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Research Article

Effect of Polycyclic Aromatic Hydrocarbons Liberated From Thermal Power Units on the Metabolic and Oxidative Status of *Raphanus sativus*

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Keywords

- Polycyclic aromatic hydrocarbons (PAH's); *Raphanus sativus*; phenolics; Antioxidant; Reactive oxygen species (ROS)

Abstract

Threats of carcinogenic environmental pollutants on vegetables and other crops can never be overlooked. This current research takes into account the impact of stress on a commonly used dietary plant *Raphanus sativus* (cultivated in close vicinity of a thermal power unit) due to liberation of polycyclic aromatic hydrocarbons (PAH's), from thermal power units. Chlorophyll content, impact on membrane integrity (MDA content) and plants antioxidant defence system (superoxide dismutase enzyme), was evaluated. Real time evidences through histochemical analysis were also generated. Impact of PAH's on the nutraceutical principles were also determined. PAH accumulation in tissues and SEM analysis for stomatal configuration was also studied. Results were clear indicative of the fact that under the influence of PAH, the oxidative homeostasis of the plant collected from thermal power unit site was significantly compromised. All results where compared to a control sample (cultivated in a site relatively free from industrial pollution). Roots and leaves of the said plant were separately evaluated. Metabolic trouble was indicated through increase in % inhibition activity of SOD to the extent of 70% in leaves. Nutraceutical principles namely ascorbic acid, α-tocopherol, phenolics and flavonoids were significantly inhibited to the extent of 66%, 43.8%, 52.7% and 43% respectively. Cumulative PAH accumulation was 68 times higher with stomata found blocked. Such dietary plants cultivated under the influence of PAH pollution are under intense metabolic trouble and compromised in their nutraceutical richness and also serve as the store house of such environmental pollutants which then infringe into the human biological system.

ABBREVIATIONS

PAHS: Polyaromatic Hydrocarbons; ROS: Reactive Oxygen Species; SOD: Superoxide Dismutase; TPC: Total Phenolic Content; TFC: Total Flavonoid Content

INTRODUCTION

In the last few decades there have been a significant rises in industrialization, urbanization and global population as well. With this increasing industrial invasion; various environmental threats have cropped up which are potentially dangerous. Among such threats are coal-based power sector industries in which burning of coal leads to the production of various entities of contaminants such as polycyclic aromatic hydrocarbons [1]. Polycyclic aromatic hydrocarbons (PAH's) are persistent organic pollutants derived from incomplete combustion of organic compounds such as fossils, smoking tobacco, burning fuels, and incinerating wastes. PAH's can quickly accumulate on plant surfaces, especially on leaf surfaces [2]. Leaves and roots are vulnerable targets for any types of contaminants such as heavy metals and PAH's. Recently; Mandal et al., have critically reviewed the impact assessment of PAH on leaves with especial mention to edible and medicinal plants. The said article also analyzed research trends (2006-2016), of PAH's on various plant parts; and reported that maximum research in this regard has been done on roots [3]. In India, various coal-based power sectors are running, especially in the state of Chhattisgarh which is the power hub for central India. Allocation of such thermal-based power plant units near agriculture land could severely impact crop quality through PAH accumulation. Consumption of such PAH contaminated vegetables are likely to cause cancer, endocrine disorders, cardio vascular issues and many other health problems [4]. PAH contains 16 types of compounds which are cancerous as well as mutagenic according to the United States of Environmental protection agency (USEPA) [5]. Vegetables are considered as universal food due to their richness in nutrients, minerals, fibres, and antioxidants [6]. Among such vegetables; radish was mainly chosen for the purpose of studying the impact of PAH induced stress on the production of secondary metabolites in the said plant. Radish is commonly known as spring barley (botanical name: *Raphanus sativus*), and is a common root/leaf vegetable crop belonging to the family Brassicaceae. Globally it is consumed in forms such as pickles, salads, and curries. In India, it is most commonly used in various types of curry dishes as well as salads. Apart from the root, leaves are also used as a dietary food in Indian cuisine. Both roots and leaves have medicinal and nutritional values as well [7]. The extract of radish are used in various ailments such as GIT disorders, urinary infections, hepatic disorder, and cardiac disorder [8]. It also consists of various nutritional components such as amino acids,

fibers, minerals, carbohydrates, proteins, and flavonoids [9]. The integrity of nutritional and medicinal values of such a commonly used dietary plant can be compromised due to accumulation of PAH's liberated from the coal fired power plants located in close vicinity of such agriculture land. PAH's can severely affect the physiological and metabolic activities of such vegetables owing to induced oxidative stress resulting in dismantling of the plants oxidative balance [10].

Henceforth, the situation warrants that investigation on the nutritional and medicinal integrity of such an important dietary plant (*Raphanus sativus),* is monitored from close quarters. Literature as sufficient evidence to anticipate that PAH contaminated vegetables may be compromised in terms of nutritional contents and can also act as a channel for PAH's to infringe into the human biological system through the food chain [11]. In light of the above facts, the current study investigates the biochemical and nutritional responses of *Raphanus sativus* towards PAH induced oxidative stress along with the estimation of PAH accumulation in the said dietary plant.

MATERIALS AND METHODS

Description of the study area

The agriculture land located just outside the premises of K.S.K. Mahanadi coal based power plant unit was chosen as the sample collection site. The collection was carried out within 0.5 km radius from the boundary wall of the power plant unit. K.S.K. Mahanadi power plant unit is one of the India's largest coal based power plant unit with a maximum production capacity of 3600 MW. The vegetables grown in the identified site by the local farmers are used for personal consumption and also for selling the same at the nearby vegetable markets. The collected sample from the above described site was referred to as "sample or test group". Samples collected from a different area ("Akaltara") far away (15 km), from any possible thermal and highway vehicular pollution served as the "control group".

Sample collection and processing

Samples (vegetables), were collected from the described sample collection site during winter season (December-January), in downward wind direction. Vegetable crop was collected when it was fully ready for consumption. Collected samples (both roots and leaves of the said plant) were washed with distilled water to remove debris and soil particles. Sample processing was carried out by two different methods. In the first method, samples were dried and grinded by using mortar pestle to obtain a homogenous powder which was subjected to extraction using suitable solvent as per experimental protocols described in the latter sections. The second method; comprised of using the fresh samples as such (homogenized mix), for estimating chlorophyll content and enzymatic action. A detailed illustrative representation of entire experimental protocol has been presented in Figure 1.

Standards and reagents

A PAH Calibration Mix (consisting of 16 PAH's namely, Acenaphthene, Acenapthylene, Anthracene, Benz[*a*]anthracene, Benzo[*a*]pyrene, Benzo[*b*]fluoranthene, Benzo[*ghi*]perylene, Benzo[*k*]fluoranthene, Chrysene, Dibenz[*a,h*]anthracene, Fluoranthene, Fluorene, Indeno[1,2,3-*cd*]pyrene, Naphthalene, Phenanthrene, Pyrene) 1x1 mL, 10µg/mL each component in acetonitrile (part number: CRM47940) was purchased from TraceCERT (Supelco), nitro blue tetrazolium (NBT), gallic acid (certified reference material, TraceCERT), 2-aminoethyl diphenylborinate (Neu's reagent, assay≥97%), DPPH (2,2-diphenyl-1-picrylhydrazyl), α-tocoferol (assay ≥ 96% HPLC) and folin-denis reagent was purchased from Sigma Aldrich (St. Louis, MO, USA). Ascorbic acid, anthrone, commasine blue G, acridine orange, DAB (3,3-diaminobenzidiene) and evan's blue reagent were purchased from HIMEDIA Co. Ltd. (India). All solvents used in the extraction and chromatographic processes were purchased from Thermo Fisher Scientific (India).

Instrumentation

Extraction of PAH and nutraceutical content from plant material was performed using automated Soxhlet Extraction Unit - B811 (BUCHI, Switzerland). Effective condensation was performed with a recirculation chiller (Lab-X, Kolkata, India) maintained at 15ºC. Quantification of PAH's was performed by Gas Chromatography (Varian 363) equipped with a HP-DB-5MS column (60 x 0.25mm, 0.25µm film thickness) and coupled with

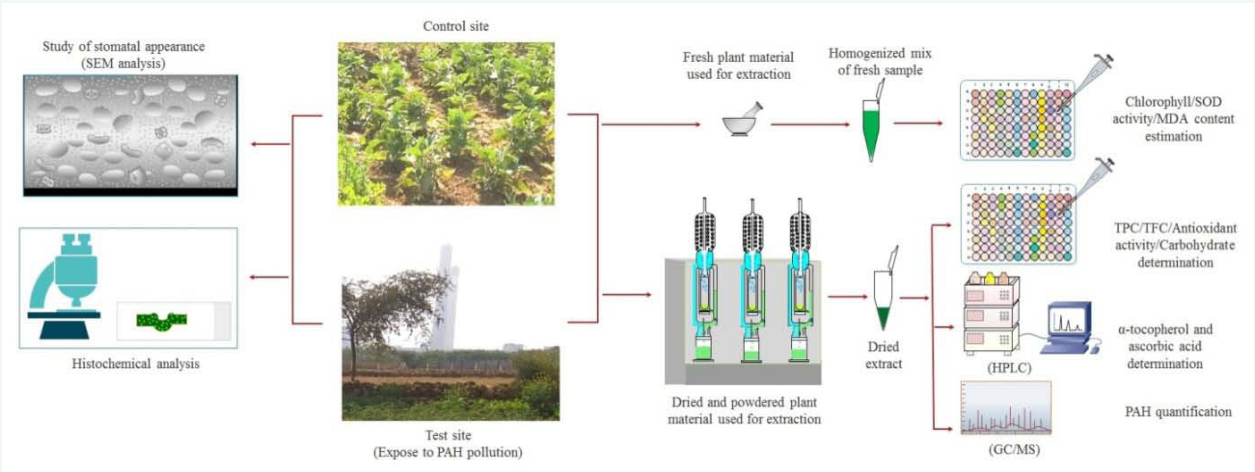


Figure 1 Illustrative representation of experimental protocol.

mass detector. Data acquisition and integration were carried out with the GALAXY chromatography software. Analytical Technologies (Baroda, India) Binary Gradient HPLC system (P3000) was used for HPLC analysis.

Estimation of Chlorophyll content

Estimation of photosynthetic pigments of leaf samples was performed according to Lichtenthaler. 1987 [12]. Briefly, fresh leaves were homogenized with 80% DMSO and supernatant was centrifuged at 10000 rpm for 5 min and collected supernatant was measured by UV-spectrophotometer at 645 nm and 663 nm for estimating chlorophyll a and chlorophyll b respectively using the below mentioned formula,

$$chl_a = 0.0127 \times A_{663} - 0.002369 \times A_{645}$$

$$chl_b = 0.0229 \times A_{645} - 0.00468 \times A_{663}$$

$$chl_{\frac{a}{b}} = 0.0202 \times A_{645} + 0.00802 \times A_{663}$$

Lipid peroxidation (Malondialdehyde-MDA Content) Estimation

Lipid peroxidation (MDA content) was performed as per the method given in the manual provided along with the assay kit (Sigma Aldrich, USA). The readings were recorded using 96 well plates at 532 nm (Multiskan Ex, Ascent software 2.6, Thermo Fisher Scientific, China).

Superoxide Dismutase (SOD) % Inhibition activity

SOD % inhibition activity was performed as per the manual given in SOD assay kit (Sigma Aldrich, USA). SOD Assay Kit-WST allows very convenient SOD assaying by utilizing Dojindo's highly water-soluble tetrazolium salt, WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2Htetrazolium, monosodium salt) that produces a water-soluble formazan dye upon reduction with a superoxide anion. The rate of the reduction with O_2 is linearly related to the xanthine oxidase (XO) activity, and is inhibited by SOD thus enabling % inhibition activity of SOD or SOD-like materials determination using colorimetric method. Since the absorbance at 440 nm is proportional to the amount of superoxide anion, the SOD activity as an inhibition activity can be quantified by measuring the decrease in the colour development at 440 nm. It may be noted that one unit is the amount of SOD that inhibits the rate of formazan dye formation by 50% [13].

Histochemical analyses

For all histochemical analysis fresh plant material was used. Cell death in leaf tissue was visualized by using Evan's blue staining method by immersing fresh leaves in 0.25 % (w/v), aqueous solution of Evans blue [14]. The procedure was vacuum infiltrated and a contact time of 6 h was provided. Leaves were then bleached with 95% v/v ethanol. Cell death could be visualized as blue patches on the leaf surface. The *in-vivo* localization of super oxide anion radical ($O_2^{\cdot-}$) was performed by immersing the fresh leaves in 0.1% nitro blue tetrazolium (NBT), solution containing 10 mM sodium azide, prepared in potassium phosphate buffer (pH 6.4) [15]. After allowing a contact time of 16 h, the leaves were illuminated until the appearance of dark blue spots and then bleached with boiling ethanol, and macro-photographed with a digital camera (Sony DSC-W380, Japan). Likewise, for H_2O_2 localization, 1% 3, 3-diaminobenzidine (DAB) solution was used to immerse the leaves and then incubated for 16 h. After the appearance of brown spots, leaves were bleached with boiling ethanol and macro-photographed [15]. The localization of the end product of lipid peroxidation which is malondialdehyde (MDA), was done by using Schiff's reagent [15].

For determination of cell permeability (pre-apoptic changes), acridine orange solution (5µgmL^{-1}) was used, thin sections of leaf samples were dipped in acridine solution and visualized by using UV fluorescence microscopy (Axio Cam ERc 5s, Carl Zeiss, Germany) [16].

For visualization of phenolic principles, Neu's reagent (2-amino ethyldiphenylborinate) was used as per the method described by Chouhan et al [17]. Briefly, a thin section of leaf samples was dipped in 1% Neu's reagent for 2-5 minutes and visualized by using UV-fluorescence microscopy for localization of phenols. Phenolic principles if present shall exhibit bright green fluorescence.

Plant cell viability assay was performed by using commercially available Plant Cell Viability Assay Kit (Sigma Aldrich, USA). The experiment was performed as per the instructions provided in the kit manual. Stained leaf samples were observed under UV fluorescence microscopy (Axio Cam ERc 5s, Carl Zeiss, Germany) by using FITC and DAPI filter [18].

Nutraceutical profiling

For nutraceutical profiling, 1 g dried leaf powdered sample was extracted through automated Soxhlet extraction unit (B-811), using methanol as solvent as per the method described by Chouhan et al. [19]. The dried extract was then used for estimation of different nutraceutical principles. Ascorbic acid was quantified by HPLC using a mobile phase composition of 0.2% metaphosphoric acid in water:methanol (90: 10 v/v), with detection being carried out at 254 nm [19]. Identification was carried out using authentic external standards. Carbohydrate estimation was carried out using anthrone reagent using glucose as the standard and absorbance were recorded with a UV spectrophotometer at 620 nm [17]. Alpha Tocoferol was determined by HPLC using mobile phase; methanol: water (98:2 v/v) with detection being done at 292 nm [19].

Phenolics, flavanoids and Antioxidant principle analysis

Total phenolic Content (TPC): TPC was determined using Folin–Ciocalteu method as described by the authors previously Chouhan et al [17]. Absorbance of standard gallic acid and samples at 765 nm was measured by an UV-spectrophotometer (calibration curve linear equation: y = 0.036x - 0.023, r^2 = 0.995). TPC in samples was expressed as mg gallic acid equivalents (GAE) per g of dried extract.

Total flavanoid content (TFC): The TFC of crude extract was determined by the aluminium chloride colorimetric method [19]. The absorbance was measured at 510 nm and TFC was calculated using the calibration curve equation: y = 0.006x + 0.005, r^2 =

0.994. The results were expressed as mg gm^{-1} of dried extract quercetin equivalents (QUE). The following formula as described by the authors in their previous publication was used for the calculation of TPC and TFC [11].

Equation 1: TPC/TFC = (c*V)/m

c = value of "x" which reflects the concentration

V = final volume including dilution factor (if any)

m = weight of the main extract in g

Antioxidant analysis

The DPPH radical scavenging assay was used to determine the antioxidant activity of the extract at 100µg/mL [17]. Absorbance of samples and control were measured at 510 nm with methanol as the blank.

$$Equation\ 2: Scavenging\ activity\ (\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100\%$$

where, $A_{control}$ is the absorbance of the control and A_{sample} is the absorbance of the test sample.

PAH extraction and enrichment

For extraction of PAH's from plant samples; 1 g powdered samples were extracted using automated Soxhlet extraction unit B-811 as per the method described by the authors in their previous publications using dichlomethane as solvent for 120 min along with 30 min of rinsing [11]. For enrichment of PAH's from extract, column chromatography method as described by the authors earlier was used using 30 mL DCM: hexane (1:1 v/v) as the mobile phase [11]. Collected eluent (10 mL) was concentrated and reconstituted by using 2 mL acetonitrile for PAH quantification using GC-MS.

PAH Quantification

PAH analysis was carried out through a well-established protocol using GC-MS as per the method described by Meudec et al [20]. Briefly, the initial oven temperature was 50ºC, upon analysis, it was then held for 5 min, and then raised steadily to 300 ºC at 12 ºC min^{-1} with a final holding time of 20 min. The injector, transfer lines and ion source temperatures were set at 260 ºC, 305 ºC and 186 ºC respectively as reported by Meudec et al [20]. Injection volume of the PAH enriched sample was 1 µL (injected through autosampler using splitless mode). Helium was used as the carrier gas. Individual PAH's were identified using external standards (PAH Calibration Mix which consisted of a mixture of 16 PAH's) and subsequent calibration curve was also prepared for quantification. The final results of PAH quantification was expressed in ng g^{-1}.

Field emission scanning electron microscopy (SEM)

All the specimens were examined with a JEOL JSM-7610F (Tokyo, Japan) scanning electron microscope under high vacuum condition and at an accelerating voltage of 5.0 kV. Electron gun: Schottky type field emission (T-FE) gun

Statistical analysis

The Duncan's test at the 0.05 probability level and the paired t-test at 95% confidence level were performed for comparison of sample with control (for all evaluated parameters), using Graph Pad Prism version 7.0. Experiments were performed in triplicate and the results were exhibited as means followed by the corresponding standard deviations.

RESULTS AND DISCUSSION

Chlorophyll Content

In stress conditions, content of photosynthetic pigments can reveal vital information's. Figure 2 shows a significant decline of chlorophyll contents such as chl a chl b and total chl for samples (leaves), collected from close vicinity of thermal power unit when compared to control. A decline in chlorophyll content in leaves to the extent of 31.20%, 56.80%, and 51.68% when compared to the control were recorded for chl a chl b and total chl respectively and the results were found to be in agreement with some previously published reports by various authors [10,21-25]. The cumulative impact of PAH's may lead to the overproduction of reactive oxygen species (ROS) which may be responsible for the loss of photosynthetic pigments. Higher content of ROS may compromise the photosynthesis process occurring in the chloroplast and may cause decline in photosynthetic precursors as well [11,21]. A similar observation was also reported earlier by several authors in connection with heavy metal accumulation in plants resulting in depletion of chlorophyll content due to inhibition of δ-aminolaevulinic acid/ dehydratase activity and pheophitinization occurring in chlorophylls [15,26-28].

Lipid peroxidation (MDA content)

MDA content is an essential parameter for lipid peroxidation in plant cell. Figure 3 indicates that the MDA content of leaves had increased by 6 times whereas 2.4 times increase in roots was recorded for samples collected from PAH exposed site (close vicinity to thermal power unit) when compared to control group. MDA content is directly proportional to the damage inflicted on the plant cell membranes through oxidative stress. Similar observations were also recorded by several other authors in context to impact of PAH's on plants [16,29]. The hypothesis

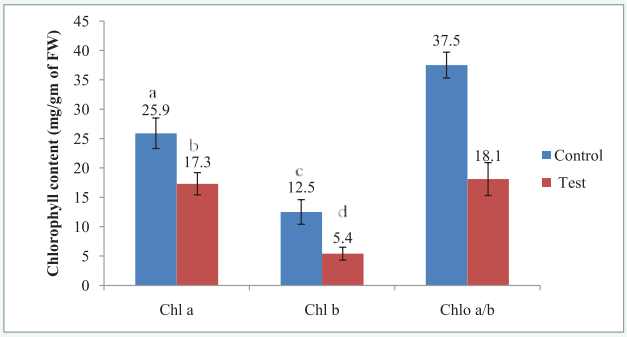


Figure 2 Impact of PAH's on chlorophyll content of leaves of *Raphanus sativus*.
Data marked with different letters are significantly different at $P < 0.05$. Results are expressed as mean ± standard deviation ($n = 3$). Control: leaves of *Raphanus sativus* collected from a site which is deemed to be free from any major industrial pollution (background sample), Test: Plant sample collected from agricultural land in close vicinity to a thermal power unit. Different letters indicate significant difference ($p < 0.05$). Values are mean ± SD

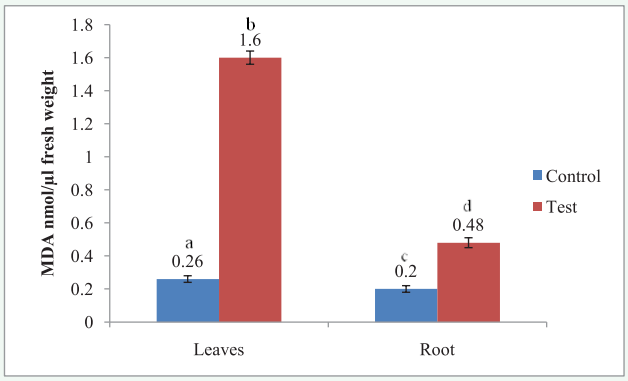


Figure 3 Impact of PAH's on MDA content of leaves and roots of *Raphanus sativus*.
Data marked with different letters are significantly different at $P < 0.05$. Results are expressed as mean ± standard deviation ($n = 3$). Control: leaves of *Raphanus sativus* collected from a site which is deemed to be free from any major industrial pollution (background sample), Test: Plant sample collected from agricultural land in close vicinity to a thermal power unit. Different letters indicate significant difference ($p < 0.05$). Values are mean ± SD.

connecting increased MDA content and damage to cell membranes has been well validated through histochemical analysis explained in the latter section.

SOD % inhibition activity

Activity of SOD is the first line of defense against oxidative stress and plays a critical role in maintaining the redox homeostasis. It eliminates the ROS threat by decomposing ROS into O_2 and H_2O_2. According to Yin et al. [29], increased SOD activity can be directly accounted for increased production of ROS. Figure 4 indicates a significant rise in % inhibition activity of SOD to the extent of 70.4% and 39.7% for both leaves and roots respectively, when compared to control. Increased % inhibition activity of SOD as per the kits working principle indicates reduced conversion of highly water-soluble tetrazolium salt (WST-1) into water-soluble formazan dye upon reduction with a superoxide anion. This indirectly indicates increased activity of SOD for quenching the superoxide anion which is formed in excess in plant samples exposed to PAH pollution. In absence of superoxide anion owing to it being quenched by SOD; the conversion of WTS-1 is reduced which can be easily detected by measuring the decrease in colour development at 440 nm. Increased SOD activity which in this case is represented as % inhibition activity is actually plants own internal defense response against PAH induced oxidative stress. This antioxidant enzyme maintains the redox homeostatic of the system by combating any excess production of ROS which are highly likely to cause cellular damage leading to cell death if not controlled. Authors have earlier cited in one of their publication that denovo synthesis or activation of enzymes by transcription and translation of some specific genes could be held responsible for the exhibition of increased activity [11].

In-vivo localization of Reactive Oxygen Species

In plants, several metabolic processes results in the formation of ROS. This section provides real time assessment and mapping of *in-vivo* localization of ROS (Figure 5), present in both sample and control group. Formation of blue formazan spot due to the reduction of NBT by superoxide anion radical ($O_2^{\cdot-}$) appeared all over the test specimen (both leaves and roots), when compared to control. Likewise, the accumulation of H_2O_2 was also found significant in the test specimens by the appearance of brown spots due to the reduction of DAB by H_2O_2. Brownish appearance was found to be more in the test sample as compared to control. In continuation to this, MDA or $OH^{\cdot}$ radical was visualized using Schiff reagent which gives a prominent pink colour in the test samples as compared to control. Overproduction PAH-induced oxidative stress can severely impact the cell structure and its organelles. By using the Evans blue staining method, visualization of blue spots as an indicative of cell death was more prominent in test specimens when compared to control. Real time conclusive evidence was obtained on *in-vivo* localisation of ROS which further validates the claim of PAH induced oxidative stress and its consequences.

Histochemical analysis

Holistic visualization of in-situ phenolic principles was carried out through 1% NPR reagent. The fluorescence intensity in leaf and root tissues (transverse sections), of control sample was qualitatively compared with test specimens (Figure 5). It may be noted that fluorescence intensity is directly proportional to the phenolic content present in the tissue. Fluorescence intensity was predominantly found to be significantly more prominent especially in the vascular regions of the leaves and meristematic regions of roots for control samples. The lower fluorescence intensity observed in the said regions for test samples (exposed to PAH pollution), is a real time histochemical based conclusive evidence for depletion of phenolic resources. The onus lies on PAH induced oxidative stress resulting in redox imbalance or overproduction of ROS [11].

Cell membrane integrity was evaluated using acridine orange solution. Leaves and roots of PAH exposed plant showed

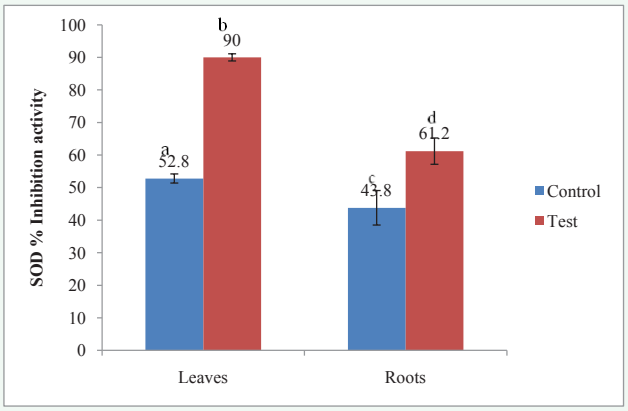


Figure 4 Impact of PAH's on the SOD activity of *Raphanus sativus* (evaluation of plants antioxidant defence system).
Data marked with different letters are significantly different at $P < 0.05$. Results are expressed as mean ± standard deviation ($n = 3$). Control: leaves of *Raphanus sativus* collected from a site which is deemed to be free from any major industrial pollution (background sample), Test: Plant sample collected from agricultural land in close vicinity to a thermal power unit. Different letters indicate significant difference ($p < 0.05$). Values are mean ± SD.

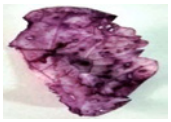
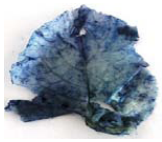
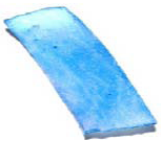
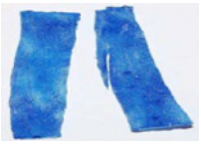
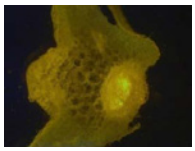
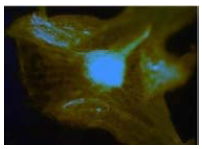
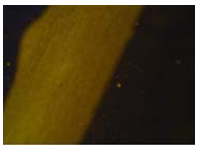
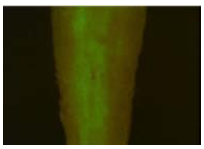
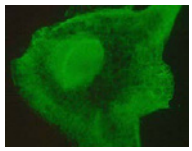
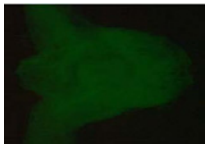
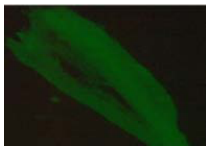
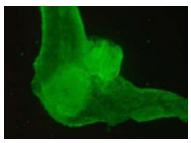
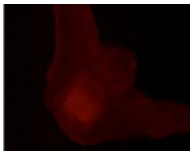
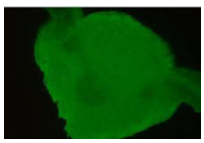
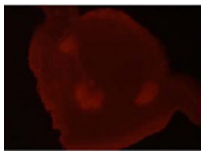
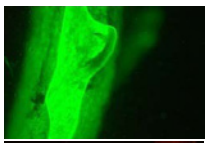
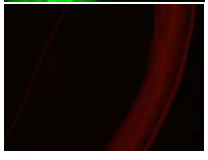
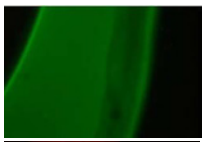
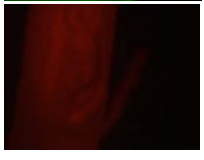
Staining method	Leaves		Roots	
	Control	Test	Control	Test
NBT				
DAB				
MDA				
EVANS BLUE				
Acridine orange				
NPR				
Plant cell viability	 	 	 	 

Figure 5 Histochemical analysis depicting in-vivo localisation of various ROS using various staining methods and its effect on membrane integrity, phenolic content and cell viability.

In-vivo localisation of ROS: NBT- highlights in-vivo localisation of super oxide anion radical through spread out of blue colour on the leaf surface. Formation of blue formazan due to reduction of NBT by the radical is indicative of the above fact. DAB- highlights the accumulation of H_2O_2 as evident from the spread out of brown spots prominently on the leaf surface of test specimens. MDA - MDA or $OH^{\cdot}$ radical was visualized using Schiff reagent which gives a prominent pink colour in the test samples when compared to control. Evans blue- visualization of blue spots is an indicative of cell death which was more prominent in test specimens when compared to control. Acridine orange - Greater accumulation of acridine orange in vascular and parenchyma portion depicted as intense orange color. NPR staining- fluorescence intensity of the leaf tissue is directly proportional to its phenolic/flavonoid contents.

considerable accumulation of acridine orange depicted as intense orange colour (Figure 5). Above results are in perfect agreement with the findings obtained for Evan's blue staining method which showed intense blue patches for PAH exposed samples indicating cell death. The claim of PAH induced ROS infliction on plant cell membranes leading to compromised cell membrane leading to dismantling the cells internal homeostasis stands validated based on the real time evidences obtained from Evan's blue and acridine orange staining methods [11].

Cell viability can be badly affected by oxidative stress. Damaged cell membrane is likely to alter cell viability as well. Two types of staining reagent were used in the kit. Fluorescein Diacetate (FDA) detects viable cells with intact cell membrane. FDA as such has no fluorescence but inside the viable plant cells they are hydrolysed to highly florescence polar compounds in presence of intracellular esterase enzyme. The florescence compounds are then retained inside the plant cell and exhibits intense green fluorescence which validates integrity of cell membrane as only viable cells with intact plasma membrane will show green fluorescence. Propidium Iodide (PI), the other component of the kit detects non-viable cells or cells with damaged cell membrane which shows red fluorescence. PI is hydrophilic molecule which can gain entry inside the cell only when the plasma membrane is damaged. Upon entry it produces a bright red fluorescence in non-viable cells. Transverse sections (both roots and leaves), of control specimens showed significantly increased bright green fluorescence indication cell viability; when compared to transverse sections of PAH exposed samples (Figure 5). Whereas in the case of PI, sample collected from the industrial site (exposed to PAH pollution), showed bright red fluorescence indicating penetration of PI dye inside the cell which can take place only through a damaged cell membrane indicating non-viable cells. Henceforth, all histochemical evidences strongly advocates the claim of damaged plasma membrane caused due to the PAH induced oxidative menace.

Nutraceutical content

Impact of PAH's on the production of nutraceuticals was estimated through quantitative evaluation of the following principles. The increase or decline of the below principles would reflect on the effect of PAH on plants metabolism with special emphasis to production of secondary metabolites.

(a) Ascorbic acid

(b) Alpha-Tocopherol

(c) Carbohydrate

(d) TPC

(e) TFC

Ascorbic acid: Ascorbic acid is abundantly present in tissues of edible/dietary plants. Its role in mitigating various oxidative responses is well established. Figure 6 shows significant decrease in ascorbic acid content for both roots and leaves for sample group (exposed to intense PAH pollution). Leaves recorded a decrease in 66% and roots showed 44.5% decline of ascorbic acid when compared to control. The decrease in ascorbic acid content could be due to increased utilization and decreased biosynthesis

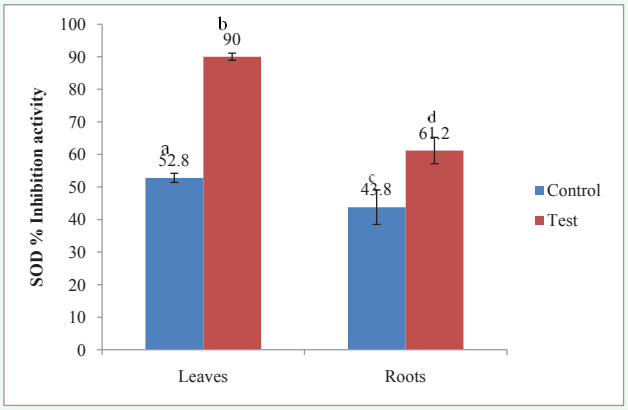


Figure 6 Effect of PAH on the production of nutraceutical principles – ascorbic acid

For ascorbic acid, response factor: (peak area/standard concentration), amount of analyte (c): (peak area of sample/response factor), final reporting concentration: (c × V/m), where "V" is the final volume including dilution factor (if any) = 2 mL and "m" is the weight of the main extract in grams [control (leaf = 0.29 g and root = 0.84 g) and test (leaf = 0.32 g and root = 0.21 g)]. Different letters indicate significant difference ($p < 0.05$). Values are mean ± SD

owing to inhibitory effects on its biosynthetic pathway because of ROS interaction [11]. Similar results were also reported by Wieczorek et al. [30], where depletion of ascorbic acid content of celery aerial parts on exposure to foliar application of anthracene was observed.

α-Tocopherol: Tocopherol are naturally occurring antioxidants which plays an essential role in counteracting oxidative stress situations such as drought, salinity, and heavy metal accumulation. Results depicted in Figure 7 indicate significant decrease in α-tocopherol content when exposed to natural PAH pollution. Leaves exhibited a decline of 43.8% and roots 25% when compared to control. This decline hints towards the inhibition of homogentisic acid and phytyldiphosphate which are well established precursors for synthesis of tocopherol in plants as stated by the authors themselves in their earlier publication [11]. Tocopherol protects membrane integrity through ROS scavenging and halting chain propagation during lipid oxidation [31]. Any decline in tocopherol content is likely to compromise membrane integrity which is very well evident in this case from the histochemical findings reported in the earlier section.

Carbohydrate: Plays a vital role in various metabolic activities such as osmo-regulation, energy production and signal generation. Change in scale of carbohydrate content virtually impacts the plant metabolism and subsequent growth. Figure 8 shows the decrease in the level of carbohydrate content of leaves (21.5% decline) and root samples (10.6% decline), collected from polluted sites when compared to control.

Total phenolics and flavonoids: Figure 9 and Figure 10 depict the scale of reduction of phenolics and flavonoid contents in leaves and roots of *Raphanus sativus.* In case of leaves severe depletion to the extent of 52.7% and 43% for phenolics and flavonoids respectively was observed. Whereas, for roots; a depletion of 39% and 31% for phenolics and flavonoids respectively was seen.

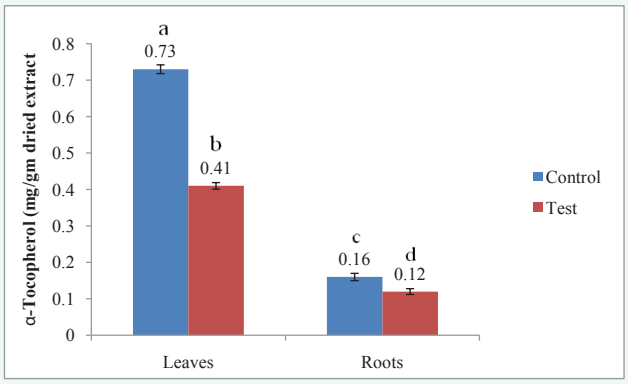


Figure 7 Effect of PAH on the production of nutraceutical principles – α-tocopherol

For α-tocopherol, response factor: (peak area/standard concentration), amount of analyte (c): (peak area of sample/response factor), final reporting concentration: (c × V/m), where "V" is the final volume including dilution factor (if any) = 2 mL and "m" is the weight of the main extract in grams [control (leaf = 0.29 g and root = 0.84 g) and test (leaf = 0.32 g and root = 0.21 g)]. Different letters indicate significant difference ($p < 0.05$). Values are mean ± SD

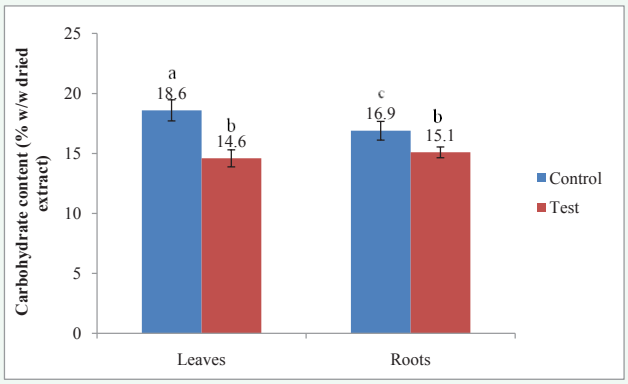


Figure 8 Effect of PAH on the production of carbohydrate

For carbohydrate, response factor: (absorbance of glucose/ concentration of glucose), amount of analyte: (peak area of sample/ response factor), final reporting concentration: (c × V/m), where V = 25 mL and "m" is weight in grams [control (leaf = 0.29 g and root = 0.84 g) and test (leaf = 0.32 g and root = 0.21)]. Different letters indicate significant difference ($p < 0.05$). Values are mean ± SD

For decoding the changing pattern of phenolics and flavonoids in response to PAH induced oxidative stress; several earlier similar published findings were studied and a hypothesis in this regard had already been published by the authors research group [11]. Every plant has its own internal mechanism to counteract internal stress conditions induced by environmental factors. The plant does so by increasing its antioxidant principles to combat the stress situations [32]. In this regard some authors have reported substantial increase in phenolics/flavonoid contents to deal with such situations by neutralizing ROS threat in order to keep the original internal oxidative balance intact [33,34]. However, when due to the induced stress conditions the production of ROS overpowers the plants internal combat mechanism, the plant then enters into a state of metabolic trouble which is exhibited through decreased production of nutraceutical/antioxidant principles as described in the above presented current findings. Depletion of phenolic resources as cited by several authors could be due to consumption or inhibition of the biosynthetic machinery of phenolics and/or due ROS induced inhibition of phenylalanine ammonia-Liaza enzyme which the principle enzyme involved in the biosynthetic pathway [33].

In order to ascertain the biological integrity of the dietary plant, the antioxidant activity was evaluated through DPPH % radical scavenging activity at 100µg/mL concentration. Table 1 indicates the decrease in magnitude of antioxidant potential of leaves and roots exposed to PAH pollution when compared to control and standard (curcumin solution at 100µg/mL). The table is self explanatory and indicates significant reduction in % radical scavenging activity for roots and leaves exposed to PAH pollution. The results are coherent with the findings of total phenolics and flavonoids content. Since mostly the phenolics/ flavonoid principles are responsible for exhibiting antioxidant activity so any decline in their total content would likely impact the antioxidant potential of the plant as well which is very well evident from the above results. It can be anticipated that if the plants antioxidant potential gets compromised; it is likely that other inherent biological activities of the plant would get compromised.

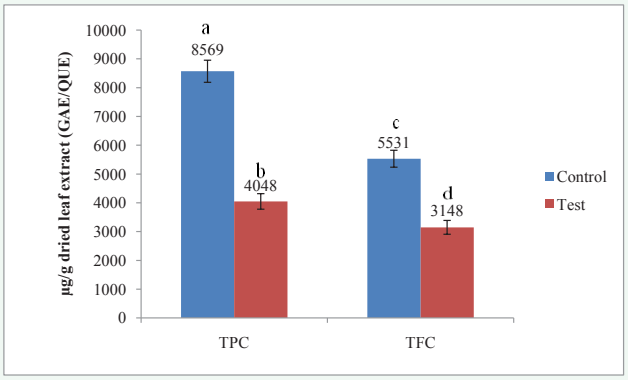


Figure 9 Effect of PAH on the production of total phenolics/flavonoids in the leaves of *Raphanus sativus*

Different letters indicate significant difference ($p < 0.05$). Values are mean ± SD

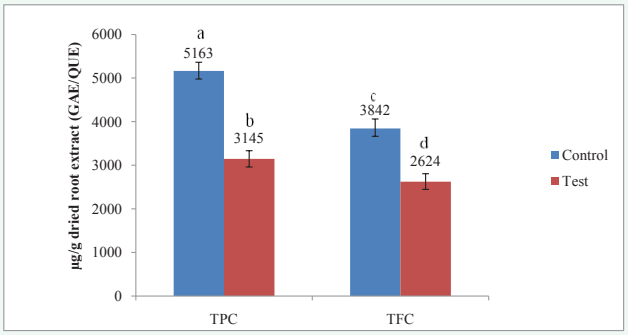


Figure 10 Effect of PAH on the production of total phenolics/ flavonoids in the roots of Raphanus sativus

Different letters indicate significant difference ($p < 0.05$). Values are mean ± SD

The presented findings regarding the response of nutraceutical principles towards PAH induced oxidative stress clearly provides conclusive evidence of decline in nutraceutical principles under oxidative stress situations. The response in leaves is more significant when compared to roots as leaves reported significantly more decline in contents when compared to roots of samples exposed to PAH pollution. This could be due to the fact that leaves are directly exposed to PAH on account of foliar deposition and also translocation of PAH through roots. So this dual exposure for leaves makes it probably more vulnerable.

PAH Quantification

The results of estimation of PAH content in leaves and roots of *Raphanus sativus* are presented in Figure 11 and Figure 12. In leaves exposed to PAH pollution a total of 08 PAH's namely; acenaphthalene, fluorene, phenanthrene, fluoranthene, pyrene, chrysene, benzopyrene and benzoanthracene were detected. Out of these only 03 (acenaphthalene, fluorene, phenanthrene), were detected in the control group with the cumulative accumulation amounting to 57.64 ng/g dried extract of leaves. Whereas, the situation in leaves exposed to PAH pollution was alarming; where cumulative accumulation of PAH was 68 times higher than control. Accumulation of benzopyrene which constituted a 7% share was the major concern as it is an extremely carcinogenic and mutagenic compound. The results were found to be coherent with earlier published reports where investigators have reported PAH accumulation in plant tissues ranging from 1.5-140 times higher in vegetables grown in close vicinity to thermal power units [11]. The picture of PAH accumulation in roots is no different either. A total of 05 PAH's were detected in root tissue of sample group out of which only phenanthrene was found in the control amounting to 46.6 ng/g dried extract of root. The cumulative PAH accumulation in PAH exposed sample was found to be 18.4 times higher. Increased depletion of nutraceutical contents was observed in leaves when compared to roots as stated in the earlier section. The above results were found to be in harmony with the observations recorded for PAH accumulation as well where significantly higher PAH accumulation was recorded for leaves.

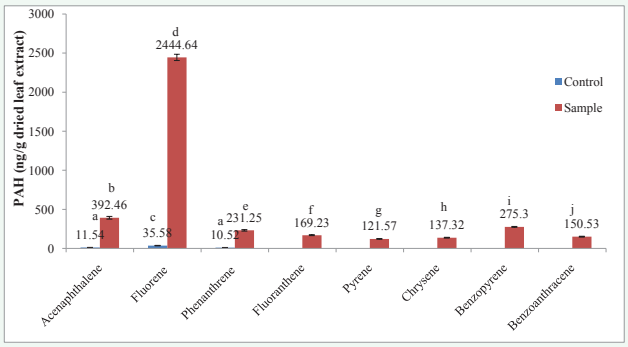


Figure 11 Estimation of PAH content in leaves of *Raphanus sativus* Formula = (C × *V*/*m*), "C" = value of "x" which reflects the concentration, to be calculated from calibration curve straight line equation. "*V*" = final volume including dilution factor (if any) (2 mL). "*m*" = weight of the main extract in g [control = 0.16 g and test = 0.36 g). Different letters indicate significant difference ($p < 0.05$). Values are mean ± SD

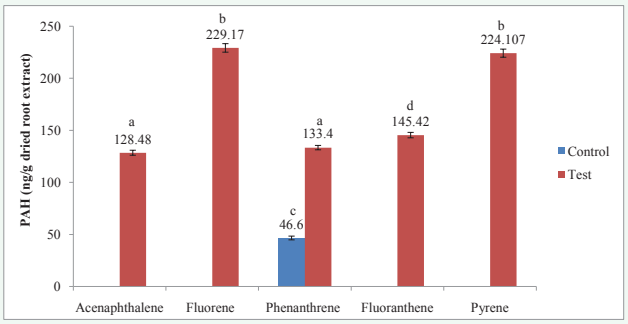


Figure 12 Estimation of PAH content in roots of *Raphanus sativus* Formula = (C × *V*/*m*), "C" = value of "x" which reflects the concentration, to be calculated from calibration curve straight line equation. "*V*" = final volume including dilution factor (if any) (2 mL). "*m*" = weight of the main extract in g [control root = 0.18 g and test root = 0.26 g). Different letters indicate significant difference ($p < 0.05$). Values are mean ± SD

It may be noted that the intensity of oxidative stress produced is directly proportional to the accumulation of PAH in plant tissues.

SEM analysis

Stomatal configuration depicting impact of PAH accumulation on stomatal opening and closing is presented in Figure 13. Surface view of stomatal structure, provides a real-time assessment indicating blockage of stomata, which could be due to deposition of PAH on the leaf surface or due to responses produced by some signaling components on stomatal opening and closing mechanism. A similar observation was reported by Quadir et al. [26], as pollutants (coal fly ash), accumulated on neem leaves and resulted in decrease of magnitude of stomatal conductance. Above results also suggest decrease in photosynthetic rate and reduction in intracellular transpiration rate as well. Above phenomenon of stomatal blockage is in agreement with low level of carbohydrate content; after all carbon is the sole source of plant metabolism and blockage in stomata results into a catastrophic chain reaction in carbon sourced metabolism. roots internal anatomy for control sample appeared healthy with cells maintained in their proper shape and size. Whereas, for test samples (exposed to PAH pollution), cells appeared distorted and a clear deviation from the healthy status can be felt.

CONCLUSION

Response of *Raphanus sativus* towards PAH induced oxidative stress revealed significant metabolic trouble which was evident from the findings of histochemical studies and production of nutraceutical principles. SOD activity and chlorophyll estimation were also in tandem to indicate towards compromised oxidative status of the plant. The said plant being an important dietary plant which is often consumed raw in the form of "salad" can present severe health threat if grown and cultivated in and around PAH polluted sites. Not only it shall present to the consumers depleted nutraceutical resources but shall also be an opportunity for various carcinogenic PAH's to infringe into the human biological system. Local administrations and power plant unit management should conduct regular monitoring by estimation of oxidative biomarkers, perform phytoremediation

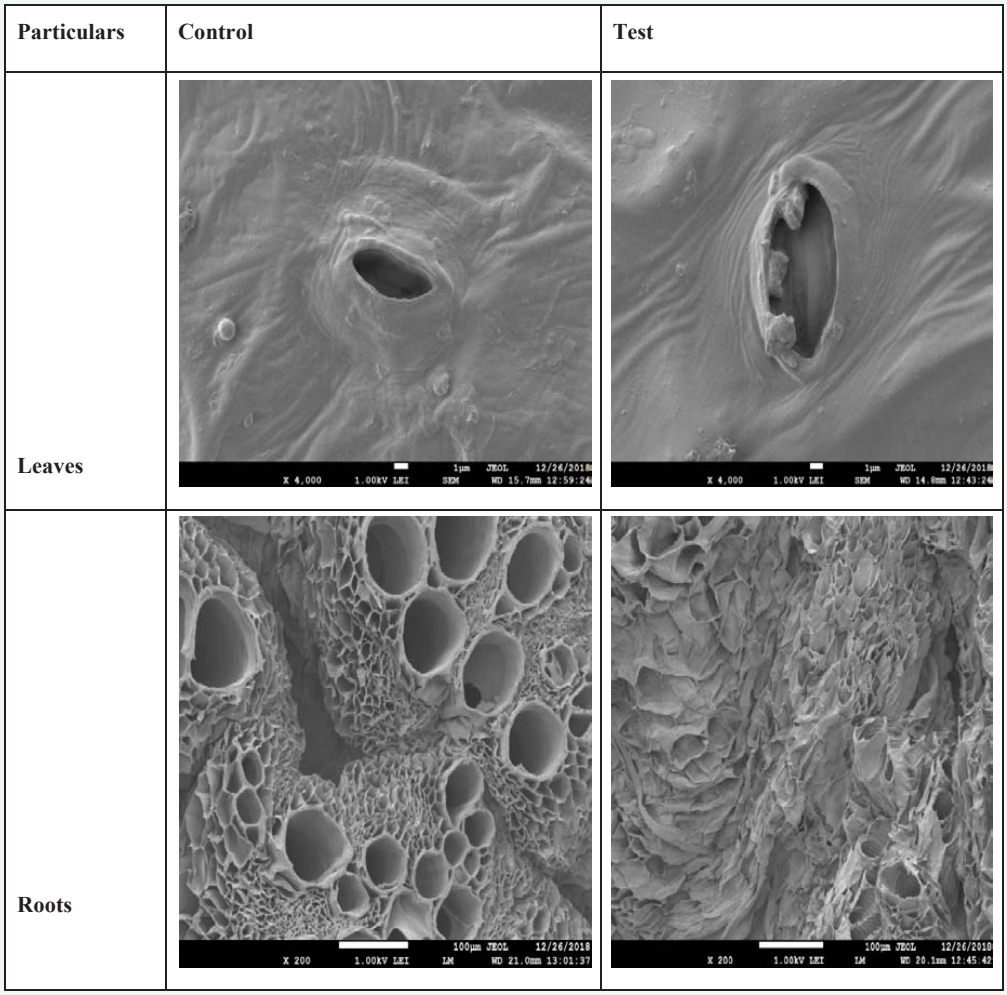


Figure 13 Scanning electron micrographs depicting stomatal status of leaves and effect on roots internal anatomy due to accumulation of PAH both externally and internally.

Table 1: Antioxidant activity in terms of % radical scavenging activity of leaves and roots of *Raphanus sativus*. Concentration used: 100 µg/Ml Different letters row wise indicate significant difference ($p < 0.05$). Values are mean ± SD.

Antioxidant activity (% scavenging activity)	Standard (Curcumin)	leaves		roots	
		Control	Sample	Control	Sample
	90±4.1[a]	75.2±3.8[b]	40.1±2.5[c]	70.7±3.6[d]	55.4±2.1[e]

and at the same time must also concentrate towards greener production. Industrialization and human safety both must be in tandem for a healthy living.

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