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In vitro propagation and antioxidant properties of hatkora (*Citrus macroptera*) from North-East India

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ABSTRACT

Satkora or hatkora is found confined in evergreen forests of N. E. India primarily are of seedling origin, therefore show a tremendous variation in their morphology among its population. Due to lack of selection of any superior germplasm, the farmers have been planting trees of seedling origin of unknown yield potential and quality. Since this method could not meet the demands of planting material, an investigation was carried out during 2016-17 at department of Biotechnology, Mizoram University to develop rapid and cost-effective *in vitro* protocols for propagation of citrus hatkora with the objective of enhancing the rate of multiplication and for maintaining the stocks of germplasm for many years. *In vitro* organogenesis of *C. macroptera* was done with the use of leaf and node explants derived from in vitro raised plants. The explants were inoculated on Murashige and Skoog media supplemented with NAA, BAP, 2,4-D and Kinetin alone or in combination. The investigation revealed that full strength MS medium is the best for in vitro hatkora seed germination. Further, maximum callus formation from the excised plant was observed on 2,4-D (2mg/L) and shoot formation on BAP (2mg/L). In addition, the antioxidant properties and the total phenolic content of the fruits was determined using was determined using DPPH method Folin-ciocalteau method revealed that the peel of *hatkora* fruit has maximum content. Therefore, There needs to be renewed focus on organogenesis using vegetative tissues like leaf explant. Diversity analysis and marker-assisted selection will go a long way in fastening the breeding and germplasm management. Research focus should be done on identification of chemical structure of all the bioactive compounds present on this high valued fruits for future use of humankind.

Key words : Antioxidant, *Citrus macroptera* Mont., *hatkora, in vitro* propagation

INTRODUCTION

The genus *Citrus* L., the sole source of the *Citrus* fruits of commerce, belongs to the orange sub family Aurantioideae of the family Rutaceae are well known for their dietary, nutritional, medicinal and cosmetic properties and are also good sources of citric acid, flavonoids, phenolics, pectins, limonoids, ascorbic acids and antioxidants (Chen *et al.*, 2017; Ahlawat *et al.*, 2018). The genus includes some of the most commercially important fruits *viz.*, mandarin (*Citrus reticulata* Blanco), sweet orange [*Citrus sinensis* (L.) Osbeck], grapefruit (*Citrus paradisi* Macf.), lemon [*Citrus limon* (L.) Burm. f.] and lime [*Citrus aurantiifolia* (Christm.) Swingle].

India enjoys a remarkable position in the "*Citrus* belt of the world" due to her rich wealth of *Citrus* genetic resources, both wild and cultivated. In India, *Citrus* represents the third most important fruit next to mango and banana and comprise about 12.41 per cent of total fruits produced in the country (Anonymous, 2017; Sangma *et al.*, 2020). North-eastern region of India is endowed with favourable agro-climatic conditions for the growth of different *Citrus* species and is

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considered as the natural home of many *Citrus* species (Chaturvedi *et al.*, 2018). A natural and undisturbed population of *Citrus* gene pool observed during collection trips from time to time confirms the assumption that this area might be the centre of origin of several *Citrus* species. A vast reservoir of *Citrus* diversity exists in wild, semi-wild form and is found scattered here and there without commercial cultivation and much care (Hazarika, 2012). *Citrus* plants growing in deep forests undisturbed by abiotic factors have also been reported from the region, thus bestowing this area with a special status of "treasure house" of *Citrus* germplasm (Sunaina *et al.*, 2018; Barbora *et al.,* 2020). As per IUCN norms, seven Indian *Citrus* species fall under the category of endangered species as indicated by threat perception analysis which include *C. indica* Tanaka, *C. macroptera* Mont., *C. latipes* Tanaka, *C. assamensis* Dutta et Bhattacharya, *C. ichangensis* Swingle, *C. megaloxycarpa* Lushington, and *C. rugulosa* Tanaka, (Hazarika *et al.*, 2017). Two species, *C. indica* and *C. macroptera* need special and immediate attention for conservation due to their endemism and high degree of threat perception (Majumdar *et al.*, 2019).

Citrus macroptera Mont., commonly known as "satkora" or "hatkora" is found confined in evergreen forests of N. E. India and moist deciduous forests of the north Himalayas and Assam (Hazarika *et al.,* 2017). A rich genetic diversity of "hatkora" exists in north-eastern region of India (Lala *et al.*, 2020). It is also reported of growing this species in semi wild form in Shella and Dawki area near Cherrapunjee in Meghalaya, Chandel district of Manipur and Mizoram. In Mizoram, *hatkora* plants are found naturally in marginal lands, forest areas and homestead gardens in semi wild and wild state. Although, this species have wider distribution in Mizoram but the commercial cultivation is confined mainly in Kolasib, Aizawl, Lunglei, Mamit, and Serchhip district, where the elevation and agro-climatic condition are lower and warmer which is suitable for its growth and yield performance.

Citrus macroptera Mont. is one of the important and popular citrus species known for its medicinal as well as therapeutic values since ancient time. Fruit is smaller than grapefruit; contains low juice, bitter in taste. The rind of the fruit can be used for flavouring dishes which has unique taste and aroma. The juice is extracted from the pulp of the fruit which when diluted with water can serve as a refreshing drink. The fruit has significant cytotoxic, antimicrobial, antihypertensive, antipyretic, and appetite stimulant potentials and is postulated to play a therapeutic role in reducing the risk of some forms of fatal oxidative stress disorders, such as liver and kidney dysfunction, cardiovascular disease, and stroke. Because of its use in traditional medicines and presence of good amount of anti-oxidants, now a day *hatkora* is gaining worldwide popularity.

Plant tissue culture has come into view as a powerful tool for propagation and improvement of many woody plant species including *Citrus*. The genetic and epigenetic mechanism of callus formation, the widespread use and knowledge of molecular mechanisms and the underlying induction of callus, deserve to be studied systematically. *In vitro* culture has the potential to eradicate diseases and provides scope for development of new cultivars through somaclonal variations (Iqbal *et al.*, 2019). Despite its rich genetic resources, scientists come across difficulties in citrus hybridization breeding due to high sterility, heterozygosity, incompatibility and nucellar embryos. Micro propagation is an important technique in citrus because it ensures maximum genetic uniformity of the resulting plants. Therefore, to build-up the stock of this very precious planting material, micropropagation assume significance and remains the only viable alternative. There are different strategies for micropropagation of *Citrus*, viz., somatic embryogenesis, adventitious shoot bud production and axillary enhancement which are routinely used. Among these, axillary enhancement using nodal segment as explant is considered best as it does not involve a callus phase, thus minimize the risk of somaclonal variation and it offers economically optimum multiplication rate. However, efforts have not yet been made to develop a method for micropropagation in *Citrus macroptera.* The rapid and cost-effective *in vitro* protocols for propagation of *Citrus macroptera* would be of great interest in this regard with the objective of enhancing the rate of multiplication and for maintaining the stocks of germplasm for many years.

In *Citrus*, both peels and seeds are an interesting source of phenolic compounds, which include phenolic acids and flavonoids. Flavonoids are represented in citrus fruits by two very classes of compounds: the polymethoxylated flavones and the glycosylated flavanones. Many flavonoids derived from citrus fruits have been reported to reduce oxidative stress, improve glucose tolerance and insulin sensitivity, modulate lipid metabolism and adipocyte differentiation (Gandhi *et al.*, 2020). Although in many commercial species of citrus, research on flavonoids and antioxidant components have been done elsewhere, but till now there is no any published work on these aspects of this highly medicinal species. With the above background informations, the present studies have been undertaken to develop a standard protocols for *in-vitro* germination of hatkora and to compare the antioxidant properties and phenolic contents among different parts of hatkora fruits.

MATERIALS AND METHODS

In vitro Seed Germination

The present experiment was carried out during 2016-17 at Department of Biotechnology, Mizoram University to develop an effective protocol for micro-propagation of *Citrus macroptera*, an endangered Citrus species of the world. Mature fruits of *Citrus macroptera* Mont. collected from farmer's field of Kolasib, Mizoram, India, in the month of December. The seeds were extracted by manual extraction the next day after measuring the size of each fruit.

The seeds were washed with water till the slipperiness is removed. After washing, the explants were soaked in 70% ethanol for 15 second. Ethanol was washed off with water for 2 or 3 times then they are taken inside the laminar air flow. The explants were then soaked with 1% Sodium hypochlorite for 15 minute and washed with sterile distilled water thrice. Again soaked in 0.1% mercuric chloride for 15 minute and washed with sterile distilled water thrice. The explants were picked up and wiped dry with sterile filter papers. The hard outer cover of the explants was removed with sterile blades and forceps while for some explants the coats were not removed. The explants were again put in sterile filter paper for proper drying. The explants were inoculated in the sterile media inside the jar. The jars were sealed with parafilm and the jars were transferred to a culture room maintained at 26±2°C and a photoperiod of 16/8 hrs of light/dark.

Tissue Culture

In vitro organogenesis of *C. macroptera was* done with the use of leaf and node explants derived from *in vitro* raised plants (Table 1). The explants were inoculated on MS 3.4 g/L (Murashige and Skoog, 1962) media supplemented with different concentrations of plant growth regulators (PGRs). In this experiment, NAA, BAP, 2,4-D and Kinetin alone or in combination were used. The treatment details are given in Table 2. All the media supplemented with 7 g/L agar. The pH of all the media were adjusted to 5.8 prior to addition of the agar and approximately 25 ml of the medium was dispensed into petriplate (Plates 1 to 6). The medium was autoclaved at 121°C for 20 minutes under 15 lb psi pressure. The chemical used for the experiment were manufactured by M/S Hi Media, Mumbai, India. Each cultured petri plate received 4 explants. Three replicates were taken for each treatment. The cultured were maintained in growth chamber at 25±1°C under 16/8 hr. (light/dark) photoperiod with light intensity of 1000 lux. The explants which showed response were transferred into media containing NAA and BAP. Sub culturing was done after every two to three weeks.

Extract Preparation

For the evaluation of antioxidant activity and total phenolic content, the dried plant parts and the juice were used. The dried plant parts were ground to fine powder with an electrical grinder and also the juice were extracted freshly. The extracts were prepared with distilled water and methanol (60%) in 1:10 (w/v).

2,2-diphenylpicrylhydrazyl (DPPH) Radical Scavenging Activity

Antioxidant property was determined using DPPH method. DPPH radical scavenging activity was carried out according to Leong and Shui (2002) with slight modifications. To various extracts (100 µL) of *C. macroptera*, 10 ml of methanolic solution of 0.3 mM DPPH (3.144 mg of DPPH was weighed and dissolved in 30 ml of methanol) was added. The mixture was then allowed to stand in the dark for 30 minutes and absorbance was measured at 518 nm. Methanol was utilized as the blank.

The DPPH radical scavenging activity was expressed as the inhibition percentage of free radical by the sample and was calculated using the formula:

DPPH radical scavenging activity (%) = (Control OD-Sample OD/Control OD) × 100

Determination of Total Phenolic Content

The total phenolic content was determined using Folin-ciocalteau method (Singleton and Rossi, 1965). 50 μL of plant extracts were mixed with 0.5 mL of folin-ciocalteau reagent (diluted ten-fold). The mixture was then incubated for 5 minutes before addition of 2.5 mL of 7.5% sodium carbonate (7.5 g of sodium carbonate was dissolved in 100 mL of distilled water). After 30-45 minutes of incubation at room temperature absorbance was measured at 760 nm using UV-Visible spectrophotometer. A mixture of distilled water, folin-ciocalteau reagent and sodium carbonate was used as the blank.

RESULTS AND DISCUSSION

Tissue Culture

When individual levels of 2,4-D (1 and 2 mg/L) was added in MS basal medium, the minimum time taken for callus induction was recorded. This might be because 2,4-D is a synthetic auxin which promote the growth of calli, cell suspensions, and organs and also regulate the direction of morphogenesis. At the cellular level, auxins control basic processes such as cell division and cell elongation. Since they are capable of initiating cell division, they are involved in the formation of meristems giving rise to either unorganized tissue or defined organs. Our study is in the line of conformity of the findings of Fathil *et al.* (2017) in *Citrus*.

The minimum number of days for shoot initiation as well as maximum shoot length was recorded on MS basal media supplemented with BAP 2 mg/L whereas the higher dose of BAP 4 mg/L had negative effect on regeneration (Table 3; Plates 1 to 9). Our results are in close conformity with the findings of Goswami *et al.* (2013) who reported that among individual levels of BAP and kinetin maximum number of shoots were recorded

Table 1. Treatments used for the study

Treatments	PGRs ((mg/L) MS media supplemented with			
	BAP	Kinetin	NAA	2,4-D
T_1	0	0	0	0
T_2	0	0	0	1
T_3	0	0	0	2
T_4	2	0	0	0
T_5	4	0	0	0
T_6	0	2	0.5	0

Table 2. Effect of plant growth regulators on leaf and node explants

Plant Growth regulators (mg/L) MS media supplemented with				Explant	No. of days taken for response	Callus (%)	Shoot (%)
BAP	Kinetin	NAA	2,4-D				
0	0	0	0	Leaf	-	-	-
				Node	-	-	-
0	0	0	1	Leaf	12	58.33	-
				Node	16	25	-
0	0	0	2	Leaf	10	100	-
				Node	13	66.67	-
2	0	0	0	Leaf	-	-	-
				Node	14	-	66.67
4	0	0	0	Leaf	-	-	-
				Node	8	-	41.67
0	2	0.5	0	Leaf	-	-	-
				Node	11	-	66.67

(-) shows absence of data.

with 0.1 mg/L BAP and 0.5 mg/L kinetin, respectively but BAP was found to be superior for shoot multiplication than kinetin.

The survival of explants (66.67%) was observed on MS media supplemented with kinetin 2 mg/L + NAA 0.5 mg/L. Kumar *et al.* (2013) reported that addition of kinetin alone in MS basal medium gave maximum survival of explants (80%) at 1.0 or 2.0 mg/L. Among BAP levels, the maximum (80%) survival of explants was recorded at 1.0 mg/L. Our study is in the line of conformity with the findings of Shah *et al.* (2020) in *Citrus* limon.

In-vitro Seed Germination

Seed germination is a response characterized by three param-eters—percentage, rate, and uniformity. Germination percentage is the number of seeds from a popula-tion that germinate. Germination rate is the "speed or velocity" of germination and can be expressed as the time it takes for a defined percentage of seed to germinate. Germination uniformity is a measure of the time it takes for all of the seeds to germinate (Niedz *et al.*, 2008).

From the present investigation, it was observed that the coated seeds did not germinate in any of the media while coated seeds germinate. The reason for germination difficulties may be due to the presence of hard seed coat which prevents germination. In both coated and uncoated seed the germination might have been better with the presence of plant growth regulators. It was also observed

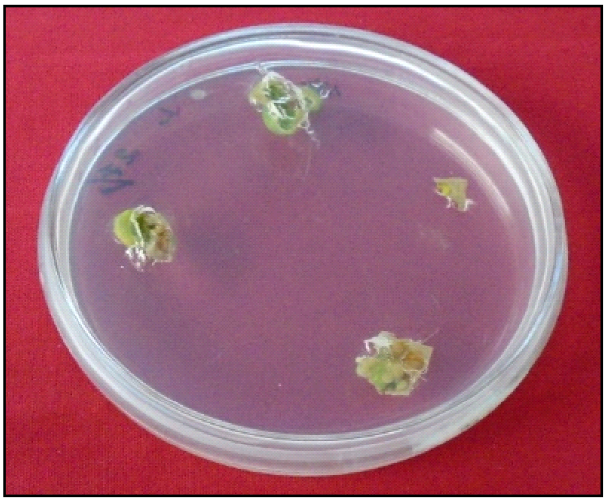

Plate 1. Supplemented with 2,4-D 1 mg/L. Formation of callus.

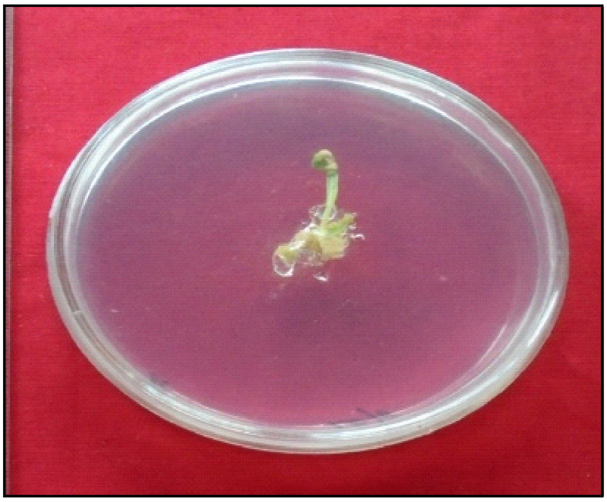

Plate 3. Supplemented with BAP 2 mg/L. Formation of shoot.

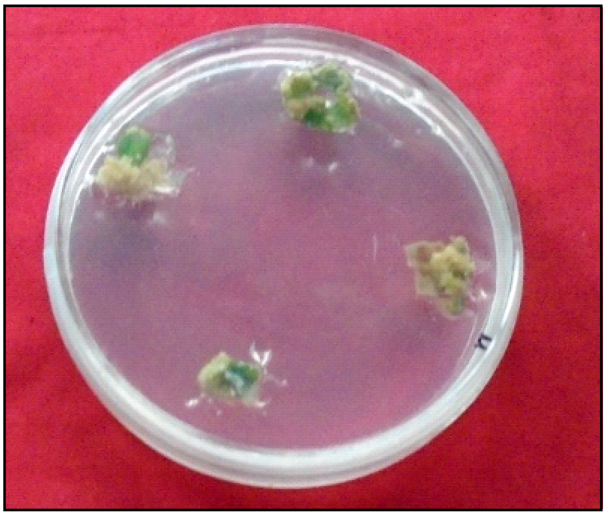

Plate 2. Supplemented with 2,4-D 2 mg/L.

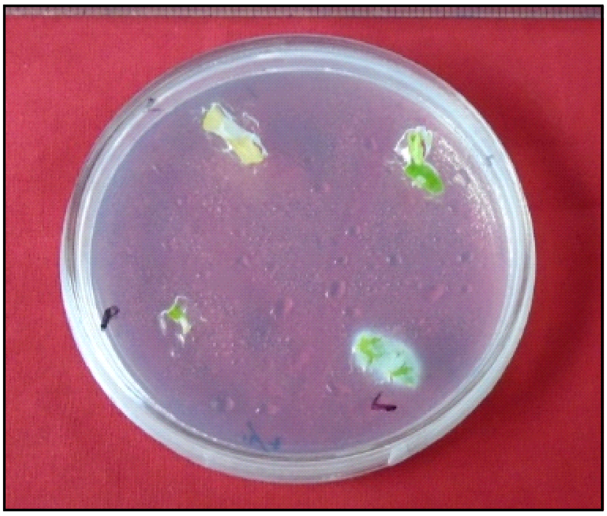

Plate 4. Supplemented with BAP 4 mg/L.

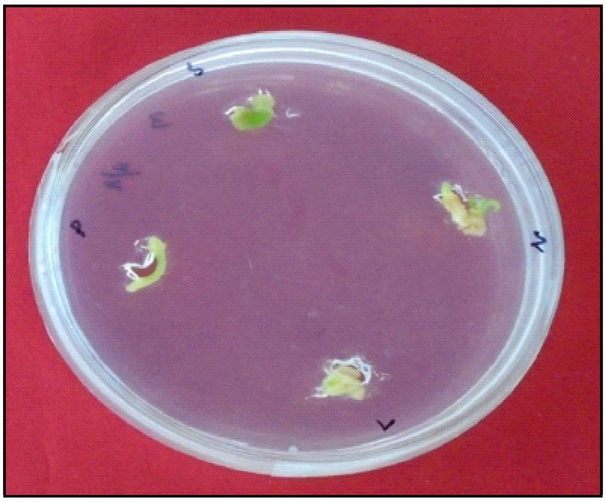

Plate 5. Supplemented with Kinetin 2 mg/L + NAA 0.5 mg/L.

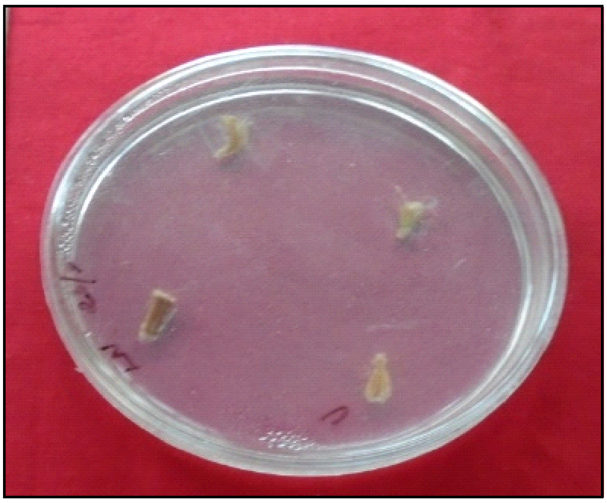

Plate 6. No response on MS basal medium.

Plate 7. Seed grown on Agar medium.

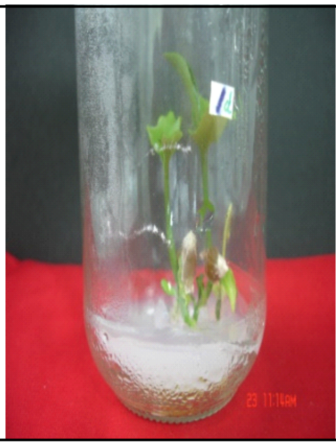

Plate 8. Seed grown on half strength MS medium.

Plate 9. Seed grown on MS medium.

that the seeds sown on full strength MS medium give the best germination and seedling growth as compared to the seeds sown on half strength MS and Agar. Since MS media contains the highest amount of nutrients required by plants the response of the seed is best as compared to other media used.

DPPH Radical Scavenging Activity

The anti-oxidant content of methanolic and aqueous extracts of fruit parts are shown in Figs. 1 and 2. *In vitro* antioxidant assay of *C. macroptera* extracts revealed the presence of antioxidant potential. Various extracts of fruit parts showed different results in the scavenging of DPPH radicals as indicated by the discoloration of DPPH. From both the methanolic and aqueous extracts of plant parts, maximum scavenging was observed from

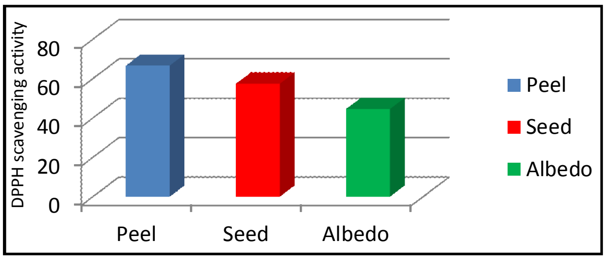

Fig. 1. DPPH radical scavenging activity of methanolic extract of plant part.

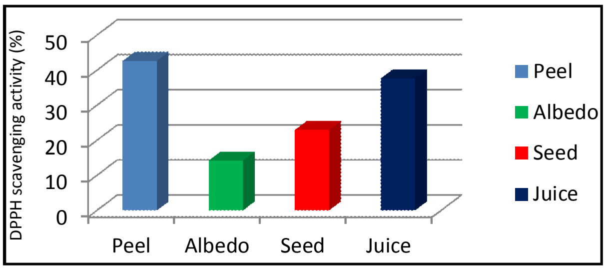

Fig. 2. DPPH radical scavenging activity of aqueous extract of plant parts.

Table 3. Morphological changes of *C. macroptera* explants (seed) on different media

Media	S. No.	Response								No. of explants showing multiple shoot
		Bulging		Cotyledon formation		Root formation		Shoot formation		
		Day of initiation	(%)	Day of initiation	(%)	Day of initiation	(%)	Day of initiation	(%)	
Agar	1	5th	50	13rd	50	17th	50	26th	50	-
	2	6th	50	14th	50	16th	50	25th	50	-
½ MS	1	4th	80	11th	80	14th	80	24th	80	1
	2	5th	80	10th	80	14th	80	23rd	80	1
	3	6th	50	10th	50	15th	50	24th	50	-
MS	1	3rd	100	8th	100	14th	100	20th	100	2
	2	3rd	80	9th	80	13th	80	19th	80	1
	3	4th	100	8th	100	12th	100	20th	100	-

(-) shows absence of data.

the fruit peel (66.7%, 42.71%) followed by seeds (44.60%, 22.96%) whereas albedo portion showed minimum scavenging activity. The scavenging activity of juice was observed as 37.71%. Citrus peels and seeds have an interesting antioxidant activity with regard to citronellal. Their extracts could be useful to prevent oxidation in fruit juices and essential oils. Our results are in close conformity with the studies of Prakash *et al.* (2016) where they reported different antioxidant properties in different parts of Citrus pulp and peel.

Phenolic Content

The phenolic content of methanolic and aqueous extract of fruit parts are shown in Figs. 3 and 4. The phytochemical analysis conducted on various extracts of *C. macroptera* revealed the presence of significant amounts of phenolic compounds. The presence of phenolic compound was found to be highest in both the methanolic and aqueous extract of peel followed by seed.

The higher phenolic compounds in the dried peel and seed might be due to the fact that the phenolic compounds in fruits are in the free form and/or associated with polysaccharides of the cell wall through hydrogen bonds between the hydroxyl groups of the phenolic compounds and the oxygen atoms of the polysaccharides (Pinelo *et al.*, 2006), and with drying there is a change in the nutritional value and in the physical and structural properties of fruits and vegetables. Even when this occurs at mild temperatures (50°C for orange peel, 45°C for grape and jabuticaba peel), polymers in the cell wall are destroyed, particularly pectic substances, but in lesser amounts than at high temperatures (Ebun and

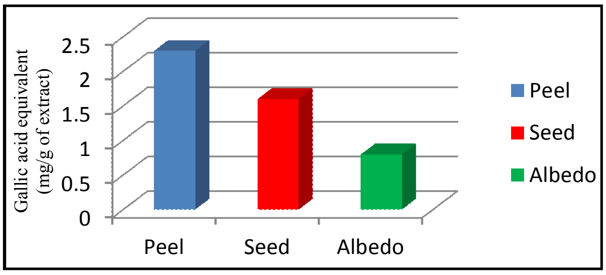

Fig. 3. Phenolic content of methanolic extract of plant parts.

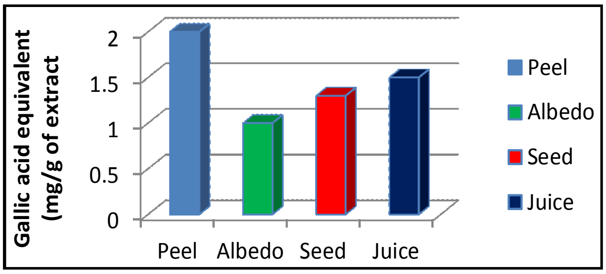

Fig. 4. Phenolic content of aqueous extract of plant parts.

Santosh, 2011). Removal of water from the hatkora fractions may therefore have caused degradation of the cell wall, with the consequent hydrolysis releasing linked phenolic compounds and making them soluble, facilitating their extraction and resulting in an increase in the levels of the compounds seen in the dried peel and seed. Recent works, reported that the total phenolic and favonoid compounds in the methanolic extracts of *Citrus* varied signifcantly depending on the different parts of the fruit used (Haraoui *et al.*, 2020).

CONCLUSION

From the results of the present investigation, it can be concluded that among all the media used, full strength MS was the best medium for germination and growth of *hatkora* seed. The investigation on tissue culture of *in vitro* propagated seeds revealed that 2,4-D induce callus formation and BAP promotes direct shoot formation. The present investigation revealed that the extracts of *hatkora* peel has the highest amount of antioxidant property and phenolic content. Future research should be oriented to develop full tissue culture protocol for complete regeneration of *Citrus macroptera*. There needs to be renewed focus on organogenesis using vegetative tissues like leaf explant, ensuring explanting year-round. Diversity analysis and marker-assisted selection will go a long way in fastening the breeding and germplasm management. In addition to this, there is still research needs for identification and characterization of all the antioxidants and phenoloics present in this endangered citrus species. Research focus should be done on identification of chemical structure of all the bioactive compounds present on this high valued fruits for future use of humankind.

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