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Synergistic Antimicrobial Effect of Tenegn Honey (*Trigona iridipennis)* and Garlic Against Standard and Clinical Pathogenic Bacterial Isolates

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Abstract: In Ethiopia, honey produced by *T. iridipennis* in combination with garlic is widely used for traditional treatment of different infections. The objective of this study was to evaluate the synergic antibacterial effect of *T. iridipennis* honey and garlic mixture against standard and clinical pathogenic bacteria. The antimicrobial activity of garlic extract, tenegn honey and mixture of tenegn honey and garlic extract against *Escherichia coli* (ATCC 25922), *Salmonella* NCTC 8385, *Listeria monocytogenes* (ATCC 19116), *Shigella flexneri* (ATCC 12022), *Shigella dysenteriae* (clinical isolate) and *Salmonella typhi (*clinical isolate) as well as *Staphylococcus aureus* (ATCC 25923) and *Streptococcus pneumonia* (ATCC 63) were determined. Inhibition zones of mixture of garlic extract and tenegn honey against all tested pathogens was significantly ($P \leq 0.05$) greater than garlic extract and tenegn honey. The diameters of zone of inhibitions against all tested pathogens ranged from 25 to 35 mm for mixture of garlic extract and tenegn honey as compared with 10 to 30 mm for chloramphenicol. There was no significant ($P \leq 0.05$) difference in MIC and MBC concentration of all antimicrobial substances against the tested Gram negative and Gram positive bacteria. The mixture of garlic extract and tenegn honey has the potential of a broad spectrum of activity against both Gram-positive and Gram-negative bacteria that leads to a new choice for the treatment of commonly encountered infectious diseases. *In vivo* study using model animals is important for further application.

Key words: Antimicrobial Activity · Garlic Extract · Pathogenic Bacteria · Synergic Effect · Tenegn Honey

INTRODUCTION

Honey has been used for treatment of different diseases starting from 2000 years ago [1]. Pure honey has shown bactericidal activity for many pathogenic organisms, including various Gram-negative and Gram-positive bacteria [2-4]. Generally, the use of honey as a medicine has continued into present-day medicine. Most of antimicrobial studies against pathogenic bacteria were investigated on honey produced by the honey bee. But the scientific investigation worked on the honey produced by stingless bee (*Trigona iridipennis*) is only limited on propolis of the honey [5].

Garlic (*Allium sativum)* is one of the most ancient cultivated herbs and is vegetatively propagated from cloves [6]. It has been used for thousands of years for cooking, medicinal and spiritual purposes around the world [7]. Garlic contains at least 33 sulfur compounds, several enzymes and minerals such as selenium [8]. It contains a higher concentration of sulfur compounds than any other *Allium* species. The sulfur compounds are responsible both for garlic's pungent odor and many of its medicinal effects. One of the most biologically active compounds, *allicin* (diallyl thiosulfanate or diallyl disulfide) is not active in garlic until it is crushed or cut; injury to the garlic bulb activates the enzyme allinase, which metabolizes alliin to allicin [9]. Allicin is further metabolized to vinyldithiines and this breakdown occurs within hours at room temperature and within minutes during cooking [10]. Allicin, which was first chemically isolated in the 1940's, has antimicrobial effects against many viruses, bacteria, fungi and parasites [7].

In Europe and India, garlic remedies are used to treat coughs, colds, hay fever and asthma. Many modern herbalists and folk healers still rely on garlic oil ear drops to heal children's' ear infection [11]. Garlic can inhibit and

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kill bacteria and fungi and it also lowers blood pressure, blood cholesterol and blood sugar. Moreover, garlic prevents blood clotting and contains anti-tumor properties. Garlic can also boost the immune system to fight off potential disease and maintain health [12]. Furthermore, it supports the activity of heart, stomach, circulation of blood and the lungs. Research suggests that garlic may help to prevent some forms of cancer, heart disease, strokes and viral infections [11]. Garlic alone can provide us with over two hundred unusual chemicals that have the capability of protecting the human body from a wide variety of diseases. The sulfur containing compounds found in garlic afford the human body with protection by stimulating the production of certain beneficial enzymes [13].

Garlic exerts have broad spectrum antimicrobial activity against many species of bacteria, virus, parasites, protozoan and fungi [14]. Crude garlic extracts exhibited activity against both Gram negative *(E. coli, Proteus spp, Salmonella, Serratia, Citrobacter, Enterobacter, Pseudomonas, Klebsiella)* and Gram positive *(Staphylococcus aureus, Streptococcus pneumoniae, Streptococcus sanguis, Group A Streptococcus, B. anthracis)* bacteria at room temperature, but there are no significant effects after the garlic is boiled for five minutes before testing [15]. According to Shobana *et al.* [16] extracts of garlic varieties inhibited the growth of enteric pathogens; *Escherichia coli*, *Proteus mirabilis, Salmonella typhi* and *Shigella flexneri.* All tested isolates of *Shigella* are susceptible to garlic extracts and no one shows resistance to garlic extracts [17].

As mentioned earlier, antimicrobial activity of honey produced by *Apis mellifera* has widely been studied [18-21]. In Ethiopia, honey produced by *T. iridipennis* is widely used for traditional treatment, such as respiratory ailments, surface infections and other diseases in line with treatments conducted using honeybee honey [22]. *T. iridipennis* honey may be effective to treat different infections at low concentrations. Therefore, the study of antimicrobial activity of *T. iridipennis* honey in combination with garlic extract is significant. This is because Ethiopian society uses *T. iridipennis* honey and garlic mixture to treat different types of diseases such as cold, cough, asthma, diarrhea and respiratory infections. Therefore, the objective of the present investigation was to study the synergic antibacterial effect of *T. iridipennis* honey and garlic mixture against standard and clinical pathogenic bacteria. The outcome of this investigation may be used as basic information to formulate antimicrobial substance from the combination of *T. iridipennis* honey with garlic extract.

MATERIALS AND METHODS

Garlic Collection and Extract Preparation: Matured fresh garlic (*Allium sativum* L.) bulbs were collected from markets in Gondar. Then the garlic bulbs were peeled, weighed (55 gm) and homogenized aseptically using a sterile mortar and pestle. The homogenized mixture was squeezed using sterile cheesecloth. Out of 55 gm garlic bulb, 18.95 gm juice which was considered as 100% in concentration was obtained [23]. From 100% garlic juice, 50, 25, 12.5 and 6.25% (v/v) were prepared by diluting the concentrated extract with appropriate volumes of sterile distilled water for determination of antimicrobial activity, MIC and MBC.

Source and Dilution of Honey: The *T. iridipennis* honey used in this study was obtained from Lie Armacheho, Southwest Ethiopia. The honey sample was aseptically filtered with a sterile mesh to remove debris. Pure (100.0%) honey was referred to as 'neat'. It was then diluted with sterile distilled water with the following concentrations; 12.5, 25.0 and 50.0% (v/v).

Test Microorganisms: All standard pathogenic bacteria cultures, namely, *Escherichia coli* (ATCC 25922), *Listeria moncytogenes* (ATCC 19116), *Shigella flexneri* (ATCC 12022), *Staphylococcus aureus* (ATCC 25923), *Proteus vulgaris* (ATCC 881), *Salmonella* (NCTC 8385) and *Streptococcus pneumoniae* (ATCC 63) were obtained from Ethiopian Health and Nutrition Research Institute (EHNRI), while *Shigella dysenteriae* (clinical isolate) and *Salmonella typhi (*clinical isolate), were obtained from University of Gondar Medical and Health Sciences Hospital. Most of them are commonly involved in causing gastroenteritis, pneumonia, wound and urinary tract infections. McFarland standard No. 0.5 was prepared according to Andrews [24].

Determination of Antibacterial Activity of Honey and Garlic Extract Mixture Using Agar Well Diffusion Assay: In brief, 2-3 colonies of the test organisms were aseptically inoculated into sterile saline solution and the turbidity was adjusted to the 0.5 McFarland's standard solution (a concentration of 1.5 X 10^8 CFU/ml) [24]. Wells of 6 mm in diameter, 4 mm deep and about 4 cm apart were punched in the Muller Hinton agar inoculated with the test organism [25]. 100µl of sample of honey and garlic extract separately with the concentration of 50% was added to the wells in the plates. The plates were incubated at 37 °C for 24 h. The mean diameters of inhibition zones were measured in mm and the results

were recorded. The inhibition zones with diameter less than 12 mm were considered as having no antibacterial activity. A positive control well was equally filled with chloramphenicol (31 µg/ml), while sterile distilled water was used as negative control.

Commercial Antibiotic Sensitivity Testing: The test microorganisms were also tested for their sensitivity against the commonly prescribed commercial antibiotics (30 µg/ml of chloramphenicol) [26] using similar methods for the determination of antimicrobial activity of honey and garlic extracts.

Determination of MIC and MBC: The minimum inhibitory concentration (MIC) of honey and garlic extracts was determined according to methods described by Shahidi [27] and Akinyemi *et al*. [28]. Extracts were diluted to concentrations ranging from 6.25 to 25% for garlic and honey and a mixture of garlic with honey (1:1 in v/v). 100 µl of the standard pathogenic and clinical bacterial inoculums were added to each dilution of honey, garlic extract and a mixture of both in nutrient broth tubes. Negative control tubes with no bacterial inoculation, were simultaneously maintained. Tubes were incubated aerobically at 37 °C for 24 h. The lowest concentration of the extract that produced no visible bacterial growth (turbidity) was recorded as the MIC. Dilutions showing no visible growth for the MIC were sub cultured onto a fresh MH agar plate and incubated at 37 °C for 24 h for the determination of minimum bactericidal concentration (MBC). The lowest concentration of the extract with no visible growth after incubation was taken as the minimum bactericidal concentration [29].

Data Analysis: All data were analyzed using SPSS version 16.0. Means and standard deviations of the triplicates analysis were calculated using analysis of variance (ANOVA) to determine the significant differences between the means followed by Duncan's Multiple range test ($p < 0.05$) when the F-test demonstrated significance. The statistically significant difference was defined as $p < 0.05$.

RESULTS AND DISCUSSION

In this study, mean of inhibition zones of mixture of garlic extract and tenegn honey against all tested pathogens was significantly ($P \leq 0.05$) greater than those of garlic extract and tenegn honey alone. The inhibition zones mean of garlic extract alone to all pathogenic bacteria was statistically ($P \leq 0.05$) greater than that of tenegn honey alone. Inhibition zones mean of chloramphenicol against all tested pathogenic bacteria was almost similar to that of mixture of garlic extract and tenegn honey (Table 1). The synergic antimicrobial effect of mixture of garlic extract and tenegn honey against all pathogenic bacteria was found to be greater than those of garlic extract and tenegn honey. That is may be the reason why the local society or community widely used the mixture of garlic and tenegn honey to treat different pathogenic bacterial infections.

The inhibition zones of mixture of garlic extract and tenegn honey, garlic extract and tenegn honey against standard and clinical pathogenic bacteria were presented in Fig. 1. The inhibition zone of mixture of garlic extract and tenegn honey against *E. coli* was 23 mm, while that of commerical chloramphenicol disk was 10 mm (Fig. 1). The synergic antimicrobial activity of mixture of garlic extract and tenegn honey against *E. coli* was significantly ($P = 0.05$) greater than commerical chloramphenicol. This implies that mixture of garlic extract and tenegn honey can serve as alternative for treatment of pathogenic bacterial infections.

The inhibition zone of mixture of garlic extract and tenegn honey against *S. aureus* (ATCC 25923) was statistically greater ($P \leq 0.05$) than other pathogenic bacteria. The inhibition zones of mixture of garlic extract and tenegn honey, against *S. flexneri* (ATCC 12022), *S. typhi* (clinical isolate) and *S. pneumoniae* (ATCC 63) were significantly ($P \leq 0.05$) less than that of *S. aureus* (ATCC 25923) but greater than those of the rest tested pathogenic bacteria. The inhibition zones of mixture of garlic extract and tenegn honey against *E. coli* and *Salmonella* NCTC 8385 were significantly ($P \leq 0.05$) less than those of the rest pathogens.

Table 1: Comparison of inhibition zones among mixture of garlic extract and tenegn honey, garlic extract and tenegn honey (50%) against standard and clinical pathogenic bacteria

Tested material	Sum of squares	Df	Meansquare	F	Sig.
Mixture of garlic extract and tenegn honey (equal volume) (50%)	424.667	7	60.667	66.182	0.00
Garlic extract (50%)	272.625	7	38.946	38.946	0.00
Tenegn honey (50)	108.000	7	15.429	15.429	0.00
Chloramphenicol (31 µg/ml)	426.667		61.445	66.256	0.00

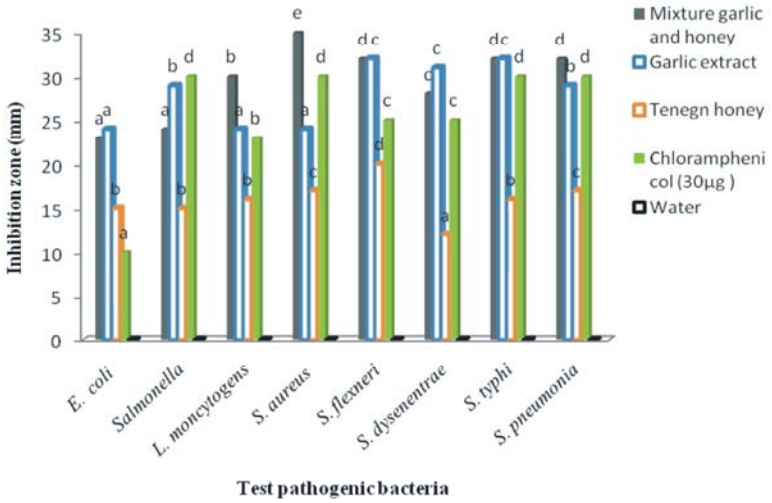

Fig. 1: Inhibitory zones of mixture of garlic extract and tenegn honey, garlic extract and tenegn honey against test pathogenic bacteria. Values are means of triplicate determinations. Values of the same color bars followed by different letters are significantly different at ($p < 0.05$)

According to this investigation, mixture of garlic extract and tenegn honey was more effective or potent against *S. aureus* (ATCC 25923), *S. flexneri* (ATCC 12022), *S. typhi* (clinical isolate) and *S. pneumoniae* (ATCC 63) than the other tested pathogens (Fig. 1). In rural area, the society traditionally use the mixture of garlic extract and tenegn honey to treat respiratory tract infections which are mostly associated with *S. pneumoniae* and *S. aureus* [30].

The inhibition zones of garlic extract against *S. flexneri* (ATCC 12022), *S. dysenteriae* (clinical isolate) and *S. typhi* (clinical isolate) were significantly ($P \leq 0.05$) greater than the rest of pathogenic bacteria. Inhibition zones of garlic extract to *Salmonella* NCTC 8385 and S. *pneumoniae* (ATCC 63) were significantly ($P \leq 0.05$) greater than those against *E. coli*, *L. monocytogenes* and *S. aureus* (ATCC 25923). The inhibition zone of tenegn honey against *S. flexneri* (ATCC 12022) was significantly ($P \leq 0.05$) greater than those against the rest of pathogenic bacteria. On the other hand, inhibition zone of tenegn honey to *S. dysenteriae* (clinical isolate) was statistically less than the rest of pathogenic bacteria (Fig. 1).

Aqueous extract of both the garlic varieties inhibited the growth of enteric pathogens; *E. coli*, *S. typhi and S. flexneri* at low concentrations [16]. According to Sadeghian and Ghazvini [17], garlic is an excellent natural antimicrobial drug that can be considered as an alternative form for treatment or prophylaxis in gastrointestinal infections.

In this study garlic was found to be effective against all pathogenic bacteria. All tested pathogens were also sensitive to tenegn honey. However, the mixture of garlic extract and tenegn honey was far effective against test pathogens than garlic extract and tenegn honey separately. The diameter of the zones of inhibition ranged from 24 to 32 mm for garlic, 25 to 35 mm for mixture of garlic extract and tenegn honey and 12 to 20 for tenegn honey as compared with 10 to 30 mm for chloramphenicol (Fig. 1), at used concentrations. The mixture of garlic extract and tenegn honey was the most effective against test pathogenic bacteria.

The diameters of the inhibition zones obtained against commonly used antibiotics were presented in table 2. The maximum inhibition zone of mixture of garlic extract and tenegn honey and garlic extract was greater than that of commercially used antibiotics against tested pathogens. Those commercial antibiotics are widely used to treat the infections caused by both Gram negative and Gram positive bacteria [31]. The mixture of garlic extract and tenegn honey may offer a new source of antibacterial agent against commonly encountered pathogens.

MIC of mixture of garlic extract and honey against *L. monocytogenes* (ATCC 19116) was 12.5% but in the rest of pathogenic bacteria, the MIC was 6.25%. On the other hand, MIC of tenegn honey against all tested pathogenic bacteria was 6.25% and that of garlic extract against *S. aureus* (ATCC 25923) was 12.5% but in the rest pathogenic bacteria, it was 6.25%.

MBC of mixture of garlic extract and honey against *S. dysenteriae* (clinical isolate) was 6.25% and in the rest pathogens MBC was 12.5%. MBC of tenegn honey against *S. aureus* (ATCC 25923),

Table 2: Antibiogram of commonly used commercial antibiotics against the test bacteria

Test pathogenic bacteria	Antibiotic	Inhibition zone (mm)
Salmonella NCTC 8385	C30	30
	CN10	30
	E15	10
	SXT	18
	Na	26
Staphylococcus aureus (ATCC 25923)	C30	32
	CN10	30
	E15	30
	SXT	20
	Na	22
Shigella dysenteriae (clinical isolate)	C30	25
	CN10	30
	SXT	10
	Na	20
	E15	15
*Salmonella typhi (*clinical isolate)	C30	20
	CN10	28
	E15	35
Streptococcus pneumonia (ATCC 63)	C30	24
	CN10	24
	E15	32
	SXT	25
	Na	23
Escherichia coli (ATCC 25922)	C30	10
	SXT	16
	Na	22
Listeria monocytogenes (ATCC 19116)	C30	23
	SXT	24
	Na	28
Shigella flexneri (ATCC 12022)	C30	25
	SXT	27
	Na	28

C = Chloramphenicol, CN = Cephalexin, E = Erythromycin, SXT = Trimethoprim-sulfamethoxazole, Na = Nalidixic Acid

Table 3: Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) determination of different concentrations of garlic extract, tenegn honey and mixture of garlic extract and tenegn honey (v/v)

	Mixture of Tenegn honey and garlic		Tenegn honey		Garlic extract	
Test organism	MIC mg/ml	MBC mg/ml	MIC mg/ml	MBC mg/ml	MIC mg/ml	MBC mg/ml
Escherichia coli (ATCC 25922)	62.5	125	62.5	125	62.5	62.5
Salmonella (NCTC 8385)	62.5	125	62.5	125	62.5	125
Listeria monocytogenes (ATCC 19116)	125	125	62.5	125	62.5	125
Staphylococcus aureus (ATCC 25923)	62.5	125	62.5	62.5	125	125
Shigella flexneri (ATCC 12022)	62.5	125	62.5	62.5	62.5	62.5
Shigella dysenteriae (clinical isolate)	62.5	625	62.5	125	62.5	62.5
*Salmonella typhi (*clinical isolate)	62.5	125	62.5	62.5	62.5	62.5
Streptococcus pneumonia (ATCC 63)	62.5	125	62.5	62.5	62.5	62.5

S. flexneri (ATCC 12022), *S. typhi (*clinical isolate) and *S. pneumonia* (ATCC 63) was 6.25%, while against, *E. coli* (ATCC 25922), *Salmonella* NCTC 8385, *L. monocytogenes* (ATCC 19116) and *S. dysenteriae* (clinical isolate) it was 125mg/ml. MBC of garlic extract against *Salmonella* NCTC 8385, *L. monocytogenes* (ATCC 19116) and *S. aureus* (ATCC 25923) was 12.5% but in rest pathogenic bacteria it was 62.5mg/ml (Table 3). In summary, 6.25% of combination of tenegn honey and garlic extract and garlic extract alone inhibited 87.5% of the total treated pathogens while 62.5mg/ml tenegn honey inhibited 100% of all pathogenic bacteria.

In this study, there was no significant (P = 0.05) difference in MIC concentration of all tested antimicrobial substances against Gram negative and Gram positive bacteria. This indicates that mixture of garlic extract and tenegn honey, garlic extract and tenegn honey have the potential of a broad spectrum of activity against both Gram-positive and Gram-negative bacteria. But we could see the variations in the sizes of the inhibition zones among the different group of bacteria.

As it was clearly presented and shown in the data for determination of MIC and MBC, the synergic antimicrobial action of garlic and tenegn honey mixture may be due to *allicin* in garlic and hydrogen peroxide in tenegn honey. Allicin is identified as the oxygenated sulfur compound, thio-2-propene-1-sulfinic acid S-allyl ester, which is generally referred to as allicin [32]. In brief, allicin interferes with RNA production and lipid synthesis. If RNA cannot be produced, or produced in less amount then protein synthesis will be severely affected. In short, no more protein synthesis takes place without the action of mRNA, rRNA and tRNA. Allicin also affects lipid synthesis and as the result phospholipids layer of the cell wall cannot be formed correctly in both Gram positive and Gram negative bacteria. All these things contribute a lot to impose on the growth of bacteria in the presence of allicin [24].

The antimicrobial role of honey is attributed to its high osmolarity, acidity (low pH) and content of hydrogen peroxide (H_2O_2) and non-peroxide components, i.e., the presence of phytochemical components like methylglyoxal [33-34]. The antimicrobial agents in honey are predominantly hydrogen peroxide, of which the concentration is determined by relative levels of glucose oxidase, synthesized by the bee and catalase originating from flower pollen [35]. The hydrogen peroxide and acidity produced by the reaction of glucose and oxygen (glucose + O_2 gluconic acid + H_2O_2) in honey might contribute a lot for inhibition and bactericidal activity against pathogenic bacteria.

The synergic effect of mixture of garlic extract and tenegn honey against pathogenic bacteria was evident in this study. Currently, the commonly prescribed commercial antibiotics are no longer effective to treat most pathogenic bacteria. Therefore, this study provides a new rationale and added inputs for the use of mixture of garlic extract and tenegn honey against infectious diseases caused by pathogenic bacteria. Further *in vivo* study using animal models and formulation of the product for treatment application are important.

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