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In Vitro Evaluation of Synergistic Anti-bacterial Effect of Raw Honey and Silk Cocoons Extract

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ABSTRACT: The study's objective is to ascertain the antibacterial activity of sericin and honey and their synergistic effect when used in combination. One of the largest challenges to world health, food security, and development progress is antibiotic resistance. It has been demonstrated that honey and sericin exhibit anti- bacterial effect on antibiotic-resistance strains. Considering the significant anti-microbial activity of honey and sericin, we have tested the synergistic anti-bacterial effect of raw honey and cocoons extract. Different concentrations of honey and sericin, the mixture was prepared to determine the anti-bacterial synergism. These samples were tested against bacterial strains of *E. coli, S. aureus, P. aeruginosa* and *Klebsiella* sp. The Kirby- Bauer disk diffusion technique was used for susceptibility testing. Over the MHA surface, the test organism was equally planted Microbial growth was assessed on MHA plates after 24 hours of incubation period. Honey and sericin inhibited the growth of all the test organisms. However, the endeavor is confronted by several challenges, standardization of raw materials is essential to account for variations, and method development must consider bacterial strains, optimal component ratio, and potential interactions. Overcoming has been done by standardization of the materials. When honey mixed with sericin, mixture showed higher antibacterial activity. Study showed that honey and sericin may be effective antibacterial future agents against resistant bacterial strains when used as combination. Sericin and honey have been found to provide health benefits for several ailments, such as microbial infections and wound healing.

Keywords: Antibacterial activity, Silkworm, cocoons, Sericin.

INTRODUCTION

Antibiotics provided a cure for bacterial infections, but not everywhere, coping with Methicillin-resistant Staphylococcus aureus and other antibiotic-resistant bacteria (MRSA) and Vancomycin- resistant pseudomonas has become a major health concern (Schneider, 2021). The efforts have been made by scientists in the different corners of the world to develop new compounds which may replace antibiotic therapy. The development of new antibiotics is difficult and expensive, requiring additional resources beyond the budget length, careful consideration of any potential side effects that could arise from their use. The usage of natural products has been reevaluated as one of the options. Among different natural substances honey, which is historically known for its non- toxicity and efficient medicinal value is the best suited substance of choice. Honey has a cherished role in the human diet because of its distinct flavour, nutritional value, and health-promoting characteristics. Although honey's antibacterial properties were discovered in 1892, there is now little evidence to support their application in modern medicine (Mohapatra *et al.,* 2011). The beekeeper's product with the greatest market potential is honey. More than 200 different chemicals can be found in honey, including 38% fructose, 31% glucose, 10% other sugar kinds, 18% water, and 3% other compounds. The product's best quality, however, is specifically the fantastic blend of chemicals in this 3%, with particular emphasis on phenolic and carotenoids compounds (Alvarez-Suarez *et al*., 2010). Using honey with an acidic pH of 3.2 to 4.5, many pathogenic organisms are inhibited, and the process of wound healing is accelerated through epithelization (Olaitan *et al*., 2007; White & Molan 2005). Honey has been shown to have antibacterial activity against a variety of antibiotic-resistant organisms, including *Combellobacter* species, *Shigella dysenteriae, Salmonella typhi*, and *Vibrio cholera* (Aurongzeb & Azim 2011).

Bombyx mori native to China represent a kept player for the global economy by providing silk with extraordinary properties. An organic polymer with a variety of amazing qualities is silk (Li *et al*., 2010) including toughness, flexibility, high strength, and biocompatibility with human organisms at an unprecedented level. Regarding the components of silk, fibroin and sericin, two major proteins, as well as a trace amount of an organic material soluble in alcohol

(Wang *et al.,* 2020). The middle silk glands of the mature silkworm larva are where the sericin protein, which accounts for 25–30% of silk proteins, is particularly biosynthesized (Aramwit *et al*., 2012). It is a family of globular proteins that are water soluble and have molecular masses between 10 and 310 KDa (Fan *et al*., 2009). Out of the yearly 1 million tonnes of fresh cocoons, there are around 50,000 tons of sericin that are thrown globally (Aramwit *et al*., 2012). Because of its nature, sericin can be utilized to make biomaterials, cosmetics, and medicinal items (Fan *et al*., 2009). Sericin also showed antioxidant, antimicrobial, antibacterial protection against ultraviolet radiations (Kato *et al*., 1998). Thus, the demand of plant and animal based antimicrobial agents is increasing day by day which forced us to design our study where antimicrobial activity of honey and silk worm cocoon extract (sericin) against few selected bacterial strains has been done. The present study was designed with the following objectives.

(i) Preparation of honey & silk cocoons extract.

(ii) Evaluation of synergistic antimicrobial effect of raw honey & silk cocoon extract.

MATERIAL AND METHODS

This project work was carried out at Kehloor Bioscience and Research Center (KBRC) Ghumarwin, Bilaspur.

A. Collection of cocoons of silkworm

The cocoons of Bombyx mori, silkworm procured from a local sericulture farm at Bilaspur and the raw honey collected from a local apiculture farmer of Bilaspur district, Himachal Pradesh.

B. Preparation of cocoons extracts (sericin)

Preparation of cocoons extract containing sericin accomplished by the modified method of (Chattopadhyay *et al*., 2005). The cocoons cut into small pieces to remove the dead larvae. 5g of chopped cocoons boiled in 200ml of distilled water for 2-3 hours. Using Whatman filter paper no. 1, the waste cocoons then filtered out to obtain sericin solution, and the aqueous solution was then collected in the screw caped tube. Two concentrations of the extract S_1 (1.5g/ml) and S_2 (2g/ml) were prepared Fig. 1. Aqueous extracts of silkworm cocoons (sericin)

(a) S_1 (1g/ml) **(b)** S_2 (1.5/ml)

Fig. 1.

C. Preparation of Honey Sample

4g of honey weighed and mixed into 1ml of sterile distilled water in an Eppendorf tube (4g/ml) and 3g of raw honey was added to 1 ml of sterile distilled water in another Eppendorf tube. The tubes then vigorously shaken and kept for the analysis.

D. Preparation of mixture of cocoons extract and raw honey

Mixture of honey and cocoons extract were prepared by mixing two different concentrations of honey with different concentration of cocoons extract. First concentration was prepared by adding 1g/ml from honey (H1) in 1g/ml of silkworm cocoon extract in a screw caped tube. Second concentration was prepared by adding 1.5g/ml of raw honey added from honey (H2) in 1.5g/ml of silkworm cocoon extract in a screw caped tube. The mixtures then shaken and kept for analysis.

E. Microbial strains used for the antibacterial analysis

Bacterial strains used for the antibacterial analysis were *E. coli*, *Klebsiella* sps., *Staphylococcus aureus* and *Pseudomonas aeruginosa*. These cultures of bacteria obtained from the KBRC laboratory, Ghumarwin.

F. In-vitro antimicrobial susceptibility testing

The antimicrobial susceptibility of the samples investigated using disc diffusion assay. The standard protocol of Kirby-Bauer disk diffusion method as described by Hudzicki followed with certain modification. The size of the zone of inhibition around the location of sample loading used to determine the results of the agar diffusion experiment.

G. MHA Medium Preparation

MHA considered the best medium for susceptibility testing of bacteria as it supports satisfactory growth of most nonfastidious bacteria and shows acceptable batch-to-batch reproductively for susceptibility testing. Also, it is low in trimethoprim, sulphonamide, and tetracycline inhibitors (Winn *et al*., 2006).

MHA was suspended in distilled water according to manufacture instructions and heated with frequent agitation and boiled for 1 minute to completely dissolve the components. The prepared medium for 15 minutes was autoclaved and cooled down at 40-50◦C. The agar was poured into sterile petri plates on a flat surface to a uniform depth of 4mm and allowed to solidify at room temperature.

H. Preparation of inoculums

Using a sterile inoculation loop, four or five colonies of a test organism suspended in 5ml of sterile saline and mixed well with the help of vortex to create a smooth suspension. The turbidity of this suspension was visually adjusted with 0.5 McFarland standards. This procedure repeated for each organism to be evaluated Fig. 2. 0.5 McFarland Standards and adjusted test organism.

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I. Inoculation of the MHA platee

A sterile swab dipped into the inoculum tube and inoculated the MHA plates by streaking the swab three times over the entire agar surface. Ensure an even distribution of the inoculums, the plates rotated approximately 60 degrees each time. The swab discarded into an appropriate container. Each MH agar plate appropriately labelled for each organism to be evaluated.

Placement of the samples and standard antibiotic discs on MHA plates: The antibiotic disc (tetracycline) as a control appropriately placed on the surface of the agar. The sterile discs (10mm, Himedia) dipped in honey and cocoons extract placed over the MHA plates. Along with these discs, disc dipped in the mixture of honey and cocoons extract also placed on the MHA plate. Discs were applied by using the aseptic technique. All the discs were placed on the surface of the agar plate with at least 24mm distance from each other. Each MHA plate appropriately labelled for each antimicrobial impregnated disc to be evaluated. After 18 hours, the plates were analysed after being immediately incubated at 37°C

Fig. 3. Deposition of samples disc on the culture.

Measurement of zone of inhibition: After 18 hours of incubation, the zones of inhibition were measured with the scale and recorded in mm.

RESULTS AND DISCUSSION

The Kirby-Bauer disc diffusion method in accordance with Clinical and Lab Standard Institute was employed in the antibacterial activity testing.

A. Activity of Honey against bacteria

The antibacterial activity of honey inhibited the growth of bacterial strains of *E. coli*, *S. aureus*, *Klebsiella* sp. And *P. auerginosa.* The maximum antibacterial activity of honey was observed against *Klebsiella sp.* followed by *S. aureus* in the concentration of 3g/ml of raw honey solution. The concentration of 4g/ml of raw honey showed the highest effective inhibitory activity against *S. aureus* as shown in Table 1 and 2.

B. Activity of silkworm cocoon extract

E. coli, S. aureus, Klebsiella sp., *and P. aeruginosa* were among the bacterial strains whose growth the silkworm cocoon extract prevented. Figure shows that the zone of inhibition for all four test species increases as cocoon extract concentration is increased.

The concentration of 1g/ml cocoon extract solution had shown lowest antibacterial activity. The maximum antibacterial activity was observed against *Klebsiella* sps.

The concentration of 2g/ml cocoon extract solution showed highest antibacterial activity. The maximum antibacterial activity was observed against *S. aureus*. It can be seen from Table 1 and 2 that zone of inhibition increases with increasing concentration of cocoon extract against all the four test organisms.

Table 1: Inhibitory activity of cocoons extract (1g/ml), raw honey (3g/ml) and their mixture.

Test organism	Zone of inhibition(mm)			
	Aqueous extract of silkworm cocoon (S_1)	Raw honey (H_1)	Raw honey + extract of silkworm cocoon (S_1+H_1)	Standard antibiotic (tetracycline)
E. coli	34	32	40	36
S. aureus	35	33	40	34
P. aeruginosa	34	32	38	33
Klebsiella sp.	39	38	40	36

Table 2: Inhibitory activity of cocoons extract (1.5g/ml), raw honey (4g/ml) and Mixture of raw honey and cocoons extra.

Test organism	Zone of inhibition(mm)			
	Aqueous extract of silkworm cocoon (S_2)	Raw honey (H_2)	Raw honey + extract of silkworm cocoon (S_2+H_2)	Standard antibiotic (tetracycline)
E. coli	49	48	51	32
S. aureus	52	50	52	34
P. aeruginosa	48	48	50	26
Klebsiella sp.	48	50	56	22

C. Anti-bacterial Synergism of raw honey and cocoons extract

When honey was mixed with cocoons extract, it showed very effective zone of inhibition in comparison with raw honey and cocoon extract solution. The antimicrobial activity of combined raw honey and silk worm cocoon extract inhibited the growth of bacterial strains of *E. coli, S. aureus, Klebsiella* sp. and *P. aeruginosa*. The maximum antibacterial activity was observed against *Klebsiella* sp. in both concentrations as shown in the Fig. 4. Antibacterial activity of honey (3g/ml), cocoons extract (sericin) (1g/ml) and their mixture against (a) *S. aureus* (b) *Pseudomonas* *aeruginosa* (c) *Klebsiella* sp. and (d) *E.coli*. Tetracycline has been included as positive control. and 4.1 Antibacterial activity of honey (4g/ml), cocoons extract (sericin) (1.5/ml) and their mixture against (a) *S. aureus* (b) *Pseudomonas aeruginosa* (c) *Klebsiella* sp. *and* (d) *E. coli*. Tetracycline has been included as positive control.

All the bacterial strains showed high sensitivity against tetracycline. The tested samples showed very effective inhibitory activity against all the strains in comparison with tetracycline.

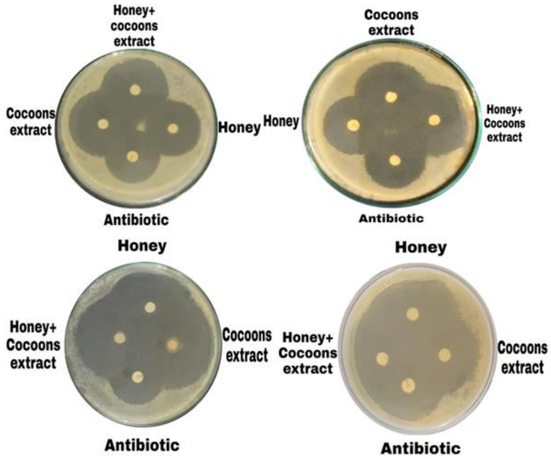

Fig. 4.

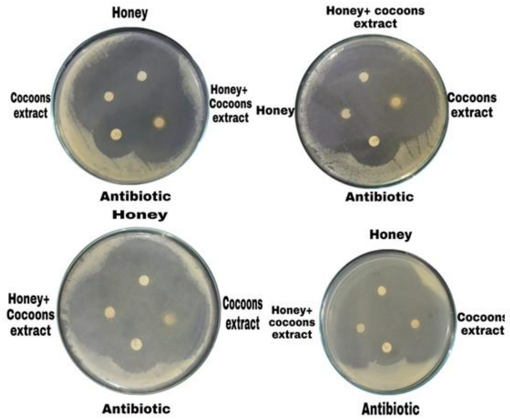

Fig. 4a.

CONCLUSIONS

The goal of the current investigation was to ascertain how honey and cocoon extract combined had an antibacterial effect on bacteria. Many studies have been published suggesting that honey (Mama *et al*., 2019) and sericin (Belhaj *et al*., 2012) exerts strong anti-bacterial activity. Additionally, as far as we are aware, no research has been done to look at the antibacterial synergism of honey and sericin. So, this is the first study to report the synergistic anti-bacterial effect of raw honey and Cocoon extract containing sericin on *E. coli, P. aeruginosa, S. aureus* and *Klebsiella sps*. (Belhaj *et al*., 2012) reported that raw honey has bactericidal effect against pathogenic micro-organisms such *as Salmonella Sp., Escherichia coli, Shigella* spp. and *Vibrio cholera.* (Abeshu and Geleta 2016) found that the honey exhibited anti-microbial, antioxidant and anti-inflammatory activities. (Abeshu and Geleta 2016) found the antimicrobial properties of honey against many bacteria. Various studies had reported antimicrobial potential of sericin present in silkworm cocoon (Mama *et al*., 2019) suggested that sericin suppress the growth of bacteria (Waraluk *et al.*, 2009). The *Bombyx mori* cocoon proteins contain several polypeptides which are amphipathic basic molecules that act as detergent on the microbial cell membranes causing death of the micro-organism by lysis (Pandiarajan *et al*., 2011). Previous studies on *Bombyx mori* sericin have reported the two antimicrobial proteins seroin-1 and serion-2 in sericin. However, conflicting results have also been obtained on sericin. According to certain studies, sericin promotes bacterial growth (Manjubala *et al*., 2020). Our findings go counter to past research that claimed sericin might encourage bacterial growth. Aurongzeb and Azim (2011) have reported that honey has inhibitory activity against 60 species of bacteria. It was found in their study that honey has the antibacterial activity against those bacteria which were resistant to some antibiotics (Amer and Abid-Alla 2021). reported that honey has anti-microbial properties against *methicillin-resistance staphylococcus aureus*.

In the present study the zone of inhibition increases with increasing concentration of honey and cocoons extract. Basualdo *et al*. (2007) reported that the bacterial effect of honey is dependent on the nature of honey and the concentration of honey. The higher the concentration of honey, the greater its usefulness as an antibacterial agent (Badawy *et al*., 2004). Depending on the type of honey, location, and botanical source, honey's antimicrobial properties will change (Sherlock *et al*., 2010). According to Johnston *et al.* (2018), gram-negative bacteria are more harmful than gramme positive bacteria. It is known that Staphylococcus aureus can develop resistance to most drugs (Mama *et al*., 2019). The efficacy of honey against *S. aureus* is dependent on the type of honey and the concentration at which it was administrated. The present study, *S. aureus* showed highest antimicrobial activity at the highest antimicrobial at the high concentration of honey in comparison with low concentration.

The results of the present study show that the bacterial strains are a bit less sensitive to the synergistic activity of honey and cocoons extract (sericin) in comparison with alone honey and cocoon extract (sericin).

The study was conducted to investigate the antimicrobial activity of aqueous extract of cocoons (sericin) and their synergistic effect when used with raw honey.

Sericin is a water-soluble protein extracted from the silkworm cocoon. After the degumming process, the silk and textile industries discard sericin as waste. The raw honey and silkworm cocoons were collected from local farmer of Bilaspur district of Himachal Pradesh. The potency of honey and sericin as an antimicrobial agent was demonstrated by the Disc-Diffusion Assay. In the present study, different concentrations of honey and sericin were prepared. The prepared concentrations of honey and sericin were mixed to determine their synergistic antibacterial effect. The bacterial strains were processed on MHA plates to enhance the growth of microbial strains. We measured the zone of inhibition produced by the discs and contrasted it with the zone of inhibition produced by tetracycline. Honey and cocoons extract (sericin) prevent the growth of the micro-organisms when applied in single and had synergistic antimicrobial effect when both the samples were mixed. The bacterial strains were processed on MHA plates to enhance the growth of microbial strains. Zone of inhibition of all the samples were observed and compared with the zone of inhibition created by tetracycline.

It was observed in the study that the mixture of Cocoons extracts and honey showed very effective inhibitory activity against all the four strains. It was observed in this study that all the four bacterial strains were a bit less sensitive to the activity of single honey and cocoon extract in comparison with mixture of honey and cocoon extract.

CONCLUSION

Finally, it was concluded that mixture of sericin and honey have efficient ability to inhibit the growth of bacterial strains. Therefore, honey and sericin could be used as a part of combination treatment for resistance bacterial infection.

FUTURE SCOPE

This study aims to determine synergistic anti-bacterial effect of honey and silk cocoons extract. Sericulture and apiculture are agro-based industries and has great capability to generate employment. The resultant by-products of silk-farming are often termed as “Sericulture waste”. The silk industries discard silk cocoons as waste after degumming process. Silkworm cocoons composed of fibroin and sericin are washed out without knowing its beneficial role. Sericin has high scientific and commercial values. Reusing cocoon waste products can help farmers' economies in addition to having a positive social, economic, and environmental impact. Silk sericin in combination with honey can be used in cosmetic formulation. When it is used in combination with Honey, it will show increased anti-bacterial effect. It could be applied to the care of burns and superficial wounds to lessen the risk of infection. It could be used as a part of natural anti-microbial therapy. Sericin combined with honey are thus suggested as a possible alternative therapy. The sericin may also form a smooth film for facial use. More research is required to use the sericin as bio-polymer films on the surface of the skin. Silk sericin films can be used in Food packaging, wound dressing materials and drug delivery carriers. Modern research comprises the use of the raw honey in edible coatings to increase the storage life of freshly cut fruits. The anti-microbial properties honey has another application that is edible coatings on fruits and vegetables.

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Conflict of Interest. None.

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