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In-Vitro Antibacterial Activity of *Excoecaria cochinchinensis* Lour's Methanol Extract and Its Combination with Commercial Drugs

(Aktivitas Antibakteri Secara In-Vitro Ekstrak Metanol *Excoecaria cochinchinensis* Lour dan Kombinasinya dengan Obat Komersial)

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Abstract : The purpose of this study was to look into the antibacterial activity of methanolic extract of *Excoecaria cochinchinensis* as well as the effect of its combination with antibiotics on the growth of *Klebsiella pneumoniae, Enterobacter aerogenes*, and *Staphylococcus epidermidis*. The antibacterial activity test was carried out using the well diffusion method, and the combined effect of the extract and antibiotics was observed using the paper strip diffusion method. At various concentrations (25, 50, 75, 100, 150, 200, and 250 mg/mL), the extract significantly inhibited the growth of *K. pneumoniae*, *E. aerogenes*, and *S. epidermidis*. The inhibitory zone's diameter increased proportionally with the extract concentration. The growth of *K. pneumoniae* and *S. epidermidis* was then classified as extremely sensitive (+++) to the addition of the extract at 200 and 250 mg/mL concentrations, meanwhile, *E. aerogenes's* with 250 mg/mL of the extract. Secondary metabolites such as oleanolic acid, arjunolic acid, scopoletin, kaempferol, quercetin, diterpenoid compounds, tannins, and other phenolics have been reported from *E. cochinchinensis* leaves and are thought to be responsible for its antimicrobial activity. The effect of the extract's combination with antibiotics was insignificant compared to their individual activity, thereby classifying them as indifferent.

Keywords: Antibacterial activity, combination effect, *Excoecaria cochinchinensis*, inhibition zone, *in vitro*.

Abstrak: Tujuan dari penelitian ini adalah untuk menyelidiki aktivitas antibakteri dari ekstrak metanol *Excoecaria cochinchinensis* serta pengaruh kombinasinya dengan antibiotik terhadap pertumbuhan *Klebsiella pneumoniae*, *Enterobacter aerogenes*, dan *Staphylococcus epidermidis*. Uji aktivitas antibakteri dilakukan dengan metode difusi sumuran, dan efek gabungan dari ekstrak dan antibiotik diamati dengan menggunakan metode difusi kertas. Pada berbagai konsentrasi (25, 50, 75, 100, 150, 200, dan 250 mg/mL), ekstrak secara signifikan menghambat pertumbuhan *K. pneumoniae*, *E. aerogenes*, dan *S. epidermidis*. Selain itu, peningkatan diameter zona hambat berbanding lurus dengan peningkatan konsentrasi ekstrak. Pertumbuhan *K. pneumoniae* dan *S. epidermidis* kemudian diklasifikasikan sebagai sangat sangat sensitif (+++) terhadap penambahan ekstrak pada konsentrasi 200 dan 250 mg/mL. Sementara itu, pertumbuhan *E. aerogenes* diklasifikasikan sebagai sangat sangat sensitif (+++) ketika diberikan 250 mg/mL ekstrak. Metabolit sekunder seperti asam oleanolic, asam arjunolic, scopoletin, kaempferol, quercetin, senyawa diterpenoid, tanin, dan fenolat lainnya telah dilaporkan dari daun *E. cochinchinensis* dan diduga bertanggung jawab atas aktivitas antimikrobanya. Efek kombinasi ekstrak dengan antibiotik tidak terjadi peningkatan signifikan jika dibandingkan dengan aktivitas secara tunggal, sehingga diklasifikasikan sebagai efek indifferent.

Kata kunci: Aktivitas antibakteri, *Excoecaria cochinchinensis*, efek kombinasi, *in vitro*, zona hambat

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INTRODUCTION

INFECTIOUS diseases were one of the biggest health problems in developing countries, including Indonesia. They attack the skin tissue, oral tract, and respiratory and digestive systems. *Enterobacter aerogene*, *Klebsiella pneumonia*, and *Escherichia coli* were gram-negative bacteria from the Enterobacteriaceae family that caused several infectious diseases such as pneumonia, septicemia, urinary tract infections, cholecystitis, cholangitis, peritonitis, meningitis, and gastroenteritis[1]. Meanwhile, *Staphylococcus epidermidis* was a gram-positive bacterium from the family Micrococcaceae that caused mild skin infections with abscess formation. It also causes urinary tract diseases, endocarditis, and infections in neonates, individuals with low immune systems, and patients with implants[2].

The advent of antibiotics made it possible to control a bacterial infection, but some pathogens quickly became resistant to the drugs, which caused unwanted side effects. This resistance was due to the inappropriate and continuous use of the same antibiotic. Consequently, natural antibacterial studies on ornamental plants that also perform as medicinal plants have been carried out by researchers, such as Sambang Darah leaves (*Excoecaria cochinchinensis* Lour) that were reported to inhibit bacteria growth. *E. cochinchinensis* was a wild plant cultivated for ornamental and medicinal purposes; its leaves were also used as traditional herbal medicine by Indonesians to treat malaria and cancer[3].

E. cochinchinensis leaf extract inhibited the growth of acne-causing bacteria, namely *Staphylococcus aureus* and *Propionibacterium acnes* with a minimum inhibitory concentration (MIC) value of 1.56 mg/mL[4]. Trang et al, also reported that its ethanolic leaf extract has antibacterial activity against *Xanthomonas oryzae* pv oryzae at a concentration of 100 mg/mL with an inhibitory zone of 20.3 mm [5]. However, there were no studies on the antibacterial activity of its methanolic leaves extract against *Staphylococcus epidermidis*, *Klebsiella pneumoniae,* and *Enterobacter aerogenes*. The purpose of this study was to find out the activity of E. cochinchinensis leaves' methanolic extract in inhibiting the growth of *K. pneumoniae*, *E. aerogenes*, and *S. epidermidis* as well as the effect of the extract's combination with 1 mg/mL ciprofloxacin and 0.25 mg/mL clindamycin on the three test bacteria.

MATERIAL AND METHODS

MATERIAL. The leaves of *E. cochinchinensis* were collected in Bengkulu City, and the three types of test bacteria used were *K. pneumoniae*, *E. aerogenes*, and *S. epidermidis*.

METHODS. Sample Extraction and Phytochemical Screening. The leaves were finely chopped, sprinkled on paper, placed in an open room, and then dried without direct sunlight for 7 days. Subsequently, methanol was added to 700 g of the dried leaves until all samples were submerged. It was macerated at room temperature, then filtered to produce residue and filtrate after 4 days. At 45 °C, the filtrate's solvent was evaporated using a rotary evaporator to produce a concentrated extract, while the residue was re-macerated 3 times to obtain the maximum extract. Approximately 180.09 g of the leaves' crude methanol extract obtained was dark green with a yield of 25.72%. The extract was then subjected to qualitative phytochemical screening using a standard method[6].

Bacteria Suspension. One ose of rejuvenated bacteria was placed in 100 mL of sterilised MHB to make the test bacterial suspension. The solution was stirred at a low speed for 1 hour before being kept at room temperature for 24 hours. As a result, a cloudy solution containing colonies of the test bacteria was obtained. The optical density of the formed suspension was measured with a Spectronic 20D at a wavelength of 625 nm and an absorbance of 0.1, which was equivalent to Mc. Farlan 0.5 (1.5×10^8 CFU/mL)[7].

Antibacterial Activity. The antibacterial activity was tested using the well diffusion method, which involved pouring 15 mL of sterilised MHA media into a petri dish containing 1 mL of the test bacterial suspension. Subsequently, the mixture was homogenised by moving the dish several times to form a figure of eight, and allowed to solidify. Seven wells were then made in each Petri dish using a 6 mm diameter cork borer, and 20 µl of 25, 50, 75, 100, 150, 200, and 250 mg/mL of the extract were added to the wells, respectively. Positive control, containing 0.25 mg/mL clindamycin and 1 mg/mL ciprofloxacin as well as negative control DMSO were added to each well, then incubated at 37 °C for 24 hours. The clear zone that formed around the wells was measured in millimetres using a calliper, and the testing procedure was repeated four times[8]. Furthermore, the average results of the bacterial growth inhibition zone were described as the bacteria's sensitivity response to the addition of extracts according to Ponce, et al[9]. When the bacterial inhibition zone was <8 mm, the test bacteria were classified as insensitive (-), 9-14 mm were classified as sensitive (+), 15-19 mm are classified as very sensitive (++) and >20 mm were classified as extremely sensitive (+++).

Combination Effect of Methanol Extract from *Excoecaria cochinchinensis* Leaves with Antibiotics. The combined effect of the extract and antibiotics was observed using the paper strip diffusion method. About 30 mL of 250 mg/mL test extract, 0.25 mg/mL antibiotic clindamycin, and/or 1 mg/mL ciprofloxacin were dripped on a Whatman (No.3) paper tape measuring 3 cm × 0.5 cm. Meanwhile, the paper tape was placed on top of the MHA agar media, containing the test bacteria by forming an angle of 90°. The Petri dishes were then incubated for 24 hours at 37 °C, and a clear zone was formed around the paper tape. The combined effect was interpreted as follows: synergism was indicated by broadening of the inhibition around the angle, antagonism was shown by narrowing around the angle, and the indifferent or additive effect was indicated by no broadening or no narrowing effect in the zone of inhibition joining at angles[10].

RESULTS AND DISCUSSION

Phytochemical Screening of *E. cochinchinensis* Leaves Methanol Extract. To identify the presence of alkaloids, flavonoids, phenolics, steroids, saponins, and triterpenoids in *Excoecaria cochinchinensis* leaves, qualitative phytochemical screening was performed using standard procedures. Table 1 showed that the leaves contain flavonoids, phenolics, steroids, and triterpenoids, but no saponins and alkaloids. Furthermore, the presence of flavonoids was indicated when a reddish-brown colour was formed after treatment with magnesium powder and concentrated HCl during the Shinoda test. Phenolics were confirmed when a green-black colour was formed after the addition of ferric chloride 10% m/v solution. Meanwhile, the presence of triterpenoids and steroids was shown by the formation of red and blue colours after drops of Liebermann-Burchard reagent. These findings are in line with previous studies showing that 37 compounds have been isolated from *Excoecaria cochinchinensis*, and most of the compounds are oxygenated diterpenoids, while others include loliolide, flavonoids, megastigmane glucosides, triterpenoids, sterols, phenolics, and other compounds[11].

Antibacterial Activity of Methanol Extract of *Excoecaria cochinchinensis* Leaves. The antibacterial activity of the crude methanol extract of *E. cochinchinensis* leaves (EC MeOH**)** at all test concentrations successfully inhibited the growth of *K. pneumoniae, E. aerogenes,* and *S. epidermidis*. The antibacterial activity was indicated by the formation of a clear zone around the well, and its intensity varied depending on the extract's concentration and the type of bacteria tested. Table 2 showed the average growth inhibition diameter of *K. pneumoniae, E. aerogenes,* and *S. epidermidis*, and the comparison of inhibition zones between concentrations and between bacteria can be seen in Figure 1.

Table 2 showed that the extract's administration inhibited the growth of the three test bacteria, namely *K. pneumoniae, E. aerogenes,* and *S. epidermidis* at all concentration levels. At the lowest concentration tested, the extract inhibited the growth of *K. pneumoniae*, *E. aerogenes*, and *S. epidermidis* respectively by 12.67 mm ± 0.32, 12.48 mm ± 0.62, and 12.56 mm ± 0. 22 (Figure 1). The inhibition zones increased as the concentration of the extract in the well increased, indicating a concentration dependent activity that varied with the type of bacteria tested. However, the negative control (DMSO) did not inhibit the bacteria's growth. The positive control 0.25 mg/mL clindamycin showed an average inhibition zone diameter of 24.35 mm ± 0.48 against *S. epidermidis*, while 1 mg/mL ciprofloxacin inhibited *K. pneumoniae* and *E. aerogenes* with zone diameter of 33.97 mm ± 0.27 and 33.50 mm ± 1.39, respectively. The growth inhibition zone diameter of *K. pneumoniae* and *E. aerogenes* bacteria after the extract's administration was smaller than 1 mg/mL ciprofloxacin. Meanwhile, the growth inhibition zone diameter of *S. epidermidis* bacteria at the highest concentration was slightly larger than the positive control of 0.25 mg/mL clindamycin. Gram positive strains reacted to the extract more strongly than gram negative strains, it could be attributed to their outer peptidoglycan layer.

Table 1. Phytochemical screening of methanol extract of *Excoecaria cochinchinensis.*

Compounds Group	Reagent	Results
Alkaloids	Wagner (I_2 + KI)	-
Flavonoids	(Mg powder + Concentrated HCl)	+
Saponins	Foam test (H_2O, shake vigorously)	-
Phenolics	$FeCl_3$ 10%	+
Steroids	(Concentrated H_2SO_4 + anhydrous acetic acid)	+
Triterpenoids	(Concentrated H_2SO_4 + anhydrous acetic acid)	+

Note: (+) Contain the tested compound and (-) compound does not contain

Table 2. **Average growth inhibition of *K. pneumoniae, E. aerogenes,* and *S. epidermidis* after application of methanol extract of *Excoecaria cochinchinensis* leaves and ± STDV.**

Sample	The average of inhibition zones (mm) ± standard deviation		
	K. pneumoniae	*E. aerogenes*	*S. epidermidis*
Negative control (DMSO)	0 ± 0	0 ± 0	0 ± 0
EC MeOH [25 mg/mL]	12.67 ± 0.32	12.48 ± 0.62	12.56 ± 0.22
EC MeOH [50 mg/mL]	14.59 ± 0.60	13.51 ± 0.33	14.95 ± 0.51
EC MeOH [75 mg/mL]	15.50 ± 0.32	14.65 ± 0.46	16.88 ± 0.91
EC MeOH [100 mg/mL]	15.67 ± 0.34	15.59 ± 0.47	18.49 ± 0.51
EC MeOH [150 mg/mL]	18.24 ± 0.41	17.61 ± 0.97	19.44 ± 0.30
EC MeOH [200 mg/mL]	22.59 ± 0.20	19.78 ± 0.21	21.78 ± 0.24
EC MeOH [250 mg/mL]	25.28 ± 0.04	20.90 ± 0.42	25.75 ± 0.10
Positive control ciprofloxacin [1 mg/mL]	33.97 ± 0.27	33.50 ± 1.39	-
Positive control clindamycin [0.25 mg/mL]	-	-	24.35 ± 0.48

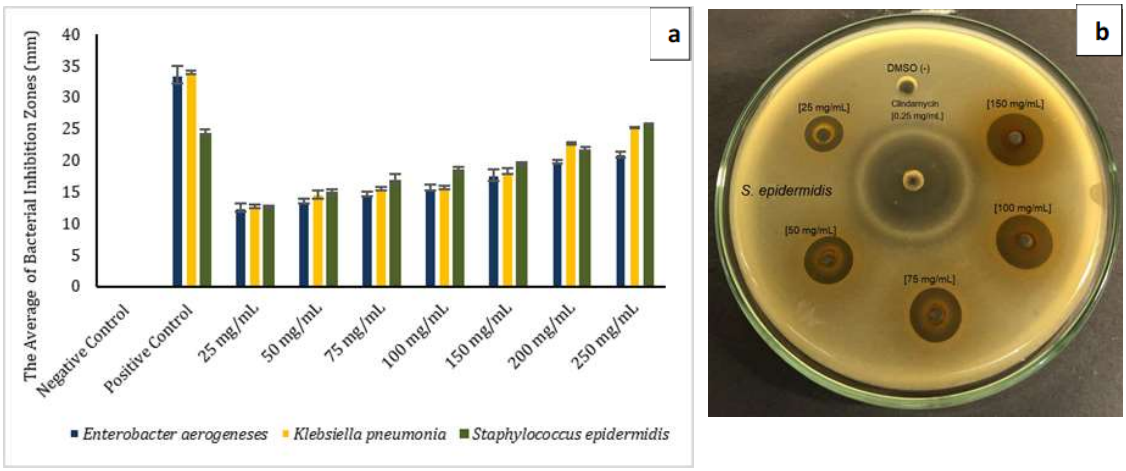

Figure 1. **Inhibition zones of *K. pneumoniae, E. aerogenes,* and *S. epidermidis* growth after treatment at various concentration of *E. cochinchinensis* crude methanol extract, (a). Average inhibition zones, (b). Representative, picture inhibition zone of *S. epidermidis* growth.**

Based on these findings, the bacteria's sensitivity response to the addition of the extract can be described according to Ponce *et al*[9]. The growth of *K. pneumoniae* and *S. epidermidis* were classified as sensitive to the crude methanol extract of *E. cochinchinensis* at concentrations tested of 25 and 50 mg/mL, and very sensitive at concentrations of 75, 100, and 150 mg/mL. While the growth of *K. pneumoniae* and *S. epidermidis* was extremely sensitive to extract concentrations of 200 and 250 mg/mL with inhibition zone diameters of 22.59 mm ± 0.20, 25.28 mm ± 0.04, and 21.78 mm ± 0.24; 25.75 mm ± 0.1, respectively. Meanwhile, the growth of *E. aerogenes* was classified as sensitive when given 25, 50, 75 mg/mL extract, and very sensitive at concentrations of 100, 150, and 200 mg/mL. The growth of *E. aerogenes* was extremely sensitive at a concentration of 250 mg/mL crude methanol extract of *E. cochinchinensis* with a diameter of 20.90 mm ± 0.42. The increase in the extract concentration also led to an increase in the average inhibition zone. This finding was consistent with Gonelimali *et al.* that the higher the concentration of an antibacterial agent, the stronger the antibacterial activity [12]. Furthermore, an antibacterial effectiveness was influenced by the concentration of its substance. Increasing the extract's concentration also increases its active compounds, which function as antibacterial, consequently, its inhibiting power was high. Although the crude methanol extract concentrations were in the range of 25, 100, 200, 300, 400, 600, 800, and 1000 times more than the standard antibiotics in this study, their zone of inhibition demonstrated clear antibacterial activity. This can happen because the active component in the extract consists of many types of compounds, so the concentration of the active component in the extract can be much lower than the standard antibiotics used. There may likewise be an antagonistic effect between some of the components contained in the extract. It was important to note that if the active components were isolated and purified, they would most likely have higher antibacterial activity than the crude methanol extract used in this study.

Secondary metabolites in plants were responsible for their antibacterial activity, and the main groups with antibacterial properties include polyphenols (flavonoids, tannins, coumarins, and quinones), phenolics, alkaloids, terpenoids, lectins, and polypeptides. Meanwhile, their antimicrobial mechanisms of action have been reported, such as disruption of microbial cell membranes by eugenol, and impairment of cell metabolism by cinnamaldehyde. Plant's antimicrobial compounds also inhibit the production of bacterial capsules, such as salicylic acid and its derivatives, as well as the weakening of virulent bacteria by controlling quorum sensing[13].

The methanol extract activity of *E. cochinchinensis* leaves inhibits the growth of *K. pneumoniae, E. aerogenes,* and *S. epidermidis* due to its metabolite content. The isolated compounds from the methanolic extract were oleanolic acid, arjunolic acid, kaempferol, quercetin, isoscutellarein, scopoletin, 3,4,5-trihydroxybenzoic acid, phloroglucinol, β-sitosterol, and daucosterol, while the leaves' water extract contained gallic acid[14]. Anthocyanin contents in *E. cochinchinensis* leaves were cyanidin 3-O-glucoside, cyanidin 3-(2″-galloylglucoside), delphinidin 3-(2″-galloylgalactoside), cyanidin 3-O- rutinoside, petunidin 3-sophoroside, and peonidin 3-gentiobioside[15]. The presence of a new rhamnofolane diterpene glucoside compound, namely excococochinoside and phenolic derivative 2-(β-glucopyranosyloxy)-3-hydroxyphenylpropanoic acid, which have moderate anti-inflammatory activity with an IC_{50} value of 12.02 μM[11].

Several studies showed that oleanolic acid, arjunolic acid, scopoletin, kaempferol, and quercetin compounds have antibacterial activity. Oleanolic acid compounds from the triterpenoid group were reported to have antibacterial activity against pathogenic bacteria in humans, such as *Streptococcus pneumonia,* methicillin-sensitive and methicillin-resistant *Staphylococcus aureus, Bacillus subtilis, B. cereus, Enterococcus faecalis, E. faecium,* and *Pseudomonas aeruginosa* [16]. Their mechanisms of action are related to the induction of the stress response, where *Escherichia coli* treated with oleanolic acid altered the synthesis of DnaK, thereby inducing the heat shock response of the species. The compounds also damage bacterial cell walls by inhibiting peptidoglycan turnover and affecting the number of muropeptides in bacteria[17].

Arjunolic acid belonging to the triterpenoid-saponin group was also reported to have antibacterial activity against *Escherichia coli* and *Shigella sonnei.* It also showed inhibitory activity against *Cryptococcus neoformans* with an IC_{50} value of 20 mg/mL. The antibacterial ability of arjunolic acid was due to the hydroxyl group's influence at C-23[18,19].

Several studies have reported that scopoletin inhibits the growth of bacteria, including *Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus cereus,* and *Escherichia coli*. Scopoletin isolated from *Lasianthus lucidus* Blume (Rubiaceae) also inhibits the growth of *Pseudomonas aeroginosa* with a MIC of 0.66 μg/mL. Its mechanism of action was indicated by the differences in the bacteria's cell walls after adding the test substance. The cell walls become swollen, the length of the cells changes, and leakage occurs, which leads to bacterial lysis. This mechanism was similar to that of β-lactam antibiotics, which inhibit penicillin-binding proteins that cause cell wall deformation, such as elongation, lesion, and lysis[20].

Kaempferol and quercetin have been tested for their activity against various types of bacteria, such as *Staphylococcus aureus, Escherichia coli,* and *Pseudomonas aeruginosa,* where they showed little or moderate inhibition. Flavonoids' antibacterial mechanisms involve the inhibition of nucleic acid synthesis, cytoplasmic membrane function, energy metabolism, attachment, and biofilm formation, as well as the inhibition of porins in cell membranes, changes in membrane permeability, and attenuation of pathogenicity. The antibacterial activity was also highly dependent on their structure, namely the substituents on the aromatic ring. The greater the extract's antibacterial activity, the more flavonoids proved to be antibacterial agents, specifically flavonoids with hydrophobic substituents such as the prenyl group[21].

Combination Effects of Methanol Extract of Sambang Darah Leaves (*Excoecaria cochinchinensis*) with Antibiotics. The interaction properties of the extract's combination with antibiotics were observed using paper strip diffusion[10]. This technique was used to determine antibacterial activity by observing patterns formed in each substance with paper strips placed on the media at a 90 degree angle. Their combination produced synergistic, antagonistic, or indifferent effects[22]. Figure 2a showed the effect of combining 250 mg/mL extract with 1 mg/mL ciprofloxacin on the growth of *K. pneumoniae*. Meanwhile, Figure 2b showed the result for the combination of 250 mg/mL extract with 1mg/mL ciprofloxacin against *E. aerogenes.* Figure 2c showed the effect of 250 mg/mL extract with clindamycin 0.25 mg/mL on *S. epidermidis*. The results revealed that the extract, 1 mg/mL ciprofloxacin, and 0.25 mg/mL clindamycin inhibited the growth of the test bacteria with the formation of a clear zone around the paper tape. However, the inhibition zone obtained from the mixture gave an indifferent effect with the formation of a clear zone area joined at the corner of the paper tape, but no widening or

narrowing was formed around the corner. The indifferent effect detected in this study was not specific to any group of organisms or class of antibiotics. Further research was needed on extract combinations with other types of antibiotics and the test concentration used was the same as the MIC for each component.

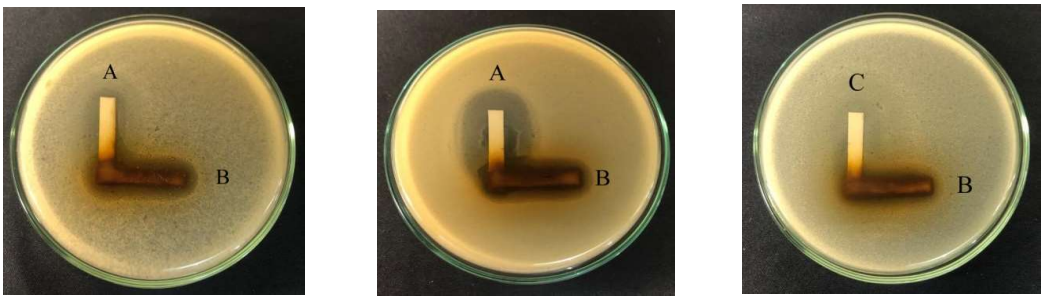

Figure 2. **The combined effect of methanol extract from 250 mg/mL of *Excoecaria cochinchinensis* leaves with ciprofloxacin 1 mg/mL and clindamycin 0.25 mg/mL using the paper tape method on the test bacteria (a) *K. pneumoniae* (b) *E. aerogenes* (c) *S. epidermidis* (Note: A = ciprofloxacin, B = methanol extract of *Excoecaria cochinchinensis* leaves, C = clindamycin).**

CONCLUSION

The administration of *Excoecaria cochinchinensis* L. leaves' methanolic extract inhibited successfully the growth of *K. pneumoniae, E. aerogenes,* and *S. epidermidis* bacteria. Infection caused by *K. pneumoniae* was difficult to treat, on the other hand we need powerful agents to combat serious nosocomial infections caused by *E. aerogenes*, and their susceptibility to *E. cochinchinensis* was a pointer to the *E. cochinchinensis* extract potential as a drug against these bacteria and support their use in traditional medicine. Furthermore, concurrent testing of the extract's combination with antibiotics had no difference compared to their individual activities. The combination was indifferent in inhibiting the growth of *K. pneumoniae, E. aerogenes,* and *S. epidermidis* bacteria.

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