Antifungal Effect of Hubballi Propolis on *Candida albicans* - An In Vitro Study

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ABSTRACT

BACKGROUND

As the longevity of human lives has increased, the geriatric population is increasing demonstrating more number of oral candidal infections due to decreased immunity. Natural products are being investigated to be used to treat oral candidiasis in place of synthetic drugs in selective geriatric cases. Propolis is one such natural product, which is time tested and developed by nature as an antimicrobial agent. Its toxicity is very less. It can be used in multiple forms. It has the antioxidant, anti-inflammatory properties apart from antibiotic properties and potency of the material improves as it matures with time. But the constituents of Propolis differ with the different sources of procurement. We wanted to evaluate the efficacy of the Propolis procured from Hubballi against *Candida albicans* in this study.

METHODS

In this invitro study, Hubballi Propolis was extracted by maceration and refluxing. Water and 70 % ethanol were used as extraction solvents. Total Phenolic and flavonoid contents were determined by using Folin - Ciocalteu spectrophotometric method and Aluminium chloride colorimetric method respectively. Antimicrobial sensitivity effect of Propolis was estimated by determining the minimum inhibitory concentration (MIC) of the samples.

RESULTS

The results of the studies revealed that propolis samples have effective antifungal activity against *Candida albicans* with MIC range from 0.01mg / ml to 0.03 mg / ml and comparable high total phenolic (TP) and total flavonoid (TF) contents ranging from 175.4 ± 5.7 to 192.2 ± 3.3 and 33.08 ± 10 to 31.73 ± 8.5 mg / ml respectively.

CONCLUSIONS

Hubballi Propolis can be used for treating Candidal infection. Further, water extract Propolis showed better in total phenolic content (TPC) and total flavonoid content (TFC) than the alcohol extract. This finding is important to overcome the disadvantage of alcohol extract and hence very useful for application in Dentistry, cosmetics and pharmaceutical industry.

KEY WORDS

Antifungal Activity, Hubballi Propolis, Flavonoid Content, Phenolic Content

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BACKGROUND

Geriatric patients due to decreased immunity are more prone to oral candidiasis, which can interfere or cause systemic diseases. At times oral candidiasis becomes resistant to any type of drug or treatment modality and it may spread to the other organs and be fatal.^{1,2} For effective reduction of oral candidiasis (denture stomatitis) in the geriatric patients, the dental treatment protocol relies on mechanical control of *Candida* biofilms, depletion of predisposing factors and antifungal drug therapy. Antifungal therapy has shown good results. The available synthetic antifungal agents along with their action on candida may have unfavourable side effects. Further candida species are developing resistance to the synthetic antibiotics.² To overcome the drawbacks of current treatment with synthetic antifungal agent, there is a shift in the research to use natural products. One such natural product or the raw material is Propolis.³ Propolis (bee glue) is a generic name.^{4,5} It is non-toxic and has no-effect level (NOEL) of 1400 mg / kg body weight / day.^{6,7} It is recommended not to be utilized by patients who are sensitive towards pollen and nectar. The compounds present in the propolis resin originate from a mixture of⁸ substances that are actively secreted by plants and are collected by bees and substances derived from bee saliva.

As the bioactive components required for propolis activity is made of components (phenolic and flavonoid) from plant and insect origin this material is least toxic and a rich source of nutrients. Hence propolis is in high demand worldwide. But its bioactive components are foraged from the vegetation by the bees, so their composition varies depending on the location and the season of collection of propolis from the hive. Hence, it is imperative that the composition of propolis obtained at different locations is verified and antifungal activity is analysed before it is used as medication in dentistry.

In this endeavour, the purpose of this study was to evaluate antifungal activity (minimum inhibitory concentration) of Hubballi Propolis. Along with antifungal activity, its total phenolic content and total flavonoid content also needs to be studied as phenolic content is considered the active component for its action. Hubballi Propolis is an unexplored propolis which is considered in this study because of the geographic features like extensive green coverage, unique and highly diverse tropical forest ecosystem in Hubballi.

METHODS

This research involved in vitro procedures to assess antifungal efficacy against *Candida albicans* and total phenolic and total flavonoid content of the propolis. The research protocol was approved by the Scientific Technical Committee, JSS Dental College & Hospital, JSS AHER, and Mysore. The study was carried out for a period of one year from Dec 2019 to Nov 2020.

Materials

Aluminum trichloride (AlCl₃), Folin Ciocalteu's phenol reagent, sodium carbonate (Na₂CO₃), gallic acid, DPPH (3, 3-

diphenyl-1-picrylhydrazyl) and ascorbic acid. All chemical and instruments were of analytical grade and available commercially. The UV-visible spectrophotometric values were recorded in UV - 500 Spectrophotometer.

Study Sample

The selection procedure of the study samples was based on the following inclusion and exclusion criteria to procure best quality propolis.

Inclusion Criteria

1. Samples collected from spring (Mar to May) to autumn (Oct to Nov) season.
2. Sample collected from certified single apiary.
3. The sample collected by manual scraping technique only.
4. The samples collected from the wooden hive.
5. Samples should have one collection date.

Exclusion Criteria

1. The samples mixed between collection dates or hives were rejected.
2. Insufficient quantity (less than 50 gms) of sample was rejected as it was difficult to carry out the analysis.

Based on the inclusion and exclusion criteria the raw propolis was collected from a government certified apiary. The sample was semisolid in consistency. It was cut in to small pieces, finely grounded, divided into two equal parts, transferred to zip lock freezer bags and labelled before storage in the freezer.

Extraction of Propolis

The chemical compounds (quantitative and qualitative) of Propolis play a significant role in its antifungal activity. It is not used in the raw form, but is purified by extraction with solvents. Therefore, the extraction techniques should not harm the important compounds, especially flavonoid and phenolic. As there are no standard protocols for extraction procedure, maceration method (Figure 1), followed by refluxing was done. Literature revealed that following these methods the chemical structure of propolis is not altered and the techniques are very simple, affordable and can be used as routine extraction procedure.⁹⁻¹⁶ Commonly used solvents are water and ethanol in different percentages. Hence in this study both water extract propolis (WEP) and 70 % ethanol extract propolis (AEP) were used to know the best extraction solvent for propolis.

Preparation of WEP

The Hubballi Propolis sample was dissolved in distilled water for the WEP sample of Propolis. The sample and solvent was standardised at the rate of 10 ml water for every 1 gm of sample. The solution was macerated (extraction of drug by allowing it to stand in contact with solvent) for 24 hrs at room temperature. Following this the samples were refluxed (to boil the substance in such a way that the vapour formed returns to

the stock of liquid after condensing) with water condenser for 3 hrs at 100 degrees boiling water and then cooled at room temperature for 6 hrs. The solutions obtained were filtered through Whatmann filter paper no. 41. The filtrate was stored in a separate container and placed in the refrigerator at 4°C. The extraction cycle was repeated twice with the residue obtained from the first filtration process. This was done to obtain all the minute trace amounts of propolis that was left over from previous cycle. Combined filtrates were concentrated and dried on boiling water bath.

From the concentrated sample (Figure 2), 1 mg / ml stock solution was prepared. This stock solution was used for analysing the total phenolic content, total flavonoid content and antifungal activity.

Preparation of AEP

The procedure for 70 % ethanol extract of propolis was similar to water extract, except that 70 % ethanol was used instead of distilled water as solvent.

Determination of Total Phenolic Content by Folin - Ciocalteu (FC) Colorimetric Method

The procedure used is based on the methods outlined by Folin-Ciocalteu. The method is based on an oxidation-reduction reaction in alkaline conditions, where the phenolate ion is oxidized while Folin's reagent is reduced, turning the solution blue. Many of the active components in propolis, such as phenolic acids and flavonoids, have a phenolic nucleus and can be evaluated by this method.¹⁶ Procedure was followed as explained in Mruthunjaya, K (2008)¹⁷ method. The content of total phenolic compounds was expressed as mg / g gallic acid equivalent (GAE) of propolis

Determination of Total Flavonoid Content by Aluminium Chloride Colorimetric Method

The Aluminium chloride colorimetric method was modified from the procedure reported by Woisky and Salatino¹⁰ and the analysis was carried. The total flavonoid content was calculated according to the standard Quercetin calibration curve. The mean of three readings was used and expressed as mg of Quercetin equivalents (QE) / g of Propolis.

Determination of Minimum Inhibitory Concentration (MIC)¹⁸

The test determines the minimum quantity of drug required to inhibit the fungal growth. It is considered as gold standard for determining the susceptibility of organism to antimicrobial agents. Procedure -

- The brain heart infusion (BHI) broth was prepared
- (Calf brain, beef heart infusion, Proteose peptone, Dextrose, Sodium chloride and Disodium phosphate final pH (AT 25 ° C) 7.4 + / -0.2)
- Serial dilution of broth and stock solution was carried out. (10 sterile eppendorf tubes were taken. In the 1st tube 300 µl of broth and 100 µl of 2 mg / ml stock solution were added and mixed well. For the 2nd tube 200µl of broth

and 200 µl of solution from the 1st tube was transferred. For the 3rd tube 200 µl of broth and 200 µl of solution from the 2nd tube was transferred. This was serially repeated up to 10th tube. 200 µl was discarded from the 10th tube. This resulted in serial dilution of the concentration of the broth and stock solution.)

- All the tubes were inoculated with constant amount of test bacteria as per the groups
- The test tubes were incubated at 37° C for 24 hrs in an incubator.
- MIC was determined by observing the turbidity after 24 hrs.
- The growth in each test tube was evaluated and recorded.
- The data was analysed by comparison with the control group

Statistical Analysis

Each procedure was carried out in triplicate. At the end of each procedure, the data were entered. Data were scrutinised and analysed using the SPSS software. Descriptive statistics, mean and standard deviations are represented in the tables.

RESULTS

Total Phenolic Content	Total Flavonoid Content
192.2 ± 0.33 mg of Gallic acid equivalent value per gram	33.08 ± 1 mg of Quercetin equivalent value per gram
Table 1. Total Phenolic Content and Total Flavonoid Content of Propolis (WEP) from Hubballi	
Values are expressed as Mean ± SD for triplicate	

Microorganisms	Concentration of Extract mg / ml									
<i>Candida albicans</i>	0.5	0.25	0.12	0.06	0.031	0.01	0.008	0.004	0.002	0.001
	S	S	S	S	S	S	R	R	R	R
Table 2. Minimum Inhibitory Concentration (MIC) of Propolis (WEP) from Hubballi										

Table 2 shows the sensitivity of *Candida albicans* to the water extract Hubballi propolis. 0.01mg / ml of Hubballi propolis is the minimum amount required to inhibit the growth of *Candida albicans*.

Total Phenolic Content	Total Flavonoid Content
175.4 ± 0.57 mg of Gallic acid equivalent value per gram	31.73 ± 0.85 mg of Quercetin equivalent value per gram
Table 3. Total Phenolic Content and Total Flavonoid Content of Propolis (AEP) from Hubballi	
Values are expressed Mean ± SD for triplicate	

Table 3 shows the total phenolic content and total flavonoid content of 70 % Ethanol extract Hubballi propolis.

Microorganisms	Concentration of Extract mg / ml									
<i>Candida albicans</i>	0.5	0.25	0.12	0.06	0.031	0.01	0.008	0.004	0.002	0.001
	S	S	S	S	S	R	R	R	R	R
Table 4. Minimum Inhibitory Concentration (MIC) of Propolis (AEP) from Hubballi										

Table 4 shows the sensitivity of *Candida albicans* to the 70 % Ethanol extract Hubballi propolis. 0.03 mg / ml of Hubballi propolis is the minimum amount required to inhibit the growth of *Candida albicans*.

DISCUSSION

The rising candidal infection in the geriatric population because of decreased immunity and harmful side effects of synthetic antifungal therapy demands alternative safe antifungal agent. Hence the present study was undertaken to assess the efficacy of Hubballi Propolis on *Candida albicans* to develop novel Propolis medicament in the future to treat Candidal infection.

Hubballi is in the northern part of Karnataka with latitude 15.36° N and longitude 75.12° E. Average maximum temperature recorded is 33° C. The results of present study showed that Hubballi Propolis sample was highly sensitive against the tested *Candida albicans*, with the MIC values 0.01mg / ml to 0.03 mg / ml (table 2 & 4) similar to Australian Propolis which had the highest activity against *Candida albicans*.

A Eralp akka et al. study report suggested that propolis can be used as an antifungal agent as their study showed better results with propolis than the commercially available CHX.¹⁹ Samet N et al. recommended commercial ethanol extracted propolis as alternative medicine for candidiasis treatment in HIV-positive patients.²⁰ VR Santos in his chapter suggests that propolis can be used as home remedies in the treatment of denture stomatitis and oral candidiasis.²² The data of previous studies showed that when propolis was combined with other antifungal agents, there was increase in their activity against the *Candida albicans*.²⁰⁻²² It can be suggested from the present study that the sample procured from Hubballi region will help in treating infections caused by *Candida albicans*.

Imaging studies with electron microscopy by Mellot et al.²³ suggested that the mechanisms of action of Brazilian green propolis on the *Candida albicans* was by rupturing of the cell wall of the fungus.

Further, the total phenolic content of Propolis procured from Hubballi ranged from 175.4 ± 5.7 to 192.2 ± 3.3 and the total flavonoid ranged from 33.08 ± 10 to 31.73 ± 8.5 mg / ml of gallic acid equivalent (Table 1 & Table 3). The examined Hubballi Propolis samples possessed total phenolic and total flavonoid contents which were in comparison with other parts of the world like Algeria, Brazil, China, Morocco, Poland, Korea and Turkey.²⁴⁻³² The results of our studies clearly indicate that tested propolis samples procured from Hubballi with high phenolic (TP) and flavonoid (TF) contents can be selected for commercial propolis products.

The next important finding in the study was that, the water as solvent used to extract Propolis from Hubballi fared better in TPC and TFC than the alcohol as a solvent. This finding of the present study helps to overcome the drawbacks of alcohol extract which has strong residual flavour and difficulty of its usage in dentistry and pharmaceutical industry. The water extract propolis results in the study were in agreement with study by Nagai et al. The reason for water extract to excel than alcohol could be the solubility of the active components of Propolis as suggested in the previous study.³³ In spite of being an invitro model, Hubballi Propolis demonstrated high antifungal activity against *Candida albicans* suggesting it to be an alternative for the treatment of Candidal infection.

Figure 1. Measured Labelled Samples Placed for Maceration after Addition of Solvent

Figure 2. Concentrated Sample Extract

CONCLUSIONS

The promising antifungal activity of Hubballi Propolis in the present study and the literature evidence on immune modulating action of propolis with less side effects helps in considering it in treating the oral candida infection of geriatric patients. Further clinical trials are needed to establish relative efficacy of propolis and synthetic drugs.

Data sharing statement provided by the authors is available with the full text of this article at jemds.com.

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