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Research Article

Antibacterial Effect of Honey and *Chromolaena odorata* on *Escherichia coli* and *Staphylococcus aureus* Isolated from Wound Infection

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Abstract

Chromolaena odorata and honey were used against *Staphylococcus aureus* and *Escherichia coli* obtained from wounds to determine the antibacterial effects of both agents against the test isolates. The study was done in the Project Laboratory of Chukwuemeka Odumegwu Ojukwu University, Uli. The isolates were obtained from wounds of the pupils of Community Primary School, Umuoma, Uli and characterized using microbiological and biochemical tests. The leaves of *C. odorata* were aseptically air-dried, ground and prepared in aqueous and ethanoic solutions. Crude extract was used. Fresh honey obtained from Ogidi market, Idemili North was prepared in different concentrations. Agar well diffusion and Minimum inhibitory concentration (MIC) was used to determine the therapeutic activity. The result revealed that honey exhibited antibacterial activities against the test organisms and zones of inhibition were obtained showing high antibacterial activity with significant increase in the concentration of the honey. Different solvents such as ethanol and cold water were used for the extraction of *C. odorata* leaves. Crude undiluted extract was also used against the test organisms. Ethanolic extract showed to be more effective than crude and cold water extracts. The MIC of ethanolic extract against *S. aureus* and *E. coli* were 62.5 mg/ml and 125 mg/ml respectively. The MIC of the *C. odorata* ethanoic extract and zone of inhibition obtained from different concentrations of honey and different extracts of *C. odorata* compares favorably with the commercially available Augmentin which justifies the therapeutic use of these agents in the medical field for treatment of ulcers, bed sores and other infections resulting from burns and wounds.

Keywords: *Chromolaena odorata; Staphylococcus aureus; Escherichia coli*; Honey; Ethanol and Cold Water

Introduction

Infections and other health related problems have been of great concern to human beings and chemotherapy is the main approach in the treatment of such conditions. Investigation into the microbial flora of wound began in the late 19th century and since the improvements in techniques have facilitated the recovery, identification and enumeration of a wide variety of microbial species [1].

Major challenges encountered with antibiotics in clinical use are resistance to antibiotics which leads eventually to failure of the Infectious diseases are known to be treated with herbal remedies/ treatment [11]. Throughout the history of mankind, even till date, natural substances continue to play a major role in primary health care as therapeutic remedies in many developing countries [2]. Over the years, there have been reports of the production of more

potent antibiotics e.g. third and fourth generation of cephalosporin by pharmaceutical companies which are not readily available and expensive. Problems of various antibiotics include low efficacy and side effects, this has led investigations into natural and potent antibacterials, and seems to be the the right step to take. There is increasing interest in the use of topical antimicrobial to wound cure. Compounds such as honey, iodine and silver have been incorporated into dressing are simply the addition of an antimicrobial to a pre-existing products [3].

Honey a sweet substance produced by bees is one of the oldest traditional medicine considered to be important in the treatment of several human ailments [3]. More recently, honey has been reported to have an inhibitory effect to around 60 species of bacterial including aerobes and anaerobes, Gram positive and Gram negative. The current prevalence of therapeutic use of ancient remedies, include honey committee on science and technology. Indeed, the medicinal importance of honey has been documented in the world oldest medical literatures, and since the ancient times it has been known to promote antimicrobial property as well as wound healing activity [3]. The healing property of honey is due to the fact that it offers antibacterial activity, maintains a moist wound condition and its high viscosity helps to provide a protective barrier and prevent infection. The antimicrobial activity in most honey is due to the enzymatic production of hydrogen peroxide.

Chromolaena odorata commonly referred to as *kirinyuh* is a weed from *Asteraceae*. It is an indigenous plant widely used for folk medicinal purposes [4]. Many rural people still depend on this plant products and herbal remedies for treating aliments. Being naturally gifted by a suitable tropical climate and fertile soil, Nigeria possess a rich flora of this tropical plants. They are seen to grow in forests, jungles, wasteland and roadsides as indigenous, naturalized and cultivated plants. They are used by indigenous people to treat wound infections and to stop bleeding. They are active and effective against many kinds of microorganisms. They possess some anti-gonorrheal, anti-inflammatory, anti helmintic, analgesic, antioxidant and antifungal activities.

Materials and Methods

Study area

The study was conducted in Community Primary School, Umuoma, Uli in Ihiala L.G.A, of Anambra State.

Sample collection

Sterile swab sticks were used to swab and obtain the organisms used in this work from six wounded pupils of Community Primary School, Uli.

Sterilization of glassware

The glass wares used were sterilized using hot air oven. The glass wares were washed with detergent and rinsed with clean water. These were air-dried and placed inverted inside the oven and set the thermostat at 160ºC for 3 hours.

Preparation of media

The media used for the study included: MacConkey agar, Mannitol salt agar (MSA) and Nutrient agar. The media were prepared according to the manufacturer's instruction and sterilized by autoclaving at 121ºC, 15psi for 15 minutes.

Isolation of organisms

The swabs were inoculated in peptone water to reactivate the organisms at 37ºC for 18 hours. After which it was re-inoculated into Nutrient agar and MacConkey agar using pour plate method and incubated at 37ºC for 24 hours. After 24 hours, the grown colonies were subcultured on fresh agar plates, characterized and identified using their colony descriptions, morphological and biochemical characteristics [5].

Characterization and identification of isolates

The isolates were sub-cultured on nutrient agar and incubated inverted at 37ºC for 24 h. the isolates were characterized and identified using their colonial and morphological description [6] and biochemical reactions. The colonial description was carried out to determine appearance of the isolates on agar media plates, their sizes, edges, consistencies and optical properties of the isolate.

Morphological characteristics

The following morphological characteristics were carried out.

Gram staining

This was carried out using the method of [5]. In this process, a thin smear was made on marked area of a grease free-clean slide, air dried and heat fixed for 3 - 4 times. The smear was covered with 2% crystal violet for 1 min and rinsed with water. It was then covered with Gram's iodine and allowed to act for I min before

rinsed with water and drained off. Acetone was used to decolorize the smear for 2 - 3 seconds and rinsed with water. The smear was counter stained with 1% safranin for 30 seconds, rinsed and air dried. A drop of immersion oil was added to the stained smear and observed under oil-immersion lens of microscope. Positive result showed dark purple while negative result showed light red color.

Motility test

This was carried out using the method described by [7]. The medium used was semi – solid agar. It was prepared by mixing 17.0 g of bacteriological agar with 20.0 g of nutrient broth in 1 liter of distilled water. The agar was dissolved by heating for 30 seconds and 10.0 ml amount was dispensed into test tubes and sterilized by autoclaving. The test tubes were allowed to set in a vertical position. Inoculation was done by making a single stab down the center of the test tubes to half the depth of the medium using a standard stabbing needle. The test tubes were incubated at 37°C for 24 h. positive result showed motile bacteria swarm and gave diffused spreading growth that was visible to the naked eyes. Negative result showed stagnancy of the bacteria.

Biochemical reactions

Catalase test

This was carried out as described in the manual of microbiology of kanika (2011). A smear of the isolate was made on a clean grease-free microscopic slide, then a drop 30% hydrogen peroxide was added on the smear. Prompt effervescence indicated catalase production.

Oxidase test

This was carried out using the method of [5]. This was done to find out if the isolate is able to produce oxidase enzyme. A small colony of the isolate was placed on a clean Whatman filter paper and 2 drops of oxidase reagent was added on it and allowed to stay for 5 seconds. Purple color indicate positive result while no color indicate negative result.

Indole test

This was carried out using the method described by [7]. Indole is a nitrogen containing compound formed when the amino acids tryptophan is hydrolyzed by bacteria that have enzyme tryptophanase. This is detected by using kovac's reagent. For this test, isolates were grown in peptone water in 500 ml of the deionized water. 10 ml of peptone water was dispensed into the test tubes. The media was then inoculated with the isolates and incubated at 37°C for 24h. 5 drops of kovac's reagents were carefully layered on 24h old pure culture. The presence of indole was revealed by the formation of red layer coloration on the top broth culture.

Methyl red test

This was carried out as described in the Manual of Microbiology by [9]. The glucose phosphate broth was prepared according the manufacturer's direction and the isolate were aseptically inoculated into the sterilized medium. This was incubated at 37°C for 48h. After incubation, 5 drops of 0.4% solution of alcoholic methyl red solution was added and mixed thoroughly and the result was read immediately. Positive test gave bright red color while negative test gave yellow color.

Voges proskauer test

his was carried out as described in the manual of microbiology by [9]. The glucose phosphate broth was prepared according the manufacturer's direction and the isolate were aseptically inoculated into the sterilized medium. This was incubated at 37°C for 48h. After incubation, 1.0 ml of 40% potassium hydroxide containing 0.3% creatine and 3 ml of 5% solution of α-naphthol was added in the absolute alcohol. Positive reaction was observed by the development of pink color with 5 minutes.

Urease test

This was carried out using the method of [5]. This was done to detect the ability of an organism to produce urease enzyme which convert urea to ammonia and carbon dioxide. This was done by inoculating the test organisms on the entire of Christensen's medium and incubated at 37°C, examined after 4 h and after overnight incubation. Positive result showed purple pink color, negative result retains the original color if the medium.

Nitrate reduction test

This was carried out using the method described by [5]. It was done to detect the production of the enzyme nitrate reductase which reduces nitrate to nitrite of free nitrogen gas. The test organism was inoculated into a 5ml of nitrate broth and incubated at 37°C for 96 h. 0.1 ml of test reagent was added. Positive test showed red coloration, negative result showed yellow coloration.

Sugar fermentation test

This was carried out using the method described by [14]. This test was carried out to know the ability of the isolates to metabolize some sugars (xylitol, glucose, maltose, lactose, sucrose, mannitol, galactose, and sorbitol) with the resultant production of acid and gas or either. One liter of 1% (w/v) peptone water was added to 3 ml of 0.2% (w/v) bromothymol blue and 9 ml was dispensed in the test tube that contained inverted Durham tubes. The medium was then sterilized by autoclaving. The sugar solution was prepared at 10% (w/v) and sterilized. 1 ml of the sugar was dispensed aseptically into the test tubes. The medium was then inoculated with the appropriate isolates and the cultures incubated at 37˚C for 48 hours and were examined for the production of acid and gas. A change in color from purple to yellow indicated acid production while gas production was assessed by the presence of bubbles in the inverted Durham tubes.

Catalase test

This was carried out using the method described in the Manual of Microbiology by [8]. The chemical used was hydrogen peroxide. A small bacterial colony was put on the surface of clean, dried glass slide then 30% hydrogen peroxide was added and mixed thoroughly. Positive test was indicated by evolution of oxygen by bubbling while negative test showed no bubbles.

Hydrogen sulfide (H_2S) production test

This was carried out using the method described by [10]. This was performed using triple sugar iron (TSI) agar. The TSI agar was prepared according to the manufacturer's instruction. This was sterilized using autoclave and allowed to cool to 45˚C. The isolates were aseptically inoculated by stabbing vertically on the medium and streaked on the top and incubated at 37˚C for 24 - 48 hours. The presence of darkened coloration was positive for hydrogen sulfide production.

Coagulase test

This was carried out using the method described by [7]. A loop full of the test isolate is smeared on a slide, mixed with normal saline and treated with a drop of serum which is then mixed together. Agglutination or clumping occurred within 5 – 10 second which showed positive result.

Source of honey

Honey used was obtained from local commercial producers in Ogidi in idemili North L.G.A of Anambra State. It did not contain any diluent or additive and had not been heated.

Source of *C. odorata* leaves

C. odorata leaves used for this study was obtained by pruning them from the bushes surrounding Deo gratis lodge, Uli.

Preparation of test organisms

This was carried out using the method of [13]. Broth culture of the isolates was centrifuged at 3000 r.p.m for 10 minutes and the supernatant was decanted. The sediment was washed twice with sterile phosphate buffer saline (PBS) and re-suspended in normal saline (0.85%) and adjusted to 10^8CFU/ml using 0.5 McFarland matching Standard which is (0.6 ml of 1% $BaCl_2.2H_20$ + 99.4 ml of 1% concentration of H_2SO_4).

Preparation of honey

The honey used were diluted with distilled water to obtain different concentrations 20%, 40%, 60%, 80% and the net honey was used (100%).

Preparation of the *C. odorata* leaves

The plant material was collected and washed to reduce microbial load to a large extent. They were further dried at room temperature for 14 days to remove water from the leaves sufficiently. After drying, it was crushed using a kitchen blender to powder and weighed.

Ethanoic extract preparation

10g of pulverized powdered leaves of plant material were weighed using an electronic weighing balance and macerated by soaking it in 250 ml conical flask containing 100 ml of absolute ethanol. The mixture was vigorously shaken manually at intervals. After 72 hours with interval shaking, the mixture was filtered using a clean Whatman filter paper into a clean 100 ml beaker and the filtrate was concentrated to semi-solid in open air.

Aqueous (cold water) extract preparation

10g of pulverized powdered leaves of plant material were weighed using an electronic weighing balance and macerated by soaking it in 250 ml conical flask containing 200 ml of distilled water. The mixture was vigorously shaken manually at intervals. After 72 hours with interval shaking, the mixture was filtered using a clean filter cloth into a clean 100 ml beaker and the filtrate was concentrated to semi-solid using a steam bath.

Crude extract preparation

A large quantity of fresh leaves of *C. odorata* was ground in a mortar and pestle and 30 ml of undiluted extract was obtained. Means of pressure was applied by squeezing the ground leaves with a clean cloth to obtain extract.

Antibacterial sensitivity test

Honey

The antibacterial activity of honey was tested in-vitro using agar well diffusion method. The test material was prepared by diluting honey in sterilized distilled water at different dilutions 20%, 40%, 60% and 80% also net honey 100%. The test organisms were inoculated using pour plate method. Wells were made using a sterile cork borer of 5.5 mm and each well was filled with 0.1 ml different concentration of the honey. The plates were incubated at 37ºC for 24 h and observed for zone of inhibitions. The in-vitro experiment was compared with the use of a sensitivity disk (Augmentin) which served as a control.

C. odorata

500mg/ml was prepared by dissolving 1g of the ethanoic extract and 1g of the aqueous extract in 2 mls of water each in 250ml beaker. The test organisms were inoculated using pour plate method. Wells were made using a sterile cork borer of 5.5 mm each well were filled with 0.1ml of the aqueous extract and ethanoic extract and incubated at 37ºC for 24h and observed for zone of inhibitions. Crude extract was not dissolved with any diluent. It was extracted undiluted and 0.1 ml of it was used to fill the wells made with a sterile cork borer of 5.5 mm. This in-vitro experiment was compared with the use of a sensitivity disk (Augmentin) which severed as a control.

Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration of the ethanoic extract on the test isolates was determined by incorporating 1 ml of different extract dilution ½, ¼, 1/8, 1/16, 1/32, 1/64 each in test tubes containing 1 ml of peptone water and 1 ml of test isolates. The presence and absence of growth was observed by turbidity.

Results

These results illustrate the antibacterial activity and the minimum inhibitory concentration of honey and *C. odorata* by exhibition of different zone of inhibition on *Escherichia coli* and *Staphylococcus aureus* collected from wound infections and presence and absence of growth that showed the MIC. *Staphylococcus aureus* shows a higher zone of inhibition.

Discussion

This study was carried out to investigate the possible antibacterial activity of honey and *C. odorata* on *Staphylococcus*

Test	*S. aureus*	*E.coli*
Catalase	+	-
Oxidase	-	-
Indole	NA	+
Citrate Utilization	NA	-
Methyl red	+	+
Voges prokeaur	+	-
H_2S production	-	-
Urease	+	-
Nitrate reduction	+	-
Coagulase	+	-
Sugar fermentation		
Glucose	+	+
Maltose	+	+
Sucrose	+	+
Mannitol	+	+

Table 1: Biochemical test of test isolates.
+ = Positive result; - **=** Negative result; NA = Not applicable.

Test	*S. aureus*	*E. coli*
Gram staining	+	-
Motility	-	+

Table 2: Morpological test of test isolates.
+ = Positive result; - = Negative result.

Test organism	Concentrations					
	20%	40%	60%	80%	100%	Control
E. coli	6.00 ± 0.41	6.67 ± 0.85	8.33 ± 0.47	11.33 ± 1.25	13.00 ± 1.41	19.00 ± 0.67
S. aureus	6.90 ± 0.54	9.00 ± 0.82	12.67 ± 1.51	13.87 ± 1.67	16.00 ± 1.74	21.05 ± 0.76

Table 3: Zone of inhibition on test isolates by honey.

Test organism	Ethanoic extract	Aqueous extract	Crude extract	Control
E. coli	29.20 ± 0.37	NA	21.50 ± 0.60	19.00 ± 0.67
S. aureus	30.60 ± 0.52	NA	20.90 ± 0.76	21.05 ± 0.76

Table 4: Zone of inhibition on test isolates by *C. odorata.*

NA = No activity

Test organism	½	¼	1/8	1/16	1/32	1/64
E. coli	-	-	+	+	+	+
S. aureus	-	-	-	+	+	+

Table 5: Minimum inhibitory concentration of *C. odorata* ethanoic extract on test isolates.

+ = Growth; - = No growth

MIC = Minimum conc. * Stock dilution

E. coli

MIC = ¼ * 500 mg/ml

MIC = 125 mg/ml

S. aureus

MIC = 1/8 * 500 mg/ml

MIC = 62.5 mg/ml.

aureus and *Escherichia coli* isolated from wounds of pupils of Community Primary School, Umuoma, Uli. In this study, different dilutions of the honey were used including the net honey to obtain different zone of inhibition. It was observed from the result obtained in table 2 that the antimicrobial effect of honey on the isolates increased significantly ($P \leq 0.05$) as the concentration of the honey is increased. This observation agrees with [11]. In the study of the antibacterial activity of honey by [11] they proposed that the effect of 5 - 10% honey on *S. aureus* which was inhibitory.

In the study, a measured concentration of different diluted extracts – ethanoic and aqueous extracts and undiluted extract and crude extract were used. Observations from the results of the zone of inhibition obtained from different extract showed that aqueous extract showed no antimicrobial activity against *S. aureus* and *E. coli*, probably because the cold water used is a polar solvent and could not extract all the bio-active ingredients in the leaves which conforms to the research of [12]. However, the ethanolic extract against the test isolates were significantly effective compared to the aqueous and crude extract, this might be due to ethanol being an organic solvent and polar solvent extracted both organic and polar constituents. The phytochemical analysis of *C. odorata* showed the presence of steroids, tannins, saponins, alkaloids and flavonoids which act against microorganisms. Studying the MIC of the extract on the *E. coli* and *S. aureus*, it showed inhibition at ¼ and 1/8 respectively.

Generally, in this study, it was observed that *S. aureus* was less resistant to honey and *C. odorata* than *E. coli* because these agents are known to be more active against Gram positive than Gram negative bacteria [13], because of the presence of peptidoglycan cell wall that it acts on or retains them.

Conclusion

It has been shown that the potency of the antibacterial activity can vary very markedly. Honey and *C. odorata* used for this research shows antibacterial activity against Gram positive and Gram negative bacteria. Natural antimicrobial agents can be recommended as substitute for antibiotics. Precautions against loss of antibacterial activities of these antimicrobial agents should be taken. The antibacterial activity of these agent could be enhanced and explored further in the field of human medicine.

Appendix I

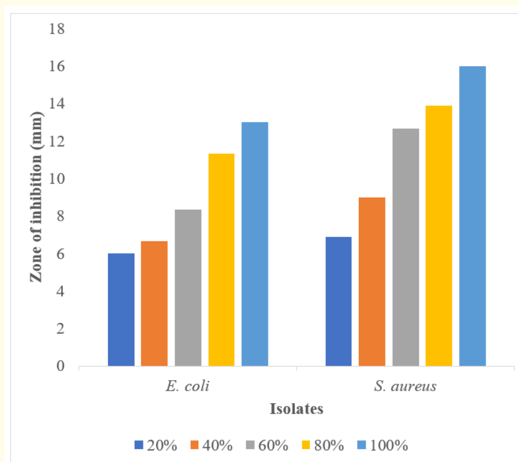

Figure 1: Zone of inhibition on the test isolates by honey.

Appendix 2

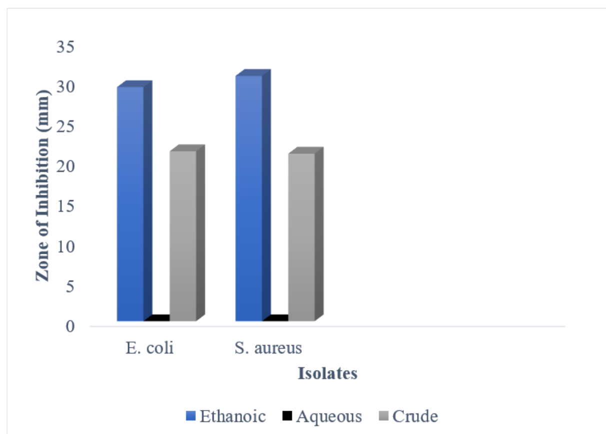

Figure 2: Zone of inhibition on test isolates by *C. odorata.*

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