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Antimicrobial activity of '*Sharbat-unnab*' a classical Unani elixir

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Abstract
Sharbat-unnab is a liquid dosage form sweet in taste, prepared from the aqueous extract of *Unnab* (*Ziziphus jujuba* fruit), being prescribed in various types of fevers, skin diseases and for detoxification and pacification of blood in case of many infective and non-infective disorders. Plant derived antibiotics may be a possible alternative to modern antibiotics and at the same time can minimize the economic constrain of the hectic process of drug development. The ingredients of the test compound were procured from authentic dealer by Regional Research Institute of Unani Medicine (RRIUM) Srinagar. The extract was dissolved in $DMSO_4$ and subject to antimicrobial evaluation at the department of Pharmaceutical Sciences University of Kashmir. Results obtained in the present study showed that the test drug possesses promising antimicrobial activity against the selected microbes. The zone of inhibition of the test drug against the *S. typhi* was highest (32 milimeters) and good against *E. coli* (20 mm). As the antibacterial activity of the test drug was promising, it may validate the classical claims about use of this drug in case of various infective conditions and further studies are mandated.

Keywords: *Sharbat-unnab*, antimicrobial activity, Unani Medicine, *Artemesia absinthium*, Dafie-ufoonat

1. Introduction
Traditional medicine has been in use since time immemorial and is being practiced in various forms in almost every part of the world particularly Asia and Africa. Traditional medicine mostly uses medicinal plants for this purpose other than mineral and animal origin drugs. Plants are the greatest friends of human beings for various reasons; they have provided us with large number of effective drugs including morphine and many anticancer drugs [1-2]. The process of Ufoonat (infection) described in Unani medicine is what we know as infection today. Unani physicians prescribe *Dafi-ufoonat* (anti-infective), *Dafi-bukhar* (antipyretic) and *Muhallil-warm* (anti-inflammatory) drugs for treating various infective conditions. The indiscriminate use of modern antibiotics not only puts humans to the risk of development of numerous dangerous side effects, but also leads rapid development of antibiotic resistance in bacteria. This process renders the antibiotics useless and also drives the health economy. The concept of infections is described in Unani medical books as process of "Ufoonat" which literally means putrefaction. Ufoonat requires moisture and heat to develop [3]. There is a long discussion on infective fevers, their causes, nature and pattern of onset, in Unani medical literature [4]. A number of herbs are being used for treating infections of various types and fevers in this system of medicine. The drugs used for this purpose have been classified as *Dafi-ufoonat* (anti-infective), *Dafi-humma* (antipyretic), *Muhallil* (anti-inflammatory) and *Musaffi-dam* (blood purifier). These classes of drugs may be the possible leads for developing natural antibiotic drugs. Many of them have been proved to have antimicrobial properties in experimental studies [5]. In this study a well-known and frequently prescribed liquid dosage form *Sharbat-Unnab (SU)* was selected for evaluation of antimicrobial activity against four selected bacteria. SU is a compound drug with Unnab (*Ziziphus jujuba*) fruit as main ingredient. The reason for studying this drug was its use in febrile conditions and as blood purifier in Unani system of medicine. There are many such indications of this medicine which point out to its possible activity against bacteria and fungi. After preparing *SU* as per standard procedure mentioned in Unani Pharmacopeia [6-8]. Its hydro-alcoholic extract was obtained and subsequently tested for antimicrobial activity. The antimicrobial activity of the test drug was evaluated against four microbes, viz. *Staphylococcus aureus*, *Salmonella typhi*, *Bacillus subtilis* and *Escherichia coli*. A combination of gram positive and gram negative bacteria were used so that the test drugs could be evaluated for their possible anti-microbial activities against both classes of bacteria.

2. Materials and Methods

2.1 Plant material: The ingredients of Sharbat Unnab (SU) were procured from authentic dealer by Regional Research Institute of Unani Medicine (RRIUM) Srinagar. SU was prepared as per standard procedure [9] and guidelines in the Pharmacy section of Regional Research Institute of Unani Medicine Srinagar.

Table 1: Ingredients of Sharbat-e-Unnab

S. No.	Ingredients	Quantity
1.	Unnab Vilayati (*Ziziphus jujube* fruit)	0.46 kg
2.	Shakar safed (white sugar)	0.933kg to 1.39 kg
3	Water	1.86 litres

2.2 Identification of ingredients and the compound formulation: The ingredients of the test compound where identified prior to compound preparation and subsequently the compound formulation was also identified and authenticated by Dr Mohd Afsahul Kalam, Asst. Professor, Department of Ilmul Advia (Pharmacology), RRIUM Srinagar. The specimen of the ingredients and the compound drug was preserved in the phyto-chemistry lab. With voucher number S-2016.

2.3 Preparation of Extracts: The ingredients of test formulation SU were coarsely powdered in an electric grinder. The powder of each drug was extracted separately in 50% alcohol (hydro alcoholic) with the help of soxhlet's apparatus for 6 hours and then the extracts were dried in oven at 60ºC and were dissolved in DMSO (dimethyl-sulphoxide) to required concentration [10-11].

2.4 Mueller Hinton Agar (MHA)): Media was procured from HIMEDIA Mumbai in dehydrated form and reconstituted using distilled water as per directions of manufacturer. The composition of Mueller Hinton agar is as follows [12]; Meat infusion from 300g; 2.00gms/ltr Casein; acidhydrolysate 17.50gms/ltr, Starch 1.50gms/ltr, Agar 17.00gms/ltr.

2.5 Mueller Hinton Broth (MHB)

Dimethyl Sulphoxide (DMSO): This solvent was used for making test solutions of the extract and was also used as negative control [13].

Normal saline: 0.9% NS was used to make bacterial suspension of test bacteria of all the four strains used in the study.

Distilled Water: Distilled water was used for the preparation of MHA and MHB medium.

2.6 Test microorganisms: A panel of bacterial strains [bacteria: *staphylococcus aureus* (ATCC 6538P), *Bacillus subtilis* (ATCC 19659), *Eshcheria coli* (ATCC 8739), and *Salmonella typhimurium* (ATCC 13311) were used in the study. All bacterial strains were obtained from HiMedia Mumbai.

2.7 Mc Farland standard solution: For preparing a desired inoculum of 6 cfu/ml it has to be compared with 0.5 Mc Farland standard. This standard was prepared by adding 0.5 ml of 0.048 M $Bacl_2$ to 99.5 ml of 0.18 M H_2SO_4 (1%v/v) with constant stirring. After preparation the standard was transferred to sterile screw cap tubes of equal size and sealed tightly to prevent loss by evaporation. The standard solution was protected from light and stored at room temperature [14].

2.8 Revival of bacteria: Lyophilized bacterial cultures were revived as per the manufacturer's instructions [15]. Sterile MHB was poured into sterile culture tubes up to 4ml. The bacterial tube was opened under laminar hood. With the help of a cotton bud the bacteria were transferred to the broth medium and kept in incubator at 37 ^{0}C for 18 to 20hrs. Bacteria were revived on agar medium also. 20ml of sterile agar medium was poured in petri plate and after cooling down cotton bud was dipped in liquid bacteria and the bud was rubbed on the agar medium in streaking manner. The petri plate was kept in incubator at 37 ^{0}C for 18-20hrs. All the process was done under laminar hood.

2.9 Preparation of bacterial suspension: The bacterial suspensions were prepared by suspending freshly cultured bacterial strains in sterile normal saline (0.9%). The turbidity of the bacterial suspensions was adjusted to 0.5 McFarland standards on spectrophotometer at a wave length of 625nm. These suspensions were used within 30 min of preparation.

Glass ware: Sterile petri plates of 90mm, sterile culture tubes, sterile inoculating loop, sterile cork borer, funnel, beakers, conical flask, stirrer etc.

2.10 Positive control

1. Gentamicin: The Gentamicin and tetracycline antibiotic discs (SD016) were procured from HIMEDIA containing 10µg in each disc and served as positive control.

2.11 Agar well diffusion method: The bacterial activity tests were carried out using agar well diffusion assay [16]. After sterilization the tubes were cooled down so that a temperature of medium is reached above 45 to 50ºC and inoculation was done at this temperature. This inoculated medium was poured into sterile petri plates of 90mm to yield a uniform depth of 4mm. The media in petri plates was allowed to solidify under laminar hood. After solidifying the media sterile cork borer of 6mm in diameter was used to form uniform wells into each plate. Each petri plate contained four wells with the 40 micro liter volume of each concentration (10mg/Ml, 5mg/Ml,2.5mg/Ml and 1.25mg/Ml) of hydro-alcoholic extract of the test drug. The extract was dissolved in DMSO which also served as negative control. The positive control test discs of gentamycin (10 microgram per disc) and tetracycline (10microgram per disc) was also placed in petri plate. All the petri plates were kept for one hour for pre-diffusion of extracts before incubating. After 18 to 20 hours in incubator at 37^{0}C the plates were observed for zone of inhibition. The antibacterial activity of extract was measured by antibiotic scale and compared with the standard against the test microorganisms.

2.12 Minimum Inhibitory Concentration (MIC) determination

1. Macro Broth dilution method
2. Agar dilution method
3. For testing the minimum inhibitory concentration of the test drugs two tests broth dilution and Agar dilution were performed for cross checking the results [17].

2.13 Macro Broth Dilution Method: The MIC of test drug was determined by macro broth dilution method [18]. The different concentrations of the extract was prepared in DMSO and then 1ml of each concentration was poured into 5ml sterile Muller Hinton media tubes and then 1 micro liter of each bacterial suspension was poured into the MHB medium

and mixed thoroughly. Known inoculum of only positive control in one tube under laminar hood was taken. All the tubes were kept in incubator at 37 degree centigrade for 18 to 20 hours and then MIC was determined. MIC was determined as the lowest concentration of extract inhibiting visible growth of each organism in the tube.

2.14 Agar Dilution Method: 10ml of sterile MHA was poured in sterile petri plates under laminar hood and then the plates are allowed to solidify. 1ml of each extract at different concentrations was poured into plates along with 1ml of bacterial suspension. After pre-diffusion of extracts for 1 hour the plates were kept in incubator for 18 to 20 hours at 37°C. The MIC was determined by measuring the visible zone of inhibition of micro-organisms at different concentrations after comparing it with the growth at the bacterial spot [19-20]

3. Results

3.1 Microbial load: The microbial load of the extracts was simply checked by placing the sample drugs on sterile petri plates under Laminar hood and were kept in incubator for 24 hours duration and then examined for microbial contamination. No microbial contamination was shown by any extract after incubation period.

3.2 Antimicrobial activity: The antimicrobial activity of the test drug was carried at the department of Pharmaceutical sciences, University of Kashmir. The agar well diffusion method was employed to test the antimicrobial activity of the test drugs. After preparation of the agar medium as per standard procedure it was inoculated with test bacteria. The inoculated media was poured into sterile petri plates of 90 mm to yield a uniform depth of 4mm. The media in petri plates was allowed to solidify under laminar hood. After solidifying the media sterile cork borer of 6mm in diameter was used to form uniform wells into each plate. Each petri plate contained four wells with the 40 micro liter volume of each concentration (10mg/Ml, 5mg/Ml, 2.5mg/Ml and 1.25mg/Ml) of the extract. The extracts were dissolved in DMSO which also served as negative control. The positive control test discs of gentamycin (10 microgram per disc) and tetracycline (10 microgram per disc) was also placed in petri plate. All the petri plates were kept for one hour for prediffusion of extracts before incubating. After 18 to 20 hours in incubator at 37^{0}C the plates were observed for zone of inhibition. The antibacterial activity of extract and (SU) were measured by antibiotic scale and compared with the standard against the selected pathogens viz. *Staphylococcus aureus*, *Salmonella typhi*, *Bacillus subtilis* and *Escherichia coli*. The test drugs showed significant zone of inhibition against the selected microbes (Table 2,3 and 4).

HA extract of *Ziziphous jujuba* showed highest zone of inhibition (30mm) against S. aureus and 23mm zone of inhibition against *E.coli*.

3.2 Minimum inhibitory concentration (MIC): The minimum inhibitory concentration of the test drug was determined using Macro Broth dilution method and Agar dilution method.

The Minimum bactericidal concentration (MBC) of the same drug was calculated as 400mcg. The HAEZJ extract also showed MIC at 100 mcg to 200mcg against *S. aureus* and its MBC against the same organism was 400mcg. HAEZJ also showed MIC of 400mcg against *S. typhi*. MIC of the test drug were only determined for those bacterial strains against which the dugs showed good antibacterial activity.

The slight higher MIC and MBC of the test might be due to the fact that it is not a pure chemical but a crude extract with number of different chemicals. The actual chemical responsible for the antimicrobial activity of the test drug after separation might show the same activities at a much smaller concentration.

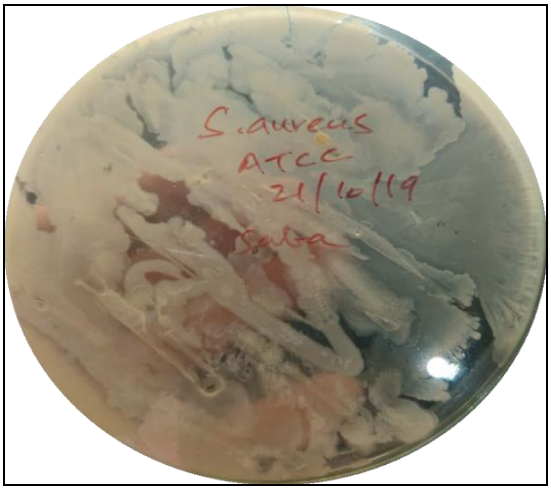

Fig 1: Media plate showing extensive growth of *S. aureus*

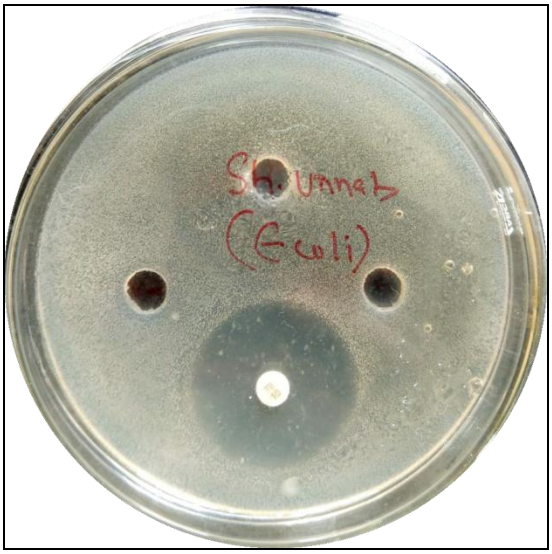

Fig 2: Zone of inhibition of test drug SU aginst *E. coli* compared with tetracycline

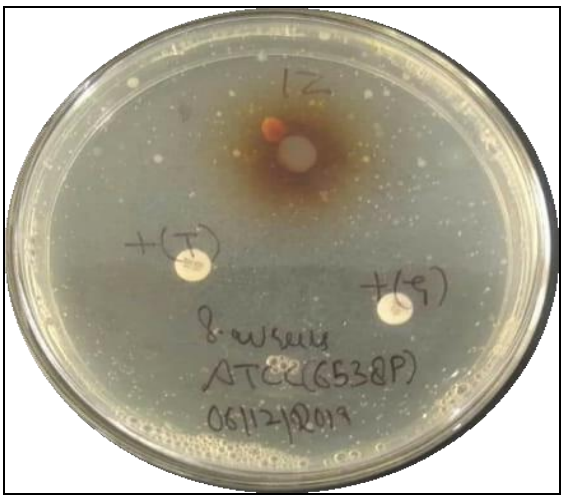

Fig 3: Agar plate showing inhibition of *S. aureus* by HAEZJ

Table 2: Antimicrobial activity of Hydro-alcoholic extract of *Ziziphus jujuba* (HAEZJM) against the four test bacteria

Microbes	Concentration of test drug (HAEZJM)(mg/ml)				Standard drug(positive control) (10µg)	
	1Z	2Z	3Z	4Z	TC	GM
	Zone of inhibition (mm)					
S.aureus	30	23	16	11	32	31
B. subtalis.	23	20	15	14	37	32
E.coli	23	21	18	14	24	23
S. typhi	25	22	16	13	38	30

Note: 1Z=10mg/ml, 2Z= 5mg/ml, 3Z= 2.5mg/ml. 4Z= 1.25./ml, TC is Tetracycline and GM is Gentamicin.

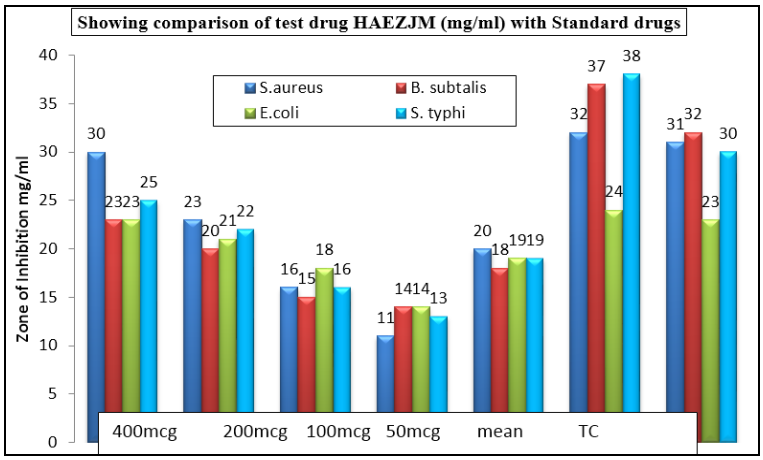

Fig 4: Comparison of test drug (HAEZJ) activity with standard drugs

Table 3: Comparison of antibacterial activity of HAEZJ and standard drugs against *Staphylococcus aureus*

Concentration of test drug (HAEZJ)	Zone of Inhibition (mm)	Zone of inhibition by test drug	
		Tetracycline (10µg)	Gentamicin (10µg)
10mg/ML	30	33	32
5mg/ML	23	30	30
2.5mg/ML	16	34	31
1.25mg/ML	11	31	31

Table 4: Comparison of antibacterial activity of HAEZJ and standard drugs against Salmonella typhi

Concentration of test drug (HAEZJ)	Zone of Inhibition (mm)	Zone of inhibition by test drug	
		Tetracycline (10µg)	Gentamicin(10µg)
10 mg/ML	25	37	30
5 mg/ML	22	39	28
2.5 mg/ML	16	38	34
1.25 mg/ML	13	38	28

From the above graphical analysis, representing the antimicrobial activity of test drug HAEZJM and standard drugs (tetracycline and gentamicin) against the four test organisms, it is evident that corresponding to each test organisms the maximum zone of inhibition is due to standard drugs tetracycline and gentamicin in comparison to average level zone of inhibition of test drugHAEZJM at different concentrations. It is also worth to note here, that with increase in concentration in test drugHAEZJM the zone of inhibition tends to increases against all the test organisms used in the study.

4. Discussion

Plants and other natural sources of complex range of chemicals can be promising in development of new drugs. Many researchers focus on identifying leads for drug development from chemicals like oils, saponins, alkaloids, and tannins etc. which occur naturally in plants in a diverse range [21-22].

There are a number of plant based drugs which show a wide range of antimicrobial activity, including activity against gram positive and gram negative as well as antifungal activities [23]. This fact forms basis for conducting researches in this direction, to continue our search for better and safe alternative antibiotics. Keeping in view the new challenges and trends for developing effective antibiotic substances, some authors suggest to search and develop antibiotic drugs from natural sources. Traditional medicines, including Unani medicine primarily use drugs of natural origin. Therefore they may be able to provide us a lead for developing new antibiotics [24-25].

We are on war with bacteria, while we develop new weapons to destroy them; they form new tactics to protect them. Pathogenic bacteria develop resistance to antibiotics through many ways, either by deactivating the drug or by protecting themselves from effect of the antibiotic drugs on them. Bacterial resistance may be a threat not only to human civilization but animals also, in case new drugs could not be developed against mutating bacteria. In that scenario natural drug sources may either be used on holistic approach basis to aid antibiotics in their action through synergism, or they can be a good alternative source of antibiotic leads owing to presence of vast range of active metabolites in them. Plants have shown antibacterial activity against many pathogens, and

also have been shown to act against multidrug resistant strains of NDM-1 group and others. All these findings encourage further studies on natural sources of drugs for development of alternative antibiotic agents [26].

In this study a compound formulation. Sharbat Unnab was selected for evaluation of antimicrobial activity. This syrup is being prescribed in various types of fevers, skin infections, and as blood purifier. A drug can be either directly antipyretic or indirectly by killing or stopping the growth of pathogens in the body which cause fever. As nature has provided plants with lots of chemically different constituents ranging from painkillers to anticancer drugs, our goal is to identify and separate the pharmacologically active constituents from plants traditionally used for different ailments since ages. The concept of infections is described in Unani medical books as process of "Ufoonat" which literally means putrefaction. Ufoonat requires moisture and heat to develop [27]. A number of herbs are being used for treating infections of various types and fevers in this system of medicine. Different drug classes in unani medicine are prescribed for infective conditions which may provide leads for developing herb based antibiotics.

The hydro-alcoholic extracts of the test drug showed significant zone of inhibition against the selected microbes. It showed highest activity against *staphylococcus aureus.* The test drug showed minimum activity against Bacillus subtilis, which is a good indication in other sense. The *B. subtilis* bacterium is basically more friendly then pathogenic to humans [28]. It has been proven to be normal probiotic organism, residing normally inhuman gut as a nonpathogenic and friendly commensal. As the test drug showed less activity against this strain, it indicated that the use of this drug in humans may not possibly harm the friendly bacteria in the intestines. Further studies on the test drug may be conducted to explore its antibiotic potential.

5. Conclusion

Following conclusions may be drawn from this research work.

The test drug Sharbat Unnab posseses significant antimicrobial activity.

Highest activity of HAE of *Sharbat-e-Unnab* was against Staphylococcus aureus *Sharbat-e-Unnab* in its original form showed significant activity against *E. Coli*. This form of the test drug could not be evaluated against other strains, as it was too viscous to be poured in the micropipette.

The MIC of HAEZJ ranges between 5 to 10mg/ML against *S, Typhi* and MBC of 400µg against the same bacterium.

The test drug showed no activity against *Bacillus subtilis*, indicating that oral intake of this drug may not be disturbing the normal flora of intestines and *B. subtilis* being one of them.

As the antibacterial activity of the test drugs was promising, it validates the classical claims about use of these drugs in case of various infective conditions.

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Conflict of Interest The authors express no conflict of interest

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