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In-vitro multiplication techniques of *Crataeva nurvala* Buch. Ham., a potential medicinal plant for the pharmaceutical industry

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Abstract

Crataeva nurvala Buch. Ham., is a medium-sized deciduous tree commonly used in different medicinal systems which belongs the family Capparidaceae. *Crataeva nurvala* is the perennial sources of herbal products and biomolecules of interest. It is also used as ornamental tree for landscaping and gardening because of its beautiful flowers. *In-vitro* propagation techniques will not only help in standardization of plant materials but also large-scale propagation and long-term preservation of the valuable plant. The suspension cultures are expected to provide a sustainable alternative to indiscriminate and destructive ways of harvesting from the wild, which often leads to the complete decimation of the population. Moreover, suspension cultures as a source of biomolecules or herbs are not season-dependent and are produced under controlled environmental and nutritional conditions; temporal and spatial variations in quality are unlikely.

Key words : *Crataeva nurvala*, micropropagation, medicinal plants, Capparidaceae, lupeol, *In-vitro* multiplication.

About 8 species of the genus *Crataeva* is distributed in various parts of all over the world out of these eight four are found in India[25]. The plant *Crataeva nurvala* (Family Capparid aceae) is one among the eleven species within the family, including rare herbs, trees, and shrubs are frequently distributed in the warm, tropical, and occasionally arid regions of both hemispheres[15], Bopana and Saxena[7]. *C. nurvala* is commonly distributed in China, Indonesia, Malaysia, Myanmar, Sri Lanka, including India. This plant is indigenous to Southern Himalayan range Karnataka, Kerala and Tamilnadu; it is commonly grown in Assam, Bengal, Sikkim, Central India, Andaman & Nicobar Island and near river banks in Kanara and Malabar[15,35]. *Crataeva nurvala* Buch. Ham. popularly known as “three-leaved caper” (in English), Varun (Sanskrit), Borun (Bengali), Barna (Punjabi) and Baruna (Hindi); *C. magna, C. religiosa* or *C. roxburghii* are the synonyms

of *C. nurvala*. The flowers of *C. nurvala* blooms in march and converted in to fruits in June month every year[18,21]. *C. nurvala* is deciduous, medium sized tree having branched head; and leaves are trifoliated with petioles. The average bark length is usually 6-15 cm long, 3- 10 cm broad and 5-15 mm thick in diameter. Outer bark is moderate greyish to brownish and inner whitish brown to pale in colour; having a rough outer and suave inner appearance (Bhattacharjee et al., 2012; Soosamma at al., 2010; Kirtikar and Basu, 2005; Bal et al., 2016; Chopra et al., 1969; Khattar and Wal, 2012).

In Ayurveda system *C. nurvala* has different names based on their qualities such as- due to bitter taste of leaves known as 'Tikta saka'; expulsive property of renal calculus 'Setu briksh'; younger leaves for many days 'Kumarak' and due to its whitish flower 'Sweta puspa[5,23,24,32]. *C. nurvala* has been widely used as traditional medicine to cure cardiac and lungs weakness, respiratory complications, blood flow, memory loss, joint problems, fever, wound healing, and weak immune system[6,17,22]. Lupeol worked as cholesterol-lowering agent and also exhibits anti-inflammatory, anti-proliferative, anti-invasive, anti-angiogenic, anti-microbial, and anti-protozoal, properties. It is also reported that lupeol is therapeutically efficient in wound healing, and in the treatment of diabetes, cardiovascular disease, kidney disease, and arthritis[28]. A large number of pharmaceutical products using *Crateva* as one of the components are developed by various pharmaceutical companies available in the market. Bark of *C. nurvala* is used in the treatment of urinary disorders (as diuretic), including urolithiasis, prostatic hypertrophy, neurogenic bladder and urinary infections; as well as uterine and gastrointestinal problems. Traditionally, the juice extracted from bark of *C. nurvala* is provided to women after labour pain, and also applied to scrofulous enlargements of glands when root bark extract is mixed with honey[11,13]. *In-vitro* propagation results a possibility for the largescale season-independent propagation and pathogen-free production of clonal plants of *C. nurvala*, which is important for the standardization of plant material. For establishment of a complete tissue culture protocol, donor plant & explant collection, selection, and surface sterilization are the preliminary steps, followed by culture establishment, maintenance and multiplication, rooting and acclimatization, and successful transfer of plants are desired steps.

Selection of explants, surface sterilization and choice of culture vessel :

Selection of suitable explant is an important step for *in-vitro* culture establishment as source, type of explant, size, and age of mother plant play a noteworthy role in establishment of an aseptic culture. Explants, including leaves, internodes, cotyledonary nodes, cotyledons, hypocotyls and root segments have been used for the micropropagation of *C. nurvala* [4,34]. Walia *et al*.,[33] reported that thirty-year-old tree was used to collect the nodal explants when cultured on MS medium supplemented with 2.22 microM BAP produced multiple shoots, which elongated reasonably on the same medium. Surface disinfection is a crucial step for the establishment of an uninfected plant tissue culture protocol[31]. Nodal segments with axillary buds were sterilized and inoculated aseptically on culture medium. and studied the impact on shoots initiation, proliferation and elongation of the explants when combines with 6-benzylami-

nopurine (BAP) and kinetin (kin) as well as the effect of Indole-3-butyric acid (IBA) and Indole-3-acetic acid (IAA) on root formation[1]. Based on the explant type surface disinfection varies, which involves choice of disinfectant, concentration and duration of treatment. For example, young or juvenile explants require lesser time and concentration of disinfectant when compared with mature explants. In *Crataeva* species, explants are generally treated with 0.1 % mercuric chloride ($HgCl_2$) [1–3 min], 70 % ethanol [30 s] followed by a rinse 4–6 times with sterilized distilled water (SDW). Other variants include a protocol by Shirin and Maravi[27], who used a combination of cetrimide (a detergent and antiseptic) and 0.05 % Tween-20 for 10 min followed by a rinse with SDW; explants were further treated with ethanol [30 s] and 0.1 % $HgCl_2$ [6–10 min] and finally rinsed with SDW 3–4 times. Panwar *et al*.[20] used two antioxidants, 283.5 µM ascorbic acid and 260 µM citric acid, during surface disinfection to prevent explant browning[13]. The most commonly used vessel for *C. nurvala* culture was borosilicate glass test tubes (size 25x150 mm), and Erlenmeyer flasks (size 100–250 ml) for culture multiplication; similarly, the present investigation was also completed[4,13,25,28].

In vitro multiplication :

A number of media were tested for inducing multiple shoots and for callus induction in the nodal and inter-nodal explants, on basal media (BM) [MS + Agar (0.8%) + (3%) Sucrose] adjuvated with following hormonal combinations and the data was recorded in (Table-1, Fig. 1). Babbar *et al*.,[4], concluded that Murashige and Skoog's (MS) or 6-benzylaminopurine (BAP) at different concentrations were used to develop the seedling-derived explants viz. cotyledonary nodes, epicotyl nodes, hypocotyl segments, first pair of leaves, cotyledons, and root segments.

Table-1. *In vitro* multiplication and callus induction from nodal explants

S. No.	Media composition	Response and morphology	Degree of callusing	Shooting response
1.	BM	Callusing	+	Nil
2.	BM+2,4-D (0.5 mg/l)	Callusing	++++	Nil
3.	BM+2,4-D (1 mg/l)	Callusing	+++	Nil
4.	BM+2,4-D (1.5 mg/l)	Callusing	+++	Nil
5.	BM+2,4-D (2 mg/l)	Callusing	++	Nil
6.	BM+BAP (0.25 mg/l)	Callusing	+	6-9 shoots
7.	BM+BAP (0.5 mg/l)	Callusing	++	9-11 shoots
8.	BM+BAP (1 mg/l)	Callusing	+	4-5 shoots

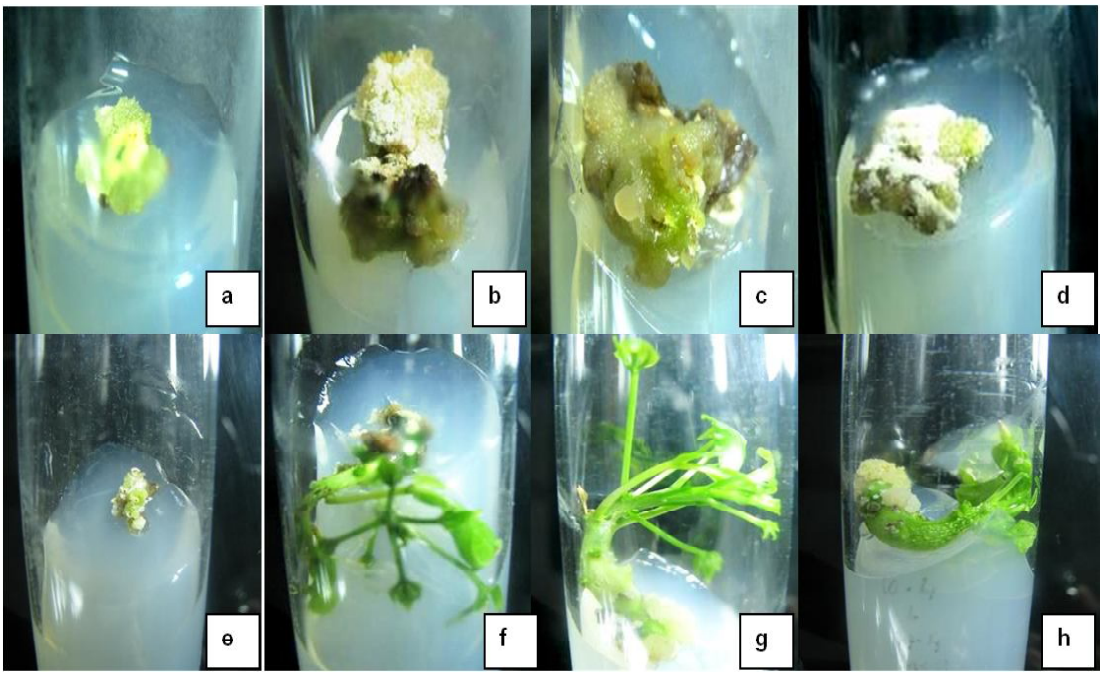


Fig. 1. Effects of different concentrations and combinations of auxin and cytokinin adjuvated to BM containing 3% sucrose, on the nodal and explants from mature tree of *Crataeva nurvala*. **(a)** BM, **(b)** BM+2,4-D (0.5 mg/l), **(c)** BM+2,4-D (1 mg/l), **(d)** BM+2,4-D (1.5 mg/l), **(e)** BM+2,4-D (2 mg/l), **(f)** BM+BAP (0.25 mg/l), **(g)** BM+BAP (0.5 mg/l), **(h)** BM+BAP (1 mg/l)

Of the different media tested for *in vitro* multiplication, BM media supplemented with 0.5mg/l BAP was found to be the best in terms of number of shoots obtained (9-11 in number) (Table-1, Fig. 1 (g), 2 (a). Each one of the shoots was inoculated on fresh media to obtain more shoots (Fig. 2 (b)), hence ensuring a continuous supply of the explants in and out of the growing season. Callus was induced in the nodal and inter−nodal explants on media with a number of combinations out of which MS supplemented with 2mg/l 2, 4-D was found to be the best (Table-1, Fig. 1 (b) and 2 (c)). Walia *et al*.,[33] reported that addition of casein hydrolysate pointedly increases average number of shoots per explant and the maximum number of shoots regenerated on medium supplemented with 100 mg per liter casein hydrolysate. Shoots could be rooted on 1/2 MS supplemented with 0.11 and 0.54 microM NAA.

Maintenance of media :

Above mentioned eight combinations were again tried for maintenance of the obtained calli of which, BM+2, 4-D (0.5 mg/l) was found to be the best (Fig. 3).

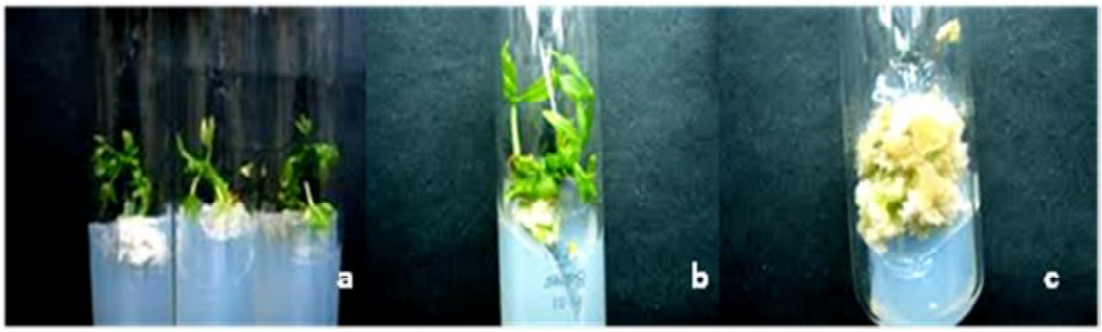


Fig. 2. **(A)** Multiple shoots of *Crataeva nurvala* formed from nodal explants on SCN1 (MS+0.5mg/l BAP); **(B)** An elongated shoot of *Crataeva nurvala*; **(C)** Callus induction

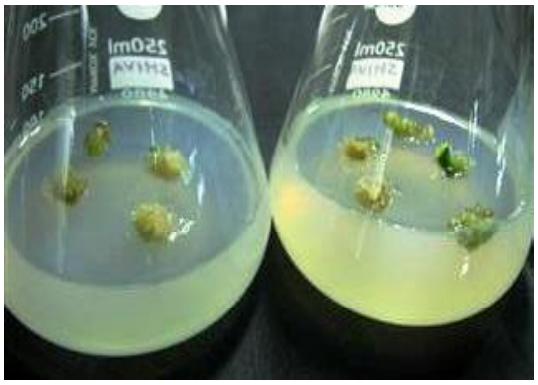


Fig. 3. Callus maintained on BM+2, 4-D (0.5 mg/l)

Callus harvesting and study on biomass content :

Calli grown for different durations on different media were harvested and weighed for their fresh and dry weights as recorded in Table-2.

Phytochemical analysis :

Phytochemical analysis of different plant parts was done to compare the lupeol content. This was followed by comparison of lupeol in callus raised and sub-cultured on varying culture media.

Comparison of lupeol in different plant parts :

Bark, seeds, nodes and leaves of *Crataeva nurvala* were compared for their lupeol contents, which were expressed in terms of weight percentage (Fig. 4, 6(A-D)). Highest weight percentage was noted in the bark, which was followed by the leaves, nodes and seeds.

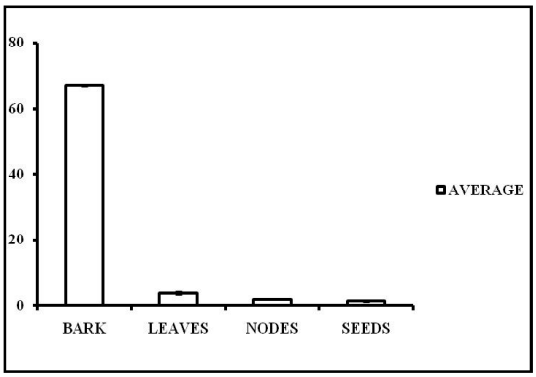


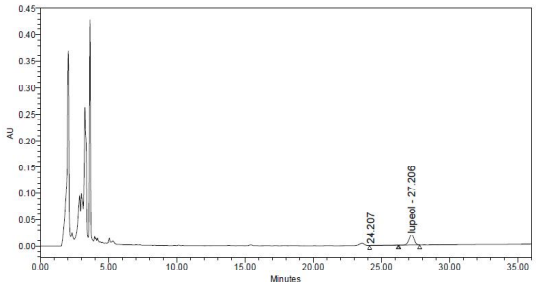
Fig. 4. Weight percentage of lupeol in different plant parts

Effect of different hormones on lupeol content in media for varying durations :

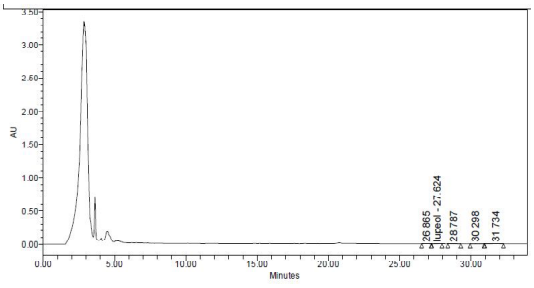
Comparison of lupeol in calli raised on media with varying hormonal combinations for different durations was done to get the effect of these factors on the weight percentage of the bioactive compound (Table-2, Fig. 5 (A-J)).

Table-2. Lupeol assay values determined by HPLC in bark and calli grown different media for different time periods

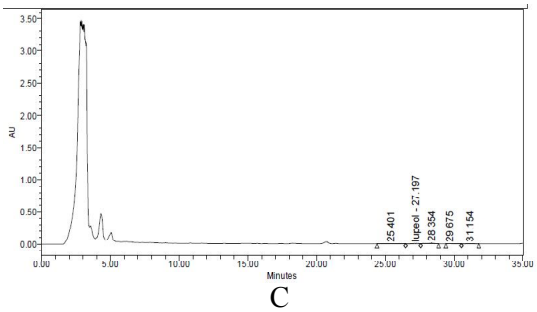
S. No.	Media composition	Weeks	Fresh weight (g)	Dry weight (g)	Amount (µg/ml)	w/w%
1.	Bark	-	-	-	103.441	0.103%
2.	MS+0.2mg/l 2,4-D	2	1.6	1.426	14.627	0.014%
3.	MS+1mg/l 2,4-D	2	2.86	2.038	27.424	0.027%
4.	MS+2mg/l 2,4-D + 1mg/l NAA	2	3	1.95	24.383	0.024%
5.	MS+0.2mg/l 2,4-D	4	3.262 g	2.042 g	13.962	0.013%
6.	MS+1mg/l 2,4-D	4	3.102 g	1.978 g	18.916	0.018%
7.	MS+2mg/l 2,4-D + 1mg/l NAA	4	1.988 g	1.398 g	13.399	0.013%
8.	MS+0.2mg/l 2,4-D	6	2.715	2.155	33.976	0.033%
9.	MS+1mg/l 2,4-D	6	1.5775	1.5425	28.738	0.028%
10.	MS+0.2mg/l 2,4-D	8	1.9125	1.3875	20.016	0.020%



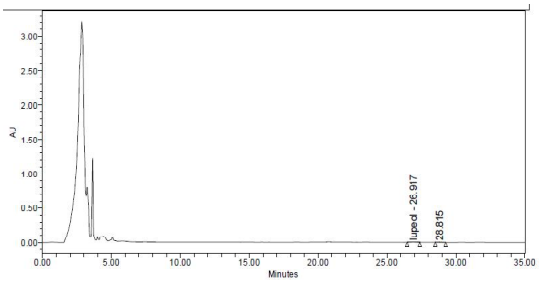
A



B



C



D

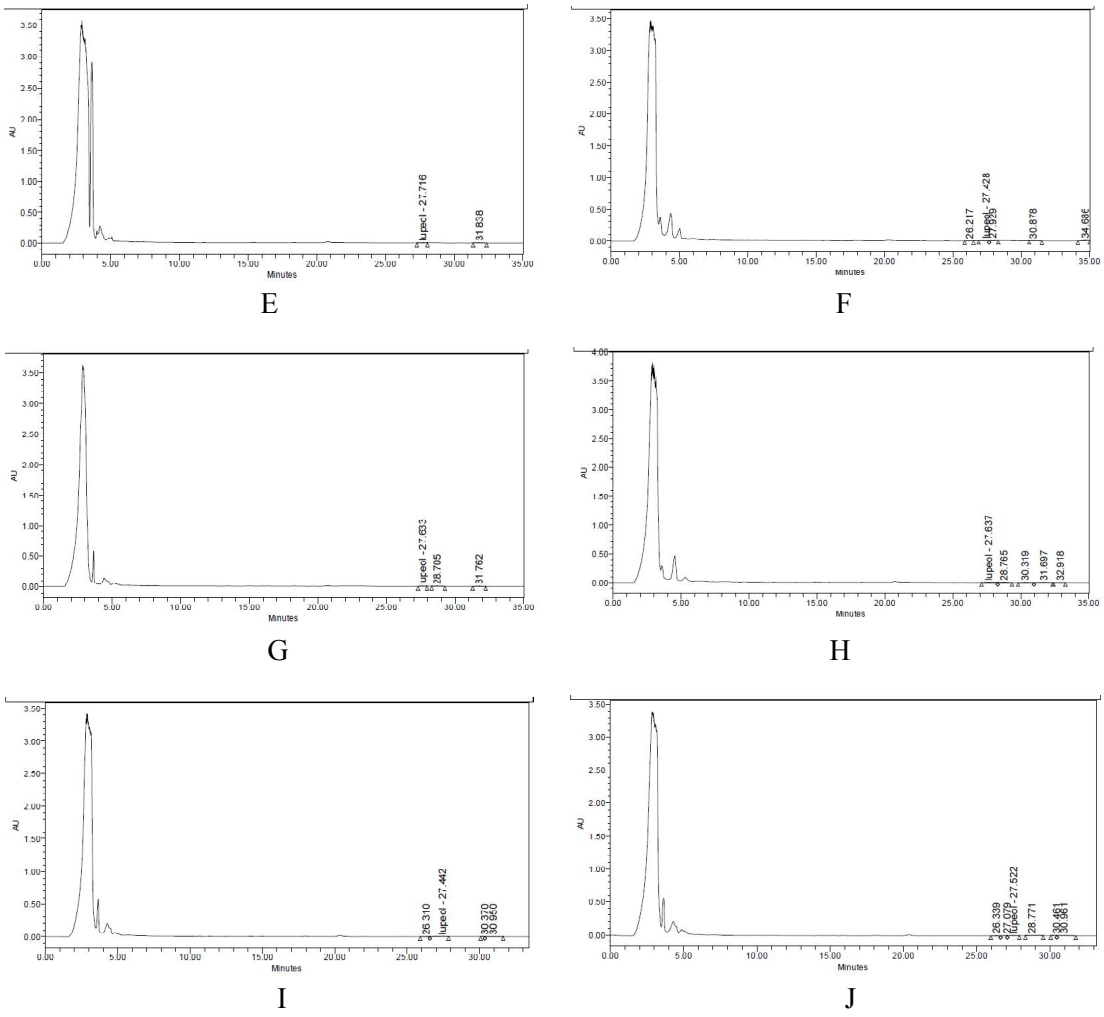


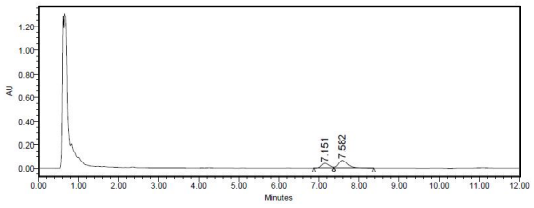
Fig. 5. (A-J) HPLC profiles of calli samples grown on varying culture media

Effect of elicitors on callus morphology and biomarker content :

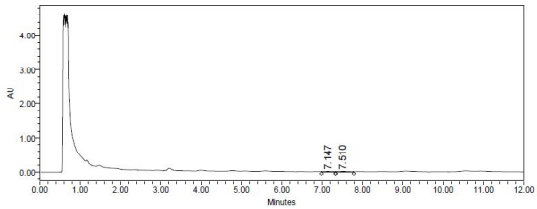
Basal media supplemented with fixed concentration of 2, 4-D (2 mg/l) was used to study effect of varying concentration of SNP (0-4 µM) on callus morphology and biomarker content. Fresh and dry weight of the calli obtained, exhibited a regular reduction with increasing SNP concentration (Table-3). When subjected to phytochemical test, lupeol content in terms of weight percentage was seen to get almost doubled in 2 µM (0.7184) as compared to control (0.4576). However, in 4 µM callus sample, this content was seen to have dropped lower than the control (0.3139) (Table 3, Fig. 6(G-I), 7).

Table 3. Effect of SNP treatment on biomass growth

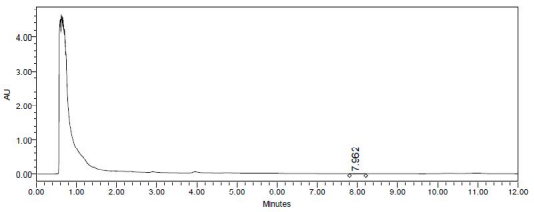
S. No.	Code	SNP (µM)	FW (mg)	DW (mg)	DW/FW	Lupeol Content (µg/µl)	Total Lupeol (in dry sample) (µg)
1.	SCN2	0	951	115.6	0.1215	0.4576	52.8985
2.	SCN13	2	868.2	79.7	0.0917	0.7184	57.2564
3.	SCN14	4	368.7	42.1	0.1141	0.3139	13.2151



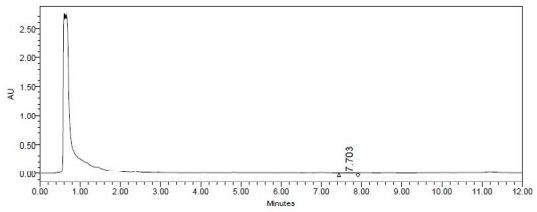
(A) Bark



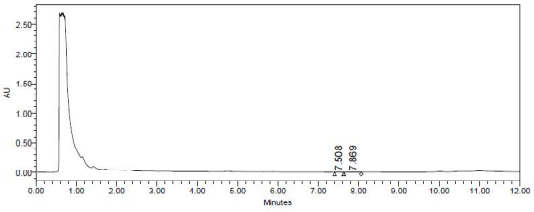
(B) Leaves



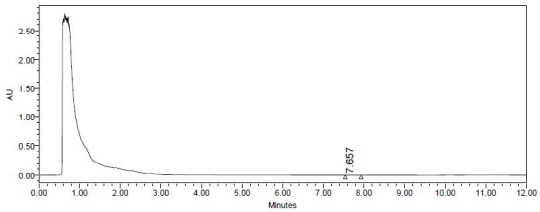
(C) Nodes



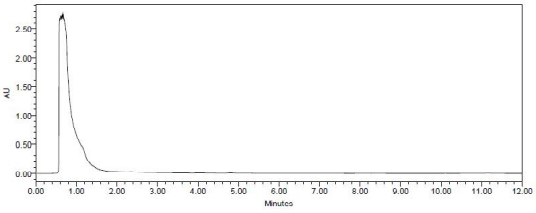
(D) Seeds



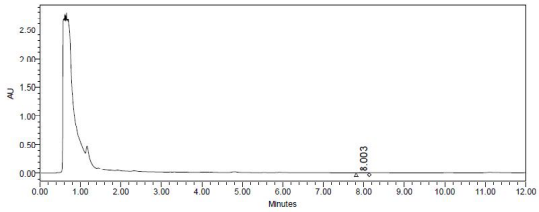
(E) Fresh calli



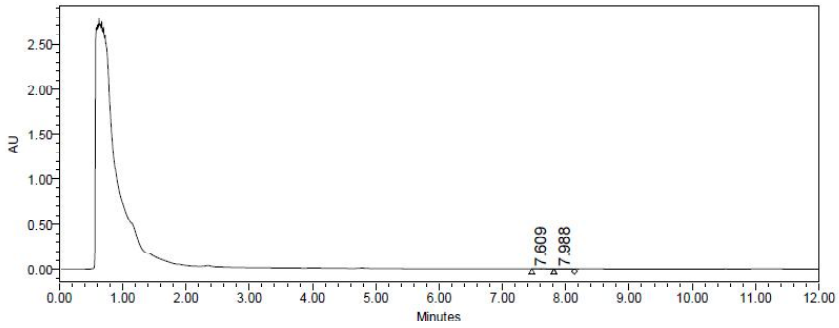
(F) Brown calli



(G) SCN2



(H) SCN13



(I) SCN14

Fig. 6. (A-I) HPLC profiles of different plant parts and calli samples

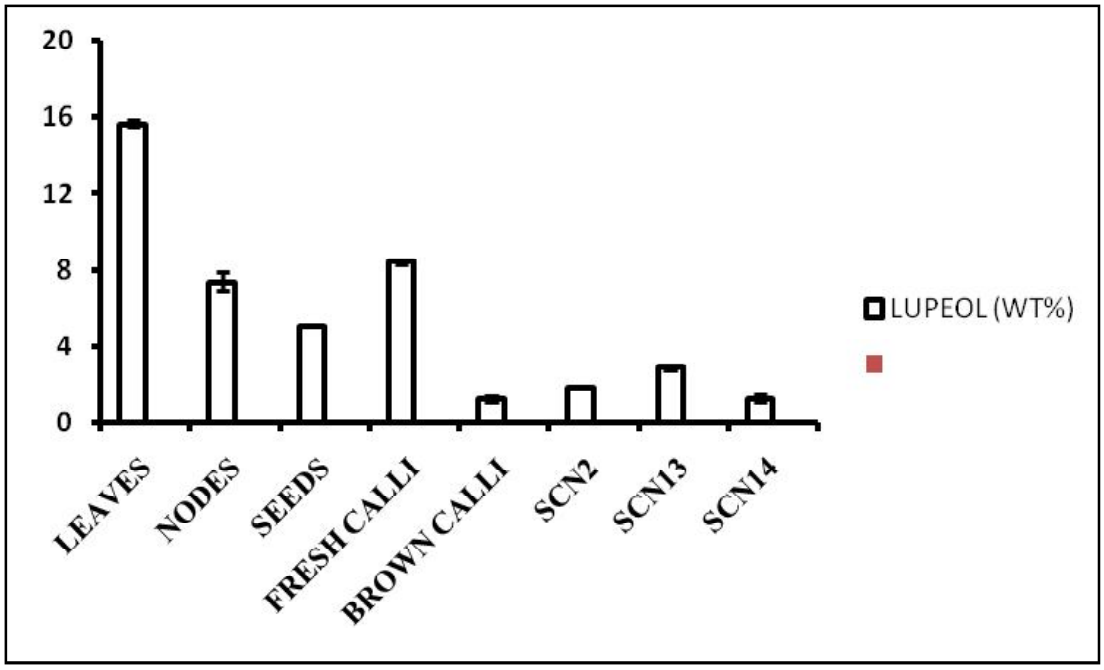


Fig. 7. Comparison of lupeol content in different plant parts and calli samples

Suspension culture of C. nurvala :

The callus was further taken to suspension stage where cell growth was measured as a function of

a. Cell number
b. Optical density
c. pH of the solution
d. Fresh cell weight
e. Cell morphology

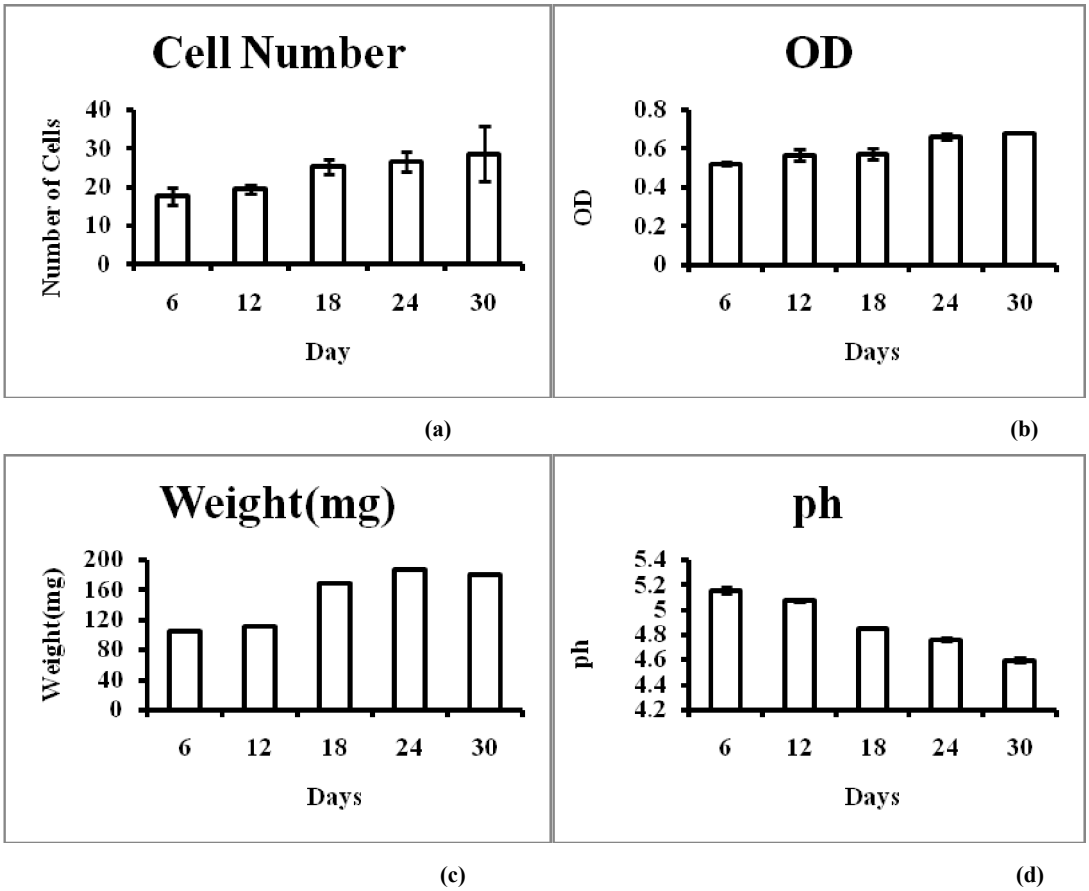


Fig. 8 (a) Cell number, (b) Optical density, (c) Fresh cell weight and (d) pH of the cell suspension of *Crataeva nurvala*

The cell number and corresponding optical density of the suspension was seen to increase with increasing number of days as noted on an interval of 6 days for 30 days (Fig 8(a) and (b)). The fresh weight of the harvested cells was seen to increase till 24th day of observation, after which it demonstrated a slight decrease (Fig 8(c)). The ph of the solution showed a regular decreasing trend with increasing number of days (Fig 8(d)). The phytochemical analysis did not detect any lupeol content in any of the tested samples. This could probably be because of the little amount of cell mass taken. Increasing cell mass in suspension is being taken, so that comparison of the cells in suspension and calli can be made with each having equal initial weights.

Ascertaining anti-proliferative activity in C. nurvala cells :

To evaluate the anti-proliferative effect of *C. nurvala* cell extract, A549 cells

were seeded in 96 well plates at concentration of 1×10^4 cells/well and treated with varying concentrations of cell extract (0.25-1mg/ml) for 96h. Then the medium was removed carefully using suction pump and the cells were fixed in 100% methanol. After removing the fixative, cells were washed with PBS and stained with 0.02% aqueous solution of crystal violet at room temperature for 2 min. Crystal violet solution was removed and the plates were washed gently with water to remove extra stain with intermittent tapping. Finally, 100 µl DMSO was added to lyse the cells and dissolve the crystal violet. Absorbance was measured at 540nm using micro plate reader (Biotek, USA). Intensity of crystal violet staining was directly proportional to number of adherent cells.

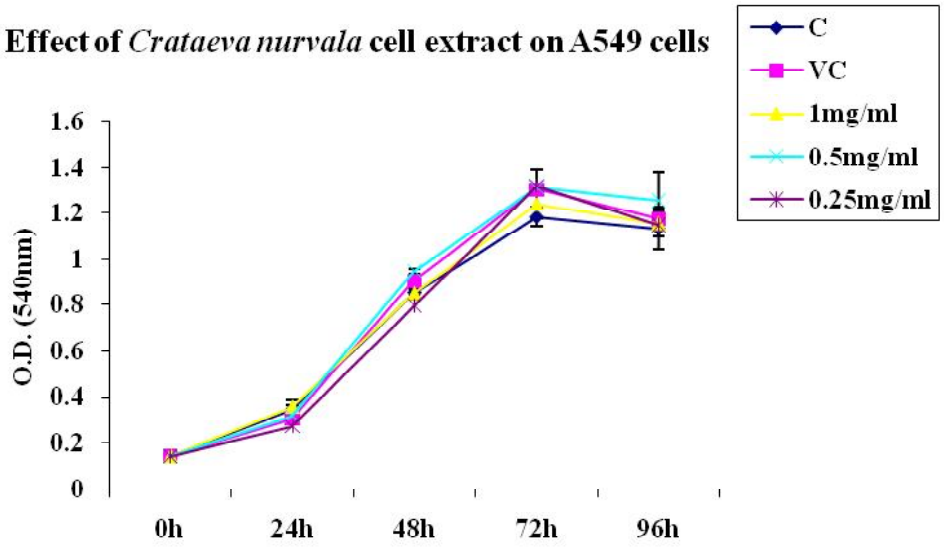


Fig. 9 Effect of *Crataeva nurvala* cells extract on A549 cells

No significant anti-proliferative activity could be noted in the cell extract as compared to the control and vehicular control for the entire range of tested concentration and duration.

The presence of the active principle in the bark sample (67µg/µl) verified our selection of extraction and estimation procedure. The lupeol content for the leaves, nodes and seeds was estimated to be 3.9107, 1.8398 and 1.261µg/µl. This is the first report of the presence of lupeol in calli (0.3002-2.099µg/µl). The bark being composed of dead cells entirely is the source of lupeol in this plant. It is thus expected that cells will exhibit lupeol only in later stages. These observations however provide evidence for the presence of lupeol in calli grown for 14-55 days in different culture media. Fresh calli (2.099µg/µl) was seen to have better lupeol content than browning calli (0.3002µg/µl). Callus treated with NO using SNP as the donor was seen to have an increased lupeol content at the concentration of 2µM (0.7184µg/µl) as compared to control (0.4576µg/µl). Lupeol content was, however, seen to have dropped to

levels lower than the control (0.3139µg/µl) at 4µM concentration of SNP. This sums up the conclusion to the treatment of 2µM SNP as the best treatment for obtaining maximum lupeol content. In suspension, despite increasing number of cells, fresh weight and optical density with increasing number of days, no lupeol was detected[2,13]

The molecular markers, used for identifying genetic variation in regenerants [10,31], have only been used to examine the variation between mother plants and in vitro culture of *C. magna* using inter simple sequence repeat (ISSR) analysis[8]. Pathogen indexing is also very important parameter since there are reports about persistent latent endophytic contaminants (reviewed by Leifert and Cassells[16], Kher *et al*.[12]

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