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ANTIOXIDANT AND ANTI-MDR MICROBIAL ACTIVITY OF RED GINGER AND BETEL LEAF COMBINATIONS WAS DRIVEN BY PHENOLIC CONTENTS

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ABSTRACT

A combination of red ginger and betel leaf is widely used in Baturraden, Indonesia, to treat *masuk angin*. This study aimed to standardize and characterise volatile oil constituents of red ginger and betel leaf crude drugs, and determine the antioxidant and antimicrobial activities of their extracts and oils. Crude drug quality was assessed in accordance with Indonesian Herbal Pharmacopoeia (IHP) specifications. Volatile oils were analysed by gas chromatography–mass spectrometry (GC-MS). Volatile oils and ethanol extracts were prepared in five combinations with varied ratios. Antioxidant activity was determined using standard total flavonoid content (TFC), total phenolic content (TPC), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity, and ferric reducing antioxidant power (FRAP) assays. Antimicrobial activity was evaluated by the microdilution method. All crude drugs met IHP quality standards. Red ginger oil was dominated by camphene, eucalyptol, and zingiberene, whereas betel leaf oil primarily contained β-selinene, eugenol, and curlone. Extract's antioxidant activity varied with red ginger-betel leaf ratio, with Formula A yielding the highest activity. TFC of the extracts correlated moderately with DPPH scavenging activity and strongly with FRAP. Volatile oils weakly inhibited both multidrug-resistant (MDR) *Staphylococcus aureus* and *Candida albicans*, while extracts showed moderate, ratio-dependent activity. Formulas A and D exhibited the highest microbial inhibitory activity, which was strongly correlated with TPC. Optimising herbal ratios enhances bioactivity against MDR pathogens, with phenolics driving antimicrobial effects and flavonoids key to antioxidant activity. Validated crude drug quality bridges traditional Indonesian practice with pharmacopeial standards, guiding future formulation design.

Keywords: Essential Oils, Flavonoids and Phenolic Compounds, MDR Pathogens, *Piper betle*, Polyherbal Formulation, *Zingiber officinale var. rubrum*.

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INTRODUCTION

Polyherbal formulations are widely used in traditional Indonesian medicine. The use of combinations of multiple herbal materials is widespread across the country, with those from Java and Bali being the best-known. However, the phytochemical composition, bioactivity, and standard profiles of many polyherbal formulations have not been sufficiently characterized.[1] One such preparation is the combination of red ginger (*Zingiber officinale var. rubrum* Theilade) and betel leaf (*Piper betle* L.), which is widely used in Baturraden, Central Java, to manage *masuk angin.*[2] It is a culturally recognised illness concept associated with symptoms such as diarrhoea, fatigue, fever, headaches, nausea, and vomiting.[3] Scientific approaches

can be applied to treat *masuk angin* and address its observed symptoms. A holistic treatment concept employs multiple strategies to address *masuk angin*, which aligns with the numerous components of polyherbal formulations. Some components serve as the main pharmacological ingredients for treating specific symptoms, while others act as supporting ingredients with analgesic, anti-inflammatory, antimicrobial, antioxidant, or immunomodulatory activities.[4,5] Red ginger and betel leaf are prominent ingredients in Indonesian ethnomedicine. Both plants have been evaluated for their pharmacological activities and the constituents responsible for those. Red ginger is traditionally used to treat cancer, colds, coughs, flatulence, headaches, indigestion, muscle pain, nausea, and vomiting, and to support digestion and immune health. These uses are attributed to its volatile (mainly ar-curcumene, camphene, geranial, neral, sesquiphellandrene, and zingiberene) and non-volatile (apigenin, fisetin, morin, naringenin, quercetin, rutin, caffeic acid, cinnamic acid, ferulic acid, gallic acid, tannic acid, and vanillic acid) constituents. These compounds have been associated with antihyperlipidemic, antihypertensive, antihyperuricemic, antimicrobial, cytotoxic, and immunomodulatory activities.[6] On the other hand, betel leaves are used in traditional treatments for colds, conjunctivitis, cough, rheumatism, stomach ache, and wounds, and also act as anthelminthics, astringents, carminatives, and stimulants. These traditional uses are attributable to the volatile allylpyrocatechol, chavibetol, curlone, eugenol, hydroxychavicol, trans-caryophyllene, α-selinene, β-selinene, and γ-cadinene, as well as non-volatile apigenin, catechin, kaempferol, myricetin, quercetin, rutin, caffeic acid, ferulic acid, gallic acid, and p-coumaric acid. The confirmed pharmacological activities of betel leaf include analgesic, anticancer, antimicrobial, antioxidant, hepatoprotective, and neuroprotective.[7] Antioxidant and antimicrobial activities played a supporting role in the *masuk angin* treatment. Both red ginger and betel leaf contained considerable levels of flavonoids and phenolic compounds, which were related to their antioxidant activities.[8,9] However, the effects of combining red ginger and betel leaf on antioxidant activity, which is relevant to *masuk angin* symptoms, have not yet been evaluated. In addition to demonstrating the supporting activity in *masuk angin* treatment, the antimicrobial activity evaluation conducted in this study also aimed to address antibiotic resistance. The tested microorganisms, patient-isolated multidrug-resistant (MDR) *Staphylococcus aureus* and *Candida albicans*, are among the most clinically significant opportunistic pathogens. A previous study reported that Thai-originated betel leaf essential oils rich in eugenol and hydroxychavicol showed good antibacterial activity against canine-isolated methicillin-resistant Staphylococcus strains. Similarly, red ginger extracts also demonstrated inhibitory activity against methicillin-resistant *Staphylococcus aureus.*[10] The antimicrobial activity of red ginger and betel leaf is closely related to the essential oil content and other bioactive components, such as flavonoids, tannins, and polyphenols.[11,12] However, the antimicrobial profile of the red ginger-betel leaf combinations against the MDR *Staphylococcus aureus* and *Candida albicans* has not yet been evaluated. Furthermore, the chemical constituents of their essential oils have not yet been reported.

Standardisation is imperative to ensure the safety, effectiveness, and quality of herbal medicines. The Indonesian Herbal Pharmacopeia (IHP) specifies the quality characteristics of crude drugs and their extracts. Standardizing red ginger and betel leaf crude drugs according to IHP monographs, particularly their content aspects, supported their pharmacological activities relevant to their medicinal uses. Both plants contain high levels of flavonoids and phenolic compounds, which support antioxidant and antimicrobial activities, key mechanisms in supporting *masuk angin* management.[6,7] Despite the promising individual bioactivities, the influence of different red ginger-to-betel leaf ratios on antioxidant and antimicrobial activities has not been systematically studied. No prior work has compared the antimicrobial effects of the ethanolic extracts with those of the volatile oils in this combination. Furthermore, an integrated approach combining pharmacognostic standardisation, phytochemical profiling, and bioactivity evaluation is lacking. This research aligns most closely with Sustainable Development Goal (SDG) 3, specifically targets 3.3 (combating communicable diseases) and 3.8 (access to safe, effective medicines). By addressing antibiotic resistance and promoting herbal standardization, the study contributes to universal health coverage and evidence-based traditional medicine. Therefore, the purposes of this study are to standardize red ginger and betel leaf crude drugs according to IHP specifications, characterize the chemical constituents of their volatile oils, evaluate the effects of different combination ratios on antioxidant properties (total flavonoid content, total phenolic content, 2,2-diphenyl-

1-picrylhydrazyl (DPPH) radical-scavenging activity, and ferric reducing antioxidant power) of ethanolic extracts; and determine the antimicrobial activity of both extracts and essential oils against patient-isolated MDR *Staphylococcus aureus* and *Candida albicans.*

EXPERIMENTAL

Materials

Crude drugs of red ginger and betel leaf were purchased from the Centre for Medicinal Plant and Traditional Medicine Research (Balai Besar Penelitian dan Pengembangan Tanaman Obat dan Obat Tradisional, Karanganyar, Indonesia). Patient-isolated multidrug-resistant *Staphylococcus aureus* (resistant to Ciprofloxacin and Erythromycin, among others) and *Candida albicans* (resistant to Voriconazole and Amphotericin B, among others) were obtained from the Laboratory of Parasitology, Faculty of Medicine, University of Indonesia (Jakarta, Indonesia). Aquadest, ethanol, hydrochloric acid, and chloral hydrate were from Merck (Darmstadt, Germany). The 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), acetate buffer, aluminium chloride, DPPH, Folin & Ciocalteu's phenol reagent, gallic acid, iron (III) chloride hexahydrate, quercetin, and Trolox were from Sigma-Aldrich (Darmstadt, Germany). Mueller Hinton broth (MHB) and potato dextrose broth (PDB) were from HiMedia Laboratories (Mumbai, India). Gentamycin sulfate and fluconazole were from Bernofarm (Sidoarjo, Indonesia)

Standardisation of Red Ginger and Betel Leaf Crude Drugs

The qualitative macroscopic and microscopic morphology, as well as the quantitative parameters of loss on drying, total ash, acid-insoluble ash, ethanol-extractable, water-extractable, and chemical content, for both red ginger and betel leaf crude drugs were analyzed using the official methods in the IHP. The obtained values for each parameter were standardised to their respective specifications in the IHP.[13]

Distillation and Analysis of the Volatile Oils

The volatile oil was isolated using a conventional water- and steam-distillation apparatus. Briefly, 2.0 kg of the ground crude drugs were placed in a distiller containing about 4 L of distilled water. The apparatus was heated for 5.5 hours. The oil was collected and dried over anhydrous sodium sulfate. The distillation yield (v/w) was calculated. The chemical composition of the obtained volatile oil was analyzed by a Shimadzu Gas Chromatography-Mass Spectrometry (GC-MS) (Kyoto, Japan). Separation was achieved using a SH-Rxi-5Sil MS column (30 m × 0.25 mm × 0.25 μm). The GC operating conditions were as follows: injector temperature, 250 °C; injection mode, split (split ratio 1:20); carrier gas, helium at a constant linear flow rate of 1.0 mL/min. The oven temperature was programmed to ramp from 60 °C to 240 °C at 4 °C/min, with a final hold of 5 min. The mass spectrometer was operated in electron impact ionisation (EI) mode with an ion source temperature of 230 °C and an interface temperature of 250 °C. The ionisation energy was set at 70 eV, and the mass scan range was 40-500 m/z. The total ion chromatogram (TIC) was recorded. Constituents were identified by comparing their recorded mass spectra with reference spectra in the Wiley Registry of Mass Spectral Data (9th edition). The relative percentage amount of each component was calculated from the GC peak areas without the use of correction factors and is expressed as percent area.

Evaluation of the Antioxidant Properties

Powdered red ginger and betel leaf crude drugs were weighed and mixed to prepare five combinations (Table-1). The ethanolic extract was prepared from each combination using the official remaceration method in the IHP, as described previously.[14] Briefly, the crude drugs were macerated in 70% aqueous ethanol at a 1:10 ratio. The mixture was kept at room temperature for 24 hours with occasional shaking. The extract was filtered, and the marc was remacerated with fresh solvent at a 1:5 ratio for 24 hours. The combined filtrates were concentrated under reduced pressure at 40 °C using an IKA rotary evaporator (Staufen im Breisgau, Germany) to obtain the crude extract. A stock solution was prepared by dissolving the extract in ethanol to a final concentration of 10 mg/mL, from which dilutions were made to obtain the appropriate sample solutions.

The total flavonoid content (TFC) in the extract was determined using the aluminium chloride colourimetric method, following a previously described method with slight modifications.[15] 0.5 mL of an

appropriately diluted sample solution was mixed with 1.5 mL of ethanol, 0.1 mL of a 10% aluminium chloride solution, 0.1 mL of a sodium acetate solution, and 2.8 mL of water.

Table-1: Preparation of Red Ginger and Betel Leaf Combinations

Combinations	Ratio of crude drugs (%)	
	Red ginger	Betel leaf
Formula A	0	100
Formula B	25	75
Formula C	50	50
Formula D	75	25
Formula E	100	0

The absorbance was read at 415 nm using a Thermo Scientific UV-Vis Spectrophotometer (Waltham, United States) after incubation at room temperature for 30 minutes. A standard curve ($y = 0.0085x + 0.1897$, $R^2 = 0.983$) was calculated from the concentrations and absorbances of the quercetin solutions. The TFC was calculated accordingly and expressed as mg of quercetin equivalents (QE)/g of extract. The total phenolic content (TPC) was determined using the Folin-Ciocalteu colourimetric method as previously described.[16] In brief, 0.1 mL of appropriately diluted sample solution was mixed with 7.9 mL of water and 0.5 mL of Folin-Ciocalteau reagent. 1.5 mL of saturated sodium carbonate was added, and the mixture was incubated at room temperature for 2 hours. The absorbance was read at 764 nm. A standard curve ($y = 0.0069x + 0.1936$, $R^2 = 0.986$) was plotted from the concentrations and absorbance of gallic acid solutions. The TPC was calculated from the curve and expressed as mg of gallic acid equivalents (GAE)/g of extract. The free radical-scavenging capacity of the extract was determined using the previously reported DPPH assay.[16] 0.5 mL of appropriately diluted sample solution (or ethanol as a blank) was mixed with 5.0 mL of DPPH solution. The mixture was incubated in the dark at room temperature for 30 minutes. The absorbance was measured at 517 nm. The percentage of inhibition was calculated using the formula as follows:

$$\%\ Inhibition = \frac{(Absorbance_{blank} - Absorbance_{sample})}{Absorbance_{blank}} \times 100$$

A standard curve ($y = 0.2438x - 1.759$, $R^2 = 0.991$) was calculated from Trolox solution concentrations and their corresponding % inhibition. Results were expressed as μmol of Trolox equivalents (TE)/g extract. The ferric reducing antioxidant power (FRAP) assay was conducted by freshly preparing the FRAP reagent and mixing 3.99 mL of the aliquot with 0.21 mL of the appropriately diluted sample solution. The mixture was incubated at 37 °C for 10 minutes, and the absorbance was measured at 594 nm accordingly. A standard curve ($y = 0.0028x - 0{,}0362$, $R^2 = 0.994$) was calculated from Trolox solution concentrations and absorbances. FRAP was expressed as μmol of TE/g extract.

Evaluation of the Antimicrobial Activity

$$\%\ Inhibition = \frac{(OD_{growthcontrol} - OD_{testsample})}{(OD_{growthcontrol} - OD_{sterilitycontrol})} \times 100$$

Data Analysis

Statistical analysis was performed using IBM SPSS Statistics software (Version 26.0, Armonk, USA). The mean values of TFC, TPC, DPPH scavenging activity, and FRAP, as well as % inhibition against tested microbes between combinations of red ginger and betel leaf, were compared using a one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. Correlations between the content (TFC and TPC) and the activities (DPPH scavenging activity, FRAP, and % inhibition) of the combination extracts were calculated using Pearson's correlation test. The difference and correlation were considered statistically significant at p-value >0.05.

RESULTS AND DISCUSSION

In the present study, the crude drugs of red ginger and betel leaf were subjected to quality control assessment to comply with the respective monographs in the IHP. The botanical identity was verified by the similarity in microscopic and macroscopic morphology, as well as in the TLC profile (Fig.-1 and 2).

Red ginger and betel leaf crude drugs were of good quality, with the values of all physicochemical characters meeting the specified standard in the IHP (Table-2). Both crude drugs complied with all relevant quantitative quality parameters, including loss on drying, total ash, acid-insoluble ash, and extractive values, as specified in the IHP for red ginger and betel leaf.[13] Confirmation that the crude drugs were of good quality enables accurate characterisation of their phytochemical profile and biological activities. The pharmacopeial-compliant ash values and loss on drying indicated that the crude drugs are free of excessive soil and other inorganic contaminants, as well as moisture, which could pose safety risks during use. The confirmed good quality of the extractable matter, volatile content, and total flavonoid content supports the validity of the bioactivity findings, where the observed antioxidant and antimicrobial activities resulted from the crude drug's intrinsic phytochemical constituents. This pharmacopeial compliance is a critical first step toward developing standardised herbal phytopharmaceuticals, a direct contribution to SDG Target 3.8 (access to safe, effective, quality medicines).

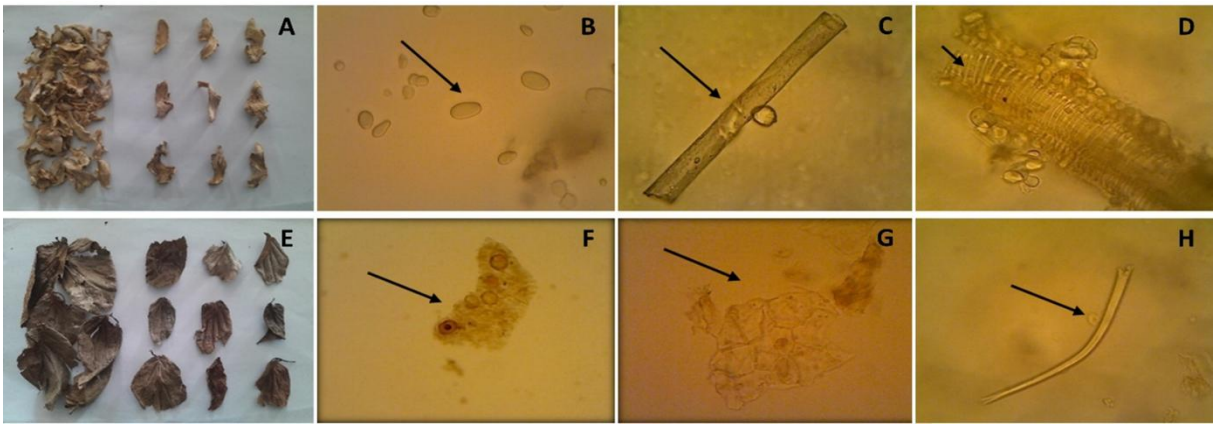

Fig.-1: Morphological Characters of Red Ginger (upper panel) and Betel Leaf (lower panel) Crude Drugs Showed Macroscopic (A, E) and Selected Microscopic Diagnostic Fragments, i.e., Starch Granules (B), Sclerenchyma (C), Xylem (D), Lower Epidermis with Idioblast (F), Upper Epidermis (G), and Covering Trichome (H)

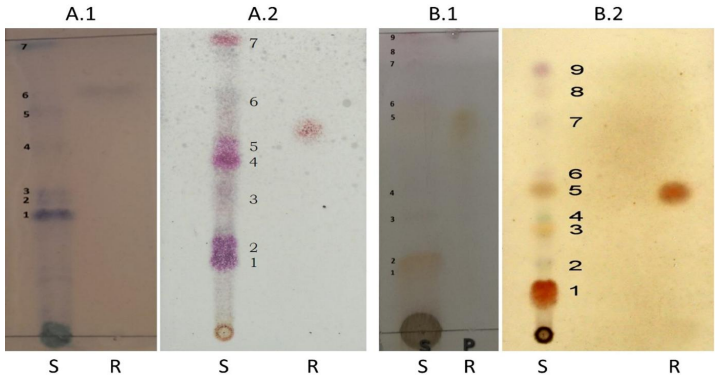

Fig.-2: TLC Profiles of the Red Ginger (A.1) and Betel Leaf (B.1) Crude Drugs and their Respective Standards (A.2, B.2). S, Crude Drug Extract; R, Reference Compounds

Table-2: Pharmacognostic Characters of the Crude Drugs

Parameters	Value (%)	
	Red ginger	Betel leaf
Loss on drying	7.90±0.75	8.17±0.33
Total ash	4.06±0.03	3.19±0.18
Acid-insoluble ash	0.39±0.16	1.03±0.05
Ethanol extractable	14.9±0.66	21.24±2.96
Water extractable	18.95±0.19	25.23±2.01
Volatile content	1.11±0.09	-
Total flavonoid content	-	7.05±0.17

The yields for red ginger and betel leaf oil distillations were 0.55% and 0.16%, respectively. The chromatograms showed 38 and 41 compounds in red ginger and betel leaf oils, respectively. The main compounds of red ginger oil were camphene (12.08%), eucalyptol, ar-curcumene, neral, and zingiberene. The major constituents of betel leaf oil were β-selinene (9.65%), eugenol, curlone, α-selinene, trans-caryophyllene, and γ-cadinene. The compounds detected in both red ginger and betel leaf oils with areas greater than 1% were α-pinene, β-pinene, β-bisabolene, and curlone (Table-3).

Table-3: Major Compounds of Red Ginger and Betel Leaf Oils

Retention time (min)	Compound names	Peak area (%)	
		Red ginger	Betel leaf
3.360	α-Pinene	4.07	2.26
3.599	Camphene	12.08	2.54
4.013	β-Phellandrene	-	4.49
4.075	β-Pinene	1.16	1.45
4.326	Myrcene	2.12	-
5.320	Sabinene hydrate	-	1.84
5.381	Eucalyptol	11.62	-
7.100	Camphor	2.29	-
8.584	Borneol	3.67	-
9.862	β-Citronellol	1.24	-
10.063	Geranial	4.61	-
10.252	Neral	8.93	-
10.368	Geraniol	3.31	-
12.106	Copaene	-	1.27
12.410	Eugenol	-	8.82
12.708	α-Bergamotene	-	1.24
12.836	trans-Caryophyllene	-	6.81
13.344	α-Humulene	-	4.60
13.667	γ-Cadinene	-	6.34
13.774	α-Curcumene	-	3.75
13.780	Ar-Curcumene	9.37	-
13.869	β-Selinene	-	9.65
13.909	Zingiberene	5.03	-
13.992	α-selinene	-	8.59
14.065	β-Bisabolene	4.23	1.78
14.281	γ-Muurolene	-	4.36
14.307	Sesquiphellandrene	4.68	-
15.204	Caryophyllene oxide	-	2.10
15.238	Bisabolol	1.49	-
15.530	Zingiberenol	1.87	-
15.554	(-)-Epoxycaryophyllene	-	1.67
15.768	α-Bisabolol	1.19	-
16.260	Curlone	4.80	8.60
16.497	Carotol	1.93	-
	Hydrocarbons	42.74	62.90
	Oxygenated compounds	46.95	19.26
	Total compounds with area >1%	89.69	82.16

The distillation yield of red ginger and betel leaf oils was likely linked to the storage location of volatile oils within plant tissues. Red ginger accumulated oils in the idioblasts and secretory cavities within the parenchymal tissue, that is, in the much deeper, inner part of the rhizomes.[17] On the other hand, betel leaf's essential oils are stored in the superficial glandular trichomes and parenchymal idioblasts.[18] This leads to higher oil loss from the betel leaf at a higher rate than from the red ginger during crude drug processing, resulting in lower oil yields. These yields fall within the reported ranges for these species, which underlined the appropriateness of the applied distillation parameters. For example, red ginger oil

yield was 0.08-1.91%, depending on the plant's growing location, processing, and extraction method.[19–21] Similarly, reported yields for betel leaf oil distillation ranged from 0.11 to 0.65%.[22–24] The dominant compounds identified in red ginger oil largely align with the established chemotypes for the Indonesian red ginger. Neral and camphene were dominant in Magelang-originated red ginger oil, but that from Pacitan was mainly constituted of neral, camphene, and eucalyptol. Zingiberene and curcumenes were identified as the main components of red ginger oil from Banten.[20,25] The major compounds identified in this study were consistent with the typical chemotypes of betel leaf oils. Padang- and Klaten-originated oils shared similar characteristics, including a high level of trans-caryophyllene.[26,27] Moreover, the Indian betel leaf oils were dominated by eugenol and characterized by high fractions of chavibetol, δ-elemene, and β-bourbonene.[23,28]

The ratio of red ginger and betel leaf extract significantly affected the combination's antioxidant property parameters. Formula A, which contained betel leaf only, showed the highest value for TFC (5.67±0.11 mg QE/g), TPC (12.24±0.5 mg GAE/g), DPPH scavenging activity (49.42±2.00 mmol TE/g), and FRAP (257.62±0.99 mmol TE/g). However, Formula A showed TPC values comparable to those of Formula D (12.23±0.49 mg GAE/g) and Formula E (12.45±0.43 mg GAE/g). Formula A was also similar in DPPH scavenging activity to Formula B (49.32±0.68 mmol TE/g) (Fig.-3). The TFC was moderately correlated with the DPPH scavenging activity (R-value = 0.527) and strongly correlated with the FRAP (R-value = 0.831) across all combinations.

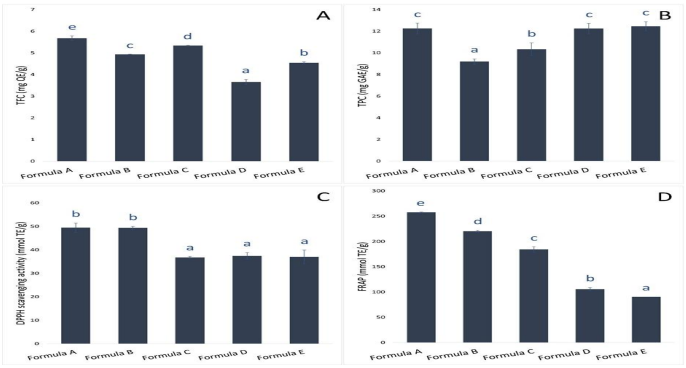

Fig.-3: Profiles of TFC (A), TPC (B), DPPH Scavenging Activity (C), and FRAP (D) of the Combination of Red Ginger and Betel Leaf Extract

The variation in antioxidant activity across the red ginger and betel leaf combinations is consistent with the qualitative and quantitative profiles of flavonoids and phenolic compounds present in each plant. Betel leaf, which dominated the higher-flavonoid combinations (Formula A and Formula B), contained abundant quercetin, kaempferol, apigenin, and myricetin.[29,30] Flavonoids, mainly quercetin, were also found in red ginger.[31] These compounds are known for their high hydrogen-donating capacity, which further stabilises radicals and thereby contributes to their DPPH-scavenging activity.[32,33] The higher TFC of betel leaf compared with red ginger likely underpins the significantly higher DPPH activity observed in Formulas A and B than in the other combinations. Both red ginger and betel leaf were rich in phenolic compounds. Red ginger contained major phenolic compounds, including gingerols, paradols, shogaols, and zingerone.[6] Betel leaf also contained considerable amounts of allylpyrocatechol, chavibetol, chavicol, eugenol, gallic acid, and hydroxychavicol.[34] The phenolic compounds in betel leaf support rapid free-radical neutralisation, as demonstrated by their high DPPH scavenging activity.[33] The FRAP assay quantified the electron-donating capacity of the antioxidants in the extracts. The decrease in FRAP values with increasing proportions of red ginger indicated the stronger ferric-reducing capacity of betel leaf than red ginger. High levels of catechin, epicatechin, quercetin, and hydroxychavicol as dominant components of betel leaf are consistent with the findings. These compounds have high reducing capacity because they can delocalize electrons and have low redox potentials.[35] Therefore, betel leaf is the primary contributor to the antioxidant mechanisms related to reducing capacity in the combination of red ginger and betel leaf. The strong positive correlation between TFC and FRAP underscores the direct role of flavonoids in reducing-related antioxidant mechanisms. These compounds are highly reactive in this single-electron

transfer mechanism.[36] In contrast, the moderate correlation between TFC and DPPH scavenging activity implies that non-flavonoid phenolic compounds play a dominant role in hydrogen-atom transfer to neutralise the DPPH radical.[37,38] Our findings suggest that betel leaf is the primary contributor to the antioxidant activity in the combination of red ginger and betel leaf. The ratio of red ginger and betel leaf did not affect the inhibitory activity of the volatile oils against MDR *Staphylococcus aureus* and *Candida albicans*. The oil weakly inhibited both microorganisms to a comparable extent. On the other hand, the combinations of red ginger and betel leaf extracts showed a moderate inhibitory activity against the microbes. The ratio of the components significantly affected the activity, with Formula A and Formula D showing the most potent effects (Table-4, 5). The *Staphylococcus aureus*-inhibitory activity of the combination extracts was strongly and positively correlated with TPC (R-value = 0.757).

Table-4: Antimicrobial Growth Inhibition Activity of Red Ginger and Betel Leaf Volatile Oil Combinations

Combination	Microbial growth inhibitory (%) at concentrations of					
	125 µl/ml	62.5 µl/ml	31.25 µl/ml	15.63 µl/ml	7.81 µl/ml	3.91 µl/ml
Against MDR *Staphylococcus aureus*						
Formula A	25.16±2.24[a]	20.89±2.03	8.30±11.97	22.44±14.74	12.72±8.71	17.22±8.50
Formula B	31.24±5.58[a]	27.43±3.89	20.32±9.28	35.15±9.56	20.06±9.22	20.55±9.26
Formula C	25.31±3.48[a]	21.58±0.49	14.07±5.88	13.35±6.25	18.19±14.73	3.82±5.60
Formula D	26.86±6.25[a]	20.71±1.44	14.07±6.59	19.58±6.25	31.53±16.45	12.13±6.99
Formula E	29.88±0.20[a]	16.01±2.70	11.51±6.26	6.91±11.74	16.28±1.94	2.41±1.55
Negative control	0					
Positive control	84.41±0.09[b]					
Against MDR *Candida albicans*						
Formula A	33.57±2.22[a]	18.58±4.85	17.23±3.95	26.36±8.42	16.56±4.91	18.86±6.33
Formula B	28.93±7.75[a]	24.61±3.47	21.11±5.83	18.399±6.83	16.93±7.90	14.03±2.02
Formula C	36.95±4.12[a]	34.56±4.11	29.11±5.33	26.96±4.68	24.77±6.00	21.06±2.30
Formula D	31.18±1.83[a]	30.55±1.79	28.60±1.69	24.99±3.80	22.26±4.18	19.63±3.80
Formula E	30.44±1.59[a]	23.63±2.25	20.06±4.74	18.79±5.42	17.84±5.42	13.78±4.68
Negative control	0					
Positive control	80.76±0.05[b]					

Different superscripted alphabets in the same column, in bold, indicate significantly different inhibitory activity against the tested microorganisms

Table-5: Antimicrobial Growth Inhibition Activity of Red Ginger and Betel Leaf Extract Combinations

Combination	Microbial growth inhibitory activity (%)					
	125 µg/ml	62.5 µg/ml	31.25 µg/ml	15.63 µg/ml	7.81 µg/ml	3.91 µg/ml
Against MDR *Staphylococcus aureus*						
Formula A	50.87±6.22[b]	39.63±0.65	36.95±4.77	27.77±2.94	25.90±6.19	20.14±3.04
Formula B	27.32±2.84[b]	25.13±1.14	17.17±12.99	15.83±5.80	11.69±2.22	10.25±1.25
Formula C	45.03±2.20[b]	38.21±3.72	25.25±3.98	22.98±3.06	19.36±5.79	19.04±4.68
Formula D	49.09±6.21[b]	38.39±4.43	31.45±4.92	26.36±4.52	17.97±2.77	14.04±9.32
Formula E	40.59±4.59[a]	30.29±6.18	22.65±7.91	15.35±4.67	11.33±2.72	11.33±2.53
Negative control	0					
Positive control	87.86±0.07[c]					
Against MDR *Candida albicans*						
Formula A	51.28±1.43[b]	46.08±5.59	35.20±6.67	28.26±4.76	20.10±3.22	18.84±6.10
Formula B	45.59±6.52[ab]	39.22±5.46	36.70±3.09	27.73±2.92	20.79±6.56	24.32±1.41

Formula C	47.83±2.25[ab]	43.08±0.39	36.83±2.20	31.10±2.65	23.91±3.77	16.89±2.01
Formula D	50.91±0.64[b]	41.69±3.21	36.14±1.93	26.85±4.09	24.48±8.04	15.90±6.32
Formula E	44.78±2.63[a]	34.43±4.45	28.99±10.18	23.63±3.59	19.49±2.32	9.69±0.60
Negative control	0					
Positive control	78.86±0.02[c]					

Different superscripted alphabets in the same column, in bold, indicate significantly different inhibitory activity against the tested microorganisms

Our findings demonstrated differences in antimicrobial efficacy between volatile oils and ethanolic extracts of red ginger and betel leaf combinations. The volatile oils produced weak inhibition, irrespective of the red ginger-to-betel leaf ratio. The major constituents of red ginger and betel leaf oils, including camphene, eucalyptol, ar-curcumene, eugenol, and trans-caryophyllene, are known to possess antimicrobial properties, including against antibiotic-resistant bacteria and fungi.[39,40] However, their efficacy strongly depends on concentration, oil dispersion, and solubility in aqueous media. In the microdilution assay performed in this study, the antimicrobial compounds in the oils may be present at concentrations below the effective range due to limited aqueous solubility. This might reduce contact between the oils and the microbial cells. Moreover, volatility and rapid loss of some monoterpenes during handling can lower their observed activity.[41] On the other hand, ethanolic extracts exhibited moderate, ratio-dependent activity, with Formula A and Formula D showing the most potent inhibition. Ethanolic extracts contained flavonoid and phenolic compounds that are more compatible with aqueous test conditions in the microdilution assays. The comparable antimicrobial activity against both microbes exhibited by Formula A and Formula D was consistent with the TPC data. Formula D, despite containing a majority of red ginger, contained a similar number of phenolic compounds and, hence, a comparable antimicrobial capacity. A strong, positive correlation between the MDR *Staphylococcus aureus*-inhibitory activity and TPC implied that phenolic compounds were the primary determinant of antibacterial activity. These compounds inhibited microbial growth by disrupting microbial membranes, chelating essential metal ions, inhibiting enzymes, and generating oxidative stress.[42] These effects were considered to have broad-spectrum inhibitory potential, effective against the MDR phenotypes used in this study. The highest antimicrobial activity in Formulas A and D might indicate that eugenol and related compounds from betel leaf are the primary antimicrobial agents. In addition, different proportions of red ginger and betel leaf may exert different interaction effects. In Formula D, additive or synergistic effects likely occurred, with phenolic compounds from betel leaf enhancing the extract's activity despite its lower proportion. Conversely, antagonistic interactions were likely to occur in Formula B and Formula C, which exhibited lower observed inhibition percentages against the tested microorganism. The different activities resulting from variations in the component ratio are commonly reported, and synergistic effects can be achieved using several optimisation approaches.[43] For example, combinations of turmeric, Java tea, seed-under-leaf, ginger, and cinnamon have shown antagonistic antioxidant effects.[44] A particular ratio in combinations of moringa, cinnamon, and black cumin produced synergistic antimicrobial effects.[45] The limitation of this study was reliance on per cent-inhibition endpoints rather than minimum inhibitory concentrations (MICs) to express the antimicrobial potency of combinations of red ginger and betel leaf extracts and volatile oils. This reliance hindered the direct comparability with other studies. The study also tested against a limited number of clinical isolates. Future studies should identify the specific phenolic compounds responsible for activity and perform MIC and interaction-effect evaluations. Our study contributes to the pharmacopeial standardisation of red ginger and betel leaf crude drugs, identifying betel leaf as the primary driver of antioxidant activity and phenolic compounds as key determinants of anti-MDR effects. By demonstrating ratio-dependent bioactivity and linking it to specific phytochemicals, we provide a mechanistic framework for optimising herbal formulations. These findings support the development of affordable, standardised plant-based therapies against MDR pathogens, addressing a critical gap in ethnopharmacological evidence. This research directly supports SDG target 3.3, which aims to combat communicable diseases by addressing antimicrobial resistance through testing patient-isolated MDR *Staphylococcus aureus* and *Candida albicans*, two WHO priority pathogens. The demonstrated moderate

activity of standardized extracts provides a scientific basis for developing affordable, accessible herbal therapeutics that could reduce reliance on failing antibiotics. Furthermore, the pharmacopeial standardization aligns with SDG target 3.8 to ensure access to safe, effective, high-quality medicines.

CONCLUSION

Red ginger and betel leaf crude drugs met IHP quality standards, enabling a valid assessment of bioactivity. The main constituents of red ginger oil were camphene, eucalyptol, and zingiberene, while those of betel leaf oil were β-selinene, eugenol, and curlone. Betel leaf was the primary contributor to antioxidant activity via flavonoids (strongly correlated with FRAP), whereas non-flavonoid phenolics defined DPPH scavenging. Ethanolic extracts exhibited moderate, ratio-dependent antimicrobial activity against MDR *Staphylococcus aureus* and *Candida albicans*, with Formula A and Formula D being the most potent, strongly correlated with total phenolic content. Volatile oils showed weak inhibition regardless of ratio, likely due to poor aqueous solubility. This study provides a mechanistic framework for optimising red ginger–betel leaf ratios to enhance bioactivity. Our findings support SDG targets 3.3 and 3.8, providing a scientific basis for developing affordable, standardised plant-based therapies against MDR pathogens.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

The present work is related to *SDG-3: Good Health and Well-Being*. All the authors contributed significantly to this manuscript, participated in reviewing/editing and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

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