PHENOL COEFFICIENT TEST OF COMBINATION EXTRACT OF LEAVES AND PEEL OF CITRUS HYSTRIX DC IN VITRO AS AN ALTERNATIVE ANTISEPTIC

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ABSTRACT
Citrus hystrix DC (C.hystrix) contains several antimicrobial compounds that have the potential as antiseptic candidates. This study reports the effectiveness of C.hystrix leaf and fruit peel (LPE) combination extract against several microbial isolates in vitro through a phenol coefficient test (KF). KF test on C.hystrix LPE suspension, 70% alcohol control, 5% phenol with test microbe, in 1:20 dilution; 1:30; 1:40; 1:50; 1:60; 1:70; 1:80; 1:90; 1:100; 1:110; 1:150; 1:200; 1:250 which was observed at 5, 10, and 15 minutes and the KF value was calculated. The effective KF value is 5%=1 phenol equivalent. The results showed that the KF value of the control and LPE *C*.hystrix treatments for Staphylococcus aureus = 1.25; Escherichia coli=1.00; Pseudomonas aeruginosa=1.00; and Candida albicans= 1.00. In conclusion, the combination of leaf extract and C.hystrix fruit peel has effectiveness as an antimicrobial which has the potential to be used as an alternative antiseptic.

KEYWORDS
Alternative antiseptic; Citrus hystrix DC; Combination extract; In vitro; Phenol Coefficient test

INTRODUCTION

Infectious diseases that are transmitted through hands are still a health problem in wetland areas. Transmission of infectious diseases in wetland areas is associated with community hygiene, especially the behavior of washing hands using antiseptic substances. Generally, infection transmission often occurs due to the use of water that has been contaminated with bacteria and direct contact with hands or the presence of bacterial contaminants on the skin surface[1,2].

The report on the identification of the types of bacteria found in the hand swab samples of the people living around the riverbanks of Banjarmasin City, found the types of bacteria S. aureus, S. epidermidis and E. coli in the hand samples. and E. coli and S. typhi bacteria in stool samples[1,3]. Types of yeast Candida albicans (C. albicans) can also be transmitted through the hands or skin with abnormalities. Candida albicans yeast transmission between people can occur when using public tubs or toilet facilities[4] or during a flood disaster[5].

The transmission of infectious agents from person to person can be prevented by the use of antiseptics, which play a role in reducing or killing microbial colonization found on the surface or skin tissue[5]. The use of antiseptic substances when washing hands, which are alcohol-based or made from natural ingredients, is intended to minimize the transmission of infectious agents. The active ingredient is alcohol which has high effectiveness against viruses, bacteria, and fungi. Alcohol is one type of antiseptic that is often used, by damaging cell membranes, inhibiting enzyme performance and denaturing microbial cell proteins[6]. Alcohol is flammable and can dry out your hands. In addition, the use of alcohol at high levels has the impact of increasing the risk of viral infections that trigger

inflammation of the digestive tract[7,8]. Prolonged use of alcohol can harm the skin such as causing burning, irritation, dry skin, and cannot be used on wound skin[9]. Efforts to reduce the impact of alcohol use, is to use natural antiseptic preparations. Alternative antibacterial substances can play a safer and more effective role and are cheaper than synthetic drugs[10].

The effectiveness of an antiseptic agent in inhibiting the colonization of bacterial growth, can be measured based on the phenol coefficient test. In the phenol coefficient test, the coefficient value is compared with 5% phenol as a comparison, which has known effectiveness as a disinfecting agent. The value of a good phenol coefficient is equivalent to 1; effectiveness gets better if the value of phenol coefficient 1. Laboratory standard microbial isolates that can be used in the phenol coefficient test include S.aureus, E.coli, P.aeruginosa, and S.typhi and the yeast C.albicans[11,5]. The phenol coefficient test can also be applied to herbal preparations that have antibacterial activity[11]. The test results of the combination treatment of Cananga odorata flower and Averrhoa bilimbi fruit infusion against S.aureus and against S.typhi resulted in different magnitudes of inhibition and phenol coefficient values[12].

Citrus *C.* hystrix DC (C.hystrix*)* has been used by the community as medicine; C.hystrix can be used to treat coughs, mouthwash, and as an antiseptic. Phytochemical test results in C.hystrix, various secondary compounds with antibacterial properties were obtained, in the form of citronellol, limonene, and geraniol essential oils; as well as flavonoids, phenolics, terpenoid alkaloids, and tannins[13,14,15]. The results of phytochemical tests on the eatanol extract of the leaves and skin of C.hystrix fruit were found to have active compounds of flavonoids, tannins, phenols, and alkaloids[11]. In a single dosage form, C. hystrix was able to inhibit various test bacteria including E.coli[13,14] 96% ethanol extract of *C.* hystrix bark at 100% treatment, could inhibit E.coli with an inhibition zone of 10.67mm[14], but the resulting inhibition zone was still under positive disk control[15,16,17]. Budiarti et al mentioned that the effect of treatment with 100% C. hystrix fruit peel infusion can reduce the number of S. E.coli, but the effect is still not equivalent to 70% alcohol treatment. Efforts to increase the antibacterial effect in addition to using suitable solvents for extraction dosage forms as well as in combination/mixed forms. Results of *in vitro test combination* treatment of ethanol extract of leaves and bark of C. hystrix 12.5%, 25%, 50%, and 75% (w/v) had different effects on S.aureus, S.epidermidis, E.coli, and P.aeruginosa; the combination of 75% (1:1) produced the largest inhibition zone in the four test bacteria and was equivalent to the positive control[11]. Test results for hand sanitizer preparations combined with 75% Ocimum basilicum leaf extract and C.hystrix peel extract 25%, showed effective antiseptic properties in reducing the number of test bacteria compared to other formulas[18]. Availability C.hystrix in the community there are quite a lot, but its use as an antiseptic preparation in the form of a combination has not been widely informed. The results of previous studies became the basis for examining the antibacterial activity of the ethanol extract of the combination of leaves and skin of C. hystrix DC. fruit as an alternative antiseptic candidate preparation. This study aims to observe the effectiveness of antiseptic preparations from a combination of C. hystrix leaf and peel extract preparations in vitro through the phenol coefficient test. The effectiveness of herbal preparations as antiseptic candidates was observed based on the comparison of the phenol coefficient of the combination of C. hystrix leaf peel extract and 5 % phenol solution as a comparison substance. Laboratory standard microbial isolates tested were S.aureus, E.coli, and P.aeruginosa, as well as on the yeast C.albicans.

RESEARCH METHOD

Research Material

The plant material used in this study was Citrus hystrix, the test plant species obtained from areas in South Kalimantan. The isolates of the tested bacteria studied were Staphylococcus aureus ATCC 29523 Escherichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 27853, and Candida albicans ATCC 10231. The microbial isolates tested are a collection of the Microbiology Laboratory of the Faculty of Medicine, ULM Banjarbaru. The tested microbial suspension was made homogeneous by growing on BHI media and made equivalent to 0.5 Mac Farland solution ($1.5x10^6$ CFU/ml).

The test media used were Nutrient Agar (NA), Nutrient Broth (NB), and Brain Heart Infusion (BHI). The solvent in the manufacture of extracts and series of dilutions is sterile distilled water. The

controls used in the phenol coefficient test were 0.002% chlorine and 5% phenol.

Production of Ethanol Extract of Leaves and Peel of *C.hystrix*

The extraction method used for this research is maceration. Each 1.000 gram sample of leaf powder and fruit peel of *C. hystrix* put into the maceration tool, then 96% ethanol solution is poured slowly into the maceration tool. The maceration process is carried out within 3 x 24 hours by stirring until evenly distributed, every 1 x 24 hours the filtrate is filtered, and the solvent is replaced with a new one. After that, the extract was put into a *rotary evaporator* at a temperature of 60ºC until a thick ethanol extract was obtained, then evaporated in a water bath so that a constant weight was obtained. Extraction results can be stored in the refrigerator at a temperature 4ºC[19].

Preparation of Treatment Dilution Series on Phenol Coefficient Test

Each of 26 sterile tubes were prepared for the combination treatment of extracts leaves and peels of C. hystrix 100% (1:1) and 5% phenol control and labeled 1-26. In each tube 1-13, 2 ml of test extract treatment and 5% phenol control were added, and sterile distilled water was added from each tube 1- 13, namely 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 13, 18, and 23 ml; Then each tube was homogenized. Furthermore, in each tube containing the extract and test control, 2 ml aseptically pipetted and put into tubes 14-26. The results obtained that the dilution series of each treatment and 5% phenol control test was 1:20; 1:30; 1:40; 1:50; 1:60; 1:70; 1:80; 1:90; 1:100; 1:110; 1:150; 1:200; and 1:250. The series of treatment dilutions were made in the same steps for each treatment of the bacteria tested[11].

Phenol Coefficient Test

A suspension of test bacteria (S. aureus, S. epidermidis, E. coli, and P. aeruginosa), and the fungus Candida albicans were prepared on tube. racks and sterile test tubes containing NB media that have been labeled according to the dilution along with the length of contact time (5, 10, and 15 minutes), as well as equipment such as spirit lamps, micropipettes, and loops. 0.5 ml of the suspension of the test bacteria was put into a tube containing the test treatment (leaf and peel extract of C. hystrix and 5% phenol) in various dilution series starting from the tube with a dilution of 1:20 to 1:250, then homogenized. After 5 minutes, 1 ose was taken from each test dilution series tube and put into each test tube containing the test dilution series with a contact time of 5 minutes, then the ose was sterilized with a spirit lamp. After the second 5 minutes, 1 ose is taken from each test treatment dilution series tube into the test treatment dilution series test tube with a contact time of 5 minutes (total contact time is 10 minutes), then the ose is sterilized with a spirit lamp. After the third 5 minutes, 1 ose was taken from each test treatment dilution tube into the test treatment dilution series test tube with a contact time of 5 minutes (total contact time 15 minutes), then the ose was sterilized with a spirit lamp. The same steps were carried out for the treatment of each test bacteria. All the tubes were incubated at 37°C for 24 hours, then observed for turbidity. The presence of bacterial growth (+) was indicated by the medium becoming cloudy, and the absence of growth of the test microbes (-) indicated by the medium remaining clear. Furthermore, the value of the phenol coefficient is calculated using the following formula[20].

Phenol Coefficient

$$= \frac{\left\{\begin{matrix}\text{The lowest dilution of phenol that kills bacteria}\\ \text{the lowest dilution of antiseptic that kills bacteria}\end{matrix}\right\}}{\left\{\begin{matrix}\text{the highest dilution of phenol that kills bacteria}\\ \text{the highest dilution of antiseptic that kills bacteria}\end{matrix}\right\}}$$

Data Analyst

Phenol coefficient value and descriptive analysis.

RESULTS

The effectiveness of an antimicrobial substance as a good disinfectant is equal to or greater than the 5% phenol liquid coefficient, which is equal to 1 (one). Observations of the phenol coefficient test in each of the tested treatments can be seen in tables 1 and 2. In the treatment that showed no microbial growth, the effectiveness was calculated through the formula for the phenol coefficient value.

The results showed that the phenol coefficient value of alcohol and the ethanol extract of C. hystrix leaves and citrus peel (LPE) treatment was the same as the 5% phenol coefficient value. So

that it can be said that the effectiveness of the extract is the same as the antiseptic substance. It was found that the phenol coefficient (FC) value of the extract was slightly higher in S.aureus than the other microbes tested. The FC values obtained sequentially were: S.aureus=1.25; E.coli= 1.00; P.aeruginosa=1.00; and C.albicans = 1.00.

This study shows that there is a difference in the effectiveness of the treatment, based on the magnitude of the phenol coefficient value. The preparation of the extract as an antiseptic is effective, if the value is equal or more to the comparison of phenol 5%. The effectiveness of an antimicrobial substance as a good disinfectant is equal to or greater than the 5% phenol liquid coefficient, which is equal to 1 (one). Observations of the phenol coefficient test in each of the tested treatments can be seen in tables 1 and 2. In the treatment that showed no microbial growth, the effectiveness was calculated through the formula for the phenol coefficient value. The average value of the phenol coefficient in each treatment is shown in Table2. This study shows that there is a difference in the effectiveness of the treatment, based on the magnitude of the phenol coefficient value. The preparation of the extract as an antiseptic is effective, if the value is equal or more to the comparison of phenol 5%. The results of the higher phenol coefficient value between the combined treatment of C. hystrix extract and *phenol 5*%, namely Staphylococcus aureus. The same coefficient value with 5% phenol is in Escherichia coli, Pseudomonas aeruginosa and Candida albicans.

DISCUSSIONS

In this study it can be proved that combination extract preparations can produce effectiveness as an antiseptic. This is due to the role of the bioactive compounds contained in the combination extract, which work synergistically. The effectiveness of the extract can match 5% phenol which has been tested as an antiseptic that has a good effect on various microbes. Phenol is the standard of antiseptic strength. The mechanism of action of phenol as a bacteriostatic antiseptic works by interaction between phenolic compounds and bacterial cells through an absorption process involving hydrogen bonds. At low concentrations, phenol will form protein complexes with weak bonds and decompose immediately, followed by penetration of phenol into bacterial cells and causes protein deposition and denaturation. Meanwhile, at high concentrations of phenol will cause coagulase of bacterial cell proteins and cytoplasmic membranes to undergo lysis[12].

Effectiveness as an antimicrobial and antiseptic preparation, due to the role of these compounds metabolites secondary contained in C. hystrix. The active compounds in the leaves and skins of C. hystrix fruit include essential oil compounds citronellol, limonene, and geraniol, as well as flavonoid compounds, alkaloids, and tannins. [21] Citronellol, limonene and geraniol compounds are terpenoids that can suppress the growth of microorganisms by inhibiting the metabolic processes of bacterial cells. Citronellol, limonene and geraniol compounds have a granyl acetate structure with alcohol and aldehyde groups that play a role in denaturing bacterial cell proteins. Citronellol compounds can cause protein denaturation in bacteria and can inhibit bacteria. Limonene compounds inhibit bacterial growth by denaturing proteins and inhibiting cell growth and causing bacterial cell death. Meanwhile, geraniol compounds are able to disrupt cell membrane permeability[22].

The active compounds of flavonoids can inhibit the topoisomerase II (DNA gyrase) enzyme which is an important enzyme in the process of replication and transcription of bacterial DNA, so that bacterial growth will be disrupted. The main content of flavonoids in C. hystrix is naringin which is found in the skin and fruit and has a function as a strong antioxidant and inhibits the function of cell membranes, so that the bacteria die[23]. In this study, it can be proven that the combination treatment of extracts can produce good antimicrobial effectiveness, resulting in a phenol coefficient value that is equivalent to or higher than the 5% phenol control. These results indicate a synergistic effect as informed from previous studies. The results of the hand sanitizer preparation test combination of Ocimum basilicum leaf extract 75% and C. hystrix DC bark extract 25%, showed an effective antiseptic power in reducing the number of test bacteria compared to the formula other[18]. The results were relatively the same, namely the combination treatment of Averrhoae blimbi and Cananga odorata[12] infusions and the combination of Mimosa pudica and Cyperus rotundus infusions[24] demonstrated its effectiveness as an alternative antiseptic preparation.

Table 1. Observation Results of Phenol Coefficient Test Treatment of Combination of Leaf and Peel Extract of C. hystrix , 5% Phenol, 70% Alcohol Against S.aureus Bacteria

Sample	Treatment Concentration	Repeat 1			Repeat 2			Repeat 2			Phenol Coefficient
		5	5	5	10	10	10	15	15	15	
C.hystrix Combination Extract	1:20	-	-	-	-	-	-	-	-	-	**1.125**
	1:30	-	-	-	-	-	-	-	-	-	
	1:40	-	-	-	-	-	-	-	-	-	
	1:50	-	-	-	-	-	-	-	-	-	
	1:60	-	-	-	-	-	-	-	-	-	
	1:70	-	-	-	-	-	-	-	-	-	
	1:80	-	-	-	-	-	-	-	-	-	
	1:90	+	-	-	-	-	-	-	-	-	
	1:100	+	-	-	+	+	-	+	-	-	
	1:110	+	+	+	+	+	-	+	+	-	
	1:150	+	+	+	+	+	+	+	+	+	
	1:200	+	+	+	+	+	+	+	+	+	
	1:250	+	+	+	+	+	+	+	+	+	
70% alcohol	1:20	-	-	-	-	-	-	-	-	-	**1**
	1:30	-	-	-	-	-	-	-	-	-	
	1:40	-	-	-	-	-	-	-	-	-	
	1:50	-	-	-	-	-	-	-	-	-	
	1:60	-	-	-	-	-	-	-	-	-	
	1:70	-	-	-	-	-	-	-	-	-	
	1:80	-	-	-	-	-	-	-	-	-	
	1:90	+	-	-	-	-	-	-	-	-	
	1:100	+	+	-	+	-	-	+	-	-	
	1:110	+	+	+	+	+	-	+	+	-	
	1:150	+	+	+	+	+	+	+	+	+	
	1:200	+	+	+	+	+	+	+	+	+	
	1:250	+	+	+	+	+	+	+	+	+	
Phenol 5%	1:20	-	-	-	-	-	-	-	-	-	**1**
	1:30	-	-	-	-	-	-	-	-	-	
	1:40	-	-	-	-	-	-	-	-	-	
	1:50	-	-	-	-	-	-	-	-	-	
	1:60	-	-	-	-	-	-	-	-	-	
	1:70	-	-	-	-	-	-	-	-	-	
	1:80	+	-	-	-	-	-	-	-	-	
	1:90	+	-	-	-	-	-	-	-	-	
	1:100	+	+	-	+	-	-	+	-	-	
	1:110	+	+	+	+	+	-	+	+	-	
	1:150	+	+	+	+	+	+	+	+	+	
	1:200	+	+	+	+	+	+	+	+	+	
	1:250	+	+	+	+	+	+	+	+	+	

Table 2. Observation Results of Phenol Coefficient Test Treatment of Leaf and Peel Extract Combination of C. hystrix, Phenol 5%, Alcohol 70% Against E.coli, P.aeruginosa, and C.albicans

Sample	Treatment	Repeat 1			Repeat 2			Repeat 2			Phenol
	Concentration	5	5	5	10	10	10	15	15	15	Coefficient
C.hystrix Combination Extract	1:20	-	-	-	-	-	-	-	-	-	**1**
	1:30	-	-	-	-	-	-	-	-	-	
	1:40	-	-	-	-	-	-	-	-	-	
	1:50	-	-	-	-	-	-	-	-	-	
	1:60	-	-	-	-	-	-	-	-	-	
	1:70	-	-	-	-	-	-	-	-	-	
	1:80	-	-	-	-	-	-	-	-	-	
	1:90	+	+	-		-	-	-	-	-	
	1:100	+	-	-	+	+	-	+	-	-	
	1:110	+	+	+	+	+	-	+	+	-	
	1:150	+	+	+	+	+	+	+	+	+	
	1:200	+	+	+	+	+	+	+	+	+	
	1:250	+	+	+	+	+	+	+	+	+	
70% alcohol	1:20	-	-	-	-	-	-	-	-	-	**1**
	1:30	-	-	-	-	-	-	-	-	-	
	1:40	-	-	-	-	-	-	-	-	-	
	1:50	-	-	-	-	-	-	-	-	-	
	1:60	-	-	-	-	-	-	-	-	-	
	1:70	-	-	-	-	-	-	-	-	-	
	1:80	-	-	-	-	-	-	-	-	-	
	1:90	-	-	-	+	-	-	-	-	-	
	1:100	+	+	-	+	-	-	+	-	-	
	1:110	+	+	+	+	+	-	+	+	-	
	1:150	+	+	+	+	+	+	+	+	+	
	1:200	+	+	+	+	+	+	+	+	+	
	1:250	+	+	+	+	+	+	+	+	+	
Phenol 5%	1:20	-	-	-	-	-	-	-	-	-	**1**
	1:30	-	-	-	-	-	-	-	-	-	
	1:40	-	-	-	-	-	-	-	-	-	
	1:50	-	-	-	-	-	-	-	-	-	
	1:60	-	-	-	-	-	-	-	-	-	
	1:70	-	-	-	-	-	-	-	-	-	
	1:80	-	-	-	-	-	-	-	-	-	
	1:90	+	-	-	-	-	-	-	-	-	
	1:100	+	+	-	+	-	-	+	-	-	
	1:110	+	+	+	+	+	-	+	+	-	
	1:150	+	+	+	+	+	+	+	+	+	
	1:200	+	+	+	+	+	+	+	+	+	
	1:250	+	+	+	+	+	+	+	+	+	

Effectiveness on Gram positive bacteria is generally better than Gram negative. The type of bacteria inhibited, the structure of the bacterial cell wall, penetration and bonding of antibacterial compounds can affect the activity of antibacterial substances based on the variation of the phenol coefficient value obtained. Gram-positive bacteria tend to be more sensitive to antibacterial compounds such as flavonoids, saponins, and tannins. This is because the structure of the cell wall of gram-positive bacteria is relatively simpler than the structure of the cell wall of gram-negative bacteria. The cell wall composition of gram- positive bacteria consists of more than 50% peptidoglycan and teichoic acid content which is polar and low lipid content (1-4%) which is non-polar. Meanwhile, flavonoids, saponins and tannins are polar compounds[12]. Therefore, this antibacterial compound more easily penetrates the cell membrane of gram- positive bacteria. While gram-negative bacteria have bacterial cell walls that are more difficult for compounds to penetrate by flavonoids, saponins and tannins because they consist of high lipid content (11-22%) which are non-polar and peptidoglycan content is only about 5-10% which is polar. and the outer cell membrane which functions as a selective defense of toxic compounds entering and leaving the cell. In addition,

gram-negative bacteria have an outer membrane consisting of phospholipids (inner layer) and non-polar lipopolysaccharides (outer layer). This is what causes polar secondary compounds (flavonoids, saponins and tannins) to be more difficult to enter into gram-negative bacteria cells so that their antibacterial activity is less strong than gram-positive bacteria[12].

The gram-negative outer membrane consists of three layers, namely lipopolysaccharides (LPS), lipoproteins, and phospholipids. In phospholipids there are porins which are formed from proteins. Porins are channels through which some molecules can pass. This outer membrane serves as a barrier against antibiotics, digestive enzymes, and dry conditions, but cannot be a barrier to all substances. The main factors of cell wall damage are lipopolysaccharide (LPS) and porin. Antibacterial compounds that work by penetrating LPS (lipopolysaccharide), hydrophilic molecules will more easily pass through LPS than hydrophobic molecules. Gram-negative bacteria have hydrophilic properties, namely carboxyl, amino acids and hydroxyl. The mechanism of antibacterial action of each secondary metabolite compound is different. Secondary metabolite compounds inhibit the growth of bacteria starting by damaging the cell wall. Polar compounds can penetrate polar peptidoglycan, as well as some polar compounds such as phenolic compounds that can break the peptidoglycan bond in the bacterial wall. Antibacterial compounds that are able to react with porins (trans membrane proteins) on the outer membrane of bacteria and bacterial cell walls will form strong polymer bonds, resulting in the destruction of the porin. Damage to the porin which functions as a place for entry and exit of nutrients will cause the permeability of the bacterial cell wall to decrease so that bacterial growth is inhibited, or the bacterial cell will die[25].

Some of the roles of secondary compounds in fungi can be explained as follows. Flavonoid group compounds are strongly suspected of being able to inhibit fungal growth by inhibiting the synthesis of fungal nucleic acids and inhibiting the division and proliferation of fungal cells[26,27]. Compounds of the tannin group in the extract can produce glycosyltransferase enzymes that affect the integrity of the composition of the fungal cell wall of C.albicans. monosaccharide units into polysaccharides, making it easier for other compounds to synergize to work to destroy nucleic acid synthesis[26,28]. The process of inhibiting the growth of C.albicans requires more and more specific metabolites compared to inhibiting other microbes, because the cell wall structure of C.albicans consists of 5 layers and the outer layer of the plasma membrane contains lipids in the form of ergosterol which is a double phospholipid membrane which function to resist lysis due to osmotic pressure[29,30].

This in vitro study proves that C. hystrix extract has effectiveness as an antiseptic. The development of C. hystrix as an alternative antiseptic preparation needs to be supported based on research, both in combination extract preparations with different plant species, as well as in vitro and organoleptic testing.

CONCLUSION

Based on the phenol coefficient test, the combination of leaf extract and C.hystrix fruit peel has antimicrobial effectiveness which has the potential to be used as an alternative antiseptic.

For further research, you can explore more about the stability, viability of the extract as an antiseptic, and its organoleptic properties according to safety and health standards.

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