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A REVIEW ON LICORICE

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ABSTRACT: *Licorice (Glycyrrhizaglabra L) is an important herb used in almost all systems of medicine. The author tries to present in this article a comprehensive review on all aspects of Licorice.*

INTRODUCTION

Licorice, *Glycyrrhiza glabra* L. belongs to the Family – Fabaceae; Tribe – Astragaleae. The name *Glycyrrhiza* is of Greek origin which means "sweet wood". Since ancient times, licorice finds an important place in Chinese medicine. It was considered to rejuvenate those consuming it for longer periods [Chopra et al 1958]. Licorice is referred to by Theophrastus. Romans cultivated it after thirteenth centuryand called it as *Radix dulis*. In UK it is in cultivation from sixteenth century. [Trease and Evans 1983]. In Indian medicine; licorice is one of the principal drugs of *Susruta.* It is referred to as *Mulethi, Malahatti, Yastimadhu* and in Malayalam called as *Erattimathuram.* In Japan, commonly known as *Kanzo.* Species other than *G.glabra* were also found to yield compounds of commercial value.

In virtue of its importance in food and pharmaceutical industry, licorice was extensively subjected to scientific investigations. Attempts were made to understand the biochemistry of licorice and its derivatives. New compounds were derived from this plant utilizing new and improved analytical methods. Recent advents in molecular biology and biotechnology led to the efforts to improve/increase the yield of compounds which are of economic importance.

Occurrence

Different varieties of *G. glabra* are known to yield commercial drugs [Trease and Evans 1983].

G. glabra var. *typica* Reg. *et. Herd.* The plant is about 1.5 m high bearing publish – blue flowers. The underground portion consists of long root and thin rhizome or stolons and penetrates the soil upto a depth of 1m or more. It is the source of Spanish licorice and is grown in Spain, Italy, England, France, Germany and the USA.

G. glabra var. *glandulifera* Wald. *et* Kit. is the source for Russian licorice and grows wild in Central and Southern Russia. The underground portion consists of large root stock bearing numerous long roots but no stolons.

G. glabra var *B.violacea* Bioss. is Persian licorice which bears violet flowers. It is available in Iran, Iraq and in the valleys of Tigris and Euphrates. The other

macrosopic and microscopical characters were detailed by Trease and Evans [1983].

G.lepidota was reported to grow in rich soil of low or moist land in the valleys or on the plains 20 to 4000 feet throughout California [Jepson 1970].

Cultivation of Licorice

Licorice can be cultivated or obtained from wild plants. The plants are known to grow well in deep fertile sandy soils near streams in the subtropics. Dry seasons are beneficial to the crop and thrives well in warm regions where the annual rainfall is not more than 50cm. Fertile sandy of sandy-loan soils devoid of any stones are optimal for licorice. Manuring is not required unless the soil is not fertile [Singh *et al* 1984]. *G.uralensis,* another species of *Glycyrrhiiza* known to yield commercial products was reported by Nadezhina *et al* [1981] to grow in dense sands.

G.glabra was found to exist under wide range of soil salinity [Mirkin *et al* 1971]. Drought resistant variety of *G. glabra* was reported by Aprasidi [1978] from the flood plains of river Amudarya. Khafizova [1978] recorded the highest yield of roots and top growth of Golodnaya steppe and Amudarya populations of licorice in chloride sulphate soils of Uzbek, USSR.

Mohammad and Rehman [1985] compared the cultivation of licorice in irrigated and rainfed sanddunes. Survival percentage was more in irrigated sands. To achieve stable yields of licorice, irrigation of *G.glabra* was a must in oasis region sands [Durmeshev 1986]. It was reported by Osipov [1987] that variations in the yield of root mass of licorice was caused by different hydrogeologic regimes of Amudarya flood plain. Increase growth was recorded where subsoil waters were at a depth from 177cm to 195cm.

Reclamation of desert sands upon cultivation of licorice was demonstrated in sands adjacent to oases in the Russian desers [Kel'dzhaev and Gladyshev 1982]. A study carried out by Varganov and Gladyshev [1981] revealed the utility of cultivation of licroce on oasis sands in stabilization of sand and quick improvement of soil. Similar report was also on record [Mohammad and Rehman 1985] which shows the stabilization of sanddunes after cultivation of licorice.

Though inter-plantation of carrot, potato or cabbage crops along with licorice is feasible for the first two years, it is discouraged in the view of the increase in weed population [Singh *et al* 1984].

Reports are available on the cultivation of licorice in India. It was found to grow well in Patiala, Hissar of Haryana State [Singh 1964], Uttar Pradesh [Uniyal *et al* 1978] and in South India [Ahmad and Khaleefathullah 1986]. *Abrus precatorius* Linn. Is commonly called as wild licorice, Indian licorice or licorice bush. Though the roots of this plant are found as a substitute for genuine roots, it was not recommended by Chopra *et al* [1958] due to the toxic properties associated with it.

Propagation

Propagation is generally performed by employing 15cm long stolons with two or three buds obtained from the planting stock. Old crowns were also used as planting materials [Singh *et al* 1984]. Germination capacity of *G.glabra* seeds at different stages was studies by Gladyshev and Kerbabaev [1967]. Seeds in waxy-ripe stage were found to show highest germination

rate. Slow germination of scarified seeds of licorice was revealed from the studies of Yaskonis [1976].

The cuttings planted and irrigated in the ridges of 45-60 cm in height facilated root development. Poor quality of licorice yield resulted when the plants were allowed to set seeds. Removal of flowers immediately after their emergence yielded good quality of licorice [Singh *et al* 1984]. Seed germination of *G. uralensis*, as reported by Liu [1987] depends on the thickness and texture of soil.

Licorice root production was successfully enhanced by treating cuttings with 0.0025% succinic acid followed by the application of nitrogen and phosphorus. The root weight was found two fold in three-year old plants [Badalov 1978].

Different harvesting procedures were detailed by Singh *et al* [1984]. Regeneration occurred from underground root system after harvesting and were ready for further harvesting after two to five years.

The water product of licorice after extraction was found useful as animal feed [Yumatni *et al* 1980]. It was reported by Timofeyer [1984] that the waste yields inorganic substances and vitamins.

Diseases and Pests of Licorice

Only few reports are available on the disease and pests of licorice. Gentrospora acerina caused licorice rot [Bank 1963]. Viral infection of licorice was documented by Blattny *et al* [1950]. *Heterodera glycyrrhizae* [Narbaev 1987] and *Acanthoscelides aureolus* [Boe *et al* 1988] were the pests of licorice reported from USSR and USA respectively. According to CIMAP [1982], *G.glabra* plants were affected by weevil, *Myllocerus* undecimpustulatus.

Developmental studies in Licorice

Galimova [1978a] conducted studies on male and female gametophyte development in *G.glabra* and *G. uralensis.* Microsporogenesis and development of gametophyte of *Glycyrrhiza* was investigated by Ashurmetov *et al* [1979]. Embryological features in spontaneous hybrids of *Glycyrrhiza* revealed normal male and female gametophyte development and seed production. Superior seed quality was also reported from such hybrids [Ashurmetov and Sakhibaeva 1985]. Factors affecting the seed productivity in *G.glabra* and *G.uralensis* were assessed by Galimova [1978b]. Crossess of *G.glabra* and *G.ralensis* produced viable fruits and seeds. Galimova and Murinova [1985] further found that these hybrids were of immense use from cultivation studies.

Mikailov and Mirzaliev [1978] demonstrated distinctive morphological characters such as color of flowers, their size, weight, shape of pod as well as yield and number of seeds to be helpful in identification of useful forms of *G. glabra*. Based on anatomical characters, Sukhova and Kel'dzhaev [1987] differentiated licorice grown in sands and natural conditions in Amudarya alluvial plain [USSR]. They showed an increase in volume of stalk and root in *G. glabra* grown in sands. To identify the crude drugs present in licorice, a detailed key was developed by Zeng *et al* [1988] by evaluating the morphological and microscopically similarities.

Cytogenetics of Licorice

The diploid chromosomes complement of Glycyrrhiza glabra was established as 2n = 16 [Darlington and Wylie 1955]. Morphological and biological polymorphism in different populations of *G.glabra* were reported by Tashmukhamedov and Aprasidi [1977]. Karyological investigations were carried out in three species of *Glycyrrhiza*, namely *G. glabra, G. lepidota* and *G. echinata* by Barghi and Siljak-Yakovlev [1990]. Inspite of their morphological variations, these species revealed a stable chromosomes number supporting the earlier investigations [Taylor and Taylor 1977; Mikailov and Mirzaliev 1978; Pauzner and Tashmukhemdov 1978; Magulaev 1980; Ashurmetov and Karshibaev 1982]. However, total and relative lengths of chromosome pairs and centrometric indices differed among species. Ashurmetov *et al* [1979] recorded normal meiosis in six different species of *Glycyrrhiza*.

Licorice Phytochemistry

The major constituents of licorice are triterpenoids and flavonoids. Apart from these, occurrence of other classes of compounds in small quantities has also been reported. The Sweetness of licorice is due to glycyrrhizin [a triterpenoid compound]. Presence of flavonoids gives yellow colour to licorice. Quantitative differences could be observed in the compounds derived from various sources of licorice. Analytical methods such as TLC, GLC, HPLC were employed for the separation of constituents of licorice. Further evaluation and determination were carried out using spectrophotometric, mass spectrophotometric, and mass spectrophotometric and NMR procedures.

Flavonoids

Afcher *et al* [1980] isolated and identified licuroside, liquiritigenin, isoliquiritigenin and liquiritin from Iranian *G. glabra var. glandulifera.* Epigeal portions of *G. glabra* yielded new flavonoids galangin, naringenin, tioxy-isoflavone and dioxy-flavone apart from pinocembrin and glabranins [Baturov *et al* 1986]. Structure of licoricidin, a prenylated isoflavan isolated from Si-pei licorice was characterized by Fukai *et al* [1988]. They also studied the structure of six isoprenoid substituted flavonoids from Xibei licorice [Fukai *et al* 1989]. Licoflavanone isolated from the leaves of *G. glabra* [Fukai *et al* 1988] was found to possess antimicrobial property. Neolicuroside, a new chalcone glycoside was isolated by Miethin and Speicher-Brinker [1989] from the roots of *G. glabra.* Two flavanon glycosides have been isolated by Yahara and Nishioka [1984] from *G. uralensis.*

Nakanishi *et al* [1985] isolated flavonoid glycosides from *G. uralensis* roots. On the basis of irradiation experiments, Shiozawa *et al* [1989] revised the structure of glycyrol and isoglycyrol from *G. Uralensis* roots. Gancaonins, the prenylated flavonoids, were isolated from aerial parts of *G.uralensis* and *G.palladiflora* and characterized by special studies [Fukai *et ali* 1990a, b].

Studies of Ghisalberi *et al* [1981] showed that aerial parts of G.acanthocarpa yielded four isopreny-lated resocrcinol derivatives. *G.eurycarpa*, new species was reported from Gansu province in China. Liu and Liu [1989] for the first time isolated glycyroside, an isoflavone diglycoside, apart from liquiritigenin, isoliquiritigenin and schaftoside. Cultured cells of *G.echinata,* also called as pseudoglycyrrhiza, yielded two flavonoids viz. echinatin and licodione

and their structure were evaluated by Ayabe *et al* [1980].

Triterpenoids

The biosynthetic pathway of glycyrrhizic acid was demonstrated by Fraz *et al* [1983]. Glabranin A and B were isolated from *G.glabra* root. Varshney *et al* [1983] elucidated their structure employing TLC and HPLC. Shu *et al* [1987] determined the structure of uralenolide, a new triterpenoid lactone from *G. uralensis.* Employing a chromatographic system, a new triterpene namely glyuranolide was isolated from the crude sapogenin of *G.uralensis* [Jia *et al* 1989]. Recently, Mirhom *et al* [1990] isolated a new triterpenoid from the roots of *G. echinata*. Licorice roots yielded 5-penta cyclic triterpenoids [Elgamal *et al* 1990].

Polysaccharides

A neutral polysaccharide, glycyrrhizin, was isolated by Shimizu *et al* [1990] from the roots of *G. uralensis.* Similarly Tomoda *et al* [1990] characterized two polysaccharides, glycyrrhizans UA and UB from the same source.

Phenolic compounds of Licorice

Hatano *et al* [1989] isolated licopyranocoumarin, an anti HIV phenolic compound from Si-pei licorice from the north – western region of China. Four new phenolic compounds were derived from the roots of *Glycyrrhiza* species. Kiuchi *et al* [1990] elucidated their structure on the basis of spectroscopic and chemical studies. Similarly, phenolic constituents of *G.uralensis* were studied by Fukai *et al* [1991].

Other constituents of Licorice

Phrrolo – pyrimidine alkaloid and tetrahydroquinoline alkaloids were isolated from the roots of *G.uralensis* [Han dna Chung 1990; Han *et al* 1990]. Licocoumarone, a new benzofuran derivative from licorice was isolated and characterized by Demizu *et al* [1988]. Antimicrobial glepidotin, a bibenzyl from American licorice, *G. lepidota* was reported [Mitscher *et al* 1983; Sitaraghav *et al* 1989]. Volatile flavor components were isolated by Miyazawa and Kameoka [1990] from *G. glabra* var. *glandulifera* growing in north-east China.

Isolation and Determination of Licorice Constituents

Various methods employed to isolate the constituents of licorice were modified from time to time in accordance with improvement in technology. The traditional method of licorice extraction involves the shredding of dried roots and stolons and extraction with hot water. The aqueous extract was then allowed to concentrate after evaporation in vacuum pans. A paste with 18-25% moisture was obtained and moulded to sticks or blocks of required size and shape [Singh *et al* 1984].

Modern continuous extraction plants have been developed in later years [Masters 1972; Molyneux 1975]. An equipment was devised by Muraviev *et al* [1985] for gravitational multistep extraction of solid-liquid system to extract licorice roots to yield higher quantities of biologically active agents. Stepanova [1985] demonstrated the usefulness of fermentation of harvested plant material. Fermentation led to an increase in the yield of triterpene saponins from *G. glabra* when compared to the non-fermented sample.

An array of analytical methods are on record for the determination of licorice derivatives. High performance liquid chromatography [HPLC] was applied to estimate the content of *Glycyrrhiza* in traditional Chinese drug 'Kampo" [Akada *et al* 1978]. Sulfostyrene cationic exchanger was used by Manyak and Muraviev [1984] for the spectrophotometric determination of glycyrrhizic acid in *Glycyrrihiza* roots and drug extracts. Sagara *et al* [1985] developed a simple precise method to determine glycyrrhizin in *Glycyrrhiza* radix employing ionpair HPLC. Using bilayer column chromatography, determination of glycyrrhizhin in cosmetic lotions and creams was demonstrated [Mikami *et al* 1988]. Spectrophotometric quantitation of hexoses and pentoses [free and linked to polysaccharide chain] from *G.glabra* extracts was reported by Riccio and Riviera [1988]. A second derivative [D2] spectrophotometric method was developed [Song *et al* 1990] recently to detect the total glycyrrhizic acid in *G. glabra* using ion-pair extraction technique. Zeng *et al* [1990a] devised a rapid HPLC method for simultaneous separation and determination of flavonoids and coumarins from licorice.

Chemical Synthesis / Modification of Licorice Derivatives

Attempts were made on chemical modification, biotransformation of licorice phytochemicals to obtain more derivatives of pharmacological importance. A perusal of literature, however, reveals no reports on direct chemical synthesis of licorice products.

Glycyrrhetinic acid was subjected to chemical modification to derive deoxyglycyrrhetol with a view to eliminated pseudoaldosteronism, a side effect accompanied with glycyrrhetinic acid administration. Chemically derived deoxyglycyrrhetol was found to lact aldosteronic effect, while it maintained the therapeutic activities [Shibata *et al* 1987]. Chemical synthesis of glycopeptides from glycyrrhizhic acid was demonstrated from glycyrrhizhic acid was demonstrated by Tolstikov *et al* [1989]. The derivatives were shown to possess anti-inflammatory activity and stimulating effects on hormonal factors of immunity. Chemical synthesis of glycyrrhizin from glycyrrhetinic acid was achieved by coupling of methyl glycyrrhetate with per-O-acetylated glycosyl bromides of mono and di-saccharides [Hirooka *et al* 1989]. The synthesized glycosides were found to be beta-type on the basis of NMR studies.

Amano[1] [1984] obtained patent for biotransformation of glycyrrhetic acid to 3-epiglycyrrhetic acid employing intestinal bacteria. The compound was found to have same effect as glycyrrhetinic acid excepting pseudo-aldosteronic effect. Biotransformation was attempted in virtue of the difficulty associated with the synthesis of compounds by chemical means.

Conversion of glycyrrhetinic acid to 18-beta-glycyrrhetic acid by *Streptomyces* G 20 [isolated from soil] was demonstrated by Sakano and Ohshima [1986] and their structures were demonstrated. Daiichi-Pharm3 [1986] employed *Chainia antibiotica* to transform glycyrrhetinic acid to its derivatives which have pharmaceutical value. Recent investigations of Tanaka *et al* [1990] suggested the use of soil bacterium, *Pseudomonas saccharophila* strain 11, for industrial use to convert glycyrrhizin to glycyrrhizic acid. The maximum yield was attributed to the higher beta – glucurudinase activity possessed by this bacterial strain when compared to its counterparts.

Licorice in *vitro*

Efforts were made to increase the yield in licorice, employing plant tissue culture techniques. Shah and Dalal [1982] attempted for *in vitro* multiplication under various cultural conditions employing modifications of MS medium. Their trails yielded successful establishment of plantlets and found 15-20 fold increase in multiplication rate when compared to propagation through stolon cuttings. Similarly, Syrtanova and Mukhitdinova [1984] tried colonel propagation of *G. glabra* and *G. uralensis.*

Investigations were also carried out to derive commercially important phytochemicals from licorice. Wu *et al* [1974] reported the absence of glycyrrhizin in suspension cultures of licorice. Hayashi *et al* [1988] recorded similar observations in callus and cell suspension cultures of *G. glabra.* The cells failed to produce detectable amounts of glycyrrhizin though the intermediate compounds such as betulinic acid, beta-amyrin were detected. They speculated the absence of glycyrrhizin production was to be due to interruption in the biosynthetic pathway of glycyrrhizin. Their later studies also revealed the failure of suspension cultures to produce glycyrrhizin after exogenous supplementation of 18 β-glycyrrhizic acid [Hayashi *et al* 1990a]. Triterpenoid biosynthesis in the cultured tissues of *G.glabra* var. *glandulifera* was ascertained by Ayabe *et al* [1990]. Their studies demonstrated the quantitative differences in the metabolic alterations in the intermediate products of glycyrrhizin biosynthesis in stolon segments, roots and callus cultures. However, in suspension cultures of *G.glabra,* accumulation of soyasaponins could be observed and their production was found to be influenced by culture strains and growth harmones [Hayashi *et al* 1990b].

Callus cultures of *G. uralensis* under optimal conditions were found to yield formononetin, isoliquiritigenin, echinatin, liquiritigenin, p-hydroxy benzoic acid and isobarachalcone [Kobayashi *et al* 1985]. It was Ko *et al* [1989] to demonstrate the production of glycyrrhizin in *Agrobacterium rhizogenes* transformed hairy roots of *G.uralensis.* Contrary to this, Saito *et al* [1990] reported the absence of glycyrrhizin in a similar investigation.

Though contradicting reports are available on the production of glycyrrhizin through plant tissue culture techniques, patents were obtained by commercial establishments. Adventitious roots of licorice produced *in vitro* [PCC Technol[10]] were demonstrated to contain glycyrrhizic acid. Babcock-Hitachi[2] developed a novel procedure to regenerate plants from calli which yielded glycyrrhizic acid. Production of glycyrrhizic acid by *A.rhizogenes* transformed *G.uralensis* tissue was claimed by Mitsui-Toatsu-Chem[9].

In vitro studies on *G.echinata* revealed the production of flavonoids. Investigations of Ayabe *et al* [1980] led to the isolation of echinatin and licodione from *G.echinata*. They studies the biosynthetic pathway apart from characterization of the flavonoids. *G.echinata* cells when transferred to fresh medium or immobilized, exhibited a rapid transient accumulation of retrochalcone, echinatin in both cells and the medium [Ayabe *et al* 1986a]. According to their further studies, addition of yeast extract or calcium alginate beads stimulated the production of echinatin and retrochalcone which was found to be under the influence

of O-methyl-transferases [Ayabe *et al* 1986b, 1987].

Suspension cultures of *G.glabra* [Dorisse I 1988] and *G. echinata* [Ushimaya *et al* 1989] were found to effectively biotransform papaverine hydrochloride and phenylcarboxylic acids respectively.

Licorice in Food industry

Licorice is widely used as a flavouring agent especially in tobacco industry. In combination with sugar, the sweetness increased by 100 times. In pharmaceuticals and medicinal tea it not only acts as a flavouring agent but also reduces the unpleasant taste of other constituents. It is used as a sweetner and flavouring agent in low caloric and non-cariogenic food. It gives sparkle and aroma to confectionary products and beer respectively. Licorice serves as a preservative in food industry. Excessive consumption, however, leads to harmful consequences.

Pharmacological Activities of Licorice

Though the major use is as a flavouring agent in food industry, licorice finds its place in pharmaceuticals too owing to its diverse pharmacological activities. According to Chinese Materia Medica, licorice increases the physical strength and cures wounds. In India, licorice powder mixed with fat and honey is applied to cuts and wounds [Singh *et al* 1984].

It has been attempted to use licorice for the treatment of common ailments, for eg. Spasms, to dreaded diseases like AIDS, which is the present days challenge to scientists for its effective management.

Aleshinskaya *et al* [1964] reported the usefulness of cabonoxolone, a derivative of glycyrrhizic acid as an anti-inflammatory agent. Glycopeptides synthesized from glycyrrhizic acid [Baltina *et al* 1988], glyderinine, a derivative of *G. glabra* [Azimov *et al* 1988] and glycyrrhizin [Ichikawa *et al* 1989] exhibited anti-inflammatory effects in animal models. Glycyrrhizin reduced hepatotoxicity in experimental animals exposed to Paraquat [Kim and Hong 1988]. According to larkworthy [1977], deglycyrrhinized licorice was useful for the treatment of both peptic and duodenal ulcers, supporting the earlier studies [Li *et al* 1960; Takagi *et al* 1963]. Antiulcerogenic activity in mice and rats by glycyrrhitinic acid derivatives were reported by Yano *et al* [1989]. Glycyrrhiza also protected the rat liver damage caused by Ischemia [Nagai *et al* 1991].

The antioxidative property of *Glycyrrhizai* flavonoids and glycyrrhizic salts in liver tissues were on record [Syrov *et al* 1987; Ju *et al* 1989; Abdugafurova *et al* 1990]. It was Berger and Holler [1957], Jo *et al* [1986] who demonstrated the analgesic activity of licorice in animal cells. Hashiguchi *et al* [1990] suggested the use of glycyrrhetinic acid as an alternative to local anaesthetic.

Glycyrrhetinic acid inhibited the activity of 11 beta-dehydrogenase resulting in a blockade in the conversion of cortisol to cortisone in human [Mackenzie *et al* 1990]. Mineralocortecoid effect of licorice derivatives was reported by Stewart *et al* [1990] in healthy volunteers. Isoliquiritigenin prevented the diabetes related complications in rats [Aida *et al* 1990].

Sureshkumar and Prabhakar [1990] reported the cario-active property of licorice. Zinc deficiency in children was effectively managed by licorice extracts [Qiao *et al*

1987]. Licorice served as a brain tonic for mental disorders [Upadhyaya 1986]. Glycyrrhizin was found useful in the treatment of skin diseases like eczema and dermatitis as reported by Hayakawa *et al* [1987]. Glycyrrhizin reduced the morphine induced harmful effects *in vitro* systems [Huh *et al* 1988].

Non-mutagenic property of glycyrrhitinic acid in Chinese Hamster V-79 H_3 cells was reported by Tsuda and Okamoto [1986]. Antimutagenic potential of *Glycyrrhiza* extracts and glycyrrhizin against methyl cholanthrene, imidazole and diethyl nitrosamine-induced mutagenicity was demonstrated in mammalian cells [Tanaka *et al* 1987]. Investigations of Minematsu *et al* [990] on Shosaiko-to-go-Keishikash-hakuyaku-to [TJ-960] revealed the protective effects against valpronic acid-induced anomalies in rats.

Protective role of licorice crude extracts against radiation-induced lethal damage in mice was recorded by Ohta *et al* [1987]. Glycyrrhiza flavonoids are reported to possess radical scavenging capacity [Hatano *et al* 1988; Ju *et al* 1989] in various test systems.

Anticarcinogenic activity of licorice in mammalian cells was on record. Abe *et al* [1987] reported that glycyrrhizin and glycyrrhitinic acid inhibited melanogenesis in cultured B 16 melanoma cells. Benzanthracene and tetradecanoyl phorbol-13 acetate [TPA] activated carci nogenesis was found inhibited by glycyrrhizin in mouse skin tumors [Yasukava *et al* 1988; O'Brain *et al* 1990]. It was also shown that inhibition of protein kinase was the cause for the suppression of carcinogenicity in these cell lines. Observations of Mashiba and Matsunaga [1990] revealed that glycyrrhizin in combination with diethyl dithio carbamate inhibited the *in vitro* proliferation of mammalian tumor cells.

Licorice exhibited antibacterial activity. Isoflavonoids and related susbstances from *G. glabra* var. typical and prenylated falvonoids from *G.lepidota* demonstrated their antimicrobial activity [Mitscher *et al* 1980]. Investigations carried out by Hattori *et al* [1986] revealed the inhibitory property of *G. uralensis* derivatives on cariogenic bacterium, *Streptococcus mutans.* On the other hand, glycyrrhizin stimulated the growth of *Eubacterium* strain GLH, a human intestinal bacterium [Akao *et al* 1988].

Derivatives of licorice exhibited inhibitory action against different classes of virus. A triterpenoid component of *G.glabra* [Pompei *et al* 1980] and *Glycyrrhiza* polysaccharides [Chang *et al* 1989] were shown to possess antiviral properties against DNA and RNA viruses. It was reported by Ohtsuki and Iahida [1988] that direct binding of glycyrrhizin to virus causes a dose-dependent direct inactivation of virus associated kinase and hence the reduction of viral infectivity. Segal and Pisanty [1987] suggested the use of glycyrrhizin gel along with iododeoxyuridine to reduce the healing time in patients with herpes of lips and nose. Glycyrrhizin was also found useful as an additive agent, in chemotherapy of herpes zoster patients [Aikawa *et al* 1990].

Glycyrrhizin was shown to have antiviral activity against Hepatitis A virus replication in vitro. A dose-dependent inhibition of HAV antigen and HAV infectivity was reported [Crance *et al* 1989, 1990]. They suggested the utility of *Glycyrrhiza* for chemotherapy of acute Hepatitis A. According to Hayashi *et al* [1989], treatment of chronic Hepatitis patients with

glycyrrhizin was effective without any side effects.

Recent investigations further revealed a significant antiviral property of licorice derivatives against Human Immuno deficiency Virus [HIV]. Glycyrrhizin showed a dose-dependent inhibition of the replication of HIV-1 in MOLT-4 cells. It was suggested by Ito *et al* [1988] that suppression of HIV replication was due to the inhibitory action of glycyrrhizin on protein kinase C. Administration of glycyrrhizin i.v. for a period of more than 30d in individuals with AIDS resulted in inhibition of HIV replication in vitro [Hattori *et al* 1989c]. According to Mori *et al* [1990], glycyrrhizin treatment to individuals with asymptomatic carriers of AIDS and AIDS related complexes prevented them to develop AIDS.

Licorice extracts were found to exert their effect on plant virus also. A dose dependent inhibitory effect of licorice extract was found in spinach mosaic virus [Zaidi *et al* 1988].

Insecticidal property of licorice was on record. Erion and Mahrous [1983] showed the toxic effects of *G.glabra* root extracts on Spodoptera littoralis.

Pharmacological Studies on Drugs Containing Licorice

Glycyrrhiza was found to be a constituent in various Chinese and Japanese herbal drug formulations. These drugs were in use for a wide range of ailments.

"TCM-WM", a Chinese traditional drug, reduced biliary gastritis in patients [Gouzeng 1987]. Reduction of hepatotoxicity was reported by Kim *et al* [1986] upon administration of 'Sosiho Tang". Brunner's glands of duodenal ulcerated rats exhibited reduced β-glucuronidase activity. Nadar and Pillai [1989] observed an increase in betaglycyronidase after administration of "Shanka Bhasma" and thus showed a protection against duodenal ulcers. "TJ-8014", a Japanese herbal drug found to have antinephritic action in rats [Hattori *et al* [1989a,b].

Antidiabetic effect of "Ganshao Jiantong" tablets was reported by Wang [1986] in diabetes mellitus patients. Plasma adrenaline levels reduced when Amagaya and Ogihara [1989] administered rats with "Shosaiko-to".

Parikh *et al* [1984] studies revealed 50% improvement in Schizophrenia patients after treatment with an indigenous drug [G K 022]. The herbal drug 'Sipmidojuskam" showed sedative, antipyretic, analgesic, anticonvulsive, antiedemic properties in mice. It reduced blood pressure and caused dialation of blood vessels. It also caused relaxing effect in smooth muscles of digestive organs in rats [Hong *et al* 1989]. Sedative effect of "Sanayangsohap-Won" and "Woohwang-poryong-hwan" was reported by Lee and Han [1986] in experimental animals.

The herbal drug "Lactare" improved the sizes of mammary gland and teat in guinea pigs and goats [Narendranath *et al* 1986]. Their clinical trials also showed that lactare benefited the lactation in mothers and improved appetite in mothers. Further evaluation revealed ed no toxic effects. Capsules of lactare were also found to exert similar effect [Sholapurkar 1986].

"Tiao Wei Cheng Qi Tang" decoction along with liquid diet was effective for bowel cleansing and Zhang *et al* [1986] found this

method to be cheap and convenient with no side effects. Eye drops and capsules consisting of *G. glanra, Berberis aristata* and *Curcuma longa* were reported to be highly effective in curing all kinds of allergic conjunctivitis without any recurrence of clinical symptoms [Athneria *et al* 1987]. Licorice extracts enhanced the absorption of iron in intestinal segments of rats as revealed from the studies of El-chobaki *et al* [1990]. They recommended it as a preventive agent to iron deficiency anemia in both children and adults and as a bioavailability source of medicinal iron.

Safety Evaluation of Drugs Containing Licorice

Investigations were carried out to ascertain the toxic effects, if any, of drug formulations containing Licorice.

"Sairei-to" and "Saiboku-to", the Chinese herbal medicines, were found non-toxic to rats after 90 days of administration. No changes occurred in body weight, food intake, urological, haematological, ophthalmological and pathoanatomical features. [Kiwaki *et al* 1989a,c]. Similarly, Minematsu *et al* [1989] conducted a study on the effect of "Junjentaiho-to". The drug was shown to have no toxic effects in mice and rats as assessed from death rate, abnormal symptoms, difference in body weight, food intake, urological, haematological, ophthalmological and histopathological data.

Teratological evaluation of "Sairei-to" was carried out by Kiwaki *et al* [1989b] on rats. Their studies revealed no harmful effects of the drugs as evidenced by the absence of increase in fetal mortality and inhibition of fetal growth. No developmental and reproductive deformities of offsprings recorded, thus, exhibiting non-teratogenic effect of the drug.

Patents of Licorice

Owing to the economic importance of licorice, attempts were made for commercial production of licorice derivatives. Recent advents in biotechnological procedures led to either improvement or enhancement of licorice derivatives.

Irkutsk State Medical Institute[5,6] obtained patent on extraction of licorice from ground roots. Stable crude drugs were prepared by Gelia-Shinyaku[4] from *Glycyrrhiza* radix employing two surfactants viz, polyoxy ethylene curing, castor oil and polyoxy ethylene polyoxy propylene condensate.

Biotransformation studies on licorice constituents were conducted to derive pharmaceutical compounds. Amano[1] patented for biotransformation of glycyrrhitinic acid to 3-epiglycyrrhetic acid employing intestinal bacteria. The compound was found to have same effect as that of glycyrrhitinic acid. Further it has no pseudo-aldosteronic effect which glycyrrhitinic acid generally possesses. Biotransformation was attempted since it was difficult to derive the compound by chemical means. Similarly, Daiichi Pharm3 employed *chainia antibiotica* to transform glycyrrhitinic acid to its derivatives which are antiallergic, anti-inflammatory and antitumerogenic.

Tsumura-Juntendo[13] demonstrated the antitumor effect of *Glycyrrhiza* extracts in combination with oyster shell powder. Benzopyranone glycosides possessing antianemic activity were isolated from *G. glabra* [Juntendo Inc[7]]. Eye drops produced by Tsumura-Juntendo[14], where licorice extract was one of its constituent was found

effective for the treatment of conjunctivitis. Licorice extract in combination with other herbal drugs prevented tooth decay by inhibiting the growth of *Streptococcus mutans*. Takasge Perfum[12] suggested the use of this drug formulation in chewing gum, tooth paste and mouth washes.

Compounds derived from licorice were also of importance in food industry. Aqueous extracts of starchy material of licorice yielded edible sweetner upon incubaction with cyclodextrin [Kabushiki[8]]. The product, a sweetner, found suitable for incorporation into low calorie or non-cariogenic food and beverages. It has a milder flavor than glycyrrhizin without unwanted flavors or medicinal odors. Ueno[15] prepared phytoncides from licorice, which were of use as food preservatives. Antioxidant and antimicrobial substances from licorice [Ueno[16]] were also useful as food preservatives.

Extracts of licorice find their place in cosmetic preparations too. Rohto[11] developed a hair tonic preparation from licorice which was claimed to promote hair growth and also effective in preventing hair loss.

Patents on Licorice Cell Cultures

In vitro production of adventitious roots by *Glycyrrhiza* cells was demonstrated by PCC-Technol[10]. These roots were found to contain glycyrrhizic acid. Babcock-Hitachi[2] developed a new method to regenerate plants through in vitro manipulation from calli of licorice rhizome. The regenerated plants were reported to possess glycyrrhizic acid. Production of glycyrrhizic acid from *Agrobacterium rhizogenes* transformed *Glycyrrhiz uralensis* tissues *in vitro* was demonstrated by Mitsui-Toatsu-Chem[9].

Market Potential of Licorice*

Spain supplied the world's requirement of licorice until 1970. The other countries which are major commercial producers of licorice are : Spain, France, Italy, Afghanistan, Iran, Iraq, Turkey, Syria, Lebanon, Israel, USSR and China.

The data presented in the following table depicts the import of Licorice in India.

Year	Quantity [in tones]	Value [in lakhs of Rs.]
1976-77	364.42	9.85
1977-78	752.16	28.97
1978-79	578.98	21.86
1979-80	642.77	26.20
1980-81	286.88	13.37
1981-82	515.63	30.04
1982-83	574.41	29.38

1983-84	1165.00	68.80
1984-85	372.23	22.15
1985-86	1273.57	78.53
1986-87	401.84	19.79

The data show the increasing requirements of licorice and sudden spurt in its requirement was seen during the period 1983-84 and 1985 – 86. However, India too entered the international market by exporting licorice, though it is to a lesser extent. The export statistics show that during the years 1984 – 85 and 1985- 86, our country exported one tonne of licorice in each year which is equivalent to Rs.0.16 and Rs. 0.18 lakhs respectively. The cost of powdered and raw licorice/kg in 1989 was US$2.2 and US$0.9 respectively.

Source: *Current Research on Medicinal and Aromatic Plants* [Quarterly Journal Published by Central Institute of Medicinal and Aromatic Plants, Lucknow].

Future Prospects of Research on Licorice

From the preceding part of this text, the economic potential of licorice is unequivocally evident from its highly diverse intrinsic pharmacological properties apart from its use in food industry. With further advancement in knowledge on its pharmacological activities and use in food industry, the commercial requirement of licorice will certainly increase in future. The statistical data on licorice imports shows the increasing demand. To conserve foreign exchange and to meet the increasing demand, it is highly necessary to focus our attention on licorice research. Attempts on various aspects can be made to indigenize the commercial production of licorice. The goal can be achieved, in a broad manner, by the application of plant breeding techniques, micropropagation and metabolite production *in vitro.* Many investigations manifest that a large number of food products / medically important compounds of plant origin have been obtained through plant tissue culture, direct enzyme application and utilization of whole cells in biocatalysts in fermentation technology.

1. Cultivation and Plant Breeding Techniques

Investigations on licorice cultivation reveal that the plant requires sandy or sandy loam soils with rainfall less that 50cm per year. Manuring was not required when soils were fertile [Singh *et al* 1984]. Irrigated sands were found beneficial for the cultivation of licorice [Mohammad and Rehman 1985]. Its existence under wide salinity and drought conditions was on record [Mirkin *et al* 1971; Aprasidi 1978]. Soils containing chloride sulphate were reported to be advantageous for licorice cultivation [Khafizova 1978]. Licorice cultivation helps the reclamation/improvement of soil condition [Varganov and Gladyshev 1981; Kel'dznaev and Gladyshev 1982; Mohammad and Rehman 1985].

Though earlier reports on its cultivation in India are available [Chopra and Kapoor 1952; Kapoor *et al* 1955; Singh 1964, Verma 1969; Uniyal *et al* 1978; Chandra 1970], subsequent reports for the past

decade are scandy. Singh *et al* [1984] proclaimed in their view that inspite of the attempts made on its cultivation, commercial production was not achieved in our country.

From the agronomical point of view, it is necessary to explore the suitable locations in our country for its cultivation on a large scale basis for the commercial propagation of licorice. Morphological features which were found useful to identify the useful forms of licorice as described by Mikailov and Mirzaliev [1978] and Zeng *et al* [1988] may be utilized and cultivation can be attempted with such plants by adopting appropriate propagation methods.

Not much importance was given to breeding experiments on licorice, as evidenced by very few reports on this theme. Perhaps the longer duration to attain flowering stage would have restricted such studies. To overcome this problem, prtoplast fusion techniques may be of immense use.

2. How can Biotechnology Help Us to Improve Cultivation Methods and Commercial Production of Useful Compounds of Licorice?

The impact of plant tissue culture on agriculture and pharmaceutical industries is tremendous. The plant tissue culture techniques have become powerful tools for studying basic and applied aspects of plant sciences. These methods have found wide rage of applications from propagation of plants to the use of bioreactors and immobilized cell technology.

Micropropagation

A recent review by Balaj *et al* [1988] enunciated the usefulness of in vitro techniques of higher plants for industrial production of medicinally important compounds. *In vitro* micropropagation techniques were found advantageous to supersede the problems encountered with propagation by conventional methods. The benefits associated with this technology are, increase in the rate of propagation, rapid multiplication of plants which in a particular climate do not promote germination of seeds, availability of plants throughout the year, uniformity of selected genotypes, production of uniform clones and conservation of genetic resources. Additionally, this type of multiplication procedure of medicinal plants avoid the problems connected with the loss of biosynthesis pathways in dedifferentiated tissues *in vitro.*

Different types of clonal propagation methods are in use for the micropropagation of various crops. The most routinely used practice is direct propagation from existing meristems, through which identical plants with desired characters can be obtained [Bajaj 1988].

Attempts have already been made on the micropropagation of *Glycyrrhiza* in this direction [Shah and Dalal 1982; Syrtanova and Mukhitdinova 1984]. Attention can now be focused on the utilization of somatic embryoids derived from callus and production of "artificial seeds" for the multiplication of licorice. Cryopreservation of germplasm and cultured tissues would also meet the industrial requirement of licorice.

Metabolite Production *in vitro*

Apart from the use of micropropagation techniques, other plant tissue culture procedure may also be considered. From a biotechnological point of view, exploitation of cells suspension cultures provide an appropriate system for the production of secondary metabolites on an economical

scale in bioreactors. The metabolic pathways involved in secondary metabolism are often complex in nature and living cells have become the only source as it is also turn with licorice, since the approaches on chemical synthesis of licorice derivatives were unsuccessful. The following are the few familiar procedures to increase the yield of medicinally important compounds.

Selection of High Yielding Cells for Culture

The variability in any population for any trait has long been recognized. The production of a secondary metabolite by a plant is no exception. Hence, the selection of a variety with a high yield of a desired natural product is a prerequisite for the development of cell culture system [Misawa, 1985; Hoekstra *et al* 1988; Rhodes *et al* 1988; Roberts 1988]. An admirable example has been the selection of Catharanthus roseus plants with high yields of vindoline, ajmalicine and serpentine [Zenk *et al* 1977]. Continuous selection was also found necessary to maintain high yielding cell cultures [Deus and Zenk 1982].

Hayashi *et al* [1990b] reported that the accumulation of soyasaponins in *Glycyrrhiza glabra* suspension cultures was under the influence of culture strains employed. This clearly shows the importance of selection of high yielding cells. Similar types of studies can be performed to derive other important constituents of licorice. Investigations, however, reveal contradictory reports on the production of glycyrrhizin under culture conditions [Wu *et al* 1974; Hayashi *et al* 1988; PCC Technol[10]]. These reports perhaps demonstrate the varietal difference to play an important role. Hence, meticulous screening procedures to identify high yielding strains and their utilization *in vitro* might help to derive increased yield of licorice products.

Optimization of Environmental Conditions

The yield of secondary metabolities can be improved by the optimization of culture conditions [Mantell and Smith 1983]. Changes in hormonal concentration in culture medium enhanced the alkaloid production from Catharanthus roseus [Deus and Zenk 1982], *Thalictrum minus* [Nakagawa *et al* 1986] and Rauvolfia serpentine [Yamamoto and Yamada 1987]. Other environmental factors are also critical. Dissolved oxygen content of the medium influenced the production of secondary metabolites [Breuling *et al* 1986]. Light and temperature also play a significant role [Courtois and Guern 1980; Ohlssen *et al* 1983; Misawa 1985; Hobbs and Yeoman 1988]. Metal ions were also found to influence secondary metabolism [Threlfall and Whitehead 1988].

In many instances, synthesis of secondary metabolites was in response to abiotic and biotic stress [Timmermann *et al* 1984]. Fungal elictors of secondary metabolism have been effective to increase the phytochemicals [Funk *et al* 1987; Di Cosmo *et al* 1987; Holden *et al* 1988; van der Heijden *et al* 1988]. Special attention can be paid to find optimal environmental conditions for the production of licorice derivatives in vitro by supplementing the medium with hormones, elicitors and altering other environmental conditions.

Supplementation with Precursors and Biotransformation

Supplementation of the media with appropriate precursors or related compounds, in few cases, stimulates the

production of secondary metabolites. Addition of amino acids enhanced the production of tropane alkaloids, indole alkaloids nad ephedrine [Reinhard and Alfermann 1990; Misawa 1985; Roberts 1988]. However, to achieve maximum benefits of medium supplementation, it is necessary to have a thorough comprehension on the biosynthetic pathway of phytoproducts which are generally complicated.

Biotransformation has been extensively applied in the fermentation industry [Misawa 1985] and a classical example of this approach was the conversion of beta-methyldigitoxin to beta-methyl digoxin.

Fungal metabolism of licorice derivatives was reported by Tahara *et al* [1985]. A thorough screening of various microorganism led to the isolation of an enzyme preparation [Muro *et al* 1986] from a strain of *Aspergillus niger* strain GRM3 which effectively catalyzed the hydrolysis of glycyrrhizic acid.

The influence of active principals of fungal derivative in enhancing the licorice secondary metabolites in vitro could be assessed. Biotransformation studies carried out by Hayashi *et al* [1990a] revealed the failure of glycyrrhizic acid production in vitro. Further strengthening of the investigations in this direction might help in increasing the in vitro production of *Glycyrrhiza* secondary metabolities.

Enzyme Isolation and Localization

This is an important aspect for the complete exploitation of plant biotechnology for the production of secondary metabolites. However, a sound knowledge on the characteristic of enzymes involved in the biosynthesis of these products is highly essential. Though this area is still infancy, few reports are available [Robers 1988]. Similar type of studies have not been carried out so far in licorice. Though these investigations are of academic importance, they unravel the metabolic pathways involved in secondary metabolism which ultimately have great impact on biotechnological approaches of licorice.

Induction of Mutants

In biotechnology, induction of genetic mutant strains of micro organisms is ubiquitous and auxotrophic and regulatory mutants are used extensively to produce amino acids, nucleotides, antibiotics etc. The impediments for such as approach with the plant system is due to the lacuna in the knowledge on the regulatory mechanisms of biosynthetic pathways of secondary metabolism. However, attempts were made by employing chemical and physical mutagents [Misawa 1985] and extension of such studies may be beneficial to derive secondary metabolites of licorice.

Plant Cell Immobilization

The development of techniques for plant cell and enzyme immobilization has increase the flexibility of plant cell biotechnology for the production of pharmaceuticals. Brodelius and Nilsson [1980] described the two major methods of plant cell immobilization – adhesion and entrapment. In the former type, plant cells from suspension culture will spontaneously bind to a suitable matrix in bioreactor. Polyurethane foam particles have been found to support dense cell masses and other matrices have also been successfully used [Mavituna and Park 1985; Rhodes *et al* 1985, 1987]. In the entrapment techniques, plant cell immobilization is an inert gel or bead matrix [alginate, acrylamide, carageenan, chitin or chitosan] is one of the most successful

cultivation methods [Nabajima *et al* 1986; Rosevear and Lambe 1986]. Increased of secondary metabolic production and recovery are through possible. [Morris *et al* 1985*;* Yeoman 1986].

The advantages of this technology are - reduced rate results in increased levels of secondary metabolite production, greater cell to cell contact increases levels of secondary metabolites, chemical composition of the medium can be optimized to maximize product and less production costs which is an important criteria for commercial utilization. Immobilized plant cells can also be employed for biotransformation, eg. [-] codeinone to [-] codeine and digitoxin to digoxin. Precursors and elicitors can be employed [Roberts 1988].

The exciting developments in immobilized technology encouraged investigators to employ similar methods for licorice. Favourable observations were found by Ayabe *et al* [1986a]. When *G.echinata cells* immobilized, the accumulation of flavonoids, retrochalcone and echinatin transiently increased in both cells and media. Similar attempts with other species of *Glycyrrhiza* may be of immense use to accumulate other constituents of licorice.

3. How can Recombinant DNA Technology Help in Genetic Manipulation of Licorice?

The recent past has seen the development of techniques by which goes may be transferred into plants. In addition to the established methods, new and refined methods for manipulating DNA *in vitro* will offer many exciting and novel opportunities. Fundamental questions regarding the control of plant secondary metabolism can be tackled and ultimately, the ability to influence secondary product accumulation, both qualitatively and quantitatively, in plants and in tissues grown *in vitro* should be possible.

Hairy Root Cultures

Transformed root cultures (also known as 'hairy' root cultures) are derived from tissues after injection with *Agrobacterium rhizogenes* and have a number of attractive features regarding the synthesis of plant secondary products. These include ease of culture in vitro using simple media lacking phytohormones, reproducible and predictable levels of product synthesis and genetic stability over prolonged periods of growth *in vitro* features which are not usually associated with cell suspension or callus cultures. The advantages of transformed root tissues have been reviewed by many investigators [Flores *et al* 1987; Hamill *et al* 1987; Weising *et al* 1988].

The state-of-the art of transformation of roots by *A.rhizogenes,* the exciting developments in other phytoproducts and the benefits associated with this technology opened new vistas for similar type of studies on licorice. Efforts were taken to gain more yield of *Glycyrrhiza* products which are of commercial importance. Ko *et al* [1989] was successful in demonstrating the production of glycyrrhizin in *A.rhizogenes* transformed hairy roots of *G.uralensis.* Similar was the report from Mitsui-Toatsu-Chem[9]. The production of glycyrrhizic acid from transgenic roots of *G.uralensis* was patented by them. Investigations carried out by Saito *et al* [1990], however, manifest the absence of production of glycyrrhizin in transformed roots of *G.uralensis.*

As this area is in its infancy, with regard to licorice detailed investigations will certainly help the efficient utilization of the *A.*

rhizogenes transformed roots. Such studies will throw more light on the production of licorice derivatives *in vitro* and ultimately benefit the industry.

Utilization of Vectors

Apart from the *Agrobacterium* another vector of potential utility in plant genetic engineering is the cauliflower mosaic runs. Recent evidence indicates that the cauliflower mosaic virus is capable of vectoring in a certain amount of DNA in recombinant form. The limitation to use this virus as plant vector is its limited host range.

Microinjection and Encapsulation

Micromanipulation of isolated protoplasts for the purpose of micro injection of recombinant DNA fragments appears to be gaining acceptance as a potent tools to engineer the plant cells. Injection of liposome encapsulated DNA into protoplasts was also evaluated by investigators. These approaches would avoid the complexities of constructing a recombinant vector system.

Protoplast and Organelle Fusion

Protoplasts isolated were allowed to fuse under in vitro conditions and the resultant hybrid protoplasts were shown to regenerate into hybrid plants (Vasil and Vasi 1980). Advantages to such fusion systems include the ability to recognize and micromanipulate the hybrid cells, use of selective markets to distinguish true hybrids. Mitochondria and chloroplasts when incubated with protoplasts under appropriate conditions can be taken up by the stable manner for several cell generations. As a considerable number of secondary metabolites are formed in these organelles, the potential for organelle transplantation appears most promising.

It is evident from the literature that genetic engineering approaches for the improvement of licorice is meager. Success can be achieved with licorice too by employing these methods. However, a clear establishment of the molecular mechanisms involved in the metabolic pathways of its derivative is a prerequisite for an effort usage of the most appropriate recombinant [DNA] methods.

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