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Bacterial isolates from surgical wound infection and their susceptibility reaction to *Cnidoscolus aconitifolius* leaf extract and honey

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Abstract
The management of surgical wound infections has no doubt become more challenging due to widespread resistance to available antibiotics and a greater incidence of infections caused by notable bacterial strains. This therefore demands that a renewed effort be made to select suitable antimicrobial therapy from plant derived substances that will aid in the prompt healing of infected surgical wounds. In the present study antimicrobial activity of *Cnidoscolus aconitifolius* leaf and honey are investigated against *Pseudomonas aeruginosa*, *Pseudomonas fluorescence*, *Staphylococcus aureus* and *Escherichia coli*. These isolates were obtained from the wound laboratory, microbiology department, University of Port Harcourt Teaching Hospital, Nigeria. *C. aconitifolius* extract was prepared using methanol and water as extraction solvents. The samples were subjected to phytochemical and antimicrobial examinations using standard methods. Results of phytochemical analysis indicated the presence of alkaloids, saponins, tannins, flavonoids, carbohydrates and steroids. Antimicrobial effects of *C. aconitifolius* leaf extract, honey, extract/honey combination was in decreasing order: *P. fluorescence* > *P. aeruginosa* > *S. aureus* > *E. coli*. The variable sensitivity to leaf extract, honey, extract/honey combination seems to relate to the different resistance levels between the microbial species. The susceptibility test carried out using commercial antibiotics shows *P. fluorescence* and *P. aeruginosa* as the most susceptible, while *E. coli* was least susceptible. This may be due to their differences in cell wall composition, metabolism, nature, resistance to antibiotics or local environmental factors. The study highlights the role of natural product in broadening antimicrobial effectiveness against drug resistant organisms.

Keywords: *Cnidoscolus aconitifolius*, honey, organisms, antibiotics, surgical wound infection, susceptibility, resistance

1. Introduction
A surgical wound is considered infected when the surgical site (usually made by a scalpel during surgery) is invaded by disease-causing agents [1]. The disease-causing agents could be from endogenous or exogenous sources, colonizing and multiplying in the exposed subcutaneous tissue [2-5]. Despite the effort and advances in wound management, surgical wound infection remains one of the most common health challenges in many parts of the world, especially Africa [5]. One amongst several options available for the treatment of infected surgical wounds caused by bacteria is the use of antibiotics [6]. However, there's seemed to be concerns about the resistance of these wound pathogens to most of the available antibiotics [7, 8]. Acquired resistance to these antimicrobials may arise from a combination of several mechanisms including change in permeability target, accumulation of multiple genes, each coding for resistance to a single drug, within a single cell, increased expression of genes that code for multidrug efflux pumps, extruding a wide range of drugs [7]. Previous report suggest that persistence of pathogenic microorganisms may occur in an antibiotic treated patient because they get into a physiologically resistant state without any genetic changes [7]. As a result, there are situations where infected patients cannot be treated promptly resulting in complications or even death [1, 9].
The importance of surgical wound infection in human and cost to economy is enormous. The enormity is in terms of complications of the illness, anxiety it causes, increase in patients discomfort and may lead to death as previously reported [1, 9]. The identified problem therefore demands that a renewed effort be made to select suitable antimicrobial therapy that will aid in the prompt healing of contaminated surgical wounds. Several authors have reported on therapeutic efficacy of some of these medicinal plants having significant healing power, either

in their natural state or as the source of new pharmaceuticals [10-13]. The healing is likely due to the production of bioactive compounds of high structural diversity, that serve as defense agents against invading microorganisms [14-16]. The most important of these bioactive compounds of plants are tannins, alkaloids, steroids, flavonoids, phenolic compounds, gums, fatty acids and resins which can produce definite physiological action on body [9]. A good number of the medicinal plant have been adapted in the management of varieties of conditions such as fever, ulcer, convulsion, tumour, spasm, inflammation, pain [14], including the treatment of fungal infections [17], gastrointestinal nematodes [18] and sexually transmitted infections [19]. Many countries of the world today have integrated, plant-based medicines as an integral part of their primary health care system [20], yet more still need to be done to source for potential new antimicrobials.

Cnidoscolus aconitifolius is one amongst several plant used in traditional medical practice in Nigeria for a number of ailments including haemorrhoids, alcoholism, insomnia, gout, acne, kidney stones, skin disorders, arthritis, obesity, brain and vision improvement [21]. In most part of the world, *C. aconitifolius* shoots and leaves are reported to have been taken as a laxative, circulation stimulant, diuretic and for improving digestion, stimulating lactation, improving brain function and memory, strengthening fingernails and darkening greying hair [22]. Few of the popular common names of the plant are Spinach Tree, Tread Softly, Cabbage Star, Chaya, Chicasquil, Devil nettle and Tree-spinach [22]. *C. aconitifolius* is a fast-growing leafy perennial shrub of the family Euphorbiaceae commonly found in the tropics [23]. The plant could often grow to 3 m (10 ft.) in height, and 2 m (6.5 ft.) in width but some may reach up to five or six meters tall [22]. It has succulent stems which exude a milky sap when cut and grow well in most soil conditions including moist, well-drained soil [22]. Leaves are dark green, alternate, simple, slick surfaced often with some hairs and palmately lobed. Each leaf is 6 to 8 inches across and is borne on a long slender petiole (leaf stem). Where the leaf stem connects to the leaf, the leaf veins are fleshy and cuplike. Wood of young stems is soft, easily broken, and susceptible to rot [22]. Previous studies show that *C. aconitifolius* contain phenols, saponins, cardiac glycosides, phlobatannin, high fibre content, antioxidant properties against paracetamol toxicity, ameliorative effect on anaemia and increases erythrocyte osmotic fragility induced by protein energy malnutrition [23].

Honey is one of the oldest traditional medicines considered to be important in the treatment of several human ailments [24]. According to Moore *et al*. [25] honey is obtained from the secretions of the living parts or excretions of plants which the honey bees known as *Apis mellifera* collects and store. Their use as nutrient, drug and ointment has been widely reported by several authors [24, 26, 27]. The high osmotic nature, ability to produce hydrogen peroxide, naturally low pH (3.2–4.5), phytochemical factors such as tetracycline derivatives, ascorbic acid, peroxides, benzoic acid, phenols, terpenes, amylase, benzyl alcohol and fatty acids are factors that confers potency against pathogenic organisms [26, 28]. Earlier studies have shown effectiveness of honey in wound dressing, burns and skin ulcers [26, 27]. According to Lusby *et al*. [27] honey speeds up growth of new tissues and help to heal wound, reducing pain and odour quickly.

Though honey is used widely in traditional medicine, its use in modern medicine seems to be limited because of variation in the antimicrobial activity of some natural honeys [24]. The variation is reported to be as result of spatial and temporal variation in sources of nectar and as such requiring identification and characterization of the active principles [24]. Since resistance has become a global health issue, the integration of traditional medicine such as the use of honey and/or medicinal plants could be an alternate option to broaden the spectrum of antimicrobial activity against drug resistant bacteria [26]. We, therefore, under took this study to evaluate the antimicrobial activity of the extracts of *Cnidoscolus aconitifolius* leaf alone and in combination with honey against organisms isolated from surgical wound infections.

2. Materials and Methods

2.1. Plant sample

Fresh sample of *Cnidoscolus aconitifolius* leaf was collected from Ogbatai, Woji in Obio Akpor, South-south region, Nigeria. The sample was authenticated at the herbarium (FHI) forestry research institute of Nigeria, Ibadan, Oyo State, South-west region with an herbarium number of 109457.

2.2. Preparation of plant extract

The collected material was rinsed severally with clean tap water to make it dust and debris free and subjected to drying in a dark place at room temperature for about 2 weeks. The dried leaf was ground in electric chopper and fine plant powder-soaked methanol and water separately for 1 week with subsequent stirring, then filtered with a Whitman's filter paper. The filtrate was dried on a porcelain crucible and placed on a water bath set at 40 ^{o}C until all the solvent had vapourized. After solvent evaporation, the extract was weighed and preserved at – 4 ^{o}C until ready for use [29].

2.3. Honey sample

Fresh honey sample was collected from Abakpa, Enugu, South-eastern region, Nigeria. The sample was labelled and transported to pharmaceutical microbiology laboratory, University of Port Harcourt for examination and storage.

2.4. Culture media

The media used in this study includes, Nutrient Agar, Nutrient broth, Muller Hinton agar, Muller Hinton broth, MacConkey Agar, Simon's Citrate Agar, Cetrimide Agar (Lab M Limited, England) and constituted according to manufacturer's specification. Sterilization was by autoclaving at 121 °C for 15 min and maintained in molten form until ready for use [30, 31].

2.5. Commercial antibiotic used

The antimicrobial agents used were commercially prepared antibiotic disc from Abtek Biologicals Ltd; (Lot/Batch Number: RC09/P). They include, cefalexin 30 µg, ampicillin 10 µg, streptomycin 10 µg, cotrimoxazole 25 µg, amoxicillin-clavulanate 20/10 µg, gentamycin 10 µg, pefloxacin 5 µg, nalidixic acid 30 µg, ofloxacin 5 µg, rifampicin 5 µg, amoxicillin 20 µg, norfloxacin 10 µg, chloramphenicol 30 µg, ciprofloxacin 5 µg, erythromycin 15 µg, levofloxacin 5 µg.

2.6. Test organisms

Test organisms (*Pseudomonas aeruginosa*, *Pseudomonas fluorescence, Staphylococcus aureus and Escherichia coli*) were obtained from the wound laboratory, microbiology department, University of Port Harcourt Teaching Hospital, Nigeria. The organisms were collected in sterile agar slants, labelled with the date and transported immediately to the laboratory for analysis.

2.7. Authentication of isolates
The test organisms were cultured onto selected culturing media and incubated for 24 hours at 37 ºC to obtain colonies. After overnight incubation, colonies were authenticated using colony characteristics, gram reaction of the organisms and biochemical test following standard procedure [32].

2.8. Standardization of test organisms
A loopful of the purified culture was collected using a flamed and cooled sterile wire loop, introduced into sterile peptone water in universal bottles. The bottles were shaken, and the turbidity compared with that of the McFarland standard. 0.5 McFarland Standards is equivalent to approximately 1.5 x 10^8 CFU/mL of microbial suspension, when the turbidity values of the suspensions match optically or visually [33, 34].

2.9. Phytochemical tests
Phytochemical component screening tests and identification of flavonoids, carbohydrates, cardiac glycosides, anthraquinone glycosides, saponins, tannins, alkaloids, carotenoids were carried out on the extracts using the standard procedures as described [35-38].

2.10. Antimicrobial assay
Antimicrobial activities of *C. aconitifolius* leaf extract and honey were determined using the well diffusion method previously described in the standard of the National Committee for Clinical Laboratory Standards [39, 40]. Standardized cultures of four bacterial isolates (*P. aeruginosa*, *P. fluorescence*, *E. coli* and *S. aureus*) were seeded onto Muller Hinton agar (MHA) pour and allowed to set on the bench. Wells measuring 6 mm in diameter were aseptically cut in the agar plate using a cork borer. Each well respectively filled with 0.2 mL of extract or honey by using a pipette in aseptic condition as previously described [41]. The plates were preincubated at 4 ºC for 2 hours to allow uniform diffusion into the agar. After preincubation, the plates were incubated at 37 °C for 24 hours. The results were observed by measuring the zone of inhibition [42, 43].
In addition, commercial antibiotics were used as positive control to determine the sensitivity of the test organisms. Sensitivity tests were performed following modified Kirby-Bauer disc diffusion method as recommended by the Clinical Laboratory Standards Institute (CLSI) [44]. Using a swab, the standardized organisms were inoculated onto a Mueller Hinton agar and disc containing antibiotics placed equidistance to each other as previously described [44, 45]. Uninoculated plates containing only the media and antibiotic disc were used as blank to compare the different samples. The plates were incubated in inverted position at 37 ºC for 24 hours and the resulting inhibition zone diameter (IZD) interpreted using CLSI protocols [39, 46, 47].

3. Results
3.1 Phytochemical tests
Table 1 represents methanolic and aqueous reactions to various phytochemical test carried out. As shown, the methanol extract gave positive reactions for flavonoids, tannins, alkaloids, carbohydrates, steroids, carotenoids, saponins and negative for anthraquinone. Similar result was recorded for the aqueous extract for carotenoids which tested negative.

Table 1: Phytochemical Screening of *Cnidoscolus aconitifolius* Leaf Extract

Phytochemical constituents	Extract	
	Methanolic	Aqueous
Flavonoids	+	++
Tannins	+	+
Alkaloids	++	+
Anthraquinone	-	-
Carbohydrate	+	++
Steroids	+	+
Carotenoids	+	-
Saponins	+	+

Key: + = Present in moderate amounts, ++ = Present in high amounts, - = absent

3.2 Antimicrobial assay
3.2.1 Cnidoscolus aconitifolius leaf extract and honey
The isolates *P. aeruginosa*, *P. fluorescence*, *E. coli* and *S. aureus* were tested for their antimicrobial activity to *C. aconitifolius* leaf extract and honey and the results of inhibition zones measured in millimetre are presented in Fig. 1 – 4. The results presented showed honey with the widest inhibition zone diameter of 17, 13.7, 12.2 and 11.7 mm respectively for *P. aeruginosa*, *P. fluorescence*, *S. aureus* and *E. coli*. The test with extract alone gave an inhibition zone diameter reading of 8, 9, 1, 8 mm (methanol extraction) and 3, 5, 2, 4 mm (aqueous extraction) for *P. aeruginosa*, *P. fluorescence*, *E. coli* and *S. aureus* respectively. While the test involving extract in combination with honey gave a zone of inhibition diameter of 10, 11, 7.5, 8.5 mm (methanol extraction) and 5, 8, 5, 5 mm (aqueous extraction) for *P. aeruginosa*, *P. fluorescence*, *E. coli* and *S. aureus* respectively.

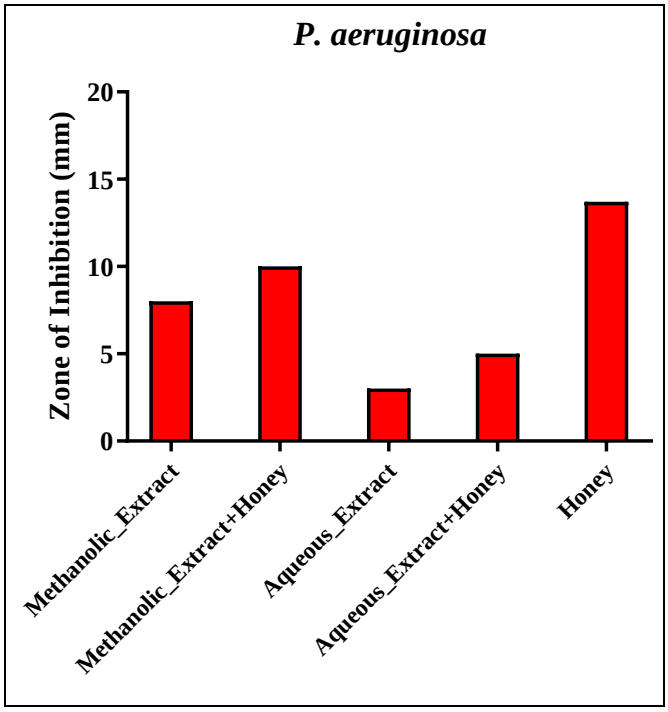

Fig 1: Column chart showing zone of inhibition produced by *C. aconitifolius* leaf extract alone, extract/honey combination and honey alone against *P. aeruginosa*

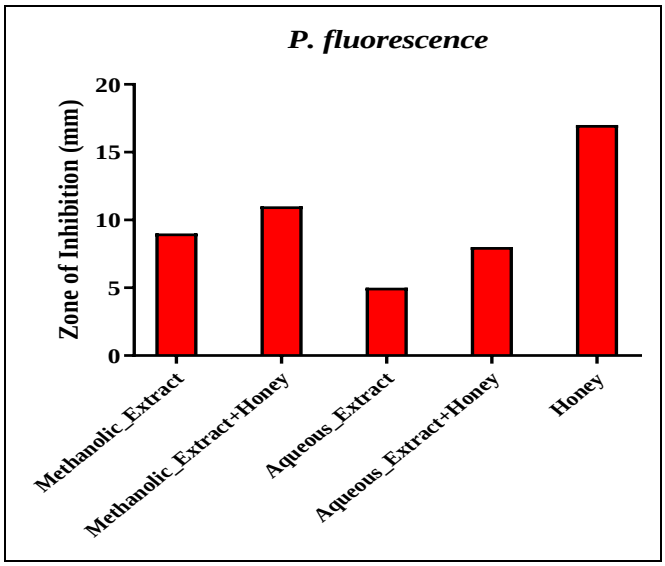

Fig 2: Column chart showing zone of inhibition produced by *C. aconitifolius* leaf extract alone, extract/honey combination and honey alone against *P. fluorescence*

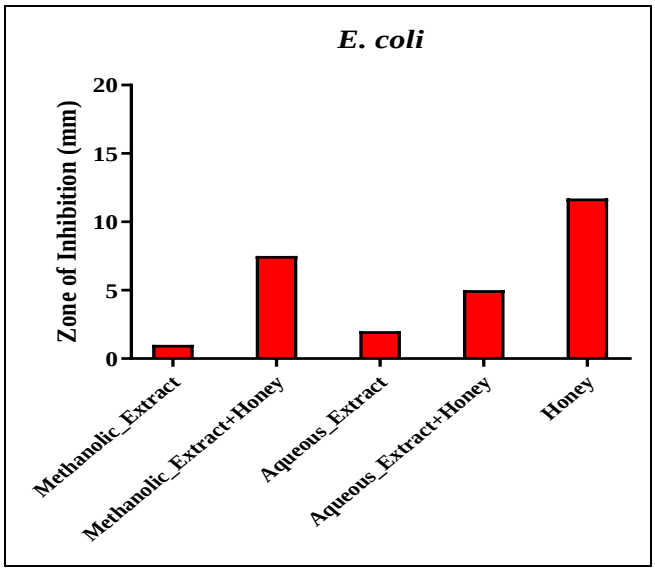

Fig 3: Column chart showing zone of inhibition produced by *C. aconitifolius* leaf extract alone, extract/honey combination and honey alone against *E. coli*

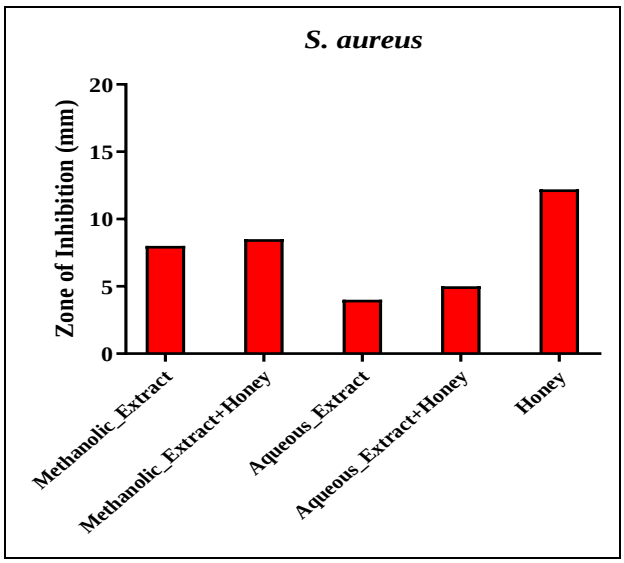

Fig 4: Column chart showing zone of inhibition produced by *C. aconitifolius* leaf extract alone, extract/honey combination and honey alone against *S. aureus*

3.2.2 Commercial antibiotics

All four isolates *P. aeruginosa*, *P. fluorescence*, *E. coli* and *S. aureus* were tested for their susceptibility to commercially available antibiotics and the pattern of their sensitivity measured in millimetre are presented in Fig. 5 – 8. Using the interpretative chart derived from the zones of inhibition of standard organisms according to the Clinical Laboratory Science Institute (CLSI), the zone size of each antibiotic was interpreted, and the isolate was reported as being "susceptible", "intermediate" or "resistant" [39, 48]. As shown, *P. aeruginosa* and *P. fluorescence* are susceptible to cefalexin, ampicillin, amoxicillin-clavulanate, gentamycin, ofloxacin showing intermediary susceptibility to streptomycin, resistance to pefloxacin, with varied susceptibility to ciprofloxacin and cotrimoxazole. *E. coli* was resistant to all test antibiotics (Fig. 7). *S. aureus* was susceptible to streptomycin, chloramphenicol but resistant to gentamycin, ampicillin, rifampicin, amoxicillin, erythromycin, levofloxacin and showing intermediary susceptibility to norfloxacin and ciprofloxacin (Fig. 8).

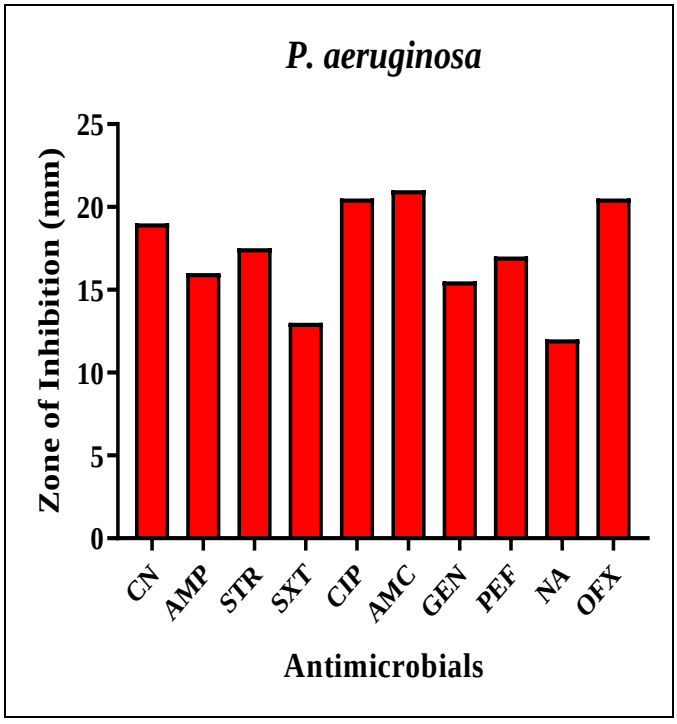

Fig 5: Column chart showing zone of inhibition produced by commonly used antimicrobials against *P. aeruginosa*. The result shows that *P. aeruginosa* was susceptible to cefalexin, ampicillin, ciprofloxacin, amoxicillin-clavulanate, gentamycin, ofloxacin and resistant to pefloxacin, nalidixic acid while streptomycin, cotrimoxazole showed intermediate effect.

Key: CN = Cefalexin, AMP = Ampicillin, STR = Streptomycin, SXT = Cotrimoxazole, CIP = Ciprofloxacin, AMC = Amoxicillin-clavulanate, GEN = Gentamycin, PEF = Pefloxacin, NA = Nalidixic acid, OFX = Ofloxacin.

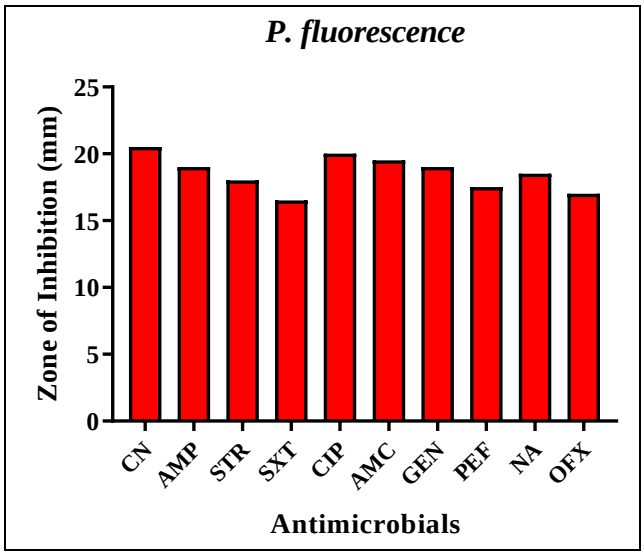

Fig 6: Column chart showing zone of inhibition produced by commonly used antimicrobials against *P. fluorescence*. Result indicates susceptibility to cefalexin, ampicillin, streptomycin, amoxicillin-clavulanate, gentamycin, nalidixic acid, ofloxacin showing intermediary susceptibility to ciprofloxacin, cotrimoxazole and resistant to pefloxacin.

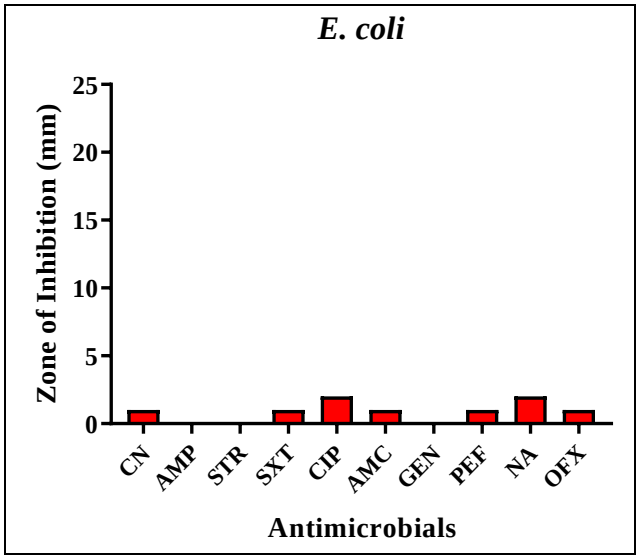

Fig 7: Column chart showing zone of inhibition produced by commonly used antimicrobials against *E. coli*. As shown, *E. coli* was resistant to the test antibiotics.

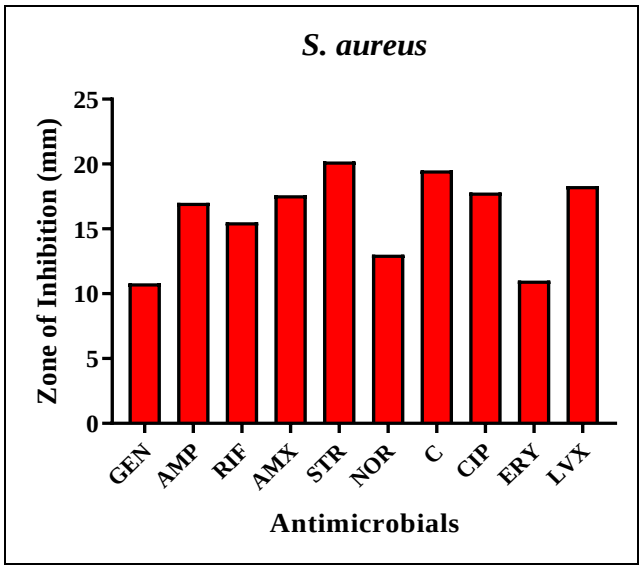

Fig 8: Column chart showing zone of inhibition produced by commonly used antimicrobials against *S. aureus*. From the result *S. aureus* was susceptible to streptomycin, chloramphenicol, intermediary susceptibility to norfloxacin, ciprofloxacin and resistant to gentamycin, ampicillin, rifampicin, amoxicillin, erythromycin, levofloxacin. Key: GEN = Gentamycin, AMP = Ampicillin, RIF = Rifampicin, AMX = Amoxicillin, STR = Streptomycin, NOR = Norfloxacin, C = Chloramphenicol, CIP = Ciprofloxacin, ERY = Erythromycin, LVX = Levofloxacin

4. Discussion

The clinical relevance of infections caused by drug-resistant organisms has been outlined in several studies [5, 7, 49-52]. Such organisms are reported to presents worse clinical outcomes than those caused by non-resistant strains of the same organisms [49]. This is very important not only in terms of increased trauma to patient but also because of its burden on cost and the increasing demand for cost-effective management within the healthcare system as previously reported [5]. The extent of the above outcomes is reported to be more pronounced as disease severity, strain virulence, or host susceptibility rises [49]. The present study was carried out to evaluate the possible antimicrobial activities of *C. aconitifolius* leaf extract alone and its combination with honey to multi-drug resistant organisms isolated from surgical wound infections. *P. aeruginosa*, *P. fluorescence*, *E. coli* (gram negative) and *S. aureus* (gram positive) were the predominant organisms in the present study similar to previous report by Bowler *et al*. [2], Emele *et al*. [53], Mama *et al*. [54], Okoli *et al*. [55].

The phytochemical analysis of the leaf extract indicated the presence of alkaloids, saponins, tannins, flavonoids, carbohydrates and steroids. Previous report has indicated that these metabolites are usually responsible for the pharmacological activities of most medicinal plants as well as being a possible precursor for clinically useful drugs [16, 42, 55-59]. For example, various human physiological activities, such as stimulation of phagocytic cells, host-mediated tumour activity, and a wide range of anti-infective actions, have been assigned to tannins [60]. Flavonoids is reported to target microbial membrane, peptides which are often positively charged and contain disulphide bonds. It inhibits growth by the formation of ion channels in the microbial membrane or competitive inhibition of adhesion of microbial proteins to host polysaccharide receptors [61]. It is possible that one or more of these active compounds may have contributed to the observed inhibition which may also have overlapped due to the solvent used for screening [9]. This suggests that the constituents of this leaf may play a valuable role as alternative therapy in wound healing as previously reported [55].

Results of antimicrobial assay showed that honey alone had considerable effect on growth of all four isolates compared to the extract alone and in combination with honey. Whilst honey gave inhibition zone diameter ranging from 11.7 to 17 mm, extract alone (using methanol and aqueous) gave a zone of inhibition ranging from 1 and 9 mm and extract in combination with honey gave a zone of inhibition ranging from 5 to 11 mm. The aqueous extracts exhibited the least antimicrobial effect as compared to methanol extract. From the results, antimicrobial effect of *C. aconitifolius* leaf extract, honey, extract/honey combination to the test organisms was in decreasing order: *P. fluorescence* > *P. aeruginosa* > *S. aureus* > *E. coli*. The variable sensitivity of different microorganisms to leaf extract, honey, extract/honey combination seems to relate to the different resistance levels between the microbial species [43]. High antimicrobial activity expressed by honey alone is probably due to the enzymatic production of hydrogen peroxide, high osmotic nature, naturally low pH, phytochemical factors such as tetracycline derivatives, peroxides, amylase, fatty acids, phenols, ascorbic acid, terpenes, benzyl alcohol and benzoic acid [24, 26, 62]. The result of this study seems to agree with several other in vitro and *in vivo* studies showing high antimicrobial potential of honey against some important pathogens [24, 26, 62, 63]. As shown, honey/extract combination did not produce a significant effect as compared to when honey is used alone. This could be attributable to number of factors including inactivity of the extract itself, dilution of the honey by the extract, nature of the bacterial isolate and concentration of honey used [26]. The minimal antimicrobial activity recorded in methanol and aqueous extracts could be due to low concentration of antimicrobial compounds in these extracts or the fact that the antibacterial compound(s) may not have been extracted by these solvents [9]. Previous report by Cowan [61] indicates the most commonly used solvents (ethanol and methanol) may not demonstrate the greatest sensitivity in yielding antimicrobial chemicals on an initial screening. Hence the need for further investigation into the disparity as the search for new antimicrobials intensifies [61].

Similarly, the susceptibility test carried out using commercial antibiotics for gram negative and positive organisms showed that *P. fluorescence* and *P. aeruginosa* are susceptible to cefalexin, ampicillin, amoxicillin-clavulanate, gentamycin, ofloxacin showing intermediary susceptibility to streptomycin, resistance to pefloxacin, with varied susceptibility to ciprofloxacin and cotrimoxazole. *S. aureus* was susceptible to streptomycin, chloramphenicol but resistant to gentamycin, ampicillin, rifampicin, amoxicillin, erythromycin, levofloxacin and showing intermediary susceptibility to norfloxacin and ciprofloxacin. As shown in Fig. 7, *E. coli* was least sensitive compared with the other test bacteria. This may be due to their differences in cell wall composition, metabolism, nature, resistance to antibiotics or local environmental factors [43]. Similarities of the test organisms in relation to their susceptibility to the plant extracts is in agreement with previous studies by Oskay and Sarı [43]. The most significant effect in this study is the sensitivity expressed by antibiotic-resistant *E. coli* to honey alone, combination of honey and *C. aconitifolius* leaf extract. This suggest the non-interference of an antibiotic resistant organism with the anti-microbial action of plant extracts, indicating that these extracts and/or honey might have different modes of action on test organisms [43].

5. Conclusions

This study confirms that, multi drug resistance *E. coli* and *S. aureus* strains are important problems in hospitals and treatment options seems to be limited for patient visiting University of Port Harcourt Teaching Hospital. Our findings show that *C. aconitifolius* and honey combinations, can be efficiently used in the treatment of multi drug resistance *E. coli* and *S. aureus* surgical infections. It should be noted that *C. aconitifolius* in combination with honey did not produce significant activity against *P. aeruginosa* and *P. fluorescence*. Therefore, further studies should be performed since *in vitro* experiences are limited for *C. aconitifolius* and honey combinations. Since *E. coli* strains are resistant against majority of antibiotics tested, surveillance results should be considered when selecting empirical therapy that will be applied to patients with surgical wound contamination.

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