Isolation, identification and evaluation of antioxidant, anti-inflammatory and antimutagenic properties of bio-active pigment from *Rhodococcus corynebacterioides* SCG11

doi.org/10.33500/ijambr.2020.08.001

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Article History

Received 16 January, 2020
Received in revised form 20 February, 2020
Accepted 25 February, 2020

Keywords:

Bio-pigments,
Rhodococcus corynebacterioides,
Antioxidant,
Anti-inflammatory,
Antimutagenic,
GC-MS.

Article Type:

Full Length Research Article

ABSTRACT

In an examination during the course of this study, yellow-orange producing bacterium was isolated from pharmaceutical effluent to assess its antioxidant, anti-inflammatory and antimutagenic properties. Morphological and molecular (16S rRNA) characterization identified the isolate as a Gram-positive bacilli *Rhodococcus corynebacterioides* and assigned as SCG11. The 16S rRNA sequence was submitted to NCBI Gen Bank with accession number: KU995335 and gene ID: 1015917315. The phylogenetic investigation with most extreme probability technique indicated 100% closeness with *Rhodococcus* spp. PBTS1. Thin layer chromatography was done for extracted pigment with various solvents system; acetonitrile and water (8:2, v/v) was observed to be the best solvent system and indicated distinct separations. The pigment was additionally purified through silica gel column chromatography and purified pigment was exposed to GC-MS examination. GC-MS results revealed the presence of ~29 compounds out of which hexadecanoic acid methyl ester (peak area % = 5.75), n-hexadecanoic acid (peak area % = 7.59) have antioxidant properties, n-hexadecanoic acid (peak area % = 7.59) and ergotaman-3',6',18-trione, 9,10-dihydro-12'-hydro-2'-methyl-5-(phenyl methyl)-, (5'α,10α)- (peak area % = 3.19), ornithine (peak area % = 6.84) have anti-inflammatory properties and Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- (peak area % = 3.78) has antimutagenic properties.

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INTRODUCTION

Pigments are synthetic or chemical compound which assimilate and reflect just certain wavelengths of visible light whose nearness in the cell or tissue gives it characteristic color. Pigments have assortment of hues (Tibor, 2007) and are utilized as additives, colorants, flavorings in ventures like sustenance, textile cosmetics,

pharmaceutical and printing enterprises. They additionally have application in restorative field, for example, as therapeutic agents as an antioxidant, anti-tumor, anti-inflammatory, anti-microbial, anti-arthritis and different properties.

Synthetic pigments cause more environmental hazards compared to natural pigments; this awareness resulted in strong consumer demand for natural pigments/colors. Natural colors are generally extracted from fruits, vegetables, roots these are also called as bio-colors (Pattnaik et al., 1997). The major sources being the

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plants (Mizukami et al., 1978) and microorganisms (Cross and Edinberry, 1972). Plant pigments have drawbacks like weather dependency, low yield, cost and photo, thermo instability or adverse pH (Bhat and Marar, 2015). All the problems can be addressed by laboratory cultivation of pigment producing microbes.

Microorganisms are prolific producers of pigments and they are ubiquitous in nature, produce various pigments like carotenoids, melanin, flavins, monascins, violacein and indigo (Duffose, 2009; Goodwin and Britton, 1988). It is known that aerobic and facultative anaerobic bacteria are pigmented, as molecular oxygen is essential for pigment production. In bacteria, pigment formation is associated with morphological characteristics, cellular activities, pathogenesis, protection and survival. Pigments are produced by bacteria to absorb UV radiation or to quench oxygen free radicals and also helps in survival in stress conditions.

Rhodococcus, belongs to the family *Nocardiaceae*, they are generally non-motile, intracellular aerobic, Gram-positive, weakly acid-fast coccobacilli. Colonies are salmon-pink to red colored and teardrop shaped or coalescent mucoid. The colony size ranges from 2 to 4 mm after 4 days of growth on solid media (Al Akhrass et al., 2012). There are 34 named species in the genus. Most of these bacteria are non-pathogenic and found in a broad range of environments, such as soil, groundwater, animal dung, and plants. According to literature survey done, there are not many reports available on utility of these microorganisms for pigment production and its application. Hence, the present work was undertaken to produce microbial pigments and evaluate their antioxidant, anti-inflammatory and anticancer properties.

MATERIALS AND METHODS

Isolation of pigment producing bacteria

Effluent samples were collected from various industries such as food industry, sugar cane industry, paper and pulp industry, distilleries and pharmaceutical industries. Effluents were serially diluted, and it was inoculated onto the nutrient agar, as a selective screening technique, agar plates were exposed to UV radiation for 1–3 min before incubation. The UV treatment decreases the undesirable (non-pigmented) microbes and construct microbial library that includes only pigmented microbes.

Identification and characterization of isolates

The morphology of isolated microorganism was studied macroscopically by observing colony features (color and texture) and microscopically by Gram staining and biochemical test.

16S rRNA sequencing

Bacterial genomic DNA was isolated using the InstaGene™ Matrix Genomic DNA isolation kit. 1 µL of template DNA added in 20 µL of polymerase chain reaction (PCR) solution and amplification of 16S rRNA was performed using 27F/1492R (5'-AGAGTTTGATCMTGGCTCAG-3') and (5'-TACGGYTACCTTGTTACGACTT-3') primers used for bacteria, and then PCR performed with below conditions: Initial denaturation 94°C for 2 min and then 35 amplification cycles at 94°C for 45 s, 55°C for 60 s, and 72°C for 60 s. Final extension at 72°C for 10 min. DNA fragments were amplified about 1,400 bp in the case of bacteria. A positive control (*Escherichia coli* genomic DNA) and a negative control were included in the PCR. Unincorporated PCR primers and dNTPs were removed from PCR products by using Montage PCR Clean up kit (Millipore). The PCR product was sequenced using the 518F/800R primers.

Sequencing reactions were performed using a ABI PRISM® BigDye™ Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems). The 16S rRNA sequence was blast using NCBI blast similarity search tool. The phylogeny analysis was done by sequencing with the closely related sequence of blast results performed, followed by multiple sequence alignment.

Extraction of pigments

The isolated pigment producing bacterial cells were grown in nutrient medium for 24 h pursued by centrifugation at 10000 rpm for 20 min (REMI C-24 cooling axis). Both the supernatant and bacterial cell pellets were extracted utilizing 95% (v/v) methanol in the proportion of 1: 5 (supernatant) or until the point when the pellet was drab, that is, finish color extraction has been accomplished. The bacterial cell pellet was then disposed of while the supernatant was first extracted by utilizing ethyl acetate followed by concentrating, utilizing rotary evaporator at 50°C with chiller temperature set at underneath 10°C. The pigment concentration process was completed until around 1% (v/v) of the underlying dissolvable volume was left in the dissipation jar. The concentrated pigment was then transferred into glass Petri dishes preceding drying for 3 days using vacuum drier.

Thin layer chromatography

Chromatography was performed on commercially available pre-coated silica gel plate (TLC Silica gel 60 F₂₅₄, (MERK)). An aliquot of extracted pigment was loaded

on to silica gel TLC plates (10 cm × 5 cm). The plates was developed in ascending direction with 4 different combinations of mobile phases were used toluene/ethylacetate/methanol (8:1:1, v/v), acetonitrile/water (8:2, v/v), methanol /water (9:1, v/v) and acetic acid/chloroform (1:9, v/v). After completion of chromatographic run, plate was air-dried, and the spots on the plate were visualized by exposure to UV light.

GC-MS/MS analysis

GC-MS/MS analysis of extracted pigment was performed using a Scion 436-GC Bruker model coupled with a Triple quadrupole mass spectrophotometer with fused silica capillary Column BR-5MS (5% Diphenyl/95% Dimethyl poly siloxane), 30 m × 0.25 mm ID × 0.25 µm df. The experiment was programmed with total run time 37.5 min, helium was used as a carrier gas at a constant flow rate of 1.0 mL/min, split less flow 1 mL/min. Injection volume was 2 µL with scan mass range 50 – 500. The column oven temperature program was as follows: 80°C hold for 2 min, Up to 160°C at the rate of 20°C/min-No hold, Up to 280°C at the rate of 5°C/min-No hold, Up to 300°C at the rate of 20°C/min-10 min hold, Injector temperature 280°C. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. Peaks were analyzed using data analysis software NIST- version 2011 (Srinivasan and Kumaravel, 2016).

Determination of biological activities

Determination of Antioxidant assay

So as to decide antioxidant properties of the pigment were performed by following strategies ferric ion reducing antioxidant power (FRAP), 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and ABTS tests were utilized.

DPPH radical scavenging assay

The free radical scavenging activities of pigment was performed by using 1,1-diphenyl-2-picryl-hydrazyl (DPPH). The assay was carried out as per Hatano et al., (1998), 1 mL of extracted pigment was added to 0.2 mM of 1 mL DPPH radical. The mixture was shaken vigorously, allowed to stand for 30 min in the dark and the absorbance was measured at 517 nm against a blank. Butylated hydroxytoluene (BTH) was used as a positive control. The capability to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = \frac{[A_0 - A_1]}{A_0} \times 100$$

Where, A₀ is the absorbance of the control reaction; and A₁, absorbance in the presence of the sample.

Ferric ion reducing antioxidant power (FRAP) assay

The assay was carried out as reported by Benzie and Strain (1996) with some modifications. FRAP reagents were freshly prepared by mixing 25 mL acetate buffer (300 mM, pH 3.6), 2.5 mL 2,4,6-Tris (2-pyridyl)-S-triazine (TPTZ) solution (10 mM TPTZ in 40 mM HCl) and 2.5 mL FeCl₃ (20 mM) water solution. Pigment sample (150 µL) was dissolved in methanol (0.5 mL) and added in 4.5 mL of freshly prepared FRAP reagent and stirred. After 5 min, absorbance was measured at 593 nm, using FRAP solution as blank. A calibration curve of ferrous sulfate (100-1000 µM) was used and results were expressed in µM Fe²⁺/mg dry weight extract. The relative activity of the samples was compared to L-ascorbic acid.

ABTS radical scavenging activity

2,2-azinobis-3ethylbenzothiazoline-6-sulfonic (ABTS) acid assay was performed according to Govindappa et al. (2011) with little modifications, two different stock solutions, 7.4 mM ABTS and 2.6 mM potassium persulphate were prepared separately. The working solution was prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in dark. The solution was diluted by mixing 1 mL ABTS solution prepared using 50 mL of methanol, in order to obtain absorbance 1.1 ± 0.02 units at 734 nm. Approximately 1.5 mL of pigment sample was mixed with 2.5 mL of ABTS solution and the mixture was left at room temperature for 2 h in dark. The capability to scavenge the ABTS radical was calculated using the following equation:

$$\text{ABTS scavenging effect (\%)} = \frac{[A_0 - A_1]}{A_0} \times 100$$

Where, A₀ is the absorbance of the control reaction; and A₁, the absorbance in the presence of the sample.

In vitro anti-inflammatory activity

Inhibition of bovine serum albumin (BSA) denaturation

Anti-inflammatory activity was performed as per Mizushima and Kobayashi (1968) and Sakat et al. (2010) with slight modifications. The reaction mixture contained of test pigment and 1% aqueous solution BSA. The sample was incubated at 37°C for 20 min and then heated to 50°C for 30 min after cooling the samples the

turbidity was read at 660 nm through spectrophotometer. Percentage inhibition of protein denaturation was calculated as follows:

$$\% \text{ inhibition} = \frac{[\text{Abs control} - \text{Abs sample}]/\text{Abs control}] \times 100}$$

Where, Abs control is the absorbance without sample, Abs sample is the absorbance of pigment/standard.

Preparation of red blood cells (RBCs) suspension

Fresh whole human blood (10 mL) was collected and transferred to the centrifuged tubes. The tubes were centrifuged at 3000 × g for 10 min. and were washed three times with equal volume of normal saline. The volume of blood was measured and re-constituted as 10% v/v suspension with normal saline (Sakat et al., 2010).

Heat induced hemolysis

The reaction mixture (2 mL) consisted of 1 mL of test sample solution and 1 mL of 10% RBCs suspension, instead of test sample only saline was added to the control test tube. Diclofenac sodium was used as a standard drug. Reaction mixture was incubated in water bath at 56°C for 30 min, and was allowed to cool down. The reaction mixture was centrifuged at 2500 × g for 5 min and the absorbance of the supernatants was taken at 560 nm. Percent membrane stabilization activity was calculated by the formula:

$$\% \text{ inhibition} = \frac{[\text{Abs Control} - \text{Abs sample}]/\text{Abs control}] \times 100}$$

Human red blood cell (HRBC) membrane stabilization assay

HRBC was conducted by collecting fresh blood from individual and mixed with equal volume of Alsever solution (anti-coagulation) and it was then centrifuged at 3000 × g for 15 min. The packed cells were washed with isosaline and a 10% suspension was made with isosaline. To which 0.5 mL of pigment, 1 mL phosphate buffer, 2 mL hyposaline and 0.5 mL HRBC suspension was added and incubated for 30 min at 37°C, and then centrifuged at 3000 × g for 20 min. Absorbance was measured at 560 nm. Without pigment was considered as control.

$$\% \text{ inhibition} = \frac{[\text{Abs Control} - \text{Abs sample}]/\text{Abs control}] \times 100}$$

Antimitotic activity

Antimitotic activity assay was performed as per

Shwetha et al. (2012) using *Allium cepa* (Onion) root with slight modification. *A. cepa* bulbs were sprouted in water for 24 h at room temperature. The uniform root tips of Onions were selected for the study. These roots were dipped in the pigment for 48 h. Water was used for dilution. After 48 h, the root tips were fixed in the fixing solution of acetic acid and alcohol (1:3). Squash preparation was made by staining with acetocarmine stain. Morphology and the number of the cells were observed under the microscope (40×). In all 350-400 cells were counted and cells manifesting different stages of mitosis that is interphase, prophase (P), metaphase (M), anaphase (A) and telophase (T) were recorded. The mitotic index was calculated using the following formula (Subhadradevi et al., 2011).

$$\text{mitotic index} = \frac{P + M + A + T}{\text{Total cells}} \times 100$$

Anti-proliferative activity

Evaluation of ant proliferative activity of pigment was performed by yeast *Saccharomyces cerevisiae* as a model according to Shwetha et al. (2012). Yeast inoculum was prepared by inoculating yeast to potato dextrose broth and incubated at 37°C for 24 h, it was referred as seeded broth. Cell viability assay was performed with 2.5 mL of potato dextrose broth and 0.5 mL of yeast inoculums in four separate test tubes. The first test tube distilled water and in other test tubes the pigment extracts (200 µL) were added. All the tubes were incubated at 37°C for 24 h. In the above cell suspension, 0.1% methylene blue dye was added in all tubes and they were observed under low power microscope. The number of viable cells, those does not stain and look transparent with oval shape while dead cells get stained and appeared blue in color were counted in 16 chambers of hemocytometer and the average number of cells were calculated. The percentage of cell viability was calculated using the formula (Subhadradevi et al., 2011).

$$\% \text{ cytotoxicity} = \frac{\text{No. of dead cells}}{\text{No. of viable cells} + \text{No. of dead cells}} \times 100$$

RESULTS AND DISCUSSION

Isolation of bacteria

A total 15 different microbial strains were isolated from different effluent samples on the basis of colony morphology, pigment production and duration of UV treatment. All isolates were primarily screened for pigment production and one bacterial strain showing maximum resistant to UV light with yellow orange pigment production was selected and further characterized by morphological and biochemical tests. As

Figure 1. Isolation of pigment producing bacteria. **A**, Pigment producing bacteria incubated at normal light; **B**, pigment producing bacteria incubated under UV light; **C**, *R. corynebacteriodes* SCG11.

Table 1. Colony morphology and Biochemical characteristics of *R. corynebacteriodes* SCG11.

S/N	Colony characteristics	Observation
1	Colony appearance	Yellow-orange
2	Form	Circular
3	Elevation	Convex
4	Surface	Smooth
5	Margin	Entire
6	Morphological characteristics	
7	Gram stain	Gram positive
8	Cell shape	Rod
9	Motility	Non-motile
10	Spore	Non sporulation

Biochemical tests

Hydrolysis	Tests	Observation
01	Aesculine	-ve
02	L-Tyrosin	-ve

Utilization as sole source of carbon and energy

03	Citrate	+ve
04	Gluconate	+ve
05	Lactate	-ve
06	Sucrose	+ve
07	Trehalose	+ve
08	D-Xylose	-ve
09	myo-Inositol	+ve
10	Mannitol	+ve
11	D-Sorbitol	+ve
12	2,3-Butanediol	+ve
13	1,2-Propanediol	+ve
14	m-Hydroxybenzoate	-ve
15	p-Hydroxybenzoate	-ve

Utilization as sole source of carbon and nitrogen

16	Acetamide	-ve
17	L-Alanine	-ve
18	Proline	+ve
19	Serine	+ve
20	Ornithine	+ve
21	Acetate	+ve
22	Adenine	+ve
23	Glucose	+ve
24	Testosterone	+ve
25	Urea	+ve
26	Adonitol	-ve
27	Adipic acid	-ve
28	iso-amylalcohol	-ve
29	L-arabinose	-ve
30	Lactose	-ve
31	Maltose	-ve
32	Raffinose	-ve
33	Rhamnose	-ve
34	Casein	-ve
35	Elastin	-ve
36	Gelatin	-ve
37	Hypoxanthine	-ve
38	Xanthine	-ve

per the colony characteristic and biochemical test the bacteria were identified as *Rhodococcus* sp. and it was designated as *Rhodococcus* SCG11. Their distinguish feature was depicted in Table 1, and its colony morphology in Figure 1.

Figure 2. Phylogenetic analysis of *R. corynebacterioides* SCG11.

Phylogenetic analysis

The *Rhodococcus* SCG11 acquired from the morphological and biochemical character was subjected to 16S rRNA sequencing so as to distinguish the organisms at the species level. The sequence obtained after performing sequencing studies were blasted using the BLAST search tool from NCBI's GenBank nucleotide database ([www http://blast.ncbi.nlm.nih.gov/](http://blast.ncbi.nlm.nih.gov/)) to retrieve comparative taxa for further investigations. The blast search results of *Rhodococcus* SCG11 spp. sequence data were shown the greatest identity with maximum score from the blast search were selected with most extreme score taxa. The impact looks were chosen, and accessible sort strains were incorporated for building the phylogenetic trees by neighbor joining strategy. The

phylogeny resulted (Figure 2) more than 20 known representative strains of *Rhodococcus*. It had 100% matches with many isolates in the BLAST search of NCBI's GenBank nucleotide database. The maximum score was similar to *R. corynebacterioides* and E value is 0.0. Hence the isolated organism was identified as *R. corynebacterioides* and designated as *R. corynebacterioides* SCG11. Further, *R. corynebacterioides* SCG11 sequence was submitted to NCBI Gene Bank with an Accession Number KU995335 and gene ID: 1015917315.

Extraction of pigment

Out of the 4 different solvent systems, methanol was found to be the suitable solvent to extract and solubilize

Figure 3. Methanol extracts of pigment from *R. corynebacteriodes* SCG 11.

Figure 4. TLC plates of the extracted pigment from *R. corynebacteriodes* SCG11 with different combinations of solvent system and observed under UV light. A, Toluene, ethyl acetate and methanol (8:1:1); B, acetonitrile and water (8:2); C, methanol and water (9:1); D, acetic acid and chloroform (1:9).

maximum pigment from the pellet (Figure 3). Chemical composition of the pigment generally decides the choice of organic solvent and total extraction of pigment. Lipophilic pigments are soluble in organic solvents, such as chloroform, hexane, acetone, petroleum ether and methanol.

TLC and GC-MS analysis

TLC and GC-MS analysis revealed the presence of bioactive compounds in the pigment extract (Figure 4A).

From the figure (Figure 4C), the methanol and water (9:1) solvent system gave a lot of tailing in the TLC. The other solvent systems used, that is, acetonitrile and water (8:2), toluene, ethyl acetate and methanol (8:1:1), chloroform and acetic acid (9:1) showed a better separation since it is a mixture of polar and nonpolar solvents. But toluene, ethyl acetate and methanol (8:1:1) (Figure 4A) system have 4 to 5 distinct bands on plates. While pigment was subjected to GC-MS analysis, revealed that the presence of ~29 (Figure 5 and Table 2) compounds out of which hexadecanoic acid methyl ester (peak area % = 5.75), n-

Figure 5. GC-MS analysis of the extracted pigment from *R. corynebacteriodes* SCG11 showing 19 predominant peaks.

Table 2. GC-MS analysis identified bioactive compounds in the extracted pigment sample of *R. corynebacteriodes* SCG11.

S/N	RT	Name of the compound	Molecular formulae	Molecular weight	Peak area %
1	5.42	Pyridine, 1-acetyl-1,2,3,4-tetrahydro-	C ₇ H ₁₁ NO	125	2.62
2	7.18	Ornithine	C ₅ H ₁₂ N ₂ O ₂	132	6.84
3	8.21	Pyrrolidin-2-one, 5-heptyl-	C ₁₁ H ₂₁ NO	183	0.24
4	8.71	Pyrazine, 2-methyl-3-(methylthio)-	C ₆ H ₈ N ₂ S	140	2.66
5	9.83	L-Proline, 1-methyl-5-oxo-, methyl ester	C ₇ H ₁₁ NO ₃	157	4.13
6	10.14	2H-Pyran-2-one, tetrahydro-6-propyl-	C ₈ H ₁₄ O ₂	142	1.25
7	12.09	Conhydrin	C ₈ H ₁₇ NO	143	0.33
8	12.38	Thieno[2,3-c] furan-3-carbonitrile, 2-amino-4,6- dihydro-4,4,6,6-tetramethyl-	C ₁₁ H ₁₄ N ₂ OS	222	1.75
9	12.54	3-Methyl-1,4-diazabicyclo [4.3.0] nonan-2,5- dione, N-acetyl-	C ₁₀ H ₁₄ N ₂ O ₃	210	2.25
10	13.08	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-	C ₇ H ₁₀ N ₂ O ₂	154	8.55
11	13.79	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	C ₁₁ H ₁₈ N ₂ O ₂	210	3.78
12	15.05	L-Leucine, N-cyclopropylcarbonyl-, methyl Ester	C ₁₁ H ₁₉ NO ₃	213	0.35
13	15.12	Hexadecanoic acid, methyl ester	C ₁₇ H ₃₄ O ₂	270	5.75
14	15.41	5,10-Diethoxy-2,3,7,8-tetrahydro-1H,6H- dipyrrolo[1,2-a:1',2'-d]pyrazine	C ₁₄ H ₂₂ N ₂ O ₂	250	2.49
15	15.59	n-Hexadecanoic acid	C ₁₆ H ₃₂ O ₂	256	7.59
16	17.06	1-Benzyl-4-piperidineacetonitrile	C ₁₄ H ₁₈ N ₂	214	4.41
17	17.54	9-Octadecenoic acid (Z)-, methyl ester	C ₁₉ H ₃₆ O ₂	296	5.20
18	17.70	4-Methyl-5-(4-methoxyphenyl) pyrimidine	C ₁₂ H ₁₂ N ₂ O	200	7.86
19	17.85	2,5-Piperazinedione, 3-methyl-6- (phenylmethyl)-	C ₁₂ H ₁₄ N ₂ O ₂	218	3.02
20	18.07	Trans-13-Octadecenoic acid	C ₁₈ H ₃₄ O ₂	282	2.41
21	18.31	dl-Alanyl-l-phenylalanine	C ₁₂ H ₁₆ N ₂ O ₃	236	3.94
22	19.71	Pyrrolo[1,2-a]pyrazine-3-propanamide, 2,3,6,7,8,8a-hexahydro-1,4-dioxo-	C ₁₀ H ₁₅ N ₃ O ₃	225	3.78
23	20.21	Fumaric acid, decyl 2-dimethylaminoethyl ester	C ₁₈ H ₃₃ NO ₄	327	0.38
24	20.97	Ergotaman-3',6',18-trione, 12'-hydroxy-2'- methyl-5'-(phenylmethyl)-, (5'α)-	C ₃₃ H ₃₅ N ₅ O ₅	581	1.89
25	21.35	9-Octadecenamide, (Z)-	C ₁₈ H ₃₅ NO	281	8.04
26	21.63	Ergotaman-3',6',18-trione, 9,10-dihydro-12'- hydroxy-2'-methyl-5'-(phenylmethyl)-, (5'α,10α)-	C ₃₃ H ₃₇ N ₅ O ₅	583	3.19
27	23.56	Hexadecanoic acid, 1-(hydroxymethyl)-1,2- ethanediyl ester	C ₃₅ H ₆₈ O ₅	568	2.09
28	26.15	Cholestan-3-ol, 2-methylene-, (3β,5α)-	C ₂₈ H ₄₈ O	400	1.04
29	27.70	18,19-Secoyohimban-19-oic acid, 16,17,20,21- tetrahydro-16-(hydroxymethyl)-, methyl ester, (15β,16E)-	C ₂₁ H ₂₄ N ₂ O ₃	352	2.16

Table 3. GC-MS analysis shows some important bioactive components peaks from the extracted pigment of *R. corynebacteriodes* SCG11.

S/N	GCMSpeaks	Name of compound	Structure	Peak area	RT	Biological activity
1	7	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-		3.78	13.79	Antibacterial activity, Anti-cancerous activity
2	8	Hexadecanoic acid, methyl ester		5.75	15.12	Antioxidant, Hypocholesterolemic, Nematicide, Pesticide, Antiandrogenic flavor, Hemolytic, 5-Alpha reductase inhibitor, component of pheromone
3	10	n-Hexadecanoic acid		7.59	15.59	Antioxidant, hypocholesterolemic, Anti-inflammatory, Antibacterial

hexadecanoic acid (peak area% = 7.59) have antioxidant properties, n-hexadecanoic acid (peak area% = 7.59) and ergotaman-3',6',18-trione,9,10-dihydro-12'-hydro-2'-methyl'5-(phenyl methyl)-,(5'α,10α)- (peak area % = 3.19), ornithine (peak area % = 6.84) have anti-inflammatory properties, Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- (peak area % = 3.78) has antimitotic properties. Some important bio-compounds and their biological activities are shown in Table 3.

Antioxidant assay

The pigment extracted from *R. corynebacteriodes* showed positive results for all the three antioxidant assays. The bio-active compounds having the antioxidant property that was identified by GC-MS analysis (Table 2) and the pigment itself could contribute to the overall antioxidant property of the pigment extract.

Synthetic antioxidants play an important role in preventing major degenerative diseases caused by free radicals and protecting foodstuffs from lipid oxidation (Kumaran and Karunakaran, 2007). However, with increasing documentation of possible adverse effects of some synthetic antioxidants on human health, there is an increasing interest in finding natural and biologically produced antioxidants. Further, the emergence of bacterial strains resistant to clinically used antibiotics and changing patterns of susceptibility to clinically available antimicrobial agents require continuous updating of knowledge concerning the treatment of diseases caused by such pathogens (Brandão et al., 2010).

The antioxidant properties were determined as percentage radical scavenging activity using three different methods and it was compared with standard ascorbic acid. *R. corynebacteriodes* SCG11 pigment showed maximum activity in DPPH assay 64.10% where as in FRAP and phosphomolybdenum assay 57.11 and 31.94% activity respectively. Ascorbic acid was used as the standard (Figure 6). *R. corynebacteriodes* SCG11 pigment was showed good result in DPPH assay as it reacts with a hydrogen donor, the reduced (molecular) form (DPPH) is generated, accompanied by the disappearance of the violet color. Therefore, the absorbance diminution depends linearly on the antioxidant concentration (Pisoschi and Negulescu, 2011). The DPPH and FRAP assays gave comparable results for the antioxidant activity measured in methanol extract. The FRAP technique showed high reproducibility, was simple, rapidly performed and showed the highest correlation with both ascorbic acid and total phenolic (Thaipong et al., 2006). Antioxidant activities measured in methanol extract obtained using DPPH, FRAP, assays from a single extract were measured three times to test the reproducibility of the assays (Thaipong et al., 2006).

Anti-inflammatory assay

Inflammation is the reaction of living tissues to injury, infection or irritation. Bacterial infections cause an increased number of neutrophils, which produce an oxidative burst at the site of microbial invasion. The lysosomal enzyme released during inflammation

Figure 6. Comparative antioxidant activity of extracted pigment from *R. corynebacteriodes* SCG11.

Table 4. Effect of *R. corynebacteriodes* SCG11 pigment on HRBC membrane stabilization.

Concentration of pigment ($\mu\text{g/mL}$)	(membrane stabilization) % protection	Diclofenac sodium (% protection)	Albumin denaturation	Proteinase inhibition
50	92.26 $\pm$ 0.12	95.17 $\pm$ 0.26	75.46 $\pm$ 0.18	70.24 $\pm$ 0.21
100	94.25 $\pm$ 0.21	96.51 $\pm$ 0.16	78.51 $\pm$ 0.21	79.54 $\pm$ 0.31
200	96.28 $\pm$ 0.16	97.12 $\pm$ 0.21	81.25 $\pm$ 0.15	84.23 $\pm$ 0.23
300	98.44 $\pm$.25	99.12 $\pm$ 0.23	88.23 $\pm$ 0.23	87.19 $\pm$ 0.14

produces a variety of disorders. The extracellular activity of these enzymes is said to be related to acute or chronic inflammation. The non-steroidal drugs act either by inhibiting these lysosomal enzymes or by stabilizing the lysosomal membrane. Since HRBC membrane is similar to lysosomal membrane, the study was undertaken to check the stability of HRBC membrane predict the anti-inflammatory activity (Yoganandam et al., 2010).

The uncontrolled release of reactive oxygen species is assumed to be responsible for certain pathological conditions as heart attacks, septic shocks and rheumatoid arthritis. Administration of agents, which decrease the neutrophil accumulation in inflamed areas, might be a remedy in these cases.

The human RBC cell membrane has been especially helpful as a model for investigations of membrane structure. RBC do not contain any nuclei or internal membranes, so they are utilized as a source from which pure plasma membrane can be effectively isolated for biochemical examination. The erythrocyte plasma membrane resemblances to the lysosomal membrane and hence the stabilizing effect of drugs on erythrocyte membrane may correlate with its lysosomal membrane

stabilizing effect (Amujoyegbe et al., 2012). The lysosomal membrane stabilization prompts the hindrance of arrival of the incendiary arbiters and resulting restraint of the process of inflammation (Omale and Okafor, 2008). In the membrane stabilization examine, the erythrocytes are tested with various hemolytic boosts like heat, osmotic shock and free radicals (Takebayashi et al., 2007). Table 4 shows that *R. corynebacteriodes* SCG11 pigment demonstrated strong stabilizing effect on human RBC membrane. The membrane stabilizing effect of *R. corynebacteriodes* SCG11 pigment extract was concentration-dependent and ranged from 92.26 to 98.44%. Whereas a standard anti-inflammatory drug Diclofenac showed the maximum inhibition of (99%) at the concentration of 300 $\mu\text{g/mL}$. The result exhibited by the pigment is almost on par with the synthetic anti-inflammatory drug diclofenac. This inhibition may be due to chemical constituent of the pigment possible inhibiting release of lysosomal content of the scavenging cells of the immune system at the site of injury.

Also, the pigment exhibit significant levels of anti-proteinase activity 87.2% similar results were observed from the findings of Govindappa et al. (2015).

Figure 7. Antimitotic activity of pigment from *R. corynebacterioides* SCG11 on onion root tip method. Normal mitotic phase: A, prophase; B, metaphase; C, anaphase; D, chromosomal bridge lagging chromosome during anaphase; E, displacement of chromosome during metaphase; F, chromosomal clumping during metaphase.

Figure 8. Anti-mitotic activity assay of extracted pigment from *R. corynebacterioides* SCG11.

Antimitotic assay

The antimitotic activity was screened using *A. cepa* root meristematic cells which have been used extensively in screening of drugs with antimitotic activity (Abhang et al., 1991; Latha et al., 1998). The roots of all plants have distinguished regions, one of them being the region of cell division that lies beyond the root cap and extends a few mm after that. Cells of this region undergo repeated divisions. The rate of cell division is higher in this region

compared to that of the other tissues. This region is called the meristematic region (Dutta, 1971). This division is similar to the abovementioned cancer division in humans. Hence, these meristematic cells can be used for preliminary screening of drugs with anticancer activity.

The results of the antimitotic activity was observed and depicted in Figures 7 and 8. We have found that *R. corynebacterioides* extract induced antimitotic activity at various levels of cell cycle namely (1) arrest of cells at interphase with large nucleoli and binucleoli; (2)

Figure 9. Ant proliferative assay of extracted pigment from *R. corynebacterioides* and yeast cells are model organism, under the microscope. a, Colorless yeast viable cells; b, arrow showing the viable and dead cell. Dead yeast cell staining blue.

Figure 10. Anti-proliferative assay of extracted pigment from *R. corynebacterioides* SCG11.

metaphase with irregular chromosomal distribution and inter chromosomal gap; (3) chromosomal stickiness and bridge at anaphase; (4) chromosomal stickiness and cell shrinkage at telophase; (5) cell shrinkage at prophase surrounded by normal cells; (6) abnormal anaphase with vagrant chromosomes. Chromosomal fragmentation at late prophase and chromosomal separation at anaphase was observed and compared with normal mitotic phases of interphase, metaphase, anaphase, prophase and telophase.

Anti-proliferative assay

In vitro ant proliferative and cytotoxic assay was studied using yeast as a model system (Clarke et al., 1993). Figures 9 and 10 shows yeast cells when visualized under the microscope apoptotic cells are characterized by the number of morphological changes such as cell shrinkage, membrane blebbing, chromatic condensation and formation of apoptotic bodies (Sadananda et al., 2013; Channabasava and Govindappa, 2014;

Zimmerman et al., 2001). The pigment extract of *R. corynebacterioides* tested positive for anti-proliferative activity and this can be further exploited as an anti-cancer or anti-tumor drug.

Conclusion

The pigment produced from *R. corynebacterioids* SCG11, is subjected to Thin layer chromatography with various solvents system, acetonitrile and water (8:2, v/v) was observed to be the best solvent system and purified pigment was exposed to GC-MS examination. Analysis revealed that the presence of ~29 compounds and these compounds are compared with standard data base with respect to their retention time and mass spectra of each compound and standard available in spectra. The main bioactive compounds which responsible for anti-oxidative and anti-proliferative would be the presence of these compounds namely hexadecanoic acid methyl ester (peak area % = 5.75), n-hexadecanoic acid (peak area% = 7.59) have antioxidant properties, n-hexadecanoic acid (peak area% = 7.59) and ergotaman-3',6',18-trione,9,10-dihydro-12'-hydro-2'-methyl-5-(phenyl methyl)-, (5'α,10α)- (peak area % = 3.19), ornithine (peak area % = 6.84) have anti-inflammatory properties, Pyrrolo[1,2- co a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- (peak area % = 3.78) has antimitotic properties. The pigment exhibit profound antioxidant and anti-inflammatory activities may be due to a combination of these or any individual compound. Further study is needed to exact individual compounds for their structural elucidation towards potent antioxidant and anti-inflammatory potentials for therapeutic purposes.

ACKNOWLEDGEMENT

Authors wish to Thank Dr. Govindappa, Professor and chairman, Department of Botany Davangere University, for his valuable suggestions and constant help throughout the manuscript preparation.

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