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PHYTOCHEMICAL AND ANTIMICROBIAL EVALUATION OF *Cnidoscolus aconitifolius* LEAF EXTRACT AND HONEY AGAINST *Klebsiella pneumoniae, Streptococcus pneumoniae* ISOLATED FROM SPUTUM SAMPLE

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ABSTRACT

Plant materials and their products are increasingly being used across the world as natural substitute for treating various ailment. The role of these plants in pharmaceutical, alternative medicine and natural therapy have been well documented. However, there's the need to carry out further investigation on such plants to provide basis for their acceptable use as well as improve the quality of healthcare. Additionally, the prominence of multidrug resistant bacterial strains, has necessitated the search for alternative natural therapy. In the present study phytochemical and antimicrobial of *Cnidoscolus aconitifolius* leaf is investigated against *Klebsiella pneumoniae* and *Streptococcus pneumoniae* clinical isolates using standard methods. *C. aconitifolius* leaf extract was prepared using methanol and water as solvents. Carbohydrate, alkaloids, steroids, flavonoids, saponins, tannins were detected in both methanol and aqueous extract. According to the findings of antimicrobial assay, honey produced the highest zone of inhibition of 12.7, 11.5 mm respectively against *K. pneumoniae* and *S. pneumoniae*. Both aqueous and methanol extract in combination with honey produced satisfactory sensitivity, while extract alone showed relatively poor antimicrobial activity against the two isolates. The variable sensitivity to extract, honey, extract/honey combination seems to relate to the different resistance levels between the microbial species. While the antimicrobial effect of extract/honey combination may be attributed to the improved absorption of bioactive compounds in combination than when they are given in isolation. The study highlights the need to further explore the use of plant derived compounds in the fight against illnesses caused by resistant bacterial strains.

KEYWORDS: Phytochemical, Antimicrobial, *Cnidoscolus aconitifolius*, sputum, resistance, honey.

INTRODUCTION

Antibiotics are ideally the most important weapons in fighting infections caused by bacteria and there is no doubt they have greatly improved the quality of human life since their introduction.[1,2] Unfortunately, these antibiotics have begun to show ineffectiveness against several pathogenic organisms, due to increasing antimicrobial resistance.[2,3] Resistance to an antimicrobial happens when a microbe evolves a protective defence against their use. A phase described as natural in microbial evolution, with many pathogens having the capacity to acquire a different genetic material or mutating into new and sometimes dangerous forms.[4] Antimicrobial resistance as reported in literature is one amongst several setbacks to successful treatment of several illnesses including major respiratory infections.[5] The frequency of occurrence has become a serious threat to clinical medicine, with increased morbidity and mortality as a result of treatment failures.[5,6] In addition to the significant negative impact on clinical outcome is the huge impact on the economy.[2,5] Thus, identification and characterization of the alternative bioactive compounds from natural products may provide the needed therapeutic potential for prompt healing of infections caused by major respiratory pathogens.[7] Already, the use of plants with medicinal value is gradually being considered as potential sources of natural agents.[2,8,10] Many of these plant extracts have in time past been screened as natural substitute for treating many infectious diseases.[9] A number of studies involving plant materials revealed the presence of bioactive compounds which are known to exhibit medical and physiological activities.[11] Antimicrobial activities exhibited by plants extract against different species of bacteria has been established by several authors.[1,11-14] The results of different studies reveal dramatic variation in antimicrobial effectiveness of medicinal plants depending on the phytochemical characteristics of plant families and subfamilies.[10,11,14-16] Presently, effort have been focused on expanding the

spectrum of antimicrobial agents from potentially effective, healthy safer and natural products. Within these contexts is the use of *Cnidoscolus aconitifolius* leaf extract and honeybee for possible treatment of sputum infection.

Cnidoscolus aconitifolius belonging to the family Euphorbiaceae is known to contain phenols, saponins, cardiac glycosides, phlobatannin, high fibre, antioxidant properties against paracetamol toxicity, ameliorative effect on anaemia and increases erythrocyte osmotic fragility induced by protein energy malnutrition.[17] The shoots and leaves have been widely used for a variety of medicinal purposes such as laxative, circulation stimulant, diuretic and for improving digestion, stimulating lactation, improving brain function and memory, strengthening fingernails and darkening greying hair.[12,18] In Nigeria, this plant is used in traditional medical practice for treatment of insomnia, gout, haemorrhoids, alcoholism, arthritis, acne, kidney stones, skin disorders, obesity, brain and vision improvement.[19] Some of the popular names of *C. aconitifolius* are Spinach Tree, Tread Softly, Cabbage Star, Chaya, Chicasquil, Devil nettle and Tree-spinach.[18] *C. aconitifolius* is a fast-growing leafy perennial shrub commonly found in the tropics.[17] Earlier report indicates that the plant could grow to 3 metres in height, 2 metres in width and sometimes grow as tall as 6 metres having succulent stems that exude a milky sap when cut.[18] It can grow well in most soil conditions including moist, well-drained soil.[18] Leaves of *C. aconitifolius* plant are alternate, simple, slick surfaced, dark green 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).

For many years, honey has had a valued place in traditional medical practice as nutrient, drug and/or ointment.[20-22] Several authors have reported a substantial variation in the potency of different honey. However, they all agree their potency is largely due to their ability to produce hydrogen peroxide, phenolic compounds, change in pH, osmotic pressure (exerted by the honey) all of which may work individually or in synergy.[20,22] Other factors shown to influence the potency of honey include the geographical, seasonal, botanical source as well as harvesting, processing and storage conditions.[20,22] The high health-promoting benefits in the use of honey is an important factor for consideration. The benefits include it effectiveness in wound dressing, burns, skin ulcers, inflammations, overcoming liver, cardiovascular and intestinal problems and the treatment of some types of infectious diseases.[21,22] The healing property of honey has been attributed to high sugar content (high osmolarity), naturally low pH, phytochemical factors, content of hydrogen peroxide and non-peroxide components like methylglyoxal.[20,21,23]

Most sputum infections have been associated with bacterial pathogens particularly members of Gram positive like *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Staphylococcus aureus* and Gram negative like *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Haemophilus influenzae*, *Legionella pneumophila*.[6,24,25] Research concerning the efficacy of *Cnidoscolus aconitifolius* leaf and honey against drug resistant bacteria are scanty in Nigeria. Therefore, the present study was aimed at screening the phytochemical and antimicrobial activity of *Cnidoscolus aconitifolius* leaf extract and honey against *Klebsiella pneumoniae*, *Streptococcus pneumoniae* isolated from sputum samples of patients attending outpatient clinic at the University of Port Harcourt Teaching Hospital, Nigeria.

MATERIALS AND METHODS

Culture media

The media used in this study includes, Nutrient Agar, Nutrient broth, MacConkey Agar, Eosin Methylene Blue agar, Blood agar, Muller Hinton agar, Muller Hinton broth, (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.[26,27]

Test organisms

Clinical isolates of *Klebsiella pneumoniae*, *Streptococcus pneumoniae* were obtained from sputum laboratory, Department of Microbiology, University of Port Harcourt Teaching Hospital (UPTH) Rivers State, Nigeria. The isolates were transported to pharmaceutical microbiology laboratory, University of Port Harcourt and sub-cultured onto selected culturing media (Nutrient agar, MacConkey agar, Eosin Methylene Blue Agar, blood agar) and incubated for 24 hours at 37 ^{o}C. After overnight incubation, colonies were authenticated using colony characteristics, gram reaction of the organisms and biochemical test following standard procedure.[28-30] Stock cultures were maintained on slopes of modified nutrient agar at 4 ^{o}C and sub-cultured routinely until ready for use.

Inoculum preparation

Stock cultures of test organisms (*K. pneumoniae*, *S. pneumoniae*) were used for the preparation of culture suspension. The bacterial inoculums were prepared with 2 to 3 hour broth culture of *K. pneumoniae* and *S. pneumoniae* adjusted to a turbidity equivalent to 0.5 McFarland Standard.[31,32]

Honey sample

Honey sample used in this study was collected from Abakpa, Enugu, South-eastern region, Nigeria

Collection and identification of plant material

Cnidoscolus aconitifolius plant materials were collected from Ogbatai, Woji Town, Obio Akpor council area, South-south region of Nigeria. The leaf sample was identified at the herbarium (FHI) forestry research institute of Nigeria, Ibadan, Oyo State with an herbarium

number of 109457. The leaf specimen was labelled to include date of collection, locality and their medicinal uses.

Preparation of leaf extract

C. aconitifolius leaves were plucked off from the stem and washed in tap water before drying at room temperature for up to three weeks. After drying, leaves were placed into a blender to be grounded into powder. The grounded leaves materials were transferred onto a closed tight container and stored at – 4 ^{o}C until ready for use. Extraction was performed by macerating 100 g of *C. aconitifolius* powder separately in 900 mL of 95 % cold methanol and water for one week. The extract was filtered and concentrated to a small volume to remove all the solvent using a rotary evaporator at 40 °C as previously described. [33] After solvent evaporation, the extract was weighed, and percentage yield was calculated using the following formula: Extract yield (%) = $W_R/W_S \times 100$ (where W_R is weight of extracted plants residues after drying and W_S; weight of plant sample before drying).[13,34]

Phytochemical analysis

The grounded leaf materials of *C. aconitifolius* was analysed for the presence of flavonoids, carbohydrates, anthraquinone glycosides, saponins, tannins, steroids, alkaloids, carotenoids using the standard phytochemical testing procedures earlier described.[35-38]

Antimicrobial activity of plant extract and honey

In vitro antimicrobial activities of *C. aconitifolius* leaf extract and honey were performed using the well diffusion method previously described.[39,40] Standardized cultures of *K. pneumoniae* and *S. pneumoniae* isolates were inoculated onto Muller Hinton agar (MHA) pour and allowed to set on the bench. Using a cork borer, 6 mm diameter wells were aseptically cut in the agar plate and each well filled with 0.2 mL of extract or honey.[41] The plates were preincubated at 4 ^{o}C for 2 hours to allow uniform diffusion into the agar and thereafter incubated at 37 °C for 24 hours. After incubation, the plates were examined, and any zone of inhibition measured in millimetre.[11,42] To determine the sensitivity of test organisms selected commercial antibiotics were used as positive control. Tests was performed following modified Kirby-Bauer disc diffusion method as recommended by the Clinical Laboratory Standards Institute.[43] Using swab sticks, the standardized organisms were inoculated onto a Mueller Hinton agar and disc containing antibiotics placed equidistance to each other as previously described.[43,44] The commercial antibiotic discs were those from Abtek Biologicals Ltd; (Lot/Batch Number: RC09/P). They include cefalexin 30 µg, ampicillin 10 µg, streptomycin 10 µg, cotrimoxazole 25 µg, ciprofloxacin 5 µg, amoxicillin-clavulanate 20/10 µg, gentamycin 10 µg, pefloxacin 5 µg, nalidixic acid 30 µg, ofloxacin 5 µg for *Klebsiella pneumoniae* (Gram-negative bacterium). *Streptococcus pneumoniae* being gram-positive bacterium was tested against gentamycin 10 µg, ampicillin 10 µg, rifampicin 5 µg, amoxicillin 20 µg, streptomycin 10 µg, norfloxacin 10 µg, chloramphenicol 30 µg, ciprofloxacin 5 µg, erythromycin 15 µg, levofloxacin 5 µg. Uninoculated plates containing only the media and antibiotic discs were used as blank to compare the different samples. The plates were incubated in inverted position at 37 ^{o}C for 24 hours and the resulting inhibition zone diameter (IZD) interpreted using CLSI protocols.[39,45,46]

Statistical analysis

A one-way analysis of variance was used to calculate probabilities and determine significance of data obtained. A p-value of less than or equal to 0.05 is considered to be statistically significant.[47,48]

RESULTS AND DISCUSSION

Phytochemical analysis

Phytochemical analysis to characterize the phytochemical constituents in methanol and aqueous leaf extracts of *Cnidoscolus aconitifolius* was performed as previously described.[34, 36] It's the simplest technique for detecting bioactive compounds that are present in plant extract and the results obtained are presented in Table 1. As shown, carbohydrate, alkaloids, steroids, flavonoids, saponins and tannins were detected in both methanol and aqueous extract of the plant leaf tested. From the result, anthraquinone was negative whereas carotenoids tested positive in methanol extraction only.

Table 1: Phytochemical profile of *Cnidoscolus aconitifolius* leaf extract.

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

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

The phytochemicals or bioactive compounds identified in this study are known to exhibit medical, physiological activities,[11,49] and may be responsible for the antimicrobial activities observed in the study.[10] Previous studies indicate the limitless ability of medicinal plants to synthesize aromatic substances, most of which are phenols or their oxygen substituted derivatives.[50] In nature, thousands of these compounds function to influence plant germination, growth, survival, and reproduction. For example, quinones and tannins are key constituent in plant pigmentation while terpenoids are responsible for odours in plants.[50] Flavonoids found in vegetables, flowers, stems, fruit, nuts, seeds, wine, honey, tea are reported to be abundant in

photosynthesising cells and used to treat human diseases.[51] Earlier report by Biswas *et al.*[11] indicate that flavonoids act by forming complexes with extracellular and soluble proteins as well as bacterial cell walls. Other bioactive compounds like saponins (which are glycosides) have inhibited the growth of most Gram-positive organism while catechol and pyrogallol (are hydroxylated phenols) have shown toxic effects against most microorganisms.[50] These bioactive compounds are important leads for the development of a pharmacologically acceptable antimicrobial agent for the treatment of infections caused by resistant bacterial strains.[10,11,50,51]

Antimicrobial activity of plant extract and honey

Figures 1 to 6 presents the antimicrobial activity of aqueous and methanol extract of *Cnidoscolus aconitifolius* leaf and honey in comparison with selected commercial antibiotic. As shown in Fig. 1 to 4, honey produced the highest zone of inhibition of 12.7, 11.5 mm respectively for *K. pneumoniae* and *S. pneumoniae*. The test involving extract in combination with honey gave a zone of inhibition diameter of 10, 6.5 mm (aqueous extraction) and 11.5, 7.5 mm (methanol extraction) for *K. pneumoniae* and *S. pneumoniae* respectively. While the test with extract alone produced inhibition zone diameters of 2, 3 mm (aqueous extraction) and 1, 3.5 mm (methanol extraction) for *K. pneumoniae* and *S. pneumoniae* respectively.

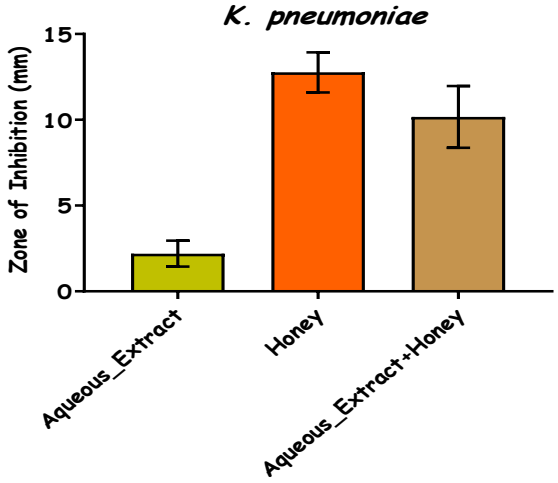

Fig. 1: Zone of inhibition produced by aqueous extract of *Cnidoscolus aconitifolius* leaf, honey and *C. aconitifolius* leaf extract/honey combination against *K. pneumoniae*.

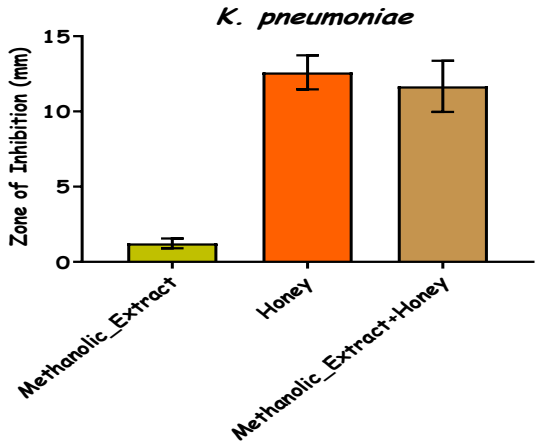

Fig. 2: Zone of inhibition produced by methanol extract of *Cnidoscolus aconitifolius* leaf, honey and *C. aconitifolius* leaf extract/honey combination against *K. pneumoniae*.

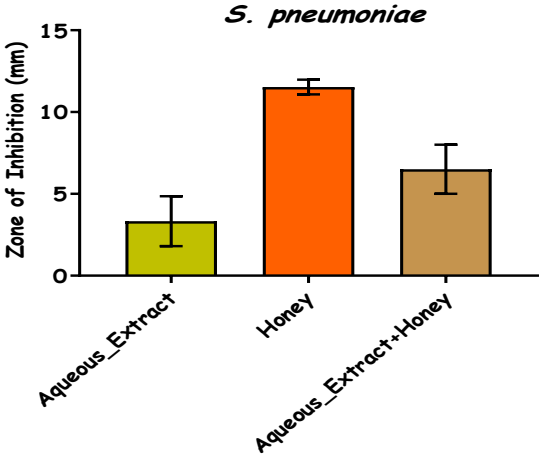

Fig. 3: Zone of inhibition produced by aqueous extract of *Cnidoscolus aconitifolius* leaf, honey and *C. aconitifolius* leaf extract/honey combination against *S. pneumoniae*.

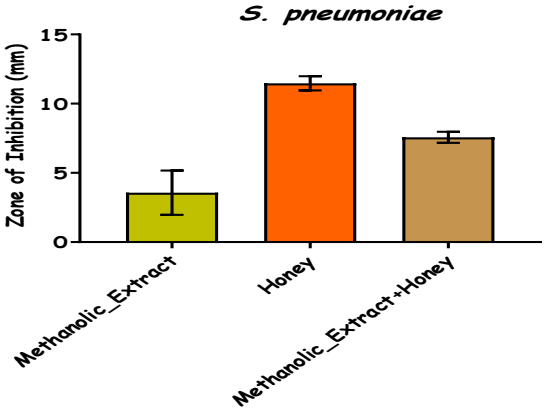

Fig. 4: Zone of inhibition produced by methanol extract of *Cnidoscolus aconitifolius* leaf, honey and *C. aconitifolius* leaf extract/honey combination against *S. pneumoniae*.

The results presented showed honey as being more potent, having a higher antimicrobial activity against the two isolates. Both aqueous and methanol extract in combination with honey produced satisfactory sensitivity, while the extract alone showed relatively poor antimicrobial activity against the two isolates. This results seems to compare favourably with related studies by Aibinu *et al*.,[52] Dahiya and Purkayastha,[53] showing varying antimicrobial activities of different plants extract against some Gram-positive and Gram-negative bacteria using methanol and aqueous extracts. Biswas *et al*.[11] also attributed variation in the activity of plant extract to extraction period, temperature of extraction, solvent concentration and polarity, solvent-to-sample ration. In this study, *K. pneumoniae* (with inhibition zone diameter of 1 mm in methanol extraction and 2 mm in aqueous extraction) was a little bit more resistant to the extract alone than *S. pneumoniae* (with inhibition zone diameter of 3.5 mm in methanol extraction and 3 mm in aqueous extraction) probably because of the differences in their cell wall. *K. pneumoniae* seems to have permeable barrier that restricted the penetration of the plant extract as compared to *S. pneumoniae*.[11] Also, the ability of honey to inhibit bacterial growth with high efficacy when tested alone and/or in combination with the extract may be attributable to phytochemical factors such as tetracycline derivatives, peroxides, amylase, fatty acids, phenols, ascorbic acid, terpenes, benzyl alcohol and benzoic acid.[20,54] Other factors could be their high osmotic nature, naturally low pH, the enzymatic production of hydrogen peroxide, the presence of secondary metabolites and improved absorption of bioactive compounds from *C. aconitifolius* leaf extracts and honey than when they are given in isolation.[11,54,55] This seems to agree with other related studies showing high antimicrobial potential of honey against some important pathogens.[20,54]

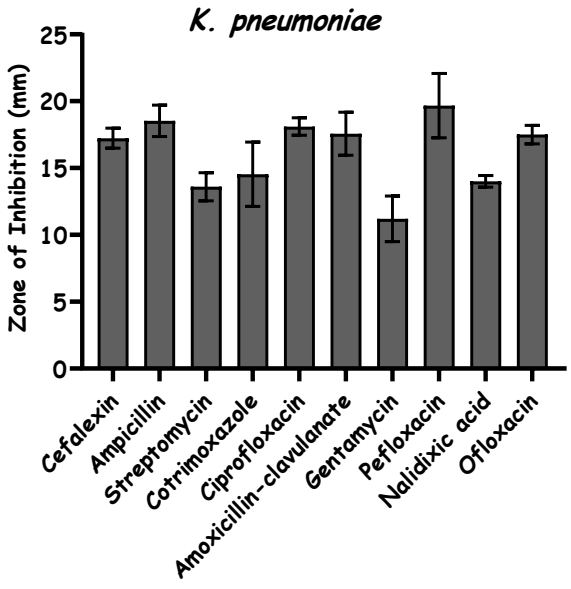

Fig. 5: Zone of inhibition produced by selected commercial antibiotics against *K. pneumonia*.

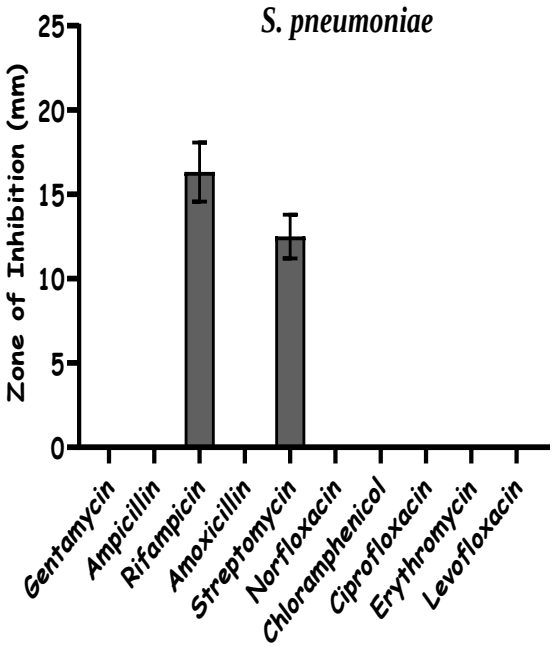

Fig. 6: Zone of inhibition produced by selected commercial antibiotics against *S. pneumoniae*.

Results of susceptibility test carried out using selected commercial antibiotics as positive control for Gram negative and positive organisms are presented in Fig. 5 and 6. The two isolates are presented as being "susceptible", "intermediate" or "resistant" to each antibiotic following the interpretative chart derived from the zones of inhibition of standard organisms in the Clinical Laboratory Science Institute.[39,56] As shown in Fig.5, the sensitivity result reveals that *K. pneumoniae* is susceptible to ampicillin, ofloxacin only showing intermediary susceptibility to cefalexin, streptomycin, ciprofloxacin, amoxicillin-clavulanate, nalidixic acid and resistant to cotrimoxazole, gentamycin, pefloxacin. In Fig. 6, *S. pneumoniae* is shown to have intermediary susceptibility to rifampicin only and resistance to gentamycin, ampicillin, amoxicillin, streptomycin, norfloxacin, chloramphenicol, ciprofloxacin, erythromycin, levofloxacin. This result is also consistent with the emerging antimicrobial resistance worldwide.[10] The outer membrane of *K pneumoniae* (gram-negative bacteria) is made up of phospholipids and lipopolysaccharides and therefore act as a barrier to the entry and response of most antimicrobial agents. The thick, multi-layered peptidoglycan component in the cell wall of *S. pneumoniae* (Gram-positive bacteria) seems not to allow for selective permeability of an antimicrobial agent.[10] Susceptibility of *K pneumoniae* to ampicillin, ofloxacin shown in Fig. 5 may be due to lesser use of these antibiotics as compared to other antibiotics. Other factors may include change in permeability target, metabolism, resistance by mutation, overexpression of efflux systems, composition of medium or condition of growth.[10,42,57,58]

CONCLUSIONS

This study demonstrates the effect of methanol and aqueous extract of *Cnidoscolus aconitifolius* leaf and honey combinations against *Klebsiella pneumoniae*, *Streptococcus pneumoniae* isolated from sputum samples. The antimicrobial effect may be attributed to the improved absorption of bioactive compounds from *C. aconitifolius* leaf extracts and honey than when they are given in isolation. The findings further support their various applications in traditional medical practice. Since *in vitro* experiences are limited for *C. aconitifolius* and honey combinations, further studies would be required in order to develop an acceptable antimicrobial agent for the treatment of infections caused by bacterial strains.

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