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Original Article

NATURAL INHIBITOR OF AGRONOMICALLY REPELLENT PLANT TOWARDS CLINICAL ISOLATE OF CHLORAMPHENICOL RESISTANT-SALMONELLA TYPHI

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ABSTRACT

Objective: This study was purposed to determine the most effective inhibition among those repellent plants i.e. The leaves of kirinyuh (*Chromolaena odorata*), kenikir (*Cosmos caudatus*), bandotan (*Ageratum conyzoides*), grass teki (*Cyperus Cyperus rotundus*), lemongrass (*Cymbopogon citratus*) and suren (*Toona sureni*) towards *S. typhi* clinical isolate.

Methods: The ethanolic extracts of sixt plants were obtained by maceration method using 70% ethanol. Phytochemical screening was done using the standard methods as described by Farnsworth. The inhibition of the repellent leaves ethanolic extracts to chloramphenicol resistant-*S. typhi* clinical isolate assayed using the agar diffusion method and statistically analyzed by ANOVA followed by the Duncan test. The most potential plant was further determined by investigating the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) value using the microdilution test.

Results: As the result, all ethanolic leaves extracts contain alkaloids, flavonoids, and tannins, except that tannins were not found in *C. rotundus* and *A. conyzoides*. However, all extracts had the activity to inhibit the growth of *S. typhi*. *T. sureni* leaves extract evidently showed the strongest inhibition with MIC value in the range of 1.5625<x≤3.125 mg/ml and the MBC value in the range of 6.25<x≤12.5 mg/ml. The ratio of MBC/MIC≤4, thus, *T. sureni* leaf extract may be classified as a strong bactericidal agent.

Conclusion: In summary, *T. sureni* extract leaves achieved the most appreciable value of MIC MBC and considered as the bactericidal agent which has strong potential to be a novel anti-typhoid fever agent.

Keywords: Repellent, *Toona sureni*, *Salmonella typhi*, Chloramphenicol, MIC, MBC

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INTRODUCTION

Typhoid fever caused by *Salmonella* typhi is a prevalent disease in Indonesia and remains a global public infection worldwide, causing the highest mortality rates, approximately 250.000 deaths annually [1-4]. This foodborne disease cases are aproximately occur more than 90% in South and Southeast Asian countries [3]. The resistance of Salmonella species to chloramphenicol began to be reported in 1972 and continue to develop to form multidrug-resistant strains [5, 6]. The development of bacterial multidrug resistance to all the three first-line drug i.e. chloramphenicol, ampicillin and trimethoprim-sulfamethoxazole, contributes to the increasing number of typhoid fever cases annually [7]. Quinolone or 3rd generation cephalosporins are recommended to replace those first-line drugs [8]. Nevertheless, the use of fluoroquinolones cannot be arbitrary in people who are at high risk, including people with a history of aneurysms and certain genetic disorders associated with blood vessel alteration, high blood pressure, and the elderly [9]. Therefore, nowadays many people use medicinal plants as complementary or alternative medicine which are considered safe for the health care system. Several studies exploiting the effectiveness of medicinal plants have also strengthened the superiority of herbal medicines to be developed as antimicrobial products.

Naturally, plants continue to improve or develop new defense strategies against pests and pathogens. This phenomenon can be studied to obtain new antimicrobials by utilizing the compounds contained in these repellent plants. It has been studied that the compounds produced in response to plant defense repellents can be as plant proteinase inhibitors (PIs) that exert toxic, repellent or/and anti-nutritive effects on herbivorous insects, such as Groundnut (*Arachis hypogaea* L.) and rice plants [10-13]. Lately, these peptides have been isolated from several plant and agronomically produce protection against microbes by disturbing the microbial cell membrane [13-15]. In this study, six repellent plants that grow in Indonesia were studied, specifically: Kirinyuh leaf (*C. odorata*), Kenikir leaf (*C. caudatus*), Bandotan leaf (*A. conyzoides*), Grass Teki leaf (*C. rotundus*), Lemongrass leaf (*C. citratus*) and Suren (*T. sureni*) leaves. Those plants are traditionally reported to be agronomically repellent plants which produce substances to exploit defense reactions against pests and pathogens. Except PIs, the significantly antimicrobial effect of those repellent plants therapeutic substances of also might come from their phytochemical components. It was reported that Kirinyuh plants contain active compounds that act as antimicrobial or antibacterial against *Staphylococcus aureus* [16]. Kenikir plants have antibacterial activity against *Bacillus subtilis, S. aureus, Escherichia coli, Pseudomonas aeruginosa,* and *Candida albicans* [17]. The ethanol extract of Bandotan leaves contains alkaloids, saponins, and tannins that can inhibit the growth of *E. coli, S. aureus, P. aeruginosa* and *Streptococcus pyogenes* [18]. The ethanol extract and essential oil of the Teki grass also reported has antibacterial and antifungal activity against Gram-positive and negative bacteria [19, 20]. The methanol extract and the ethyl fraction of Suren stem wood have antifungal and antibacterial activity [21]. The common phytochemicals that significantly causes effective results on bacterial infection are alkaloids, flavonoids, tannins and saponin. The phytochemical screening of our studied plants revealed the presence of those phytochemicals. The effectiveness of antibacterial compounds of those repellent plants was explored in this study to be further developed as alternate medicine in typhi fever disease by determining the most effective inhibition among those repellent plants towards chloramphenicol resistant-*S. typhi* clinical isolate.

MATERIALS AND METHODS

Materials

Different leaves of plants: Kirinyuh (*Chromolaena odorata*), Kenikir (*Cosmos caudatus*), Bandotan (*Ageratum conyzoides*), Grass Teki

(*Cyperus rotundus*), Lemongrass (*Cymbopogon citratus*), and Suren (*Toona sureni*), obtained from Jatiroke Garden, Sumedang, Indonesia. Those plants have been authenticated at Herbarium Bandungense, Bandung Institute Technology. The *Salmonella typhi* clinical isolate used in this study is our cultures collection isolated from market food. Various bacterial growth media were used, such as *Salmonella-Shigella Agar* (SSA-Oxoid), *Mueller Hinton Agar* (MHA-Himedia), and *Mueller Hinton Broth* (MHB-Oxoid).

Sample collection, processing, and extraction

The leaves of each repellent plant were rid with double distilled water and dried. The dried leaves then cut into small pieces and pounded coarsely into powder. The powder is then sieved through an aluminium sieve (1 mm) to obtain particles in uniform size, then weighed for 500 g and macerated in 4L of 70% technical ethanol. The macerate was kept in the maceration vessel for 3 x 24 h and stirred after 6 h; then the vessel was tightly closed for up to 18 h. The macerate was collected every 24 h then the solvent was added to replace the accommodated solvent and then it closed again tightly. The obtained macerate was then concentrated using a rotary evaporator to obtain a thick extract.

Phytochemical screening

A small portion of the thick extract was evaluated to detect phytochemical metabolites, which include alkaloids. Flavonoids. tannins. and saponins using standard methods [22].

Preparation of bacterial suspension

One Ose of *S. typhi* colonies from a surface of slant agar containing SSA media were taken and put into±2 ml sterile physiological NaCl 0.95%. The bacterial suspension was then homogenized at room temperature. The turbidity of the suspension was compared to McFarland 0.5 solution to achieve the equivalent of 1.5 x 10^8 cfu/ml [23].

Antibacterial activity test

The inhibition effects of the leaf extracts were analyzed using the agar diffusion method with the perforator technique. The extract was dissolved with 5% dimethylsulfoxide to achieve the concentration used in this study which include: 100, 200, 400 and 600 mg/ml. A total of 20 µl bacterial suspension was put into a sterile petri dish, then 20 ml of MHA media was added. Then, the petri dish was shaken slowly until homogeneous and allowed to solidify. The test medium was perforated using perforating holes with a diameter of 6 mm. A total of 50 µl of the extract with a certain concentration variation was inserted into the well. The test medium was incubated at 37 °C for 18 h and the inhibitory diameter zone was measured using a caliper [23].

Statistical analysis

The effect of the extract on the difference in the inhibition diameter was statistically analyzed using the ANOVA test, followed by Duncan test.

MIC and MBC determination

The determination of the MIC value was carried out on the most active extract using the microdilution method. All wells of each row were filled with 0.1 ml sterilized MHB. Sequentially, all wells were filled with 0.1 ml of bacterial suspension (equivalent to 1x10^5 cfu/ml) and plant extract serially diluted to achieve a concentration ranging from 100 to 1.953125 mg/ml except well 1 and 12, served as the negative and positive control. Microtiter plate was incubated for 24 h at 37 °C. The obtained turbidity was observed to determine the MIC value of the extract which is indicated by a clear test medium in the well with the smallest concentration. Then the wells that was set as the MIC range were sub-cultured in a volume of 10 µL onto the surface of MHA in petri dish and incubated for 24 h at 37 °C. The smallest concentration which showing no colonies or at least 5 colonies (99.9% inhibition) was determined as the MBC value [24].

RESULTS

The yields of the ethanol crude leaf extracts of the six plants are presented in table 1. *Toona sinensis* had the highest yield (22.20%) followed by *A. conyzoides* (20.50%), *C. caudatus* (17.10%), *C. citratus* (16.10%), *C. rotundus* (9.84%) while *C. odorata* provide the lowest yield (8.00%). The results of the phytochemical analysis provided evidence of the presence of alkaloids, flavonoids, saponins and tannins in the leaf extracts of all repellent plants, except that tannins were not found in *C. rotundus* and *A. conyzoides*.

Table 1: Yield of extract and phytochemical contents

Plant	% yield	Phytochemical contents			
		Alkaloids	Flavonoids	Saponins	Tannins
Chromolaena odorata	8.00	+	+	+	+
Toona sinensis	22.20	+	+	+	+
Cymbopogon citratus	16.10	+	+	+	+
Cyperus rotundus	9.84	+	+	+	+
Ageratum conyzoides	20.50	+	+	+	-
Cosmos caudatus	17.10	+	+	+	-

Notes: (+) presence; (-) absence

The antibacterial activities of the six plant extracts are demonstrated in table 2. As the results, all repellent plants extracts exerted potent inhibition against the tested bacteria. Of the plant extracts, *T. sureni* extract showed the most active antibacterial while *C. citratus* extract had the lowest mean total inhibition. This information was in line with the percentage of extraction yield.

Table 2: Antibacterial activity

Plant	Diameter of inhibition (mm) in certain concentration (mg/ml)			
	600	400	200	100
Chromolaena odorata	20.99±0.68	19.48±0.88	19.03±0.66	17.84±0.15
Toona sureni	31.70±1.20	28.60±0.27	26.61±0.04	24.24±0.02
Cymbopogon citratus	13.49±0.16	11.89±0.12	11.10±0.13	8.96±0.44
Cyperus rotundus	13.74±0.44	12.16±0.42	10.99±0.05	9.26±0.19
Ageratum conyzoides	14.15±0.13	13.35±0.04	12.26±0.21	10.69±0.26
Cosmos caudatus	16.82±0.64	15.30±0.84	13.72±0.98	12.66±0.75

S. typhi clinical isolate gave different sensitivity responses to repellent plant extracts at the same concentration. To observe the extent of the difference in the inhibitory potential of each extract, a statistical analysis was carried out as shown in table 3. Effect of the extract on the difference in the inhibition diameter was statistically analysed using the ANOVA test, followed by Duncan test.

Table 3: Statistical analysis result

	Sum of squares	Df	Mean square	F	Sig.
Between Groups	17.832	23	0.775	276.541	0.000
Within Groups	0.067	24	0.003		
Total	17.900	47			

The results of the ANOVA test above obtained the value of Sig. Sig. (0.000)<0.05 means that there is a difference in the inhibitory potential of the ethanol plants extracts at a concentration of 10, 20, 40 and 60 %w/v. Then further tests were carried out using Duncan to determine the most potential extract with the following results. presented in table 4. The Duncan test results revealed that *T. sureni* leaf was the most active extract based on its significantly different result from other extracts.

Table 4: Duncan test result

Plant extract	Concentration (mg/ml)	Mean	Symbol
C. odorata	100	1.784±0.016	j
	200	1.903±0.066	k
	400	1.949±0.088	k
	600	2.100±0.069	l
C. caudatus	100	1.267±0.076	efg
	200	1.373±0.098	gh
	400	1.531±0.084	i
	600	1.683±0.064	j
A. conyzoides	100	1.070±0.026	b
	200	1.226±0.021	def
	400	1.336±0.005	fgh
	600	1.416±0.013	h
C. rotundus	100	0.927±0.019	a
	200	1.099±0.006	bc
	400	1.216±0.042	cde
	600	1.375±0.045	gh
C. citratus	100	0.897±0.045	a
	200	1.111±0.013	bcd
	400	1.190±0.012	cde
	600	1.350±0.016	gh
T. sureni	100	2.425±0.002	m
	200	2.662±0.005	n
	400	2.861±0.028	o
	600	3.171±0.121	p

Notes: Different letters indicate there is a difference meanwhile the same letters means that there is no significant difference effect.

MIC and MBC of plant extracts were determined to compare the effect of each plant extracts on the microorganisms growth. The MIC values for the extracts ranged between 3.125-12.5 mg/ml and for the MBC values were in the range of 12.5-50.0 mg/ml, presented in table 5. *T. sureni* leaf extract with a MIC of 3.125 mg/ml and MBC of 12.5 mg/ml was the most potential plant extract against *S. typhi* clinical isolate. Whereas the lowest value for MIC and MBC was resulted by *C. citratus*, as same as with the results of the antibacterial activity test.

Table 5: MIC and MBC values

Plant	MIC values (mg/ml)	MBC values (mg/ml)
Chromolaena odorata	12.50	25.00
Toona sureni	3.125	12.50
Cymbopogon citratus	25.00	50.00
Cyperus rotundus	25.00	25.00
Ageratum conyzoides	25.00	25.00
Cosmos caudatus	12.50	25.00

DISCUSSION

Salmonella typhi clinical isolate used in this study was isolated from market food because they are related with foodborne illnesses. Approximately more than 95% of Salmonella cases in human cases involve consumption of contaminated food and 30% of all deaths caused by Salmonella was associated with foodborne disease [25]. The bacterial isolate used in this study was a chloramphenicol resistant strain. This is in line with the fact that resistance of Salmonella species to chloramphenicol began to be reported in 1972 and continue to develop to form multidrug-resistant strains [5, 6]. The development of bacterial multidrug resistance to all three first-line drug contributes to the increasing number of typhoid fever cases annually [7]. The increasing prevalence of multi-drug-resistant (MDR) Salmonella serotypes has a major impact on the effectiveness of antibiotic treatment and the mortality rate of Salmonella infections. The failure of those drugs to be developed recently has shifted treatment trend to plant-based products. Bioactive compounds from plants can simultaneously react with target site of pathogen in several strategy [26-28]. As repellent plant, plants develop defenses against insect and pathogen attacks by producing secondary metabolites [29], or synthesis such compounds in quite high concentrations, which act as natural insecticides e. g Pl [30, 31]. As reported this study, the resulted anti-salmonella activity of all leaf extracts may thus be due to the presence of alkaloids, flavonoids, tannins and saponins. The results of the phytochemical

analysis provide evidence of the presence of those interesting compounds in the leaf extract of all repellent plants, except that tannins were not found in *C. rotundus* and *A. conyzoides*. Similar study has also shown antibacterial activity against *S. typhi* in the presence of the same secondary metabolites in plant extracts, especially higher accumulated in the leaves than other parts of *Cassia petersiana* Bolle [32]. Flavonoids are phytochemical compounds that have been shown to have a broad antibacterial spectrum with different mechanisms [33-37]. Several studies had been reported various antibacterial mechanism of flavonoid including the inhibition of nucleic acid synthesis, interfere the function of cytoplasmic membrane and energy metabolism, reduce bacterial adhesion to form biofilm, interrupt porin, and reduce membrane permeability [38-42]. Moreover, certain flavonoids have been studied to inhibit certain bacterial enzymes that play a role in bacterial resistance; for example chloramphenicol acetyl transferase (CAT) enzyme produced by *S. typhi* is responsible in chloramphenicol resistance in *S. typhi*. This enzyme covalently bound acetyl groups of acetyl-CoA to chloramphenicol thus restrained chloramphenicol binding to bacterial ribosomes [40, 43-47]. Many plants which biosynthesize saponins was also found to inhibit the growth of *S. typhi* clinical isolate by disturbing permeability of bacterial membrane cells [48-51]. The integrated of other phytochemical substance in all leaf extracts had strengthened their antibacterial potency. Alkaloids also have an antibacterial mechanism that is almost the same as other phytochemical compounds found in all extracts of this plant such as inhibition of bacterial cell wall synthesis, bacterial metabolism, nucleic acid and protein synthesis, also disturbing the permeability of bacterial cell membrane [52, 53]. In addition, plant-derived tannins have also been used as natural antibacterial substances by interfering biofilm formation, thus reducing bacterial virulence [54, 55]. The antibacterial activity of all plant extracts increased linearly as the concentration of the extracts increased. Presumably this indicates that higher concentrations may have greater bacterial inhibitory potential. This could be due to the polar nature of the active antibacterial agent. Of all tested plant extracts, an ethanol extract of *T. sureni* leaf demonstrated the most potent inhibition against *S. typhi clinical isolate*. In addition to the complete phytochemical components, the *T. sureni* extract's yield is also obtained as the highest rendemen among other extracts and this data is thought to affect the level of each phytochemical substance it contains.

In addition to the role of those secondary metabolites, protease inhibitors produced by repellent plants play a very important role in reducing the pathogenicity of *S. typhi*. From clinical relevance, protease enzymes play an important role in the virulence of pathogenic bacteria. Extracellular proteases are responsible for the destruction of host tissues and degradation of host defense proteins [56]. Meanwhile, intracellular protease enzymes, such as cysteine protease GtgE from *S. typhi* are required to regulate the production and secretion of virulence factors, as well as to regulate stress responses that are essential for their survival in the host. This enzyme is an important component for Salmonella infections to infect host cell by modifying the regulation of host defense mechanisms. GtgE act specifically to cleavage Rab32 *in vitro*, which Rab32 is responsible in regulating lysosome-related organelles (LRO) biogenesis. Therefore, this enzyme protects *S. typhi* from degradation after being succeed to be phagocytosed by inhibiting the *S. typ*hi-containing vacuole, which also act as an LRO. Hence, it can be claimed that the inhibition of GtgE is the critical solution to overcome the infectious mechanism of *S. typhi* [57]. Therefore, proteases are reported as prime targets for the development of antibacterial drugs that are currently being studied to be obtained from plants. Agreeing with this, the exploration of protease inhibitor from plants has been promoted, such as Moringa oleifera leaves, Cassia fistula, and Lawsonia inermis seeds [58-60]. Historically, the leaves and bark of *T. sureni* are usually used to control mites, stink bugs, caterpillars, and aphids because they contain surenin and surenolactone [61]. *C. citratus* leaves are also used as insecticides to kill cabbage leaf caterpillars [62]. *C. rotundus* leaves are also often used as biopesticides for plant-disturbing insects [63]. *A. conyzoides* leaves can also be used as a natural pesticide because they contain pyrolizidine alkaloid compounds [64, 65]. *C. caudatus* plant is widely used as a pesticide to kill crickets [66]. *C. odorata* plants are also used as pest and disease control in plants because they contain vegetable mollusks [67]. Those data strengthened the evidence of protease inhibitors are present in all studied plant extracts. Similarly with the antibacterial activity result, *T. sureni* ethanolic extract inhibited the highest activity against *S. typhi* with the lowest MIC ($0.15625 \leq x \leq 0.3125$% w/v) and MBC ($0.625 < x \leq 1.25$% w/v) value which can be interpreted has strong activity [68]. Antibacterial agents are considered as bactericidal agents when the ratio MBC/MIC≤4 and bacteriostatic agents when the ratio MBC/MIC>4 [32]. For the *T. sureni* leaf extract, the ratio MBC/MIC≤4, suggesting that it may be classified as bactericidal agent.

CONCLUSION

Our findings shed light revealed an evidence that all studied repellent plant extracts provide antibacterial activity against clinical isolate of chloramphenicol resistant-*S. typhi*. This due to the detected antibacterial phytochemical substances and the probably presence of protease inhibitors in the extracts. Of all plants, *T. sureni* extract leaves achieved the most appreciable value of MIC MBC and considered as the bactericidal agent which has strong potential to be a novel anti-typhoid fever agent. The findings of the extract's potential to inhibit chloramphenicol resistant-*S. typhi* has become a novelty in the typhi fever drug discovery which can complement chloramphenicol's work of action or others conventional antibiotics.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

Declared none

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