Herbal extracts with a broad spectrum of therapeutic effects are mostly mixture of various bioactive substances, unlike allopathic where a single active substance is identified to show its bioactivity. Many publications on phytoceuticals have reported them to be suitable for long-term and for follow-up treatment of chronic diseases. The phytoceuticals may have complementary or supplementary and rarely inhibitory interaction of the given phytodrug, and more proper and scientific evaluation including its application has been warranted. There are reports where single known substance was unable to give bioactivity when compared with phytoceuticals, substantiate above view (Butcher, 1977).

It is well known that the action of a plant extract as phytocomplex may substantially differ from that of the pure active molecules. The search for the active principles in a given plant extract may be unrewarding if it does not show pharmacological activity. The complexity of plant extracts makes a difficult task that requires a huge analytical effort and manufacturing skills to produce well-defined, standardized phyto-preparations and to ascertain their mechanisms of action.

The medicinals or officinales are integral part of the natural biodiversity and history of traditional knowledge of a country (Okigbo et al., 2008) and modern pharmacopoeia still contains about 25% drugs derived from plants and many others, which are synthetic analogues built on prototype compounds isolated from plants. Interest in medicinal plants as a re-emerging health aid has been fuelled by the rising costs of prescription drugs, uncontrollable residual toxicity in the maintenance of personal health and the bio-prospecting of new plant-derived drugs (Lucy & Edgar, 1999).

Medicinally important plants harvested on a mass scale for commercial use, have depleted the resources and the conservation of these valuable genotypes is imperative. In vitro culture techniques came up as an alternative to the problem, which offered a
viable tool for mass multiplication and germ-plasm conservation (Li et al., 2004; Koilpillai et al., 2010).

The propagation of plants through plant tissue culture (PTC) is based on the principle of totipotency (Steward, 1959; Staba, 1980; Staba, 1985). The PTC in its infancy between the 1940s and 1960s was marked as new technique, Later this technique was utilized for extraction of specific value biochemical in variety of plant species, which has now assumed a major significance in metabolomics (Thorpe, 2007).

Totipotency is universal characteristic but its expression is species specific and limited to special type of cells (Torrey, 1966; Staba, 1980), which are found at various developmental stages of plant (Steeves & Sussex, 1957; Murashige et al., 1972; Cheng & Voqui, 1977). Selection, source, morphogenetic stage and age of explant including tissue or organ used have been important determinant of the degree of success of PTC (Endress, 1994).

Media play an important role, addition or deletion of certain growth regulators result into differentiation and or regeneration vary considerably; usually high concentration of 2,4-D promotes callus formation rather than differentiation (Fujii, 1970) and different types of organogenesis could be obtained by varying the concentration of cytokinins in the medium (Skoog & Miller, 1957). The relative higher concentrations of cytokinin induces shoots, the lower concentration of cytokinin relative to auxin, roots are induced and at the intermediate concentration the tissue may grow as unorganized callus (Nishi et al., 1968). Kawase (1964) demonstrated seasonal response in rooting of branch cuttings, which was later shown to be a function of cambial activity and auxin production in many woody species. Despite the fact that auxin and nutrition level as well as the rooting response, suggest a probable existence of certain balance between certain auxin and nutrition (Nanda, 1970).

The type and concentration of auxin and cytokinins and their relative ratio in the culture medium also control the biosynthesis and accumulation of secondary
metabolites. The accumulation of phenolics, coumarins, flavonoids and lignans were stimulated in the presence of low auxin levels, especially NAA (King, 1976; De-Eknamul & Ellis, 1985b, or 2, 4-D (Sugano et al., 1975). Increasing the auxin concentration either stimulated (Zenk et al., 1977) or inhibited phenolic production (Ibrahim & Edgar, 1976; Sohai & Shuler, 1984).

In the present study it has been observed that juvenile and actively dividing plant respond effectively in vitro condition due to vigorous vegetative development stage and absence of reproductive structure formation. Even in juvenile stage, tissue and organ regeneration has been more with the younger and actively dividing tissues (Endress, 1994; Reinert & Bajaj, 1977).

Various physical and chemical factors effect the growth of the plant tissue cultures and in order to achieve rapidly proliferating undifferentiated callus mass from highly organized multicellular system, use of optimum combination and concentration of plant growth hormones (auxin and cytokinins) is very essential (Skoog and Miller, 1957; Mantell & Smith, 1983; Varshney, 2009; Chen & Gao, 2013).

2, 4-D, IAA, IBA, NAA, BAP, Kn and purine derivative have been used successfully for callus culture of a number of plant species (Reinert & Bajaj, 1977). Formation of different colored callus has been observed by many workers and it is well documented by Ibrahim (1988).

Panigrahi et al., 2006 reported formation of multiple shoots along with little callusing when media supplemented with BAP 2.0(mgL-1) NAA 0.5 (mg L-1). Besides these there are no earlier attempts of tissue culture in H. auriculata

Plant tissue culture is a well-known biotechnological tool for the rapid propagation of medicinal plants for the purpose of commercialization (Kitto, 1997), conservation (Anis et al., 2007) and cryopreservation (Decruse et al., 1999). Somatic embryogenesis and organogenesis have been the common pathways for the clonal propagation of
superior medicinal plant species (Gary and Brent, 1986). There are some scanty reports on tissue culture of *Hygrophila* species.

Varshney *et al* (2009) reported somatic embryogenesis when media supplemented with BAP: NAA (1:0.5). Cinar *et al* (2013) reported adventitious shoot regeneration in *H. polysperma* when media was supplemented with Kn : IBA (0.10-1.60: 0.10mg/L). Panigrahi *et al* (2006) reported formation of multiple shoots along with little callusing when media supplemented with BAP 2.0(mgL-1) NAA 0.5 (mg L⁻¹). Besides these there are no earlier attempts of tissue culture in *H. auriculata*.

In present investigation callus was raised from nodal segments of *H. auriculata* grown on media NAA: Kn (1:1, 1:2 mgL⁻¹), BAP: IBA (0.5: 2 mgL⁻¹) and BAP: NAA (1: 0.2 mgL⁻¹), respectively unlike other reports mentioned above. In *H. quadrivalvis* callus proliferated from nodal segments and leaf explants grown on media NAA: Kn (0.3: 0.3, 0.3:2, 2.5:4, 1:2 mgL⁻¹), BAP: Kn (1:1 mgL⁻¹), IBA: BAP (2:2 mgL⁻¹) and BAP: NAA (2:0.2 mgL⁻¹).

In the present investigation when media supplemented with BAP: NAA (3: 0.3, 4: 0.4 mgL⁻¹), IBA: IAA (3: 4 mgL⁻¹) callusing along with multiple shoot initiation was observed. Media containing IBA (2 mgL⁻¹) direct regeneration of root from leaf explants was observed in *H. auriculata*.

In *H. quadrivalvis* when media supplemented with BAP: Kn (2: 0.2 mgL⁻¹), IBA: NAA: Kn (2: 0.1: 0.2 mgL⁻¹) callusing along with multiple shoot initiation was observed. Media supplemented with IBA: BAP (2: 0.5 mgL⁻¹) callus along with shoot and root was observed. When nodal explants cultured in a media supplemented with 2, 4-D (3mgL⁻¹) direct regeneration of shoot observed. This observation was not in agreement with findings of Kaminek *et al* (1987, 1997).
Media supplemented with IBA (2, 4 mgL$^{-1}$) direct regeneration of shoot and root from leaf explants was observed in *H. quadrivalvis*. Media containing IBA (2 mgL$^{-1}$) direct regeneration of root from leaf explants was observed in *H. auriculata*.

There is no uniform and clear definition of growth of plant cell cultures and dry weight or fresh weight methods have been in use for determining GI, because of its preciseness, accuracy in observing variation (Grossmann K, 1988). Several workers have established the unorganized static cultures of different plants on different medium and observed the sigmoid growth pattern of the callus culture (Agarwal & Kamal, 2004a; Agarwal & Kamal, 2004b; Staba, 1980; Endress, 1994). In the present study a sigmoidal pattern of growth curve was observed in *H. auriculata* and *H. quadrivalvis*. The maximum growth index was achieved at the 6th week of subculture indicating the exponential growth phase. Minimum growth index was observed at 2nd week of subculture. An increase in GI after supplementation of various growth regulators finds support from the observations that the growth of tissue, sometime depends upon the culture medium and also controlled by the environmental and biological factors like pH, dose and combination of growth regulators used (Barz et al, 1977; Heble, 1985; Schripsema et al, 1990).

Biochemical studies of the individual plant parts is a necessary prerequisite in order to evaluate their importance in the overall metabolism of the plant, as well as the role of specific substances that may be produced as direct or indirect products of metabolism in same physiological processes. Hence carbohydrates, proteins, amino acids, chlorophyll, vitamins, hormones, phenol etc are very essential for plant without which the plant life is hampered.

In the present study Maximum content of total soluble sugars was present in callus of *H. auriculata* (182 mg/ gdw) and minimum in callus (14.5 smg/ gdw) of *H. quadrivalvis*. Maximum content of starch was in root (127 mg/gdw of *H. auriculata* and minimum in root (59 mg/ gdw) of *H. quadrivalvis* which was at par with stem.
(61.69 mg/gdw) of the plant. Total protein content was maximum in seeds (710 mg/gdw) and minimum in root (200 mg/gdw) of *H. quadrivalvis*. Lipids were maximum in seeds (26 mg/gdw) of *H. quadrivalvis* and callus (11 mg/gdw) of *H. auriculata* had minimum content which was at par with root (12.1 mg/gdw). Total phenol content was highest in seeds (7.50 mg/gdw) of *H. auriculata* and lowest in root (2.0 mg/gdw) of *H. quadrivalvis* which was at par with stem (2.2 mg/gdw). Similar results were observed by others which have evaluated the primary metabolites of different medicinal plants (Schulz & Baranska, 2006; Vijayvergia & Kumar, 2007; Tanwer & Vijayvergia, 2010).

Metabolites are often concentrated in seeds and are needed for physiological development because of their role in basic cell metabolism. The difference in the content may be attributed to the various geographical niches in which these plants grow as suggested by Butcher (1977). Most of the bioactive compounds derived from primary metabolites (amino acids, carbohydrates, and fatty acids) are generally categorized as secondary metabolites (Bonner & Varner, 1965). Significant advances in our understanding for the synthesis of these secondary metabolites have been achieved through the use of plant cell cultures.

There are number of examples where not only primary metabolites but also secondary metabolites produced in cell cultures. This would discount the objection sometimes raised to higher plant cell cultures as potential commercial biosynthetic systems in that they were very slow growing. If a large biomass could function to release over a precursor, then the time required to grow up the biomass would be of small economic consequences. Further, the ability in the close system of constantly moving the metabolite could mimic a biological “sink” preventing any “staling” effect (toxicity) by preventing accumulation.

Plant cell culture may serve as an alternative industrial source of phytochemicals. The study of compartmentation mechanism together with metabolic studies, improvement
of culture media and selection of cell lines is particularly relevant in order to increase
the production of phytochemicals.

It has been emphasized that secondary metabolites have not been systemically
assayed in the culture medium (Petiard & Courtois, 1983). The kinetics of cell death
compared to the kinetics of metabolite excretion in most cases showed the excretion
of phytochemicals contributing to the overall dynamics of the metabolites. Secondary
metabolites are usually not distributed uniformly within the whole plant (Wiermann,
1981). Some are restricted to specific organs, other to specific tissues. The knowledge
of the biosynthetic pathways of secondary compounds and precise sites of
accumulation is still scanty.

Coordination and cooperation among various cells are prerequisites for the existence
of highly developed organisms and require structural and functional specialization of
certain cells. The cell differentiation follows specific biochemical and morphological
principles in the formation of morphologically specialized cells in multicellular
organisms. The formation of secondary plant products is endogenously controlled and
is integrated parts of a differentiation process (Endress, 1994).

Flavonoids are a series of related water soluble phenolic glycosides derived from
aromatic amino acids, have also been reported to have pathological significance in
plants by providing resistance to the plants against pests and insects (Cruckshank &

Apigenin from *Glycine max* and *Acacia pinnata* (Dongmo *et al*, 2007), apigenin,
luteolin, quercetin and chrysoerial from *Petroselinum hortense* (Kreuzaler &
Hahbrock, 1973), quercetin from Tephrosia purpurea (Khanna *et al*, 1976; Oleszek
and Stochmal, 2002), apigenin, luteolin, quercetin and kaempferol from *Indigofera
tinctoria* (Kamal & Mangla, 1990) and luteolin quercetin and kaempferol in
*Trigonella polycerata* (Kamal & Yadav, 1991), luteolin and apigenin from *Genista*
Discussion

*tinctoria* (Luczkiewicz et al, 2004), kaempferol, quercetin and rutin from *Pongamia pinnata* (Marzouk et al, 2008) and *Melilotus neapolitana* (Fiorentino et al, 2007) and also from tissue culture of other fabaceous plants have been reported (Butcher, 1977; Endress, 1994; Kamal et al, 2007).

The flavonoids usually present in their free form and bound form as glycosides are located at reactive sites. Therefore, the difference in content between free and bound forms shows their involvement at resting and active stages, thus giving higher or lower recovery of free and/or bound flavonoids (Harborne et al, 1975).

In the present investigation flavonoids were more in their free form in all plant parts. Quercetin presence in experimental plant in vivo was confirmed with Hussain et al (2012). Callus culture of *H. auriculata* quantitatively has more content (2.97 mg/gdw) than the other plant parts, it is in confirmation with several other reports where the secondary metabolites are exceeding than the parent plant production in vivo (Butcher, 1977; Zenk, 1978; Khanna, 1982; Fujita & Tabata, 1987; Mathur, 2001). Total flavonoid content (free & bound) was maximum in seeds (2.27 mg/gdw) and minimum in root (0.71 mg/gdw) of *H. quadrivalvis*. This is like the views of some workers that cultured plant cells do not accumulate secondary metabolites as readily as their parent plants (Barz & Ellis, 1981; Dougall, 1981; Berlin, 1983; Ellis, 1984; Heinstein, 1985a, b). Comparative study of both plants the total flavonoid content (free & bound) was maximum in callus (2.97 mg/gdw) of *H. auriculata* and minimum in root (0.71 mg/gdw) of *H. quadrivalvis*. Sometime quantity and type of flavonoids produced in vitro are restricted when compared with the intact plant, may be due to the presence of degradative enzymes in cultures (Hosel et al, 1972; Barz et al, 1974; Butcher, 1977).

Phyto-metabolites that appear first in different morphogenetic callus are phenolics and flavonoids. Cells producing them generally do not need specialized structures i.e. common parenchyma cells and are less complex (Constable et al, 1974). Media
components and composition are also important factors which results in product formation and accumulation. Amongst all the media components hormones, have received greatest attention. There is sufficient evidence to indicate that type and concentration of auxins and cytokinins, as well as their relative ratios in the culture medium, control the biosynthesis of secondary metabolites. Auxin especially NAA and 2,4-D affect the metabolite production in cell cultures, 2,4-D generally inhibits various metabolites but there are few reports that indicated that 2,4- D stimulate the production of polyphenols in certain cases (Sakuta & Komamine, 1988).

Increase in flavonoid content in the callus culture in the present investigation may be attributed to the combinations and concentrations of phytohormones used in the culture medium, which was in confirmation with the studies of Kamal & Yadav (1992) and Kamal & Mangla (1990).

In many plant species alkaloids get accumulated in seeds relatively high concentration as a chemical defense. During germination alkaloid get metabolized and their nitrogen is reused for seedlings metabolism (Wink & Witte, 1985). Preliminary report in presence of alkaloids in *H. spinosa* has been shown (Patra et al., 2009; Dash et al., 2012).

Trigonelline, a pyridine alkaloid serves as a storage form of nicotinic acid (Blaim & Wanner, 1960) was reported from any plants. Iribarren and Pomilo (1983) reported hypoglycaemic and hypocholesterolaemic activities of trigonelline. Khanna and Jain (1972) reported 5.2% of crude trigonelline mass in *Trigonella foenum-graecum* while 0.38% trigonelline from seeds of *T.foenum-graecum* was reported by Kuhn and Gerhard (1943). Samola and Gerhard (1983) reported (0.8% to 1.1%) trigonelline from green coffee, Kaushik and Khanna (1990) reported 0.014% from *Dolichos lablab* seeds and 0.0068 % of trigonelline from its callus cultures and Mathur (1992) reported 0.7% trigonelline in 6 weeks callus of *Vigna sinensis*. 
In the present investigation presence of trigonelline in *H. auriculata* and *H. quadrivalvis* have been reported first time. Among various plant parts maximum trigonelline content was observed in callus (2.47 mg/gdw) followed by seeds (2.36 mg/gdw), aerial part and minimum in roots of *H. auriculata*. In *H. quadrivalvis* plant parts, maximum trigonelline content was observed in seeds (2.33 mg/gdw) followed by callus (2.28 mg/gdw), roots and minimum in aerial part. On comparing of both plants maximum trigonelline content was observed in callus (2.47 mg/gdw) and minimum in root (1.21 mg/gdw) of *H. auriculata*. In tissue culture trigonelline was reported from *Trigonella foenum-graecum* (Khanna & Jain, 1972; Antony et al, 1975; Willeke et al, 1970, Radwan & Kokate, 1980), *Pisum sativum* and *Glycine max* (Tramontano et al, 1985), *Trigonella polycerata* (Yadav, 1988; Kamal et al, 1996), *Dolichos lablab* (Kaushik & Khanna, 1990), *Vigna sinensis* (Mathur, 1992), *Momordica dioica* (Katariya, 2010) and *Moringa oleifera* (Mathur & Kamal, 2012).

Use of precursor in culture medium to enhance the end product has been advocated (Staba, 1980). Nicotinic acid acts as precursor in the biosynthesis of trigonelline (Johnson & Lin, 1953), Khanna and Jain (1972) observed an increase in growth and trigonelline in tissue culture of *T. foenum-graecum* fed with nicotinic acid in 8 week old callus cultures. Later, Khanna et al (1989) reported 2 fold increase in 6 week old callus obtained from 50% nicotinic acid fed medium in *Allium cepa* callus cultures.

In the present studies, similar results were observed in both experimental plants. In *H. auriculata* and *H. quadrivalvis* callus cultures trigonelline content increased 1.42 and 1.14 fold, respectively in 6 weeks old tissue fed with nicotinic acid, whereas the growth of callus decreased remarkably, which may be due to enhanced trigonelline content that checks cell proliferation by cell arrest as suggested by Lynn et al (1978).

Sterols are ubiquitous in higher plants and their plant tissue cultures. They are known to be the starting material for the synthesis of a number of plant steroids, which are pharmaceutically important group of compounds, as sex hormones, corticosteroids and oral contraceptives. According to Stumpf and Cohn (1981) the meristmatic part of
the plant is most active in sterol synthesis. As the tissue ages, the rate of biosynthesis of metabolites decreases, but the sterols and alkaloids on the contrary continues to increase until the plant starts to senescence. This increase in sterols content may be due to a loss in primary metabolism and accumulation of secondary metabolites such as steroids and alkaloids (Grunwald, 1981) may be attributed for higher content at 6-8 weeks after fresh subculturing and maintenance of tissue cultures for more than 6 months by periodic subculturings.

Generally, the younger and growing tissues of plants synthesize higher amount of sterol (Stumpf & Cohn, 1981). The rate of general metabolism decreases with the age of the tissues, but the sterol level continued to increase until the plant senescence (Grunwald, 1981). The individual sterol levels may be affected with plant development, such as the ratio of β sitosterol to stigmasterol, which showed that meistmatic and storage tissues with high β sitosterol and low stigmasterol (Hartmann & Benveniste, 1974).

Presence of stigmastanol in roots of *Asteracantha longifolia* has been reported (Quasim & Dutta, 1967). Aerial parts of *A. longifolia* have been reported to contain lupeol, stigmasterol and butelin (Quasim & Dutta, 1967). In present study the maximum amount of steroidal content was maximum in seeds (2.36 mg/gdw) of *H. auriculata* and minimum in roots (0.93 mg/gdw) of *H. quadrivalvis*. Seeds act as storage organ (storage tissues) find support of observation of Hartmann and Benveniste (1974) and Grunwald (1981).

Sapogenins are C_{27} steroids, widely distributed in a number of families. Steroidal sapogenins, the pharmaceutically important natural products have been reported from a number of plants (Blund –den et al, 1975; Kadkade et al, 1976; Sharma & Kamal, 1981; Kamal & Sharma, 1984; Mangla & Kamal, 1989). Diosgenin, used in contraception is industrially important, which is either microbially or chemically converted to medicinally useful steroids commercially extracted from underground
portion of various *Dioscorea species* (Staba, 1977). It had also been reported from *Costus* sps, *Solanum* sps, *Trigonella* sps (Staba, 1977; Hardman, 1980; Sharma & Kamal, 1982; Kamal *et al.*, 1997; Kamal & Yadav, 1992), *M. charantia* (Agarwal & Kamal, 1998), *M. diocia* (Katariya, 2010), *Kallostroemia* (Chattopadhyay *et al.*, 1983), *Costus* (Prasad and Janakiammal, 1983), *Solanum* (Bhattacharya *et al.*, 1980), *Smilax* (Kar & Sen, 1984) species. Nino *et al.*, (2007) reported the percentages of diosgenin obtained from the seventy four accessions of *Dioscorea polygonoides* ranged from 0.02 to 2.64%, which was significant since there are several reports where the diosgenin contents was very low, for example: *D. polygonoides* (0.2%; Coursey, 1967), *Dioscorea prazeri* (1.92% Mahato *et al.*, 1981) and *Dioscorea villosa* (1.3%; Martin, 1969). Other steroidal sapogenin, tigogenin has also been reported by several workers (Kier & Gisvold; Vijay *et al.*, 2005).

In the present investigation presence of diosgenin and tigogenin was confirmed both in *vivo* and *in vitro*, which has not been reported earlier in *H. auriculata* and *H. quadrivalvis*. Among the plant part, total steroidal content was maximum in callus of *H. auriculata* (5.73 mg/gdw) and lower in roots. In *H. quadrivalvis* seeds steroidal content was maximum (6.07 mg/gdw), followed than callus, aerial part and roots. These results were not in agreement with Netien *et al.*, (1967) who reported that secondary plant products may not persist on subculture or that the products produced might chemically change on repeated subculture (Boulanger *et al.*, 1973; Kodama *et al.*, 1980; Mehra, 1990; Kamal & Mangla, 1993).

In present investigation diosgenin and tigogenin has been isolated from morphogenetic stage, callus associated with root and shoot. *H. quadrivalvis* had maximum content of diosgenin and tigogenin (2.72 mg/gdw and 2.24 mg/gdw) in comparison to (2.40 and 2.12 mg/gdw) steroidal content present in *H. auriculata*.

Use of precursors or an intermediate to be transformed biosynthetically in to the desired product has been advocated (Staba, 1985; Kamal & Mehra, 1995). Cholesterol
is one of the intermediate compounds in biosynthetic steps leading to formation of diosgenin. Diosgenin is produced from various species of *Dioscoera* cells, yielded less than 1% (Kaul & Staba, 1968) and from 1 to 2.5% dry weight if grown on media containing cholesterol and 2,4-D (Kaul et al., 1969). *Dioscorea deltoidea* cells incorporated cholesterol (Stohs et al., 1969) that led to formation to diosgenin (Tomita et al., 1970; Tomita & Uomori, 1974).

In present investigation six month old maintained callus cultures of in *H.auriculata* and *H.quadrivalvis*, were transferred to MS medium supplemented with different treatment doses (0.025, 0.05, 0.1 mM) of cholesterol and later harvested at different time intervals. All primary metabolisms possibly were channeled as precursor at the onset of secondary metabolic routes in the direction of secondary product formation (Harazdina & Jensen, 1992). Diosgenin recovery in the precursor fed tissue was significant in *H.auriculata*; (5.22 mg/gdw) and *H.quadrivalvis* (5.93 mg/gdw) comparative to control. This may due to incorporation of cholesterol acting as precursor in the biosynthesis of diosgenin finds support of work of Kaul & Staba (1968), Stohs et al (1969), Tomita et al (1970) and Staba (1977).

It has been known for long time that cultures are heterogeneous collection of cells and their composition changes with successive cultures (Yeoman et al., 1982). The synthesis of many secondary products is associated either with specialized differentiated cell types or organized tissue system. Other factors responsible for the loss of *in vitro* biosynthesis capacity may be loss of availability of precursors with passage of subculturings and accumulation of degradative enzymes (Butcher, 1977). Bailey (1970) showed a decline in pisatin (phytoalexin) production in *Pisum sativum* after prolonged culture. Hosel et al (1972) and Barz et al (1974) reported a decrease in flavonoid content in suspension cultures of *Cicer arietinum, Phaseolus aureus, P. hortense* and *G. max* due to presence of degradative enzymes as well as loss of precursors. Moreover, it is generally accepted, not all the cells in the cultures are
active in product synthesis. Thus gave differential yield of secondary metabolites at different time intervals (Fowler, 1986).

In the present study variation in diosgenin content at various morphogenetic stages finds support from above mentioned in vitro reports on product accumulation.

It has been shown during last decades that secondary product formation can be triggered in cell culture due to environment stress. Treatment in cell cultures by elicitors of fungal, bacterial origin or plant cell wall resulted in increased formation of flavonoids, stilbenes, terpenoids, alkaloids etc. In some instances chemically more define “elicitors” including heavy metal salts, bioregulators and DNA active substances results in similar inductions of product formation. (Hadwiger & Schwochau, 1971; Wink & Witte, 1983; Stoessel, 1984; Frischknight & Bawmann, 1985).

Production of secondary metabolites in tissue culture can be enhanced by use of elicitors (Wolters & Eilert, 1982; 1983; DiCosmo & Misawa, 1985; Verpoorte et al, 1991; Eilert, 1988). Elicitor in a cell cultures offer a novel approach for rapid accumulation of certain secondary metabolites which can improve the efficacy of product via induction of various enzymes or by providing additional biosynthetic pathway. Such treatment could lead to substantial change in cellular metabolism.

The time required for activation of biosynthetic pathway is plant specific, but there are some evidence that amount and type of elicitors may effect or modulate the time course (Kombrink & Hehlbrock, 1986). In related plant species which form structurally related compounds in response to elicitation the time course of accumulation are similar.

Effect of various biotic such as fungal filtrate of *Pythium aphanidermatum* (Kamal et al, 1995), ergosterol etc and abiotic elicitors such as UV, pH, heavy metal ion and
Discussion

chemicals has been reported for induction of various secondary metabolites (Heinstein, 1985a; Collinge & Susarenka, 1987; Mukandan & Hjorosto, 1990; Roewer et al, 1992; Namdeo et al, 2002; Satdive et al, 2007; Alves et al, 2007).

Salicylic acid, an abiotic elicitor, is basic compound of salicylate an important group of pharmaceutical agents. It is involved in several processes in plants and has an important signaling role in plant defense against pathogens inducing systemic acquired resistance, which in turn leads to the formation of bioactive compounds (Verberne, 2000). It has been observed that application of exogenous biotic and abiotic elicitors induce expression of defense related genes leading to the production of specific secondary metabolites thereby providing partial protection against pathogen (Eilert, 1988).

Enhanced metabolite yield by elicitation is a result of a complex interaction between the elicitor and the cultured plant cell. In this process number of enzymes are involved which may or may not induce various biosynthetic pathways (Hahlbrock & Scheel, 1989). Salicylic acid acts as a signal compound in the induction of plant defense mechanism thereby increasing the production of secondary metabolites which supports the studies of Kamal et al (1995). Moreover, salicylic acid might also affect the transient transcription and translation of enzymes needed for the biosynthetic pathway of the metabolite of interest (Eilert, 1988; Memelink, 2000), which is also in confirmation with the observation of Verpoorte (2000) that certain biosynthetic pathways are inducible at the gene level by external signals and such signals can induce complete pathway.

In the present study, six month old maintained callus cultures of *H.auriculata* and *H.quadrivalvis* were fed with different dose of SA and SNP in the medium. Diosgenin recovery in the tissue was highly significant in *H.auriculata*; (1.23 times in SA and 1.05 times in SNP) and in *H. quadrivalvis* (1.45 times in SA and 1.14 times in SNP) comparative to control. Increase in production of diosgenin shows confirmation with
the studies of Rokem *et al* (1984) and also with the findings of Kamal *et al.*, (1995); Mathur (2012) that SA might be acting as signal compounds in the induction of plant defense mechanism thereby increased the production of diosgenin in the present investigation.

Free radicals are derivatives of oxygen ("Reactive Oxygen Species", ROS) these are superoxide anion (O$_2^-$), perhydroxy radical (HOO$^-$) and hydroxyl radical (HO$^-$) and derivatives of nitrogen ("Reactive Nitrogen Species", RNS) such as peroxynitrite radical. High pro oxidant activity of free radicals is known as oxidative stress. These radicals are formed by a one electron reduction process of molecular oxygen. ROS initiates the lipid peroxidation of the membrane and damaging the phospholipids, lipoprotein by propagating a chain reaction cycle (Pryor, 1973).

Several methods such as ORAC (Oxygen Radical Absorbance Capacity), TRAP (Total Radical- Trapping Antioxidant Parameter), TOSC (Total Oxidant scavenging Capacity), TAC (Total Antioxidant Capacity), DPPH radical scavenging assay, FRAP (Ferric Reducing Antioxidant Power), CUPRAC (Copper Reduction), HG (Haemoglobin glycosylation) assay and TEAC (Trolox Equivalent Antioxidant Capacity) have been developed for measuring the total antioxidant capacity of plants, these assays differ in their chemistry (generation of different radicals and/or target molecules) and in their mechanism where end points are measured (Pellegrini *et al.*, 2000). The most commonly used ones are those involving chromogen compounds of radical nature that stimulate the reductive oxygen species. These methods are popular due to their ease, speed and sensitivity. The presence of antioxidants leads to the disappearance of these radical chromogens. These assays have presented distinct challenges in evaluation of purified individual compound, mixed extracts, endogenous food matrices, herbs, supplementation of vitamins and other chemicals/medicines. Optimization of right method/s of application & evaluation is the need of time (Pellegrini *et al*, 1999).
Antioxidant activity and mechanisms are system dependent which vary with radical targets, individual v/s total antioxidant concentrations and solvent of extraction (Barclay et al., 1999), presence of competing metals, pH and presence of oxygen (Rice-Evans, 1996) in flavonoids and phenolic acid preparations and testing methods (Finley, 2005; Prior et al., 2005; Schaich, 2005). Therefore, different antioxidant compounds act through different mechanism; therefore no single method can fully evaluated the antioxidant activity.

Antioxidant reacts with DPPH free radical, the electron becomes paired off and bleaching of the colour stochiometrically in methanol solutions that depends on the number of electrons taken up. This diversity in such methods is due to the complexity of analyzed substrate (Sanchez-Morino, 2002; Badmis et al., 2003; Kamal et al., 2011, Kamal et al., 2012).

In the present study, four different fractions (MF, HF, DCF, EAF) of *H. auriculata* and *H. quadrivalvis* plant parts and callus culture using DPPH radical scavenging assay were analysed and observed that some fraction show better AE. Present findings are in agreement with the report of workers who observed antioxidant effect of *in vivo* plant parts of *Hygrophila* sps (Hussain et al., 2009; Biswas & Bhattacharya, 2012, Kanhere et al., 2013).

Haemoglobin glycosylation (HG) is an *in vitro* non-enzymatic method. Being an oxidation reaction, an antioxidant is expected to inhibit the reaction (Pal & Dutta, 2006; Mathur, 2012). Glyco-haemoglobin is formed throughout the circulatory life of RBC by the addition of glucose to the N-terminal of the haemoglobin β chain. This process which is non-enzymatic reflects the average exposure of haemoglobin to glucose over an extended period. The degree of haemo-glycosylation *in vitro* in the presence of different concentration of fraction can be measured.
A group of medicinal plants possessing nutritious and tonic functions were chosen as model plants, and their extracts were evaluated for their antioxidant capacities using the FRAP assay. The FRAP is a simple assay that gives fast, reproducible results (Benzie & Strain, 1996). It is versatile and can be readily applied to both aqueous and alcoholic extracts of plants. In this assay, the antioxidant capacity is measured based on the ability to reduce ferric ion to ferrous ion, and the results were expressed as μmol ferrous iron equivalent per gram of sample.

In comparative study of both plants maximum AE was observed in MF of callus ((42.63± 0.71) which was at par with MF (40.53± 1.01) of seeds of *H. auriculata* and which was at par with MF (39.99± 0.18) of *H. quadrivalvis* seeds and minimum in HF (7.15± 0.52) of *H. quadrivalvis* roots.

A group of medicinal plants possessing nutritious and tonic functions were chosen as model plants, and their extracts were evaluated for their antioxidant capacities using the FRAP assay. The FRAP is a simple assay that gives fast, reproducible results (Benzie & Strain, 1996). It is versatile and can be readily applied to both aqueous and alcoholic extracts of plants. In this assay, the antioxidant capacity is measured based on the ability to reduce ferric ion to ferrous ion, and the results were expressed as μmol ferrous iron equivalent per gram of sample.

Highest activity was observed in MF (220± 1.99) of seeds and minimum in EAF (9.00± 0.62) of *H. auriculata* roots. Present findings are in agreement with the report of workers who observed antioxidant effect of *in vivo* plant parts of Hygrophila sps (Shanmugasundaram & Venkataraman, 2006). In *H. quadrivalvis* plant maximum content was obtained in HF (150± 0.49) of seeds which was at par with MF (148± 1.4) of callus and minimum in HF (11.04± 0.48) of roots. In the present study methanolic extracts of dried plant parts and callus tissue showed higher AE, when compared with reference vitamins (A, C, E). These results are in confirmation with Kahkonen *et al* (2001) that sometimes extracts show synergistic actions in comparison to the
individual compounds due to their distinct antioxidant and pro-oxidant properties. Moreover, combinations of metabolites concentration in the given extract also play a complimentary or supplementary role in determining the bioactivity.

Among the flavonoids when quercetin was used as a reference compound the loss of color (Fade percentage) indicated the free radical scavenging efficiency of the substances (Tapia et al, 2004; Lima et al, 2009) in both fractions (free & bound) which was significant change occurred. In present investigation results of *H. auriculata* and *H. quadrivalvis* plant parts and callus culture are in confirmation with this report.

Plant cells respond to various biotic and abiotic elicitors by activating a wide array of reactions (viz., ion fluxes across the plasma membrane, synthesis of reactive oxygen species-ROS and phosphorylation and de-phosphorylation of proteins). These are all putative components of signal transduction pathways that lead to elicitor-induced defense responses, e.g., the activation of defense genes and hypersensitive cell death (Dietrich et al, 1990; Baker & Orlandi, 1995). It has been suggested that ROS alone cannot mediate a sufficient disease resistance response in plants, but in combination with nitric oxide (NO) can function synergistically to activate a stronger response (Wang & Wu, 2005). Therefore, NO is a diffusible and bioactive signaling molecule (Neill et al, 2003; Chun-Hua et al, 2007).

Effect of elicitors was determined on the seeds and *in vitro* grown six months old maintained callus cultures (Kamal et al, 2007). The different treatment doses of salicylic acid, sodium nitroprusside (0.025, 0.05, 0.075 & 0.1mM), ascorbic acid (0.05 & 0.1mM) and sodium chloride (50 & 100 mM) showed significant increase in enzymatic activity after 6, 12, 18 & 24h time intervals (Yadav, 2010).

NO which is often the end product of sodium nitroprusside is an interesting signaling molecule having homeostatic properties for the coordination and synchronization of cellular metabolism (Lamattina et al, 2003). It has been shown that NO and reactive
oxygen species (ROS) can considerably impact plant growth and development (Apel & Hirt, 2004; Lamotte et al, 2005). NO and ROS can interact with each other, and these interactions can be cytotoxic (resulting in cellular death) or cytoprotective, depending on the concentrations and situation (Wink & Mitchell, 1998).

Among them, nitrogen species such as ammonium, nitrate and NO have been implicated in root growth and proliferation. It has also been reported that NO plays a central role in the lateral root formation (Correa-Aragunde et al, 2004; Creus et al, 2005). Moreover, NO has been involved in adventitious root formation, protects plant cells from photooxidative damage (Beligni & Lamattina, 2002) and retards cell death induced by abscisic acid (Zhou et al, 2005), gibberellins (Beligni et al, 2002) and cytokinin, 6-benzylaminopurine (Carimi et al, 2005).

Superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) are the most protective enzymes to remove reactive oxygen species. SOD catalyzes the dismutation of superoxide radical to generate O$_2$ and H$_2$O$_2$, and POD and CAT catalyze the conversion of the produced H$_2$O$_2$ to H$_2$O and O$_2$ (Asada, 1999). Both aerobic and anaerobic organisms possess superoxide dismutase enzyme, which catalyze the breakdown of superoxide radical (Shirwaiair et al, 2007). SOD is a kind of isoenzyme generally existent in the organs and tissues of animals and plants. It can prevent senescence and organism injury caused by active oxygen. It can catalyse exceed oxide anion to oxidation, reduce the damage of membrane system, increase the quality of crop and enhance the crop’s ability of resistance to stress. The result indicated that leaves have a higher SOD content than root. Because chloroplast is an important of plant photosynthesis cell organ. When chloroplast absorbs high-energy continuously, it will be likely to appear oxide anion. In order to keep plant grows normal, plenty of SOD which can clear away oxide anion to prevent plant damaged.

Catalase exerts a dual function, decomposition of H$_2$O$_2$ to give H$_2$O and O$_2$, and oxidation of H ion donors. In the ultraviolet range H$_2$O$_2$ shows a continual increase in absorption with decreasing wavelength. The difference in absorbance per unit time is
Discussion

a measure of catalase activity (Aebi, 1984). Increase in CAT activity could be due to higher production of H$_2$O$_2$. It is possible that CAT activity, which in turn would protect SOD inactivation by H$_2$O$_2$, causes an increase in SOD activity (Sepici-Dinkel et al, 2007).

The peroxidase enzyme reacts anaerobically with some substrates like pyrogallol, with H$_2$O$_2$ as an electron acceptor associated with biochemical and physiological processes in cell formation, fruit development and ethylene biosynthesis and various stresses (Matamoremoros et al, 2003).

In the present study the effect of an in vitro graded supply of sodium nitroprusside (SNP) as NO producer and the relationship between nitric oxide and other elicitor responses, e.g., H$_2$O$_2$ production, the activation of antioxidant defenses and effect on various other antioxidative enzymes was observed. Sodium nitroprusside and salicylic acid are the important group of pharmaceutical agents. The application of exogenous salicylic acid induces expression of defense related genes and provides partial protection against pathogen (Eilert, 1988). Salicylic acid (SA) is a natural and hormone-like signal molecule for the activation of plant defenses, and regulates a large variety of physiological processes in plants (Klessig & Malamy, 1994; Wang & Li, 2006). SA can regulate the activities of intracellular antioxidant enzymes such as SOD, POX and increase plant tolerance to environmental stresses (Senaratna et al, 2000; Sakhabutdinova et al, 2004), apoplastic antioxidant enzymes when plant leaves exposed to cold stress and induction of stress tolerance by affecting apoplastic proteins (Tasgin et al, 2003, 2006).

In present study the effect of an in vitro graded supply of SNP as NO producer and the relationship between NO and other elicitor response H$_2$O$_2$ production, activation of antioxidant defenses and effect on various other antioxidative enzymes was observed. SNP and SA are the important group of pharmaceutical agents. The application of exogenous SA induces expression of defense related genes and
provides partial protection against pathogen (Eliert, 1988; Katariya, 2010; Mathur, 2012). The NO scavenging activity in *H. auriculata* and *H. quadrivalvis* increased with increasing concentration of Aa (except in *H. auriculata* seeds at 0.1 mM after 6h) and NaCl (except in seeds of *H. quadrivalvis*).

The CA activity increased with increasing concentration of Aa and NaCl in both plants. SOD activity increased with increasing concentration of SNP and NaCl. The POX increased with increasing concentrations of NaCl treatment doses in both of plants.

Plant-based antimicrobials represent a vast untapped source for medicines and further exploration of plant antimicrobials is needed as antimicrobials of plant origin have enormous therapeutic potential. They are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects of synthetic antimicrobials (Iwu *et al.*, 1999). They may act as lead compounds for the pharmaceutical industry or as the base for the development of new antimicrobials (Adegoke & Adebayo 2009; Mathur *et al.*, 2012).

There are some earlier attempts on antimicrobial activity on various species of *Hygrophila*. Boily and Vampuyvelde (1986) and Vlientick *et al.* (1995) examined the antimicrobial activity of an ethanolic extract of the leaves, stem, fruits and root of *Hygrophila auriculata* reported that the leaves exhibited potent anti-microbial activity. In the present investigation initial screenings of the *H. auriculata* and *H. quadrivalvis* for possible antimicrobial activities was done using crude methanolic extracts. In present study *H. auriculata* and *H. quadrivalvis* showed antimicrobial activity against *E. cloacae, S. aureus, S. viridens* and *E. coli*. Similarly, in case of fungal strains both plants showed potent activity against *Aspergillus niger*, and *Trichoderma harzianum*. 
Platelets play a key role in thrombus diseases. Since there are receptors and other target molecules on platelet membranes, the present study developed a novel approach, which could be used to analyze and evaluate the binding of drug receptors under an artificial physiological environment while screening anti-platelet aggregation activities (Hong et al., 200; Kamal et al., 2012).

Increased platelet aggregation (PA), as a result of increased platelet sensitivity to agonists \textit{in vivo}, contributes to the initiation and progression of atherosclerosis and to the occurrence of thrombotic events (Lusis, 2000; Ross, 1999). Platelet aggregation, which is associated with an increased release of reactive oxidative species (Freedman & Keaney, 1999) and platelet-vessel wall interactions (Adams et al., 2000), results in damage to the vascular endothelium (Cai & Harrison, 2000). The atherosclerotic disease process is thought to begin with the impairment of endothelial function (Adams et al., 2000) followed by the increased accumulation of oxidized LDL (ox-LDL) within the wall underlying the damaged endothelium. Unregulated uptake of ox-LDL by tissue macrophages leads to the transformation of these macrophages into foam cells, which then build up within the arterial wall causing intimal thickening and the formation of fatty streak lesions (Nordestgaard et al., 1995). In addition to their role in the initiation of atherosclerosis, platelets also contribute to the progression of the disease by releasing various growth and chemotactic factors that accelerate the proliferation and migration of smooth muscle cells (Ross, 1999). Therefore, reducing the activity of platelets would potentially reduce development and progression of CAD.

In the present investigation various extracts of seeds and leaves of \textit{H. auriculata} and \textit{H. quadrivalvis} gave significant antiplatelet activity. \textit{H. auriculata} seeds aqueous extract show significant activity as compare to \textit{H. quadrivalvis} seeds aqueous extract may be due to accumulation of certain bioactives in particular organ.

It was observed that aqueous extracts were more potent than EtOH extract and prolonged clotting about 32.00 times in PT Assay when compared with standard and
10.50 times when compared with APTT Assay. The minimum aqueous treatment doses at 100µgmL⁻¹ was found to be quite significant in PT Assay and a linear increase in APTT Assay was observed in EtOH extract with increment of treatment doses up to 2000 µgmL⁻¹, which may be due to release of some metabolites with time and alcoholic concentration used, needs further investigation and identification of alleged bioactive/s. Progression in clotting time in seconds was observed in PT seconds indicated that minimum dose was efficient.

The aqueous extract of *H. auriculata* and *H. quadrivalvis* seeds prolonged the clotting time maximum up to 480 sec and 433, respectively which was much higher than the *M. malabathricum* (180 sec) as per the findings of Manicam *et al* (2010) and *P. niruri* 28 times higher than control (Kamal *et al*, 2012) thus indicated that *H. auriculata* plant is more potent than *H. quadrivalvis* and the both plants are more potent than *M. malabathricum* and *P. niruri*. Present study highlighted that the anticoagulant activity of seeds and leaves aqueous extract of both plants affect the intrinsic pathway of a coagulation cascade by causing clotting factor (s) deficiency. Consequently extracts of both plants proved a potential herbal based anticoagulant candidate and demonstrated remarkable activities when subjected to a series of *in vitro* coagulation screening procedures.

For the present study concluded that *H. auriculata* and *H. quadrivalvis* are a good source of important bioactives such as flavonoids, alkaloids, steroids, non-enzymatic and enzymatic antioxidant components. The fractioned extractives using different solvents analysed for their antioxidant potential using radical scavenging (DPPH), HG and FRAP assay gave significant antioxidant activity. Evaluation of bioefficacy (anticoagulant) of these metabolites showed pharmacological importance. In future it could be promising to investigate the underlying molecular mechanism/s associated and also long term toxicity studies, if any, in different animal models as well as on human to ascertain there therapeutic ventures.