In India, high drug yielding species of genus *Ephedra*, namely *E. gerardiana* and *E. nebrodens* grow at higher altitudes. Due to over exploitation of the plant from wild populations, *E. gerardiana* is now listed as an endangered species (Gupta and Sethi, 1983). The species used in present investigation is *E. foliata* which contains traces of ephedrine (O’Dowd et al., 1993).

*E. foliata* is a xerophytic plant and it grows under adverse soil and climatic conditions such as high light intensity and high temperature.

This species can be exploited for medicinal purposes by increasing the alkaloid content through biotechnology and tissue culture.

### 2.1 Sterilization methods:

One of the most important techniques which determine the success of any tissue culture experiment is sterilization of explants prior to inoculation. The explants when collected from field are usually contaminated with various micro-organism; which can spoil the set experiments by contaminating cultures. The common sterilizing agents used to overcome this problem are 5-10% Sodium hypochloride, 75-95% ethyl alcohol and 0.01-0.1% mercuric chloride. These were used for removal of surface contaminates. Generally, 15-20 min. treatment followed by washing explants several times with sterile water is applied (Rout et al., 2000). 20% (v/v) commercial bleach solution (Clorox) was used by Hegazi and El-Lamey (2011) for surface sterilizing *E. alata* stems. The sodium hypochlorite (0.5%) containing few drop of Tween 20 was used to sterilize stems of *Ephedra* by Parsaeimehr *et al.* (2010a). Bavastin treatment (0.1% w/v) for 10-15 minutes was given to explants prior to *HgCl₂* (0.1% w/v) treatment for 3-4 minutes followed by washing with sterile water (Lodha *et al.*, 2014, 2014a).
2.2 Micropropagation-

Traditionally Ephedra is propagated by seeds or vegetative methods (Krishnamurthy et al., 1965; Morton, 1977). However, the success rate of traditional methods is very low as in case of E. foliata, proper seed setting is problem due to high ratio of male over female plants (Lodha et al., 2014). Same way authors like Sanaenosuke and Kurihara (1967) reported that in 4 year old Ephedra altissima and Ephedra distachya only 20% and 3% cuttings showed rooting.

As a general attempt to develop micropropagation protocol O’Dowd and Richardson (1993) studied 10 Ephedra species. The nodal segments were used as explants and MS medium was supplemented with 0.05µM IBA and 0.05 µM kin. The number of shoots increased with increasing cytokinin concentration but the shoot length decreased. The rooting was found of poor quality when the shoots were grown on agar solidified medium. Even, when cultured on 0.1 - m µM NAA or high levels of IBA, cultures produced basal callus as well as hyperhydricity was also observed.

The alkaloids were not found in any shoot culture experiments in Ephedra (O’Dowd, 1991).

The factors affecting shoot multiplication of Ephedra procera were studied by Mousavi et al., (2012); their results proved that axillary buds were more significant than axial buds. 0.05 mg/l BA and 0.01 mg/l IBA were most suitable for length, fresh weight and number of axillary shoots. Whereas, 0.1 mg/l kin along with 0.01 mg/l IBA was the best concentration for all studied indices and 0.1 mg/l kin was the best concentration for shoot multiplication and over all BA was better as compared to kin. The main shoot length decreased when concentration of BA was increased in the medium.

Watanabe et al., (1996) used shoots of E. gerardiana grown in fields as well as shoots grown aseptically as explants for In vitro regeneration. The excised meristematic tissue from axillary buds was inoculated on MS medium supplemented with 0.5-1 mg/l Kin for 3-4 weeks for shoot development. Woody plant medium with 5 mg/l BAP was used for multiple shoot formation of shoots developed on MS medium. ½ Woody plant medium was effective to induce rooting.

Lodha et al. (2014a) for the first time successfully regenerated plantlets from callus cultures of Ephedra foliata female plants; callus was induced from node,
internode, apical shoot tip, scaly leaf and root as explants. However, axillary buds were proved best explants as it induced callus with green, proliferative and organized nature. MS medium was supplemented with additives like Ascorbic acid, adenine sulfate, citric acid and arginine. Shoot bud differentiation and multiplication was achieved on media with different concentrations of Kin (0.1-2 mg/l) alone or in combination with BA (0.1-1.5 mg/l) and NAA (0.1-0.5 mg/l). 0.5mg/l each of 2,4 D and Kin was found best for callus growth and shoot bud differentiation was maximum on 0.25 mg/l each of Kin and BA with 0.1 mg/l NAA.

Recently Aziani and Hashemloian (2015) in their experiments on *E. intermedia* for shoot induction found highest shoot regeneration response on 10 mg/l NAA supplemented with 2.5mg/l kin. In the same report they observed significant difference in alkaloidal contents between Ephedra organs; medium containing 15 mg/l NAA along with 10 mg/l kin showed more alkaloidal contents than Ephedra seeds and shoots.

The plant regeneration from nodal segments as explants was achieved through in vitro techniques by Lodha *et al.* (2014) in *Ephedra foliata* female plants 1.5 mg/l BA was most effective for shoot bud induction and Kin was found less effective as compared to BA for multiple shoot induction. The effect of BA for shoot bud induction has also been studied in *Pinus radiata* (Zhang *et al.*, 2010), *Terminalia belerica* (Phulwaria *et al.*, 2012), *Caralluma edulis* (Patel *et al.*, 2014). The success of shoot bud induction using BA as growth regulator was may be due to its stability and as it is easily metabolized by plant tissues (Letham and Palni, 1983).

However, in *Ephedra foliata* cultures the shoots regenerated only on Cytokinins were weak and therefore were repeatedly transferred to medium fortified with various nitrogen sources viz. NH$_4$ NO$_3$ and KNO$_3$ in combination with (NH$_4$)$_2$SO$_4$. Addition of (NH$_4$)$_2$SO$_4$ improved growth of shoots. (Lodha *et al.*, 2014a).

**2.3 Somatic Embryogenesis**

Early experiments on somatic embryogenesis were carried out independently by Steward *et al.* (1958) and Rinert (1959) (Jimenez, 2001). Their studies confirmed predictions made by Haberlandt on cellular totipotency i.e. embryos can arise from single cell (Kiyosue *et al.*, 1993).
Somatic embryogenesis has been reported in at least 200 Gymnosperm and angiosperm species (Raemakers et al., 1995). The studies on somatic embryogenesis and somaclonal variation had been done on Norway spruce by Fourre et al. (1997). Two alternative pathways or somatic embryo origin has been reported by Bozhkov (1997). Lapp et al. (1995) reported micropropagation of Pinus monticola by adventitious shoot from mature embryos.

Most of the coniferales lack ability to produce plantlets from somatic embryos and hence, it is problem for commercial utilization (Tatorus et al., 1991). The somatic embryos can be induced from Gnetum ula but embryos could not be matured as reported by Augustine and D’Souza (1997). 2, 4-D was found playing important role in the same work.

The requirement of 2, 4-D for induction of embryogenic growth in conifers have been reported by different workers like Chalupa (1985), Bekkaoui et al. (1987). The importance of Cytokinins is notable when we consider the somatic embryogenesis in different gymnosperms. Chaves et al. (1992a, b) reported major role of Kin as growth regulator in embryogenesis in cycads. High levels of Cytokinins are also required in conifers (Fowke et al., 1995).

Sankhla et al. (1967) states that Ephedra embryo culture studies have not been undertaken and somatic tissues were also neglected to potential of embryo development (O’Dowd and Richardson, 1993 a, b). Dhiman et al. (2010) reported somatic embryogenesis in Ephedra foliata through callus obtained from seeds as explants; where both auxins and Cytokinins were found to be essential for morphogenesis. It was also reported in callus cultures of Ephedra foliata obtained from stem as explant by Dhiman and Sharma (2010). Sharma et al. (2012) reported that when TDZ was used in media; internodal and nodal segments as explants induced somatic embryos and buds. Garla et al. (2011) showed effect of different concentrations of growth regulators on In vitro callus induction and shoot regeneration from it. Callus obtained from nodal explant was used for shoot induction in which medium containing 7 mg/L kinetin resulted in maximum number of shoots per explant.
In *Pinus taeda* (Mott and Amerson, 1981) also low auxin and low cytokinin were required for morphogenesis. Saha *et al.* (2008) highlights importance of trace elements for embryo induction in *Pinus ovate*.

### 2.4 Callogenesis-

No alkaloidal contents were reported from the callus cultures of *E. foliata* regenerated on 2.3 µM Kin and 54 µM NAA by Ramawat and Arya (1979a) even though on the same medium *E. gerardiana* reportedly showed yield of 0.17 % in callus cultures. Addition of 22 µM 2, 4 D reduced the yield to 0.13% (Ramawat and Arya, 1979b) and replacing NAA with IBA boosted yield of ephedrine to 0.3 % of dry weight.

These reports could not be reproduced in experiments carried out by O’Dowd *et al.* (1998).

Shukla (1980) elevated the quantities of ephedrine and pseudoephedrine three folds in callus cultures of *E. foliata* when cultures were exposed to different light wavelengths and increased alkaloidal yield was observed in blue/red light.

Induction of callus and comparative determination of five phenolic compounds production on *Ephedra alata* was studied by Hegazi and El-Lamey (2011) in which, 2,4D as auxin and Kin as cytokinin each at concentration of 1 mg/l were used for callus induction. L-Phenylalanine was used as precursor at concentrations 12.5, 25, 50 and 100 mg/l and casein hydrolysate was used as elicitor at concentrations of 0.5, 1, 1.5 and 2g/l. Casein hydrolysate when added to basal medium; increased callogenesis frequency was observed in Deep water rice (Khaleda and Al-forkan, 2006).

Parsaeimehr *et al.* (2010) in his experiments studied three *Ephedra* species viz. *E. strobilacea*, *E. procera* and *E. pachyclada* and found that NAA 1.5 mg/l and Kin 1 mg/l; NAA 2 mg/l with Kin 1 mg/l and NAA 2mg/l with Kin 0.5 mg/l were more effective for callus induction in above species respectively. In the same experiment he confirmed that Kin was more effective than BAP for callus induction and fresh weight significantly increased when NAA was used as auxin along with Kin. Total phenolic content were determined using Folin Ciocalteau test. The total...
phenolic content were highest in *E. strobilacea* wild plant and callus culture (504.9±41.51 and 114.61±15.13 µmol equivalent catechin/g extract respectively).

Sankhla and Sankhla (1967a) reported callus initiation in *E. foliata* from seeds at mature embryo stage used as explant. The White’s medium with 2, 4 D, Kin & coconut milk were used to fortify it. However, No morphogenesis was observed from callus. Singh *et al.* (1981) obtained haploid *E. foliata* plantlets from callus obtained using female gametophytes as explants. They concluded MS medium with 20% sucrose and 10% coconut milk was most suitable for callus initiation, growth and morphogenesis. Kinetin was most suitable for stimulation of callus growth and root differentiation whereas in presence of NAA with kin shoot bud differentiation was inhibited. BAP was found to induce shoot buds.

The effect of shoot bud initiation by BAP was also highlighted by Bhatnagar and Singh (1984); where high BAP concentration along with NAA induced shoot buds and kin on the other hand was effective for callus and root formation. Similar effect of BAP was also reported in *Biota orientalis* (Thomas *et al.*, 1977), *Picea glauca* (Campbell and Durzan, 1975), *Thuja plicata* (Coleman and Thorpe, 1977).

Parsaeimehr *et al.* (2010a) reported Kin along with high 2,4D induced shoot buds but continuous presence of 2, 4 D was not necessary. Kin was better than BAP for callus initiation.

Callus cultures produced relatively higher alkaloid content than contents of field grown plants in *Ephedra foliata* (Lodha *et al.*, 2014).

**2.5 Cell suspension cultures**

Cell suspension culture studies have been proved important not only for increased biomass but also accumulation of secondary metabolites. Different authors have worked to establish suspension cultures successfully.

Cell suspension culture of *E. foliata* was first reported by Khanna and Uddin (1976) and Uddin (1977). There are many reports on Suspension cultures studies of *Ephedra* species by Khanna and Uddin (1976), Uddin (1977), O’Dowd (1991), O’Dowd *et al.* (1993). In all cases medium used was MS medium supplemented with Auxins and Cytokinins. Procedure used for suspension culture was routine i.e. period
of growth on solid medium followed by selection of a friable callus as a starter inoculum in liquid culture. O’Dowd (1991) studied 12 species for suspension culture where he found that friable callus could be encouraged by frequent subculture of callus. For the initial formation of suspension culture, a relatively high inoculums density was preferable. However, several *Ephedra* species failed to produce Ephedrine and Pseudoephedrine and synthesized them in trace amounts as found by O’Dowd *et al.* (1998) in suspension cultures.

Callus from *E. andiana*, *E. fragilis*, *E. intermedia*, *E. major*, *E. saxatilis* readily adapted to growth in liquid medium. Suspension cultures established from green callus had a greater rate of survival. *E. fragilis* grew almost at equal rate, same colour and friability as of *E. saxatilis* yet suspension culture of *E. fragilis* could not be established (O’Dowd, 1991). In the same report by O’Dowd (1991), *E. gerardiana*, *E. minima* and *E. saxatilis* formed relatively fine suspensions with few aggregates greater than 50 cells. In contrast *E. andina*, *E. fragilis subsp. Camlypoda*, *E. intermedia* and *E. major* formed cell aggregates up to 4 or 5mm in diameter.

Praveen and Murthy (2010) developed suspension culture system for improved cell biomass production and enhancement of withanolide A. The effect of macronutrients and nitrogen source on cell growth and withanolide A production using cell suspension cultures was given by Nagella and Murthy (2011). Enhancement in the production of Withanolide A, Withanone and Withaferin A in hairy root cultures using Methyl jasmonate and Salicylic acid as elicitors was carried out by Sivanandhan *et al.* (2013).


### 2.6 Elicitation and precursor feeding-

Pioneering work of Mitra and Kaul on production of reserpine from *Rauwolfia serpentina* marked the beginning of production of secondary metabolites in India (Sarin, 2005).
The enhanced increase in secondary metabolite production is usually associated with increase in activities of flavonoid pathway enzymes; phenylalanine ammonia lyase and chalcone isomerase (Dixon et al., 2002)

Different levels of plant secondary metabolites can be induced with use of jasmonate elicitors with different chemical structures (Qian et al., 2004a, b). The effect of Jasmonic acid, methyl jasmonate and salicylic acid elicitation on cell growth in cell suspension cultures as well as on hypericin and hyperforin production has been demonstrated by various workers (Sirvent and Gibson, 2002; Walker et al., 2002; Liu et al., 2007a.b). Methyl jasmonate proved best for increased production of anthocyanin in suspension cultures of Vitis vinifera (Qu et al., 2011), for gymnemic acid (Veersashree et al., 2012), aromatic compounds and monoterpenoids (Grover et al., 2012), ginsenosides (Lu et al., 2001), saikosaponin (Aoyagi et al., 2001), Hypericum perforatum (Wang et al., 2015)

Salicylic acid acts as endogenous regulatory molecule which plays important role in plant’s resistance against pathogens and triggers secondary metabolite production. (Wang et al., 2004). There are many reports on enhancement of secondary metabolites using salicylic acid as elicitor namely, in Catharanthus roseus (Godoy- Hernandez and Loyola- Vargas, 1997), Hyoscyamus muticus (Mehmetoglu and Curtis, 1997), Taxol in Taxus chinensis (Wang et al., 2004), Hypericum perforatum (Gadzouska et al., 2013)

Plant growth regulators not only regulate plant growth but also influence the secondary metabolite production in different ways in various higher plants (da Rocha et al., 2005). Casein hydrolysate (CH) is an organic nitrogen source containing organic amino acids mixtures and is elicitor for secondary metabolite production (Hegazi and El-Lamey, 2011). CH stimulated ephedrine accumulation in callus cultures of Ephedra alata. Amongst the concentrations tested, maximum ephedrine was produced on 0.5mg/l casein hydrolysate and gradually decreased upon increase in concentration.

Triterpenoids in in Scutellaria baicalensis (Yoon et al., 2000), ginsenoside in Panax ginseng (Lu et al., 2001), gymnemic acid (Veersashree et al., 2012)
Aspergillus Niger and Fusarium orthoceras in Vitis vinifera (Qu et al., 2011), Endophytic fungi were isolated, identified, cultured and used as elicitor in production of inophyllum in Calophyllum inophyllum (Pawar et al., 2011)

Precursor feeding for the enhancement of secondary metabolites has been reported in different plants. Sairam and Khanna (1971) reported that phenylalanine and tyrosine increased secondary metabolites in Datura tatula. Shikonin derivatives were stimulated to grow in high percentage upon L-phenylalanine addition in experiments carried out by Muzukami et al. (1977). L-phenylalanine is precursor of ephedrine as it directly incorporated in nitrogen of ephedrine as reported in book written by Evans (1999). The increased production of taxol in Taxus cuspidata cultures (Fett-Neto, 1994).

Phenylalanine and tyrosine synthesize phenolic compounds via Shikimic acid pathway (Farah and Donangelo, 2006).

Ramawat and Arya (1979a) studied effect of various amino acids like L-phenylalanine, DL-methionine, glycine, serine, aspartic acid with IBA and NAA on ephedrine production from Ephedra gerardiana callus cultures. Cultures grown on aspartic acid, serine or leucine showed poor response in terms of growth. L-phenylalanine at concentration 0.1 g/l yielded highest ephedrine. IBA was best auxin for ephedrine production (Ramawat and Arya, 1979b) however, Kin was better than BAP for ephedrine and pseudoephedrine production (Parsaeimehr et al., 2010a)

According to Yamasaki et al. (1973) amino acids L-phenylalanine, methionine and glycine play important role in ephedrine biosynthesis. Ramawat (1978) in his Ph.D. thesis tried to explain the reason for increase in ephedrine production by these amino acids as elicitors. At high auxin and nitrogen levels in cultures amino acids are converted to proteins but when auxins and nitrogen is exhausted; the protein synthesis stops and the amino acids used for synthesis of ephedrine. L-phenylalanine with IBA was also found best for increased production of alkaloid contents in, Ephedra gerardiana (Ramawat, 1978; Ramawat and Arya, 1979) Ephedra foliata (Lodha et al., 2014)
2.7 Extraction, Analysis and determination of secondary metabolites in *Ephedra* species-

The main alkaloid components are the optically active distereoisomers of ephedrine *viz.* L- ephedrine, D-pseudoephedrine, D-norpseudoephedrine, L-norephedrine, L- methylephedrine and D-methylpseudoephedrine. The crude drug quality depends on presence of alkaloidal content (Cui *et al.*, 1991). Various workers have attempted to quantify these alkaloids using various techniques like gas chromatography (Yamasaki *et al.*, 1974), HPLC (Sagara *et al.*, 1983; Moriyasu *et al.*, 1984). However, the methods were not appropriate in terms of accuracy, resolution or sensitivity as these could determine only three, four or five out of six alkaloids. Zhang Jian-Sheng *et al.* (1988) developed a HPLC method to determine all six alkaloids in samples but according to Cui *et al.* (1991) this method was not full proof as sensitivity to determine L- methylephedrine and D-methylpseudoephedrine was very low.


Many times HPLC involve use of SDS in mobile phase to increase theoretical plate number and resolution but ephedrine separation depend on quality and concentration of SDS used (Okamura *et al.*, 1999; Sheu and Huang, 2000). Kim *et al.* (2003) described use of $^1$H –NMR spectroscopy for quantitative analysis of *Ephedra* alkaloids. They claimed the process as rapid and involved no pre-cleaning steps. This is advantage over HPLC and GC methods, which require clean up procedures and derivatization procedures to enhance separation sensitivity and to check inhibition by other compounds.


Cui *et al.* (1991) claimed GC/MS method in which all the six alkaloids were derivatized by trimethylsilylation of hydroxyl and amino groups using *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA). The trimethylsilyl (TMS) derivatives of
three distereoisomers were identified. This study was carried out on 12 species of *Ephedra* from China. Likewise, other derivatives were also used for such identifications, these includes pentafluoropropionyl- derivatives (Marchei *et al.*, 2006), Trifluoroacetyl- (Coutts *et al.*, 1980), carbon disulphide- (van der Merwe and Hendrikz, 1995).

The difficulties during separation of these alkaloids were also highlighted by NIST ephedrine alkaloid standard reference material in which values are calculated based on independent methods (Sander *et al.*, 2005).

Ranieri and Ciolino (2008) combined selective extraction solvent ammoniacal chloroform with a two stage derivatization method developed by Spyridaki *et al.* (2001). In this new GC MS method two derivatization stages were applied; first; with N- methyl, N-trimethylsilyl trifluoroacetamide (MSTFA) and trimethylsilylimidazole (TMSI) and second stage with N-methylbistrifluoroacetamide (MBTFA). The O-trimethylsilyl and N-trifluoroacetyl are derivatives for ephedrine, pseudoephedrine, norephedrine and norpseudoephedrine and O-trimethylsilyl for N-methylephedrine and N-methylpseudoephedrine.

According to Hu and Zazhi (1994) general chromatography methods were not sufficient for analysis of ephedrine in callus cultures as it has requirement of long separation time and weak separation efficiency problems. Liu and Sheu (1992) developed capillary electrophoresis method for determination of ephedrine alkaloids to overcome this problem. Capillary electrophoresis has advantage of high speed separation, requires small amount of sample proved it an advantageous technique. Li *et al.* (1999) found that the capillary used to get block by precipitate in run buffer at high pH. So, a new method of capillary electrophoresis was developed in which acetonitrile was added as modifier in Tris-NaOH- H₃PO₄ buffer. This addition increased plate number and migration behavior of hydrophobic analytes was also improved. Combination of capillary flow injection with UV detection was established for ephedrine and pseudoephedrine determination by Chen *et al.* (2003). Non aqueous capillary electrophoresis technique gained importance in recent years. Differential selectivity, high efficiency, better solubility of compounds in organic solvents than in water and shorter analysis of time made it an important technique.
for applications in pharmaceuticals (Barthe et al., 2002; Unita et al., 2003; Dong et al., 2005). Derivation of ephedrine and pseudoephedrine in non-aqueous media was first time reported by Dong et al. (2006). Non-aqueous capillary electrophoresis with laser-induced fluorescence method was developed for separation and determination; 4-chloro-7-nitrobenzo-2-oxa-1,3-diazol was used as derivatization agent.

Four non-protein amino acids with cyclopropane ring namely (2S, 3S, 4R)- and (2S,3R,4S)-2-(carboxycyclopropyl) glycine; (2S,3S,4S)-2-(carboxycyclopropyl) glycine were identified and characterized by Starratt and Caveney (1995) from seeds of E. altissima and cis-3,4methanoproline from seeds of E. foeminea & E. foliata.

Starratt and Caveney (1996) reported quinolone-2-carboxylic acids like kynurenic acid, 6-hydroxykynurenic acid and 6-methoxykynurenic acid from E. foemina and E. foliata. Kynurenic acids are also reported from Ginkgo biloba as constituents of leaves (Drieu, 1986).

Hikino et al. (1982) fractioned crude extract of Ephedra roots to obtain Ephedradine A, B, C and D as active pronciples from basic fractions. During the process they observed the hypotensive activity of acidic fraction also. This acidic fraction was a polyphenol termed as Ephedrannin A; its structure was given by same workers using NMR studies.

Ephedroxane is an anti-inflammatory principle reported in 9 Ephedra species by Konno et al. (1979).

Miyazawa et al. (1997) investigated volatile components of Ephedra sinica. The volatile oils were extracted using hydrodistillation and diethyl ether as solvent. The extracted volatile oils were analyzed using GC MS and NMR. Total 146 volatile compounds were present in GC and out of which 71 were identified by MS.

The flavonoid content of Ephedra had been reported by various workers these include di-C-glycosylflavones (Wallace et al., 1982), Flavonol-3-O-glycosides (Wallace et al., 1982; Chumbalov and Chemeneva,1977) and proanthocyanidins (Friedrich and Wiedmeyer,1976). Nawwar et al. (1984) reported isolation and identification of flavonoids from Ephedra alata. The two new flavonoids Herbacetin 8-methyl ether 3-O-glucoside-7-O-rutinoside and herbacetin 7-O-(6"-
quinylglucoside) were reported in addition to Vicenin II, Lucenin III, kaempferol 3-
ramnoside, quercetin 3-rhamnoside. Herbacetin 7-glucoside was also reported in
_E.lomatolepsis_ (Zakiroeva et al., 1983) and In _E. equisetina_ herbacetin 8-methyl
ether 3-glucoside was reported by Chumbalov and Chemeneva (1977). Ichikawa et
al. (2003) developed method for quantitative analysis of pseudoephedrine and
ephrine in herbal medicines using Solid phase extraction and ion pair reversed
HPLC method.

2.8 Biosynthesis of Ephedra alkaloids

Presence of ephedrine and pseudoephedrine alkaloids had been reported in
many plant species other than _Ephedra_ for example in _Sida cordifolia_ (Ghoshal et
al., 1975),_Catha edulis_ (Kalix,1991), _Roemeria refracta_ (Southon and
Buckingham,1989), , _Taxus baccata_ , _Aconitum napellus_ (Duke,1986), _Hamelia
patens_ (Chaudhari and Thakur,1991). Based on this wide spread presence; O’Dowd
et al. (1998) suggested that these alkaloids could be derived from same primary
metabolite.

Most of the research in this regard has been focused to find main source of C_6
ring, the C_3 side chain, the amino group and methyl group.

It was originally thought that, the complete aminophenylpropanoid system of
Ephedrine was derived from phenylalanine. However, Yamasaki _et al._ (1973) found
that phenylalanine in _Ephedra_ provided only C_6-C_1 unit. The reason behind
considering phenylalanine as a potential precursor is its structure having C_6-C_3 ring
with amino group on C_2 carbon of C_3 side chain (O’Dowd _et al._, 1998). [^{14}C] labels
from phenylalanine could be found in l-ephedrine from _Ephedra_ and _Catha edulis_
(Shibata&Imaseki, 1956; Yamasaki _et al._, 1973; Grue-Sorensen and Spencer, 1988
1989). Shibata and Imaseki (1953) reported phenylalanine as an efficient precursor
of d-norpseudoephedrine.

Conversion of l-phenylalanine to cinnamic acid is catalyzed by Phenyl
Ammonia Lyase enzyme by elimination of ammonia and cinnamic acid can be
hydrolysed by β-oxidation into benzoic acid and acetic acid. These generalized steps
in higher plants; even though not reported in _Ephedra_ separately, were assumed to
be present in them. (O’Dowd, 1998).
Yamasaki et al. (1973) proved the incorporation of [14C] labelled benzoic acid with more efficiency than [14C] labelled Phenylalanine in ephedrine in *Ephedra*. He also suggested that benzoic acid might be derived by alternative route like shikimic acid. O’Dowd et al. (1998) found the labelled benzoic acid incorporation in ephedrine and pseudoephedrine form whole plants of *E. minima*. Benzoic acid could have provided C₆-C₁ unit to ephedrine and norephedrine and other C₂ unit could be condensed by pyruvic acid (Grue-Sorensen and Spencer, 1988).

Proposed biosynthetic pathway for *Ephedra* alkaloids based on experiments of Grue-Sorensen and Spencer (1988) was reviewed by O’Dowd et al. (1998) as given below:
Phenylalanine

\[ \text{Phenylalanine} \rightarrow \text{Phenylalanine ammonia lyase} \]

Cinnamic acid

\[ \text{Cinnamic acid} \rightarrow \text{Acetic acid} \]

Benzoic acid

\[ \text{Benzoic acid} \rightarrow \text{Pyrubic Acid} \]

\[ \text{H}_2\text{O} + \text{CO}_2 \rightarrow \text{Pyruvic Acid} \]

Phenylpropanedione

\[ \text{Phenylpropanedione} \rightarrow \text{Amino group from another amino acid} \]

\[ \text{Transamination} \rightarrow \text{H}_2\text{O} \]

Cathinone

\[ \text{Cathinone} \rightarrow \text{Reduction with } \text{NADH} + \text{H}^+ / \text{NADPH} + \text{H}^+ \]

\[ \text{NAD}^+ / \text{NADP}^+ \text{ (Cofactors ?)} \]

Norephedrine/Norpseudoephedrine

\[ \text{Methylation with } \text{S-adenosylmethionine} \]

Ephedrine/Pseudoephedrine
2.9 Other activities-

There are many reports on antimicrobial activities of Ephedra. Ephedra major inhibit aflatoxin production by Aspergillus parasiticus (Bagheri and Bigdeli, 2009). Antibacterial activity of Ephedra has been shown against various bacteria like Staphylococcus aureus, Bacillus anthracis, B. diptheriae, B. dysenterie, B. typhosus and Pseudomonas aeruginosa as well as volatile oils from Ephedra showed inhibitory activity against Asian influenza virus (Soltan and Zaki, 2009; Ramawat and Arya, 1976)

Bonjar (2004) confirmed antimicrobial effects of E. intermedia while studying antiyeast activity of some medicinal plants of Iran.

Parsaimehr et al. (2010) carried out experiments to compare three Ephedra species (E. strobilacea, E. procera and E. pachyclada) and their callus cultures for their potential to show antibacterial, antifungal, antioxidant activity. These activities were related to total phenolic content. Overall highest antimicrobial activity was showed by E. strobilacea methanolic extracts. However, the methanolic extracts of E. procera showed highest antifungal activity against Candida albicans and E. pachyclada showed it against Klebsiella pneumoniae. The callus cultures of these species showed lower antioxidant activity than wild plants. Ephedra gerardiana showed antimicrobial activity against E. coli at concentration 15mg/ml.

Kim et al. (2010) for the first time proved potential of Ephedranin A and B as inhibitors of inflammatory mediators like NO, TNF- α and 1L-1β through suppression of NF-kB and p38 MAP kinase in LPS stimulated RAW2647 cells. This study also marked Ephedranin B as a potential candidate for human inflammatory diseases as it shows similarity with other characterized phytochemicals like curcumin, resveratrol etc.

Review of literature revealed that E. foliata is a potential therapeutic gymnosperous species. It has been noticed that female plant of this particular species is highly potent therapeutically. Despite being most of the previous literature survey was pertaining to the micro-propagation and secondary metabolite of female plant only and male Ephedra foliata has not been studied so far. Hence, in vitro studies along with enhancement of secondary metabolite of male Ephedra foliata has been investigated and reported in the present investigation.

Page | 26