Original Article

PRODUCTION AND CHARACTERIZATION OF GALLIC ACID WITH *Streptomyces olivochromogenes* ISOLATED FROM CASHEW NUT SHELL LIQUID (CNSL) DEPOSITED SOIL

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Abstract

Phenolic compounds are a major type of phytochemicals and among them, phenolic acids are the most potent biologically active compounds. Cashew nutshell liquid (CNSL) is a by-product that serves as a rich source of phenolic acids. Gallic acid is a major category of phenolic acids and is notable for its antimicrobial, antiviral, and antifungal properties as well as for industrial applications. The global annual requirement of gallic acid is around 8000 tonnes. Currently, the industrial production of gallic acid is facilitated by acid hydrolysis of naturally derived gGallotannins. Since the present industrial process demands higher production costs along with low product yield and release of huge volumes of toxic effluents as by-products, an enzyme dependant eco-friendly production process for gallic acid is mandatory. Microorganisms can serve as substitutes for gallic acid production since they are endowed with the capability to degrade tannic acid by producing tannase. The bacteria *Streptomyces olivochromogenes*was found to be capable of producing gallic acid in the present research. The gallic acid thus produced was found to possess effective antioxidant properties, thereby preventing protein denaturation in cells. The viability of the cancer cells was found to be significantly reduced. Reduction in oxidative stress along with upregulation of the apoptotic gene BAX coupled with the downregulation of the anti-apoptotic gene BCL2 is supposed to be the underlying mechanisms behind the anticancer activity of gallic acid. Our research manifests the therapeutic efficiency, especially the anticancer property, of gallic acid produced by *Streptomyces olivochromogenes*.

Keywords: Gallic acid, *Streptomyces olivochromogenes*, FTIR, Anticancer, DPPH.

Introduction

The term “phytochemicals” refers to a wide variety of biologically active natural compounds having pharmaceutical and nutritional attributes (Joselin *et al*., 2012; Sukumaran *et al*., 2014; Satya et al., 2017; Mariyammal *et al*., 2023). Phenolic compounds are a type of phytochemicals endowed with at least one hydroxylated benzene ring. The representatives of this huge and dissimilar cluster of chemical compounds are typically categorized based on the number of carbon atoms in their chemical structures. Phenolic acids are a vital and copious subgroup of phenolic compounds possessing the basic chemical structure of C_6-C_1 (hydroxybenzoic acids) or C_6-C_3 (hydroxycinnamic acids), comprising a phenolic ring and a carboxyl substituent (Joselin *et al*., 2013; Siah *et al*., 2016; Pengelly and Bone, 2020).

Gallic acid (3, 4, 5-trihydroxy benzoic acid) is a widely distributed category of phenolic acids in the plant kingdom. This colorless or faintly yellow crystalline compound has widespread relevance in the food and pharmaceutical industries. For research purposes, various chromatographical methods have been applied for the isolation of gallic acid from different plant species such as *Quercus* spp. and *Punica* spp. (Fernandes and Salgado, 2016). Research findings have showcased the pharmacological significance of gallic acid as a radical scavenger. This compound has been substantiated to be effective in preventing and curing several diseases, where oxidative stress acts as a major causative agent, encompassing cancer, hepatic ailments, cardiovascular diseases, neurodegenerative disorders, and also in aging (Kaur *et al*., 2005; Karamac*et al*., 2005; Nikolic, 2006). Gallic acid and its ester derivatives are widely used as flavoring agents and preservatives in the food industry (Choubey *et al*., 2015).

Cashew nutshell liquid (CNSL) is a major by-product of the cashew kernel industry and also a major environmental pollutant. At the same time, it is a rich source of various types of phenolic compounds. This raw material has numerous applications such as the production of polymers, paints, varnishes, and various other products.(Lomonaco*et al*., 2017).The huge carbon residues present in CNSL-deposited soils help the microbes in the biosynthesis of Tannase. (Vinod Viswanath*et.al.2015*)

Gallic acid can act as an effective antibacterial, antifungal, and anti-viral agent. It can restrain the adherence, biofilm formation, and motility of *Chromobacteriumviolaceum*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Streptococcus mutans* (Kang *et al*., 2008; Borges *et al*., 2012; Shao *et al*., 2015). This compound can also disorder the stability of the cell membrane in Gram-positive and Gram-negative bacteria and alter the charge, hydrophobicity, and permeability of the membrane surface (Teodoro *et al*., 2015). In the case of fungi, gallic acid serves as a nonionic surfactant and can obstruct the selectively permeable nature of the cell membrane in fungi (Kubo*et al*., 2003). Gallic acid can inhibit the dimerization of HIV-1 integrase, HIV-1 transcriptase, and HIV-1 protease (Kratz *et al*., 2008; Singh and Pal, 2015; Ahn*et al*., 2016), attachment, replication and penetration of HCV (Zuo*et al*., 2005; Salas *et al*., 2014; Hsu *et al*., 2015; Govea-Salas *et al*., 2016). It also causes distraction in *Haemophilus influenza* A and B particles (Lee *et al*., 2016).

The microbial enzyme, tannase (Tannin-acyl-hydrolase, E.C. 3.1.1.20), has greater significance in the industry. This enzyme facilitates the hydrolysis of ester and depside bonds in hydrolyzable tannins including tannic acid.The Tannase enzyme is employed in the beverage industries to eradicatechill haze development in beer and wine (Aguilar*et al*., 2001; Aguilar *et al*., 2007). In addition to this, this enzyme is extensively applied to diminish the antinutritional aspects of poultry and animal feed together with the detoxification of food and the treatment of industrial effluents (García-Conesa*et al*., 2001; Belmares*et al*., 2004; Rodríguez-Durán *et al*., 2011). Tannase is also employed in the manufacture of instant tea and gallic acid, a substrate for antioxidant propyl gallate production and trimethoprim synthesis (Kar *et al*., 2002; Lu *et al*., 2008).

The major microbial sources of tannase as per different research findings are fungi (Aguilar *et al*., 2007), mainly *Aspergilii*, *Penicilii*, *Fusaria*, and *Trichoderma* (Raghuwanshi*et al*., 2011). A vital dilemma in the employment of different fungal strains for industrial purposes is the slower degradation rate of these strains (Selwal*et al*., 2010). On the other hand, only fewer strains of bacteria are effective in tannase production so fay researchers. Such bacterial strains include some species of *Bacillus*, *Corynebacterium* sp., *Lactobacillus* sp., *Serratia* sp. (Rodríguez *et al*., 2008), *Enterococcus* (Goel *et al*., 2005), *Streptococcus* (Jiménez *et al*., 2014), *Pseudomonas* (Selwal*et al*., 2010).

The global yearly requirement for gallic acid is around 8000 tonnes. Since the natural formation levels are unable to cope with this demand, industrial production is the only way to satisfy the global constraints (Lokeswari, 2010). In the current day scenario, the majority of the industrial production of gallic acid is by acid hydrolysis of naturally derived gallotanins. Since the acid hydrolysis procedure demands high costs, lower product yield, and production of huge volumes of toxic effluents, an enzyme-dependent eco-friendly method for gallic acid production is preferred. Microorganisms are the preferred substitute for gallic acid production since they possess the capability to break down tannic acid by producing tannase (Bajpai and Patil, 1996; Banerjee and Pati, 2007; Aguilar-Zárate*et al*., 2014).

In the present-day scenario, the production of gallic acid is a high-cost and lengthy process with low product yield. Hence the emergence of innovative scientific methods associated with gallic acid synthesis is critical forreducing production costs with improved product yield. Based on these circumstances, our research work is concentrated on the production of gallic acid under anaerobic conditions by the isolated bacterial strain, *Streptomyces olivochromogenes*, along with the valuation of the biological aspects of gallic acid produced by this bacterium.

Materials and methodology

Sample Collection and Isolation of Bacterial Strains

Cashew Nut Shell Liquid (CNSL) deposited soil samples were collected from Cashew factories across Kanyakumari District, Tamil Nadu, India.The Bacterial strains were isolated on nutrient agar medium (5 g of peptone, 5 g of sodium chloride, 1.5 g of beef extract, 1.5 g of yeast extract, and 15 g of agar per liter at pH 7.4) by serial dilution method and incubated at room temperature (30ºC) for 48 h. Based on colony morphology and pigmentation bacterial isolates were selected and pure cultures were prepared.

Screening of gallic acid-producing bacteria by the tannase enzyme assay

The Tannase enzyme activity of the isolates was estimated as a screening method for gallic acid-producing bacteria, as per the method developed by Mondal et al. (2001). The reaction mixture comprised 0.3 mL of tannic acid (0.5% in 0.2M sodium acetate buffer, pH 5.5) and 0.1 mL of the enzyme, incubated at 50 °C for aperiod of 20 min. The enzymatic reaction was ceased by the addition of 3 mL of BSA solution, which precipitates the persisting tannic acid. The tubes were then subjected to centrifugation (5000 x g 10 min) and the resultant precipitate was dissolved in 3 mL SDS-triethanolamine solution. One mL of FeCl3 reagent was added to each tube and was incubated for 15 min at room temperature for the stabilization of color. The absorbance was measured at 530 nm using UV/Vis spectrophotometer. One unit of enzyme activity denotes the amount of enzyme mandatory to hydrolyze 1 mM of tannic acid in 1 min under assay conditions. The selected bacterial isolate was named KMD41

Molecular identification of the strain KMD41

Thegenomic DNA was isolated from the strain by the phenol-chloroform method (Maniatis et al., 1982). Then 16S rRNA gene was amplified using the universal eubacterial primers 27F and 1492R (Lane, 1991).The PCR amplicon was visualized using agarose gel electrophoresis. Sequencing PCR wasdone with an ABI PRISM Big Dye terminator v3.1 cycle sequencing kit and the sequencing was done in AB 3730 DNA analyzer(Life Technologies, CA, USA). The sequences obtained wereviewed with ABI sequence scanner v1.0, compiled, and aligned usingBioEdit version 7.0.9.0.(Hall, 1999).

Confirmation of Gallic acid production by *Streptomyces olivochromogene*

The isolated bacteria *Streptomyces olivochromogenes*was grown on half-strength nutrient broth supplemented with 0.5 % tannic acid. Following 48 h of incubation, the tannic acid production was established by color reaction with 1% $FeCl_3$. 2ml of culture supernatant was mixed with a few drops of 1% $FeCl_3$ and was observed for the appearance of black-brown, black-green, or black-blue color. The color development confirms the Gallic acid production (Kawakubo *et al*., 1991; Kawakubo *et al*., 1993). Uninoculated nutrient broth serves as the negative control.

Gallic Acid Extraction

Gallic acid was isolated using the organic solvent ethyl acetate. The fermented material was removed, water added, and heated to about (60-70)° C. because gallic acid is soluble at this temperature, and not in cold water. Then it was cooled to room temperature. The organic solvent was then added to it and the whole mixture was taken in a separating funnel. The mixture was immediately mixed thoroughly by vigorous shaking. Gallic acid is soluble in an organic solvent, comes into the organic phase and the rest of the matter remains in the aqueous phase. The aqueous phase was discarded and the organic layer was collected. This process was continued till the entire gallic acid came out into the organic layer. The collected volume of the organic layer was now taken for the separation of gallic acid from ethyl acetate in the rotary vacuum evaporator. The pressure and temperature at which this separation was done were 200 mbar and 70° C. Alternatively, the ethyl acetate layer was extracted with diethyl ether, and the ether layer was evaporated in a rotary evaporator to obtain pure gallic acid. The yield of the gallic acid obtained was found to vary from 65.4 to 94.8%. Studies were conducted for the optimization of various environmental parameters for obtaining maximum gallic acid production.

Quantification of Gallic acid

The Standard and extracted gallic acid were quantified using the procedure developed by Osawa and Walsh (1993).The breakdown of tannins was monitored during the fermentation process by evaluating the quantity of released gallic acid. The brown coloration of the medium indicates the presence of Gallic acid. The Absorbanceat 440 nm was recordedusinga spectrophotometerandthe quantity of gallicacidinthe mediumwas detectedusinga calibration curve.

Gallic acid production/ fermentation

The production/ fermentation of gallic acid was performed using standard methods with some minor modifications (Bajpai and Patil, 2008; Beniwal *et al*., 2010). Fermentation was performed using 250 mL flasks containing 150 mL of medium with the composition (in g/L): $FeSO_4 \cdot 7H_2O$, 0.01 M $NaNO_3$, 3 M K_2HPO_4, 1 M $MgSO_4 \cdot 7H_2O$, 0.5 M KCl, 0.5; tannic acid (1 %) and starch (5 %). The initial pH of the production medium was adjusted to 6 using 1 M NaOH or 1 N HCl and was then sterilized (121 °C for 15 min).

The culture medium was inoculated with 1 % of inoculum. The flasks were then incubated at 30 °C for the fermentation time of 48 h. Samples were withdrawn at 4 h interval post the first 12 h of fermentation followed by the initiation of tannase activity. The clarified supernatant thus obtained was used for the analysis of tannase activity, gallic acid synthesis, and tannic acid degradation.

Gallic acid Production optimization

Gallic acid production optimization was carried out by growing *Streptomycesolivochromogenes*in diverse culture settings such as in various carbon sources, dissimilar nitrogen sources, different pH ranges, divergent temperatures, etc. Optimization procedures were performed as per standard methods with minor modifications (Lokeswari and Raju, 2007; Patil et al., 2011)

- Different Carbon sources (0.2 % each of glucose, sucrose, maltose, lactose, and starch)
- Different nitrogen sources (peptone, tryptone, beef extract, and yeast extract)
- Different temperatures (room temperature, 37^0C, and 45^0C)
- And diverse pH (4 - 9).

Mass culturing of KMD-41 in optimized media

Mass culturing of KMD-41 was performed by inoculating 100 µl of the KMD-41 culture broth into 100 ml of production media taken in a 250 ml Erlenmeyer flask containing half-strength nutrient broth supplemented with tannic acid (0.5 %), glucose (0.2%), beef extract (0.2%;pH-9) and incubated for 48 h at 37°C. Gallic acid production was then quantified. Uninoculated nutrient broth served as the negative control.

Estimation of Gallic acid production using the optimized media

The disintegration of tannins was examined throughout the fermentation process by assay of released Gallic acid (Osawa and Walsh, 1993). Absorbance at 440 nm was read using a spectrophotometer and the quantity of gallic acid in the medium was detected using a calibration curve

Fourier Transform Infrared Spectroscopy (FTIR):

FT-IR analysis was done to identify thefunctional groups of the active compounds in the sample based on the peak values in the region of IR radiation. The sample extracted from the selected microorganism was mixed with potassium bromide (KBr) in an adequate ratio to make a transparent pellet and it was pulverized in a motor and pestle using a hydraulic pressure instrument. The pellet developed was kept in a sample holder and IR rays were passed across it. Characterization was executed under the following constraints:

- Spectral range, 4000-400 cm-1 to substantiate the functional groups of the extracted polymer.

FTIR analysis was performed using Perkin Elmer 1750 FT-IR spectrometer.The results obtained were evaluated for the detection of functional groups.

DPPH radical scavenging assay

The free radical scavenging activity of the test sample against stable 2, 2- diphenyl 2- picrylhydrazyl hydrate (DPPH) was estimated as per the method of Brand-William *et al*., (1995) with slight modifications. DPPH reacts with antioxidant compounds and undergoes reduction by accepting hydrogen atoms. The change in color (from deep violet to light yellow) thus occurred and was measured spectrophotometrically at the optical density of 515 nm using a UV-visible spectrophotometer. The reference standard used was ascorbic acid.

Radical scavenging activity was calculated by the following formula,

$$\text{Percentage inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

Protein denaturation inhibition assay

The anti-inflammatory property of the given sample was identified using the protein denaturation assay. The assay was performed as per the methods of Mizushima and Kobayashi (1968) and Saka*et al*., (2010) with some minor modifications. The reaction mixture (0.5 ml) was constituted of 0.4 ml bovine serum albumin BSA (3% aqueous solution) and varying concentrations of test samples. The samples were incubated at 37ºC for 20 min. After incubation, 2.5 ml phosphate buffered saline (pH 6.3) was added to each tube and then heated at 80ºC for 10 min. The absorbance of the samples was measured spectrophotometrically at 660nm.

The percentage inhibition of protein denaturation was calculated as follows:

Percentage of inhibition = [(Absorbance of Control – Absorbance of Sample) / Absorbance of Control)] x100

Anticancer efficacy screening by the MTT assay

The MTT assay was done according to the method used by Mosmann*et al.,* 1983, to study the anticancer efficacy of the extracted Gallic acid.

The human skin cancer cell line, SKMEL, was sourced from the cell repository of NCCS, Pune, India, and used for the study

The cell viability was calculated using the following formula:

$$\textbf{Percentage of cell viability} = \frac{\textbf{Average absorbance of treated}}{\textbf{Average absorbance of control}} \times \textbf{100}$$

IC 50 value

The IC_{50} value is the half-maximal inhibitory concentration of the sample. The IC_{50} values were calculated using the equation for slope (y = MX + C) obtained by plotting the average absorbance of the different concentrations of the test sample (6.25-100 µg/mL) using the software Microsoft Excel.

Gene expression studies using cell lines

Total RNA was extracted using TRIzol reagent and used as a template for the production of cDNA which was employed in reverse transcriptase PCR for the quantification of BCL2,BAX,and B-Act transcript levels according to the method of Chomczynski and Mackey (1995) with minor modifications.

The PCR product obtained was checked for its quality and quantity by using a UV-Visible spectrophotometer at 260 nm and 280 nm, followed by agar gel electrophoresis which showed DNA bands at 3000 bp on a comparative study with a 100 bp DNA ladder. Standard scientific protocols were followed along with some minor modifications (Kobayashi *et al*., 2000; Yamaguchi *et al*., 2003; Ghasemi*et al*., 2018). Gene expression patterns were analyzed using a gel documentation system [GELSTAN 4X Advanced -The Mediccare Scientific]

Primers used in this study are listed in the table below;

Oligo Name	**Sequence**
BCL2 F	5'-AAGACCCCAGCACACTTAGC - 3'
BCL2 R	5'-GTACAGGGAAACGCACCTGA -3'
BAXF	5'-AGGTTTGGGGCCACTATCTC -3'
BAXR	5'-GATCTGAAGATGGGGAGAGGG -3'
B-ActF	5’-CTGACCGAGCTGGCTAC-3’
B-ActR	5’-CCTGCTTGCTGATCCACA-3’

NB: The concentration of Gallic acid used in this study is the IC50 value of Gallic acid obtained from the MTT assay

Results and discussion

Tannase enzyme assay

Tannase is a vital enzyme that catalyzes the degradation of gallotannins and ellagictannins, the two types of hydrolyzable tannins. The Tannase enzyme particularly facilitates the hydrolysis of ester and depside bonds of hydrolyzable tannins, resulting in the release of glucose and gallic or ellagic acid. The different bacterial isolates (numbered sequentially from 1-5) were primarily screened for evaluating their tannin-degrading capability through the action of the tannase enzyme. The maximum concentration of glucose released (indicating tannin hydrolysis) was 875.5µg/mL by the isolate numbered 4. Hence for further assays, this isolate was used and it was named -41. The results are presented in Table no:1

Table 1: Different bacterial isolates and glucose-producing capability from tannins.

SL.NO:	Name of the isolate	Absorbance at 540 nm	The concentration of glucose released by 1 mL of the crude enzyme (µg/mL)
1	1	1.573	393.25
2	2	3.191	797.75
3	3	3.199	799.75
4	**4**	**3.502**	**875.5**
5	5	3.109	777.25

Molecular identification of the strain KMD41

The genomic DNA from strain KMD 41 was isolated using the phenol-chloroform isoamyl alcohol extraction method. The genomic DNA integrity was checked by agarose gel electrophoresis

A260/A280 was found to be 1.91 (1.8 for pure DNA) and hence the DNA was found to be compatible with performing downstream molecular biology works. The 16S rRNA gene was amplified successfully from the DNA and sequencing was done. The strain was identified as *Streptomyces olivochromogenes* with the help of BLAST and the 16s rRNA sequence was submitted to NCBI Genbank under the Accession number MN620387.

Gallic acid extraction(Organic solvent method)

The extraction procedure of gallic acid was done by Organic solvent Ethyl Acetate in Separating Funnel. The characteristics of gallic acid extracted by this method are represented in figure no: 1 and table no: 2 below.

The extracted gallic acid was poured into a petri dish and dried and then used for quantification and characterization.

Fig 1: Gallic acid extracted from KMD-41

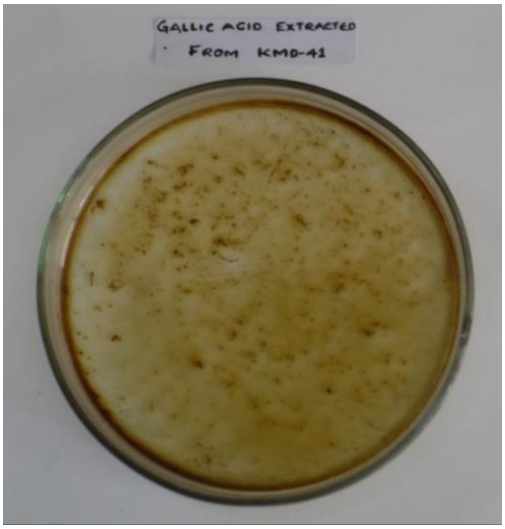


Table 2: Characteristics of the extracted gallic acid

Sample	**solvents**	**Consistency**	**Colour**	**W1(g)**	**Yield (g/L)**
KMD-41 (gallic acid)	70% acetone	Semi solid	Brown	0.108	21.6

Optimization of the growth media

Gallic acid production under different conditions was tested. These varying conditions include different carbon sources (0.2 % each of glucose, sucrose, maltose, lactose, and starch), different nitrogen sources (peptone, tryptone, beef extract,t and yeast extract), different temperatures (room temperature, 37^0C, and 45^0C), and diverse pH (4 - 9). Based on the results obtained under these varying conditions, gallic acid production by *Streptomyces olivochromogenes*was found to be the maximum with glucose and beef extract as the carbon and nitrogen sources respectively at the temperature of 37^0C with a pH of 9.

- The results are presented below in Tables 3-6. And Figures
- The optimization Chart is represented in Chart no:1
- The Final Optimized condition for the production of Gallic Acid is presented in Table 7

Table 3: Different Carbon Sources

Parameters		**Absorbance at 440nm**	**The concentration of gallic acid (µg/ml)**
Different carbon source (PH 7, 37^0C)	**0.2% glucose**	**1.940**	**388**
	0.2% sucrose	1.250	250
	0.2% maltose	1.049	209.8
	0.2% lactose	1.206	241.2

	0.2% starch	1.108	221.6

Table 4: Different Nitrogen Source

0.2% glucose + different nitrogen source (PH 7, 37^{0}C)	Peptone	0.796	150.18
	Tryptone	0.930	175.47
	Beef extract	**1.006**	**189.81**
	Yeast extract	0.753	142.07

Table 5: Different Temperature

0.2% glucose + 0.2% beef extract + different temperatures (PH 7)	Room temperature	0.859	171.8
	37^{0}C	**1.091**	**218.2**
	45^{0}C	0.865	173

Table 6: Different pH

0.2% glucose + 0.2% beef extract + different PH (37^{0}C)	pH 4	0.560	112
	pH 5	0.617	123.4
	pH 8	2.402	480.4
	pH 9	**3.441**	**688.2**

Chart 1: Optimization chart for gallic acid production

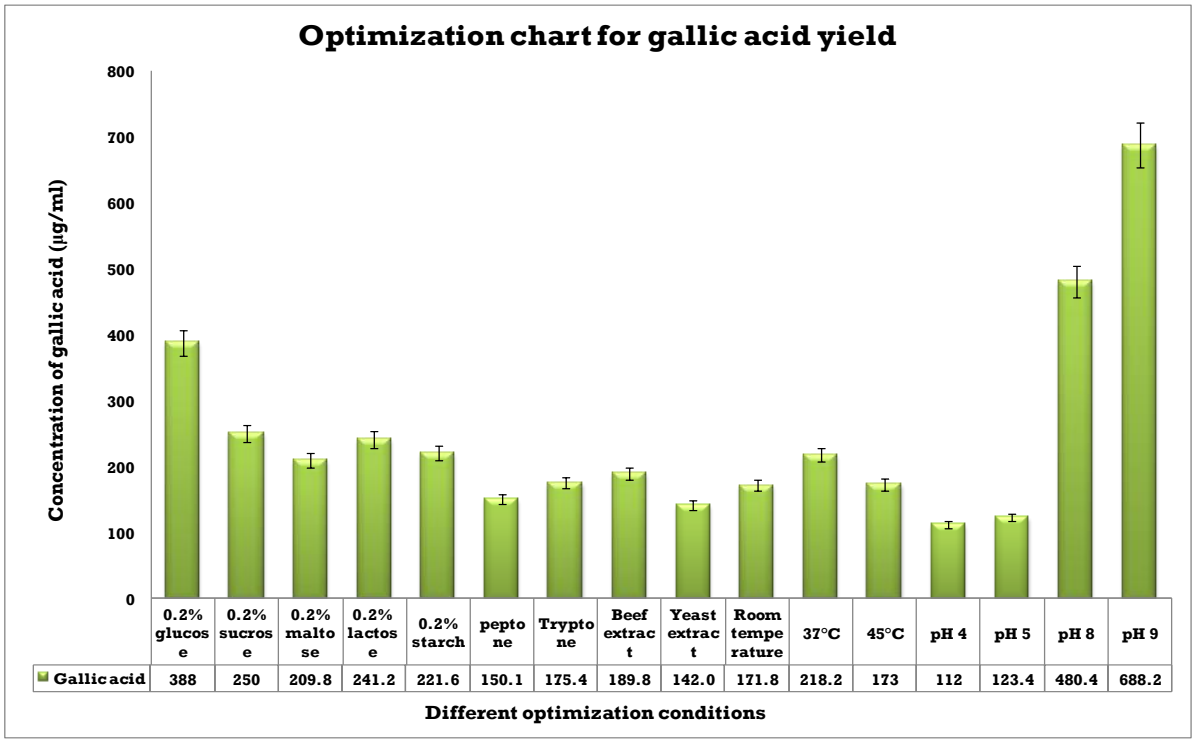


Table 7: Final optimized conditions for gallic acid production

Carbon source	**Nitrogen source**	**pH**	**Temperature**
Glucose	Beef extract	9	37^0C

Gallic acid assay (quantification)

The brown coloration of the medium indicates the presence of Gallic acid.

The result is depicted in Figure no:2 and the values of Standard and extracted Gallic Acid is presented in Table no: 8 and 9.

The Gallic acid Standard Curve is depicted in Chart No:2

Fig 2: Quantification of gallic acid (optimized media)

Standard- Gallic acid

Table 8: Gallic acid Standard quantification results

Sl. no	Concentration (µg)	Absorbance at 440 nm
1	10	0.044
2	20	0.098
3	40	0.307
4	80	0.405
5	100	0.510

Chart 2: Standard Gallic Acid Curve

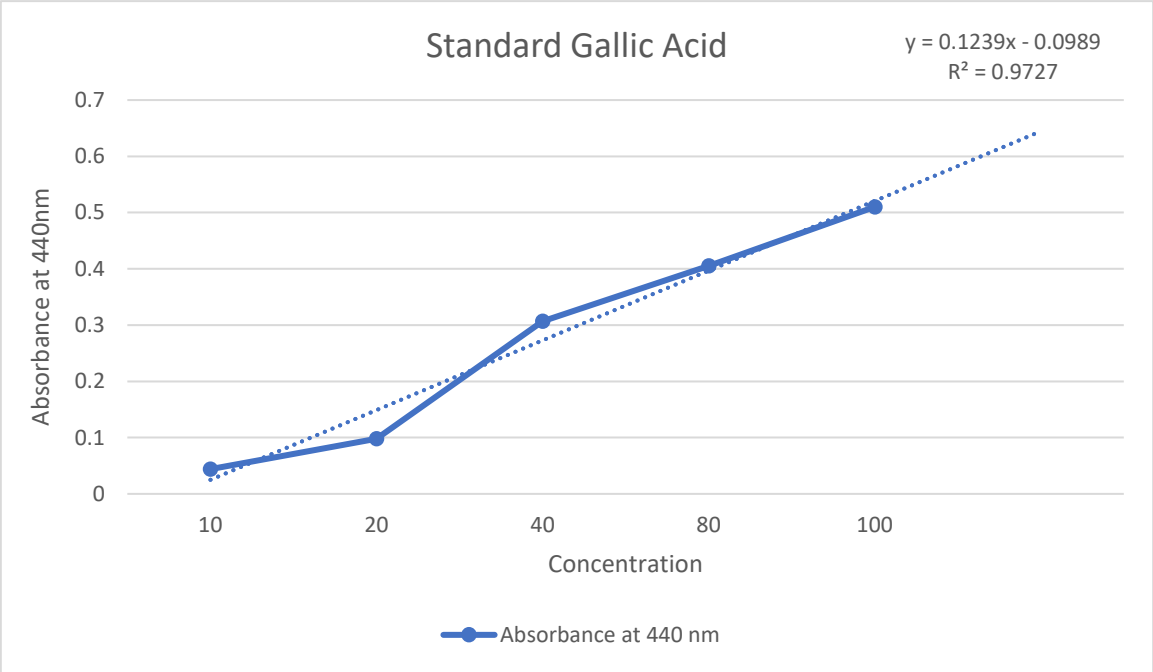


Table 9: Extracted Gallic acid quantification results

	Absorbance at 440 nm	Concentration of gallic acid (µg/ml)	Concentration of gallic acid (mg/L)
KMD-41 (Optimized media)	2.652	530.4	0.5304

Mass culturing of KMD-41 in optimized media

KMD-41 mass cultured in optimized media is represented in figureNo 3

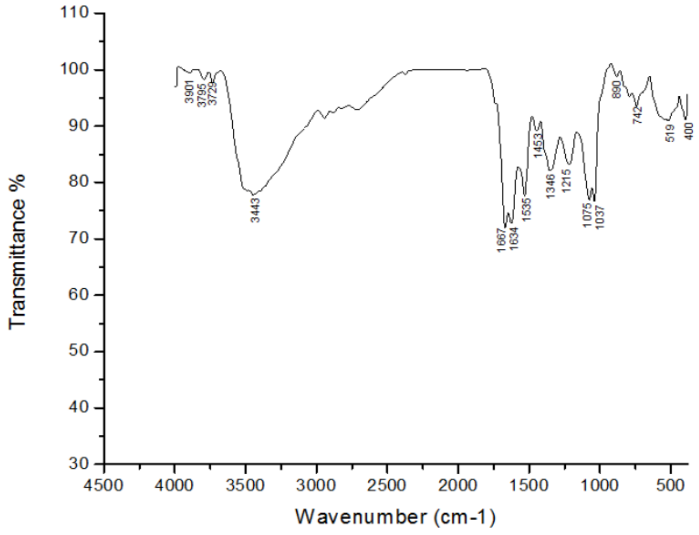
Fig 3: KMD-41 mass cultured in optimized media

FTIR characterization

The Chemical structure of polymers was examined by using the Fourier transform infrared spectroscopy (FTIR). The FTIR graph is represented in Figure no: 4

The FTIR analysis of all the polymers is summarized as follows; A strong broad peak at ~ 3901–3400/cm is attributed to the stretching vibration of the O–H bond. A strong peak at ~ 1767–1037/ cm is attributed to the vibration mode of C = O which was observed in the IR spectra of the polymers. Besides that, there is a sharp band at ~ 1635/cm which indicated the presence of C=C of alkene in the spectrum. The two peaks located in the range of 1453–1346/cm are due to the presence of –C–H bending. The peaks observed between 1215/cm and 1037/cm indicated the presence of O-C stretching vibration. The peak at 890/cm is due to the stretching of C–O–O.The peak at 742/cm is due to Methylene ―(CH2)n― rocking. The peak at 519-400/cm is due to Aryl disulfides (S-S stretch).

Fig 4: FTIR graph



DPPH radical scavenging activity

Concentration-dependent enhancement in percentage inhibition of DPPH free radical scavenging was elicited by Gallic acid extracted from *Streptomyces olivochromogenes*.The IC 50 value of the compound was found to be 28.60 µg/ml. The results are presented in tables 10 &11. And Figures

The impact of antioxidants on DPPH is considered to be due to their hydrogen-donating capability (Huang*et al*., 2005). The DPPH assay is characterized by the reduction of violet-colored DPPH solution to a yellow-colored product, diphenylpicryl hydrazine, by the addition of the sample in a concentration-related manner.

The Results show that gallic acid has an analogous free radical scavenging capability in comparison with the reference standardThe observations from our research implicate the potent hydrogen donating capability of gallic acid obtained from the bacteria *Streptomyces olivochromogenes*.

Table 10: Percentage inhibition of DPPH free radical scavenging by the standard ascorbic acid.

Standard	Concentration (µg/ml)	Absorbance at 515nm	% of Inhibition
Control	-	0.9	
Ascorbic acid (Standard)	1.56	0.82	8.89
	3.12	0.81	10.00
	6.25	0.77	14.44
	12.5	0.66	26.67
	25	0.49	45.56
	50	0.06	93.33
	100	0.06	93.33
	200	0.06	93.33
	400	0.06	93.33
	800	0.06	93.33
IC50	**26.56**		

Table 11: Percentage inhibition of DPPH free radical scavenging by Gallic acid (from *Streptomyces olivochromogenes*)

Sample	Concentration (µg/ml)	Absorbance at 515nm	% of Inhibition
Control	-	0.9	
Gallic acid (from *Streptomyces olivochromogenes*)	1.56	0.85	6.00
	3.12	0.80	11.22
	6.25	0.79	12.22
	12.5	0.70	22.22
	25	0.58	40.00
	50	0.10	88.56

	100	0.10	88.89
	200	0.10	88.89
	400	0.10	88.89
	800	0.10	88.89
IC 50	**28.60**		

Protein denaturation inhibition activity

Concentration dependant enhancement of protein denaturation inhibition was elicited by Gallic acid (from *Streptomyces olivochromogenes*). The IC 50 value of the sample was found to be 83.53 µg. The results of protein denaturation inhibition are represented in tables 12&13.

Protein denaturation mainly occurs through the enhanced production of free radicals. The free radicals are mainly categorized as reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are essential for the maintenance of normal metabolism in cells.

in the body due to various abnormalities such as phagocytosis, inflammatory reactions, ionizing radiation, etc., the free radical production will be magnified, resulting in deleterious effects. Some of the highly reactive free radicals include alkoxyl radical (RO), hydroxyl radical (OH), superoxide anion (O_2-), peroxyl radical (HOO), nitric oxide radical (NO), nitrogen oxide(NO_2), as well as potent non-radicals, such as hydrogen peroxide (H_2O_2), ozone (O_3), and oxygen singletpollutants, which are endowed with the capability of damaging the redox status (oxidation-reduction status) in cells (Belles *et al*., 1999; Meyer et *al*., 2003; Kang et *al*., 2009).

The imbalance of the redox mechanism will result in free radical chain reactions and the main target of such free radicals is proteins. Damage to proteins includes the oxidation of Cys residues to disulfide bonds, oxidation of Met residues to Metsulphoxide, and formation of carbonyl groups in side chains by the oxidation of arginine, lysine, proline, histidine, serine, and threonine residues. Oxidative reactions can also damage the peptide backbone of proteins (Feigin*et al*., 2017; Salinthone*et al*., 2007). The overall result is the denaturation of proteins. The protective potential of gallic acid in the current study is due to the capability of this compound in preventing oxidative stress by maintaining the redox balance and the results from the DPPH scavenging assay strongly confirm this.

Table 12: Percentage inhibition of protein denaturation by Diclofenac standard

Standard	**Concentration (µg)**	**Absorbance at 660 nm**	**% of Inhibition**
Control	-	0.745	-
Diclofenac	6.25	0.66	12.08
	12.5	0.63	15.30
	25	0.61	17.85
	50	0.52	30.20
	100	0.28	62.82
IC50	**78.37**		

Table 13: Percentage inhibition of protein denaturation by Gallic acid (from *Streptomyces olivochromogenes*)

Sample	**Concentration (µg)**	**Absorbance at 660 nm**	**% of Inhibition**
Control	-	0.745	
	6.25	0.69	7.38
Gallic acid	12.5	0.65	12.75
(from *Streptomycesolivochromogenes*)	25	0.62	16.78
	50	0.52	30.20
	100	0.31	58.39
IC50	**83.53**		

Cytotoxicity detection by the MTT Assay

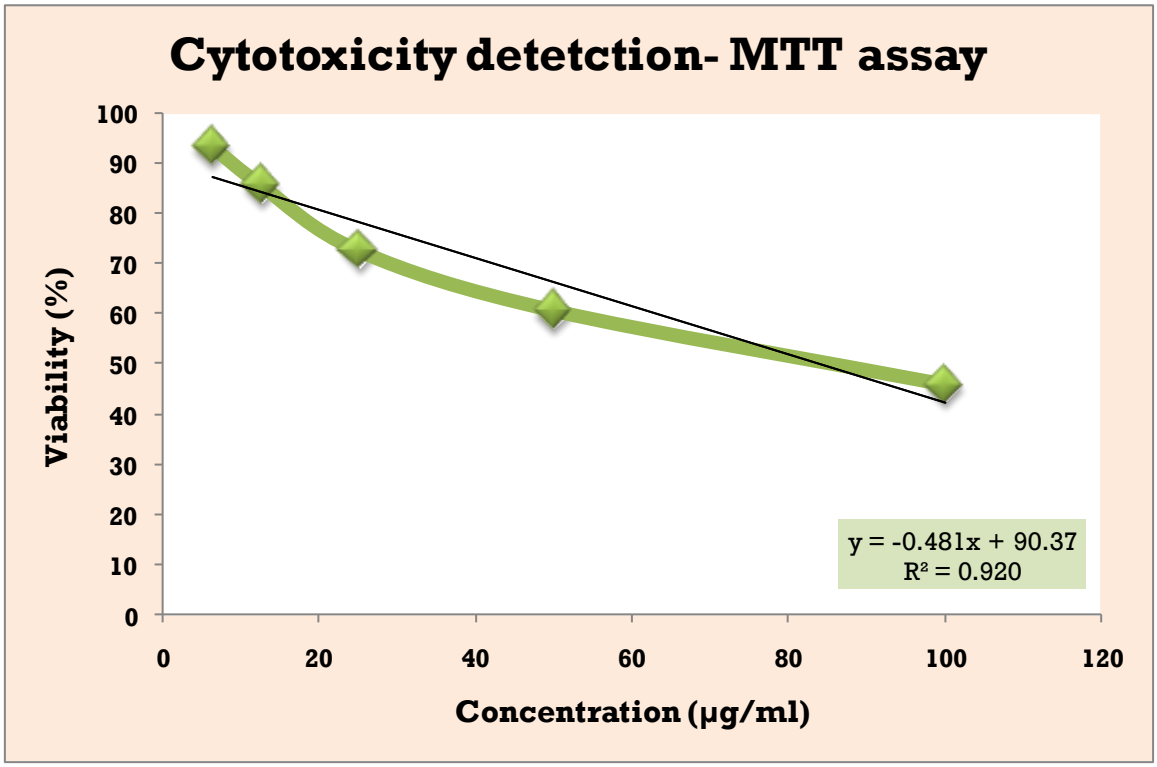
Dose-dependant reduction in cell viability was observed in SKMEL cancer cells administered with different concentrations of the sample. The IC_{50} value was obtained as 83.93 µg/mL of the sample.

The results are presented in Table 14 and graphically represented in Chart 3

Table 14: Percentage of cell viability in SKMEL cells as obtained from the MTT assay.

Samples **Concentration (µg/ml)**	**Average**	**Percentage of viability**
Control	0.666	
6.25	0.623	93.54
12.5	0.571	85.78
25	0.484	72.67
50	0.404	60.66
100	0.305	45.84
IC 50	**83.93**	

Chart 3: Graphical Representation of the cell viability analysis of SKMEL cells which were subjected to the administration with different concentrations of gallic acid from *Streptomyces olivochromogenes.*

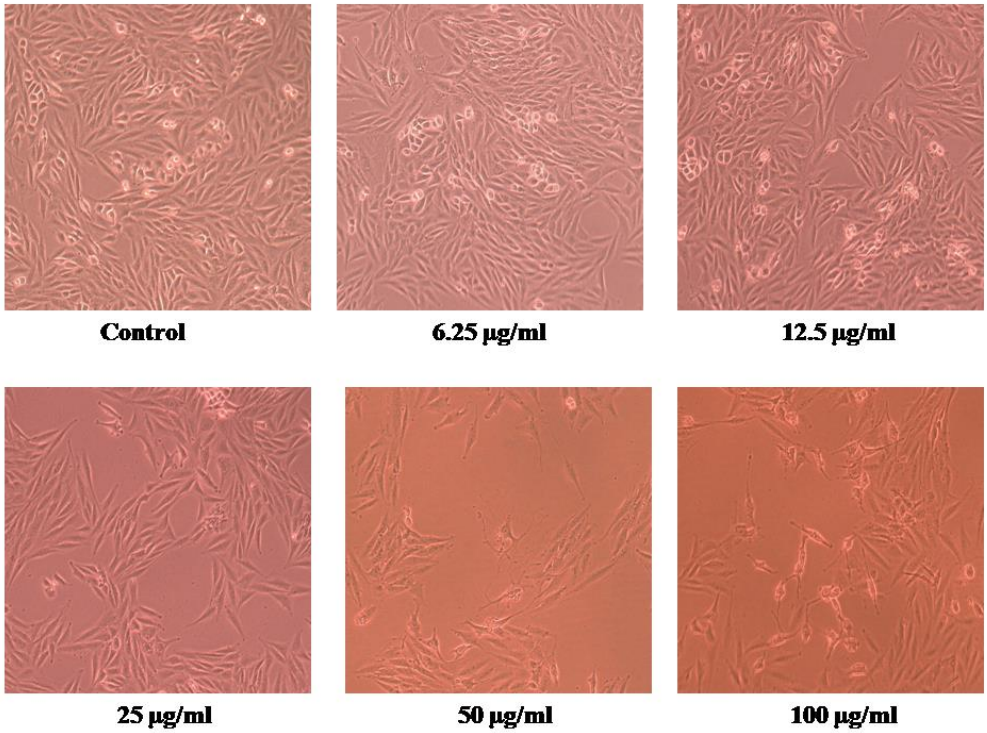


Cell Morphology

The morphology of SKMEL cells subjected to the administration of varying concentrations of gallic acid is represented in Figure5. The figure indicates a concentration dependant reduction in cell number. Changes in cell morphology as well as detachment of the cells from the surface can also be observed. All these observations along with the MTT results indicate the cytotoxic potential of gallic acid on SKMEL skin cancer cell lines.

The study found a significant dose-dependent reduction in the viability of gallic acid-treated groups of SK-MEL cells from the MTT assay. This indicated that as the concentration of gallic acid increases, the cellular metabolic status along with cell viability of the skin cancer cells has been adversely affected. Reduction in cell number as well as morphological alterations as observed from the cell morphology analysis also indicates the toxic effect of gallic acid on SKMEL cells.Hence the capability of gallic acid in inhibiting oxidative stress as observed from the DPPH antioxidant and protein denaturation assays is expected to be the main causative agent in diminishing the cancer cell growth.

Figure 5: Morphology of SKMEL cells subjected to the administration of varying concentrations of gallic acid



Gene expression studies

The BCL2 geneis generally considered to be an apoptosis suppressor gene. BCL2 averts apoptosis via a direct impact on mitochondria and decreases the cell membrane permeability and connections with other proteins (Zha*et al*., 1996). Research investigations on different tumor cell lines *in vitro*indicate that the excessive expression of BCL2 averts cell death (Findley *et al*.,1997).

Molecular-level research has pointed out the upregulation of the BAX gene during apoptosis (Ghasemi*et al*., 2018).BAX is accountable for the mitochondrial pathway of apoptosis (Paul *et al*., 2018). In cancer cells, stimulation of the BAX gene commences cell death. BAX overexpression is found to be the vital process behind apoptosis induction by chemotherapeutic agents (Kobayashi *et al*., 2000).

Administration of the sample gallic acid was found to result in the under-expression of the BCL2 gene as well as the BAX gene over-expression. The expression patterns as observed may promote the apoptosis of SKMEL skin cancer cells, thus indicating the anticancer activity of gallic acid. The results obtained from the gene expression results are in accord with the cytotoxicity experiment, the MTT assay. Thus,concluding that the gallic acid obtained from *Streptomyces olivochromogenes*has effective anticancer potential.

The results are expressed in the figures and tables below (Fig. 6& 7; Tables- 15 - 19).

Figure 6: RNA quantification image

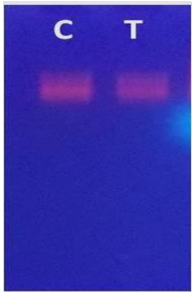


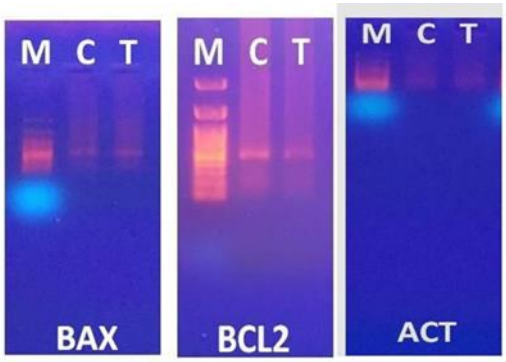
Table 15: RNA quantification result

Total RNA quality and quantity				
	A 260 nm	A 280 nm	A260/A280	**RNA concentration (ng/µl)**
Control	0.129	0.069	1.86	**516**
Gallicacid (56µg/ml)	0.135	0.076	1.77	**540**
Total RNA concentration (ng/µl) = A260 × 40ng/µl×100 (df) **Pure RNA A260/A280= 2.0**				

Table 16: DNA quantification results

cDNA		
	A260nm	**DNA concentration after RT PCR (ng/µl)**
Control	0.412	**2060**
Gallic acid (56µg/ml)	**0.319**	**1595**

Figure 7: Expression pattern of the genes, BAX, BCL2, and ACT.



M- DNA ladder, C- untreated sample, T- Gallic acid (56 µg/ ml)

Table 17 Expression results of the BAX gene

		BAX	
	A260nm	DNA concentration after gene Expression PCR (ng/µl)	**Remarks**
Control	0.169	845	-
Gallic acid (56 µg/ml)	0.175	875	**Upregulated**
Total DNA concentration (ng/µl) = A260 × 50ng/µl×100 (df)			

Table 18 Expression results of the BCL2 gene

		BCL2	
	A260nm	DNA concentration after gene Expression PCR (ng/µl)	Remarks
Control	0.207	1035	-

Gallic acid (56µg/ml)	0.175	875	Under expression
Total DNA concentration (ng/µl) = A260 × 50ng/µl×100 (df)			

Table 19 Expression results of the Beta gene

Beta Actin		
	A260 nm	**DNA concentration after gene expression PCR (ng/µl)**
Control	0.153	**765**
Gallic acid (56 µg/ml)	**0.144**	**720**

Conclusion

The research investigation showcased the capability of the bacteria *Streptomyces olivochromogenes* to produce gallic acid in ample quantities and the beneficial aspects of this microbial product in therapeutic areas. Results obtained from the FTIRstudies confirmed the product as gallic acid. The substantial antioxidant property of gallic acid was confirmed by the DPPH antioxidant assay and this antioxidant potential was found to be effective in safeguarding proteins from denaturation. The capability to maintain redox status along with the potential to act at the gene level (BAX and BCL2) may be the underlying reasons for the anticancer potential of gallic acid. Based on our findings, we suggest that more in-depth explorations will result in the development of gallic acid from *Streptomyces olivochromogenes* as an effective therapeutic agent.

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