JOURNAL OF APITHERAPY, 2019
VOL 6, NO. 1, PAGE 1–5
10.5455/ja.20190812081333

ORIGINAL RESEARCH

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Determination of antimicrobial susceptibility of ethanol, methanol, and acetate extracts of processed honey

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ABSTRACT

Background and Aim: Honey is one of the oldest traditional medicines considered as the remedy for microbial infections. It is also recognized as an efficacious tropical antimicrobial agent in the treatment of burns and wounds. The aim of the study was to determine the antimicrobial susceptibility of ethanol, methanol, and acetate extracts of processed honey.

Materials and Methods: The processed honey sample was obtained from local market in Yenagoa. Test organisms were Gram-positive (*Staphylococcus aureus* and *Bacillus cereus*) and Gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella typhi*) bacteria and fungi (*Aspergillus niger*, *Mucor mucaralis*, *Tricophyton tonsurance*, *Microsporum ferrogenium*, and *Aspergillus flavus*). And, 10-fold serial dilutions were made and plated and antimicrobial activity was evaluated using the agar well-diffusion method.

Results: The results of antibacterial activity of the extracts against tested organisms show the diameter of inhibition zone ranging from 4 to 25 mm for *S. aureus*, 14.5 to 22 mm for *E. coli*, 16.9 to 20 mm for *S. typhi*, 1 to 17 mm for *B. cereus*, and 12.6 to 20 mm for *P. aeruginosa*, while for antifungal activity show the diameter of inhibition zone ranging from 10.2 to 15 mm for *A. niger*, *M. mucaralis* (4 to 7 mm), *T. tonsurance* (8 to 11 mm), *M. ferrogenium* (4 to 18 mm), and *A. flavus* (2 to 8 mm).

Conclusion: From the result, test bacteria were found to be more susceptible compared to fungi. The study showed that the honey has antimicrobial activity against test organisms and provides alternative therapy for the inhibition of these pathogenic microorganisms.

ARTICLE HISTORY

Received August 12, 2019
Accepted September 07, 2019
Published October 05, 2019

KEYWORDS

Acetate; ethanol; honey; methanol; microbial sensitivity test

Introduction

Natural products and their derivatives (including antibiotics) represent more than 50% of all drugs in clinical use in the world. According to World Health Organization estimates, about 80% of people living in developing countries rely on harvested wild plants for some part of their primary health care [1]. There are several reports on the antimicrobial activity of different herbal extracts in different regions of the world [2,3]. Due to the side effects and the resistance that pathogenic microorganisms have developed against antibiotics, recently much attention has been paid to extracts and biologically active compounds isolated from natural species used in the herbal medicine.

Honey is a natural substance derived from honey bees, *Apis mellifera* Linn. (Family: Apidae). It is a thick, syrupy, translucent, pale yellow or yellowish brown liquid which has a characteristics odour and a sweet, faintly acidic taste that assists in healing infected wounds, whether burns, ulcers, surgical incisions, or diabetic lesions [4,5]. The use of

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honey by the traditional medicine in the treatment of infections has been practiced since the origin of humanity, and honey is one of the oldest traditional remedies that medicine considers important in the treatment of several diseases [6,7].

The current antibiotic resistant microbial species, for example, *Pseudomonas* and *Klebsiella species* resistance to gentamicin, amikacin, and ceftazidine [8], and toxicity to conventional therapy is attributed to an evolutionary response to the widespread and indiscriminate use of antimicrobials [9,10]. Multidrug resistant bacteria poses treatment problem resulting in high morbidity, mortality, and increased health care costs [11], which have led to resurgence of ancient remedies. Honey has been used as a medicine in many cultures for a long time. However, it has a limited use in medicine due to lack of scientific support. It has been rediscovered by the medical profession and it is gaining acceptance as an antibacterial treatment of topical infections resulting from burns and wounds. It is well established that honey inhibits a broad spectrum of bacterial species. More recently, honey has been reported to have an inhibitory effect to around 60 species of bacteria, including aerobes and anaerobes, Gram-positives, and Gram-negatives [12]. There are many reports of bactericidal as well as bacteriostatic activity of honey and the antibacterial properties of honey may be particularly useful against bacteria, which have developed resistance to many antibiotics [13].

The aim of the research was to determine antimicrobial susceptibility of ethanol, methanol, and ethyl acetate extracts of processed honey.

Materials and Methods

Materials

Autoclave, water bath, Hot air oven, Microscope, Glass petri dishes, test tube inoculating needle, wire loop, Bunsen burner flame, biosafety cabinet, sterile swab stick, whatman filter paper no.102, measuring cylinder, conical flask, cotton wool, aluminum oil, forcep, and spatula.

Sample collection

Processed Honey was bought from local market in Yenagoa Bayelsa state, Nigeria.

Processed honey extraction

The extraction reagents were methanol, ethanol, ethyl acetate, and dimethyl sulphoxide/acetone nitrile. Ten grams of the honey sample was placed in a beaker and 25 ml of methanol added and mixed by vortexing. It was centrifuged at 3,000 rpm for 10 minutes. The supernant was collected and transferred to a stoppered test tube by filtration. The resulting supernant was evaporated to dryness with a gentle stream of nitrogen and reconstituted with 10 ml dimethyl sulphoxide and was mixed by vortexing. The same was repeated for that of ethanol and ethyl acetate.

Preparation of dried filter paper discs

Whatman filter paper no. 102 was used to prepare discs approximately 5 mm in diameter was perforated by a perforator, which are placed in a petri dish after sterilization in autoclave.

Processed honey extract disc placement

Processed Honey disc containing 3 ml (3 µl) concentration and as well as honey were made using filter paper and then placed on the plates using sterile forcep. One sterile antibiotic disc was placed on the surface of an agar plate, using a forcep. The forcep was sterilized by immersing in alcohol for 10 minutes each time before placing another antibiotic disc. The disc then was gently pressed with the forcep to ensure complete contact with the agar surface. The discs were placed away from the edge of the plates so that it is easily measured. Once all discs are in place, the plates were inverted, and placed in a 37°C incubator for 24 hours.

Bacteria/ fungi suspension preparation

Media used: Nutrient agar, buffered peptone water, shigella salmonella agar, macconky agar, bacillus agar, cetrimide agar, and all media were prepared according to the manufacturer's direction. Using a sterile inoculating loop and needle for bacteria and fungi, respectively, through aseptic techniques, the test organisms of each colony was taken from the subculture plate. The organism was suspended in 4ml of normal saline and vortexed for overall suspension. Mcfarlad standard solution was used as a reference to adjust the turbidity of individual bacterium isolate in the suspension (1×10^8). And, 10-fold serial dilutions was made and plated for the antimicrobial sensitivity test.

Inoculation of isolates on the nutrient agar plate proper

A sterile swab stick was dipped into the bacterial/ fungi suspension and the test organisms were suspended in 4 ml of buffered peptone water. The swab was rotated against the side of the tube using firm

pressure to remove excess fluid, but the swab was not dipped wet. The dried surface of the nutrient agar plate was inoculated by streaking the swab over the entire agar surface by rotating the plate at 60° each time to ensure an even distribution of the inoculum.

Results

Antibacterial susceptibility

Antibacterial susceptibility of Gram-positive bacteria, such as *Bacillus cereus* and *Staphylococcus aureus* shows the diameter of inhibition zone of 8.8, 17, 1 and 4, 25, 5 mm for ethanol, methanol, and ethyl acetate, respectively. While Gram-negative bacteria, *Pseudomonas aeroginosa*, *Salmonella typhi*, and *Escherichia coli* shows diameter of inhibition zone for ethanol extract 12.6, 16.9, and 14 mm, methanol extract 13, 20, and 22 mm, and ethyl acetate extract 20, 18, and 15 mm, respectively (Tables 1 and 2).

Antifungal susceptibility

Antifungal activity shows *Aspergillus niger*, *Mucor mucaralisi*, and *Tricophyton tonsurance* with diameter of inhibition zone of 11, 7, and 8 mm for ethanol extracts, methanol extract (15, 6, and 11 mm), and (10.5, 4, and 8 mm) for ethyl acetate. *Microsporum ferrogenium* and *Aspergillus flavus* shows diameter of inhibition zone of 5 and 8 mm for ethanol, 4 and 2 mm for methanol, and 18 and 4 mm for ethyl acetate extract respectively (Tables 3 and 4).

Discussion

In recent years, there has been an increasing search for new antimicrobial compounds due to the lack of efficiency, side effects, and interaction with other drug taken by patients. Besides, the antibacterial and antifungal used is seemed to be resistant toward pathogens as the lesion recurrent. Thus, this fact has driven the search for new antimicrobial agent from the natural compounds. Honey is one of the natural food products that have gained much attention among modern societies due to its multi-faceted properties, such as antioxidant, antimicrobial, anti-inflammatory, immunomodulatory, and anti-cancer effects [14].

The result of susceptibility of the extracts of processed honey having varying degree of antibacterial activity against Gram-positive species, such as *S. aureus* and *B. cereus* and Gram-negative species, such as *E. coli, Pseudomonas aeruginosa*, and *S. typhi* using methanol, ethanol, and ethyl acetate as shown in Table 2. Among the honey extracts studied, methanol shows maximum antibacterial activity, especially against *S. aureus*, *E. coli*, and *S. typhi*. The mean diameter of inhibition zones produced by the extract was

Table 1. Biochemical characteristics of Isolates used for the test.

S/N	Test Organisms	Biochemical Test										
		Gram rxn	Indole	MR	VP	Citrate	Slant	Butt	CO_2	H_2S	Catalase	Oxidase
1	*P. aeroginosa*	−ve	−ve	−ve	−ve	+ve	A	A	−ve	−ve	+ve	−ve
2	*B. cereus*	+ve	−ve	−ve	−ve	−ve	A	A	−ve	−ve	+ve	+ve
3	*S. typhi*	−ve	−ve	+ve	−ve	−ve	−ve	A	−ve	+ve	+ve	−ve
4	*E. coli*	−ve	+ve	+ve	+ve	−ve	−ve	+ve	+ve	−ve	+ve	−ve
5	*S. aureus*	+ve	−ve	+ve	+ve	+ve	A	A	−ve	−ve	+ve	−ve

+ve = Positive; −ve = Negative; A = Absent; R × n = Reaction; MR = Methyl red; VP = Voges Proskauer; H_2S = Hydrogen sulfide; CO_2 = Carbon dioxide, S/N = Serial number.

Table 2. Antibacterial activity of Processed Honey (Diameter of inhibition zone in mm) (Means ± SD).

S/N	Test organisms	Extracts		
		Ethanol	Methanol	Acetate
1	*P. aeroginosa*	12.6 ± 0.70	13 ± 0.30	20 ± 0.51
2	*B. cereus*	8.8 ± 0.74	17 ± 0.42	1 ± 0.24
3	*S. typhi*	16.9 ± 0.57	20 ± 0.68	18 ± 0.65
4	*E. coli*	14.5 ± 0.88	22 ± 0.69	15 ± 0.32
5	*S. aureus*	4 ± 0.47	25 ± 0.44	5 ± 0.21

S/N = Serial number.

Table 3. Antifungal activity Processed Honey (Diameter of inhibition zone in mm) (Means ± SD).

S/N	Test organisms	Extracts		
		Ethanol	Methanol	Acetate
1	*A. niger*	11 ± 0.77	15 ± 0.62	10.2 ± 0.50
2	*M. mucaralis*	7 ± 0.51	6 ± 0.25	4 ± 0.26
3	*T. tonsurance*	8 ± 0.69	11 ± 0.44	8 ± 0.49
4	*M. ferrogenium*	5 ± 0.42	4 ± 0.75	18 ± 0.58
5	*A. flavus*	8 ± 0.65	2 ± 0.32	4 ± 0.75

S/N = Serial number.

Table 4. Cultural characterization and identification of fungi isolates.

S/N	Test isolates	Pigmentation/culture characterization
1	*A. niger*	White, dull yellow (reverse) raised
2	*M. mucaralis*	Whitish grey, white (reverse)
3	*T. tonsurance*	Grey brown, dark brown (reverse)
4	*M. ferrogenium*	Creamy to buff coloured surface (no reverse)
5	*A. flavus*	Green, cream (no reverse), curled and raised

S/N = Serial number.

25, 22, and 20 mm, respectively. For ethyl acetate, *P. aeruginosa* shows the highest diameter of inhibition zone (20 mm) and *S. typhi* (18 mm). Meanwhile, ethanol extract shows the least antibacterial activity on the test organisms. This finding is in accordance with the works of Jerlin [15], Banfitebiyi et al. [16], Marcela et al. [17], and Zobida et al. [18].

The growth of fungi was also inhibited by the extracts of honey although to a lesser extent when compared to bacterial. Among the honey extracts, methanol extracts show the maximum diameter of inhibition zone of 15 mm for *A. niger* and 11 mm for *T. tonsurance.* Followed by ethanol extract with 11 mm for *A. niger* 8 mm for *T. tonsurance* and *A. flavus.* Ethyl acetate extract shows the diameter of inhibition zone of 18 mm for *M. ferrogenium* and 10.2 mm for *A. niger* (Table 3). This result is in agreement with reports of Zubaidah et al. [19], Wisal [20], and Siti et al. [21].

The susceptibility of some of these fungi to honey is of significance, as most of the fungi have been implicated in cases of immuno-compromised patients who frequently develop opportunistic infections [22]. As a general rule, an antimicrobial is considered active against both bacteria and fungi, if the zone of inhibition was greater than 6 mm [23]. Methanol extracts of the honey sample performed better than other extracts because the most antimicrobial active components in the sample are saturated organic molecules, which are non-polar. Phytochemical profile of an extract determines polarity of the compounds being extracted in a given solvent. Hence, for active lipophilic constituents that do not extract into the other extracts, methanol extraction provides more consistent antimicrobial activity which is promising method in future drug development. The rising interest in the use of natural products are mainly due to the expanding problem of antibiotic resistance in many bacterial species and the fact that some quarters of the population could have experienced some of the possible adverse side effects of many pharmaceutical products [24].

Conclusion

The study allowed us to determine the antimicrobial susceptibility of extracts of methanol, ethanol, and ethyl acetate of processed honey sample. From the result, methanol extract showed more potent activity than other organic extracts. The bacteria tested were found to be more susceptible compared to the fungi. The study showed that the honey has antimicrobial activity against test organisms. This confirms to us that bee origin honey has an important antimicrobial activity and can provide alternative therapy for the inhibition of these pathogenic microorganisms which can be used in modern medicine for the treatment of infections. Thus, the results obtained with the extracts of processed honey sample open very interesting perspectives to continue this work on the evaluation of its antiparasitic activity and the synergy action with synthetic antibiotic drugs. Also, a study on isolation of *Candida albicans* which is relevant in human mycosis is recommended.

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