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ANTIBACTERIAL (WOUND INFECTING BACTERIA) AND ANTICANCER ACTIVITY OF DIFFERENT TYPES OF HONEY AND IT'S COMPOUND CHARACTERIZATION

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ABSTRACT

Honey is the natural sweet substance from nectar or blossom or from the secretion of the living parts or excretion of plants which the honeybees collect and store. It was widely used in traditional medicine but it use in modern medicine is limited because of the lack of scientific support. Among its several uses, honey is used for the treatment of many infections and also used effectively as wound dressing including surgical wounds, burns and skin ulcer. In this study four different honey samples were collected and investigated for its antimicrobial activity using disc diffusion and well diffusion methods and anticancer activity against HeLa Cell Line.

KEYWORDS: MDR (Multi Drug Resistant), MIC (Minimum Inhibitory Concentration), Fetal Bovine Serum (FBS), (PBS)-Phosphate Buffered Saline.

INTRODUCTION

The use of traditional and herbal medicine to treat infection was practiced since the origin of mankind and in the past it was probably the only available method to be used for that. Various plants and their extracts have already been in use for the treatment requiring antimicrobial activity and one of the popular natural antimicrobial substances described in the ancient medicine in honey (Abstons *et al.*, 2000). Natural products such as honey have potential anticancer and antibacterial activity (Moore *et al.*, 2001). Most microorganisms do not grow in honey because of no water activity (Kowsalya., 2012).

Honey is a food product which is collected from various plants and processed by honey bees (*Apis mellifera*). Honey has been used as traditional medicine for centuries in different cultures, not only for its nutritional value but also its healing properties. Honey has been tested and approved scientifically for its functional and biological properties like anti-oxidant, anti-inflammatory, anti-bacterial, anti-viral, anti-ulcerous activities, anti-lipid and anti-cancer properties (Irish *et al.*,2008*;* Temaru *et al.*, 2007*;* Gheldof *et al.*, 2002*;* Estevinho *et al*., 2008; Wang *et al*., 2002; Swellam *et al*., 2003; Orsolic *et al*., 2005; Boukraa *et al*., 2008). It has been reported that those minor ingredients are the ones that are responsible for medical and biological activities of honeys in the treatment of infections burns and wounds an ulcers (Moumbe *et al*., 2013). This study was aimed to study the antibacterial and antiproliferative activity of honey on HeLa cell lines.

MATERIALS AND METHODS

Collection of different types of honey

Three honey samples Lion Honey, Dabur Honey & Natural honey were used in this study. Lion & Dabur honey samples were obtained from local super market (vin supermarket) in Coimbatore and the natural honey obtained from Karaikudi Sarvodaya Sangh. Raw unprocessed honey samples were stored in the room temperature. The three honey samples were dispensed into sterile cork-screwed containers.

pH

pH of almost all honeys are naturally acidic and this will inhibit the activity of many microorganisms. The pH of three honey sample was measured using pH paper and also confirmed with pH meter ELICO L1617.

Moisture

For the identification of moisture content AOAC., 1990 method were used, 1gm of different honey sample were taken separately and incubated at 105°C for one hour. After incubation weight of three honey samples was measured.

Ash Content

For the identification of ash content AOAC., 1990 method were used. 1gm of different honey samples placed in each beaker and the sample was incubated at 105° to estimate the ash content of the sample. After incubation weight of three honey samples were measured.

Sodium, Potassium & Calcium Estimation

Samples were converted to ash and these ashes were used for the identification of Sodium, Potassium and Calcium content. 1gram of ash was mixed with 2ml of 15 N HCL and incubated for 10-15 minutes. Then the OD was taken at 589 nm, 768 nm and 620 nm for Sodium, Potassium and Calcium respectively using Spectrophotometer.

Total antioxidant analysis: (Phosphomolybdic method)

One ml of different honey samples were mixed with 1ml of 1mM reaction mixture containing H2SO4-0.6 Molar, Sodium phosphate-28mm, Ammonium molybdate-4mm. After the addition of reaction mixture the tube were mixed well and incubated for 90 minutes at 50°C. After cooling OD value were taken at 695 nm in Spectrophotometer. Blank were prepared without the sample .Ascorbic acid used as a Standard to calculate the total antioxidant mg/g.

Collection of wound pathogens

Isolation and Identification of Bacteria

Wound samples are collected from Abirami Private Hospital, Coimbatore. By using a sterile cotton swab, samples were collected from wound, immersed in a container of transport medium. On collecting samples from wounds, special precaution should be taken to prevent contaminating specimen with commensal organisms from the skin. The isolated bacterial strains were identified as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Klebsiella pneumoniae.*

Sub culturing of microorganism

After conformation of microorganisms the pathogen were sub cultured using nutrient broth separately and incubated at 37°C for 24 hrs. After incubation the tubes were stored at 4°C for further study.

Well Diffusion Method

The plates were prepared using sterile Muller Hinton Agar. The surface of the four plates was inoculated using a 60µL of standardized inoculums suspension of the isolated bacteria and allowed to dry. Wells with 5.0 mm diameter prepared using a cork borer and then filled with the test honey (30 µL). The plates were incubated at 37ºC for 24 hours for clear, circular inhibition zones around the wells. The diameter of zones of inhibition of the wells was measured by measuring them in millimeters (mm) in at least 2 directions perpendicular to

each other (90°). The mean of diameters of inhibition zone for each well and honey sample was calculated.

Disc diffusion method

Disc preparation: Whatman filter paper was taken at small circle size. Six different honey samples (Natural, Lion, Dabur, Natural+Dabur, Natural+Lion, Lion+Dabur). The whatman filter paper dipped in each honey samples and incubated in room temperature for 24 hrs. The honey dipped whatman filter paper was dried.

Disc diffusion method using Honey discs

Mullen Hinton Agar (MHA) plates were prepared. The MHA was poured and allowed for solidification.60 µl of standardized bacterial suspension was poured and evenly inoculated the MHA plates using sterile cotton swabs. They were allowed to dry for 3-5 minutes. Thereafter all honey discs were placed on the plates and pressed gently to ensure complete contact with agar. A distance of at least 15 mm was maintained from the edge of the plates to present over lapping of inhibition zones. After 15mins of the placement of disc, the plates were incubated for 24 hrs at 37°C.After incubation period the inhibition zone was measured.

Disc diffusion method using commercially available antibiotics

The sterile swab was taken and moistens with broth (culture) and it was pressed against the sides of tubes to remove excess fluid and was swabbed evenly throughout the medium. The surface of the plate was allowed to dry. Using sterile forceps, antimicrobial discs Ofloxacin, Cefazidine, Norfloxacin, Chloramphenicol, Ampicillin) were placed with some distance and pressed slightly. The plates were inverted and incubated at 37°C for 24 hrs.

Bactericidal/bacteriostatic effects of honey

To check this 60 ml of sterile nutrient broth were prepared and add 100µl of sample and incubate for 24 hrs. After incubation OD were taken at 600nm using Spectrophotometer. To confirm the bactericidal and bacteriostatic activity of honey 1ml of the above culture were added to 9ml of broth (N) without honey and incubated for 24 hrs after incubation 0.1 ml of this above culture was transferred into the nutrient agar plates were incubated for 24 hrs.

Anticancer activity of honey (Hela cell line)

MTT reagent (powder)

MTT (3 - [4, 5- dimethylthiazol - 2 - yl] - 2, 5- diphenyltetrazolium bromide) is a yellow colored water soluble tetrazolium dye. Mitochondrial enzyme lactate dehydrogenase produced by metabolically active cells reduces MTT to water-insoluble formazan crystals. When dissolved in appropriate solvent, these formazan crystals exhibit purple color.

He La Cells were grown in DMEM medium supplemented with 10% fetal bovine serum (FBS) (Hi Media, Mumbai), Cells were incubated in a humidified incubator contain 5% CO2 at 37ºC. After 24hrs the cells were seeded in to 96 well plate along with different honey sample (Dabur, Natural, Lion, Dabur+Lion, Dabur+Natural, Lion+Natural) then the cell culture suspension was washed with 1 X PBS (Phosphate Buffered Saline) and then added with 20µl MTT solution to the culture flask. It is then incubated at 37°C for 3 hours, removed all MTT solution, washed with 1 X PBS and added with 100 µl DMSO to each culture flask and incubated at room temperature for 30 minutes until all cells get lysed and homogenous color was obtained. The solution was then transferred to centrifuge tube and centrifuged at top speed for 2 minutes to precipitate cell debris. Debris was dissolved using DMSO. OD was measured at 540 nm using DMSO blank. Then the percentage viability was calculated using the percentage of viability formulated.

DNA damage study

3 µl of *E.coli* DNA and 3 µl of sample were mixed and 4 µl of Fendons' reagent also added and incubated in room temperature for 3 to 4 hrs. After incubation the sample was run in1% agarose gel electrophoresis under 50V.

Gel preparation

Agarose was taken and dissolved in 15ml distilled water containing 1x TAE and melted in oven. The agarose solution was cooled to 45°c. To this, 2 µl of ethidium bromide was added and the solution was poured into the casting tray with comp placed in it and allowed to solidify. DNA sample were loaded and the band were observed in UV transilluminator along with control DNA.

RESULTS AND DISCUSSION

The normal pH of honey is about 3.4 to 6.1 The result showed the Dabur honey has normal pH-4.0, Lion honey has 4.5. Natural honey has pH-4.0. Hassan., **(**2010) reported the high

acidity of honey also plays an important role in the system which prevents bacterial growth. Oyeleke., (2010) reported that honey is characteristically quite acidic, the pH value ranged between 3.2 and 4.5, which is low enough to inhibit the growth of pathogens.

The moisture content of different types of honey were (before 1 hr) Dabur 1.8%, Lion dates 1.02%, Natural honey 1% after 1 hr incubation Dabur 0.05%, Lion honey 0.07% and Natural honey 0.03%.

The results showed that the ash content of following honeys before incubation Dabur 1.8%, Lion honey 1.02%, Natural honey 1% after one day incubation Dabur 0.58%, Lion honey 0.59%, Natural honey0.75%.

The sodium content of the honey samples are Dabur (58 mg/g), Lion dates (41 mg/g), Natural honey (40mg/g). The potassium content of the samples are Dabur (1mg/g), Lion dates(2mg/g), Natural honey (13mg/g). The calcium content of the samples are Dabur (31mg/g), Lion dates(16mg/g), Natural honey (6mg/g).

Total antioxidants of the honey samples are Dabur (42mg), Lion dates(99mg), Natural honey (203mg). Ayesha Firdose *et al*., (2016) reported that Total antioxidant capacity of the extracts was calculated using inhibition against ascorbic acid.

Antibacterial activity

Well diffusion method

The inhibition zone diameter of different dabur, lion and natural honey were determined for *E.coli, s.aureus, K.pneumonia, P.aeruginosa* both dabur+lion and lion honey were highly effective against *S.aureus.* Dabur and natural+dabur honey were highly effective against *K.pneuminia.* Dabur and natural honey were highly effective against *P.aeruginosa.* Dabur+lion and dabur+natural honey were highly effective against *E.coli* (Plate1).

Kingsley.,(2001) reported the results of antibacterial activity of different honey types against *Staphylococcus aureus*, *Streptococcus pyogenes*, *Corynebacterium pseudotuberculosis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Escherichia coli* and reported that all honey types at 20.30% showed inhibition of bacterial growth.

Disc diffusion method

Disc diffusion method using commercially available antibiotics

The results showed among the three honey samples Ofloxacin have a maximum zone of inhibition (3mm) and Amphicillin have a minimum zone of inhibition (1mm) against *S.aureus.* Norfloxacin have a maximum zone of inhibition(2mm) Chloramphenical have minimum zone of inhibition(0.5nm) against *K.pneumonia.* chloramphenicol have a maximum zone of inhibition (2mm) and Cefatoxin have minimum inhibition(1mm) against *E.coli*. Norfloxacin have a maximum zone of inhibition(2mm) and Amphicillin have a minimum zone of inhibition against *P.aeruginosa* (Plate2).

Hassan., **(**2010) reported *Aeromonas schubertii* is resistant to the most antibiotics used in his experiment except Ceforperazone, Ofloxacin and Ciprofloxcaicn.

Disc diffusion method using honey as an antibiotic disc

The results showed on (Plate3)dabur+Lion honey disc have a high antibacterial effect against *E.coli*. Dabur honey disc have a high antibacterial activity against *K.pneuminia*. Dabur, lion+dabur honey disc have high antibacterial activity against *S.aureus*. Dabur and lion +dabur honey disc have high antibacterial activity against *P.aeruginosa*.

Bactericidal/bacteriostatic effects of honey

Honey have a bactericidal and bacteriostatic effect (Plate4 and Plate5). The inhibitory action of dabur, lion and natural honey on the tested bacterial strains *S.aureus, K.pneumonia, E.coli and P.aeruginosa.* Dabur+natural honey had a high bactericidal effect(0.30) against *S.aureus.* Dabur+lion honey had a high bactericidal activity (0.24) against *E.coli*. Dabur+natural honey had a high bacteriocidal activity (0.28) against *K.pneumonia*. Dabur+lion honey had a high bactericidal activity (0.27) against *P.aeruginosa*. Control OD of all sample *S.aureus*(0.49), *K.pneumonia*(0.69), *E.coli*(0.65) *and P.aeruginosa*(0.37). Araya Gebereyesus Wasihun and Berhe Gebreslassie Kasa., (2016) reported the bacteriostatic activity of red honey from Atsbi area against *P. aeruginosa, E. coli* and *K. pneumonia*. Willix *et al*.,(1992) reported that Minimum concentration of honey (%, v/v) in the growth medium needed to completely inhibit the growth of various species of wound-infecting bacteria.

Anticancer activity of honey

Generally honey contains anticancer activity. (Fig:1 and Fig:2) The following honeys were demonstrated using HELA CELL LINE Dabur honey (42.11), Natural honey (30.44), Lion

honey (47.42), Dabur+Lion honey (61.35), Dabur+Natural (30.91), Lion+Natural (15.930), cell line(1.902). Tualang honey showed antiproliferative effect on OSCC and HOS cell lines by inducing early apoptosis(Othman.,2012).

DNA damage study

DNA bands are observed under uv transilluminator. DNA nicking assay was performed and the DNA protector was analyzed. The DNA was protected using the honey and no nicking was seen.(Fig:3).

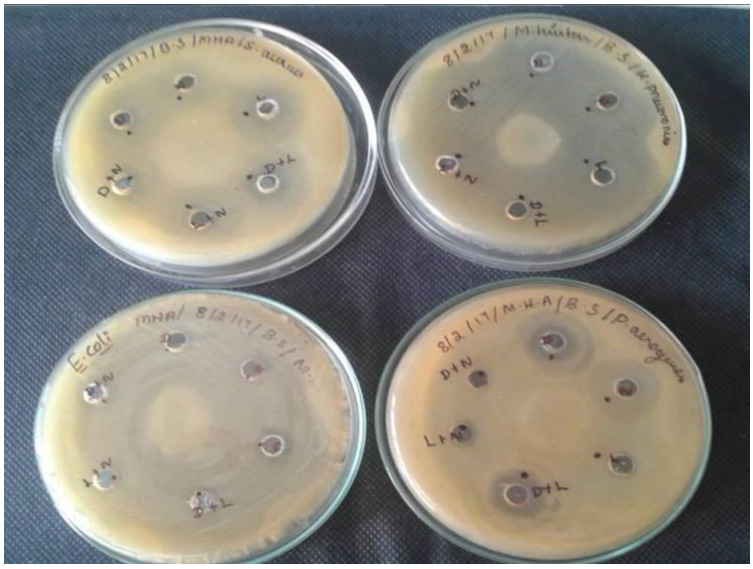
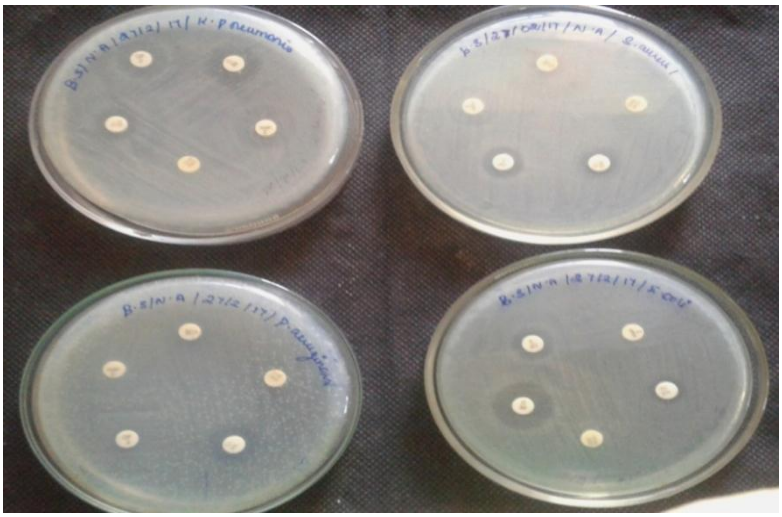


Plate 1: Well diffusion method.

Note: A=Dabur honey, B=Natural honey, C=Lion honey, D=Dabur+Lion honey, E=Lion+Natural honey, F=Dabur+Natural honey.

Culture: Plate1= *S.aureus*, Plate2=*K.pneumoniae*, Plate3=*E.coli*, Plate4=*P.aeruginosa*.



Plat 2: Disc diffusion method (commercially available antibiotics).

Antibiotics: A=Ofloxacin, B=Cefazidine, C=Norfloxacin, D=Chloramphenicol, E=Ampicillin

Organisms: Plate1=*K.pneumoniae*, Plate2=*S.aureus*, Plate3=*P.aeruginosa*, Plate4=*E.coli*.

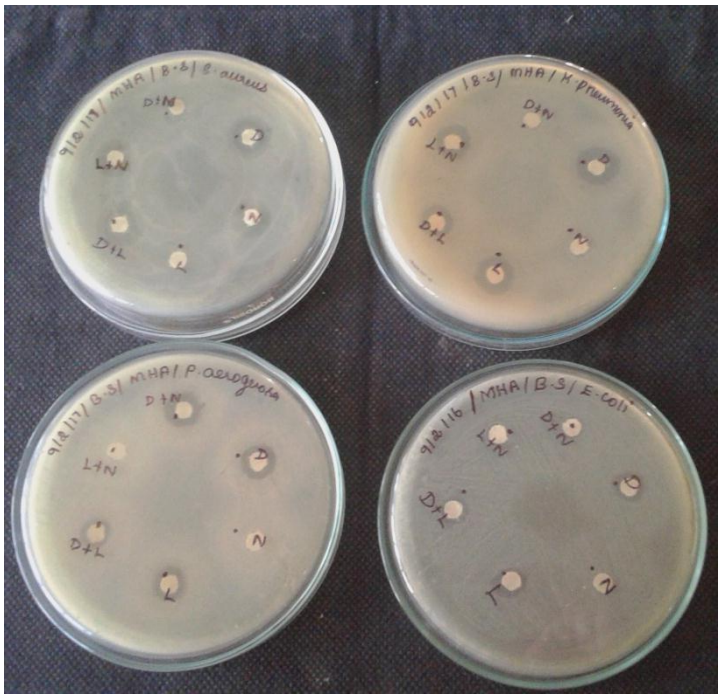


Plate 3: Disc diffusion method using honey

Note: A=Dabur honey, B=Natural honey, C=Lion honey, D=Dabur+Lion honey, E=Lion+Natural honey, F=Dabur+Natural honey.

Culture: Plate1= *S.aureus*, Plate2=*K.pneumoniae*, Plate3= *P.aeruginosa*, Plate4= *E.coli*.

Plate 4: Bactericidal and bacteriostatic activity.

Organism: Plate1 & 2=*K.pneumoniae*, Plate3&4= *E.coli*.

Honey samples: Plate1=Dabur+natural honey, Plate2=Dabur honey, Plate3=Dabur+Lion honey, Plate4=Dabur honey.

Plate 5: Bactericidal and bacteriostatic activity

Organisms: Plate5=*S.aureus*, Plate6=*S.aureus*, Plate7=*P.aeruginosa*, Plate8=*P.aeruginosa.* Honey samples: Plate5=Dabur+Natural honey, Plate6=Dabur honey, Plate7=Dabur honey, Plate8=Dabur+Natural honey.

Anticancer activity of honey

Fig 1: Preparation of DMEM medium.

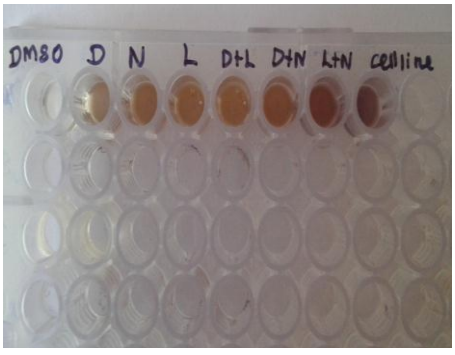


Fig 2: DNA damage study, Sample in 96 well plate.

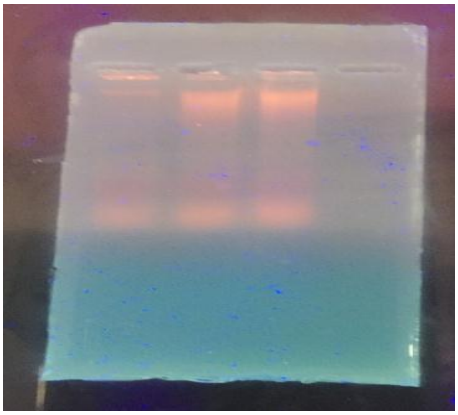


Fig 3: DNA damage study.

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