



Antioxidant, Anti-inflammation, Antimicrobial Properties of *Henckelia fischeri* using HaCaTa Human Skin Keratinocytes

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ABSTRACT

Henckelia fischeri an endemic plant enrich with medicinal uses which in the family Gesneriaceae. It is used as a folk medicine for cold, cough and fever by the local people. The solvents ethyl acetate and ethanol is used for the study, where ethanol show high results compare to the ethyl acetate. Antioxidant, anti-inflammation and antimicrobial activity is seen in different solvents of leaf extracts. The antioxidant assay was estimated with DDPH, FRAP and ABTS. The anti-inflammation was evaluated with Membrane stabilizing and anti-hemolytic assay. The bacterial strains used were *Staphylococcus aureus* (MTCC9760) and *Enterococcus faecium* (MTCC2729), *Klebsiella pneumoniae* (MTCC2403) and *Salmonella typhimurium* (MTCC3224). The fungal stains were *Candida tropicalis* and *C. albicans*. The bacterial and fungal strains were treated with different concentration i.e 10 µL to 30 µL, positive and negetative contol. This study also deals with the HaCaT Cell line which is skin human keratinocytes to prove the anti-aging property in it. The research work focus to evaluate the anti-oxidant, anti-inflammation and antimicrobial properties present in the selected medicinal plant and to record the cell activity acting against the aging property. And the results explains the phytopharmaceutical values of the plant.

Keywords: Ethylacetate, Ethanol, Antioxidant, Antimicrobial, Anti-inflammation and HaCaT.

INTRODUCTION

Plants has been a vital birth place of medicine from centuries, with a vast array of bioactive compound exhibiting antioxidant, anti-inflammatory and anti-microbial properties. According to WHO, medicinal plants are the vital source for many bioactive compounds. The increasing prevalence of

genetic and chronic disorders like, heart diseases, intestinal problems, early maturation of skin and its disorders has led to a growing absorption in therapeutic prospective of plant-derived bio-based products¹. Oxidative stress, inflammation and microbial interactions are the essential factors benefaction to the development and progression of these aliments.



Antioxidant are the important material that human body needs naturally, and this antioxidant naturally produce by plants. Free radicals are an effective neutralization by antioxidants which prevents the cell and tissue from oxidative stress and damage. Similarly, Anti-inflammation is essential need to our human body because inflammation is not a deadly disease but the major issues faced by many peoples of the world. This inflammation may be the symptom of many diseases and sometimes it acts as a disease. Anti-inflammatory amalgamation help to regulate the natural defense, reducing Inflammation and promoting tissue repair.² Elsewhere antimicrobial activity is the important property where it rules an essential role in the production of multiple diseases and it will also the necessity activity that each needs to fight against even basic diseases.³

The plant kingdom offers a rich source of antioxidants, antimicrobial and anti-inflammatory agents, including flavonoid components, compounds of phenolic acids, terpenoids, even in alkaloids. These phytochemicals have been shown to modulate various cellular pathways, including antioxidant defense systems, inflammatory signaling cascades, and cell survival mechanisms.⁴

Gesneriaceae family plants have an anti-skin diseases and anti-aging properties in them. This plant is also has a capacity to rule a work against early maturation. Hence this work also deals with the HaCaT cell and its viability. The HaCaT is a Human Skin Keratinocytes.⁵

Henckelia fischeri (Gamble) A. Weber & B. L. Burtt is a Gesneriaceae plant. It is locally known as "Parai otti", the plant is commonly used for the diseases like fever, cold and cough by the local people. The plant is herbaceous and found in the Southern Western Ghats of Tamil Nadu.

This research was designed to evaluate an anti-oxidant, Anti-inflammatory and antimicrobial activities of a choosed plant species traditionally used in (Traditional medicine/Local health practices). And also to enchance the cell viability in HaCaT cells. We employed various *in vitro* assays to understand an antioxidant, anti-inflammatory and antimicrobial potential of *Henckelia fischeri* extracts, with a focus on (Specific bioactive compounds or mechanisms)⁶. Our findings contribute to the understanding of

the medicinal properties of *Henckelia fischeri* and highlight its capacity as a wellspring of natural antioxidants, anti-inflammatory and antimicrobial agents. This study gives an idea related cytotoxicity effect of HaCaT- Human Skin Keratinocytes. And this plant is not well explored in the research point of you. So the main hypothesis is to explore.

MATERIALS AND METHODOLOGY

Gathering and Determination of the sample

Sample is assemble from the Savarikkadu, Southern Western Ghats of Tamil Nadu. The sample is collected with the help of local peoples. The authentication of the assembled medicinal plants is done in BSI of Coimbatore, Tamil nadu, India.

Study plant and its Medicinal Uses Systematic position

Class : Magnoliopsida (or) Dicotyledonae
Order : Lamiales
Family : Gesneriaceae
Genus : *Henckelia*
Species : *H. fischeri*

The genus *Henckelia* is available in North east area of Asian Continent and those are endemic to certain regions of India. 70 species in worldwide and 35 species found in India. The plant is perennials, rosette-forming and it is a hairy herbs with Rootstocks. The roots are adventitious and thin. Leaves are winged with 12-20 in numbers arranged 4 whorls. Flowers are small with Dark to pale lilac in colour. Capsules-cylindrical, straight or randomly curved. Seeds- numerous with dark brown in colour. Flowering will occur from the month of may to october whereas Fruiting will occur from the season July to December.⁷

Ethnomedicinal Uses: Commonly used for cold, cough and fever. But it also have properties like antioxidant, antimicrobial and anti-inflammation.

Sample Extraction

The leaves were cleaned under running water, allowed to dry in the shade at room temperature, and then ground into a fine powder. The extraction process begins with a pack of 30 g of powdered dried leaf, treated with 300 milliliters of ethyl acetate and then the same powdered dried

leaf is treated with 300 milliliters of ethanol that is soluble in a Soxhlet apparatus. The temperature is kept at its boiling point until the extraction is finished. Following the drying process of the plant's extracted leaf sample, the crude was then gathered. Using the product, the additional analysis was completed.⁸

Anti-oxidant Investigation-*In vitro* **Quenching activity DPPH Assay**

An antioxidant estimation was estimated with the donation of hydrogen or through the ability of radical scavenging. The samples were treated with different concentration along with the methanolic solution of DDPH. Here the standards used are rutin and BHT which was shaken vigorously. And the test tubes along with negative control was kept in 27°C for 20 minute. The sample was readed at 517nm along with blank. Here the blank act as a methanol.⁹

Scavenging activity ABTS Assay

ABTS Assay was sustained with the help of decolourization of samples. Taking of 2.4mM Potassium persulfate is mixed with 7mM ABTS aqueous solution which is diluted in ethanol and equilibrated at 30°C which is readed at 734nm.¹⁰

FRAP Assay

Freshly prepared FRAP reagent was incubated for 37°C. Again the sample is treated with distilled water and methanol, after that it is incubated in water bath for 30 min at 37°C. The optical density reading was calculated at 593nm along with the reagent blank in spectrophotometer at the end of incubation.¹¹

Anti-Inflammatory Investigation-*In vitro* **Membrane-Stabilizing assay**

Extract is incorporated with HRBC solution of 0.5 mL and Hypotonic solution of 4.5 mL which contains diverse concentration of bergapten (10, 30, and 100 µg/mL). Triplicate is method of this demonstration. The combination is incubated for 10 min in room temperature along with the positive and negative control. The centrifugation was also done at 3000rpm at 10 minute. The supernatant is considered as a hemoglobin content which is measured at 540nm in spectrophotometer. The formulae used to estimate the percent of inhibition of hemolysis was¹²

$$\% \text{inhibition} = \frac{\text{absorbance}(\text{control}) - \text{absorbance}(\text{test})}{\text{absorbance}(\text{control})} \times 100$$

Anti-hemolytic analysis

Evaluation of ferrous ion induced hemolysis in extracts were investigated. 2.5 µM ferrous sulfate is combined with RBC suspension was incubated with at 37°C for 30 min then addition of phosphate buffered saline (PBS) 4 mL with the samples. The centrifugation was done at 2500rpm for 5 minute. The hemolysis were calculated with the use of spectrophotometer by at 540nm. The control sample is calculated without the extract.¹³ Calculation of anti-hemolysis percent was done by the equation.

$$\% \text{Inhibition} = 100 \times (1 - \text{OD sample}) / \text{OD control}$$

Antimicrobial Analysis **Strains of Bacteria**

The Biotechnology Department from Bharathiar University located in Coimbatore on condition that the four strains of dangerous bacteria used in this study. The Strains of bacteria used are *Staphylococcus aureus* (MTCC9760) and *Enterococcus faecium* (MTCC2729), *Klebsiella pneumoniae* (MTCC2403) and *Salmonella typhimurium* (MTCC3224)

Disk Diffusion Method

The extraction of plant material was diluted in DMSO of 1 mL. Muller Hinton agar were freshly make ready and an isolated organism were inoculated overnight in the Nutrient broth in a sterile condition. The plates used to lawn with swabs. The sterile discs contain 30 µL of plant extract and then the plates are allowed to incubate overnight at 37°C in a sterile condition¹³. Antifungal Activity Antifungal was done with the fungal *Candida tropicalis* and *Candida albicans*. A sterile filter paper disc at the concentration of 10 mg/mL was taken.¹⁴ And the method was done in triplicate and also repeated twice.

Development of Cell

The cell line HaCaT is an Human Skin Keratinocyte Cell Line which were secure from NCCS, Pune. Dulbecco's Modified eagle medium is the method which was used to yield the cell line in large scale. The cells are maintained in the media of DMEM along with 10% FBS and 1% anti-biotic anti-mycotic solution in the temperature, 5% CO₂ and 18–20% of O₂ at 37°C for 3 days.¹⁵

MTT Assay

The major purpose of the analysis of is

to determine the MTT Assay of Proliferation cell and its cytotoxicity, it is colorimetric assay. It is done based on the reduction of coloured yellow water soluble Tetrazolium Dye MTT, and this dye redacts to formazan crystals. The cells formed in the region of mitochondrial lactate dehydrogenase that reduces the MTT to a formazan crystals where it forms a colour purple here the number of viable cell is proportional to its intensity and those cells are estimated using spectrophotometer in 570nm.^{15,16}

Cytotoxicity and cell proliferation–*In vitro* analysis

The HaCaT is A Human Skin Keratinocyte cell line, here the seed of 200 μ L in a well plate is allowed to grow for about 24 hours. And then it is incubation was done in 5% of CO_2 atmosphere for 24 hours. After the completion of the incubation period the media was removed from the cells are allowed for MTT reagent for 0.5 mg/mL in concentration. Over 3 h of incubation removing of MTT reagent and addition of DMSO is done. Then the reading of absorbance was done in spectrophotometer or an ELISA reader at 570nm wavelength. And the viability of cell was estimated using different Concentration (6.25, 12.5, 25, 50, 100 and 200 μ g/mL). The rapid reducing of cells can be seen in the different concentrations of the sample. The growth inhibition percentage was estimated by using the concentration of the extract need to inhibit cell growth by 50% value i.e. IC_{50} .^{16,17}. The graph curve shows the rapid decreasing of affected cell. And the % of cell viability was determine by the equation.

$$\text{Percent of viability cell} = \frac{\text{Mean absorb of treated cells}}{\text{Mean absorb of untreated cells}} \times 100$$

RESULTS

Anti-oxidant Investigation¹⁸

DPPH Assay

DPPH assay is the familiar activity meant for the evaluation of the Antioxidant content in a sample. In the analysis the presences of anti-oxidant is identified by the decolourize. And the standard used here is rutin and BHT. The results of DDPH assay shows the ethyl acetate is high compare to ethanol. And the Fig and Table 1 shows the results of DDPH scavenging assay.

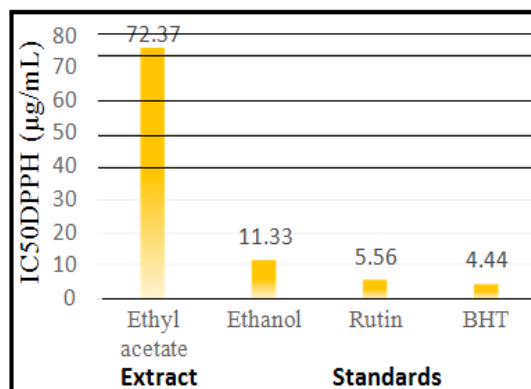


Table 1: DPPH Assay

Specimens	Distillation	DPPH% Assay IC ₅₀ (µg/mL)
Standard	Ethyl acetate	72.37
	Ethanol	11.33
	Rutin	5.56
	BHT	4.44

Fig. 1 and Table 1. Showing the DPPH Assay of the selected Sample

ABTS Assay

ABTS Assay is also a measure of decolourization of the specimen. In ABTS Assay the BHT and rutin is acted as a standard. And the sample is evaluated in triplicate and after incubation the measurement of sample is at 734nm. As the results Ethanol shows high activity compare to ethyl acetate, where the ethyl acetate is 98194.44 and ethanol is 112222.2. It is proved that plants have enormous chemical compounds in them. Here the Fig and Table 2 shows the results of ABTS Assay of Sample.

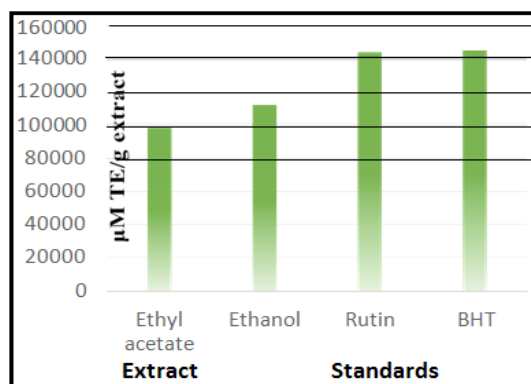


Table 2: ABTS Assay

Samples	Extracts	ABTS(µg TE/g extract)
Standard	Ethyl acetate	98194.44 ± 2926.57
	Ethanol	112222.2 ± 636.46
	Rutin	144166.7 ± 416.6
	BHT	145347.2 ± 636.4

Fig. 2 and Table 2. Showing the ABTS Assay of the selected sample

FRAP Assay

The estimation of FRAP Assay results that an ethyl acetate shows high activity than ethanol. And Rutin and BHT is used as a standard. Fig and Table 3 indicates the results of the selected samples in the following analysis.

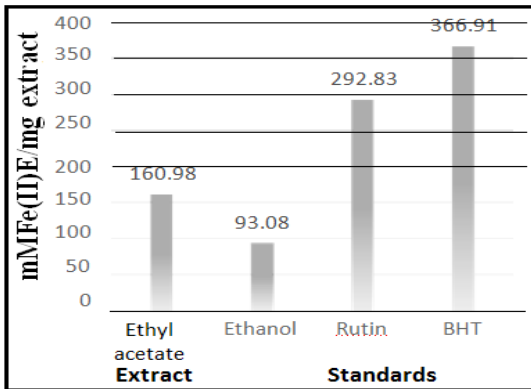


Table 3: FRAP Assay

Samples	Extracts	FRAPmM Fe(II)/mg extract
	Ethyl acetate	160.98±5.6
	Ethanol	93.08±2.38
Standards	Rutin	292.83±4.46
	BHT	366.91±3.5

Fig. 3 and Table 3. Showing the FRAP Assay of the selected sample

**Anti Inflammatory Activity¹⁹
Membrane-Stabilizing assay**

The membrane stabilizing assay is carried out in triplicates with the negative control. The incubation of the sample is done for 10 min at room temperature which after done for 10 min centrifugation at 3000rpm. As the results are calculated, where the ethanol (46.46) shows the high activity compare to ethyl acetate (32.25). Fig and Table 4 is the results of membrane stabilizing assay.

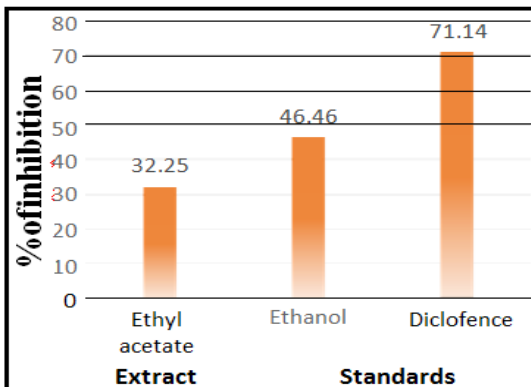


Table 4: Membrane Stabilizing assay

Samples	Extracts	% of inhibition
	Ethyl acetate	32.25 ± 0.09
	Ethanol	46.46 ± 0.34
Standard	Diclofenac	71.14 ± 0.42

Fig. 4 and Table 4. Showing the Membrane-Stabilizing assay of the selected sample

Anti-hemolytic activity

Hemolysis is among the common method to investigate the anti-inflammation activity. Hemolysis was estimated by the measure of OD reading was analyzed for the supernatant in 540nm in the spectrophotometer. As a results, Where the Fig and Table 5 explains the Anti-hemolytic activity ethanol sample shows high response compare to the ethyl acetate of the value 55.6 and 25.95 respectively.

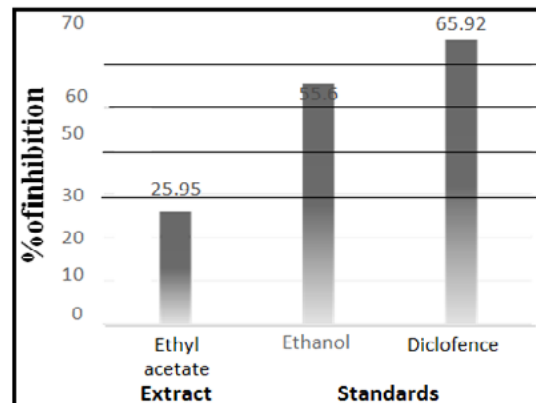


Table 5: Anti-hemolytic activity

Samples	Extracts	% of inhibition
	Ethyl acetate	25.95±0.25
	Ethanol	55.6±0.1
Standard	Diclofenac	65.92±0.11

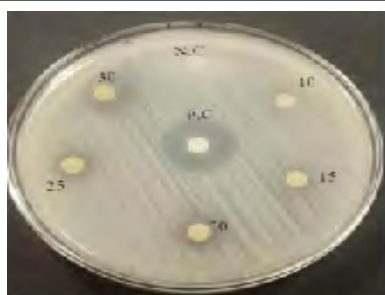
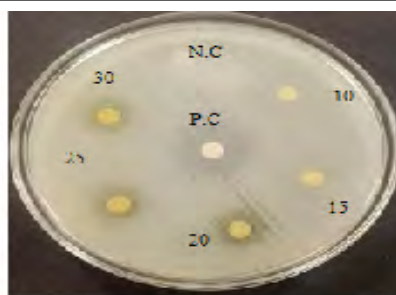
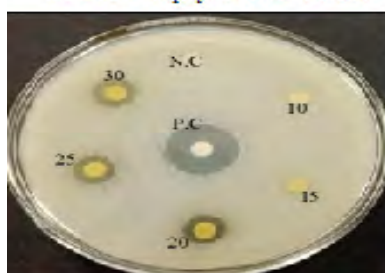
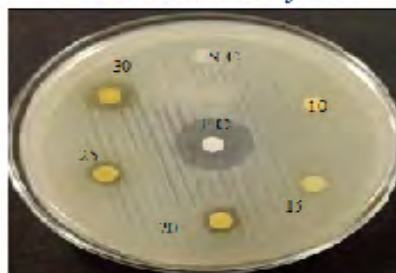
Fig. 5 and Table. 5 Showing the Anti-hemolytic activity of the selected sample

Antimicrobial Activity²⁰

The antimicrobial activity was done in both the samples such as ethyl acetate and ethanol. The work was carried out in 2 Gram+ve bacteria such as *Staphylococcus aureus* and *Enterococcus faecium* and Gram-ve bacteria like *Klebsiella pneumonia* and *Salmonella typhimurium* and in 2 fungal organisms namely *Candida tropicalis* and *Candida albicans*. And, results was listed below in Table 6 to 9 and Picture 1-12.

Table 6: Showing the Antibacterial activity in ethyl acetate of the selected sample

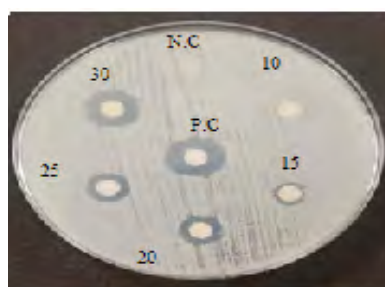
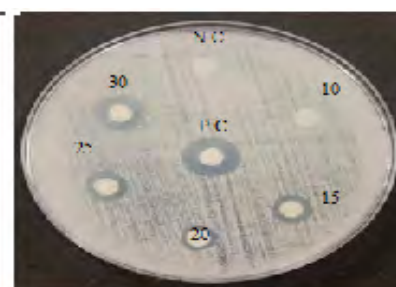
Extract Concentration (10 mg/mL)	Zone of inhibition (<i>Staphylococcus aureus</i>)	Zone of inhibition (<i>Enterococcus faecium</i>)	Zone of inhibition (<i>Klebsiella pneumoniae</i>)	Zone of inhibition (<i>Salmonella typhimurium</i>)
Negative control (DMSO)	-	-	-	-
10 μ L	-	-	-	-
15 μ L	-	-	-	-
20 μ L	10	10	10	8
25 μ L	11	11	10	8
30 μ L	12	10	11	10
Positive control (Streptomycin)	19	17	18	19

Picture-1 *Staphylococcus aureus*Picture-2 *Enterococcus faecium*Picture-3 *Klebsiella pneumoniae*Picture-4 *Salmonella typhimurium*

Picture 1- 4. Showing the Antibacterial activity in ethyl acetate of selected sample

Table 7: Showing the Antifungal activity of ethyl acetate of selected sample

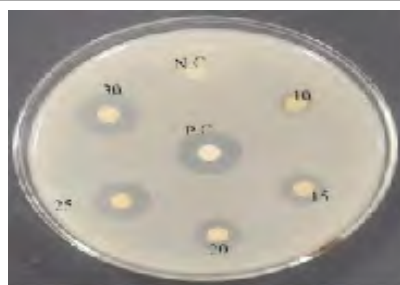
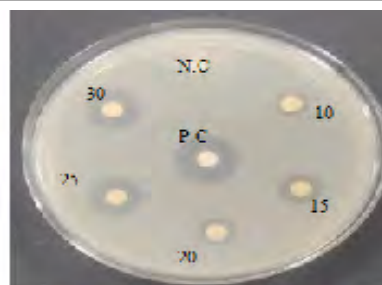
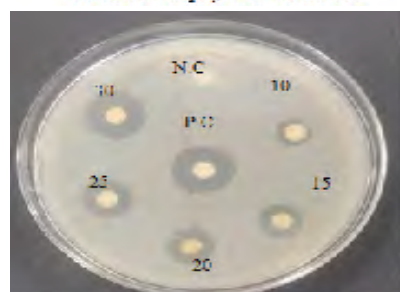
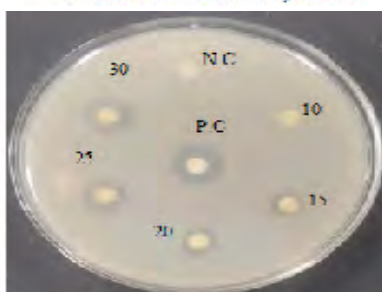
Extract Concentration (10 mg/mL)	Zone of inhibition (<i>Candida tropicalis</i>)	Zone of inhibition (<i>Candida albicans</i>)
Negative control(DMSO)	-	-
10 μ L	-	-
15 μ L	7	8
20 μ L	10	7
25 μ L	10	9
30 μ L	13	11
Positive control (Fluconazole)	14	14

Picture. 5 *Candida tropicalis*Picture. 6 *Candida albicans*

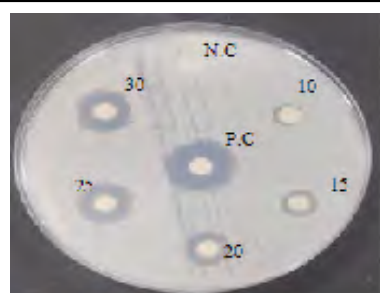
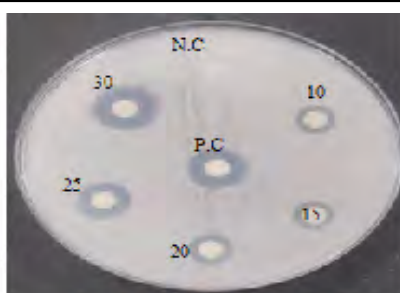
Picture 5 & 6. Showing the Antifungal activity in ethyl acetate of selected sample

Table 8: Showing the Antibacterial activity in Ethanol of selected sample

Extract Concentration (10mg/mL)	Inhibition Zone (<i>Staphylococcus aureus</i>)	Inhibition Zone (<i>Enterococcus faecium</i>)	Zone of inhibition (<i>Klebsiella pneumoniae</i>)	Zone of inhibition (<i>Salmonella typhimurium</i>)
Negative control (DMSO)	-	-	-	-
10 μ L	9	9	9	-
15 μ L	12	11	12	8
20 μ L	13	10	13	9
25 μ L	13	14	15	12
30 μ L	16	15	16	13
Positive control (Streptomycin)	18	17	18	14

**Picture 7: *Staphylococcus aureus*****Picture 8: *Enterococcus faecium*****Picture 9: *Klebsiella pneumoniae*****Picture 10: *Salmonella typhimurium*****Picture 7- 10. Showing the Antibacterial activity in Ethanol of selected sample****Table 9: Showing the Antifungal activity in Ethanol of selected sample**

Extract Concentration (10mg/mL)	Zone of inhibition (<i>Candida tropicalis</i>)	Zone of inhibition (<i>Candida albicans</i>)
Negative control (DMSO)	-	-
10 μ L	6	8
15 μ L	9	9
20 μ L	12	10
25 μ L	13	12
30 μ L	13	12
Positive control (Fluconazole)	18	15

**Picture 11: *Candida tropicalis*****Picture 12: *Candida albicans*****Picture 11- 12. Showing the Antifungal activity in Ethanol of selected sample**

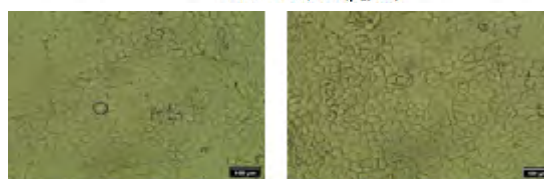
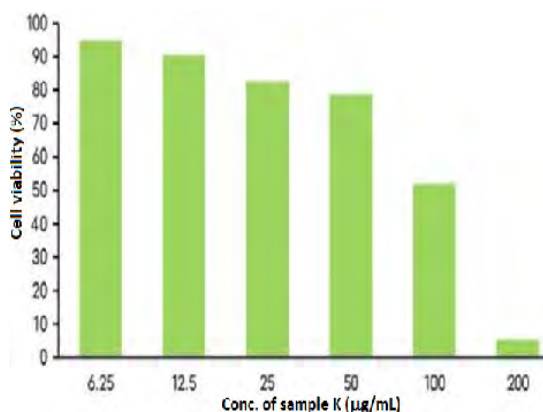
Cytotoxicity and cell proliferation—*In vitro* analysis (HaCaT-Human Skin Keratinocyte cell)

To appraise the source in *H. fischeri* on development of HaCaT, The cells were studied in the different concentration such as 6.25, 12.5, 25, 50, 100 and 200 $\mu\text{g/mL}$ and the reduction of toxic cells have taken place in the increasing of concentration. Where the concentration 6.25 shows 94.65% of cells, 12.5 shows 90.25% of cells, 25 shows 82.37% of cells, 50 shows 78.40%, 100 shows 51.87% and 200 shows 5.08%. So this rapid decrease indicates that the sample has the capacity of anti-aging property in it. And the results was listed below in Table 10, Fig. 6 and Picture 13 to 19.²¹

Table 10: MTT Assay of HaCaT in Ethanol extract

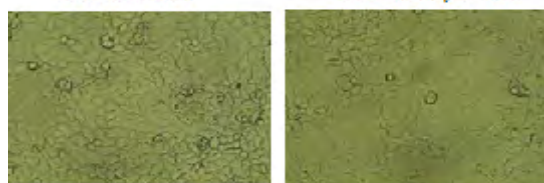
Culture condition	% of Viability Cell
Untreated	100
6.25 $\mu\text{g/mL}$	94.65
12.5 $\mu\text{g/mL}$	90.25
25 $\mu\text{g/mL}$	82.37
50 $\mu\text{g/mL}$	78.40
100 $\mu\text{g/mL}$	51.87
200 $\mu\text{g/mL}$	5.08

$\text{IC}_{50} = 103.16 \mu\text{G/ML}$



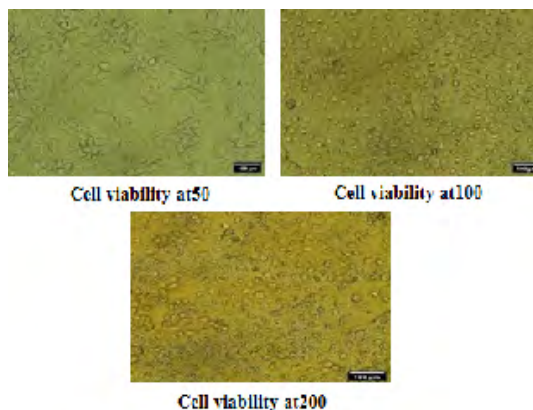
Untreated cells

Cell viability at 6.25



Cell viability at 12.5

Cell viability at 25



Picture 13 to 19. MTT Assay of HaCaT in Ethanol extract

DISCUSSION

The results secured from the followed activities proves that the selected medicinal plant sample has an enormous properties of antioxidant, anti-inflammation and antimicrobial content whereas the HaCaT cells prove that the sample has the ability of work against the aging and skin diseases. Now-a-days the world facing an indirect problem which is aging at early stage. So this observation helps the researchers to find a new beginning to proceed^{22,23}. And the above estimation have also discuss about the different assay like DDPH, FRAP and ABTS in the antioxidant activity which ensure that the selected solvent both ethanol and ethyl acetate of *Henckelia fischeri* (Gamble) A. Weber & B. L. Burt has a wealthy source of antioxidant in it. Whereas the anti-inflammation property was done in 2 assay including Anti-hemolytic and Membrane stabilizing assay proves that the sample inhibits the nature against inflammation.²⁴ The microbial work was carried out in both *Gram+ve* and *Gram-ve* bacteria and fungal which proves that specimen work against them in a sterile condition and proves that it is antimicrobial specimen.^{25,26} With the following results the work undergone to the check the cell viability in a fibroblast cells, the cell specified was HaCaT it is a Human Skin Keratinocyte and this cell was allowed to identify its viability at different concentration such as 6.25, 12.5, 25, 50, 100 and 200 $\mu\text{g/mL}$. Where the decreasing of cell can be seen, in other words increasing of concentration leads to increase of cell viability.²⁷ Hence from the discussion part, the study reveals that the specimen selected has countless medicinal resources and they have also proved the presences of secondary metabolites like phenol, tannin, flavonoid and Alkaloid which is the lead to the pharmaceutical world.

CONCLUSION

The study can be terminate the leaf extracts from the selected medicinal plant *H. fischeri* (Gamble) A. Weber & B. L. Burt of Gesneriaceae, has the property of antioxidant, anti-inflammation and antimicrobial activity in the high quantity. And this study is conclude that the ethanol solvent of plant extract has rich activity compare to that of ethyl acetate in all the three properties such as antioxidant, anti-inflammation and antimicrobial. The HaCaT shows the highest rate of proliferation at the 200 µg/mL concentration. And futher work was going in the same plant. Hence this plant has high medicinal uses and this could utilize in the diagnosis of numerous diseases. Therefore, additional investigation is necessitate to pinout and pindown the compounds present in the plant extract prior to clinical use and to establish the therapeutic value.

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Bones of Contention

The authors have no Bones of contention in this work.

Ethical Consideration

This study does not contribute any investigations on animals or human subjects.

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