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Antimicrobial Activity of Propolis Extract on URT Infections in Pediatric Patients Admitted to Al-Thowrah Hospital, Hodeidah City, Yemen

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Abstract: The study was aimed at determining the causal microbial agents of URT infections in children and the susceptibility of isolates to antibiotics and propolis as a natural antimicrobial substance. A total of 17 throat swabs was obtained from children patients (aged up to 11 years) who were diagnosed with upper respiratory tract infections attending Al-Thawrah hospital, Hodeidah City. Samples were collected between April and June, 2011, cultured and the isolates were characterized by standard microbiological procedures. Of the 17 samples, 9 children had positive cultures with *Streptococcus pyogenes* having the highest prevalence (52.9 %), followed by 2 *Haemophilus influenzae* (11.8 %) and 6 isolates were identified as *Candida albicans* (35.3 %). The highest rate of *Streptococcus pyogenes* isolates was from the age group of 8 - 11 years. *Haemophilus influenzae* was not isolated in age group ≤ 3. The highest presence of *Candida albicans* was recorded in the age group ≤ 3. All isolates of *S. pyogenes* were susceptible to ciprofloxacin, amoxacillin and cephalixin. Some isolates showed resistance to ampicillin and erythromycin. The two isolates of *H. influenzae* were sensitive to ciprofloxacin, lincomycin and cephalixin, while erythromycin was ineffective. Nystatin recorded positive antifungal activity against the isolates of *C. albicans.* Propolis antimicrobial activity revealed that all isolates were sensitive. The growth of *S. pyogenes, H. influenzae* and *C. albicans* was inhibited by using propolis at MIC of 200 mg/ml with zones of inhibition of 24, 17 and 19 mm, respectively. The efficacy of propolis as a treatment of URT infections in 41 pediatric patients was also tested. An enhanced antimicrobial effect was obtained by using a mixture of propolis and goat's milk. A complete remission of both streptococcal and candidal symptoms was attained in a shorter time (2-5 days) than the time of recovery achieved by using each agent singly in all children. The findings suggest that propolis mixed with goat's milk is a very effective antimicrobial agent for the treatment and management of throat infections caused by bacterial and candidal species in children.

Key words: Pediatric · Throat Infection · Bacteria · Candida · Antibiotic-Resistant · Propolis · Antimicrobial Activity

INTRODUCTION

An upper respiratory tract infection (URTI) is a nonspecific term used to describe acute infections involving the nose, paranasal sinuses, pharynx, larynx, trachea and bronchi [1]. URTIs such as sore throat, ear ache, laryngitis, common cold, otitis media and sinusitis are the most frequently occurring infections of all human diseases and among the leading causes of health services worldwide and have been frequently documented [1-3]. Recurrent URTIs in children constitute a serious problem worldwide. Adults develop an average of two to four colds annually [1]. It has been reported that the majority of URTIs are of viral origin with rhinovirus, parainfluenza virus, coronavirus, adenovirus, respiratory syncytial virus and influenza virus accounting for most cases [4, 5]. Apart from viruses, bacterial pathogens have been reported to cause URTI and these include *Haemophilus influenzae, Staphylococcus aureus, Streptococcus pneumoniae, Klebsiella pneumoniae, Pseudomonas aeruginosa* and some Enterobacteriaceae [6, 7].

The overuse and misuse of antibiotics for URTl in patients is widespread and fuelled by public attitudes and expectations heralding the emergence of resistance by microorganisms [8]. Due to resistance to antibiotics by

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pathogens, recent research has been directed towards the use of traditional medicine/natural products for treatment and control of infections. Propolis is one of such products that are being tested on pathogens. It is a natural composite of balsam produced by honey bees (*Apis melifera*) from the gum of various plants [9]. The medicinal and antimicrobial properties of propolis have been widely reported and have a long history [10-14].

Due to the increasing rate of antibiotic resistances by most bacteria of respiratory infections, treatment and management of URTls which increase the risk of mortality and morbidity in patients have become difficult in the study area. Therefore, the antimicrobial activity of propolis, a product from honey bee, was reported in this study for its possible use for the treatment and control of different oral infections.

MATERIALS AND METHODS

Collection of Samples: A total of 17 throat swab samples from pediatric patients with throat infections attending Al-Thawrah hospital, Hodeidah City, Yemen was collected by trained personnel and samples were transported to the Microbiology Laboratory of the Department of Medical Laboratories, Faculty of Medical Sciences, Hodeidah University, Hodeidah City, Yemen for microbiological analysis. The diagnosed infections for the purpose of this study were common cold, acute pharyngitis, acute tonsillitis and oral candidiasis. The age of patients was up to 11 years and they gave their informed consent for this study. The samples were collected between April - June, 2011.

Microbiological Analysis: A loopful of each sample was inoculated into blood agar, chocolate agar and Sabouraud agar and incubated at 37°C for 24 - 48 h aerobically except for chocolate agar in which plates were incubated microaerophically [15]. After incubation, macroscopic and microscopic examinations of colonies were carried out, which were sub-cultured on the appropriate slants and stored at 4°C for biochemical and culture characterization for identification [16].

The Used Antibiotics: Ampicillin 10 µg, penicillin 10 µg, erythromycin 10 µg, ciprofloxacin 5µg, lincomycin10 µg, amoxacillin 25 µg and cephalixin 30 µg were used in the sensitivity test against the bacterial isolates. Nystatin 50 µg was used as antifungal against the isolated yeast.

Antibiotic Sensitivity Test: The antibiotic sensitivity of the isolates was determined by using the modified Kirby Bauer disc diffusion method [17]. All tests were performed on Muller-Hinton agar and were interpreted after incubation for 24 h at 37°C. The inhibition zones diameters (mm) measured around each disk were interpreted on the basis of guidelines published by the Clinical and Laboratory Standards Institute [18].

Extraction of Propolis: Propolis was obtained from a honey bee market located in Hodeidah City, the study area. The market is known for the collection, processing and selling of honey bee products. The whole sample of propolis (20 g) was frozen, ground and homogenized prior to beginning extraction. The dried propolis samples (20 g) were ground into fine powder, then mixed with 20 mL of 80% aqueous ethanol and shaken at 70 °C for 30 min. After extraction, the mixture was diluted to 100 ml with distilled water in a volumetric flask and finally centrifuged. The obtained ethanol extract of propolis (EEP) was used for antimicrobial tests [19, 20].

Antimicrobial Activity of Propolis: The Kirby-Bauer disc diffusion method was used as described [17]. Briefly, a small single well isolated colony was emulsified in 2 ml sterile saline and incubated at 37°C for 4 h to obtain the growing culture and the turbidity was adjusted to 0.5 Mc Farlandstandard. A sterile cotton swab with the adjusted suspension was used to evenly spread the entire surface of the Mueller- Hinton agar (Biotec Lab Ltd, UK) plates to obtain uniform inocula. The plates were dried for 2 - 4 min. Propolis impregnated disc were applied to the surface of inoculated plates with sterile forceps, ensuring complete contact of disc with agar. The plates were incubated at 37°C for 18-24 h and examined for zones of inhibition to the nearest mm. Resistance and sensitivity to propolis was measured [21]. When the diameter of the zone of inhibition produced by the antibiotic agent was 16 mm or higher, it was recorded as sensitive and resistant when less than 16 mm. Minimal inhibitory concentration (MIC) for propolis against the isolates were determined using ethanol extract of propolis (EEP) in serial concentrations: 0, 25, 50, 100, 200 and 300 mg/ml. Control plates with serial concentration of ethanolic alcohol solution were also tested. All tests were performed in triplicate. The efficacy of propolis for treatment of URTIs was tested in 41 pediatric patients at different ages up to 10 years old. Treatment of the disease symptoms was tested by using 20% ethanol propolis extract, goat's milk and a mixture of both agents at different doses.

RESULTS AND DISCUSSION

The study focused on the microbial pathogens of URTIs in patients and their sensitivity to propolis. The prevalence of bacteria and yeast isolated from throat swabs of patients in different age groups was shown in Table (1). All analyzed samples (17) had positive cultures. The identified bacterial isolates included *S. pyogenes* and *H. influenzae*. The only isolated yeast was *C. albicans*. The isolates of *S. pyogenes* represented the most frequent pathogen (52.9 %). The isolation rates of 11.8 % and 35.3 % were also noted for *H. influenzae* and *C. albicans,* respectively. The highest rate of *S. pyogenes* isolates was from the age group of 8 - 11 years. *H. influenzae* was not isolated in age group ≤ 3. While, the highest presence of *C. albicans* was recorded in the age group ≤ 3. Previous studies have reported these microbes as significant causes of URTIs [2, 22].

Respiratory diseases have been reported to be more common cause of death among children than diarrhea in the developing countries [23]. Many cases of URT1s are known to respond to antibiotics. However, due to overuse and misuse of antibiotics for URTIs by patients, there is an increasing rate of antibiotic resistance by most bacterial pathogens [24]. In this study, the antibiotic susceptibility of *S. pyogenes* isolates was tested (Table 2). The data showed that ciprofloxacin, amoxacillin and cephalixin were the most effective antibiotics against the tested bacterial isolates. Regarding *S. pyogenes*, out of 9 isolates, 4 - 5 isolates showed resistance to ampicillin and erythromycin. Two isolates were not affected by penicillin and lincomycin. The data revealed that the two isolates of *H. influenzae* were sensitive to ciprofloxacin, lincomycin and cephalixin, whereas they were resistant to erythromycin. The *in vitro* testing of nystatin recorded positive antifungal activity against all isolates of *C. albicans*. High prevalence of macrolides resistance in Streptococcus pyogenes has been recorded in several European countries [25]. A significant positive correlation between macrolides use and erythromycin resistance has been found in some studies using an ecological analysis; those studies correlated treatment with macrolides either with rates of erythromycin resistance of S. pyogenes in different areas (provinces, regions, or countries) or with a temporal trend of erythromycin resistance [26, 27]. *Haemophilus influenzae* was detected in about 40% of both nasal cavity (51 patients) and throat cultures (14 patients) and approximately 20% of these were BLNAR (β-lactamase-negative ampicillin-resistant *H. influenzae*. Whereas, *Streptococcus pyogenes* was

Table 1: Age distribution of 17 patients suffering from sore throat.

Age (years)	*S. pyogenes*	*H. influenzae*	*C. albicans*	Total
≤ 3	1	0	3	4
4 - 7	3	1	1	5
8 - 11	5	1	2	8
Total	9	2	6	

Table 2: Antibiotic susceptibility testing of *Streptococcus pyogenes, Haemophilus influenzae* and *Candida albicans* isolates.

	S. pyogenes n=9			*H. influenzae* n=2			*C. albicans* n=6		
Antibiotics	S	M	R	S	M	R	S	M	R
Ampicillin	4	1	4	1	-	1	ND	ND	ND
Penicillin	6	1	2	1	1	-	ND	ND	ND
Erythromycin	4	-	5	-	-	2	ND	ND	ND
Ciprofloxacin	8	1	-	2	-	-	ND	ND	ND
Lincomycin	5	2	2	2	-	-	ND	ND	ND
Amoxacillin	7	2	-	1	-	1	ND	ND	ND
Cephalixin	7	2	-	2	-	-	ND	ND	ND
Nystatin	ND	ND	ND	ND	ND	ND	6	-	-

S: Sensitive, M: Moderately resistant, R: Resistant, ND: Not detected

Table 3: Minimum inhibitory concentrations (MIC) of ethanolic extract of propolis.

Microorganisms	Zone of inhibition (mm)	MIC (mg/ml)
S. pyogenes	24	200
H. influenzae	17	200
C. albicans	19	200

Table 4: Clinical signs of URT infections in pediatric patients at different ages.

Age (year)	Number of children	Pathogen	Clinical signs
<1	10	*C. albicans*	White plaque on the tongue (thrush), no sucking
1-1.5	6	*C. albicans*	Thrush on the tongue, pain, refuse to feed
4	5	*S. pyogenes*	Hyperemia, painful throat, hard swallowing, T. 38-39°C
6	7	*S. pyogenes*	Hyperemia, painful throat, lymphadenitis, T. 38-39°C
9	6	*S. pyogenes*	Painful throat, lymphadenitis, hard eating, T. 38-39°C
10	7	*S. pyogenes*	Hyperemia, restless, lymphadenitis, hard eating, T. 38-39°C

Table 5: *In vivo* treatment of URT infections in pediatric patients at different ages with 20% ethanol propolis extract.

			Remission	
Age (year)	Infection signs	Dose of propolis (drops x time)	Level	Days
<1	Candidiasis	20x4	_	_
1-1.5	Candidiasis	20x4	_	_
4	Hyperemia	20x4	++	3-6
6	lymphadenitis	25x4	++	3-6
9	lymphadenitis	30x4	+++	3-6
10	lymphadenitis	30x4	+++	3-7

+++: high, ++: moderate, -: no response

Table 6: *In vivo* treatment of URT infections in pediatric patients at different ages with fresh goat's milk.

Age (year)	Oral infection signs	Dose of goat's milk (vol. x time)	Remission level	Remission days
<1	Candidiasis	5 ml x 2	+++	3
1-1.5	Candidiasis	10 ml x 2	+++	5
4	Hyperemia	10 ml x 2	_	_
6	lymphadenitis	10 ml x 2	_	_
9	lymphadenitis	10 ml x 2	_	_
10	lymphadenitis	10 ml x 2	_	_

+++: high, ++: moderate, -: no response

Table 7: *In vivo* treatment of URT infections in pediatric patients at different ages with a mixture of propolis and fresh goat's milk.

Age (year)	Oral infection signs	Dose of Propolis+goat's milk (drops + vol.) x time	Remission Level	Remission Days
<1	Candidiasis	(20+5 ml) x 4	+++	3
1-1.5	Candidiasis	(20+10 ml) x 4	+++	5
4	Hyperemia	(20+10 ml) x 4	++	3-5
6	lymphadenitis	(20+10 ml) x 4	++	3-5
9	lymphadenitis	(20+10 ml) x 4	+++	3-5
10	lymphadenitis	(20+10 ml) x 4	+++	3-5

+++: high, ++: moderate, -: no response

detected at a low rate in 2.0% of nasal cavity cultures and 7.1% of throat cultures [28]. Various antifungal agents are used in treatment of oral candidiasis; however, nystatin is the treatment of choice [29].

This increasing resistance has made it difficult for the treatment and management of URTI, which increases the risk for morbidity and mortality if treatment fails to eradicate the disease. Antimicrobial activity of propolis against URTIs has been reported [1, 9]. Popolis has been shown to have antimicrobial activity against bacterial pathogens of the oral cavity, respiratory tract and intestinal tract and even against protozoa and viruses [11, 30]. The propolis extract showed antimicrobial activity against all 3 tested microbial isolates (Table 3). All control plates including those with different ethanolic alcohol concentration and the negative controls, presented regular bacterial growth. Susceptibility was assessed with reference to CLSI guidelines [18]. *S. pyogenes, H. influenzae* and *C. albicans* were sensitive to propolis at MIC of 200 mg/ml with zones of inhibition of 24, 17 and 19 mm, respectively. Our results showed that propolis extract presented "*in- vitro*" antimicrobial activity to *S. pyogenes* and *H. influenzae*. In an earlier study, *H. influenzae, K. pneumoniae, S. pneumoniae* were found to be more sensitive to propolis at MIC of 1.0, 2.0 and 2.0 ìg/ml respectively with zones of inhibition of 26, 32 and 30 mm each. *M. catarrhalis* and *S. pyogenes* were the least sensitive with MIC of 0.5 and 8.0 ìg/ml, respectively with zones of inhibition of 10 mm each [22]. Although the number of isolates used in our study might be too small to draw meaningful conclusion on susceptibility pattern, it however provided baseline data for future studies. The result of these findings are therefore of clinical and epidemiological significance.

The efficacy of propolis as a treatment of URTI infections in 41 pediatric patients was tested. Table 4 showed the clinical signs of the observed infections at different ages up to 10 years old. Babies aged less than 1.5 years were suffering from candidal thrush symptoms. In the older children (4-10 years), streptococcal infections were the most common in the form of hyperemia, painful throat and lymphadenitis. Treatment of throat infections in pediatric patients with 20% ethanol propolis extract was effective against the clinical signs caused by streptococcal infection (3-7 days), but not against candidal thrush (Table 5). In comparison, the effect of goat's milk against URT infections was also tested (Table 6). Its antimicrobial activity was only recorded against candidiasis which was completely recovered within 3-5 days. An enhanced antimicrobial effect was obtained by using a mixture of propolis and goat's milk (Table 7). A complete remission of both streptococcal and candidal symptoms was attained in a shorter time (2-5 days) in all children.

Ethanolic extracts of propolis samples showed high antibacterial activity against Gram-positive cocci; *Staphylococcus aureus*, but had a weak activity against Gram-negative bacteria; *Escherichia coli* and *Pseudomonas aeruginosa* and yeast; *Candida albicans* [12]. *Candida albicans* is susceptible *in vitro* to EPE [31, 32]. The Brazilian commercial ethanol propolis extract, also formulated to ensure physical and chemical stability, was found to inhibit oral candidiasis in 12 denture-bearing patients with prosthesis stomatitis candidiasis association [33]. Soft and purified propolis extracts possess antimicrobial activity due to phenolic compounds. They could be recommended as natural preservatives in the manufacture of pharmaceutical products [13]. A semisolid emulsion system containing propolis and/or lysozyme was tested for antimicrobial and antimycotic properties. It was reported that the application of propolis and lysozyme as active substances may increase the antimycotic and antibacterial effects of the studied preparations [14].

The total antibacterial effect of goat and sheep milk proteins was reported to be greater than the sum of the individual contributions of immunoglobulin and

nonimmunoglobulin defense proteins such as lactoferrin (LF), lactoperoxidase (LP), lysozyme and other peptides [34]. Lactoperoxidase (LP), a protein present in goat milk is found to be effective against a battery of bacteria causing cholera (*Vibrio cholera*), typhoid (*Salmonella typhi*), pneumonia (*Klebsiella pneumoniae*), dysentery (*Shigella dysenteriae*) and food poisoning (*Staphylococcus aureus*) [35]. Therefore, the enhanced remission activity of propolis mixed with goat's milk recorded in our study could be attributed to a synergistic effect of all antimicrobial components present in the tested mixture.

The result of this study is promising in the treatment and management of bacterial and yeast pathogens of URTIs. Further studies with more significant patient numbers are necessary for the statistical confirmation of these results.

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