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GC-MS analysis and somatic embryogenesis in *Benincasa hispida* (Thunb.) Cogn. Var. NeiKumbalam

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Abstract
NeiKumbalam is a medicinal vegetable and major ingredient of ayurvedic preparations. Due to the medicinal property and rare distribution, the present study was focused on the phytochemical analysis and somatic embryogenesis in NeiKumbalam. GC-MS analysis of hexane extract of NeiKumbalam fruit pulp revealed the presence of 8 major compounds including Trichloromethane (53%), 3-Pyrrolidinol (28%) and Methane oxybis [dichloro- (18%), while the major constituent of seed extract was 9,12-Octadecadienoic acid (Z,Z)- (100%). Results showed the presence of biologically and pharmacologically active compounds in NeiKumbalam fruit, among them 9, 12-Octadecadienoic acid (Z, Z)- was a 6 omega fatty acid. Callus induction was highest on MS medium supplemented with BAP1.0mg/l which showed 60±1.4% of callus induction frequency. Callus was white, friable and embryogenic. Within 14 weeks, the better number of somatic embryoids was observed on the same medium.

Keywords: NeiKumbalam, GC-MS analysis, 9, 12-Octadecadienoic acid (Z, Z)-, callogenesis, somatic embryogenesis, callus induction frequency

Introduction
Cucurbitaceae is a moderately large family of about 130 genera and 900 species. All genera are frost-sensitive and the family is confined to the warmer parts of the globe. In such regions, the plants are found in almost every vegetable garden and has important role in the diet (Jeffrey, 1980) [11]. Many researchers have paid attention towards the family because the fruits and seeds are traditionally used in various ayurvedic preparations and confectionary (Dhiman *et al*., 2012) [5]. NeiKumbalam is a medicinal vegetable including in Cucurbitaceae, mainly used for preparing Kushmanda Rasayana. It is rarely cultivated in Kerala at present. Due to this, common Ash gourd is often used as its substituent. NeiKumbalam is unique with respect to growth pattern, flowering nature, fruiting and yield character.

Gas Chromatography - Mass Spectrometry (GC-MS) is a technology for secondary metabolite profiling in plant and non-plant species. Gas chromatography separates the components of the mixture and mass spectroscopy analyzes each of the components separately. The plants include in Cucurbitaceae possess many medicinal properties due to the presence of secondary metabolites. The analysis of specific phytochemical compounds present in these plants should help to design many ayurvedic medicines. The presence of diverse secondary metabolites has been reported from *Polygonum chinense* L. that contained a triterpene compound - squalene (47.01%) and a plasticizer compound - 1, 2-benzenedicarboxylic acid, mono [2-ethylhexyl] ester (40.30%). Most of the analyzed phytochemical compounds in many medicinal plants had showed the antimicrobial, anticancer, antioxidant, antitumor, chemo preventive, pesticidal and sun screen properties (Nascimento *et al*., 2000; Mostafa *et al*., 2018) [22, 19].

Due to the increasing importance in medicine, NeiKumbalam should be conserve and multiply in nature. Protocols for *in vitro* medicinal plant production via direct and indirect organogenesis have many potential applications (Ahmad *et al*., 2010) [1]. Induction of callus and subsequent organogenesis is accomplished by the differential application of growth regulators (Tripathi & Tripathi, 2003) [28]. Callus induction from seed embryo is very fast compared to the callus proliferation from other explants like internodes, leaves etc. In somatic embryogenesis, embryo is formed from a single somatic cell / group of somatic cells which is more beneficial than the direct plant regeneration. Somatic embryos are bipolar and allowing them to form a whole plant without culturing on multiple media. The ratio of growth regulators to induce callus and somatic embryos are depending to plant species. Due to the significant role in ayurveda, the present study reveals the fruit characterization, phytochemical analysis and somatic embryogenesis in NeiKumbalam or Vaidya Kumbalam.

Materials and methods

Study material: Ripened NeiKumbalam fruit was collected from Thirurangadi, Malappuram, Kerala, India. All morphological features of fruit and seeds were observed and photographed.

GC-MS analysis: The fruit pulp and seeds of NeiKumbalam were air dried and powdered well using mortar and pestle. Hexane was used as solvent to extract the phytochemical compounds in fruit pulp and seeds. About 10 gm of powdered sample was dissolved in 30 ml hexane and air dried to evaporate the solvent. GC-MS analysis was carried out from Department of Applied Chemistry, Cochin University of Science and Technology (CUSAT), Kochi, Kerala, India.

Total protein and phenol estimation: The powdered sample of fruit pulp and seeds were used for analysis. Total protein and phenolic contents were estimated using Lowry's method (1951) [14] and Folin-Ciocalteu method respectively (Bray & Thorpe, 1954) [3].

In vitro study: The experiment was conducted in the tissue culture laboratory of the Department of Botany, KAHM Unity Women's College, Manjeri, Malappuram, Kerala, India.

Explant sterilization: The seeds were selected as explant for the present study. The seed coat was removed manually. Seeds were thoroughly washed with distilled water for 4-5 times followed by surface sterilization with 1% Bavistine for 30 minutes and 1% Tween 20 for 15 minutes. Then, the explants washed with double distilled water for 3 times. Within the laminar air flow cabinet, the explants were treated with 0.1% $HgCl_2$ for 3 minutes. Finally, the explants washed with sterile double distilled water for several times to remove all the sterilizing agents.

Callus induction and somatic embryogenesis: MS medium was used as the basal medium for callus induction (Murashige & Skoog, 1962) [20]. The proposed medium was supplemented with 2, 4-D 0.5-1.5mg/l, BAP 0.5-1.5mg/l, IAA0.5mg/l+Kin1.0mg/l and IAA1.0mg/l+Kin0.5mg/l. The pH of the media was adjusted to 5.8. The surface sterilized seeds were inoculated using the laminar air flow chamber and maintained in an environmentally controlled room at 22±2 °C with a photoperiod of 8 h daylight and light intensity of $1500\mu Em^{-2}S^{-1}$. Callus was sub-cultured to the MS medium supplemented with BAP1mg/l for somatic embryogenesis. Results were observed on the 14th week and statistically analyzed.

Results

NeiKumbalam fruit showed ovoid or spherical shape and dark green colour with white powdery surface. The size of the fruit was approximately 29 X 27cm with numerous seeds, *C.* 351. The fruit wall included three layers; outer epicarp, middle mesocarp and inner endocarp. Epicarp was very hard in matured fruit, while mesocarp and endocarp were white and fleshy. Endocarp consisted of many hard creamy-white coloured seeds (Figure 1).

GC-MS analysis of hexane extract of NeiKumbalam fruit pulp revealed the presence of three phytochemical compounds including Trichloromethane, 3-Pyrrolidinol and Methane, oxybis [dichloro-. The lowest RT value of 3.65 was observed for Trichloromethane while the area % was highest (53.4%). Only one compound (9, 12-Octadecadienoic acid (Z, Z)-) was analyzed from the seeds of NeiKumbalam with RT value of 37.9 and area % of 100 (Figure 2). In the study, the higher quantity of total protein and phenolic contents were observed in NeiKumbalam fruit pulp and seeds (Figure 3).

For callus induction, the seeds were cultured on MS medium supplemented with different combinations and concentrations of growth regulators. The callus induction was started within 5 weeks. However, the MS medium without growth hormones was never showed any callus induction, but the size of cotyledons was enlarged. Callus was white, compact and non-embryogenic when cultured on MS+2,4-D0.5-1.5mg/l, MS+BAP0.5mg/l, MS+BAP1.5mg/l, MS+IAA0.5mg/l +Kin1.0mg/l and MS+IAA1.0mg/l +Kin0.5mg/l (Figure 4). In the study, callus cultured on MS+BAP1.0mg/l was white and friable with 4-10 somatic embryoids within 10 weeks. The callus fresh weight (CFW) and callus induction frequency (CIF) were highest in callus grown on this medium. The white root like structures were first developed from this embryogenic callus, further developed many somatic embryoids (Figure 5).

Fig 1: NeiKumbalam fruit (A), NeiKumbalam fruit T.S. (B) and NeiKumbalam seeds (C).

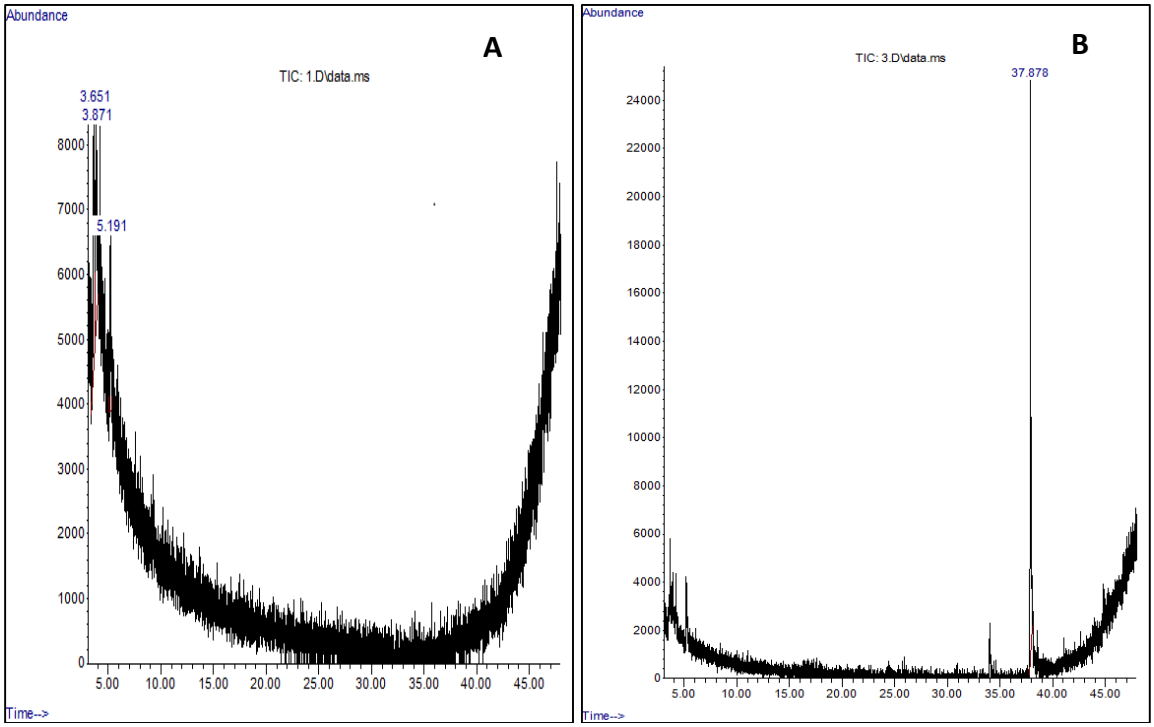

Fig 2: GC-MS chromatogram of phytochemical compounds analyzed from hexane extract of NeiKumbalam - Fruit pulp (A) and NeiKumbalam - Seeds (B).

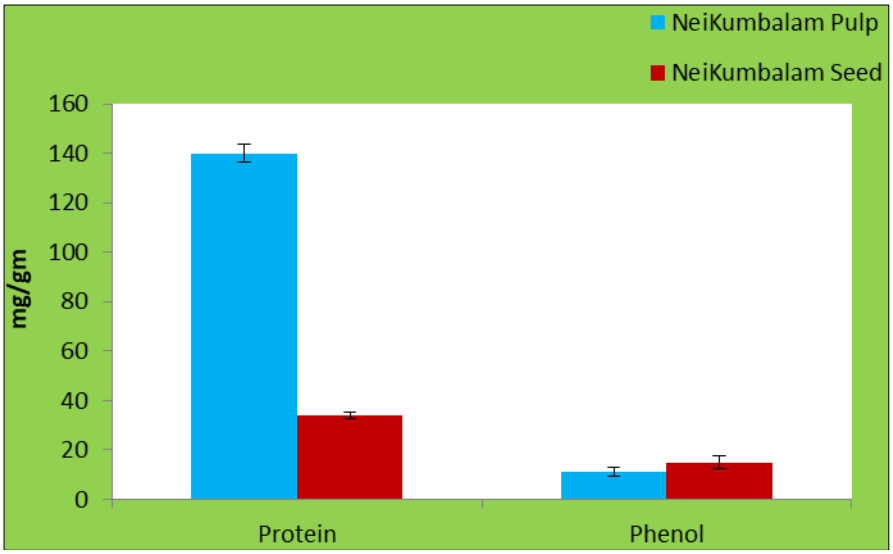

Fig 3: Protein and phenol contents in NeiKumbalam fruit pulp and seeds

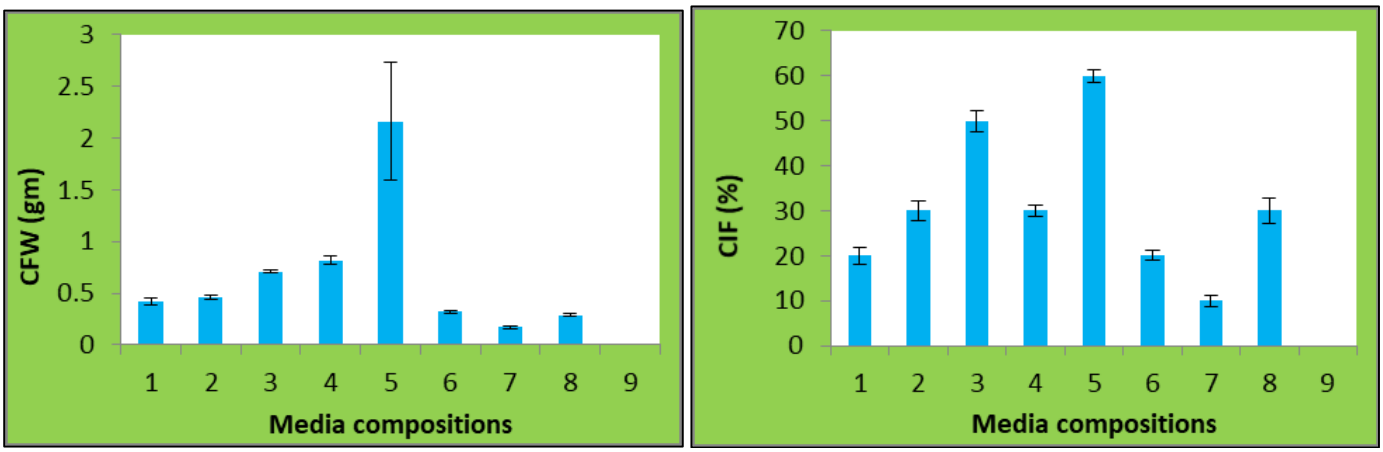

Fig 4: Callus induction in NeiKumbalam on different media compositions within 8 weeks. (1. MS+2, 4-D0.5mg/l; 2. MS+2, 4-D1.0mg/l; 3. MS+2, 4-D1.5mg/l; 4. MS+BAP0.5mg/l; 5. MS+BAP1.0mg/l; 6. MS+BAP1.5mg/l; 7. MS+IAA0.5mg/l+Kin1.0mg/l; 8. MS+IAA1.0mg/l+Kin0.5mg/l; 9. MS)

Fig 5: Somatic embryogenesis from callus of NeiKumbalam seeds cultured on MS+BAP1.0mg/l within 14 weeks.

Discussion

The vegetables are providing a healthy diet to human because they are a great source of health promoting compounds and nutrients. The health promoting compounds include different secondary metabolites like polyphenols, triterpenoids, phenolics, flavonoids, anthocyanins, alkaloids etc. Among them, most of the phytochemical compounds showed antimicrobial, antitumor and antioxidant activities (Rosa *et al*., 2009) [24]. The present study was mainly focused on phytochemical analysis and *in vitro* callogenesis of NeiKumbalam. The fruit of NeiKumbalam was morphologically different in size, shape, texture and number of seeds. It was ovoid or spherical in shape with dark green colour and white powdery surface. At maturity, the epicarp was very hard which was entirely different from Kumbalam fruit. Even though, the fruit was small in size, the endocarp included numerous seeds.

NeiKumbalam is the major component of many ayurvedic medicines like Kooshmanda Rasayana. It is due to the presence of different phytochemical compounds in fruit pulp and seeds. In the study, GC-MS analysis of hexane extract of fruit pulp and seeds were carried out to detect the phytochemical compounds. Analysis of fruit pulp revealed the presence of three phytochemical compounds; while, only one compound (9, 12-Octadecadienoic acid (Z,Z)-) was analyzed from the seeds of NeiKumbalam with lowest RT value and highest area %. The 9, 12-Octadecadienoic acid (Z,Z)- is a fattyacid which usually synthesize in the body of animals (Griinari & Bauman, 1999) [9]. Due to the presence of this fattyacid, Kooshmanda Rasayana can be enhance strength and body weight in humans. This compound was also reported in ethanolic extract of *Pothos scandens* L. and *Lens culinaris* Medik (Gallasch & Spiteller, 2000; Lalitharani *et al*., 2009) [7, 15]. Some of the phytochemical compounds present in NeiKumbalam shows antimicrobial and antioxidant properties (Rajashekar *et al*., 2012) [23]. Similarly, the different types of phytochemical compounds were detected from vegetables, fruits and many of the medicinal plants at present (Rosa *et al*., 2009; Kanthal *et al*., 2014) [24, 12]. The fruit pulp and seeds in Cucurbitaceae are good source of polyphenols and proteins that can be extracted by using different solvent system (Singh *et al*., 2016) [26]. In the study, the quantity of total protein and phenolics were higher in fruit pulp and seeds of NeiKumbalam as other vegetable fruits (Vinson *et al*., 1998; Barberan & Espin, 2001) [30].

In vitro plant biotechnology offers many unconventional techniques for crop improvement. It can be employed as an alternative means for genetic upgrading, and its applications largely depend on the reliable plant regeneration system (Lloyd & McCown, 1980; Fay, 1992) [16, 6]. In Cucurbitaceae members, the tissues could regenerate both via caulogenesis and somatic embryogenesis (Gray *et al*., 1993; Nakagawa *et al*., 2001) [8, 21]. The conservation of NeiKumbalam is highly significant due to the rare distribution. *In vitro* micropropagation is one of the best methods to conserve and multiply this medicinally valuable vegetable plant. Haque *et al*. (2008) [10] standardized the efficient protocol for plant regeneration from Kumbalam nodal explants using MS medium with 1.5mg/l BAP. In the present study, the seed embryo was used as the explant for callogenesis. Growth of NeiKumbalam is very slow in nature. Similarly, more time was taken for every *in vitro* responses from seed embryo compared to other plant explants.

Somatic embryogenesis is preferred in vegetable micropropagation because it allows production of plant without somaclonal variation. In the study, the seed was cultured on different growth media of which white, compact / friable and non-embryogenic callus was regenerated on MS+2,4-D0.5-1.5mg/l, MS+BAP0.5mg/l, MS+BAP1.5mg/l, MS+IAA0.5mg/l+Kin1.0mg/l and MS+IAA1.0mg/l+Kin0.5mg/l within 5 weeks. Similarly, Thomas & Sreejesh (2004) [27] developed a new protocol to initiate callus from Kumbalam seed embryo using 1-6µM 2, 4-D. In the study of Ugandhar *et al*. (2011) [29], the callus was regenerated from Kumbalam leaf explant when cultured on MS medium fortified with 0.75mg/l 2, 4-D. Development of efficient somatic embryos on full strength MS medium supplemented with 2, 4-D1.0 mg/l and TDZ 0.25mg/l was also observed within three weeks.

In the present experiment, somatic embryogenesis was exhibited on MS+BAP1.0mg/l within 10 weeks. The white transparent root like structures were first developed from the calli and further turned to green in colour. The type of auxin or auxin in combination with cytokinin used in the medium can greatly influence somatic embryo frequency (Merkle & Sommer, 1986) [18]. The requirement of cytokinin in addition to auxin for somatic embryogenesis was observed in *Sapindus trifoliatus* L. and *Terminelia arjuna* (Roxb.) Wight & Arn. (Desai *et al.* 1986; Kumari *et al*., 2008) [4, 13]. However, the somatic embryogenesis was induced on medium containing NAA alone in *Solanum melogena* L. (Matsuoka & Hinata, 1979; Sharma & Rajam, 1995) [17, 25].

Conclusion

NeiKumbalam fruit and seeds showed distinguishable characteristics which made this member as a distinct variety in Cucurbitaceae. The medicinal vegetable, NeiKumbalam included 8 phytochemical compounds of which 9, 12-Octadecadienoic acid (Z, Z) - was a 6 omega fatty acid. GC-MS analysis revealed the significance of NeiKumbalam in ayurveda due to the presence of this valuable secondary metabolite. *In vitro* callogenesis from NeiKumbalam seed was very slow compared to Kumbalam seeds. Within 14 weeks, the callus proliferation and somatic embryogenesis were best on MS medium supplemented with BAP1.0mg/l on which the CFW and CIF were highest.

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