The Synergistic Antibacterial Effect of *Azadirachta indica* Leaves Extract and *Aloe barbadensis* Gel Against Bacteria Associated with Skin Infection

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Abstract Combinations of two agents which create inhibitory effects that are greater than the individual effects produce a positive interaction known as synergism. This method can help in developing agents for antibacterial activity in order to treat bacterial infection. This study was done to assess the possible synergistic antibacterial effect of the combination between *Azadirachta indica* leaves extracts and *Aloe barbadensis* gel against five bacteria commonly associated with skin infection. Synergistic antibacterial activities from the interaction of both plants against *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella aerogenes* and *Streptococcus faecalis* were measured by using Kirby–Bauer disc diffusion assay. *A. indica* ethanolic extract, *A. barbadensis* gel, combination of *A. indica* ethanolic extract and *A. barbadensis* gel, and commercialize antibiotics were tested on the five bacteria. The data were subjected to statistical analysis of one-way ANOVA and Tukey's post hoc tests ($\alpha = 0.05$). The results of this study showed significant inhibition of the bacteria from the synergistic effect of *A. indica* and *A. barbadensis* compared to the commercial antibiotic. The significant results may contribute to the development of stronger antibacterial agent in skin infection treatment in the pharmaceutical industry.

Keywords *Azadirachta indica* · *Aloe barbadensis* · Synergistic antibacterial activity · Kirby–Bauer disc diffusion assay versus skin infection bacteria

1 Introduction

Many types of plants have been used in treating diseases since the early ages, for example in the Ayurvedic medicines. Each part of different plants has been collected and studied to show the antibacterial effect, and the number of plants already

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introduced to the world is estimated to be about 250,000–500,000 species (Das et al. 2014). The amount of plants that are used for medicinal purposes are less than the amount of plants that are used as food by human and animals (Das et al. 2014).

As the production of medicine is increasing and developing rapidly, the emergence of disease has also grown. One of the common diseases that is associated with human is skin disease. Skin diseases are mostly caused by antibacterial infection. For example, cellulitis is caused by β-haemolytic *Streptococci* and *Staphylococcus aureus* (Gunderson 2011). Skin infections such as impetigo, folliculitis, furunculosis, cellulitis, abscesses are caused by methicillin-resistant *S. aureus* (Ullah et al. 2016). Thus, many types of research have been done in producing antibacterial agent that can kill bacteria that cause these skin diseases.

Synergism is one of the best methods where the highly potential antibacterial agents are combined to create new and strong antibacterial activities that produce an effect greater than the sum of their individual effects. The combined effect of bacteriocin and extracts of neem leaf is being tested at present that will assist to make a formulation for microbial infection on skin (Das et al. 2014).

In this study, two types of plants, *Azadirachta indica* and *Aloe barbadensis,* were tested to evaluate the synergistic antibacterial activity potential in order to overcome skin infection caused by bacteria. *A. indica* or commonly known as neem is the native tree from India and naturalized in most of tropical and subtropical countries such as Malaysia, Indonesia and Thailand (Juss et al. 2013). Neem tree can be found in at least 30 countries in Asia, Africa, Australia as well as Central and South Americas (Patel et al. 2016). Parts of the neem tree have been used as part of traditional medicine in various locations around the world, and the antimicrobial properties of their extract and compounds have been studied widely in pharmacological aspects (Quelemes et al. 2015). One of the compounds in the neem is *Azadirachtin*, which consists of antiviral, antifungal, antibacterial and insecticidal properties (Kashyap 2014). Extracts of the neem leaf have been found to possess immunomodulatory, anti-inflammatory, and anticarcinogenic properties (Elumalai et al. 2012). Based on recent study, neem has been the object of extensive phytochemical studies, due to its strong biological effect including antibacterial activity (Sujarwo et al. 2016). The International Scientific Community has included *A. indica* as the top ten of lists of plants to be studied and used for sustainable development of the planet and the health of living beings (Kashyap 2014). Thus, *A. barbadensis* or commonly known as *Aloe vera* is one of the ancient medicinal ailments for human being. *Aloe vera* has been used in folk medicine for over 2000 years and has remained an important component in the traditional medicine of many contemporary cultures, such as China, India, the West Indies and Japan (Radha and Laxmipriya 2015). *Aloe vera* is stem less or sometimes may be a very short-stemmed succulent plant growing up to 60–100 cm tall and has thick, fleshy green leaves with some varieties showing white flecks on the upper and lower stem surfaces (Irshad et al. 2011). It is a perennial succulent xerophyte, which develops water storage tissue in the leaves to allow it to survive

in dry areas of low or erratic rainfall, and the innermost part of the leaf has a clear, soft, moist and slippery tissue that consists of large thin-walled parenchyma cells in which water is held in the form of vicious mucilage (Nejatzadeh-barandozi 2013). In recent studies, *Aloe vera* is recommended for treating all types of skin diseases (Bhat et al. 2014).

The main point of this study is to introduce new potential antibacterial source to treat skin infection by synergistic effect of two different plants without chemical interference. The usage of herbs in treating skin infection has been practised a long time ago, but based on past studies there is no specific documentation on the traditional treatment methods to cure skin diseases (Bhat et al. 2014). Nowadays, advancement of medicinal studies has increased the chances to reduce skin infection. This is only possible if various and repetitive research and development is being done.

2 Materials and Methods

2.1 Collection of Plant Materials

The leaves of *A. indica* were collected in the month of June 2016, and the leaves of *A. barbadensis* were collected in the month of August 2016 from the tree growing wildly in Bintong, Perlis, Malaysia.

2.2 Extraction of Ethanolic Compound from Azadirachta Indica *Leaves*

The ethanolic compounds were extracted according to the method used by (Abdussalam 2011). Initially, the fresh leaves were allowed to dry under shade for 14 days and ground into powder using a grinder. The powdered material was weighed using electronic weighing balance, and drying of the leaves was continued until a constant weight was obtained. An amount of two hundred and fifty grams of the powder was placed in a container and was defatted using petroleum ether, following which it was subjected to maceration using 300 ml of 95% (v/v) ethanol in order to obtain the ethanolic extract of the plant. The mixture was stirred up and kept for 24 h. The mixture was filtered, and another 300 ml of the ethanol was added to the residue and kept for another 24 h before filtration. This procedure was repeated 3 times, and the combined filtrate was subjected to rotary evaporator to obtain the crude extract.

2.3 *Extraction of* Aloe barbadensis *Gel*

The outermost part of *A. barbadensis* leaf was peeled off, and the inner part of the leaf was left in the form of vicious mucilage. The gel from the skinless leaf was stripped out, and about 5 ml of gel was obtained from single 25 cm length of *A. barbadensis* leaf.

2.4 *Test Organisms*

The micro-organisms used were *Escherichia coli* (ATCC 11303), *S. aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 10145), *Klebsiella aerogenes* (ATCC 15380) and *Streptococcus faecalis* (ATCC 29212) and were obtained from Microbiology Laboratory 5 UiTM Perlis, Arau, Malaysia, to represent skin infection bacteria.

2.5 *Sterilization of the Equipment and Disinfection*

All the equipment was disinfected with cotton wool soaked in 70% ethanol so as to maintain sterility throughout the process. Wire loop, conical flask and beaker were sterilized by hot air oven at 160 °C for 45 min, whereas moisture-insensitive materials were sterilized by autoclaving at 121 °C for 15 min (Abdussalam 2011).

2.6 *Preparation of Media*

The Mueller–Hilton agar (MHA) consisted of (gm/litre) agar 17.0 g, beef extract 2.0 g, starch 1.50 g and acid hydrolysate of casein 17.50 g. An amount of 35 g of Mueller–Hilton agar was weighed and dissolved in 1000 ml of distilled water and adjusted to pH of 7.4 ± 0.2 at 25 °C. This was sterilized by autoclaving at 121 °C for 15 min at 15 psi pressure and was used for Kirby–Bauer disc diffusion tests.

2.7 *Antibacterial Activity Assay of the Plant Extracts*

Kirby–Bauer disc diffusion assay was carried out to get the zone of inhibition that showed the antimicrobial activity of *A. indica* leaves extract, *A. barbadensis* gel, and the combination *A. indica* leaves extract and *A. barbadensis* gel towards the five microbes: *E. coli* (ATCC 11303), *S. aureus* (ATCC 25923), *P. aeruginosa*

(ATCC 10145), *K. aerogenes* (ATCC 15380) and *S. faecalis* (ATCC 29212). All the bacteria were subcultured into McCartney bottle that contains nutrient broth before being spread on the Mueller–Hilton agar. First, the agar was removed from the refrigerator, placed in incubator and let it to come down to the room temperature. In the meantime, the laboratory bench was first disinfected with 70% ethanol and the Bunsen burner was lighted up to keep up with a sterile environment. Once the agar was warmed up, the agar was removed from the incubator for culturing. Forty-five blank paper discs were used in the antibacterial assay. Fifteen blank paper discs were soaked in crude extract of *A. indica* leaves, next fifteen blank paper discs were soaked in *A. barbadensis* gel, and another fifteen blank paper discs were soaked in the mixture of crude extract of *A. indica* leaves and *A. barbadensis* gel. Six discs of 10 μg ampicillin and nine disc of 20 μg gentamicin were used as the positive control, and five blank paper discs were soaked in ethanol as negative control. About 0.2 ml of the bacterium broth culture was transferred onto the Mueller–Hilton agar medium, aseptically. Then, the broth culture of *E. coli, S. aureus and P. aeruginosa*, *K. aerogenes* and *S. faecalis* was spread with L-shaped glass spreader. For precaution, the L-shaped glass spreader was dipped into ethanol first before being used in the next spreading. Then, in order to determine their inhibitory antibacterial effect, the soaked paper discs and positive control and negative control discs were placed onto the Mueller–Hilton agar surface. Each disc was slightly pressed down into the agar medium to ensure the complete contact with the Mueller–Hilton agar. All plates were inverted and incubated at 37 °C for 24 h. The diameter of the clear zones of inhibition of the test organisms in response to the crude leaves extract of *A. indica*, *A. barbadensis* gel, mixture of the crude leaves extract of *A. indica* and *A. barbadensis* gel, gentamicin, ampicillin, and ethanol was measured in millimetres.

2.8 Statistical Analysis

Data were statistically analysed using IBM SPSS statistics version 23. A one-way analysis of variance (ANOVA) followed by Tukey's post hoc test was applied for analysis of data with the level of significance set at $p < 0.05$.

3 Results and Discussion

3.1 Results

These experiments using Kirby–Bauer disc diffusion method have shown significant results where the zone of inhibition of the five tested bacteria for the mixture of the crude leaves extract of *A. indica* and *A. barbadensis* gel was obviously larger than

the zone of inhibition by the commercialize antibiotic, the crude leaves extract of *A. indica* and *A. barbadensis* gel that were tested individually. All the five bacteria tested were susceptible towards the commercial antibiotic, the crude leaves extract of *A. indica* and the mixture of the crude leaves extract of *A. indica* and *A. barbadensis* gel (Table 1 and Fig. 1). The comparison of the antibacterial combination effect for the mixture of the crude leaves extract of *A. indica* and *A. barbadensis* gel was clarified in Fig. 1.

Table 1 Inhibitory effect of antibacterial sample on five tested bacteria

	Zone of inhibition (mm)					
	Antibacterial sample					
Bacteria	Neem extract	Aloe vera gel	Aloe vera gel and Neem extract	20 µg gentamicin	10 µg ampicillin	70% ethanol
Escherichia coli	192.82	132.16	322.16	192.54	Not tested	0
Pseudomonas aeruginosa	164.32	9,331.69	272.16	20,33.51	Not tested	0
Staphylococcus aureus	18.7	10,331.25	220.82	18,31.74	Not tested	0
Klebsiella aerogenes	18,662.36	100.82	220.82	Not tested	240	0
Streptococcus faecalis	19,670.47	10,330.47	22,332.06	Not tested	18	0

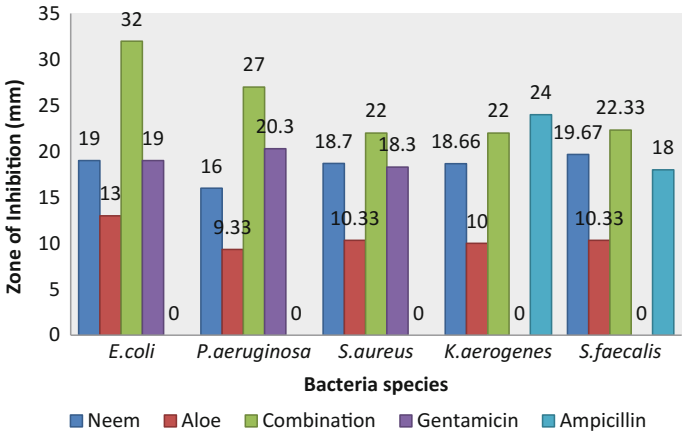

Fig. 1 Comparison of zone of inhibition of five skin infection bacteria from the inhibitory effect of neem extract, aloe vera gel, combination of neem extract and aloe vera gel, and commercialized antibiotics

3.2 Discussion

Plants are the larger source of potentially useful compound for the development of new antibacterial agent which can be tested in vitro with an antibacterial activity assay. The idea of finding synergistic antibacterial activity effect of two different plants is initiated by the need of a new type of antibacterial agent that can overcome skin infection caused by bacteria. Most of the commercial antibiotic is already ineffective against the bacteria, and there are chemical influence from the current antibiotic agent (Cock 2015). The increasing of frequency in use of antibiotics for treatment of humans and animals has developed the antibiotic resistance and multidrug resistance micro-organisms (Prasannabalaji et al. 2012). In this research, *A. indica* and *A. barbadensis* were chosen in determining the synergistic antibacterial activity against five skin infection bacteria. The results of this experiment are not compared with any other research since there is lack of research focusing on combining these two plants. Both of these plants are well known in treating skin infection. From the recent study, *Aloe vera* has been recommended for treating various kinds of skin disease (Bhat et al. 2014). The synergistic effects of both plants were measured by using disc diffusion assay on five different types of bacteria that are related to skin infection. The antibacterial activities were measured by the diameter of zone of inhibition and the larger the diameters of the zone of inhibition represent the stronger antibacterial activity. The result showed that the combination of *A. indica* leaves extract and *A. barbadensis* gel produced larger zone of inhibition on the tested bacteria compared to other individual extracts (Table 1). The largest average zone of inhibition for the combination of *A. indica* leaves extract and *A. barbadensis* gel was about 32 mm on *E. coli* which was susceptible, and the antibacterial activity on other bacteria has shown positive results. Commercial antibiotic has been used to compare with the samples which are gentamicin and ampicillin. Specific antibiotic was placed on different bacteria where the gentamicin is used on *E. coli, P. aeruginosa* and *S. aureus,* while ampicillin was used on *Klebsiella aerogene* and *S. faecalis.* Different antibiotics are designated to different bacteria in order to get optimum zone of inhibition since in past study has shown the effect of these antibiotics on the bacteria. There were large differences in the diameter of zone of inhibition for the combination of *A. indica* leaves extract and *A. barbadensis* gel compared to *A. indica* leaves extract, *A. barbadensis* gel and the Commercial antibiotic tested individually towards the five pathogenic bacteria (Fig. 1). The results of the synergistic antibacterial activity can be seen more after being analysed using ANOVA which showed that the significant value is positive since the value did not exceed $\alpha = 0.05$ and it was proceeded with Tukey's post hoc test that produced same results as shown in Table 2. The influence of diffusion of the bioactive compound from the extract into the media could be responsible for the results. The results of this finding are aligned with several

Table 2 Multiple comparison

Dependent Variable	(I) Antibacterial agent	(J) Antibacterial agent	Mean difference (I–J)	Std. error	Sig.	95% confidence interval	
						Lower bound	Upper bound
Escherichia coli	Aloe vera	Neem	−6	2.4037	0.103	−13.3752	1.3752
		Aloe neem	−19.00000*	2.4037	0.001	−26.3752	−11.6248
	Neem	Aloe vera	6	2.4037	0.103	−1.3752	13.3752
		Aloe neem	−13.00000*	2.4037	0.004	−20.3752	−5.6248
	Aloe neem	Aloe vera	19.00000*	2.4037	0.001	11.6248	26.3752
		Neem	13.00000*	2.4037	0.004	5.6248	20.3752
P. aeruginosa	Aloe vera	Neem	−6.66667	2.95647	0.14	−15.7379	2.4046
		Aloe neem	−17.66667*	2.95647	0.002	−26.7379	−8.5954
	Neem	Aloe vera	6.66667	2.95647	0.14	−2.4046	15.7379
		Aloe neem	−11.00000*	2.95647	0.023	−20.0713	−1.9287
	Aloe neem	Aloe vera	17.66667*	2.95647	0.002	8.5954	26.7379
		Neem	11.00000*	2.95647	0.023	1.9287	20.0713
Staphylococcus aureus	Aloe vera	Neem	−8.33333*	2.09054	0.017	−14.7477	−1.919
		Aloe neem	−11.66667*	2.09054	0.003	−18.081	−5.2523
	Neem	Aloe vera	8.33333*	2.09054	0.017	1.919	14.7477
		Aloe neem	−3.33333	2.09054	0.318	−9.7477	3.081
	Aloe neem	Aloe vera	11.66667*	2.09054	0.003	5.2523	18.081
		Neem	3.33333	2.09054	0.318	−3.081	9.7477
K. aerogene	Aloe vera	Neem	−8.66667*	1.51535	0.003	−13.3162	−4.0171
		Aloe neem	−12.00000*	1.51535	0.001	−16.6495	−7.3505
	Neem	Aloe vera	8.66667*	1.51535	0.003	4.0171	13.3162
		Aloe neem	−3.33333	1.51535	0.15	−7.9829	1.3162
	Aloe neem	Aloe vera	12.00000*	1.51535	0.001	7.3505	16.6495

(continued)

Table 2 (continued)

Dependent Variable	(I) Antibacterial agent	(J) Antibacterial agent	Mean difference (I–J)	Std. error	Sig.	95% confidence interval	
						Lower bound	Upper bound
		Neem	3.33333	1.51535	0.15	−1.3162	7.9829
S. faecalis	Aloe vera	Neem	−9.33333*	1.24722	0.001	−13.1601	−5.5065
		Aloe neem	−12.00000*	1.24722	0	−15.8268	−8.1732
	Neem	Aloe vera	9.33333*	1.24722	0.001	5.5065	13.1601
		Aloe neem	−2.66667	1.24722	0.162	−6.4935	1.1601
	Aloe neem	Aloe vera	12.00000*	1.24722	0	8.1732	15.8268
		Neem	2.66667	1.24722	0.162	−1.1601	6.4935

literature (Abdussalam 2011; Khan et al. 2010; Reynolds and Dweck 1999) which found that the plants possess significant antimicrobial activities against several pathogens. From the results, the finding of the synergistic antibacterial activity of *A. indica* and *A. barbadensis* looks promising to become potential antibacterial agent in treating skin infection caused by this pathogen.

4 Conclusion

From this study, it can be concluded that there is high potential of synergistic antibacterial activity from the combination of *A. indica* leaves extract and *A. barbadensis* gel against *E. coli, S. aureus, P. aeruginosa*, *K. aerogene* and *S. faecalis*. The tested bacteria *E. coli* were more susceptible to the combination of *A. indica* leaves extract and *A. barbadensis* gel. Furthermore, the inhibitory effect of the synergistic plants extract outperformed the inhibitory effect of the positive control of the 20 μg gentamicin disc, a commercialized antibiotic on *E. coli*. Thus, the use of synergistic antibacterial activity from the combination of *A. indica* leaves extract and *A. barbadensis* gel seems promising to be a potential antibacterial agent in treating skin infection caused by these bacteria. As this study was preliminary, further study is needed to be done by including minimum inhibitory concentration and using other parts of these plants and tested on clinical isolates of bacteria and resistant strains.

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