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IN VIVO AND *IN VITRO* EVALUATION OF NUTRACEUTICALS FROM UNDEREXPLOITED *LIMMONIA ACIDISSIMA* (L.) GENOTYPES

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Limonia acidissima (L.), a nutrient dense but underutilized fruit crop, has potential for value addition and commercialization. In present investigation, two genotypes of *Limonia acidissima* were procured from wild region of Bikaner and Bharatpur district (Rajasthan). *In vitro* cultures were established on MS medium supplemented with various concentrations and combinations of PGRs. Different plant parts (bark, leaves, fruit shell, pulp and seeds), callus and *in vitro* shoots were evaluated for their nutritional primary metabolites quantitatively. Maximum amount of total soluble sugar, reducing sugar, non-reducing sugar, starch, protein and ascorbic acid content was observed in pulp of both the genotypes.

Key words: *in vitro* cultures, *Limonia acidissima,* Nutritional metabolites.

Introduction

Plants have great importance due to their nutritive and medicinal value. India is origin of many fruit crops and the most of crops is limited to its growing region only. Most of underutilized fruits are in the core recipes of many ayurvedic formulations[1]. *Limonia acidissima* (L.), also known as woodapple and, kathbel belongs to family Rutaceae is a rare and an endangered tree species but equally a valued plant for its edible fruits and immense nutritional and medicinal properties. This nutrient dense but underutilized fruit tree has potential for value addition and commercialization. The different parts of plant (leaf, stem, bark, fruit, and seed) have been used for medicinal purposes[2]. The fruit is used for curative properties, which make the tree one of the useful medicinal plants of India. It has curative value for various diseases of bones and joints, bilious diseases, prevention of capillary bleeding, cold, influenza, piles, dysentery, habitual constipation and scurvy. The fruit is also used as a liver and cardiac tonic, diarrhoea and dysentery[3].The review of literature suggests many diverse pharmacological properties of the fruit including anti-diabetic[4], anti-ulcerative[5], wound healing[6], anti-tumour[7] and anti-microbial activity[8]. Due to rich in vitamin C, it is useful in preventing and curing scurvy and also serves as excellent source of antioxidant. The fruit pulp has long shelf life and can be used for the preparation of different value added products like preserve, candy, sherbet, juice, chutneys, jam, jelly and squash but inspite of all it is still under unexploited because of lack of awareness regarding its significance with respect to their nutritional and therapeutical values. The value added products of these fruits have expected to catch the national and international markets if it is properly

focused. Plant cell and tissue culture technology has been considered as a powerful tool for the biomass and bioactive compound production from medicinal plants. In the past decade, tremendous progress has been made in this area and its importance has rapidly increased because of increased need for medicinal plant substances as sources of medicine and health food ingredients[9]. Many studies have been conducted on the *in vitro* cultures for biomass and primary metabolite production for commercial purposes,[10]but so far *L. acidissima* has not been investigated for its *in vivo* and *in vitro* production of metabolites. Thus the present study has been undertaken to evaluate some nutritional metabolites *viz.* carbohydrates (total soluble sugar, reducing sugar, non-reducing sugar and starch), proteins and ascorbic acid (vitamin c) in two genotypes of this underutilized fruit crop *in vivo* and *in vitro.*

Material and Methods

Collection of plant material:

Plant samples of *Limonia acidissima* were randomly collected from wild region of Bikaner and Bharatpur district (Rajasthan) and botanically authenticated from Herbarium (Voucher specimen D.C.M.1850: DCB NO.-1219; BSI-3355), Department of Botany, Govt. Dunger college, Bikaner, Rajasthan.

Establishment of *in vitro* cultures:

Various explants *viz*. Epicotyl, hypocotyl, cotyledon and internodal segments were excised from 6-8 week old seedlings. These explants were then established and maintained by frequent subculturings after 4 weeks on MS Medium supplemented with various concentrations and combinations of kinetin and 2, 4-D for callus induction and kinetin and BAP for induction of multiple shoots. Cultures were maintained in growth chamber with regulated temperature (26±2^{o}C), relative humidity (55±5%), and light 3000 lux intensity. Data was recorded after 2, 4, 6, 8 and 10 weeks and growth indices were calculated. *In vitro* cultures were harvested at maximum GI for evaluation.

Primary metabolite estimation:

Each of the plant parts (bark, leaves, fruit shell, pulp and seed) and *in vitro* cultures were harvested at maximum GI dried, powdered and evaluated quantitatively to estimate the total content of carbohydrates (total soluble sugar, reducing sugar, non-reducing sugar and starch), protein and ascorbic acid (vitamin c) . All experiments were performed in triplicates and values were expressed as mean ± standard deviation.

Estimation of Carbohydrate:

Each of the dried samples were weighed, homogenized separately in 90 percent ethanol, centrifuged at 4000 rpm for 20 minutes and then refluxed over a steam bath for 4 hours. The extract was cooled, and recentrifuged at 3000 rpm for 10 minutes. The supernatant was collected and the residue was homogenized again in 90% (v/v) ethanol and refluxed for 1 hour and the extract was again centrifuged. The supernatant was combined with the original and dried *in vacuo*. The dried residue was dissolved in 10ml distilled water and aliquots were taken from this for estimation of total soluble sugar by procedure of Yemm and Willis[11], and total reducing sugar according to the Nelson-Somogyi method [12,13]. Starch was determined in the dry residue left after sugar extraction by the method of McCready[14]. The absorbance was measured using a UV-VIS Spectrophotometer C-119 (Systronics) set at 620 nm respectively against blank.

Estimation of Protein:

Each of the dried samples was homogenized separately in 5ml of phosphate buffer and centrifuged at 5000 rpm for 10 min. Supernatant was collected and repeated the extraction three times. Supernatants

combined and the volume was made up to 25 ml with phosphate buffer. The extract was centrifuged in 10% cold trichloro acetic acid (TCA) in equal volume (1ml phosphate buffer: 1ml 10% TCA) at 5000 rpm for 10 min. Supernatant discarded and pellet was saved. Pellets were again suspended in 5ml of cold 10% TCA and re-centrifuged for 10 min. Supernatant was again discarded and the precipitate was dissolved in 5 ml of 0.1 N NaOH and aliquots were taken from this for estimation of protein content following the procedure of Lowry [15].

Estimation of Ascorbic acid:

Each of the dried samples was weighed, crushed in ice cold CO_2 saturated water and the extract was made to a definite volume. Three ml of extract was mixed with an equal volume of buffered metaphosphoric acid. Two ml aliquot of this solution mixed with 5 ml distilled water served as reference and 2 ml aliquot in another test tube was then mixed with 5 ml of 5% 2, 6 dichlorophenol indophenol. The optical density was measured by UV-VIS spectrophotometer C-119 (Systronics) set at 546 nm against reference and the amount of ascorbic acid was calculated following the procedure of Chinoy[16].

Results and Discussion

In vitro studies:

Varied responses were observed on MS medium supplemented with different concentrations of various auxins and cytokinins. Best response towards *in vitro* multiple shoot regeneration was obtained on MS medium supplemented with BAP (0.5mg/L) and Kinetin (0.5mg/L) and profuse callusing was obtained on MS Medium Supplemented with 2,4-D (1.0 mg/L) and Kinetin (0.5mg/L). The callus was fragile, creamish green and fast growing.

Carbohydrates:

As depicted in table-1 and Fig-1 more amount of total soluble sugar, reducing sugar and non-reducing sugar was observed in BIK-S1genotype as compared to BRP-S2 genotype. Among all the plant samples tested maximum amount of total soluble sugar, reducing sugar and non-reducing sugar content was observed in pulp whereas minimum amount was found in *in vitro* shoots of both the genotypes. Highest amount of total soluble sugar (22.69±0.107 mg/g.d.w), reducing sugar (15.72±0.106 mg/g.d.w) and non-reducing sugar (6.97±0.46 mg/g.d.w) were observed in pulp of BIK-S1 genotype while lowest amount of total soluble sugar (2.68±0.12 mg/g.d.w), reducing sugar (1.08±0 .085 mg/g.d.w) and non-reducing sugar (1.60±0.21 mg/g.d.w) were observed in *in vitro* shoots of BRP-S2 genotype. Callus cultures of both the genotypes showed significantly more amount of total soluble sugar, reducing sugar and non-reducing sugar than *in vitro* shoots and *in vivo* bark as well.Maximum amount of starch was found in pulp and minimum in fruit shell of both the genotypes. Highest starch content was found in *in vitro* shoots (30.32 ±0.163 mg/g.d.w)of BRP-S2 genotype and minimum in fruit shell (2.68 ±0.053 mg/g.d.w) of BIK-I genotype. More amount of starch was observed in pulp, leaves and bark of BIK-S1 genotype whereas in case of BRP-S2 genotype more amount was observed in fruit shell, seed and callus. *In vitro* cultures showed appreciable amount of starch when compared with *in vivo* plant parts. However Vijayvergiya[17] reported highest amount of starch content (18.4mg/gdw) and sugar content (52±0.81mg/gdw) in stem of *L. acidissima*. Singh[18]evaluated maximum starch content in pulp (3.6g/100g) followed by seed (3.1g/100g) and leaf (1.3g/100g) in *Aegle maemelos* .

Protein:

Presence of protein content was found to be comparatively higher in pulp and lower in leaves of both the genotypes of

Plant Genotype	Plant Parts	Total Sugar (mg/gdw)	Reducing Sugar (mg/gdw)	Non Reducing Sugar (mg/gdw)	Starch (mg/gdw)	Protein (mg/gdw)	Ascorbic Acid (mg/100gdw)
BIK -S1	Bark	8.61 ±0.029	3.84±0.088	4.77±0.54	14.08± .061	7.43 ±0.021	55.08 ± 0.129
	Leaves	13.5±0.20	7.71±0.089	5.79±0.25	19.29 ±0.21	5.24 ±0.037	58.35 ± 0.008
	Fruit shell	18.83 ±0.62	12.33 ±0.36	6.50±0.49	2.68 ±0.053	8.65 ±0.037	60.46 ± 0.041
	Pulp	22.69 ±0.107	14.72±0.106	6.97±0.47	29.07 ±0.16	16.52 ±0.032	63.80 ±0.054
	Seed	15.52± .061	9.41±0.056	6.11±0.2	14.59 ±0.074	10.41 ±0.028	61.56 ± 0.016
	Callus	9.78±0.206	4.24±0.120	5.54±0.50	18.72 ±0.10	5.53 ±0.025	63.76 ±0.012
	Invitro shoots	3.37±0.057	1.26 ±0.02	2.11±0.20	26.52 ±0.060	7.27 ±0.014	59.45 ± 0.008
BRP- S2	Bark	5.61± 0.10	1.26 ±0.02	4.35±0.12	10.32 ±0.082	5.82 ±0.021	44.15 ± 0.043
	Leaves	10.21± 0.16	5.30± 0.10	4.91±0.34	18.41 ±0.041	4.34 ±0.037	57.29 ± 0.040
	Fruit shell	15.82±0.061	11.22± 0.021	4.60±0.24	7.33 ±0.20	6.12 ±0.016	58.27 ± 0.037
	Pulp	19.57±0.061	13.53±0.12	6.04±0.22	26.68 ±0.19	10.54 ±0.037	62.72 ±0.037
	Seed	9.75±0.040	7.52± 0.060	2.05±0.39	19.57 ±0.061	6.73 ±0.021	56.04 ± 0.027
	Callus	7.61±0.029	2.17 ±0.053	5.44±0.49	22.52 ±0.082	5.30 ±0.040	62.65 ±0.029
	Invitro shoots	2.68±0.12	1.08±0 .085	1.60±0.21	30.32 ±0.163	5.55 ± .081	58.37 ± 0.069

Table -1: Neutraceuticals *in Limmonia acidissima in vivo* and *in vitro*

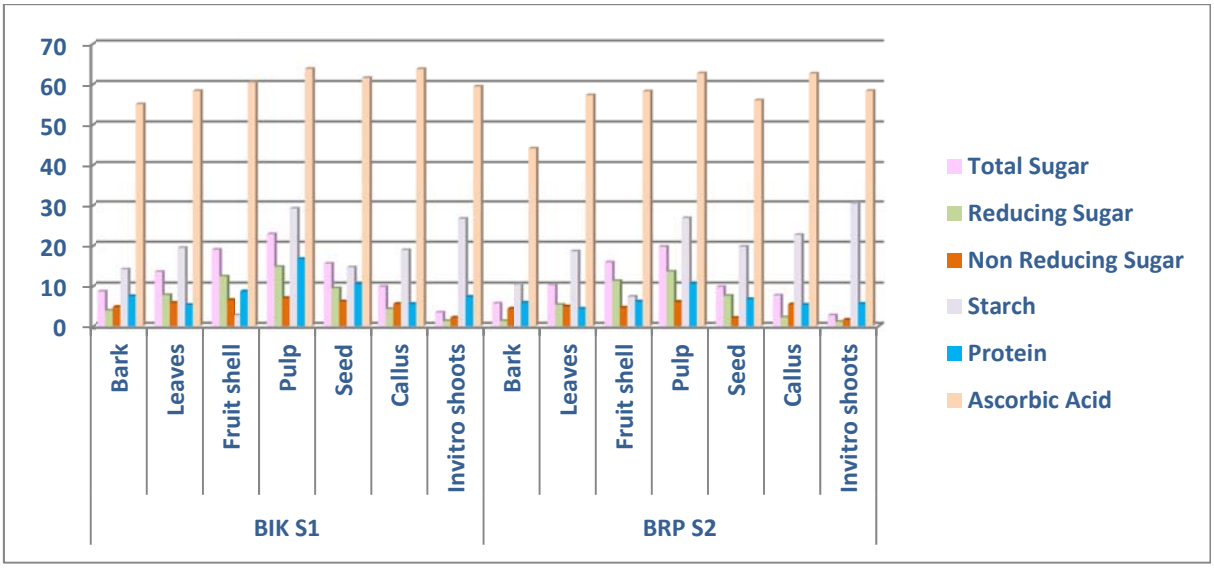

Fig.-1: Nutritional metabolites in *L. acidissima*

tested. Among samples of BIK- S1 genotype maximum protein content (16.52±0.032 mg/g.d.w) was found in pulp and minimum (5.24±0.037 mg/g.d.w.) in leaves whereas among the samples of BRP- S2 genotype maximum amount (10.54±0.037 mg/g.d.w) was found in pulp and minimum (4.34±0.032 mg/g.d.w.) in Leaves. BIK-S1genotype exhibited more amount of protein content in comparison to BRP- S2 genotype. *In vitro* cultures showed less amount of protein as compared to plant parts. Thomas [19] evaluated higher amount of protein in pulp (19.33mg/g) and lowest in bark (10.53mg/g) whereas Hemalatha and Parameshwary[20] observed 3.20 g/100gm of protein in pulp in *L. acidissima.*Ghorad[21] found 27.56% protein content in seed flour and 77% protein in pulp concentrate of *L . acidissima.*

Ascorbic acid:

Appreciable amount of ascorbic acid content was found in all the samples of both the genotypes of *L. acidissima.*Concentration of free ascorbic acid was found to be comparable in all the samples tested. Maximum and similar amount of ascorbic acid was recorded in pulp (63.80 $\pm$ 0.054mg/100g.d.w.) and callus cultures (63.76$\pm$ 0.012mg/100g.d.w.) of BIK – S1genotype.

The minimum amount of ascorbic acid was found in bark (44.15 $\pm$ 0.043mg/100g.d.w.) of BRP- S2 genotype. Among all the plant samples compared maximum amount was observed in pulp and minimum amount was observed in bark. Except bark not much variation inascorbic acid content was found in all the samples tested. *In vitro* cultures (both callus cultures and *in vitro* shoots) also showed significant amount of ascorbic acid when compared with *in vivo* plant parts.

Kumar and Deen[22] reported 6.82 mg/100g ascorbic acid in fully ripe fruit of *Limonia acidissima.* Shrestha[23] recorded ascorbic acid in *Citrus limon* (34.8 mg*), Citrus aurantium* (29.89 mg), *Citrus aurantium var. sinensis* (25.11 mg), *Citrus maxima* (61.29 mg), *Citrus paradise* (39.80 mg) and *Citrus medica* (17.4mg). Singh *et al*[24]*.,* observed maximum ascorbic acid content (48.16mg/ 100g pulp) in *Aegle marmelos.* Maximum amount of ascorbic acid has been reported in flowers (77.502 to 224.672mg/100gm) of *Moringa oleifera* then leaves and pod [25].

Conclusion

Our findings revealed that *Limonia acidissima* is a rich source of carbohydrate, protein and ascorbic acid (vitamin C) as compared to other citrus plants. It also supports that *in vitro* callus cultures also produces these metabolites in fair amount. Thus it is recommended for the use as a dietary source of neutraceuticals. The study suggests that BIK-S1 genotype is suitable for exploitation and processing of fruits and there is tremendous scope of utilizing this underexploited fruit crop in different promising value added products to the food and nutraceutical industry.

References

1. Singh KA, Pal R, Abrol G, Punetha S, Sharma P and Pandey AK 2019, Nutritional and Medicinal Value of Underutilized Fruits.*Acta Scientific Agriculture* 3 **1** 16-22.
2. Sharma P, Bodhankar SL, Thakur Desai PA 2012, Protective effect of aqueous extract of *Feronia elephantum* Correa leaves on thioacetamide induced liver necrosis in diabetic rats. *Asian Pacific Journal of Tropical Biomedicine.* 2 **9** 691-695.
3. Balick MJ Paul and Cox A 1996, Plants that heal; people and culture: The science of ethno botany. *Scientific American Library*. **73** 25 - 61.
4. Gupta R, Johri S, and Saxena A M 2009, Effect of ethanolic extract of *Feronia elephantum* Correa fruits on

blood glucose levels in normal and streptozotocin-induced diabetic rats.*Natural Product Radiance*. 8 **1** 32-36.
5. Mishra A, Arora S, Gupta R, ManviPunia RK and Sharma AK 2009, Effect of *Feronia elephantum*(Corr) fruit pulp extract on indomethacin-induced gastric ulcer in albino rats. *Trop J Pharm Res*. **8** 509-514.
6. K Ilango and V Chitra 2010, Wound Healing and Anti-oxidant Activities of the Fruit Pulp of *Limonia acidissima* Linn (Rutaceae) in Rats, *Tropical Journal of Pharmaceutical Research* . 9 **3** 223-230.
7. Saima Y, Das AK, Sarkar KK, Sen AK, Sur P 2000, An antitumor pecticpolysaccharide from *Feronia limonia*. *International Journal of Biology and Macromolecule.* **27** 333-335.
8. Senthil kumarA and VenkatesalV 2013, Chemical constituents, *in vitro* antioxidant and antimicrobial activities of essential oil from the fruit pulp of wood apple. *Industrial crops and products*. **46** 66-72
9. Park So-Young 2016, Production of biomass and secondary metabolites from plant cell and organ culture using bioreactor system in medicinal plants. *J Pharmacogn Nat Prod* **2** 3Suppl)
10. Meena, Prakash Vijay, Meena Chand Mahesh and Sharma LataSuman 2014, quantitative estimation of some metabolites and enzymes in insect induced leaf galls of *Ficus religiosa*. *International journal of life science & pharma research*. 4 **3** 01-07
11. Yemm E W and Willis A J 1954, The estimation of carbohydrates in plant extracts by anthrone. *Biochemistry Journal* **57** 508-514.
12. Nelson NA 1944, Photometric adaptation of the Somogyi method for the determination of glucose. *J. Biol. Chem.*153 **2** 375.
13. Somogyi M 1952, Notes on sugar determination, *J. Biol. Chem*.195 **1** 19-23.
14. McCready R M, Guggols J, Silviers V and Owen H S 1950, Determination of starch and amylase in vegetable. *Ann. Chem*. **22** 1156-1158.
15. Lowry, O. H., N. J. Rosebrough, A.C.Fara and R. J. Randall, 1951. J.Biol. Chem. 193 – 265.
16. Chinoy JJ 1962, Formation and utilization of ascorbic acid in the shoot apex of wheat as factors of growth and development. *Indian J. Plant Physiol*.**5** 172- 201.
17. Vijayvargia Pratima, Vijayvergia Rekha 2014, A Review on *Limonia acidissima* L. Multipotential Medicinal Plant. *Int. J. Pharm. Sci. Rev. Res.* 28 **1** 191-195.
18. Uttara Singh, Anita Kochhar and Rajbir Boora 2012, Proximate composition, available carbohydrates, dietary fibres and anti-nutritional factors in bael (*Aegle maemelos* L.) leaf, pulp and seed powder. *International Journal of Scientific and Research Publications*. 2 **4** 1-4.
19. Thomas Asha and Ponnammal N R 2005, Preliminary studies on phytochemical and antibacterial activity of *Limonia acidissima* L. plant parts. *Ancient Science of Life*. XXV **2** 57-61.
20. Hemalatha C and Parameshwari S 2019, Development and standardization of wood apple (*Limonia acidissima*) incorporated novel food products. *International Journal of Pharmaceutical Sciences and Research.* 10 **11** 5087-5093.
21. Ghorad A S, M M Humane, D T Bornare 2018, Preparation of wood apple seed protein concentrate and study of its physico-chemical analysis. *International Journal of Innovative Research and*

Creative Technology. 3 **6** 45-46.

22. Kumar Awadhesh and Deen Bhagwan 2017, Studies on Bio-Chemical Changes in Wood Apple (*Limonia acidissima* L.) Fruits during Growth and Development, *International Journal of Current Microbiology and Applied Science*. 6 **8** 2552-2560
23. Shrestha Nishanta, Shrestha Sudan and Bhattarai Ajaya 2016, Determination of ascorbic acid in different *citrus* fruits of kathmandu valley. *Journal of Medical and Biological Science Research* . 2 **1** 9-14.
24. Singh Ravi Pratap, Singh AK, Singh Anshuman, Sharma Nitesh Singh AP, Singh Kunwar Avinash P, Singh Anand and Singh Abhishek Kumar 2018, Estimates of chemical characteristics of bael (*Aegle marmelos* Correa) genotypes under SODIC soil . *International Journal of Chemical Studies*; 6 **6** 1799-1802
25. Ahmed Khondoker Shahin, Banik Rajib, Hossain Hemayet M and IsmetJahanAra2016, Vitamin C (L-ascorbic Acid) Content in Different Parts of *Moringa oleifera* Grown in Bangladesh.*American Chemical Science Journal* . 11 **1** 1-6.