader phytopharmacological literature, which underscores that combinations do not inherently guarantee improved efficacy and that each specific botanical pairing requires empirical validation, rather than the assumption of synergistic potential (Junio et al., 2011; Rasoanaivo et al., 2011).

From a translational standpoint, the persistent gap between all plant-based treatments and the clindamycin positive control indicates that, at the concentrations tested, none of the extract regimens approached the efficacy of conventional antibiotic therapy. Nevertheless, given the documented, rising resistance of *C. acnes* to clindamycin, which has been reported as high as 61.3% in some Indonesian clinical studies (Legiawati et al., 2023; Zahrah et al., 2019), even a moderate, non-resistance-prone antibacterial effect from *P. betle L* holds significant potential value. These extracts could serve as adjunctive or preventive topical agents, provided that future work optimizes parameters such as extract concentration, solvent polarity, and delivery vehicle formulation to narrow the potency gap.

This study did not quantify the concentration of individual secondary metabolites in either extract, precluding a mechanistic explanation for the observed antagonism at the compound level. Antibacterial testing was also restricted to a single organism (C. acnes) and a single extraction solvent/method (95% ethanol maceration), limiting generalizability to other acne-associated pathogens or extraction approaches. Future studies should incorporate quantitative phytochemical analysis (e.g., total flavonoid and phenolic content), test a broader concentration range and mixing ratios, and evaluate alternative solvents and extraction methods to clarify the basis of the antagonistic interaction observed.

CONCLUSION

Combined ethanolic extracts of *P. betle L* and *P. pellucida* L exhibit antibacterial activity against *C. acnes*, but combination at the ratios and concentration tested (1:1, 1:2, 2:1; fixed 15% total) did not enhance efficacy beyond that of the single P. betle 15% extract, which remained the most active plant-derived treatment (12.19 mm). Among the combinations, the 1:1 ratio produced the largest inhibition zone (9.57 mm) and was the only combination not statistically different from the clindamycin positive control in post-hoc comparison, although its absolute potency remained substantially lower. Comparison of minimum inhibitory concentration values indicated an antagonistic rather than synergistic interaction between the two extracts. These findings address the study's original hypothesis — that combination would alter antibacterial activity relative to single extracts — confirming a statistically significant difference among groups, while showing that the direction of this difference favored the single P. betle L extract rather than the combinations. Future investigations should quantify the phytochemical constituents responsible for this antagonism and explore alternative ratios, concentrations, and delivery formulations to develop a viable herbal alternative for antibiotic-resistant acne management. Suggestions and feedback from readers and reviewers on the design and interpretation of this work are welcomed by the authors.

ACKNOWLEDGMENTS

The authors thank the Microbiology Laboratory Staff, Faculty of Medicine and Veterinary Medicine, Nusa Cendana University

CONFLICT OF INTERESTS

The authors declare no conflict of interest related to this study.

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