

IN VITRO PLANT REGENERATION, ANTIOXIDANT AND ANTIBACTERIAL STUDIES ON BROCCOLI, *BRASSICA OLERACEA* VAR. *ITALICA*

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Abstract

Leaf and shoot tip explant of *Brassica oleracea* var. *italica* were inoculated on Murashige and Skoog (MS) basal medium supplemented with various concentrations of (BAP) and (IBA), alone and in combinations to achieve plant regeneration. Subsequently, antioxidant and antibacterial activities were determined from *In vitro* and *In vivo* plant parts. The highest mean numbers of shoot was observed on combinations of 1 mg l⁻¹ and 1.5 mg l⁻¹ of BAP and IBA, 9.49 and 8.69, respectively. The highest number of shoot produced (11.55) per shoot tip explant was recorded on 2.5 mg l⁻¹ BAP. In addition, highest percentage of roots (100%) and highest mean number of roots produced per leaf explant (8.47) occurred on medium with combination of 1.5 mg l⁻¹ IBA and BAP by shoot tip explant (4.51). The highest antioxidant activity (70%) and 2 mm inhibition zone in antibacterial activity were observed in leaf extract compared to Callus extract.

Introduction

Brassica is one of the most valuable vegetable families and universally important in the world that includes Broccoli, Cabbage, Cauliflower, Brussels sprouts, Collard and kale; but still not much work has been done on *In vitro* regeneration of this species. However, because broccoli heads easily turn yellow and become unmarketable within 1 to 3 days (Forney, 1995), genetic engineering is required for maintaining its quality. Furthermore, Cruciferous vegetables (such as cabbage and broccoli) contain indole-3-carbinol (I3C) and Isothiocyanates which many studies have proven I3C to be effective in helping prevent various types of cancer (Shukla *et al.*, 2004), with special attention being given to its ability to potentially reduce the risk and/or severity of prostate cancer (Garikapaty *et al.*, 2005). Therefore, researchers are looking for a suitable *Agrobacterium*-mediated transformation system in order to obtain a high ratio of transgenic plants. *In vitro* regeneration offers an opportunity for a rapid production of desirable and essentially genetically identical plants, (Ravanfar *et al.*, 2009; Msikita *et al.*, 1989; Lazzeri *et al.*, 1986). On the other hand, this species is less cultured *In vitro* condition (Ravanfar *et al.*, 2009; Widiyanto *et al.*, 2001), and also tissue-culture techniques have been used extensively for vegetable crops, the efficiency for transferring genes into plants is restricted by the choice of culturing method (Jong *et al.*, 2002). Some of the results showed some success in *In vitro* culture of *Brassica* species from hypocotyls segments, root segments, primary leaf discs, cotyledons and anther (Ravanfar *et al.*, 2009; Cao, 2003).

Various concentrations of auxins such as naphthalene acetic acid (NAA), indole butyric acid (IBA) and Indole acetic acid (IAA) have been evaluated for rooting of *In vitro* regenerated shoots of broccoli and cauliflower (Ravanfar *et al.*, 2009; Maciej *et al.*, 2006; Prem *et al.*, 1999).

Some plants may produce some antimicrobial compounds after pathogen attack such as secondary metabolites being known as phytoalexins. These compounds have demonstrated a striking activity *In vitro* against potential pathogens (Chong *et al.*, 2006). The

medicinal and nutritional values of Asparagus were confirmed in different bioactivity studies as investigations showed it to be a rich source of proteins with gullible activities. For example, antifungal activity of Asparagus was reported due to the presence of a novel Deoxyribonuclease in its seeds (Wang *et al.*, 2001).

The variety of compounds produced *In vivo* and *In vitro* plants can show different bioactivity potentials (Ambreen *et al.*, 2012, Sultana *et al.*, 2011, Ayesha *et al.*, 2010). Grzegorzcyk (2007) reported a higher antioxidant potential for acetone extract of *In vitro* plant. Rafat *et al.*, (2010) and Khorasani *et al.*, (2010) proved that different plant organs and different growth conditions showed different of antioxidant activity.

The main objectives of the present study was to study the effect of different hormones on *In vitro* plant regeneration of this species and potential antioxidant and antibacterial effects of *B. oleracea* var. *italica*.

Materials and Methods

Plant materials: Hybrid broccoli summer green (No.310 F1) supplied by Leckat Corporation SDN Bhd, Malaysia was used. In this study, seeds were stored at 3.5-4°C until used. Seeds were surface-sterilized in 75% (v/v) sodium hypochlorite containing 0.1% (V/V) Tween 20 and shook for 3 min. After five rinses in sterile distilled water, seeds were placed on germination medium containing MS medium (Murashige & Skoog, 1962) supplemented with 20gl⁻¹ sucrose and 3gl⁻¹ Gelrite (Sigma Chemical Co.) without hormone. The cultures were maintained at 25°C under a 16-8 hour's photoperiod light and dark conditions.

Culture medium: For all treatments, MS salts (Murashige & Skoog, 1962) included 3% sucrose, were solidified with 6gl⁻¹ agar and supplemented with various concentrations of IBA and BAP from 0.25 to 3.5 mg l⁻¹ in combinations and singly applied. All media were adjusted to pH 5.8 with 1 N, KOH and autoclaved at 121°C for 20 min. The media was dispensed into 60mm specimen containers in the laminar flow under aseptic condition.

Explant preparation and culture: Explants from vegetative tissues and four-week old *In vitro* seedlings were used as source of explants.

Leaf explants: Leaves were cut into approximately 5 to 8 mm square sections removing leaf ribs and any other major leaf veins. The explants (3 per plate) were arranged horizontally and pressed lightly into the surface of the culture media.

Shoot tip explants: Shoot tips (3 per plate) were cut into 5 to 8 mm, and placed on the surface of the culture medium. Leaf and shoot tip were considered of two experiments with ten replicates. All cultures were incubated at $25 \pm 1^\circ\text{C}$ using a 16-8 photoperiod in culture room. The results were recorded after ten weeks.

Plant extract: The plant samples included *In vivo* and *In vitro* grown plants and callus tissues were dried at room temperature for 4 days. After that, samples were ground to produce fine homogenous powder using an electric blender. The (3 g) fine powder was soaked in 40 ml of

95% ethanol at room temperature for three days before it was filtered through Whatman® No. 1 filter paper (Whatman International, England). The filtered solutions were evaporated to dryness by water bath at 40°C for 24 hours. The plant extracts (g l^{-1}) was dissolved in phosphate buffered saline (PBS) and kept at 4°C until required for the experiments.

Radical scavenging capacity assay: DPPH* (2, 2-diphenyl-1-picrylhydrazil) free radical scavenging capacity assay was obtained using the protocol described by Rafat *et al.*, (2010). DPPH* ($950 \mu\text{l}$) a concentration of $90 \mu\text{M}$ was mixed with $50 \mu\text{l}$ of the plant extracts (10g l^{-1}) and the volume was adjusted to 4 ml using 95% ethanol before incubation at room temperature in the dark for 120 min. Scavenging of DPPH* reduced the colour of the solution and was measured using a spectrophotometer at 515 nm. Comparison of the reduction of colour in the examined samples with the blank (solution without plant extract) was used to measure the potential of scavenging capacity of the plant extracts using the following equation (Rafat *et al.*, 2010).

$$\text{Radical scavenging capacity (\%)} = [(\text{Blank} - \text{Sample A}) / \text{Blank}] \times 100$$

Antibacterial activity assay

Disk diffusion method: Based on Ambreen *et al.*, (2012) sterile paper disks were put in the samples, sterile distilled water and kanamycin antibiotic as control for 2 hours.

Five common bacteria (*Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli* and *Pseudomonas aeruginosa*) were inoculated on the Mueller Hinton Broth (media) and the sterile paper disks were put on the media at certain distance between each other. The bacteria plates were kept in incubator at temperature 37°C overnight.

Results and Discussion

Regeneration: Subsequent to seed germination, 98% of the seeds germinated, the roots were formed three days after germination. Shoots were formed after five days. Two weeks after germination, root length was 6-8 cm and the heights of shoots were 5-6 cm. The vegetative parts such as shoot tips and leaves were cut into sections and were cultured on the media with various hormones for four weeks. Leaves and shoot tips explants were cultured on media containing hormone IBA and BAP at concentration of $0.25\text{-}3.5 \text{mg l}^{-1}$ either singly or in combinations. Leaf explants produced roots, and shoot tips explants formed shoots on MS media with hormone singly or combination after the first sub culture.

After 6 weeks, the highest average number of shoots and roots from shoot tips were counted as 11.28 shoots and 3.71 roots on MS media supplemented with combinations of 2mg l^{-1} BAP and IBA.

The highest average number of roots (Fig. 1) and shoots (Fig. 4) from shoot tip explants were observed (11.55 shoots and 4.51 roots) in MS media containing 2.5

mg l^{-1} BAP and 1.5mg l^{-1} IBA. Furthermore, this value changed to 7.5 roots (Fig. 2) and 9.49 shoots (Fig. 3) in leaf explants when cultured in the same media with combination of 1mg l^{-1} , BAP and IBA. However, in leaf explants, combination of 1mg l^{-1} IBA and BAP showed the highest average number of root formation with 9.49 roots, and this amount decreased to 8.47 roots on MS medium supplemented with of 1.5mg l^{-1} BAP from the same explants (Fig. 2).

On MS medium supplemented with 1.0mg l^{-1} IBA and 1.5mg l^{-1} BAP, the leaf explants showed the best results based on their sizes in diameter and fresh weight (13.34 g) and dry weight of callus (2.78 g). MS medium supplemented with 3.0mg l^{-1} BAP and 1.0mg l^{-1} IBA revealed 97% callus induction from shoot tip explants, and 84% of callus formation observed in combination of 3mg l^{-1} IBA and BAP.

For shoot regeneration and root formation using of leaves and shoot tips as explants, the MS media supplemented with 1mg l^{-1} BAP + 1.5mg l^{-1} IBA and 1.5mg l^{-1} IBA + 2.5mg l^{-1} BAP, respectively showed the best result. The results observed in the present study were in agreement with the previous reports by Prem *et al.*, (1999), Chong *et al.*, (2006), Maciej *et al.*, (2006), Ravanfar *et al.*, (2009), Jiin *et al.*, (2001), Jong *et al.*, (2002) and Rafat *et al.*, (2010).

Regeneration of Broccoli by using of BAP and NAA were examined by Ravanfar *et al.*, (2009) and Prem *et al.*, (1999), according to their results, BAP alone or in combinations with NAA is one of the useful cytokinin for shoot regeneration. Furthermore, Maciej *et al.*, (2006) and Ravanfar *et al.*, (2009) proved that BAP in combination with NAA and 2,4-D are more effective hormones compared with Kn and TDZ.

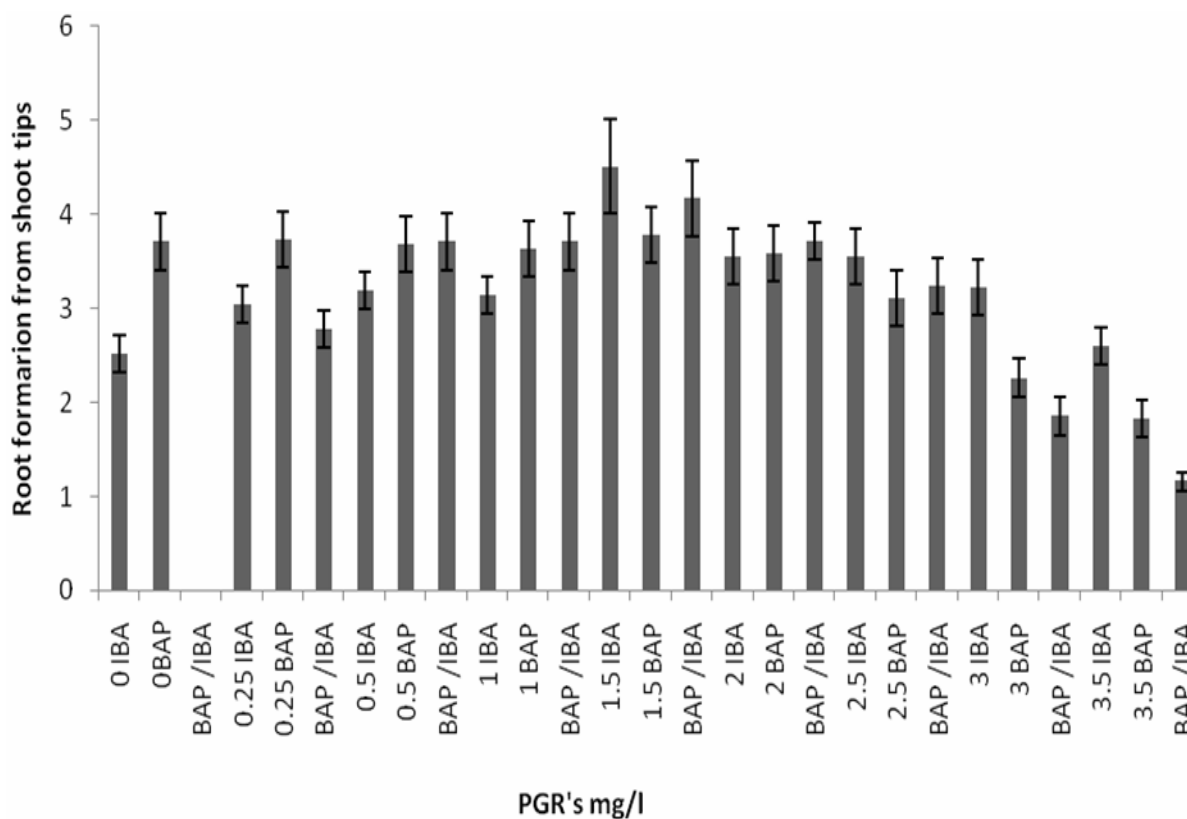


Fig. 1. Effect of BAP and IBA on root formation from shoot tip explants of *Brassica oleracea* var. *italica*.

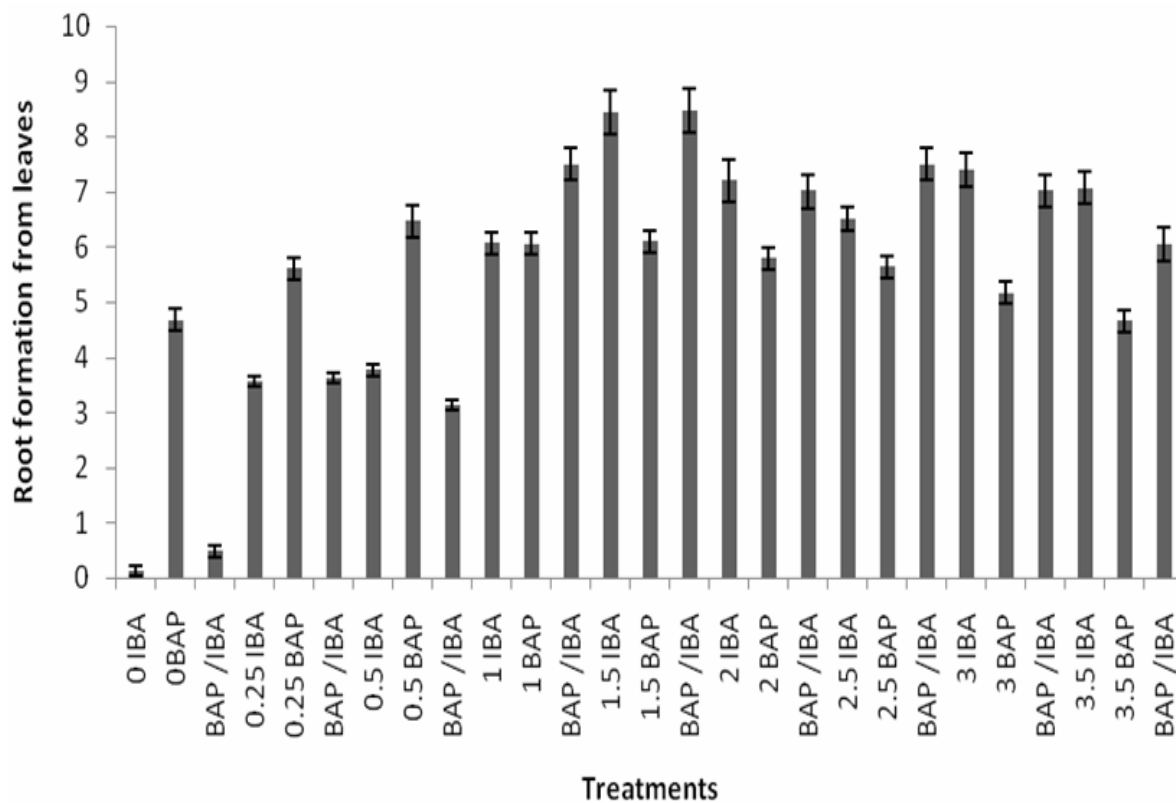


Fig. 2. Effect of BAP and IBA on root formation from leaf explants of *Brassica oleracea* var. *italica*.

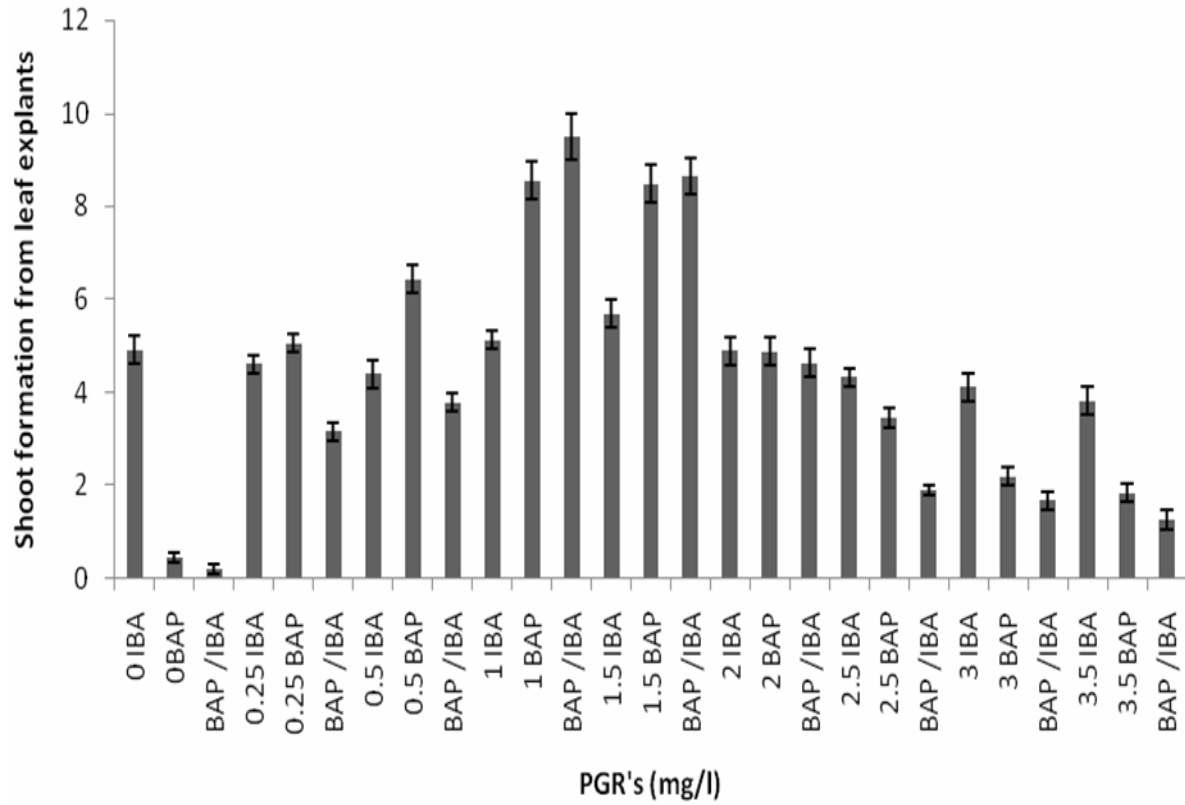


Fig. 3. Effect of BAP and IBA on shoot formation from leaf explants of *Brassica oleracea* var. *italica*.

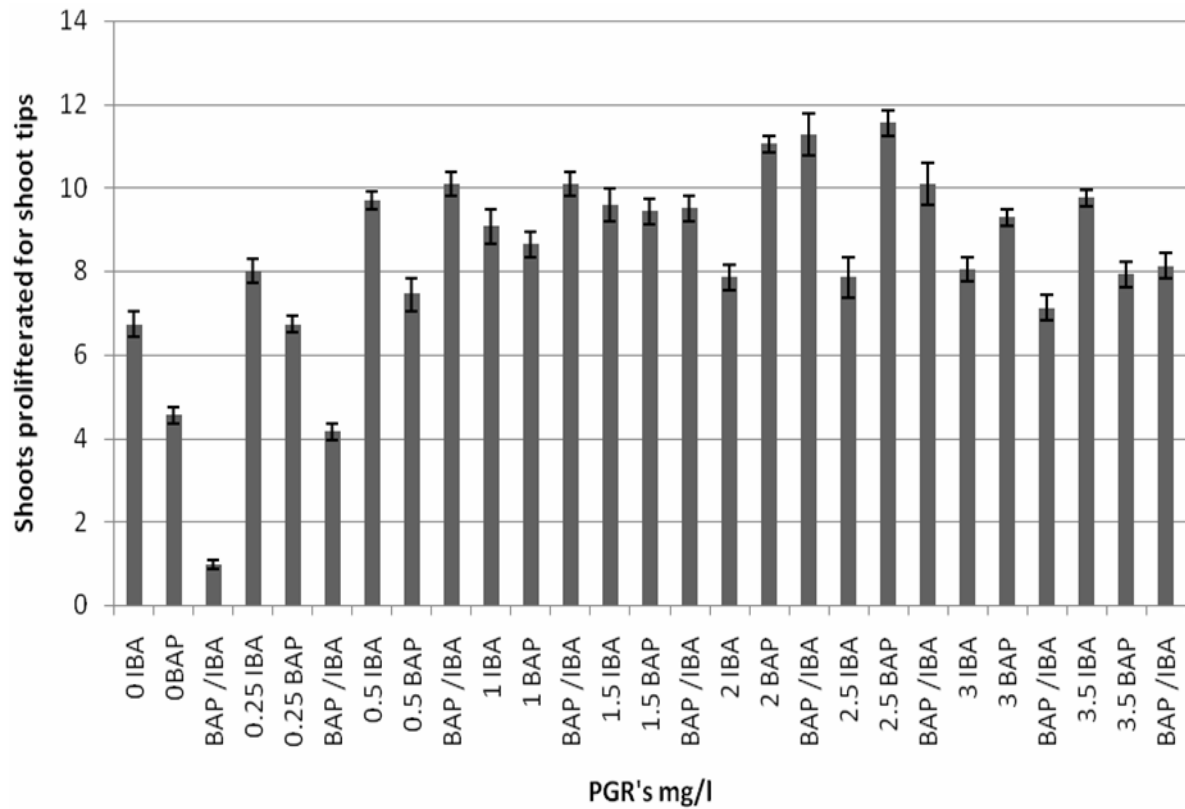


Fig. 4. Effect of BAP and IBA on shoot formation from shoot tip explants of *Brassica oleracea* var. *italica*.

Antioxidant and antibacterial activities: Antioxidant activity of examined plant extracts (*In vivo* plant, *In vitro* plant, and callus) of *Brassica oleracea* was compared with BHT and Ascorbic acid or vitamin C (1 mg l^{-1}) as the positive control. Based on the results in (Fig. 5), the free radical scavenging potential of leaf extract (70%) was higher than callus extracts (46%). In addition, the DPPH free radical scavenging activity were observed lower in two types of explants compared to the positive control, vitamin C (91%) and BHT (86%).

Only leaf extract showed inhibitory zone (2 mm) against the gram-negative bacteria, *Pseudomonas aeruginosa* at concentration of 300 g l^{-1} . This study also showed that leaf extracts gave higher results for antioxidant activity (70%) and 2mm inhibition zone

against gram-negative bacteria for antibacterial properties compared to callus extract (Table 1).

The data were analyzed by one-way ANOVA and the haemolysis percentage means of samples were compared using Duncan's multiple comparison test (DMCT) ($p < 0.05$).

Jong *et al.*, (2002) showed the different effects between antimicrobial activities. According to Rafat *et al.*, (2010) and Jong *et al.*, (2002), different parts of plant produce different compound or different amount of compounds due to their degree of differences in gene expression. Jin-Tzong (2001) proved that a proper stem or leaf processing or treatment to develop a new type of product could enhance the utilization of broccoli.

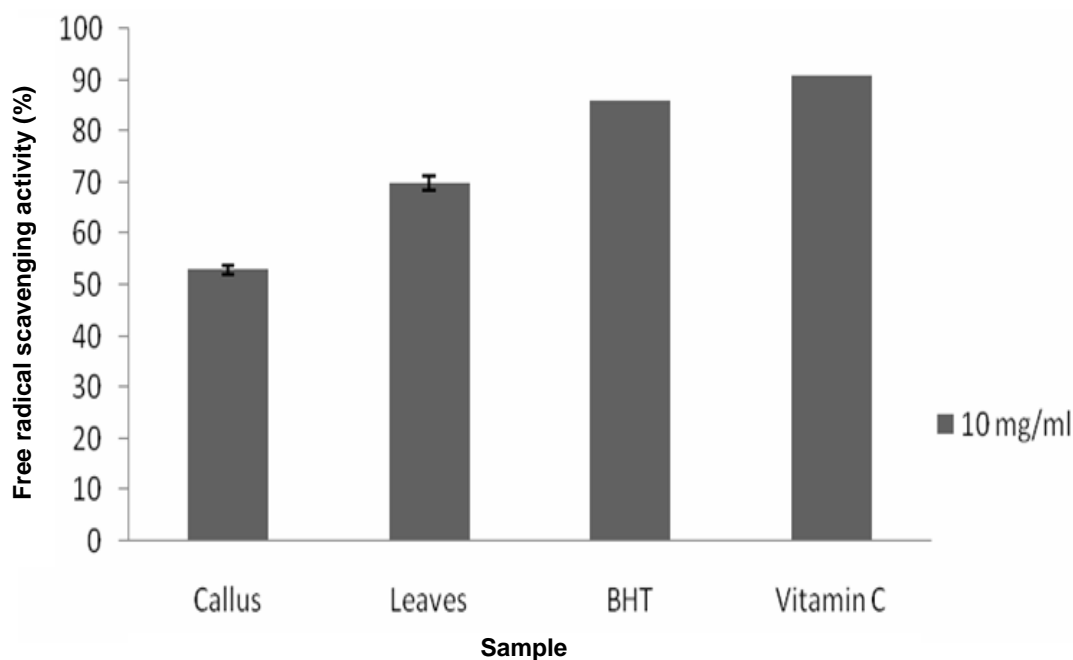


Fig. 5. Antioxidant activity of *Brassica oleracea* var. *italica* based on free radical scavenging activity.

Table 1. Inhibition zone in mm (5mm diameter of disk) as the means of triplicate of experiments.

Tested microorganisms		Types of explants	Concentration of extracts (g l^{-1})	Inhibitory zone(mm)
Gram positive bacteria	<i>Staphylococcus aureus</i>	Callus extracts	100	-
			300	-
		Leaves extracts	100	-
			300	-
	<i>Bacillus cereus</i>	Callus extracts	100	-
			300	-
Leaves extracts		100	-	
		300	-	
Gram negative bacteria	<i>Escherichia coli</i>	Callus extracts	100	-
			300	-
		Leaves extracts	100	-
			300	-
	<i>Pseudomonas aeruginosa</i>	Callus extracts	100	-
			300	-
		Leaves extracts	100	-
			300	7

- = no inhibitory zone

Conclusion

Combinations of 1mg l⁻¹ BAP and IBA on MS media showed the highest average numbers of root and shoot formation in leaf explants and combinations of 2.5mg l⁻¹ BAP and 1.5 mg l⁻¹ IBA on the same media showed the highest average of root and shoot in shoot tip explants. Only 300g l⁻¹ of leaf extract showed inhibitory zone against the gram-negative bacteria. The free radical scavenging potential of leaf extract observed to be higher than callus extracts.

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