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In Vitro Assays To Investigate The Therapeutic Activity Of Bacterial Pigments As A Clinical Approach

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ABSTRACT: The objective was to study the production of pigments by bacteria isolated from air sampling and their characteristic in therapeutic applications. The pigment-producing bacteria were isolated by air sampling and selected based on the biochemical characteristics and their ability to produce pigments within 24hours. The pigments were extracted and partially purified then characterized by Thin Layer Chromatography (TLC) and Fourier Transform Infrared Spectroscopy (FTIR). The effects of pigments were characterized by studying the antidiabetic, antioxidant, anti-inflammatory activities and stability studies of crude pigment were also conducted. The bacterial pigments ABT1Y, ABT2O, ABT3P, ABT7P and ABT9P isolated from air sampling produced yellow, orange, pink and dark- pink pigment on nutrient agar plates within 24hours at 37°C. All the isolated bacteria were Gram-positive and Catalase-positive. Thin-layer chromatography (TLC) analysis revealed the occurrence of different pigments in the crude extract of bacteria belongs to the carotenoid group. The significant features of pigments ABT1Y, ABT2O, ABT3P, ABT7P and ABT9P are anti-diabetic, anti-oxidant and anti-inflammatory activities. The pigments were stable at pH 7 to 8.5 with less degeneration of color. The results suggest the application potential of bacterial pigments in various clinical approaches.

KEYWORDS: Bacterial pigments, FTIR, antidiabetic, antioxidant, anti-inflammatory activities.

I. INTRODUCTION

Pigments are produced from plants, animals, microorganisms and can also be manufactured by chemicals artificially. The pigments have many uses in industries (food, textile, paper, cosmetics, plastic, paint) agriculture, biology (antibiotics, antimicrobial agents, anticancer agents), etc [1-4]. These day's pigments produced from living organisms have obtained more importance while synthetic pigments are toxic and show harmful effects like carcinogenicity, mutagenicity, teratogenicity, genotoxicity, cytotoxicity, neurotoxicity, and have hazardous effects on the ecosystem [5-7].

Due to several reasons, pigments obtained from the plants and animals sources are inferior to the pigments produced from microbial productions. Those reasons are; rapid growth rate, easy downstream processing, cost-effectiveness, independent of season and geographical conditions, controllable, more stable, and safe to use [8, 9].

Pigments obtained from microbes have different shades of colors like yellow, purple, pink, orange, bluish-red, red pigments, etc. Microorganisms produce various pigments like carotenoid, bacteriochlorophyll a & b, melanin, flavonoid, quinines, prodigiosin, phenazine and more specifically monascin, violacein or indigo. They own various biological activities like antioxidant, antimicrobial, anticancer, anti-inflammatory, antiproliferative, anti-obesity and are used as bio-indicators [10-12].

Protection from UV rays, acts as an antioxidant, protects from extreme heat and cold and acquisition of nutrients like iron, nitrogen, and carbon are some properties of microbial pigments [13]. The anticancer ability of bacterial pigments is reported by many studies, for example, Lin et al. in 2005 studied the activity of pigments extracted from 12 different bacteria [14]. The bacterial pigment named violacein with significant antileishmanial activity was reported by Leon et al. [15]. The antibacterial activity of pigments isolated from fifteen different bacteria was determined by

Rashid et al. in 2014 [16]. Recent studies had revealed that inflammation and oxidative stress are closely correlated with diabetes, but the mechanism involved in it is not clearly confirmed because of the dual character of oxidative stress as a signal and as a damaging agent (transcriptional control and cell cycle regulation).[17−20]

This research aimed to isolate the pigment-producing bacteria from air sampling and evaluate the anti-diabetic, anti-oxidant, and anti-inflammatory activity of bacterial pigments.

II. EXPERIMENTATION

2.1. Isolation of pigment-producing bacteria

The bacteria were isolated by air sampling using standard techniques. The Petri plate containing nutrient media were exposed to the surrounding for 5min and incubated for 48hours at $37^{o}C$. The pigment-producing bacteria were isolated and sub-cultured several times. The Identification of isolates obtained in pure cultures were characterized by morphological characteristics and various biochemical tests according to Bergey's Manual of Determinative Bacteriology [21].

2.2. Pigment extraction and partial purification

Each pigment-producing bacteria were streaked on 5 different Petri plates for pigment extraction. After incubation of 24hours the culture were scraped and collected in a conical flask. 50ml of methanol was added to the conical flask and placed in the rotary shaker for 24hours. The pigments were subjected to successive solvent extraction using methanol and centrifuge to separate the cell debris. Methanol was then evaporated at room temperature to get the crude sample.

2.2.1. Thin-layer chromatography (TLC): The extracted pigments were characterized by thin-layer chromatography (TLC). A pre-coated silica gel sheet of 0.2 mm thickness was used as a stationary phase. The spot of extracted pigment was applied on the silica gel TLC sheet. The TLC sheet was then dipped in the solvent system until the solvent reached up to 3/4th of the TLC sheet. The sheet was kept in the oven for 5 minutes for drying and the bands were observed under UV light for spot observation. The R_f value of pigments were calculated [22].

2.2.2. Column chromatography: The stationary phase of silica gel (100–200 µm) and mobile phase based on the result of TLC were used for the partial purification of bacterial pigments. The purified bacterial pigments were used for further evaluation.

2.2.3. FTIR analysis The samples were analyzed at FTIR unite (Department of chemistry), University of Pune. The functional group of pigment can be determined by FTIR analysis [23].

2.3. Antidiabetic activity by using in vitro assays

α-amylase inhibitory assay: The α-amylase inhibitory assay for different bacterial pigments were evaluated according to the method previously described by Arun Kashivishwanath Shettar et.al in 2017 [24]. Different concentration of bacterial pigments (50- 100µg/ml) was mixed with 0.5 ml of α-amylase solution (0.5 mg/ml) with 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl). The mixture was incubated at room temperature for 10 min and then 0.5 ml of starch solution (1%) in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) was added. The mixture was incubated at room temperature for 10 min, and the reaction was terminated by adding 1 ml of dinitro salicylic acid color reagent. The test tubes were placed in a water bath (100 °C and 5 min) and then cooled to room temperature. The mixture was diluted with 10 ml distilled water, and absorbance was recorded at 540 nm. The absorbance of blank and control samples were also determined. Acarbose was used as a standard drug to compare the inhibition of α-amylase. The inhibition of α-amylase was calculated using the following equation:

% of inhibition of α-amylase =Abs control – Abs sample/ Abs control X 100

Where;

Abs control corresponds to the absorbance of the solution without bacterial pigment (buffer instead of bacterial pigment) and with α-amylase solution

Abs sample corresponds to the solution with bacterial pigment and α-amylase solution.

2.4. Assay of H_2O_2 scavenging activity

The ability of bacterial to scavenge hydrogen peroxide was determined according to the method described by Jignasu P. Mehta et. al in 2013 [25]. A solution of hydrogen peroxide (2 mM) was prepared in a phosphate buffer of pH 7.4. H_2O_2 concentration was determined spectrophotometrically from absorption at 230 nm. Bacterial pigments (100 to 500µg/ml) in distilled water were added to a hydrogen peroxide solution (0.6 mL). The absorbance of H_2O_2 at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The percentages of scavenging of hydrogen peroxide of extracts of aerial parts and standard compounds are calculated by using the following equation.

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% of Scavenging H_2O_2 = Absorbance control – Absorbance sample/ Absorbance control X 100

2.5. Inhibition of protein denaturation assay

Protein denaturation has been correlated with the formation of inflammatory disorders like rheumatoid arthritis, diabetes, and cancer. Therefore the ability of a substance to prevent protein denaturation may also help to prevent inflammatory disorders. In this assay, either egg albumin or bovine serum albumin (BSA) was used as protein. The denaturation of protein was induced by keeping the reaction mixture at 55°C in a water bath for 10 minutes. A reaction mixture consists of various concentrations of Bacterial pigments 1000 µL (100-500 µg/ml), 200 µL of egg albumin and 1400 µL of phosphate-buffered saline. Distilled water instead of extracts with the above mixture was used as a negative control. Afterward, the mixtures were incubated at 37 °C for 15 min and then heated at 70°C for 5 min. After cooling the mixture under running tap water, their absorbances were measured at 660 nm. Aspirin was taken as a positive control [26]. The experiment was carried out in triplicates and percent inhibition for protein denaturation was calculated using the following equations:

% Inhibition of denaturation = Absorbance control – Absorbance test / Absorbance control X 100.

2.6. Stability test

The effect of temperature and pH on the pigment was observed at different temperatures (20, 25, 30, 37, 45 ^{O}C) and pH (1, 3, 5, 7, 9, 11, 13) range. After every 24 hours, the OD of pigments was recorded using UV Spectrophotometer.

III. RESULTS AND DISCUSSION

3.1. Isolation of pigment-producing Bacteria

The Bacteria were isolated from Nigdi, Pradhikara, District Pune (lat: 18.65355- Long: 73.77247) by air sampling method. Twelve pigment-producing bacteria were isolated and pure-cultured on a nutrient agar plate (figure. 1). Five bacteria with high pigment-producing ability were further subjected to morphological and biochemical characterization (table.1). The increase in the incubation period (36 hours) causes the pigment to get darker.

Table 1: Morphological and biochemical characteristics (+ = Positive, - = Negative)

Isolates	ABT 1Y	ABT 20	ABT 3P	ABT 7P	ABT 9P
Gram- Stain	Gram +ve	Gram +ve	Gram +ve	Gram +ve	Gram +ve
Shape	Cocci	Cocci	cocci(diplococci)	Cocci	Cocci
Colour	Yellow	Orange	Pink	Light-Pink	Dark- Pink
KOH string test	-	-	-	-	-
Motility	-	-	-	-	-
Indol	-	-	-	-	-
MR	-	+	-	+	+
VP	-	-	-	-	-
Citrate	-	-	-	-	-
Glucose	+	-	+/acid no gas	-	-
Lactose	-	-	-	-	-
Mannitol	-	-	-	-	-
TSI	+	-	-	-	-
Amylase	-	-	-	+	-
Catalase	+	+	+	+	+
Oxidase	-	+	+	-	-

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Figure 1: Pure culture

3.2. Pigment extraction and partial purification

The pigment-producing bacteria streaked on Petri plates were scraped and collected in a conical flask. 50ml of methanol was added to the conical flask and placed in the rotary shaker. The pigments were subjected to successive solvent extraction using methanol and centrifuge to separate the cell debris. Methanol was evaporated at room temperature to get the crude sample (figure. 2).

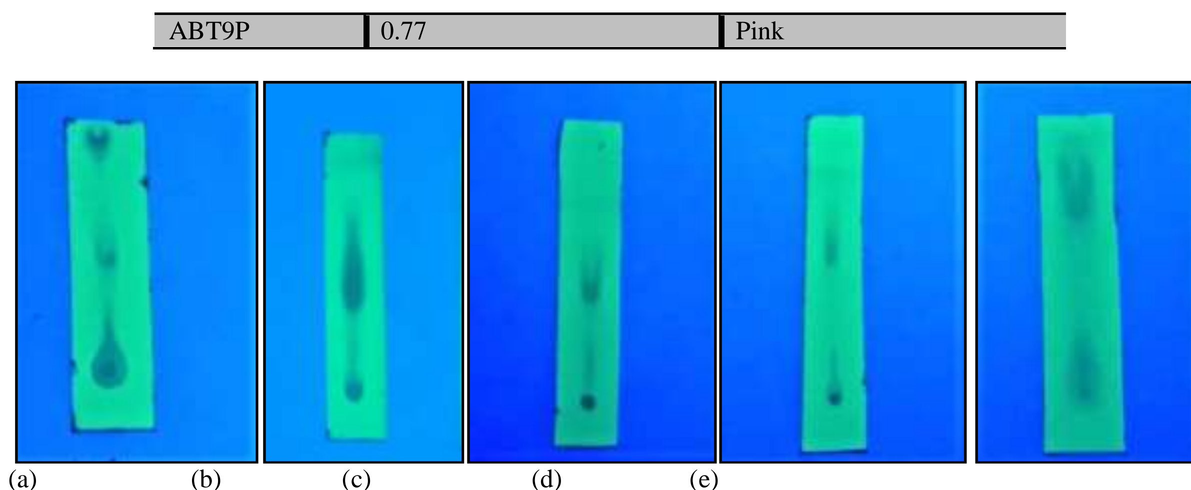
Figure 2: Methanolic extract of bacterial pigment

3.2.1. TLC: Crude methanolic extract of the pigment was spotted on a 0.20 mm precoated silica gel aluminium sheet (Merck) and developed with a mixture of chloroform and methanol in different concentrations (figure. 3). The R_f value of spots was observed (table. 2) on TLC and using the same ratio of mobile phase in column chromatography the pigments were purified. The purified fractions of pigments were given for FTIR analysis.

Table 2: (TLC) R_f value of bacterial pigments

Sample	R_f value	Band Colour
ABT1Y	0.54, 0.93	Yellow, Orange
ABT2O	0.55	Orange
ABT3P	0.44	Pink
ABT7P	0.62	Pink

ABT9P	0.77	Pink



(a) (b) (c) (d) (e)

Figure 3: TLC of crude bacterial extract:
(a) ABT1Y, (b) ABT2O, (c) ABT3P, (d) ABT7P, (e) ABT9P

3.2.2. FTIR analysis: According to the FTIR peak analysis (table. 3) and R_f of TLC the pigment can be a derivative of carotenoid. In the FTIR spectra many peaks were common in all pigments (2923-2925, 2853-2854, 1962-1994, 1650-1657, 1732-1735, 1459-1461, 1373-1375, 1214-1215, 1051-1052, 751-754, 655-667, 508-515) (figure. 4). Comparatively very few literatures is available on bacterial carotenoid pigment using FTIR spectroscopy. The complete structure of the pigment cannot be determined based on IR. A similar peak was observed in Kocuria sp. BRI 36 by Anuradha Mulik, Priyanka Kumbhar, and Rama Bhadekar in 2017 [10].

Table 3: (FTIR analysis) Wavenumber cm -1 of pigments and the bond represented by them.

Sample	Different region of peaks for FTIR analysis in wavenumber (cm-1)			
	Single Bond	**Triple Bond**	**Double Bond**	**Fingerprinting region**
ABT1Y	3405.84, 2924.48, 2854.52	2016.78	1973.60, 1733.65,	1461.02, 1373.79, 1214.59, 1052.26, 871.45, 752.21, 667.06, 521.00, 456.49
ABT2O	2925.26, 2854.24	2053.58	1982.04, 1732.68, 1652.70	1459.62, 1214.47, 1049.73, 751.03, 666.92, 512.67
ABT3P	3364.33, 2922.62, 2853.29	2190.18, 2128.47	1962.34, 1735.73, 1650.43	1459.68, 1375.42, 1214.91, 1051.91, 839.59, 755.87, 720.64, 664.12, 515.30, 469.30, 440.01
ABT7P	2924.03, 2854.74	2177.25	1994.53, 1733.18	1460.42, 1375.94, 1214.61, 1052.37, 752.07, 666.88, 523.71
ABT9P	3359.79, 2923.52, 2853.99	2132.68	1734.07, 1657.81	1459.83, 1374.89, 1215.15, 1051.47, 853.79, 754.10, 665.86, 598.65, 508.80, 477.05

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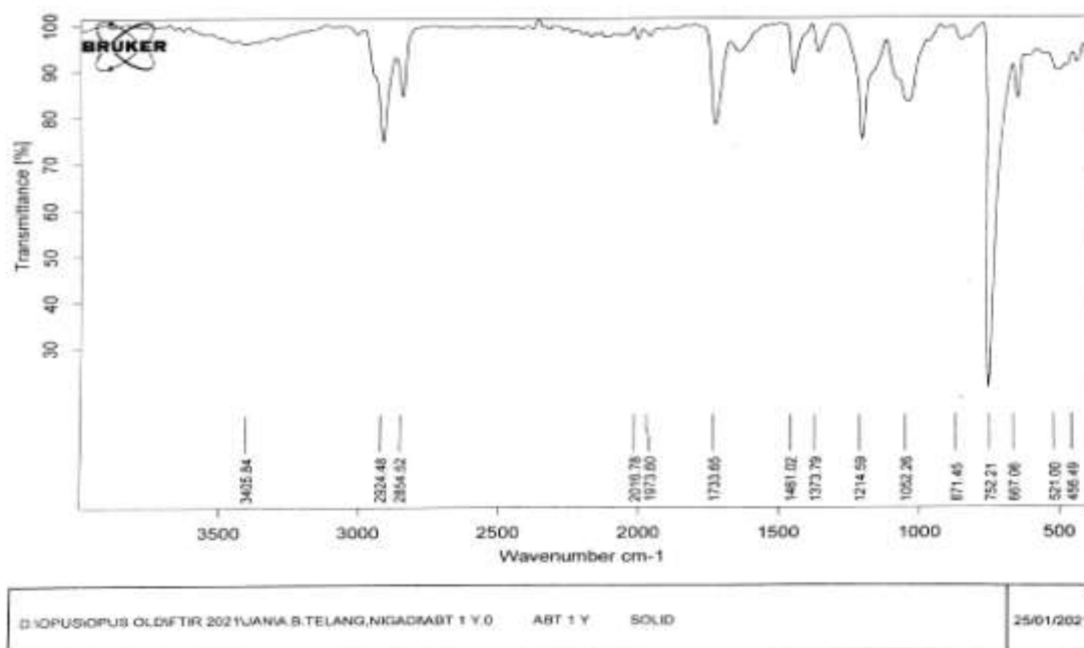

Figure 4a: FTIR analysis of pigment from ABT1Y

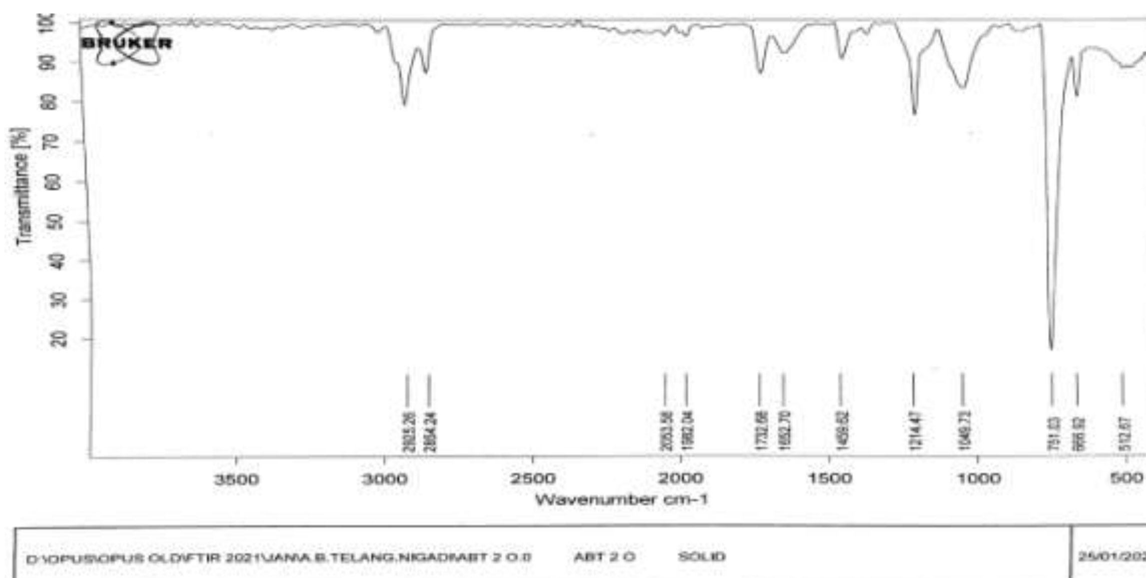

Figure 4b: FTIR analysis of pigment from ABT2O

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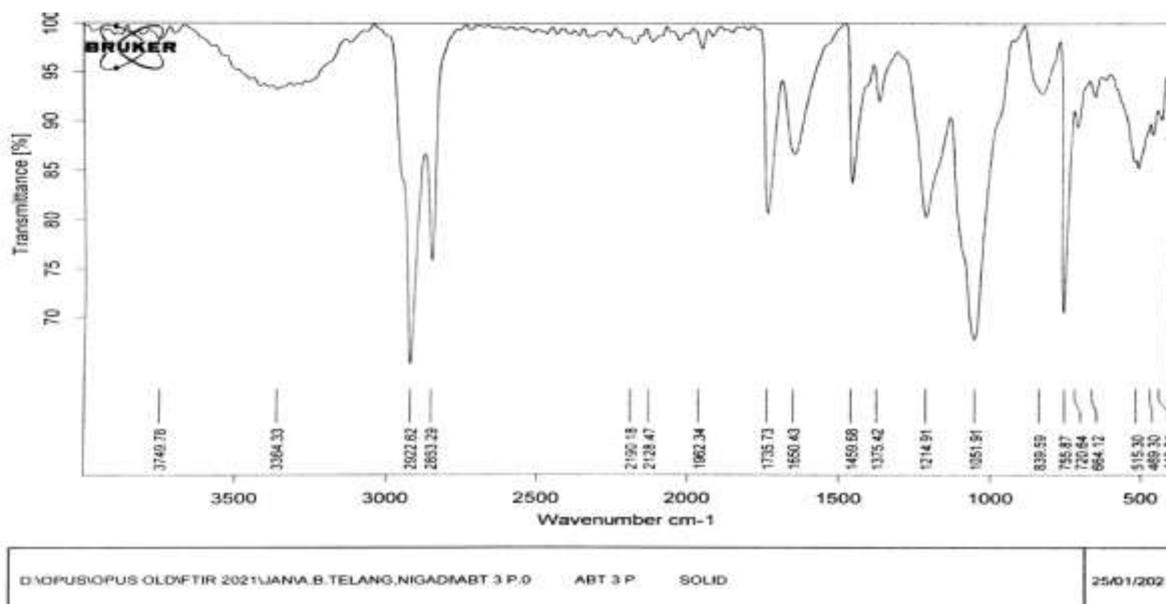

Figure 4c: FTIR analysis of pigment from ABT3P

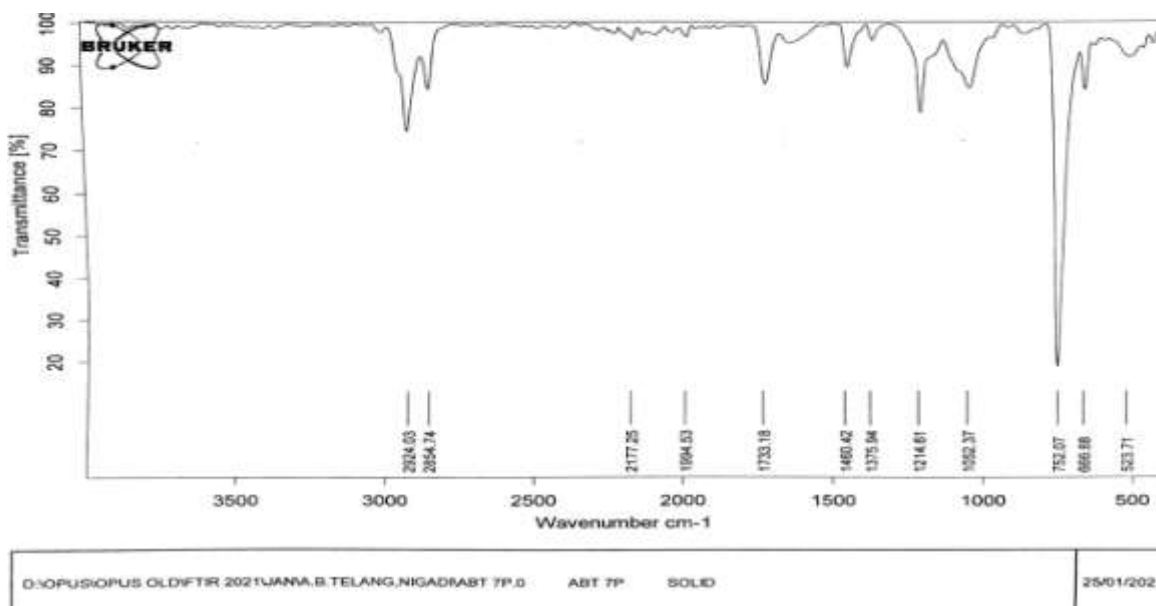

Figure 4d: FTIR analysis of pigment from ABT7P

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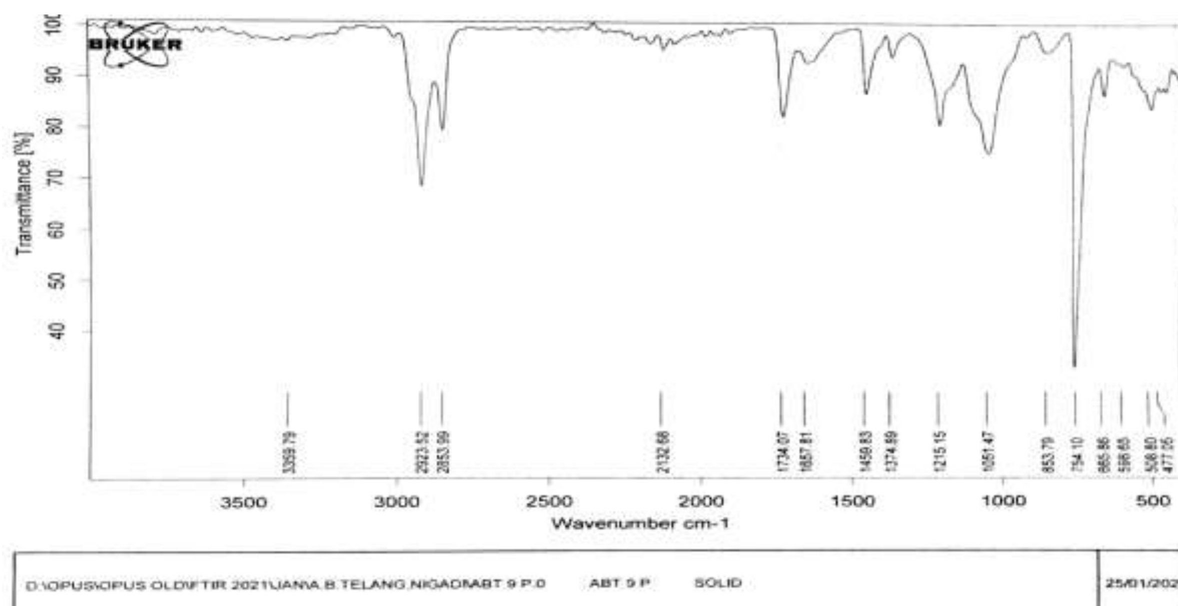

Figure 4e: FTIR analysis of pigment from ABT9P

3.3. Antidiabetic activity by using in vitro assays

3.3.1. α-amylase inhibitory assay: In vitro antidiabetic studies show that in the case of bacterial pigment ABT7P methanol extract showed comparable antidiabetic activity with 69 % of α-amylase inhibition at a concentration of 100µg/ml (table. 4) and it was less on comparison with standard i.e. 85% at a concentration of 100µg/ml.

Table 4: α-amylase inhibitory assay

Sample	% of inhibition at different Concentration	
	50µg/ml	100µg/ml
ABT1Y	32	66
ABT2O	11	25
ABT3P	21	57
ABT7P	37	69
ABT9P	13	30
Acarbose	46	85

3.4. H_2O_2 scavenging activity

Bacterial pigment when compared with ascorbic acid, the extract was less effective for scavenging H_2O_2. The ascorbic acid was able to scavenge the H_2O_2 in the concentration range 50 to 200µg/ml, whereas the concentration range of bacterial extracts was 100 to 500µg/ml. In the case of bacterial pigment, ABT3P showed the highest scavenging activity with 66 % percentage of H_2O_2 inhibition at a concentration of 200µg/ml and it was less in comparison with ascorbic acid i.e. 79% at a concentration of 200µg/ml (figure. 5).

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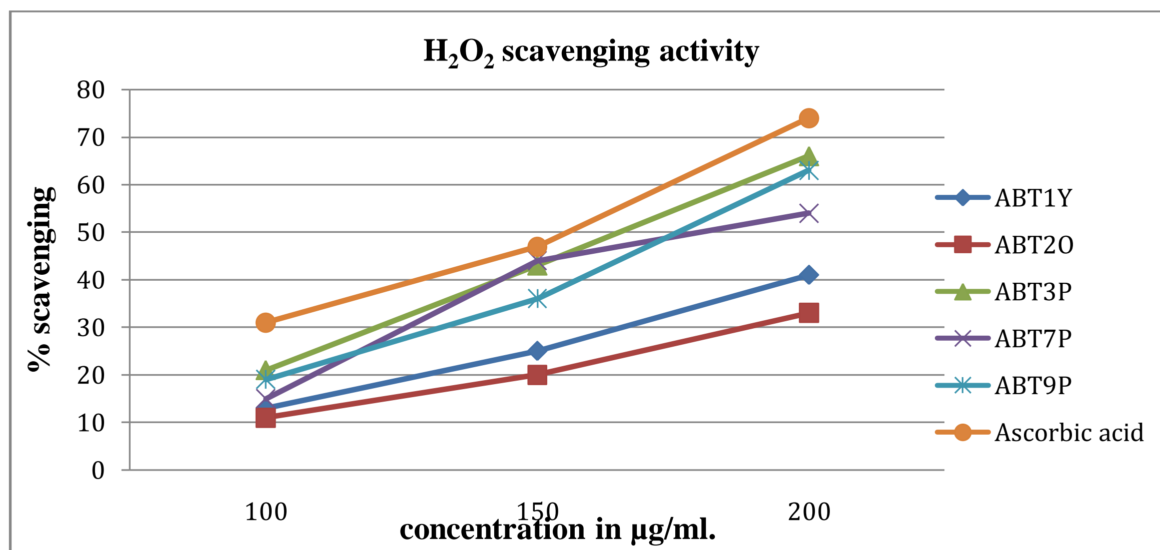

Figure 5: H_2O_2 scavenging activity

3.5. Inhibition of protein denaturation assay

The denaturation of proteins is a well-known cause of inflammation. The pigment extracts were tested against Aspirin for the inhibition of egg albumin. Maximum inhibition of 59 % was observed by ABTY1 at the concentration of 200µg/ml. Aspirin, a standard anti-inflammation drug showed the maximum inhibition of 71% at the concentration of 200µg/ml (figure. 6).

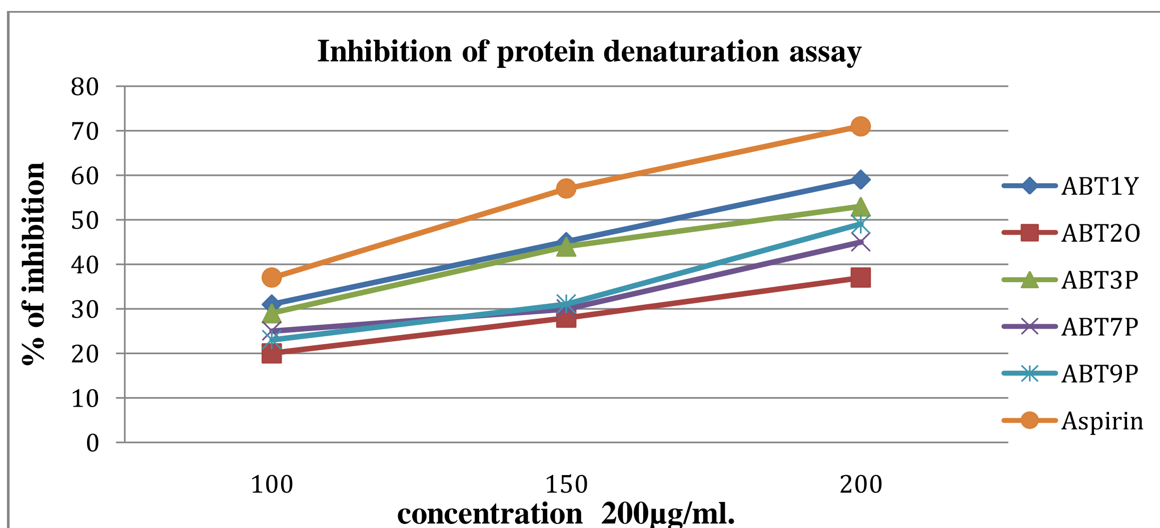

Figure 6: Inhibition of protein denaturation assay

3.6. Stability test

The pigments were unstable at pH 1, 2, 3 and denatured within 24hours at room temperature except for ABT1Y. The pigment ABT1Y was slowly degrading at pH 1 and the color fainted. The pigments were more stable at pH 7 to 8.5 at room temperature with a low denaturation rate. Temperature above 55°C affected all the pigments, whereas temperature below 30 ^{O}C was more suitable to preserve the pigments for more than a month. The temperature below 0 ^{O}C does not show any change in OD of the pigments. Later the pigments were dried to check the stability without any solvent or a buffer, the rate of denaturation was very low.

IV. CONCLUSION

The isolated pigments can be a derivative of Carotenoid. The pigments showed positive

results for antioxidant, anti-inflammatory and anti-diabetic activity hence can be utilized for therapeutic purpose. The best combination to preserve the pigments is the low temperature at pH 7 in dried form. Further studies need to be conducted for its therapeutic use.

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