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RESEARCH ARTICLE

RELATIVE EXPRESSION OF SLOW GROWTH SUPPRESSOR GENE(SGS1) UNDER THE TREATMENT OF HERBAL FORMULATION ON YEAST MODEL

***[1]Sudhakar Malla, [2]Selvam Arjunan, [1]Sanjana, W.K., [1]Anu John, B., [1]Gunna Vidyanandh and [1]Shriramwar Yogesh Gajanan**

[1]Department of Biotechnology, Indian Academy Degree College, Bangalore
[2]Department of Life Sciences, Kristu Jayanthi College, Bangalore

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ABSTRACT

Hair loss is one the dermatological disorder, estimated to effect nearly 2% of the worlds population. The problem of hair loss, greying of hair in young age is normally caused due to exposure to pollution, pesticides, imbalanced nutrition, altered gene expression and also harmful chemicals that are present in dermatological products. The present study was aimed to evaluate the expression of SGS1 gene which is homologue to human WRN and BLM gene that plays an important role in hair follicle development. The hair growth activity was studied by treating the yeast cells with herbal extracts (*Eclipta alba* and *Ficus Bengalensis* prop roots) and simultaneously inducing the cells to stress by organopesticides to investigate the inhibitory action of the herbal extracts on yeast cells. The cell viability and antioxidant assays were performed for the herbal extracts where significant results were observed in ethanol and methanol fractions. The expression of SGS1 gene was studied using RT-PCR, where the expression of the gene was found to be lowered in case of yeast cells treated with pesticides whereas the gene expression was found to be more in yeast cells that were treated with pesticides and herbal extracts. However further study at protein level is needed to conform the activity with the pure extract.

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INTRODUCTION

The miniature organ of the mammalian skin known to be the Hair, emerges from the active hair follicle composed of completely keratinized epithelial cells, renders numerous functions throughout the adult life (Lee Won Gyu, 2001). Hormones play a vital role in the cyclic hair growth process characterized by anagen (growth phase), catagen (regression phase), and telogen (resting phase) (S. Tiede *et al*, 2007). The problems of hair loss, hair growth or greying of hair at an young age can be caused when the genes responsible for hair growth cycle is altered, due to the environment factors such as exposure to pesticides, metabolic imbalance or improper nutrition (Love S. Chokotia, 2013). The defect in WRN and BLM genes were found to cause Werner's syndrome (premature aging disorder) and Bloom's syndrome (greying of hair) of normal aging in young adults. The gene WRN and BLM was found to be homologus to *Saccharomyces cerevisiae* SGS1 gene encoding for DNA helicases (Ellis *et al*.,1995; Yu C.-E *et al*,1996; Nakayama *et al*., 1984; Umezu *et al*.,1990; Watt *et al*., 1996). There occurs a physical interaction of SGS1 gene and topoisomerase III (Watt P. M *et al*,1995; Gangloff S *et al*,1994), where mutation in this SGS1 gene suppresses the growth defect caused by topoisomerase III mutation (Habibi, 2011), repeated ribosomal DNA locus undergoes increased recombination and reduce the average life span of yeast cells (Hanada *et al*.,1997; Watt *et al*., 1996; Watt *et al*.,1995; Gangloff *et al*.,1994). Hence the present study was aimed to evaluate the hair growth activity using herbal formulation (HF) which includes *Eclipta alba* and *Ficus bengalensis* (prop roots). The plant species *Eclipta alba* commonly recognized as 'Bhringaraja', belongs to the family Asteraceae (Mithun NM, 2011). The annual herbaceous plant is very well known for its traditional medicinal purposes, commonly used in polyherbal formulation for hair growth development (Cheol, 2004; Xiulan,1997). The plant is small, erect or prostate, cylindrical, grayish roots, branched and has white flower heads, commonly found in tropical or subtropical regions of the world containing many curative properties like antioxidants,

****Corresponding author: Sudhakar Malla***
Department of Biotechnology, Indian Academy Degree College, Bangalore

immunomodulatory, antibacterial, analgesic. The phytochemicals like flavonoids, alkaloids, glycosides, polyphenols, stigmasterols, demethylwedelolactone, present in *Eclipta alba,* are the essential compounds for hair growth. The plant species *Ficus bengalensis* commonly recognized as Banayan tree, belongs to the family Moraceae. The evergreen to deciduous tree, ranging upto 20m tall, wide leaves and branches spreading to 100m with aerial roots is famous for its Ayruvedic properties and most commonly seen in India. All the plant parts contains astringent, anti-inflammatory and antidiarrheal activities (Nadakarni, 2004). The phytochemicals like phytosterols, flavonoids, sterols, triterpenes, triterpenoids, tiglic acid esters were reported in *Ficus bengalensis* can be the essential components for hair growth (Daniel *et al*., 2003; Sharad *et al*., 2007). Flavonoids and phenols are polyphenolic plant pigments widely present in many plants which are a group of antioxidants, known for its efficacy for promoting hair growth, preselected for their telogen phase of hair growth. The present study demonstrated the hair growth promoting potential of the crude extracts of *Eclipta alba* and *Ficus Bengalensis* that contains phenols and flavonoids. The exact mechanism of action or the component(s) responsible for the hair growth promoting activity in the plant extract could not be established in this study. However, a number of investigators have shown that flavonoids and triterpenoids possess hair growth promoting activity. The present study aims at studying the expression of SGS1 gene of yeast by treating them with organopesticides and to try to inhibit their effect on yeast using herbal extracts of *Eclipta alba* and *Ficus Bengalensis* (prop roots). Hence the study was undertaken with a view to elucidate the ability of herbal extracts to inhibit the effect of organopesticides on hair follicle development using yeast model.

MATERIALS AND METHODS

Plant material: Plant samples of *Ficus bengalensis* (prop roots), *Eclipta alba* (bringa raja) were procured from the botanical garden of GKVK-Bangalore India. Yeast (*Sacharomyces cerevisiae*) were cultured in potato dextrose broth and maintained at 37°C for 48 hours and the culture was used for further experiments.

Extraction and fractionation: 50g of the dried pulverized powder of *Eclipta alba* and *Ficus bengalensis* was extracted via maceration with 50ml of ethanol, chloroform and methanol each using soxhlet method. The extracts were filtered and evaporated to dryness using rotatory vacuum evaporator at 40°C. The powdered material collected was weighed and stored for further use. Phytochemical analysis for phenols, flavonoids and steroids (Shieh and Tsai, 1985) was done on the extracts.

Assessment of cell viability: The yeast cells were treated with organopesticides (DDT, 50mg/ml) for about 9 days to check for the vitality and cell cytotoxicity. The treated cells were further treated with the isolated herbal extract to check for the effect of the extract. Control was maintained without treatment. Following incubation, the cells were harvested and the cell viability was examined using the trypan blue staining method.

Lipid peroxidation: Lipid peroxidation assay was performed for different time period of day 3, day 6, and day 9 to evaluate the cell viability and proliferation. The cells were treated with the 20µl extract (2mg/µl) and added with 20µl with organopesticides (50mg/ml). 150µl of water and 1ml of Thiobarbituric acid was added to the samples and the vials were incubated in boiling water bath for 20min. The contents were cooled and centrifuged at 2000rpm for 10min the supernatant was collected and the absorbance was measured at 532nm against a blank. Cells treated with the pesticide alone were treated as control.

MTT assay: MTT assay was performed for different time period of day 3, day 6, and day 9 to evaluate cell proliferation where the cells were taken in two vials one incubated with 20µl extract (2mg/µl) and 20µl of organopesticides (50mg/ml). The contents were mixed thoroughly and added with 20µl of MTT (0.5mg/ml) and were incubated for 4hrs at 37°C. 200µl of acidic isopropanol was added and incubated for 1hr at 37°C and the absorbance was recorded at 570nm.

Superoxide Dismutase assay: Superoxide dismutase assay was performed to evaluate the resistance to stress condition when yeast cells were treated with herbal extracts. Superoxide dismutase (SOD) was assayed according to the technique [18]. The enzyme SOD is extracted using extraction buffer. To 0.75ml of extract equal amount of water is added and mixed properly. The contents were mixed thoroughly and added with 1ml of 0.01M Tris-Cl (pH 8.5) and 0.5ml of pyrogallol. For blank, 1.5ml of water is used as blank together with 1ml of 0.01M Tris-Cl (pH 8.5) and 0.5ml of pyrogallol. The contents were incubated at 37^0C for 20min. The absorbance was recorded at 420nm at 0 and 20min time intervals. The rate of increase on absorbance units (A) per minute for the blank and for the test sample are calculated by the equation (A420nm/min= O.D at last interval –O.D at first interval/time in min). The % inhibition is given by the formula

(A420nm/min[blank] - A420nm/min[test] / A 420nm/min[blank] * 100.

Catalase assay: To 0.04ml of sample, add 2.6ml of 50mM potassium phosphate buffer [pH=7.0] and 0.4ml of 15mM H_2SO_4. The contents are mixed properly. Incubate the contents at 25°C for 5min. Absorbance was recorded at 420nm at 0 and 5 minute time intervals. The rate of increase on absorbance units (A) per minute for the blank and for the test sample are calculated by the equation (A420nm/min= O.D at last interval –O.D at first interval/time in min). The % inhibition is given by the formula (A420nm/min[blank] - A420nm/min[test] / A 420nm/m in[blank] * 100.

Peroxidase assay: Peroxidase assay was performed to determine Peroxidase enzymatic activity using Pyrogallol as the substrate. The yeast cells were allowed to incubate with 380µl of extract. To this 1.5ml of phosphate buffer, 770µl of 0.01M pyrogallol and 770µl of 0.005M H_2SO_4 was added and incubated for 5min at 25°C. The reaction was stopped by adding 1ml of 2.5 M H_2SO_4. The absorbance was measured at 420nm. The rate of increase on absorbance units (A) per minute for the blank and for the test sample are calculated by

the equation (A420nm/min= O.D at last interval –O.D at first interval/time in min). The % inhibition is given by the formula (A420nm/min[blank] - A420nm/min[test] / A 420nm/min[blank] * 100.

Gene expression studies

RNA isolation: The isolated yeast colonies were cultured in potato dextrose broth and incubated at 37°c for 48 hours to obtain a mid - exponential phase (OD600 = 0.5-0.7). Following the incubation, 2ml yeast culture was centrifuged at 6000rpm for about 10 minutes. To the pellet 400µl TES solution, 400µl acid phenol was added and vortexed vigorously and incubated at 65ºC for 30 to 60 minutes. The vials were placed on ice for 5minutes and centrifuged for 10min at 10000rpm, 4°C. The upper aqueous layer was transferred and 400µl of acid phenol was added and vortexed vigorously followed by centrifugation for 5minutes at top speed. To the upper aqueous phase 400µl of chloroform was added and centrifuged at 10,000rpm for 5minutes. The obtained upper aqueous layer was transferred and 40µl of 3 M sodium acetate, 1ml of icecold 100% ethanol was added to precipitate the RNA and centrifuged to obtain the pellet. The RNA pelleted was stored in 50µl RNase free H2O at at 4°C for further use. The pure RNA obtained thus obtained was checked for purity using spectrophotometer and used for RT PCR.

Gene amplification: The SGS1 gene was amplified by RT-PCR using purified RNA as a template for cDNA synthesis using reverse transcriptase (HI MEDIA) and random primers according to manufacturer's protocol. The cDNA obtained was used for amplification of SGS1. The amplification was done suing the primers (FW, 5GCACTTATAAAGCGCGGTGG3′ RV, 5TGTACAGAAGGCATGGCAGG 3).

The PCR mixture consisted of 5µl of 1X PCR Reaction Buffer, 1µl of 10mM dNTPs (200µM), 1µl of 10µM Forward Primer (0.2µM), 1µl of 10µM Reverse Primer (0.2µM) and template DNA in a total volume of 50µl. The PCR was performed with the following cycling profile: Initial denaturation at 94°C for 10 min, followed by 30 cycles of 30s denaturation at 94°C, annealing at 56°C for 30s, and extension at 72°C for 1min. The time for the final extension step was increased to 10 min for 72°C. The PCR products amplified were then qualitatively analysed on 1% agarose gel.

RESULTS

Phytochemical Screening

The herbal extracts of ethanol, methanol and chloroform were screened for phytochemical screening, where steroids were present in ethanol, methanol and chloroform fractions, flavonoids were present in methanol and chloroform extracted fractions and polyphenols were present only in the ethanol extracted fraction. The phytochemical results showed the presence of flavonoids and phenols in ethanol and methanol extract which are active compounds for hair follicle development.

Cell viability assay: It was observed that when the yeast cells were treated alone with DDT the viability of the cells drastically reduced when compared to control, simultaneously when cells were treated with extracts of methanol and ethanol the cell viability increased inhibiting the action of DDT. The ethanol extract was found to give an increased cell viability when compared to methanol extract (Fig 1). It was observed that when the yeast cells were treated alone with Endura the viability of the cells drastically reduced when compared to

Table 1. Table showing the phytochemical results of ethanol, methanol and chloroform extracts

Herbal Extract	Tannin	Saponin	Alkaloid	Cardiac glucosides	Steroids	Flavonoids	Phenolic compounds
Ethanol	–	–	–	–	+	–	+
Methanol	–	–	–	–	+	+	–
Chloroform	–	–	–	–	+	+	–

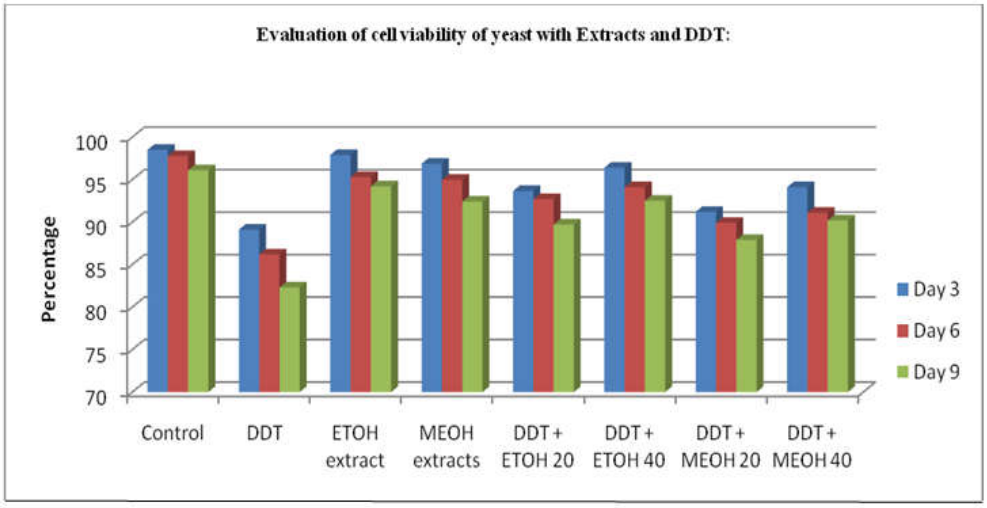

Fig. 1. Figure showing the Percentage of cell viability of yeast cells with extracts and DDT. Measured for a intervals of 3 days (day3, day6, day9). The results were expressed in percentage

control, simultaneously when cells were treated with extracts of methanol and ethanol the cell viability increased inhibiting the action of Endura. Ethanol extract was found to give an increased cell viability when compared to methanol extract (Fig 2).

Peroxidase Assay: The percentage inhibition of lipid peroxidation by ethanol and methanolic fractions showed significant results when compared to the positive control (Ascorbic acid) as shown in Table 1.

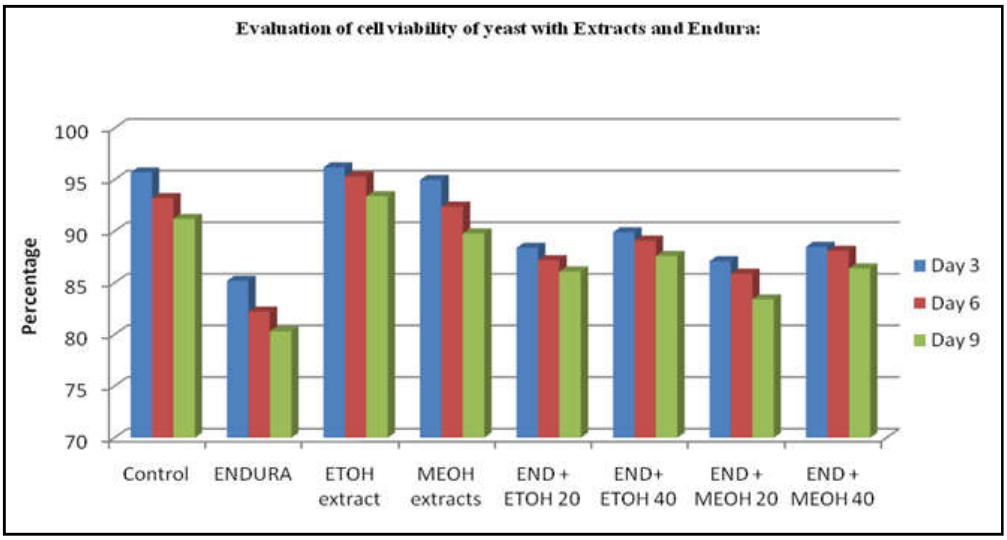

Fig. 2. Figure showing the Percentage of cell viability of yeast cells treated with Extracts and Endura. Measured for a intervals of 3 days (day3, day6, day9). The results were expressed in percentage

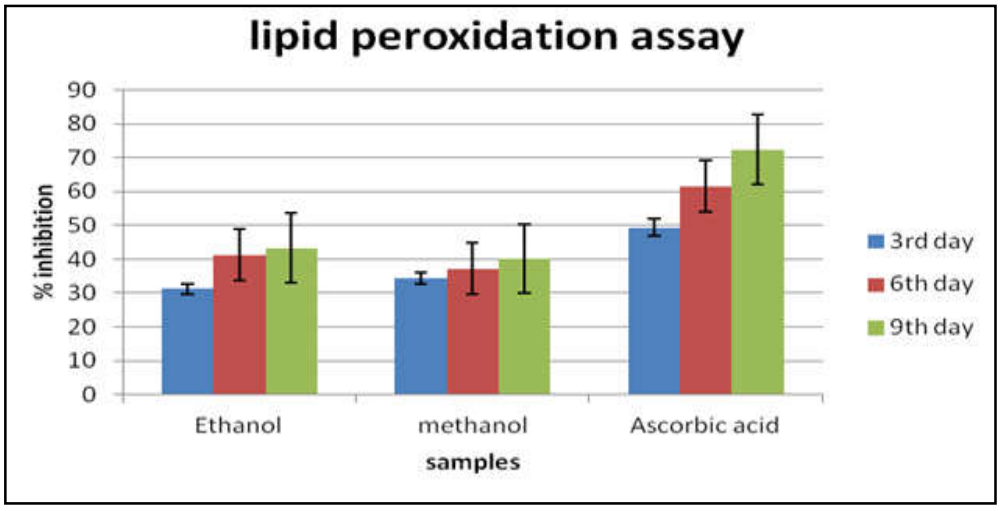

Fig. 3. Percent inhibition of lipid peroxidation of extracts using yeast cells. All the values were average of triplicates. The values were expressed as % ± s.e.

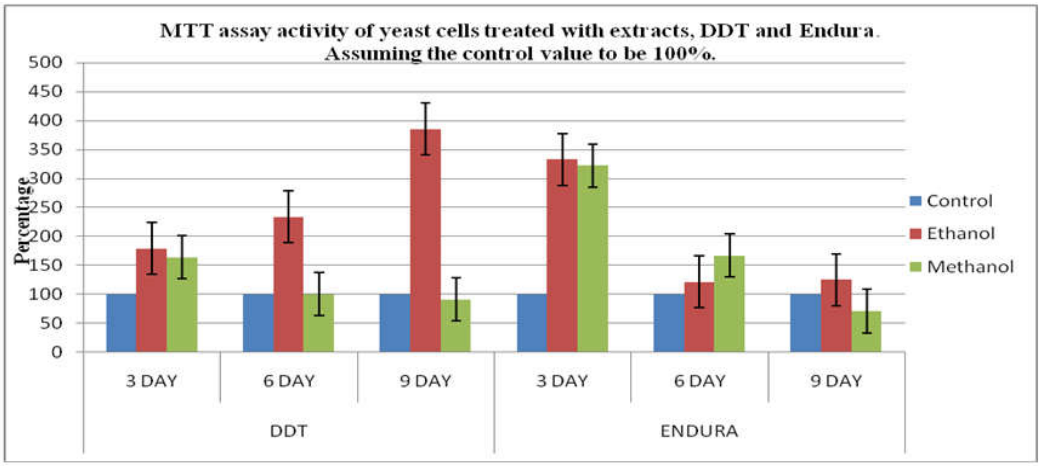

Fig. 4. Figure showing the Percentage of cell density in yeast cells treated with extracts, DDT and Endura. Measured for interval of 3 days (day3, day6, day9). Assuming the control to be 100% All the values were average of triplicates

Chloroform fraction showed no significant effect on lipid peroxidation when compared with the control and methanol fractions. Both the ethanol and methanol fractions showed increase in the percent inhibition upto 6th day and thereafter remained stable. The activity was found to increase with days and at the same there was no effect observed on prolongation.

MTT Assay: The cell density and viability of the cells were found to increase when treated with herbal extracted fractions of ethanol and methanol, where as when the cells were induced to stress with pesticides the MTT activity decreased. Simultaneously when the cells were treated with herbal extracts and pesticides, the herbal extracts inhibited the action of organo pesticides and thus increased MTT activity was observed (Fig 4).

SOD, POX AND CAT ASSAY: When the herbal extracts were used to check the SOD activity, ethanol extracted fractions were found to show more inhibition effect when compared to methanol extracted fractions. The ethanol extracts provided an important defense against the toxicity of the superoxide radicals when compared to methanol.

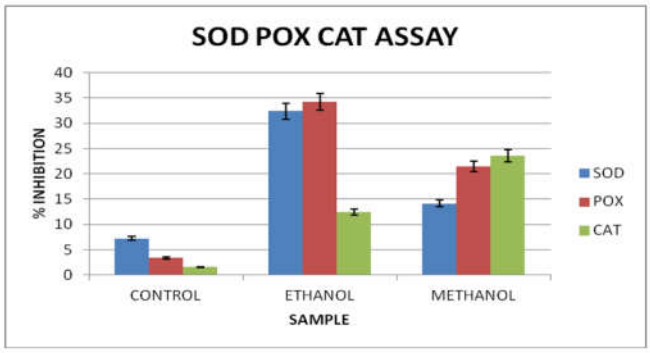

Fig. 5. Figure showing the percentage inhibition in SOD, POX and CAT assay. All the values were average of triplicates. The values were expressed as percent ± s.e

The increased amount of peroxidase present in the ethanol extracts catalysis the reduction of hydrogen peroxidase to water, rendering it to be harmless and thus provides tolerence to stress condition. Catalase being an ubiquitinase antioxidant enzyme degrades the hydrogen peroxidase into water and oxygen, rendering it to be harmless and thus provides tolerance against stress conditions. Therefore ethanol's extracts was found to have more catalase activity. The ethanol extract showed positive results for SOD, POX and CAT assay. Both the ethanol and methanol showed significant results. But the ethanol fraction showed higher response in terms of activity. The percent inhibition of Ethanol extract for SOD, POX and CAT assay were 32.38 ± 0.32, 34.23 ± 0.31and 12.43 ± 0.51 respectively. The percent inhibition of methanol extract for SOD, POX and CAT assay were found to be 14.12 ± 0.43, 21.45 ± 0.51 and 23.56 ± 0.55 respectively.

Gene expression studies

The gene expression was studied using RT PCR using the primers for SGS1 gene. The expression of the gene was found to be lowered in case of yeast cells treated with pesticides, whereas the SGS1 gene expression obtained from the yeast cells treated with pesticides and herbal extracts was found to more expressed than the control. However further study at protein level is needed to conform the activity. But the expression under the treatment seems to be promising for further study.

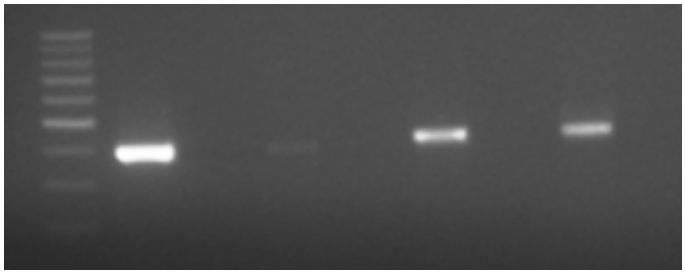

Fig. 6. Gel showing the PCR amplification of expression of SGS1 gene. The samples were run on 2% agarose gel using 200bp molecular marker. Lane M: Molecular Marker; Lane 1: Control; Lane 2: Negative control; Lane 3: DDT treatment; Lane 4: Negative control; Lane 5: Methanol extract; Lane 6: Negative control; Lane 7: Ethanol extract; Lane 8: Negative control

DISCUSSION

The study is done on SGS1 gene that is slow growth suppression 1 gene of *saccharomyces cerevisiae*, involved in hair follicle development. Defective in these gene causes premature aging disorder Werner's syndrome and Bloom's syndrome characterized by the premature appearance (greying of hair) of normal aging in young adults. Hence the present study was aimed to evaluate the hair growth activity using herbal formulation to inhibit organopesticides treated on yeast cells and to increase the hair follicle development. The herbal extracts were assayed for phytochemical screening, where steroids were present in ethanol, methanol and chloroform fractions, flavonoids were present in methanol and chloroform extracted fractions and polyphenols were present only in the ethanol extracted fraction. The present study demonstrated the hair growth promoting potential of the crude extracts of *Eclipta alba* and *Ficus Bengalensis* that contains phenols and flavonoids. The obtained extracts were used for the cell viability assay, Lipid peroxidase assay, MTT assay, showed significant results in Ethanol and Methanol fractions than that of the chloroform fractions. The Ethanol fraction were found to have more cell viability compared to the Methanol extracts. When the cells were treated with organopesticides like DDT and Endura along with herbal extracts of ethanol and methanol fractions were found to give increased cell viability than that of cells that were treated with pesticides alone. The expression studies on SGS1 gene was done by isolating the mRNA from the yeast cells treated with herbal extracts along with pesticides.

The SGS1 gene is involved in hair follicle development. The mRNA was later converted to cDNA using reverse transcriptase and electrophoresed. The lanes were observed in the gel, where the expressions of SGS1 gene obtained from the yeast cells treated with pesticides were relatively low, whereas the SGS1 gene expression obtained from the yeast cells treated with pesticides and herbal extracts was found to more expressed than the control. When the cells were treated with

organo pesticides, the expression of SGS1 gene was suppressed but in the presence of the herbal the gene expression was rejuvenated and expressed at high level.

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