

## **Antioxidative Metabolites Synthesized by Marine Pigmented *Vibrio* sp. and its Protection on Oxidative Deterioration of Membrane Lipids**

Ravindra Pawar<sup>1</sup> • C. Mohandass<sup>1</sup> • Syed G. Dastager<sup>2</sup> •  
Yogesh M. Kolekar<sup>3</sup> • Rahul Malwankar<sup>2</sup>

1 Biological Oceanography Division, CSIR-National Institute of Oceanography,  
Dona Paula, Goa, India

2 NCIM - Resource Centre, CSIR-National Chemical Laboratory, Pune, Maharashtra, India

3 Division of Biochemical Sciences, CSIR-National Chemical Laboratory, Pune, Maharashtra, India

**Correspondence:** 403004, India; Phone: +91(0)-832-2450414;  
Fax: +91(0)-832-2450606; E-mail: cmohan@nio.org

### **Abstract**

Bacterial strain *Vibrio* sp. (PIGB184) isolated from water samples of the Arabian Sea and identified through 16SrRNA, demonstrated the production of pigmentary antioxidants with higher ABTS activities  $90.9 \pm 0.42$  % in comparison with the standard commercial pigmented antioxidant, quercetin  $88.8 \pm 1.4$ %. Antioxidative metabolites of this strain substantially inhibit the lipid peroxidation (LPO) reactions tested in sheep liver and brain. The antioxidant compounds produced by the *Vibrio* sp. (PIGB184), analysed by GC-MS, reveals that it is composed mostly of Phenol, 2,4-bis(1,1-dimethylethyl) and Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl). The interrelationship assessed between LPO, and the phenolic compounds showed significant correlation with anti LPO properties ( $R^2 = 0.9698$  to  $0.9861$ ). These compounds are responsible for obstruction of harmful radical associated biochemical reactions in biological systems. Pigmented metabolites also tested for attributive biological properties against pathogenic bacteria showed prominent inhibition towards Gram +ve organisms ( $31.25$  to  $62.5 \mu\text{g ml}^{-1}$ ). From this study, it may be suggested that the marine bacterium PIGB184 could be used as a potential bio-resource for antioxidants and needs to be worked out for mass production.

**Keywords** Marine pigmented bacteria · Antioxidants · Lipid peroxidation · Phenolics · Pathogen inhibition

## **Introduction**

Environmental stress and exposure to anthropogenic substances generate a diverse range of reactive free radicals (FR) in living systems [1]. Overproduction of such reactive radicals initiates deleterious chain reactions inside the cell and causes adverse effects on health [2]. Even though humans and animals have their own antioxidant (AO) defense system, it is necessary to supplement essential antioxidants through either external diet or medicines to counteract overproduced free radicals and associated stress conditions [1, 3]. This awareness has increased the demand of antioxidant rich natural products in human health, food and personal care at an exponential rate. Therefore exploring wealthy resources of natural antioxidants has become the major task of pharmaceutical science.

Natural phenolic compounds are well recognized for their antioxidant properties. These compounds act as reducing agents, radical scavengers and metal chelators, which helps them to take part in molecular processes like obstruction of radical chain reactions and inhibition of lipid peroxidation [4, 5]. Conversely, the pigments of bacterial origin are also known to impart protective action against oxidative stress induced cell damages [6]. For example, Lavey et al. [7] and Slade et al. [8] examined the red pigments from *Deinococcus radiodurans* for its anti-lipid peroxidation activity and demonstrated that this property imparts increased resistance to the host organism under extreme stress conditions.

This evidence indicates that, although pigments and phenolic contents have been studied independently, the exclusive role of pigmentary phenolics, particularly from marine bacterial source has not yet been well understood in terms of their biological activities. Hence, in the present study, we try to understand whether the antioxidative metabolites, including phenolics of pigmented bacteria (PIGB 184: *Vibrio* sp.) do impart the biological functions attributed towards antioxidant activities like inhibitory actions on lipid peroxidation (LPO) and growth of opportunistic human pathogens.

## **Materials and Methods**

### **Chemicals**

All chemicals used for experimental work were of an extra pure quality purchased from Sigma-Aldrich Chemical Pvt. Ltd., Bangalore. Standard antibiotics were purchased from Hi-Media Laboratories, Mumbai. For molecular analysis, the gel extraction and purification kits were purchased from QIAGEN India Pvt. Ltd., New Delhi.

### **Sampling site, sample collection and isolation of bacteria**

Seawater and Sediment samples were collected bimonthly (2010-2011; 2012-2013) from coastal locations of the states of Maharashtra (Kunkeshwar and Malvan) and Goa (Vagator and Cabo De Rama) along the central west coast of India. The water samples were collected in sterile plastic bottles, directly, while the sediment samples were collected by Van Veen grab. All the collected samples were serially diluted, spread plated in duplicates on Zobell Marine agar (ZMA) and 50% seawater nutrient agar (SWNA) and incubated at  $28 \pm 2$  °C for 48-72 h. Distinctly formed pigmented colonies were selected, and purified cultures were stored in glycerol vials at -80 °C until further analysis.

### **Antioxidant screening, strain selection, mass culturing and sample extraction**

Each pure strain of pigmented bacteria (PIGB) primarily scrutinized by DPPH-antioxidant decolourization method [9, 10]. The AO zones were noted for each analyzed culture and screening was continued until we got the high potent strain. Based on the primary screening, an aliquot (100 µl) culture of selected strain PIGB 184 was inoculated in a 2 L Erlenmeyer flask containing sterile marine broth (800 ml) and incubated on shaker incubator at 28 °C (120 rpm) for 48 h. Further, the supernatant separation and extraction (ethyl acetate) were carried out as per our earlier described method [10]. Finally, the known concentrations of samples viz. 100, 500 and 1000 µg ml<sup>-1</sup> were prepared in HPLC grade methanol and stored in -20 °C until further analysis.

### **Quantitative antioxidant analysis**

#### *Determination of total antioxidant power*

The ABTS decolourization assay [11] was employed to evaluate the total antioxidant capacity of sample extracts. The decrease in absorbance was measured at 734 nm, and the antioxidant capacity was expressed in percent inhibition. Quercetin used as a standard antioxidant and IC<sub>50</sub> value was calculated from the regression analysis.

#### *DPPH radical scavenging activity*

The DPPH free radical scavenging property of the pigmentary extract was assessed according to the method of Larrauri et al. [12]. The inhibition of DPPH free radicals by the sample extracts calculated according to the equation,

$$\text{DPPH (\%)} = [1 - (\text{Absorbance of the sample} / \text{Absorbance of the control})] \times 100.$$

### *Measurement of reducing activities*

(i) Reducing potential (RP) and (ii) Ferric reducing antioxidant powers (FRAP) of PIGB184 were determined as per the standard method of Ferreira [13] and modified ELISA plate reader method of Benzie and Strain's [14] respectively. The reducing AO activities were expressed in terms  $\mu\text{g ml}^{-1}$  of ascorbic acid equivalents (AsA Eqs).

### *Estimation of total phenolic contents*

Folin-ciocalteu's colorimetric method described by Shan et al. [15] was employed to quantify the total phenolic contents in the pigmentary extract. The absorbance of the resulting blue colour was measured at 760 nm and TPCs were quantified with reference to standard gallic acid curve.

### **Assessment of lipid peroxidation inhibition**

PIGB 184 metabolites were evaluated for their protective role against peroxidation of lipids in sheep liver (SL) and brain (SB) as per the standard protocol [16]. Briefly, fresh SL and SB samples collected from the consumer market of Panaji (Goa, India) were cleanly washed and 20% tissue homogenate was prepared in ice-cold potassium chloride (1.15% KCl). Homogenate was filtered with cheese cloth, centrifuged at  $10,000 \times g$  for 10 min at 4 °C and the post mitochondrial fractions (PMF) were obtained as per the standard procedure. The lipoprotein concentrations in SL and SB samples were determined by Lowry's [17] methods. LPO activities were estimated as per our earlier protocol [16] and the results were presented as relative % activity in comparison with the known commercial antioxidant ascorbic acid (AsA:  $100 \mu\text{g ml}^{-1}$ ).

### **Antibacterial activity of antioxidative pigments**

The antibacterial potential of antioxidative pigmentary extract (PIGB184) was evaluated by well diffusion assay on Mueller-Hinton agar media. Activities were tested against each of 5 gram negative and gram positive human pathogens. Agar plates were spread plated with individual pathogens on separate plates and wells were punctured with sterile borer. Each well was saturated with 1 mg of sample extract while control was maintained with solvent. The plates were then kept for diffusion in the refrigerator (10 °C, 4-6 h) and later incubated at 37 °C for 24-48 h. The inhibitory zones were noted down in comparison with standard second and third generation antibiotics. The minimum inhibitory concentration (MIC) and  $\text{IC}_{50}$  values were determined by 96 well microtiter plate assay method [18].

### **Purification and GC-MS analysis of antioxidative metabolites**

The crude pigmentary extract (227.3 mg obtained from 3.2 L supernatant) was subjected to Sephadex-LH20 size exclusion chromatography and eluted using HPLC methanol. Each of the total 114 (0.5 ml each) fractions obtained from an aliquot of 2  $\mu$ l was tested for antioxidant activity by DPPH spot TLC method. Related fractions were pulled together into 7 main fractions (F1-F7) and checked for AO (%DPPH) activity. Active components of potent fraction F3 were further visualized using TLC (Mobile phase- PE 10:1 MeOH) plates in duplicates. Both TLC plates were visualized under UV detector, and one of them was sprayed with DPPH solution (0.01 mM) to visualize AO compounds. Based on  $R_f$  values the corresponding AO compounds were retrieved in HPLC methanol from another TLC plate for further analysis.

Purified AO fraction (F3) was passed through gas chromatography mass spectrometry (GC-MS: Agilent 7890A). GC-MS spectra were obtained with instrument specifications like column HP-5 capillary column (30 m  $\times$  0.32 mm  $\times$  0.25 mm, J and W Scientific), carrier gas Nitrogen (flow rate 1 ml/min), sample injection port temperature 250  $^{\circ}$ C, split ratio 8:1, oven temperature 40 to 280  $^{\circ}$ C (raise rate: 5  $^{\circ}$ C/min up to 150 degrees and then 10  $^{\circ}$ C/min) with final hold at 280  $^{\circ}$ C for 3 min. Compounds were identified by comparing retention time and mass fragmentation pattern of the standards of major constituents and also by comparing the acquired mass spectra and retention indices with NIST/NBS and the Wiley mass spectral library (software version 2.0).

### **16S rRNA gene sequencing and phylogenetic identification**

The genomic DNA from potent isolate was extracted by using DNeasy Blood and Tissue kit (Qiagen) according to manufacturer's instructions. The extracted DNA was subjected to polymerase chain reaction (PCR) with universal primers 27F and 1492R [19]. DNA sequencing was performed with an automatic sequencer (Applied Biosystems 3130xl Genetic Analyzer) with the bacterial primers 27F, 518F and 1492R. The resulted 16S rRNA gene sequence data was analyzed for bacterial identity, submitted to the GenBank and the accession number was obtained (KJ396268).

### **Statistical analysis**

All the samples were analyzed in triplicate for statistical analysis, and results are expressed as mean  $\pm$  standard deviation (SD). The changes in biochemical (antioxidant) parameters were correlated with total phenolic contents, and the correlation coefficient ( $R^2$ ) values were noted down.

## Results and Discussion

The recent advances in development and application of novel bio-actives from marine organisms are the emerging areas of modern science. Targeting oceanic bacteria for antioxidant search is expected to reveal novel and cost effective candidates for sustainable health applications. Hence in this investigation, based on radical scavenging and the stress relieving property of the pigment [6, 10] we explored the pigmented bacterial flora of the Arabian Sea for their antioxidant potential.

### Antioxidant potential of pigmented bacteria

During primary screening by DPPH decolorization assay from 102 selected isolates, 46 of the pigmented bacteria were observed to secrete antioxidant metabolites. 9.80 % isolates expressed AO zones greater than 1 cm while 35.29 % were in the range of 0-1 cm. Strains isolated from seawater samples contributed more than those of sediments. PIGB 184 with 3.2 cm AO zone was selected as potent strain whereas isolates exhibiting < 3 cm zone were excluded from further quantitative analysis.

#### *Total antioxidant power*

The total antioxidant powers of the potent sample extracts (PIGB 184) analysed for quenching of ABTS<sup>+</sup> radical showed significant dose dependant activities (Fig. 1A). The pigments of PIGB 184 exhibited highest antiradical effects i.e.  $90.91 \pm 0.42$  % ( $EC_{50} 364.58 \pm 19.31 \mu\text{g ml}^{-1}$ ) at highest sample concentration ( $1000 \mu\text{g ml}^{-1}$ ) tested. The observed activities overwhelmed the antioxidant activities of the standard compound quercetin ( $88.75 \pm 1.38\%$ ). Determination of total antioxidant capacity is a prime requisite measure to analyse the efficacy of natural or synthetic AO compounds that are intimated for therapeutic applications. The ABTS radical is known to be reactive towards most of the natural antioxidants including phenolics, thiols, vitamin C and protein hydrolysates [20, 21].

The efficient ABTS radical scavenging by selected marine bacterium (Fig. 1A) and its significant correlation with phenolic contents ( $R^2= 0.9861$ ) indicates the possible existence of relative bioactives in pigmentary extracts. Considering total antioxidant activities, bacterial pigments were observed to exhibit better ABTS scavenging properties over the standard quercetin. The pigment quercetin (a plant-derived flavonoid) has used as dietary supplements in food items and beverages due to its significant antioxidant and anti-inflammatory properties [22]. Our results indicate that the marine bacterial pigments possessing excellent anti-radical powers could be used as antioxidant rich substitutes for similar health applications.

### *Radical scavenging ability*

The ability of sample extracts to scavenge DPPH free radicals was assessed in comparison with the known synthetic antioxidant BHT (Fig. 1A). PIGB 184 showed elevating AO activities i.e.  $20.45 \pm 1.32 < 34.45 \pm 1.06 < 54.04 \pm 0.51$  % DPPH scavenging with increasing sample concentrations from  $100 < 500 < 1000 \mu\text{g ml}^{-1}$  respectively. In the case of DPPH scavenging, the observed  $\text{EC}_{50}$  value was high ( $909.54 \pm 10.45 \mu\text{g ml}^{-1}$ ) as compared to that of ABTS total antioxidant power ( $53.56 \pm 0.83 \mu\text{g ml}^{-1}$ ). A recent study by Horta et al. [18] evidenced that 84.62 % of seaweed associated bacteria possess DPPH  $\text{IC}_{50}$  values  $>1000 \mu\text{g ml}^{-1}$ . In the present investigation, our strain PIGB 184 also showed  $\text{EC}_{50}$  more or less close to this value i.e.  $909.54 \pm 10.45 \mu\text{g ml}^{-1}$ . Wang et al. [23] proved that the compounds showing high ABTS activity may not exhibit similar DPPH activity and vice versa. Similarly, the unambiguous variation in ABTS and DPPH scavenging effects (Fig. 1A) were also observed in our study. More interestingly, DPPH effective concentrations ( $\text{EC}_{50}$ ) of bioactive metabolites from a bacterial origin in the present study overwhelmed the similar radical scavenging properties those obtained from higher marine organisms as well [24].

### *Reducing capability of pigmented metabolites*

The ferric reducing abilities and reducing powers of pigmentary extracts were assessed in terms of their  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$  transition abilities. The extract of PIGB 184 has significant FRAP values in the range of 0.39 to  $22.14 \mu\text{g ml}^{-1}$  AsA Eqs with  $100\text{-}500 \mu\text{g ml}^{-1}$  of sample concentration (Fig. 1A). Similarly, the reducing power (RP) tests used to measure the reducing potential of the antioxidants also gave a significant value of  $3.92 \pm 0.37$  for  $100 \mu\text{g ml}^{-1}$  sample concentration. Further, these activities were linearly increased with the antioxidant concentration  $13.53 \pm 0.89$  for  $500 \mu\text{g ml}^{-1}$  and  $26.54 \pm 1.04$  for  $1000 \mu\text{g ml}^{-1}$ . Reducing activity of any compound is a positive indication of the production of reductone or reducing compounds [16]. Elevating levels of FRAP and RP values noticed in the present study in reference to the standard ascorbic acid calibration curve indicate that our strain PIGB184 produces reductone type of compounds in a noticeable manner. Phenolic compounds are known to act as significant reducing agents, and the prominent levels of TPCs produced by our strain contributed the observed FRAP and RP values.

### **Protective role against oxidation of membrane lipids**

Protective role of PIGB 184 against the oxidation of membrane lipids tested in sheep liver and brain samples are presented in Fig. 2. Metabolites of PIGB 184 gave higher activities of anti LPO in the liver ( $12.25 \pm 3.20$  to  $81.98 \pm 0.36$ ) than in the brain ( $31.44 \pm 4.79$  to  $63.03 \pm 2.54$ ). In both cases,

the activities were observed in dose dependent manner, and it was found that higher concentrations gave more activities that linearly decreased with lower concentrations. Correa-Llanten and co-workers [6] have recently demonstrated that bacterial pigments do protect membrane lipids and help in maintaining cell integrity under stress conditions. Similarly, the bacterial extract of the red pigmented bacterium *Dienococcus radiophilus* has been shown to attenuate the LPO [7, 8] reactions. However, none of these studies could point out the possible reason behind their observed anti-LPO effect, and hence we report the protective roles of pigmentary metabolites including phenolic AO compounds against oxidative deterioration of membrane lipids in biological systems.

### **Pigmented phenolics**

The phenolic contents of the *Vibrio* were estimated in terms of  $\mu\text{g ml}^{-1}$  gallic acid equivalents (GAEs). They were in the order of  $4.27 \pm 0.05$  in  $100 \mu\text{g ml}^{-1}$  of sample extract and these contents increased with the increase in a volume of the extracts i.e.  $36.67 \pm 0.49$  for  $500 \mu\text{g ml}^{-1}$  and  $> 58.78 \pm 0.22$  for  $1000 \mu\text{g ml}^{-1}$ . Correlation analysis between total phenolic content and antioxidant parameter, presented in Fig. 3, gave significant linear curves and  $R^2$  values against ABTS (0.9861), DPPH (0.973), RP (0.9768) and FRAP (0.9698). Similarly, the anti-LOP activities also had the significant correlation with phenolics (SL:  $R^2= 0.998$ , SB:  $R^2= 0.9902$ ).

Significant correlations among the estimated amount of TPC's and AO parameters highlighted the assistive roles of phenolics in obstructing the activities of free radical associated LPO inhibitions (Fig. 3). As discussed earlier, the phenolic compounds are well documented to scavenge reactive species and take part in inhibition of lipid oxidation [4, 25]. Koren et al. [26] demonstrated that bacteria, when coated with polyphenols, acquire potent oxidant scavenging powers and help the mammalian cells against oxidative cell damage. Considerable production of phenolic contents by our strain PIGB 184 (Fig. 1B) also plays a significant role in scavenging free radicals more in the liver and than in the brain, emphasized the possible contribution of antioxidant activity in the lipid peroxidation process.

### **Inhibition of opportunistic human pathogens**

Pigmented extracts of PIGB 184 significantly inhibited the growth of 5 gram-positive and 2 gram-negative opportunistic human pathogens (Table 1). It exhibited maximum activities against *A. baumannii* ( $10 \pm 3$ ) and *P. aeruginosa* ( $8 \pm 2$ ) in terms of antibiotic zones at  $1000 \mu\text{g}$  sample concentration. However, the *S. aureus* ( $6 \pm 1$ ) and *B. megaterium* ( $5 \pm 1$ ) were found less prone to the inhibitory actions of PIGB 184 metabolites. Commercial antibiotic sensitivity discs of broad



spectrum Ampicillin and the third generation (CAZ- Ceftazidime, CPD- Cefpodoxime, CTR- Ceftriaxone) tested in comparison with PIGB 184 crude extract gave ~ 1.5 to 2.5 fold increase against the human pathogen *P. aeruginosa* (Table 1). Minimum inhibition concentrations tested with the pigmented extract of our species exhibited that gram-positive bacteria were more effectively inhibited (MIC: 31.25 – 62.5  $\mu\text{g ml}^{-1}$ ) than the gram-negative bacteria (MIC:  $\geq 1000 \mu\text{g ml}^{-1}$ ).

Extracts of the marine bacterium *Alteromonas* sp. isolated from seaweed surfaces from Peniche (Portugal) coast have been demonstrated to exhibit antibacterial properties with  $\text{IC}_{50}$  values in the range of 43.48 - 50.85  $\mu\text{g ml}^{-1}$  [18]. Similarly, an Antarctic bacterium *Janthinobacterium* sp. has been recently reported to exhibit antibacterial effects (MIC: 0.5 – 16  $\mu\text{g ml}^{-1}$ ) against multi-resistant gram-negative human pathogens [27]. Contrastingly, our pigmented strain PIGB 184 (*Vibrio* sp.), from a coastal source though showed prominent antibacterial effects against both gram-positive and gram-negative bacteria, their efficiencies were comparable or slightly more than the previous studies. An interesting observation we made was that the available literature mainly reports the antibacterial resistance towards gram-negative organisms, but our results with pigmented compounds support more action against the gram-positive pathogens. Antibiotic sensitivity and resistance may depend upon the localities and the ecosystem they live in and the metabolites produced were also distinct in respect to bioactive properties. Here in this study, it may be seen that the marine pigmentary bacterium PIGB 184 produces more of phenolic constituents that help to control the growth of more clinical pathogens.

Isnansetyo and Kamei [28] demonstrated that phenolics produced by the novel species *Pseudoalteromonas phenolica* possess bactericidal activity against *Staphylococcus aureus*. In a similar approach phenolic derivatives of *Vibrio* sp. [18] have been evidenced to exhibit antibacterial properties against *B. subtilis* ( $\text{IC}_{50}$ : 58.86  $\mu\text{g ml}^{-1}$ ) and *S. aureus* ( $\text{IC}_{50}$ :  $>1000 \mu\text{g ml}^{-1}$ ). In contrast, PIGB 184 pigmented metabolites in the present study had better inhibitions against *Bacillus* ( $\text{EC}_{50}$ : 9.36-23.69  $\mu\text{g ml}^{-1}$ ) and *Staphylococcus* pathogen ( $\text{EC}_{50}$ : 26.82  $\mu\text{g ml}^{-1}$ ). Hence, antagonistic compounds produced by our *Vibrio* sp. PIGB184 also underline the potential of bacterial phenolics towards the development of novel antibiotic agents.

### **Characterization of antioxidative metabolites and potent strain**

Purification of PIGB 184 metabolites separated by Sephadex LH20 size exclusion chromatography gave 7 major fractions (F1-F7), and AO activities tested in each fraction are shown in Table 2. DPPH guided TLC autography demonstrated the fraction F3 to have active components. GC-MS analysis of

F3 fraction showed 3 compounds: (1) Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro- [C<sub>7</sub>H<sub>10</sub>N<sub>2</sub>O<sub>2</sub>; MW: 154]; (2) Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl) [C<sub>14</sub>H<sub>22</sub>O; MW: 210] and (3) Phenol, 2,4-bis(1,1-dimethylethyl) [C<sub>11</sub>H<sub>18</sub>N<sub>2</sub>O<sub>2</sub>; MW: 206] (Fig. 4).

Similar compounds from bacterial sources have reported by earlier workers for antioxidant and antimicrobial properties. The compound Pyrrolo[1,2-a]pyrazine-1,4-dione derivatives from the marine bacterium *Bacillus subtilis* has been recently noted for its antioxidant activity [29]. Padmavathi and co-workers [30] have demonstrated that Phenol, 2,4-bis(1,1-dimethylethyl) compounds produced by *Vibrio alginolyticus* species acts as an anti-fouling agent. Synthesis of similar metabolites by our marine *Vibrio* strain PIGB 184 further strengthens our views on the possible contribution of pyrrolo and phenol derivatives towards observed antioxidant and antibacterial effects.

Information retrieved from NCBI PubChem Compound database ([CID=7311](#)) on phenol,2,4-Bis(1,1-dimethylethyl) and its polymer phenol,2,4-Bis(1,1-dimethylethyl)-,1,1,1-phosphite [31] indicated that these derivatives are employed as an intermediate oxidizing/reducing agents in the production of light-protecting and phenolic antioxidant compounds. Interestingly, PIGB184 also had a close similarity in its structural and functional properties to BHT i.e. 2,6-bis(1,1-dimethylethyl)-4-methylphenol. BHT is used as a synthetic antioxidant in food, cosmetics and medicines and is reported to have adverse effects on animal health [1]. Although we did not test our compound for similar applications, the synthesis of particular phenolic metabolites from our strain could form a natural alternative source of antioxidant compounds.

The molecular (16SrRNA) gene sequence of PIGB 184 was assessed for the closest match using BLASTn and obtained 99.9 % similarity with genera *Vibrio* (Gammaproteobacteria) submitted to the gene bank accession number KJ396268. Gammaproteobacteria group has been recently [10] reported to exhibit excellent antioxidant activities. Moreover, pigmented bacteria belonging to this group are also documented to exhibit biological activities such as antimicrobial and cytotoxic effects as well [18, 32]. It emphasizes that more inclusive bioactive studies on pigmented organisms affiliating Gammaproteobacteria could reveal novel metabolites of human use.

## Conclusions

Pigmented bacterium PIGB184 assessed for antioxidant properties demonstrated their potential towards obstruction of radical mediated chain reactions. The phenolics produced by this strain with significant inhibition of oxidative reactions in membrane proteins, and growth of some opportunistic

human pathogens, emphasizes their significance and the protective role in applying biological systems. Active synthesis of Phenol, 2,4-bis(1,1-dimethylethyl) derivatives by our strain with equally good or more properties could form an alternative to synthetic antioxidant BHT since both bear relatively similar structures and antioxidant activities. These need to be explored for their bioactive properties of human importance.

### **Conflicts of interest**

All authors declare that there are no conflicts of interest.

### **Acknowledgements**

Authors would like to thank the Director of NIO, Dr. N. Ramaiah and Dr. P. Vethamony for providing lab support. Mr. R. M. Meena is acknowledged for his help in sequence analysis. This work was supported by the projects PSC0206 and BSC0111 under CSIR Grant. The authors declare that there are no conflicts of interest. This is NIO contribution number ----.

### **References**

1. Halliwell, B., & Gutteridge J. M. (2007). Free radicals in biology and medicine. 4<sup>th</sup> edition, New York: Clarendon Press; Oxford, UK: Oxford University Press.
2. Devasagayam, T. P. A., Tilak, J. C., Bloor, K. K., Sane, K. S., Ghaskadbi, S. S., & Lele, R. D. (2004). Free radicals and antioxidants in human health: Current status and future prospects. *Journal of the Association of Physicians of India* 52, 794-804.
3. Dekkers, J. C., Van Doornen, L. J. P. H., & Kemper, C. G. (1996). The role of antioxidant vitamins and enzymes in the prevention of exercise-induced muscle damage. *Sports Medicine* 21, 213-238.
4. Kim, S. K., & Wijesekara, I. (2013). Marine-derived nutraceuticals: trends and prospects. In: Kim SK, editor. Marine nutraceuticals: prospects and perspectives. Boca Raton, FL: CRC Press, p 464.
5. Hamed, I., Ozogul, F., Ozogul, Y., & Regenstein, J. M. (2015). Marine bioactive compounds and their health benefits: A Review. *Comprehensive Reviews in Food Science and Food Safety* 14, 446-65.
6. Correa-Llanten, D. N., Amenabar, M. J., & Blamey, J. M. (2012). Antioxidant capacity of novel pigments from an Antarctic bacterium. *Journal of Microbiology* 50, 374-379.
7. Lavy, A., Neeman, Y., & Fuhrman, B. (2005). The antioxidative effect of the bacteria *Dienococcus radiophilus* against LDL lipid peroxidation. *European Journal of Nutrition* 44, 281-284.
8. Slade, D., & Radman, M. (2011). Oxidative stress resistance in *Deinococcus radiodurans*. *Microbiology and Molecular Biology Reviews* 75, 133-191.
9. Takao, T., Kitatani, F., Watanabe, N., Yagi, A., & Sakata, K. (1994). A simple screening method for antioxidants and isolation of several antioxidant produced by marine bacteria from fish and shellfish. *Bioscience, Biotechnology, and Biochemistry* 58, 1780-1783.
10. Pawar, R. T., Mohandass, C., Sivaperumal, E., Sabu, E., Rajasabapathy, R., & Jagtap, T. G. (2015). Epiphytic marine pigmented bacteria: A prospective source for natural antioxidants. *Brazilian Journal of Microbiology* 46, 29-39.

11. Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine* 26, 1231-1237.
12. Larrauri, J. A., Sanchez-Moreno, C., & Saura-Calixto, F. (1998). Effect of temperature on the free radical scavenging capacity of extracts from red and white grape pomace peels. *Journal of Agricultural Food Chemistry* 46, 2694-2697.
13. Ferreira, I. C. F. R., Baptista, P., Vilas-Boas, M., & Barros, L. (2007). Free-radical scavenging capacity and reducing power of wild edible mushrooms from northeast Portugal: individual cap and stipe activity. *Food Chemistry* 100, 1511-1516.
14. Benzie, I. F. F., & Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of 'antioxidant power': the FRAP assay. *Analytical Biochemistry* 239, 70-76.
15. Shan, B., Cai, Y. Z., Brooks, J. D., & Corke, H. (2007). The in vitro antibacterial activity of dietary spice and medicinal herb extracts. *International Journal of Food Microbiology* 117, 112-119.
16. Pawar, R., Nagvenkar, S., & Jagtap, T. (2013). Protective role of edible clam *Paphia malabarica* (Chemnitz) against lipid peroxidation and free radical. *Turkish Journal of Biochemistry* 38, 138-144.
17. Lowry, O. H., Rosbrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* 193, 265-275.
18. Horta, A., Pinteus, S., Alves, C., Fino, N., Silva, J., Fernandez, S., Rodrigues, A., & Pedrosa, R. (2014). Antioxidant and antimicrobial potential of the *Bifurcaria bifurcata* epiphytic bacteria. *Marine Drugs* 12, 1676-1689.
19. Lane, D. J. (1991). 16S/23S rRNA sequencing. In nucleic acid techniques in bacterial systematic, ed. by Stackebrandt, E. and Goodfellow, M. John Wiley and Sons Incorporation, New York, pp. 115-175
20. Walker, R. B., & Everette, J. D. Comparative reaction rates of various antioxidants with ABTS radical cation. *Journal of Agricultural and Food Chemistry* 57, 1156-1161.
21. Godinho, I., Pires, C., Pedro, S., Teixeira, B., Mendes, R., Nunes, M. L., & Batista, I. (2015). Antioxidant properties of fish protein hydrolysates prepared from cod protein hydrolysate by *Bacillus* sp. *Applied Biochemistry and Biotechnology*, 1-18. DOI 10.1007/s12010-015-1931-5.
22. Martin, K. R., & Appel, C. L. (2010). Polyphenols as dietary supplements: A double-edged sword. *Nutrition and Dietary Supplements* 2, 1-12.
23. Wang, M., Li, J., Rangarajan, M., Shao, Y., La Voie, E. J., Huang, T. C., & Ho, C. T. (1998). Antioxidative phenolic compounds from sage (*Salvia officinalis*). *Journal of Agricultural and Food Chemistry* 46, 4869-4873.
24. Hajji, S., Younes, I., Rinaudo, M., Jellouli, K., & Nasri, M. (2015). Characterization and in vitro evaluation of cytotoxicity, antimicrobial and antioxidant activities of chitosans extracted from three different marine sources. *Applied Biochemistry and Biotechnology* 177, 18-35.
25. Sun, J., Kan, F., Liu, P., He, S., Mou, H., Xue, C., & Mao, X. (2015). Screening of microorganisms from deep-sea mud for Antarctic krill (*Euphausia superba*) fermentation and evaluation of the bioactive compounds. *Applied Biochemistry and Biotechnology* 175, 1664-77.
26. Koren, E., Kohen, R., Ovadia, H., & Ginsburg, I. (2009). Bacteria coated by polyphenols acquire potent oxidant-scavenging capacities. *Experimental Biology and Medicine* 234, 940-951.
27. Asencio, G., Lavin, P., Alegria, K., Dominguez, M., Bello, H., Gonzalez-Rocha, G., Gonzalez-Aravena, M. (2014). Antibacterial activity of the Antarctic bacterium *Janthinobacterium* sp. SMN 33.6 against multi-resistant Gram-negative bacteria. *Electronic Journal of Biotechnology* 17, 1-5.

28. Isnansetyo, A., & Kamei, Y. (2003). *Pseudoalteromonas phenolica* sp. nov., a novel marine bacterium that produces phenolic anti-methicillin-resistant *Staphylococcus aureus* substances. *International Journal of Systematic and Evolutionary Microbiology* 53, 583–588.
29. Balakrishnan, D., Bibiana, A. S., Vijayakumar, A., Santhosh, R. S., Dhevendran, K., & Nithyanand, P. (2015) Antioxidant Activity of Bacteria Associated with the Marine Sponge *Tedania anhelans*. *Indian Journal of Microbiology* 55, 13-18.
30. Padmavathi, A. R., Abinaya, B., & Pandian, S. K. (2014). Phenol, 2,4-bis(1,1-dimethylethyl) of marine bacterial origin inhibits quorum sensing mediated biofilm formation in the uropathogen *Serratia marcescens*. *Biofouling* 30, 1111-1122.
31. Lee, S. (2010). Phenol, 2,4-Bis(1,1-Dimethylethyl)-,1,1',1''-Phosphite. e-EROS encyclopedia of reagents for organic synthesis. John Wiley & Sons, Ltd. DOI: 10.1002/047084289X.rn01170.
32. Kuo, Y. H., Liang, T. W., Liu, K. C., Hsu, Y. W., Hsu, H. C., & Wang, S. L. (2011). Isolation and identification of a novel antioxidant with antitumor activity from *Serratia ureilytica* using squid pen as fermentation substrate. *Marine Biotechnology* 13, 451-461.

**Table 1** Antibacterial activities of antioxidative pigmentary metabolites against opportunistic human pathogens

Sr. No.	Pathogenic strains (NCIM code)	Inhibition zones (mm)						Inhibitory concentrations ( $\mu\text{g ml}^{-1}$ )		
		PIGB 184	Broad spectrum antibiotics			Third generation antibiotics			PIGB184	
			TE	AMP	CL	CAZ	CPD	CTR	IC <sub>50</sub>	MIC
1.	<i>B. cereus</i> (2156)	7 ± 1	16 ± 2	2 ± 1	22 ± 2	3 ± 1	2 ± 1	10 ± 1	9.36	31.25
2.	<i>B. megaterium</i> (2087)	5 ± 1	30 ± 2	15 ± 4	32 ± 3	27 ± 2	11 ± 1	26 ± 2	23.69	62.5
3.	<i>M. luteus</i> (2169)	7 ± 1	18 ± 2	NA	13 ± 2	18 ± 2	6 ± 2	5 ± 2	18.56	31.25
4.	<i>S. aureus</i> (2079)	6 ± 1	26 ± 2	29 ± 2	25 ± 1	12 ± 2	8 ± 2	24 ± 2	26.82	62.5
5.	<i>S. epidermidis</i> (2493)	NA	30 ± 2	9 ± 2	28 ± 3	22 ± 1	10 ± 1	29 ± 1	NT	NT
6.	<i>A. baumannii</i> (5152)	10 ± 3	19 ± 2	NA	11 ± 1	23 ± 2	14 ± 1	29 ± 2	21.13	62.5
7.	<i>E. coli</i> (2065)	NA	16 ± 1	11 ± 1	21 ± 2	28 ± 2	14 ± 2	20 ± 1	NT	NT
8.	<i>K. pneumonia</i> (2706)	NA	16 ± 2	NA	16 ± 1	25 ± 1	12 ± 1	29 ± 2	NT	NT
9.	<i>P. aeruginosa</i> (2200)	8 ± 2	25 ± 3	6 ± 2	25 ± 3	4 ± 1	NA	8 ± 1	464.34	1000
10.	<i>S. typhimurium</i> (2501)	4 ± 1	18 ± 2	21 ± 2	27 ± 3	27 ± 2	17 ± 2	31 ± 2	273.35	1000

Values are the mean ± SD. Clinical pathogens: Sr. No. 1-5 Gram-positive and 6-10 Gram-negative bacteria. Standards: AMP- Ampicillin (25  $\mu\text{g}$ ), TE- Tetracycline (10  $\mu\text{g}$ ), CL- Chloramphenicol (25  $\mu\text{g}$ ), CTR- Ceftriaxone (30  $\mu\text{g}$ ), CPD- Cefpodoxime (10  $\mu\text{g}$ ), and CAZ- Ceftazidime (30  $\mu\text{g}$ ). NA: no activity. NT: not tested. Antibacterial investigations were carried out at National Collection of Industrial Microorganisms (NCIM), CSIR-National Chemical Laboratory, Pune.

**Table 2** Purification of PIGB 184 antioxidative metabolites by column chromatography

Experimental stage	Sample weight (mg)	Antioxidant activity	Yield (%)
Initial crude sample	227.3	54.04 ± 0.51	100.00
Fraction F1	3.2	1.83 ± 0.03	1.41
Fraction F2	10.5	6.52 ± 0.06	4.62
Fraction F3*	31.7	30.89 ± 1.88	13.9
Fraction F4	5.2	3.07 ± 0.07	2.19
Fraction F5	19.3	6.60 ± 0.05	8.5
Fraction F6	45.4	12.12 ± 0.14	19.8
Fraction F7	2.1	0.82 ± 0.00	0.92

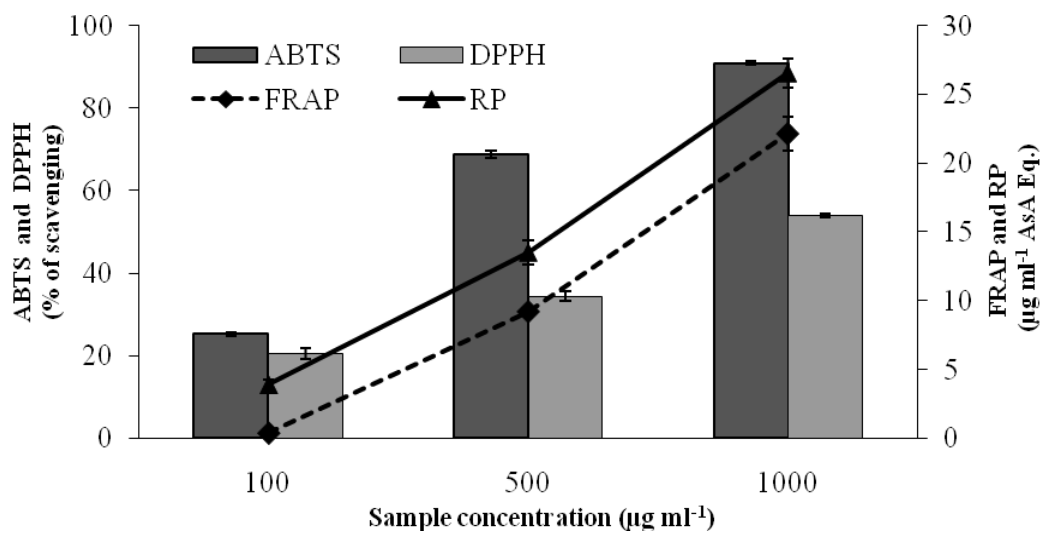
\*Represents potent fraction. Antioxidant activity was measured in terms of % DPPH scavenging

**Table 3** Molecular (16s rRNA) identity for antioxidant potent pigmented strain

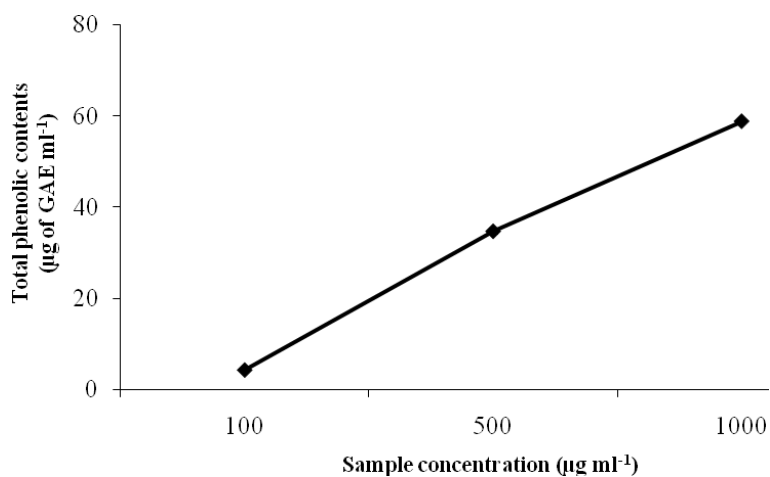
Isolate	Antioxidant zone (cm)*			Isolation source	Closest match	NCBI accession number; Similarity (%)	Phylogenetic group	Pigmentation
	a	b	b-a					
PIGB 184	0.5	3.7	3.2	Seawater	<i>Vibrio</i> sp.	KJ396268 (99.9%)	Gammaproteobacteria	Yellow

a: Colony size, b: Zone of DPPH decolourization, b-a: Final of zone of antioxidant.

(A)



(B)

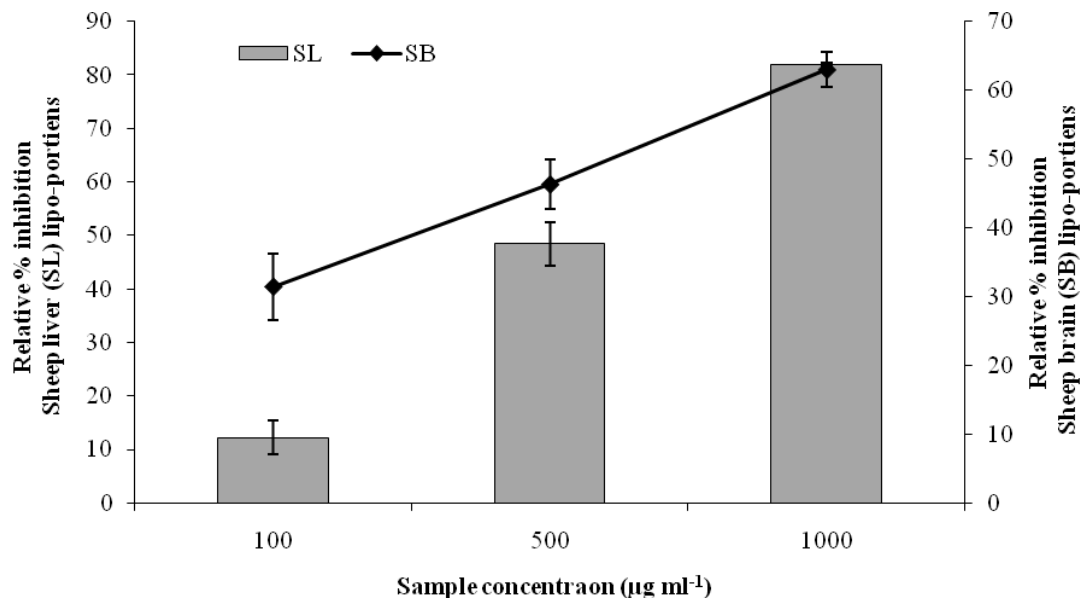


AsA Eq.: Ascorbic acid equivalents, GAE: Gallic acid equivalents.

\*

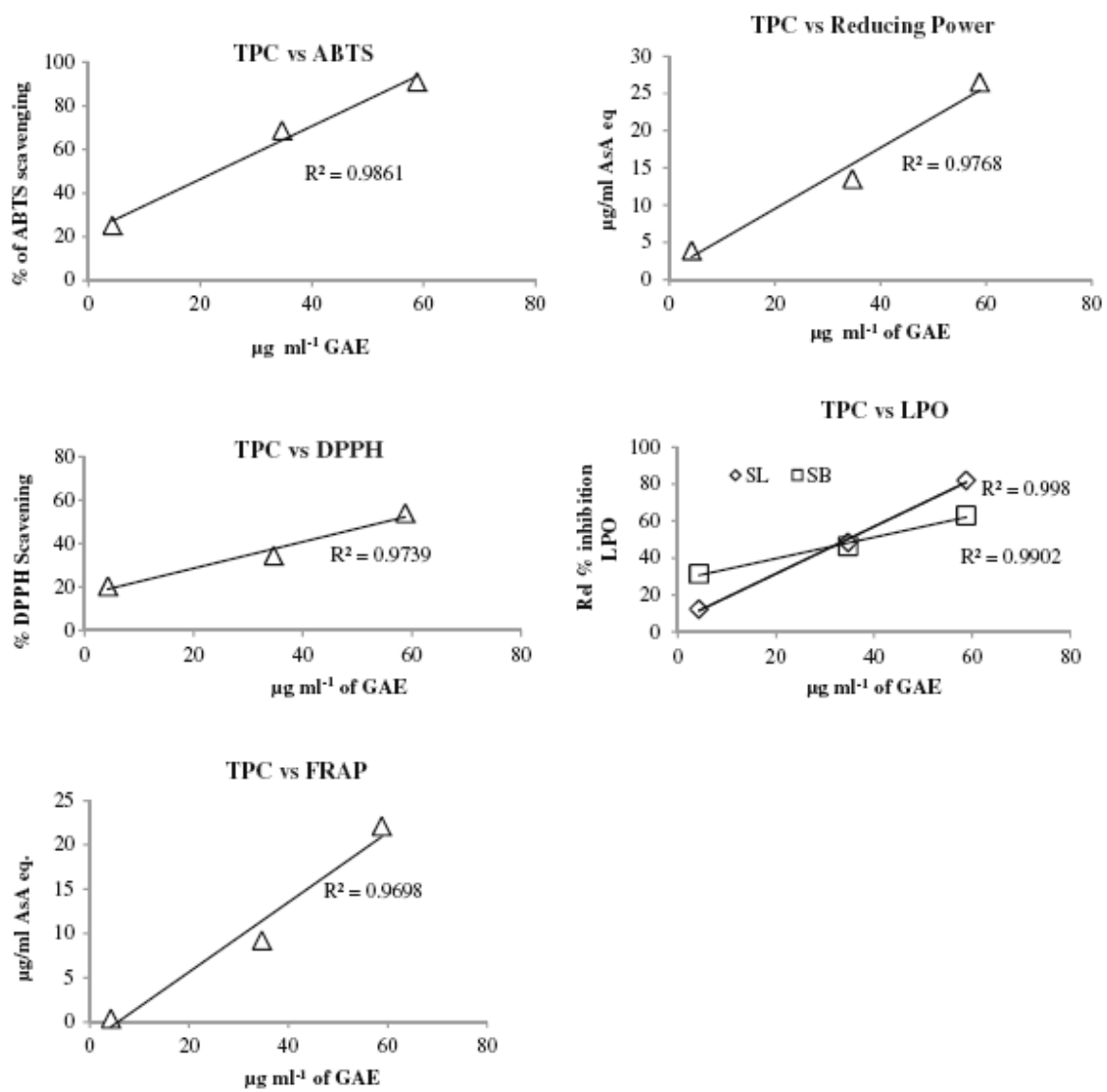
**Fig. 1** Antioxidant potential of marine pigmented bacterium PIGB 184 in terms of DPPH, ABTS, RP, FRAP (A) and TPC (B) activities. Values are mean  $\pm$  SD (n=3)



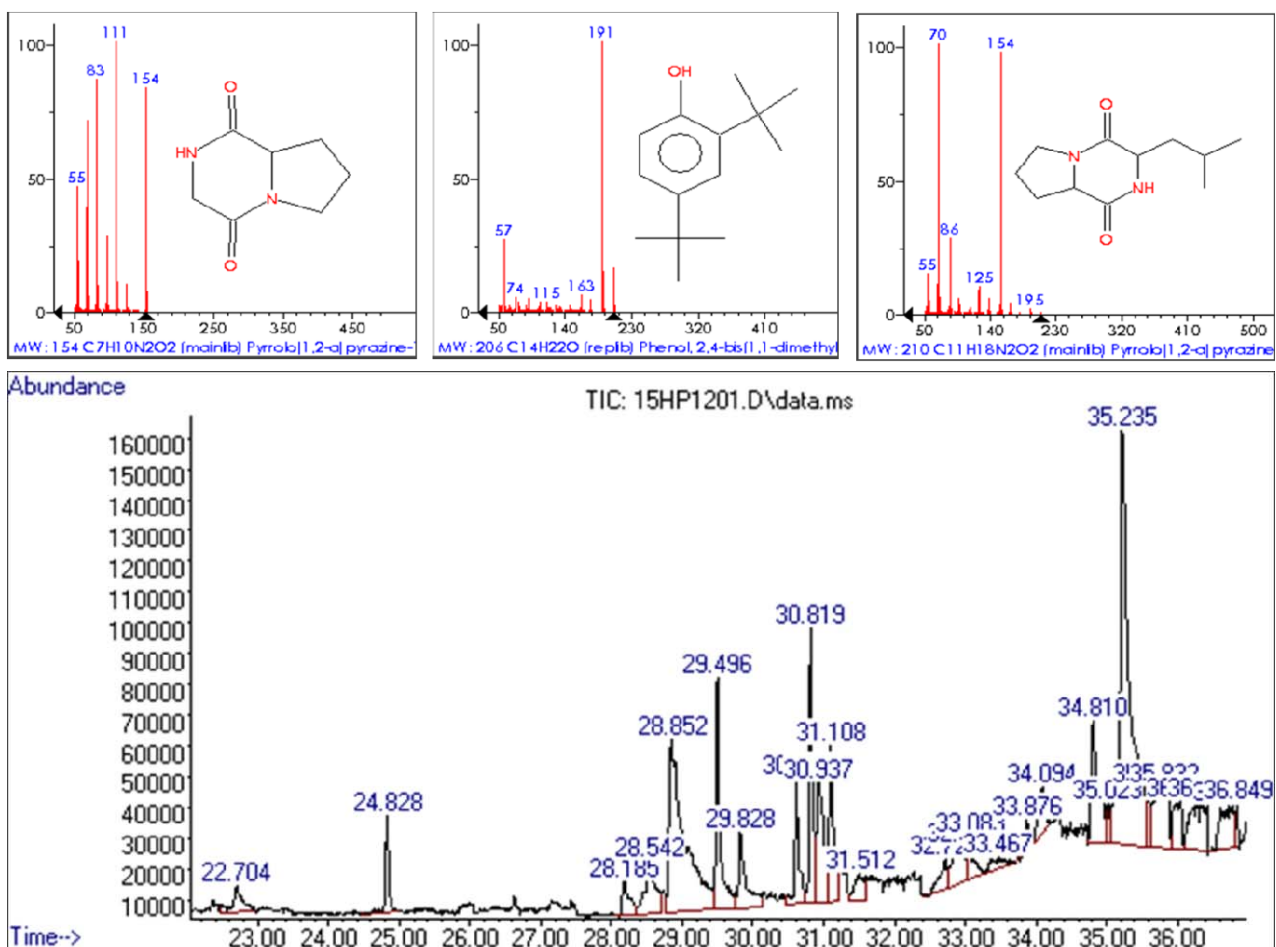


\*Relative % inhibition of LPO is presented in comparison with standard ascorbic acid (AsA:  $100 \mu\text{g ml}^{-1}$ ).

**Fig. 2 Anti-LPO activities of PIGB 184 against oxidation of sheep liver (SL) and brain (SB) lipoproteins**



**Fig. 3** Relation of antioxidant and lipid peroxidation activities with total phenolic contents



**Fig. 4 GC-MS spectrum and types of antioxidative metabolites synthesized by PIGB 184**  
 \*Presented components are from antioxidant potent fraction F3 (Please refer Table 2 for more information)