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A study on Propolis of Stingless Bees reared from the most Commercial Hub of Chennai, Tamilnadu, India

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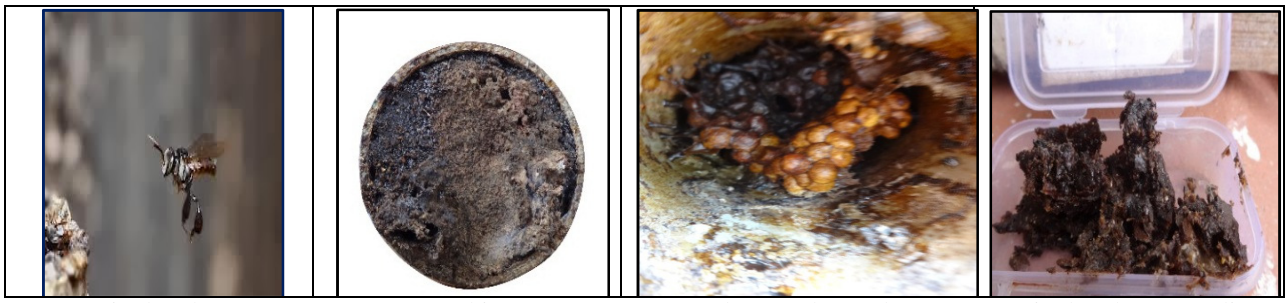
Abstract

Stingless bees are indigenous pollinators of treasured herbal plants. Propolis of stingless bees is a natural, strongly adhesive mixture made of bee secretions and plant resins. Stingless bee Propolis exhibit a wide spectrum of pharmacological activities that vary depending on the geographical location, climate, generic type of bees and availability of botanical sources for the bees to forage. In this study, we report the nature, composition, and bio-medical applications of Propolis of stingless bees, reared and collected from the most commercial hub of Chennai, Thyagaraya Nagar. Active components were best extracted with ethanol-water mixture using ultrasonication method. The results of the study revealed that stingless bee Propolis of this region is found to have anti-fungal activity and significant anti-cancer activity against A549lung cancer cells with IC $_{50}$ value of 76µg/ml. Evaluation of total phenol, flavonoid, radical scavenging ability and anti-bacterialstudies reflected the impact of the environment on the quality of Propolis of stingless bees of this region.

Keywords: Stingless bee propolis, ultrasonic extraction, polyphenols, flavonoids, anti-oxidant, anti-microbial, anti-fungal, anti-cancer activity.

Introduction

Bees are nature's gift to mankind. Bees with non-functional sting are called stingless bees. Stingless bees belong to the Apidae family. They are exclusive to tropical and sub-tropical areas[1]. Their size ranges from 2mm and not more than 5mm. In Tamil they are called as Siruthenikkal. They are the most indigenous pollinators of treasured herbalplants as they collect nectar and pollen selectively from medicinally important small herbal plants and flowers such as Coco, palm, banana, guava, papaya, mango, tamarind, thumba-poo, thengen-boo, touch-me-not plant, jackfruit tree, tulsi, teak etc. Since stingless bees lack defense organs, it protects the medicinally important honey by building their nest in a unique way, having a multilayer arrangement, providing separate provision for pollen storage, honey storage and brood rearing with small single entrance[2]. It uses a special type of resinous substance, called Propolisthat it creates on its own by mixing its own body secretions from the salivary glands with the resins and exudates collected from the leaves, trees, plants, buds etc. To seal the hive so as to avoid the danger from invasion by predators like spiders, flies, wasps, ants, lizards, etc, and to keep the hive environment aseptic[3]. Stingless bee Propolis has varied chemical composition depending on the geographical location, climate, type of bees, and the availability of vegetation in the locality, hence differs in their smell, colour and also differ in their pharmacological activity such as antibacterial[4], antimicrobial, antioxidant[5], anti-herpes, anti-ulcer, anti-hypertensive, anti-inflammatory, and also anti-cancer properties[6,7]. Owing to the high pharmacological importance, extensive studies on Propolis were made worldwide, over various regions[7-9]. In India, a few studies were reported inthe regions of Maharashtra, Karnataka, Gujarat and Uttar Pradesh[10,11]. In Tamilnadu, studies over Propolis, that too with Stingless bee Propolis is very scarce[12-15]. So, it is necessary to explore the biomedical uses and composition of propolis of different origin and various regions of Tamilnadu.



Stingless bee | Bee hive | Colony under study | Stingless bee Propolis

This main objective of this work is to study in detail the nature, chemical composition and biomedical applications– anti-bacterial, anti-fungal, antioxidant and anti-cancer activities of stingless bee Propolis, which is collected from the most commercial hub of Chennai, Thyagaraya Nagar and also to analyseany impact of the environment is there on the quality of the sample.

Material and Methods

Stingless bee propolis collection: Stingless bees were reared and collected from the most commercial hub of Chennai, Thyagaraya Nagar

Coordinates: 13.08389° N 80.27000° E. The sample was kept in a freezer for easy handling.

Ultrasonic extraction of propolis: Instrument: Wensor Ultrasonicator: 20g of Propolis was cut into small pieces and was grounded well. 200 ml of a solvent mixture containing 140ml of ethanol and 60ml of distilled water in the ratio (7:3) was added in small lots with constant stirring and subjected to ultra-sonication for about three hours for effective extraction[30]. The solution was then filtered through Whatman 41 filter paper and used as a stock solution.

Materials: Absolute ethanol, Folinciocalteu reagent, 20% sodium carbonate, Gallic acid, methanol, Aluminium chloride, potassium acetate quercetin, 1,1-diphenyl-2-picryl hydrazyl radical, Butyl Hydroxyl Toluene Agar, Mullar Hinton agar, ampicillin, potato dextrose agar, pencilline, Dimethyl sulphoxide (DMSO), Minimum essential medium (MEM), Fetal bovine serum(FBS), Phosphate buffered saline solution, Trypsin, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT), all purchased from Hi media and Sigma Aldrich.

Determination of total polyphenols: The total polyphenolic content of the ultra sonicated ethanolic extract of stingless bee Propolis was determined using Folin-ciocalteu reagent. Gallic acid was used as the standard. Different concentrations of the sample and standards were added with 1ml of Folin-phenol reagent and 1ml of 7% sodium carbonate solution. The mixture was incubated at room temperature for 1hr and 30 minutes. Absorbance was measured at 750nm for various concentrations. Total phenol content present in the sample was obtained by extrapolating the calibration curve obtained by plotting absorbance Vs Concentration of the standard and sample. The total phenolic content was expressed in µg of gallic acid equivalents (GAE) per ml of the extract.

Estimation of total flavonoid: Aluminum chloride colorimetric technique was used for flavonoids estimation. 0.5 ml of the Sample was mixed with 1.5ml of methanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1 M Potassium acetate and 2.8ml of distilled water. It was left at room temperature for 30 minutes, after which the absorbance of the reaction mixture was measured at 415 nm with a double beam UV-Visible spectrophotometer. The total flavonoid in the test sample was determined by extrapolating the calibration graph using Quercetin as the standard. Total flavonoid content was expressed in quercetin equivalents (QE).

Evaluation of antioxidant activity: 1, 1–diphenyl-2-picryl hydrazyl radical (DPPH) was used for the determination of free radical scavenging activity. BHT (Butyl Hydroxyl Toluene) was used as standard. 100µl of the sample was added to 3.7ml of absolute methanol. To this 200µl of DPPH reagent was added and the reaction mixture was kept at room temperature for 30 minutes. The absorbance of the reaction mixture was recorded at 517nm using a UV-visible spectrophotometer. The same procedure was done for the standard BHT and its absorbance was also measured.

% Antioxidant Activity =

$$\frac{\text{(Absorbance of Blank) – (Absorbance of test)}}{\text{Absorbance of Blank}} \times 100$$

Anti-microbial studies: Preparation of inoculums: Stock cultures were maintained at 4°C on Nutrient Agar Slant. Active cultures for the experiment were prepared by transferring a loop full of culture from the stock cultures into the test tubes containing nutrient broth and were incubated for 24hrs at 37°C.

Agar Disc Diffusion Method: The anti-bacterial activity of the extract was determined by disc diffusion method on Muller Hinton agar (MHA) medium. Muller Hinton Agar (MHA) medium was poured into the petri plate. After the medium was solidified, the inoculums were spread on the solid plates with

sterile swabs moistened with the bacterial suspension. The discs were placed on MHA plates and 20 µl sample of concentration 1000µg/ml, 750µg/ml and 500 µg /ml were placed on the disc. 20 µl of Standard ampicillin of concentration 1mg/ml was also placed in the disc. The plates were incubated at 37°C for 24 hrs. The quantification of microbial growth inhibition was determined by measuring the diameter of clear zones of microbial growth around the wells in the agar. DMSO was used as negative control.

Anti-fungal susceptibility test: Antifungal activity of the sample was determined by antifungal susceptibility test. Potato Dextrose Broth was prepared and the culture was inoculated andshaken for a day. 3.9gms of potato dextrose agar was dissolved in 100ml of distilled water and 1gm of agar was added. Then the medium is kept for sterilization.

After sterilization the media were poured in to sterile petri-plates, these petri-plates were allowed to solidify for twenty minutes. After solidification, the inoculums were spread on the solid plates with asterile swab moistened with the fungal suspension. The discs were placed in PDA plate and add 20 µl of sample [concentration: 2000, 1000, 500, 250, 125µg]. The plates were kept at room Temperature. Then the microbial growth was determined by measuring the diameter of zone of inhibition.

In vitro anti- cancer studies: Cell culture: Cell lines were obtained from National centre for cell sciences Pune (NCCS). The cells were maintained in Minimal Essential Media supplemented with 10% FBS, penicillin (100 µg/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 50 µg/ml CO_2 at 37°C.

Cell Viability: The anticancer activity on A549 human cancer cells was determined by the MTT assay. Cultured A549 human lung cancer cells (1×10^5 cells) were plated in 0.2 ml of the medium in 96 flat bottomed well plates. Incubated at 5 % CO_2 atmosphere for 72 hours. Then, various concentrations of stingless bee Propolis extract in 0.1% DMSO were added to the cells and maintained at 5% CO_2 incubator for 24hrs.

After incubation and washing with phosphate-buffered saline (pH 7.4), 20µl of 0.5% 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl--tetrazolium bromide (MTT) in phosphate- buffered saline solution was added to the incubated cells and further incubated for four more hours at 37°C and at 5% CO_2 atmosphere. After 4hrs of incubation, 1ml of DMSO was added. Viable cells at various concentrations (dilution method) were determined by measuring the absorbance at 540nm. The concentration required for 50% inhibition (IC_{50}) was determined graphically. The effect of the Propolis extract on the proliferation of A549cells was expressed as the % cell viability.

% cell viability =
A549 of treated cells / A549 of control cells × 100

Measurement of cytomorphological changes in A549 cells: A549 cells were pre-treated with different concentrations of the stingless bee propolis extract was incubated for 24h at 37°C in 5% CO_2 atmosphere. After incubation, the gross morphological changes in the cells were observed under an inverted microscope (Lobomed) at the magnification of 40X.

Results and Discussion

The colour and odour of Propolis sample vary from region to region and from country to country. Itis reported that Brazilian Propolis is green in colour, Taiwan Propolis a light yellowish brown incolour. In Tamilnadu, the colour of the stingless bee Propolis sample varies from light honey brown to dark brown colour[12-15]. The stingless bee Propolis collected from the most commercial place of Chennai is brownish black in colour without odour. The components are best extracted by ultrasonication method in a solvent mixture of Ethanol and water (7:3). To understand the nature of biomolecules present in the sample, FT-IR spectra of both solid stingless bee propolis sample and ultrasonicated ethanolic extract of stingless bee propolis, were taken by grinding the samples with KBr, using Shimadzu FT-IT spectrophotometer in the wavelength range from 4000-400$cm^{-1.}$

FT-IR spectrum of the solid stingless bee Propolis sample shows its complex chemical composition, having all the fundamental vibrations corresponding to aromatic C-C, C-H, C-O,N-H and O-H bonds along with the presence of alcohols, esters, amides and alkanes. However, FT-IR spectrum of anultra sonicated ethanolic extract of the sample shows only two major peaks, one at 3450cm^{-1} corresponding to the presence of hydrogen bonded O-H group and another peak at 1637 cm^{-1} corresponding to the presence C=O of acids, amides. This shows the major role played by the extraction method. When liquid samples are subjected to ultrasonic waves (Sound waves having frequencies in the range of 20 KHz to 10 MHz), no direct interaction takes place between the ultrasound waves and the chemical compounds, as acoustic wavelengths are much larger than molecular interactions[16]. However, the alternating expansive and compressive acoustic waves create oscillating called cavities[17]. These oscillating bubbles accumulate ultrasonic energy and grows to a certain size, under the right conditions, these bubbles collapse (cavitation implosion), releasing the concentrated energy stored in the bubble within a short time[18]. This bubble collapse provides an ambient condition for the complex molecules to become molecules of simple nature.

When UV-Vis spectral analysis was done using Shimadzu UV 1650 PC Spectrophotometer at the scanning range of 200nm to 500nm, a small peak was observed at 266.5nm with the absorption value of 0.5%. Absorption value is indicative of the quality of the Propolissample[4]. 0.5% of absorption intensity may be attributed to the low percentage of polyphenols and flavonoids in the sample.

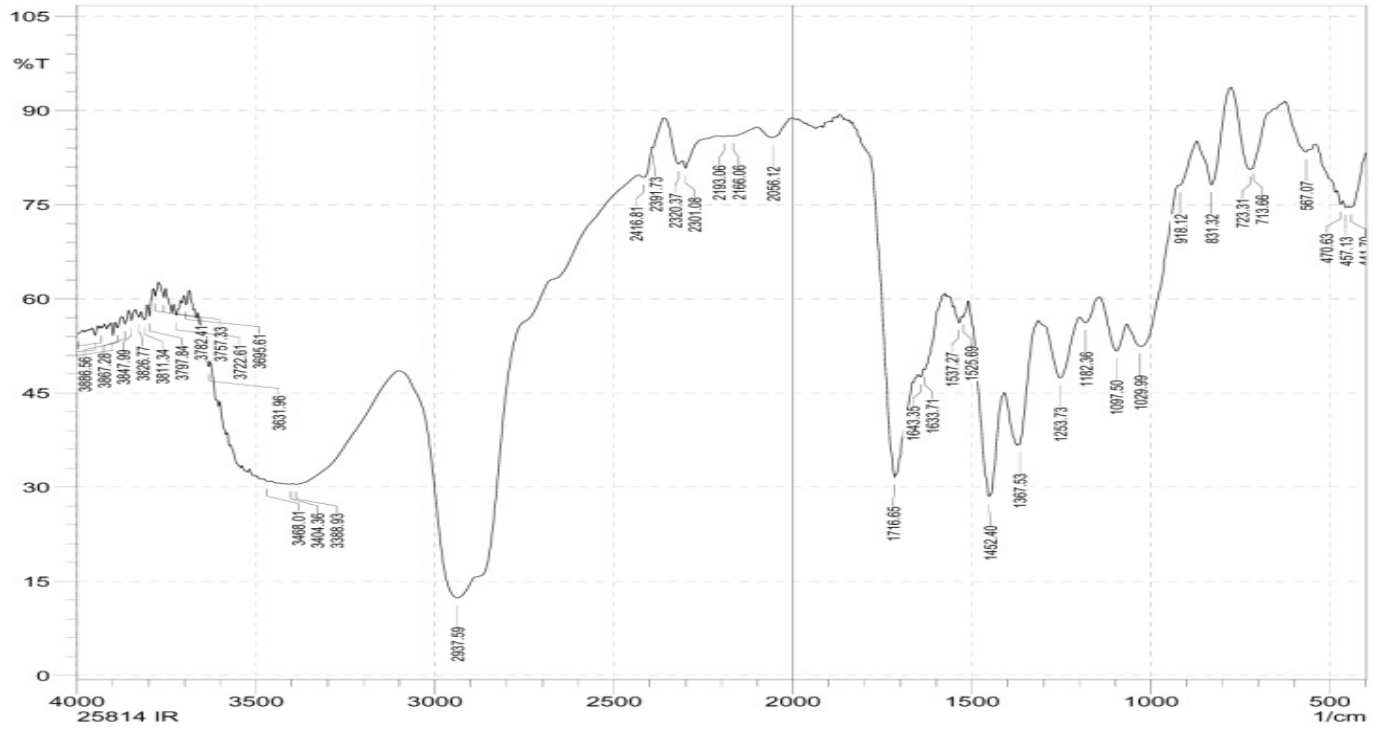


Figure-1
FT-IR spectrum of solid raw propolis sample

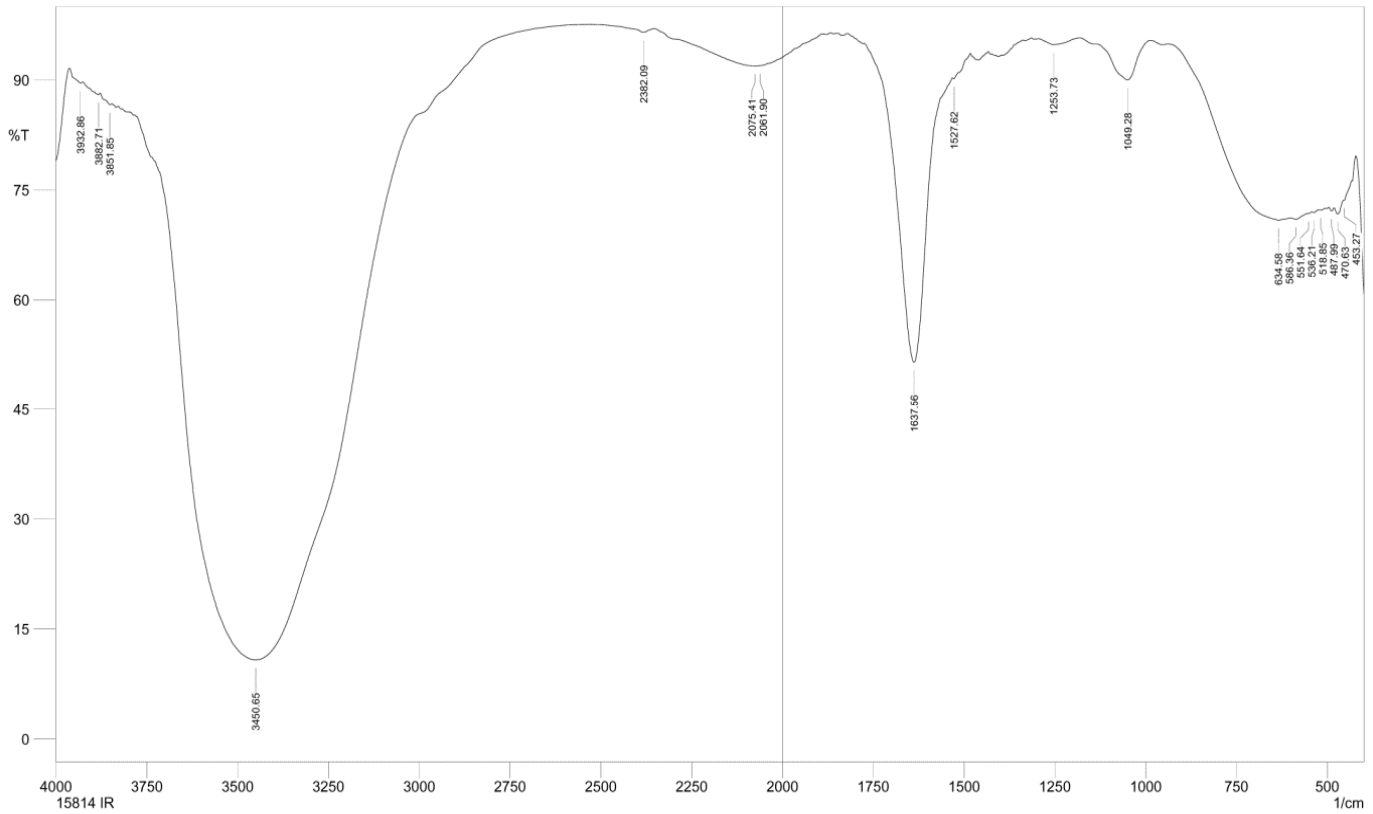


Figure-2
FT-IR spectrumof ethanolic extract of stingless bee propolis sample

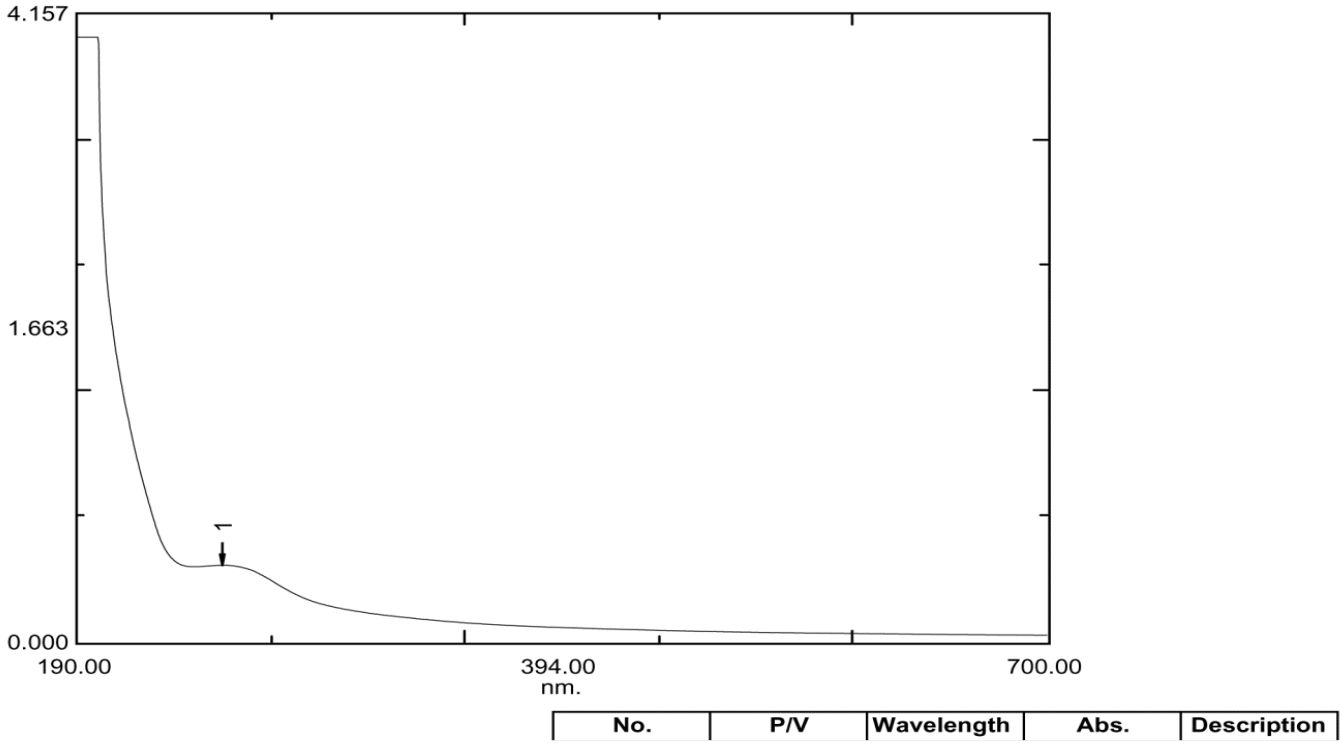


Figure-3
UV-absorption spectra of stingless bee propolis

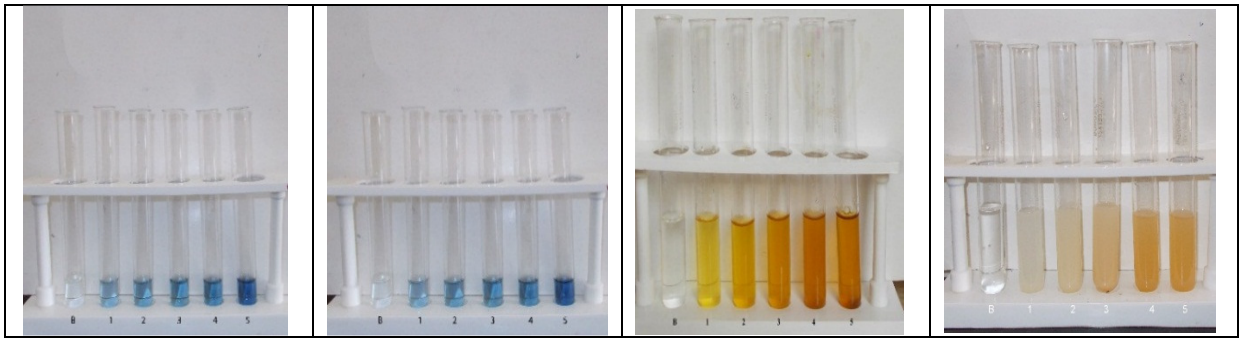


Figure-4
4A and 4B shows the phenolic content determination of the Standard Gallic acid and the sample using Folin-Ciocalteu's method. 4C and 4D shows the flavonoid content determination of the standard, Quercetin and the sample using Aluminum chloride colorimetric method

The amount of total phenols present in the sample was determined by extrapolating the calibration curve obtained by plotting absorbance at 765nm Versus various concentrations of Gallic acid standard and the sample and its amount is expressed in µgas Gallic acid equivalent (GAE) per ml of the extract. Similarly the amount of flavonoids present in the stingless bee Propolis sample was obtained graphically by extrapolating the calibration graph between absorbance at 415nm versus various concentrations of quercetin standard and the sample and its amount is expressed in µg as Quercetin equivalents (QE). The results of the study are given in table-1.

Table-1
Total phenolic and flavonoid contents

Content	Amount
Total phenolic content	10µg/ml GAE
Total flavonoid content	48µg/ml QE

The amount of poly phenols and flavonoids in the sample is found to be very less when compared to samples reported from other regions of Tamilnadu[14,15].

The results obtained from Folin-Ciocalteu's method and Aluminium chloride colorimetric method were found to be in

agreement with the antioxidant capacity of the stingless bee Propolis of this region. One of the most common methods used to evaluate antioxidant potential is based on the depletion of DPPH free radicals by the addition of scavenger compounds. DPPH is a stable free radical compound, deep violet in colour and shows an absorbance maximum at 517nm.

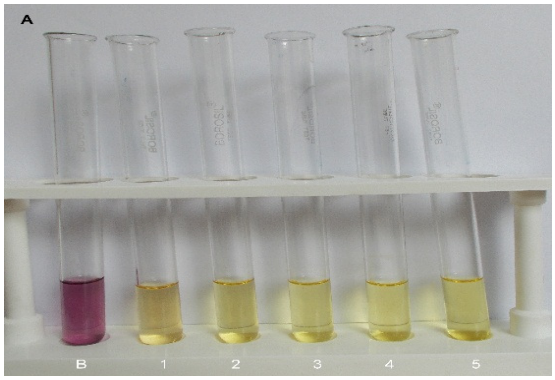


Figure-5A
DPPH radical scavenging ability of the Standard BHT (Butyl Hydroxy Toluene)

Figure-5B
DPPH radical scavenging ability of the sample at various concentrations

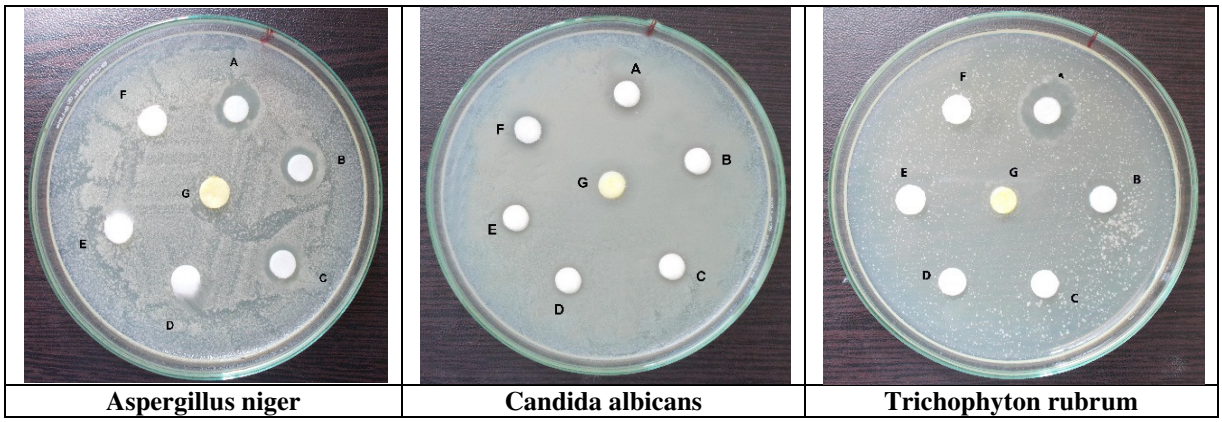
Measurements of DPPH radical consumption are related to the intrinsic ability of a substance to donate hydrogen atoms or electrons to this reactive species. The reduction of DPPH radical by the sample and the standard is monitored by measuring the decrease in absorbance at 517nm.Upon complete reduction, the absorbance at 517nm disappears. The results of the study are given in table-2.

Table-2
Antioxidant determination Blank absorbance 0.60

	100µg	**200µg**	**300µg**	**400µg**	**500 µg**
BHT absorbance	0.03	0	0	0	0
% antioxidant capacity	95	100	100	100	100
Sample absorbance	0.60	0.58	0.57	0.56	0.56
% antioxidant potential	0	3.3	5.0	6.6	6.6

In general, Propolis is known for their antioxidant property which can be associated with the active principle components present in it. Stingless bee Propolis of this region is found to exhibit only 6.6% of antioxidant activity compared to its standard BHT at 500 µg/ml, this is attributed to the less percentage of phenol and flavonoid contents of the sample which in turn depends on the location and the environment.

Anti-microbial activity: It was reported that propolis collected from different regions, in different solvents were found to be active against different microbes.



Aspergillus niger	**Candida albicans**	**Trichophyton rubrum**

Figure-6
Antifungal activity of ultrasonicated ethanolic extract of stingless bee propolis collected from Thyagaraya Nagar, Chennai, Tamil Nadu

When the anti-fungal activity of the ultrasonicated ethanolic extract of the stingless bee Propolis sample was tested against A.niger, C.albicans and T.rubrum, it showed good inhibitory activity against A. niger and C. albicans at the concentration of 500 µg and against T.rubrum at the concentration of 1000 µg table-3. Stingless bee propolis collected from this region showed reasonably good anti-fungal activity than anti-bacterial activity.

When the antibacterial activity of the sample was tested against certain gram +ve bacteria viz., Staphylococcus, Vibrio parahaemolytics, Bacillus and against certain gram –ve bacteria, namely E.Coli, Vibrio spp, Salmonella, Aeromonas, Klebsiella, proteus spp , the sample showed almost nil inhibitory activity at various concentration levels of 500 µg/ml, 750µg/ml and 1000µg/ml. This shows that chemical combinations necessary for the anti-bacterial activity were absent in the stingless bee Propolis sample of this region.

Anti-cancer activity: Many reports have indicated that different types of Propolis significantly inhibited cell growth and its cytotoxicity effect varied largely with different samples of Propolis[19, 20].When we have investigated the anti-cancer activity of the stingless bee Propolis of this region on human A549 lung cancer cells by following a serial dilution MTT assay, which is based on the ability of the live cells to reduce the yellow tetrazolium dye to a purple form azan product, it is seen that in spite of its low phenolic and flavonoid contents and high commercial environment, the stingless bee Propolis extract of this region has significantly inhibited the cell growth and reduced the proliferation of cancer cells.

A dose-dependent anti-cancer activity was noticed. There is an inverse dose relationship with cell viability that is with percentage of living cells with the concentration of the stingless bee Propolis extract. As the concentration of the extract increases, the percentage of live cancer cells decreases, and it is reflected in the absorbance value at 540nm. Graphical determination revealed that 50% of the tumor cells were inhibited at76µg/ml concentration of the extract.

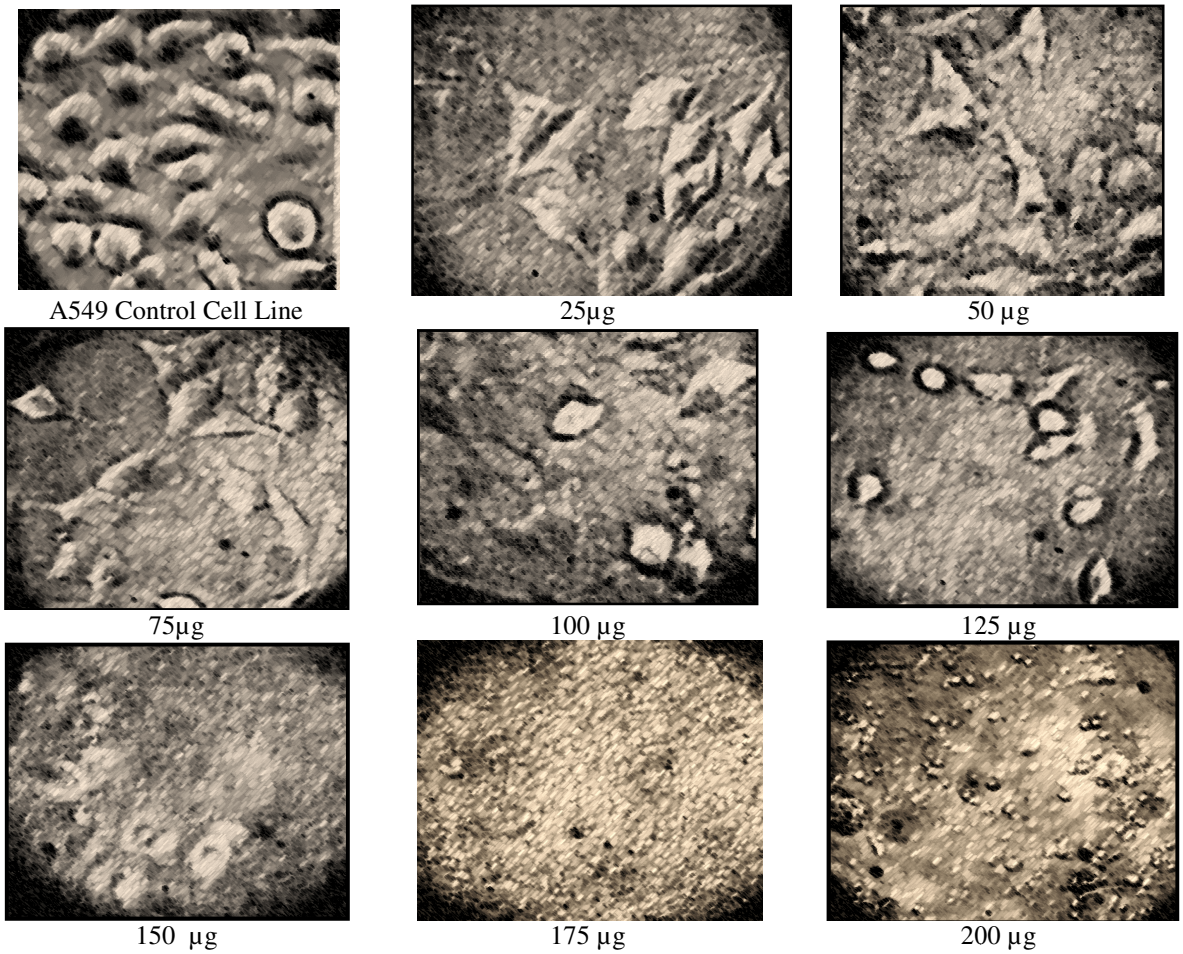
Various morphological alterations were observed when A549 human lung cancer cells were treated with various concentrations of stingless bee Propolis extract collected from Thyagaraya Nagar of Chennai. Figure-7 shows the anti-cancer effect when viewed through an inverted microscope (lobomed) with a magnification of 40X. However, no such effects were seen in control (non- treated cells).

Table-3
Anti- fungal activity

Micro organisms	Zone of inhibition in mm						
	Concentration of Stingless bee propolis extract					70% ethanol (F)	Std 20 µg/disc (G)
	2000µg (A)	1000 µg (B)	500 µg (C)	250 µg (D)	125 µg (E)		
Aspergillus niger	11	9	7	-	-	-	-
Candida albicans	7	6	5	-	-	5	10
Trichophyton rubrum	11	7	-	-	-	-	-

Table-4
Dose dependent anti -cancer activity

Concentration µg/ml	Dilution	Absorbance 540nm	% cell Viability
200	Neat	0.05	6.4
175	1:1	0.07	8.9
150	1:2	0.16	20.5
125	1:4	0.21	26.9
100	1:8	0.37	47.4
75	1:16	0.43	55.1
50	1:32	0.51	65.3
25	1:64	0.63	80.7
control	-	0.78	100



Conclusion

To our knowledge, this is the first study, which gives a detailed report on the total phenolic and flavonoids contents, spectroscopic characterization, antioxidant, antimicrobial and anti-cancer activities of propolis of stingless bees, collected from the most commercial place in Chennai, Thyagaraya Nagar. Stingless bee propolis of this region is found to be having anti-fungal activity and notable anti-cancer activity against A549 human lung cancer cells with IC $_{50}$ value of 76 µg/ml. However, less percentage of polyphenols and flavonoids and its reflection in the poor anti-oxidant and almost nil anti-bacterial activity show that the environment prevailing in this region is not conducive to the survival of these most indigenous pollinators. The results of the study cautions us to find a rational solution to this ignored blunder, by creating a proper pollution free green environment for the existence of these small creatures in the region of study as there is no substitute for these untiring workers for pollination services.

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