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Anti-Skin Bacteria Activity of Selected Plants in Malaysia

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ABSTRACT

Cosmeceuticals are topical cosmetic pharmaceutical preparations which are used to enhance the beauty through ingredients that provide additional health-related function or benefit. Natural colorants are dyes and pigmentary molecules which are extracted from nature sources such as minerals, plants and animals. A total of six plants were selected as potential candidates for this project namely *Punica granatum, Dicranopteris linearis*, *Tagetes erecta*, *Hibiscus rosa sinensis* (red and yellow) and *Hibiscus schizopethalus*. Plant samples which were freeze-dried and oven-dried were extracted using absolute methanol and subjected to rotary evaporation. For the preparation of boiled extracts, samples were boiled with distilled water for an hour prior to freeze drying. All the extracts exerted no damage to RAW 264.7 murine macrophage cell line with IC50 values more than 100 µg/ml, which was the highest concentration tested in the current study using MTT assay. A total of three species of skin bacteria namely *Staphylococcus aureus* (five isolates: Sa1, Sa2, Sa3, Sa4, Sa5), *Staphylococcus epidermidis* (five isolates: Se1, Se2, Se3, Se4, Se5) and *Chryseobacterium aquifrigindense* (five isolates: Ca1, Ca2, Ca3, Ca4, Ca5) were isolated from the skin swabs of thirty subjects with skin infection. From the results obtained from anti-skin bacterial testing using disc diffusion method, the extracts inhibited *Staphylococcus aureus* and *Staphylococcus epidermidis* but did not show any anti-bactericidal activity towards *Chryseobacterium aquifrigindense*. The highest anti-skin bacterial activity was shown by *Dicranopteris linearis* (oven-dried) and *Punica granatum* (freeze-dried and oven-dried) extracts. In conclusion, these extracts have potential to inhibit the growth of skin bacteria without causing toxicity towards the skin.

Keywords: Anti skin bacteria; Cosmeceutical; Natural colorant extract.

1.0 Introduction

Natural dyes or colorants can be derived from insects, minerals and plants. The interest towards the natural colorant which is extracted from plant sources is receiving a growing interest in food and cosmetic industries. The plant resources have a wide range of compounds for cosmetics coloring such as anthocyanin, betalain, chlorophyll and also caretenoid (Reshmi et al., 2012). Various plant pigments have been used as additives or colorants in cosmetic industry. There are some major drawbacks which are caused by the synthetic colorants and the interest towards the usage of natural colorants increases currently (Kapoor, 2005).

The synthetic colorants are harmful, toxic and cause harmful responses such as chronic allergy to human. Natural colorants offer a good alternative as in certain areas the synthetic colorants are imported from different countries, thus causing the cost to be relatively expensive (NRDC, 2012). Nowadays, due to the growing environmental and health concerns, natural colorants have emerged as a potential viable option as an alternative to substitute the usage of synthetic dyes (Boo et al., 2012). This indirectly has opened the scope for the use of natural colorants in the cosmetic field. Natural colorants are renewable and sustainable bioresource products with minimum environmental impact and known since ancient times for their use (Sivakumar et al., 2011).

Due to the poor image that animal and chemical derived products have acquired, the usage of plant extract in cosmetic formulation is increasing. The aim of the cosmetology field is to replace the harmful colorants and animal derived products, but synthetic chemicals solely will not be able to do this (Aburjai and Natsheh, 2003). A large number of plants have been identified for the color extraction and their diversified use in cosmetics (Shahid et al., 2013).

Malaysia has a rich biodiversity and harbors a huge variety of flora which has potential values. Nature has gifted Malaysia approximately 500 color yielding plants and most of the natural colorants are found in the parts of plants such as the bark, root, flower, peels or shells of plants (Sivakumar et al., 2011). Malaysians have been using plant based natural colorant since ancient times. In this current study, the extracts of *Tagetes erecta*, *Punica granatum*, Dicranopteris *linearis*, *Hibiscus rosa sinensis* (red and yellow) and *Hibiscus schizopethalus* were assessed for their cytotoxic activities to determine if the extracts are safe for topical application and also anti-skin bacterial properties which might add value to cosmeceutical application. It is anticipated that the data obtained from this study would add new knowledge and beneficiary value to natural extracts derived from Malaysian plants for cosmeceutical applications.

2.0 Materials and Methods

2.1 Plant Samples Collection and Identification

The plant samples were collected at several botanical gardens in Perak and Kuala Lumpur. The collection sites of plant samples are shown in Table 1 Only matured plant samples are collected for this study. The collected plants samples were sent to Rimba Ilmu, University of Malaya, Kuala Lumpur for authentication and identification. The plants samples were then stored carefully in 4 ºC in order to maintain the constituents before processing.

Table 1. The collection sites of the plant samples

Plant sample	Collection site
***H. rosa sinensis* (red; flower petal)**	Taiping, Perak
***H. rosa sinensis* (yellow; flower petal)**	Taiping, Perak
***T. erecta* (flower petal)**	Petaling Jaya, Kuala Lumpur
***H. schizopetalus* (flower petal)**	Petaling Jaya, Kuala Lumpur
***P. granatum* (peel)**	Taiping, Perak
***D. linearis* (leaf)**	University of Malaya, Kuala Lumpur

2.2 Preparation of Colorant Extracts

Plant samples were processed using different methods, such as oven-dried, freeze-dried and boiled. The procedures of freeze-dried and oven-dried colorant extracts preparation are shown in Figure 1 and 2, respectively.

For the preparation of oven-dried samples, fresh plant samples were washed and cut into pieces before drying at 45 ºC in oven for three days. The dried plant samples were then ground into fine powder using an electric blender (Panasonic). The fine powder was then kept in an airtight container and stored in fridge (4 ºC).

For the preparation of freeze-dried samples, the fresh samples were washed and cut into pieces before subjected to freeze drying. The plant sample which was freeze-dried was then collected and ground using an electric blender (Panasonic) to produce fine powder. The fine powder was kept in an airtight container and stored in fridge (4 ºC) (Wells, 2013).

2.3 Oven-Dried and Freeze-Dried Colorant Extracts

The colorant extract preparation for oven-dried and freeze-dried plant samples were similar as mentioned in Section 2.2. The ground fine powder of sample was soaked in absolute methanol for 72 hours in dark room. The mixture of fine powder and methanol was filtered with muslin cloth to obtain methanol solution which was further subjected to rotary evaporation at 40 °C until a viscous semi-solid methanol extract was produced. The methanol extract was then collected and exposed in fume hood to evaporate the residue of methanol. The crude extract was then kept in an airtight bottle covered with aluminum foil and stored at 4 ºC before usage.

2.4 Boiled Colorant Extracts

For preparation of boiled colorant extract, 100 g of fresh plant material which was cleaned and cut into small pieces were boiled in 1 liter of distilled water for one hour at slow heat (80 °C). The boiled colorant extracts obtained was left to cool and were then filtered using a muslin cloth before subjected to freeze-drying. The extracts were then kept in airtight bottle covered with aluminum foil and stored at 4 ºC before usage. The preparation procedure of boiled colorant extracts is shown in Figure 3.

2.4 Cytotoxicity Assay

2.4.1 Cell Culture and Medium

Cytotoxicity of colorant extracts in the present study was tested against RAW 264.7 cell line, which is a murine macrophage cell line purchased from American Type Culture Collection (ATCC). The cells were cultured in Dulbecco Modified Eagle Medium (DMEM), supplemented with amphotericin (2 mmol/L), antibiotics (100 units/mL of penicillin and 100 mg/mL of streptomycin) and heat-inactivated fetal bovine serum (FBS) solution (10 mL/100 mL). The cells were maintained at 37 °C in a humidified incubator (Astek Co, Fukuoka, Japan) containing 5 % CO_2 / 95 % air (Lee *et al*., 2013).

2.4.2 Procedure of MTT Assay

The cytotoxicity of colorant extracts was determined using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay as described previously by Teoh *et al.* (2013). Briefly, all the colorant extracts were firstly dissolved in DMSO (except boiled colorant extracts which dissolved in distilled water) to form stock solutions. Cells were seeded into 96-well plate for 24 hours before treatment with various concentrations of the extract. The final concentration of DMSO in each well was 0.5 %. Untreated cells were used as negative controls. After 72 hours of incubation, 20 µl of MTT (5 mg/ml) was added into all wells and incubated for another four hours. The medium was then removed and replaced with DMSO. The absorbance was measured at 570 nm with 650 nm as background using a microplate reader (Thermo Scientific Multiskan GO). All the colorant extracts were assayed in triplicate. IC_{50} value is the concentration of extract or positive reference standard that inhibits 50 % of the cell's growth.

2.5 Media Preparation and Anti-Bacterial Analysis

All the media were dissolved in an appropriate amount of water and autoclaved at 121 ºC, 15 psi for 20 minutes. After the sterilization, the media were cooled down to about 50 ºC and in Hetofrig CB60VS water bath (Heto Lab Equipment A/S, BirkerQld, Denmark). The cooled media were then poured into sterile petri plates. The solidified media were then packed and placed in refrigerator until used. The refrigerated plates were allowed to warm to room temperature on the bench before using.

2.5.1 Isolation of skin bacteria

Skin swab were collected from 25 selected subjects with skin disorders such as acne and fungal infection using a sterile swabsticks. Forehead, cheek and site of acne infection of subjects were swabbed and were then streaked on the one sector of the Luria Bertani (LB) agar and then a sterile inoculation loop was used to finish the streak for isolation in the remaining sectors of each plate. The streaked plates were then incubated at 37 ºC for 24 hours. The colony obtained was examined for its colony charcateristics and Gram stain was conducted. The colonies were then cultured on Mannitol Salt Agar (MSA) and biochemical tests were done. The biochemical tests wereconducted were catalase and oxidase tests. Skin-bacteria that contribute to the body odour including *Staphylococcus epidermidis* and skin diseases including *Staphylococcus aureus* were the focus of isolation.

The oxidase test was performed by adding a few drops of oxidase reagent on a piece of Whatman filter paper No.1, 11µm pore size, which had been freshly streaked with a single bacterial colony, where positive results were indicated by the change of color from colorless to purple within 20 seconds. The catalase test was performed where one single colony from a fresh culture was transferred to a glass slide followed by the addition of one or two drops of 3 % hydrogen peroxide on it. The formation of bubbles indicated positive result.

2.5.2 Skin Bacteria Identification

The selected isolates were identified by Gram staining with microscopy analysis and biochemical tests. The isolates were then subjected to 16sDNA PCR-sequencing for identity confirmation. In brief, DNA extraction was conducted for the overnight culture on nutrient agar

to obtain DNA template for amplification of 16s rDNA gene with PCR. The PCR products were purified and subjected for sequencing. Then the sequences obtained were blasted through NCBI nucleotide database for identification.

For the DNA extraction step, the bacteria were lysed by boiling the colonies of bacteria in 100 µl of ultrapure water at 99 °C for 10 minutes. The DNA concentration was then evaluated using the Nandrop 1000 Spectrophotometer. Extracted DNA was amplified by using PCR technology and the universal 16S rDNA primers 1492R (5' TACGGYTACCTTGTTACGACTT 3') and 27F (5'AGAGTTTGATCMTGGCTCAG 3'). Master mix was prepared. All reactions were carried out in the volume of 25 µl in each tube containing 5 µl of PCR buffer, 1 µl $MgCl_2$, 0.4 µl deoxyribonucloside trisphosphate, 0.1 µl *Taq* DNA Polymerase, 0.2 µl of forward and reverse primer, 5 µl of extracted DNA and 13.1 µl of ultrapure sterile water. The preparation of master mix, the addition of primers and DNA was done under strict and sterile condition (Block *et al*., 2010).

PCR was performed with the following thermocycling programme 5 minutes denaturation at 95 °C, followed by 30 cycles of 1 minute denaturation at 95 °C, 1 minute annealing at 55 °C, 1 minute extension at 72 °C, and a final extension step of 5 minutes at 72 °C. The 100 bp ladder was used together with PCR buffer as a positive control and water was used as negative control to detect false positive results through cross contamination. 5 µl of PCR products was visualized by gel electrophoresis using the agarose gel (1.5 %) and stained with gel red before the bands are viewed. The PCR products were then outsourced for sequencing to First Base Laboratories Sdn Bhd. and the sequence were blasted through the NCBI nucleotide libraries for sequencing matching. Sequences with more than 97 % similarity will be confirmed for its identity either to genus or species level (Muyzer *et al*., 1996).

2.5.3 Screening for Anti-Skin Bacteria Property

Susceptibility of the bacterial strains to the natural colorants was investigated using the disc diffusion method. Before the anti-bacterial assay was performed, all the materials were autoclaved at high pressure saturated steam at the temperature 121 ºC for 15 minutes before the antibacterial assay was started. The materials used were placed in a bio-safety cabinet and exposed to UV light for the purpose of disinfecting.

The culture suspensions were prepared from overnight culture and adjusted to 0.5 McFarland turbidity standard tubes. Muller Hinton Agar medium (20 ml) was poured into each sterile petri dish. Then the surface of the agar medium dish was inoculated with 100 µl cultures (CLSI, 2012). By using a sterile swab stick, the culture was spread on the surface of Muller Hinton agar. Vancomycin (10 µg, Oxoid) was used as a positive control in this study, empty sterilized disc impregnated with distilled water was used as the negative control. Empty sterilized discs (6 mm) were each impregnated with 10µl of natural colorants (50 µg/ml) from the stock was prepared. andwere placed on agar plates using a sterile forcep. The plates were incubated at 37 °C for 24 hours as shown in Figure 1. The diameter of inhibition zones formed on the medium was measured in duplicate using a plastic ruler. All experiments were performed in duplicate (Calis *et al*., 2009).

3.0 Results and Discussion

3.1 Extraction Yield of Colorant Extracts

The yields of colorant extracts are presented in Table 2. The percentage of colorant extract yield in the present study was calculated based on the weight of starting plant materials. The result showed that extraction with methanol for freeze-dried extract of *P. granatum* offered the highest extraction yield with the percentage yield of 20.00 %, whereas freeze-dried extract of *T. erecta* exhibited the lowest percentage yield of 2.57 %. Studies showed that the usage of methanol as extraction solvent can be resulted into higher yield (Jaishee and Chakraborthy, 2015). The anthocyanin pigments are usually extracted with organic solvents such as methanol. The result of a high yield can be concluded due to relationship between polarity of solvents and the structure of polyphenols and pigments of the colorants (Jo *et al*., 2012). The appearances of colorant extracts of the selected plants are shown in Figure 1.

Table 2. Weight of starting material, weight of yield, and percentage of yield for different plant species with different processing methods

Plant species	Processing methods	Weight of starting material (g)	Weight of yield (g)	Percentage of yield (%)
P. granatum	Freeze-dried	2000	400.00	20.00
	Oven-dried	1800	300.00	16.67
	Boiled	700	51.80	7.40
T. erecta	Freeze-dried	60	1.54	2.57
	Oven-dried	62	2.73	4.40
	Boiled	70	5.25	7.50
H. schizopetalus	Freeze-dried	125	12.00	9.60
	Oven-dried	128	10.88	8.50
	Boiled	142	15.00	10.56
H. rosa sinensis (red)	Freeze-dried	118	8.00	6.78
	Oven-dried	120	9.00	7.50
	Boiled	153	25.00	16.34
H. rosa sinensis (yellow)	Freeze-dried	195	10.00	5.13
	Oven-dried	136	11.00	8.08
	Boiled	167	17.00	10.18
D. linearis	Freeze-dried	700	81.69	11.67
	Oven-dried	500	56.45	11.29
	Boiled	400	40.00	10.00

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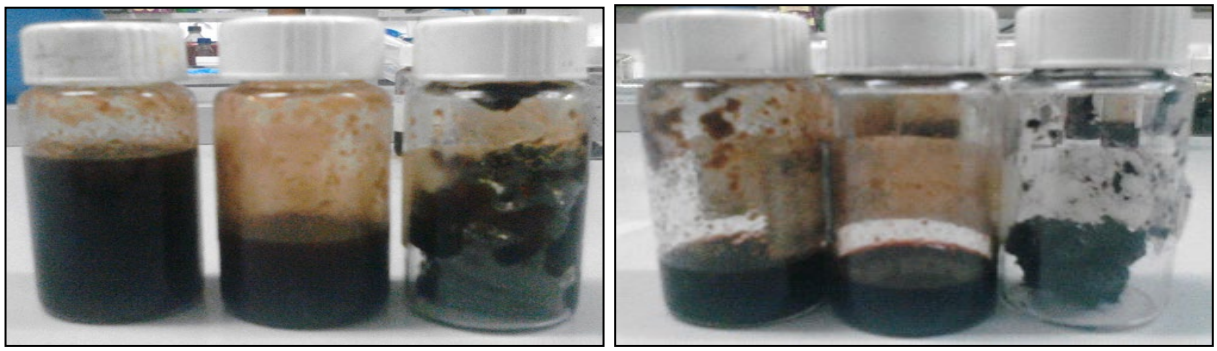
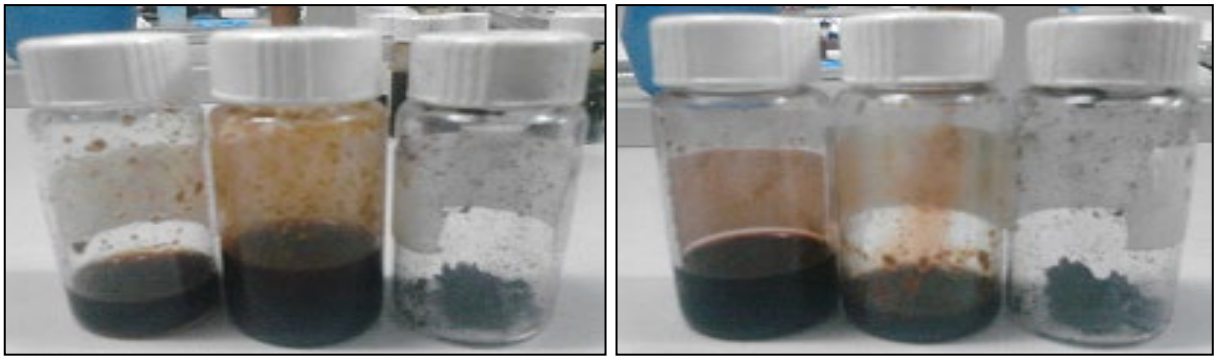


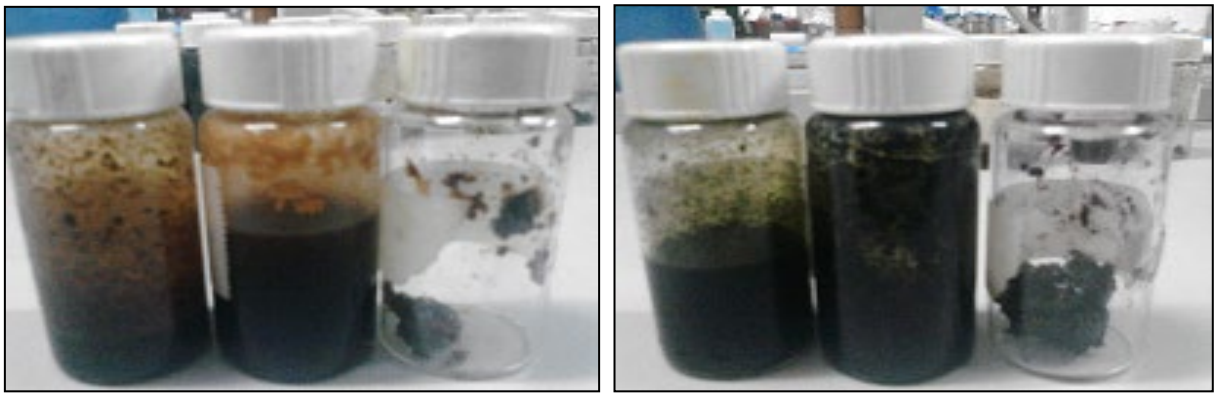
FD (Brown) OD (Brown) B (Dark Brown)
P. granatum

FD (Red) OD (Red) B (Dark Red)
Hibiscus rosa sinensis (red)



FD (Yellow) OD (Yellow) B (Yellow)
H. rosa sinensis (yellow)

FD (Red) OD (Red) B (Dark Red)
H. schizopetalus



FD (Yellow) OD (Yellow) B (Yellow)
T. erecta

FD (Green) OD (Green) B (Green)
D. linearis

FD: Freeze-dried; OD: Oven-dried; B: Boiled

Figure 1. The obtained color of extracts for all the plant materials

3.2 Cytotoxic Activities of Colorant Extracts

In current study, the cytotoxic activities of colorant extracts were evaluated against RAW 264.7 murine macrophage cells using MTT assay which measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability. The MTT assay is best known method for determining the mitochondrial dehydrogenase activities in living cells. In the assay, MTT is reduced to a purple formazon by the NADH but the purple formazon is insoluble in water and it forms a purple needle shaped like crystals in the cells (Marshall *et al*., 1995). The RAW 264.7 murine macrophage cell line was established from an ascites of a tumor induced in mouse of Abselon Leukemia Virus. This cell lines are used in this study due to the sensitivity of the cells towards toxic agents and the cells reaches confluence in a few days. From the results obtained, none of the colorant extracts was cytotoxic against RAW 264.7 murine macrophage cells as presented in Table 3. All the colorant extracts showed IC_{50} more than 100 µg/ml, which was the highest concentration tested in this assay. This shows the plant colorant extract which was used in this study are safe to be applied on skin and may not cause any toxicity. This can further conclude that the extracts do not contribute to the toxicity towards the skin.

Table 3. Cytotoxic activities (IC_{50} values) of colorant extracts against RAW264.7 cell line

Plant species	IC_{50} value (µg/ml)		
	Freeze-dried	Oven-dried	Boiled
P. granatum	>100	>100	>100
D. linearis	>100	>100	>100
H. schizopethalus	>100	>100	>100
H. rosa sinensis (Red)	>100	>100	>100
H. rosa sinensis (Yellow)	>100	>100	>100
T. erecta	>100	>100	>100

3.3 The Isolation of Skin Bacteria Strains

A total of three species of skin bacteria namely *Staphylococcus aureus* (5 isolates: Sa1, Sa2, Sa3, Sa4, Sa5), *Staphylococcus epidermidis* (5 isolates: Se1, Se2, Se3, Se4, Se5) and *Chryseobacterium aquifrigindense* (5 isolates: Ca1, Ca2, Ca3, Ca4, Ca5) were identified from the skin swab of the subjects (Table 4). All of these strains were used to test for the anti-skin bacterial properties of the natural colorant extracts. The sequence of isolated skin bacteria is illustrated in Figure 2.

Table 4. Identification of bacteria using 16s rDNA analysis, using PCR and BLASTN data sequencing

Isolate	Highest BLASTN match with bacteria	Similarity	Genbank accession number
Sa	*Staphylococcus aureus*	97 %	NR113956.1
Se	*Staphylococcus epidermidis*	98 %	NR113957.1
Ca	*Chryseobacterium aquifrigidense*	98 %	NR044334.1

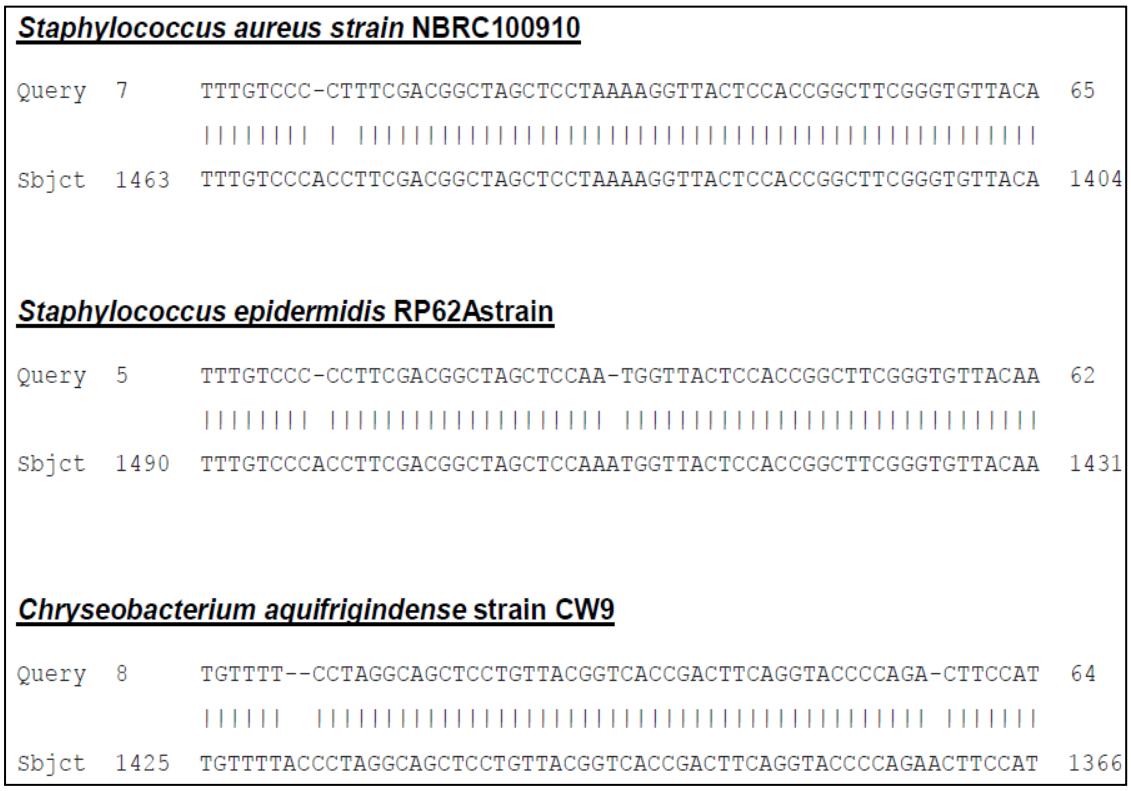


Figure 2: Sequence of isolated skin bacteria

3.3.1 Characteristics of Isolated Bacteria

3.3.1.1 *Staphylococcus aureus* and *Staphylococcus epidermidis*

Staphylococcus aureus and *Staphylococcus epidermidis* are a Gram positive, spherical bacterium approximately 1µm in diameter and it forms grape like clusters due to the process of cell division happen in more than one plane. They are non-spore forming bacteria and non-motile. *Staphylococcus aureus* and *Staphylococcus epidermidis* is often distributed as a commensal associated with skin, skin glands and mucous membrane (Otto, 2009).

On rich medium the *Staphylococcus aureus* forms medium sized golden colonies due to the presence of carotenoids and has been reported to be a virulence factor protecting the pathogen against the oxidants of immune defense system. *Staphylococcus* is a facultative anaerobe that undergoes aerobic respiration to generate energy for its usage. It also undergoes fermentation and produces lactic acid. *Staphylococcus sp*. is oxidase negative and catalase positive. There is another distinctive feature of the *Staphylococcus* is the cell wall peptidoglycan structure which contains the crosslink of glycine residue (Otto, 2009). *Staphylococcus aureus* is hemolytic on blood agar whereas *Staphylococcus epidermidis* is non-hemolytic. (Lozupone and Knight, 2009). *Staphylococcus epidermidis* produces slime layer that forms a hydrophobic biofilm. *Staphylococcus epidermidis* rarely cause pyogenic infections in humans.

The *Staphylococcus aureus* infection usually happens through the spread of the bacteria by having direct contact with the infected person. Infection is also possible when the infected

droplets through sneezing and coughing were inhaled by the individual. Staphylococcus is well known as an etiological agent of carbuncles, skin abscess and furnucles. The treatment of early staphylococcal infection includes β lactam antimicrobial drugs (Linda *et al*., 2006). Scalded skin syndrome is caused by the exfoliative toxins which are secreted on the epidermis which is a serious and a life-threatening syndrome that is caused by *Staphylococcus aureus*.

3.3.1.2 *Chryseobacterium aquifrigidense*

Chryseobacterium species are found in soil and water. It is a ubiquitous bacterium in which studies have shown that it can survive in chlorine treated water supplies. *Chryseobacterium* species are Gram negative bacilli, aerobic, oxidase positive, indole positive, non-glucose fermenters and non-motile. The bacterium grows well on Tryptic soy agar (TSA), Nutrient agar (NA) and R2 agar and does not grow on Mac Conkey agar. The colony characteristics on TSA are convex, circular, smooth, dark yellow in color and opaque with entire margin (Park *et al*., 2008).

Chryseobacterium is not a part of the human skin microbiota (Douvoyiannis *et al*., 2009). The bacterium is able to colonize the patients via contaminated objects such as medical devices such as intubation tubes, incubators for newborns, syringes and cathethers. *Chryseobacteria* have been previously reported as an agent for the pneumonia, meningitis, ocular infection and endocarditis. As an opportunistic pathogen, the *Chryseobacterium* species infects the immunocompromised patients from all age and group and the newborns (Yi *et al*., 2010).

3.4 Anti-Skin Bacteria Activities of Colorant Extracts

Plant materials were used as an alternative source as an anti-bacterial agent as for the past few decades due the increase of antibiotic resistant microorganisms. There were no anti-bacterial activities of the colorant extracts towards *C. aguifrigidense*. The colorant extracts have showed significant anti-bacterial activity against *S. aureus* and *S. epidermidis*. The zone of inhibition was measured as an indicator of the anti-bacterial activity of the colorant extract.

The most significant anti-bacterial activity was shown by the *P. granatum* freeze-dried colorant extract and *D. linearis* oven-dried colorant extract towards the *S. aureus* strain and the towards the *S. epidermidis* strain the *P. granatum* oven-dried extract showed significant anti-bacterial activity. All of tested colorant extracts shown positive inhibition towards *S. aureus* and *S. epidermidis*. However, none of the extracts were able to act against *C. aquifrigidense* isolates.

It is generally thought that the presence of anthocyanin as a major content in the natural colorants has contributed to the antioxidant and anti-bacterial properties. The presence of tannins, polyphenols and flavanoids might also be responsible for the antibacterial activity of the extracts (Chia *et al*., 2010). These mentioned compounds were found widely in the plants which have been used in this research. These plants generally use these compounds as a mode of defense against microbial activity (Mak *et al*., 2013). Previously done research on tannins have highlighted that, tannins are the major compound that is effective against the bacteria, fungi and yeast (Scalbert, 1991). This was proven based on the formation of microbial enzymes and tannin and the cell envelope transport protein complexes, which is believed to cause the inactivation of proteins and thus resulting in the inhibition of the microbial growth (Haslam, 1996).

The presence of secondary metabolites in the colorant extracts has proven to be a reason for the effective antimicrobial agent. The entire plant samples used in this study contain secondary metabolites belonging to polyketides and non-ribosomal peptide families. Previously, studies have concluded that the mechanism of antimicrobial effects involves the inhibition various kind of cellular metabolism and mechanism which is followed by the increase in plasma membrane permeability and finally the ion leakage from the cells. Plant pigments which are rich in variety of phytochemical compounds have been proven to have antimicrobial effects (Sandhu and Heinrich, 2005).

The bacteria structure plays a major role in the antibacterial activity. The Gram-positive bacteria (*S. aureus* and *S. epidermidis*) are more susceptible towards the plant colorant extracts compared to the Gram-negative bacteria (*C. aquifrigidense*) due to its outer membrane. The Gram-negative bacteria consist of lipopolysaccharide and lipoprotein which renders it to react towards the anti-bacterial substances (Ruban and Gajalakhsmi, 2012).

The highest anti-bacterial activity was observed for the *P. granatum* peel freeze-dried and oven-dried colorant extract (Table 6). The pomegranate peels are known to contain phenolic compounds including water soluble tannins which is well known for its antimicrobial activities (Machado *et al*., 2002). The high anti skin bacteria activity of *P. granatum* peel freeze-dried and oven-dried colorant extract could be due to high content of flavanols, phenolics, anthocyanins and organic acid and it was reported that in the presence of vitamin C the antibacterial activity against *Staphylococcal* strains was high (Opara *et al*., 2009). The extracts of *P. granatum* has also been found to have the ability to suppress the activity of *Propionibacterium acnes* that cause acne (Abdollahzadeh *et al*., 2011).

Besides that, the anti-skin bacteria activity of the *D. linearis* oven-dried extract towards the bacteria was also high. The freeze-dried extract and boiled extract also showed a good anti-bacterial activity. The fern is rich in phenolic compounds which had been reported to be beneficial for skin care regime which can prevents UV induced skin damage and directly prevents the colonization of bacteria on skin (Aboshoufa *et al*., 2012). Previous studies have indicated that the *Dicranopteris* species demonstrated antimicrobial activity towards *S. mutans* and *S. sobrinus* (Shin, 2010). The antimicrobial activity of *D. linearis* found in this study explained the effectiveness of with the usage of *D. linearis* to treat wound and as a folk medicine for the treatment of boils in and throat infection (Kumarpal, 2013).

The *H. rosa sinensis* and *H. schizopetalus* contains various kind of organic compounds including flavonoids, tannins, alkaloids, triterpenoids which are known to have anti-bacterial effect. The flowers of *Hibiscus* species contain a large amount of tannin which is a responsible source for the antimicrobial activity *in vitro*. The extracts or nature flowers are reported to be toxic towards the growth of microorganisms and thus leads to the death of the microorganism due to the defects in its metabolism (Upadhyay and Upadhyay, 2011). The flower of *T. erecta* also showed a moderate antimicrobial activity against *S. aureus* and *S. epidermidis* strains (Tables 5 and 6). Traditionally the marigold flowers have been applied to inhibit the inflammation caused by the skin and foot disorders such as callus, corn and hallux (Quackenbush and Miller, 1972).

From the result obtained in Table 5 and 7, the colorant extract which gave the best result is the *P. granatum* oven dried and freeze-dried colorant extract and *D. linearis* oven dried colorant extract. The extraction method also plays a major role in the anti-bacterial activity of the natural

plant sample. The freeze dried and the oven-dried colorant extracts were extracted using polar solvent, methanol and the boiled colorant extract was extracted using distilled water. Previous studies have stated that the antimicrobial activity of the extracts which was extracted with polar solvents are more effective compared to the aqueous extract due to the solubility of plant pigments and the active metabolites which are readily soluble in polar solvents. This makes the extracts more potent towards the microorganisms as it contains a rich amount of bioactive such as tannin, flavonoids, triterpenoids and flavanols in it. From this study, the plant extracts which was extracted with methanol shows anti skin bacterial activity more than the boiled colorant extract overall.

The drying method of the plant samples has effects on the photochemical constituents which are contained in the extracts. The drying method is done to preserve the plant samples by removing water. Phytochemical degradation can occur if the drying method is inappropriate. The sensitivity of the bioactives in the plant sample towards heat is the main reason of the relative instability of the phenolic compounds. Under some circumstances, some natural antioxidants are stable towards the drying process. In this study, the *D. linearis* and *P. granatum* oven-dried have given a good inhibition towards microorganisms. These showed that drying could be beneficial in protecting the phytochemical quality does not reduce the activities (Ahmed *et al*., 2014). This would bring to a conclusion that the best preservation method is the oven drying and freeze drying followed by the extraction using polar solvents.

Table 5. Anti-skin bacteria activity of colorant extracts towards *S. aureus* isolates

Colorant extract	Test bacteria (Inhibition zone)[2]				
	Sa1[1]	Sa2[1]	Sa3[1]	Sa4[1]	Sa5[1]
P. granatum (FD)	15.5±0.71	14.5±0.71	13.5±0.71	12.5±0.71	13.5±0.71
P. granatum (OD)	12.0±0.00	11.0±0.00	9.5±0.71	9.5±0.71	11.5±0.71
P. granatum (B)	14.5±0.71	13.5±0.71	13.5±2.12	13.5±2.12	12.0±2.83
D. linearis (FD)	14.5±0.71	14±1.41	14.5±0.71	13.5±0.71	13.0±1.41
D. linearis (OD)	15.5±2.12	15.5±0.71	16.0±0.00	15.0±0.00	14.5±0.71
D. linearis (B)	9.5±0.71	9.0±0.00	7.0±0.00	7.0±0.00	7.5±0.71
T. erecta (FD)	12.3±0.35	10.5±0.71	12.0±1.41	11.5±0.71	10.8±1.06
T. erecta (OD)	12.0±0.00	11.5±0.71	11.5±0.71	12.0±1.41	11.5±0.71
T. erecta (B)	8.5±0.71	9.0±0.00	8.75±0.35	8.5±2.12	8.0±0.00
H. schizopetalus (FD)	11.5±0.71	12.0±0.00	11.0±1.41	10±1.41	10.5±0.71
H. schizopetalus (OD)	11.5±0.71	11.0±1.41	12.0±0.00	11.0±0.00	13.0±1.41
H. schizopetalus (B)	11.0±1.41	11.0±0.00	11.5±0.71	11±1.41	10.5±0.71
H. rosa sinensis (FD; Red)	11.5±0.71	11.0±0.00	11.0±0.00	11.0±0.00	10.3±0.35
H. rosa sinensis (OD; Red)	11.0±1.41	11.75±1.77	12.5±0.71	11.0±0.00	12.0±0.00
H. rosa sinensis (B; Red)	10.0±0.00	11.5±0.71	12.0±0.00	11.0±0.00	11.5±0.00
H. rosa sinensis (FD; Yellow)	12.0±0.00	11±0.00	12.5±0.71	12.8±0.35	11.8±0.35
H. rosa sinensis (OD; Yellow)	12.0±0.00	11.5±0.00	12.0±0.00	11.0±0.00	12.0±0.00
H. rosa sinensis	12.5±0.71	11.5±0.71	10.0±0.00	9.0±0.00	9.25±0.35

(B; Yellow)					
Vancomycin[3]	17.0±0.00	17.0±0.00	17.0±0.00	17.0±0.00	18.0±0.00

[1]Microorganisms Sa1- Sa5 shows the five isolates of *Staphylococcus aureus*; [2]Each value represented in the table is the mean and standard deviation of the triplicate measurements of the inhibition zone of diameter (mm) including the diameter of the disc (6 mm); [3]Positive control Vancomycin (10 µg). Negative control containing only the distilled water did not show any activity; FD (Freeze-dried), OD (Oven-dried), B (Boiled).

Table 6. Anti-skin bacteria activity of colorant extracts towards *S. epidermidis* isolates

Colorant extract	Test bacteria (Inhibition zone)[2]				
	Se1[1]	Se2[1]	Se3[1]	Se4[1]	Se5[1]
P. granatum (FD)	14.0±0.00	11.5±0.71	11.5±0.71	11.0±1.41	11.5±0.71
P. granatum (OD)	13.0±0.00	14.5±0.71	16.0±0.00	14.5±0.71	13.5±0.71
P. granatum (B)	13.5±0.71	15.5±0.71	15.0±2.83	13.0±1.41	10.5±2.12
D. linearis (FD)	11.5±2.12	13.5±0.71	12.0±1.41	12.5±0.71	12.5±0.71
D. linearis (OD)	14.0±1.41	12.5±0.71	14.0±2.83	14.5±0.71	12.0±0.00
D. linearis (B)	13.5±0.71	11.0±1.41	10.0±0.00	10.5±2.12	10.5±0.71
T. erecta (FD)	11.0±1.41	11.5±0.71	10.0±0.00	11.25±0.35	11.5±0.71
T. erecta (OD)	11.0±1.41	10.5±2.12	11.0±0.00	10.5±0.71	11.75±1.77
T. erecta (B)	12.0±0.00	12.5±0.71	11.5±0.71	11.0±0.00	11.5±0.00
H. schizopetalus (FD)	10.5±0.71	10.5±0.71	11.5±0.71	11.5±0.71	10.0±0.00
H. schizopetalus (OD)	11.5±2.12	11.5±0.71	12.0±0.00	11.5±0.71	11.0±0.00
H. schizopetalus (B)	11.0±1.41	11.5±0.71	10.0±0.00	11.0±0.00	12.0±0.00
H. rosa sinensis (FD; Red)	10.80±0.35	11.25±1.06	11.5±0.71	11.0±0.00	9.75±0.35
H. rosa sinensis (OD; Red)	12.0±1.41	11.0±0.00	11.5±0.71	11.5±0.71	11.0±0.00
H. rosa sinensis (B; Red)	12.0±0.00	12.5±0.71	11.5±0.71	11.0±0.00	11.5±0.00
H. rosa sinensis (FD; Yellow)	12.0±0.00	11.75±0.35	11.5±0.71	12.5±0.71	12.0±0.00
H. rosa sinensis (OD; Yellow)	12.0±0.00	12.0±1.41	12.5±0.71	11.5±0.71	11.5±0.71
H. rosa sinensis (B; Yellow)	10.78±0.71	11.0±0.00	11.0±0.00	11.0±0.00	11.0±0.00
Vancomycin[3]	17.0±0.00	18.0±0.00	17.0±0.00	17.0±0.00	17.0±0.00

[1]Microorganism Se1- Se5 shows the five isolates of *Staphylococcus epidermidis*; [2]Each value represented in the table is the mean and standard deviation of the triplicate measurements of the inhibition zone of diameter (mm) including the diameter of the disc (6 mm); [3]Positive control: Vancomycin (10 µg). Negative control containing only the distilled water did not show any activity; FD (Freeze-dried), OD (Oven-dried), B (Boiled).

Table 7. Anti-skin bacteria activity of colorant extracts towards *C. aquifrigidense* isolates

Colorant extract	Test bacteria (Inhibition zone)[2]				
	Ca1[1]	Ca2[1]	Ca3[1]	Ca4[1]	Ca5[1]
P. granatum (FD)	NI	NI	NI	NI	NI
P. granatum (OD)	NI	NI	NI	NI	NI
P. granatum (B)	NI	NI	NI	NI	NI
D. linearis (FD)	NI	NI	NI	NI	NI

D. linearis (OD)	NI	NI	NI	NI	NI
D. linearis (B)	NI	NI	NI	NI	NI
T. erecta (FD)	NI	NI	NI	NI	NI
T. erecta (OD)	NI	NI	NI	NI	NI
T. erecta (B)	NI	NI	NI	NI	NI
H. schizopetalus (FD)	NI	NI	NI	NI	NI
H. schizopetalus (OD)	NI	NI	NI	NI	NI
H. schizopetalus (B)	NI	NI	NI	NI	NI
H. rosa sinensis (FD; Red)	NI	NI	NI	NI	NI
H. rosa sinensis (OD; Red)	NI	NI	NI	NI	NI
H. rosa sinensis (B; Red)	NI	NI	NI	NI	NI
H. rosa sinensis (FD; Yellow)	NI	NI	NI	NI	NI
H. rosa sinensis (OD; Yellow)	NI	NI	NI	NI	NI
H. rosa sinensis (B; Yellow)	NI	NI	NI	NI	NI
Vancomycin[3]	17.0±0.00	17.0±0.00	17.0±0.00	18.0±0.00	17.0±0.00

[1]Microorganisms Ca1- Ca5 shows the five isolates of *Chryseobacterium aquifrigidense*; [2]Each value represented in the table is the mean and standard deviation of the triplicate measurements of the inhibition zone of diameter (mm) including the diameter of the disc (6 mm). NI indicates no inhibition; [3]Positive control: Vancomycin (10 µg); Negative control containing only the distilled water did not show any activity; FD (Freeze-dried), OD (Oven-dried), Boiled (Boiled).

Conclusion

The plant samples in this study are *H. rosa sinensis* (red and yellow)*, H. schizopetalus, P. granatum, D. linearis* and *T. erecta*. These plant species can be used to extract colorants for the cosmetic industry as they contain high amount of phenolic compounds which are vital for natural skin and body care products. All the colorant extracts did not show cytotoxicity against the RAW 264.7 murine macrophage cells; thus, it can be concluded that these colorant may not cause toxicity against the skin. *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Chryseobacterium aquifrigidense* were isolated from the subjects with skin problems to be used in testing for anti-skin bacterial properties of the natural colorant extracts in this study. The colorant extracts showed promising anti-skin bacteria activity against S. aureus and S. epidermidis isolated from skin and this has added values to the colorant extracts for the substitution of synthetic colorants in the cosmeceutical field. The best extracts which showed good anti-skin bacterial activity in this study were the freeze-dried and oven-dried extract of *P. granatum* and the oven-dried extract of *D. linearis*. The presence of tannins, polyphenols and flavanoid in the extracts could contribute to the anti-skin bacterial activity. Freeze drying and oven drying method produced extracts with better anti-bacterial property compared to extraction using polar solvent. It is believed that freeze drying and oven drying were able to

better preserve the chemical constituents and the thus gave a more promising anti-skin bacterial property.

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