1. LITERATURE REVIEW

Echinacea is a North American plant genus found in natural populations east of the Rocky Mountains in the Atlantic drainage region of the United States and Canada. *Echinacea* species are members of the Asteraceae family, tribe Heliantheae[1] and include *E. angustifolia, E. pallida, E. simulata, E. paradoxa, E. tennesseensis, E. laevigata, E. sanguinea, E. atrorubens, E. gloriosa,* along with *E. purpurea*. The word *Echinacea* is derived from the Greek *Echinos* for sea urchin or hedgehog[2]. In 1794 the botanist Conrad Moench was the one, who gave the plant this name, a reference to the spiny appearance of the plant due to the prickly scales on its large conical dried seed head[3]. All members of the genus are herbaceous perennials having composite inflorescences typical of the Asteraceae family, consisting of both ray and disk florets, the ray florets (which can be white, yellow, pink, or purple) encircle the typically conical arrangement of disk florets, leading to the vernacular name for the genus, the coneflowers[4]. There are three closely-related species of *Echinacea* being currently used for pharmaceutical and medical purposes; *Echinacea purpurea* or purple coneflower, *Echinacea pallida* or pale coneflower and *Echinacea angustifolia* or narrow-leaved *Echinacea*[2]. Of the three *Echinacea* taxa typically used in herbal medicine, *E. purpurea* is the most widely utilized and cultivated species, and the best studied of the nine species. Given the large market and evidence for human health benefits of *Echinacea*, it would be valuable to increase concentration of the bioactive components in this plant[5].

1.1. History of *Echinacea*

The first descriptions of *Echinacea* appeared in the 18th century, first in 1737, in the *Catalogue of Plants* Fruits, *and Trees Native to Virginia*, written by John Clayton from Virginia and then in 1762, in *Flora Virginica*, published by Frederic Gronovius[3].

The first report of *Echinacea* in Western medical literature was in 1762 where it was indicated for horse saddle sores, and was briefly investigated scientifically before falling into temporary obscurity. It reemerged in the late nineteenth century when Dr. Meyer first marketed it as a "blood purifier," where after Lloyd Bros, produced the first commercial preparation based on his formulation. In 1887, *Echinacea* was also acknowledged by the American Society of Pharmacists, and has been added to its medicine register. However, *Echinacea* plant and its therapeutic properties were known and used much earlier, by numerous Indian tribes of North America. They have been all using it commonly and successfully, from skin problems to alimentary tract illnesses, fevers, respiratory and urinary system disorders. The spectrum was even broader, as the plant was used in snakes' and insects' bites, contagious diseases, various infections, venereal diseases, wound healing. Subsequently, the analgesic properties of *Echinacea* were discovered, and thus used as a painkiller and a stimulant. Traditionally, chronic skin inflammations, burns and local infections were treated with *Echinacea*. This tradition was later on passed to white settlers, who, due to the scarcity of other medicines, were somewhat forced to use the Indian remedy. Gradually, they deepened their knowledge of the medical utilization of *Echinacea* and brought the remedy to Europe. Euro-American physicians kept various *Echinacea* potions in their medicinal kits, for use against snakebite, or to counter infectious disease. *Echinacea* was described in the National Formulary of the United States from 1916 to 1950[6].

Originally, *Echinacea* was used in ethno medicine, as a broad-spectrum agent, including antidote against snail venoms. *Echinacea* was a remedy against high fevers, and abscessed, infected wounds during the American Civil War. Extracts proved to disinfect and improve the regeneration process of damaged tissues. The same extracts were administered as well in cases of burns, frostbites, bedsores and ulcerations. Moreover, *Echinacea* was administered preventively and therapeutically in patients with liver diseases and biliary tract dysfunction, as it exhibited biligenic, choleretic and spasmolytic actions. It also stimulates the secretion of digestive juices (gastric, pancreatic and intestinal)[7].

In 18th and 19th century, *Echinacea* preparations were used to treat many bacterial and virus infections, parasite diseases and mycosis (such as influenza, herpes simplex, measles, small-pox, herpes zoster, and water-pox, rubeola, angina, syphilis, diphtheria, scarlatina, malaria, upper respiratory tract diseases and sinusitis, vaginal yeast, crypto-coccosis) and even cases of rabies. Taking into account the broad spectrum of therapeutic use, in the late 19th century *Echinacea* was officially introduced to the US pharmacopoeia by John King and John Uri Lloyd. First clinical trials of *Echinacea* were conducted by Meyer and King in a Cincinnati hospital[7].

Consequently, an immensely broad medical utilization as well as a raising, public and scientific interest in *Echinacea* brought about such popularity, in the twenties of 20th century *Echinacea* had become the bestselling medical plant in the US. In Europe *Echinacea* first appeared between 19th and 20th century as a homeopathic agent, after a while however, it became a commonly administered medicine. Before the days of antibiotics, physicians used it for extremely difficult conditions such as diphtheria, tuberculosis, various cancer forms, and gangrene. *Echinacea* was mostly supplemented by sulfa drugs in the 1920's and thirties, and by penicillin in the 1940's. Use continued in Germany, however, but by the 1930's there was shortage in supply, Gerard Madaus came to the United States looking for germplasm of *E. angustifolia* [7], but mistakenly returned with *E. purpurea* seeds, beginning a long line of German cultivation. Since that time, the German market, the largest in Europe, has been dominated by *E. purpurea*. German scientists were at the forefront of *Echinacea* research throughout the twentieth century, with most of the scientific research done on *E. purpurea*. Only in the last decade or so have U.S. markets and U.S. research efforts have begun to catch up, slowly regaining stature during the 1970s. Today, numerous, immune-stimulating preparations produced from roots and herbs of *Echinacea purpurea* are available on the market[8].

1.2. Morphology and general description of *Echinacea*

The plant is hard with erect, stout branched and hairy stems. The leaves are ovate to ovate lanceolate with acute apex and serrated margins. Basal leaves and the lower stem leaves have petioles that goes on decreasing progressively as one moves up. The petioles are short and slightly winged[9].

The upper surface of the leaves is often dark green with sparse white uniseriate trichomes. The flowers are as inflorescences with crateriform to hemispherical ending into peduncles. The ray florets are dark purple, pinkish or reddish purple and linear to elliptic or obovate. The central cone is prickly. The ray florets are long and droop downward. The fruits are three or four-angled called cypselae, tan or bicolored with a dark brown band distally. The central cone (achene) becomes more pointed and prickly as the flower matures to produce yellowish brown colored seeds which germinate unevenly in 10 to 21 days. When the cones are in full seed, birds, especially finches, feed on them[9]. **(Figure 1)**

Organoleptic characteristics: Rhizomes have fibrous fracture in the exterior but smooth in the central pith. Dry roots show short fracture whereas the fresh roots show tough and flexible fracture. Aroma is Earthy and acrid slightly. The plant tastes cool with initial sweet sensation followed by bitter taste quickly and numbing and tingling in the tongue. It also has a slightly aromatic taste[10].

Figure 1: **A:** Photograph of *Echinacea purpurea* (L.) Moench**, B:** head, longitudinal section showing disc florets and receptacle scales**, C** and **D** leaves upper (adaxial) and lower (abaxial) surfaces respectively**, E:** stem and **F:** fibrous roots

1.3. The world supply of *Echinacea*

The first formal research on *E. purpurea* was done in Schwebheim (Germany) by Barnickel in 1985. Later, long-term and basic agronomic research was carried out at Bavarian State Research Center for Agricultural Sciences and Agronomy in Freising (Germany). Published results generated increasing interest in *Echinacea* in other European countries. Agronomic research and commercial cultivation extended into several European countries[11].

Commercial cultivation of *Echinacea* is mostly located in North-Western United States, and Western Canada. Austria, Germany, Russia, New Zealand, Ukraine, Yugoslavia and the Republic of South Africa have well-established cultivation of *Echinacea*, though mostly *E. purpurea* or *E. pallida*. *E. purpurea* cultivation extends as far as Ural Mountains and Altai highlands in Siberia with an increasing tendency in size and processing capacity. *Echinacea* is widely adapted and can be grown under extremely varying climatic or vegetative conditions varying from 135 days in Siberia to 365 days in tropical/subtropical environments. It is important however that cultivars be selected for different ecological zones[12].

Brazil, Chile, Argentine, and Costa Rica have established field production of *Echinacea* since 1998. Experimental fields of *Echinacea* have been established in Egypt, Botswana, and Zambia. In Tanzania, *Echinacea* is cultivated for export to Europe. Presently the Republic of South Africa has commercial production that supplies some of the Western European *Echinacea* raw material, and prepares hydro-alcoholic extracts based on cheaper sugarcane-based ethanol of African origin. Extracts are transported from Republic of South Africa to Europe and North American markets[12].

In Egypt, interest in *Echinacea* regarding its cultivation, investigation and use in pharmaceutical preparations has started since 1990. *Echinacea* was first introduced into Egypt by **Libra farming company** a subsidiary of **Sekem** Group. Cultivation began in Anshas El Raml El Sharqiyah governorate on 5 feddan, then it was extended to reach 45 feddan in 2013, accordingly several articles have been published in this regard[13-15]. The previous studies were concentrated on *Echinacea purpurea*. As a result, standardized extracts and some phytomedicines are now produced locally[16].

1.4. Tissue culture and biotechnological techniques of *Echinacea*

One of the main constraints to the use of cultivated plants as a source of their metabolites is the ability to ensure the constant and efficient supply of the compounds, since the yields are usually affected by the genetic background, as well as by the geographic location and climatic conditions at the site of cultivation, combined with the potential effect of harvest and transport methods. The use of plant tissue culture has been proposed as an alternative to conventional agriculture for the production of secondary metabolites due to the possibility of controlling the quality and quantity of the compound of interest by controlling factors affecting its synthesis and/or accumulation[17].

Plant tissue culture is defined as the process of producing callus tissue, plant organs, or intact plants using a small piece of a donor plant or even a single cell. This process consists of using artificial medium and exogenous growth hormones. Successful plant tissue culture is based on the cell theory of Schleiden and Schwann, and the concept of totipotency (a plant cell is capable of regenerating a whole plant)[17].

Recent advances in the field of plant biotechnology show the potential of using plant cell and tissue cultures as a source for the large-scale production of valuable secondary metabolites instead of using whole plants and subsequent extensive land exploitation[17].

Some of the advantages of this technology over the conventional approach of extraction of industrial phytochemicals from whole plants are:

1. In cultures, factory-type production of natural compounds can be carried out throughout the year, unaffected by the season, pests and diseases, climate, and geographical constraints.

2. The risk of crop failure due to natural hazards and the danger of extinction of some species due to their mass extraction from the natural populations are eliminated.

3. A more consistent product quality could be assured with cultivation of selected cell lines under controlled conditions.

4. Biotic and abiotic stresses (elicitors), which are known to enhance the accumulation of valuable secondary metabolites, qualitatively and quantitatively, can be introduced into the medium to improve the productivity of the cultures.

5. Due to technological advancements, several cell lines capable of synthesizing the natural compounds in quantities higher than the whole plant have been isolated some of the systems have been commercialized.

6. Cell cultures not only provide means for *de novo* synthesis of natural products but also serve as factories for bioconversion of low value compounds into high value products.

7. Novel compounds not known in intact plants may be produced by cell cultures.

8. Extraction of products from *in vitro* tissues is much simpler than the complex *in vivo* tissues because of the absence of significant amount of pigments, making efficient downstream recovery, and product yield[18]

Plant tissue culture is essentially a closed system, it can be used alone or in combination with genetic transformation as useful tools for crop improvement and for investigating the production of important secondary metabolite, *in vitro* culture may be used also to obtain virus- free plant material by excising only the meristematic dome of a contaminated plant and using it as an explant source[19].

1.5. *In vitro* production of active components from *Echinacea* plant

1.5.1. Adventitious root cultures

Adventitious root cultures have been applied in many plants due to its rapid growth, stable and continuous mass production of secondary metabolites[20]. Therefore, high product concentrations and efficiency can be reached by optimizing the *in vitro* culture conditions[21].

The effects of different concentrations of auxins, the strength of the MS medium and ammonium/nitrate contents, initial medium pH, and inoculum size on biomass increase and the accumulation of total phenols and flavonoids in adventitious roots of *Echinacea angustifolia* were studied. The roots were cultured under dark conditions for 4 weeks. IBA proved to be the best auxin for inducing root proliferation. Growth was inhibited when the initial pH was maintained below 5.0 or above 6.0. Nitrate, rather than ammonium, was more necessary for root growth and phenolics accumulations. Overall, biomass increased and total phenol and flavonoid contents were maximized under the following conditions: half-strength MS medium supplemented with 2.0 mg/l IBA, 5% (w/v) sucrose, 5:25 (mM) ammonium/nitrate ratio, pH adjusted to 6.0 before autoclaving, and an inoculum size of 10 g/l F.W. Such optimization is beneficial to large-scale production of biomass and secondary metabolites in that species[21].

Also, the effects of temperature and light irradiation on growth and production of caffeic acid derivatives (caftaric acid, chlorogenic acid and cichoric acid) in adventitious root cultures of *Echinacea purpurea* was studied. Biomass accumulation and production of caffeic acid derivatives were optimal under incubation temperature of 20°C among the different incubation temperatures tested (10, 15, 20, 25 and 30°C). Biomass of adventitious roots was highest in cultures grown under dark while accumulation of caffeic acid derivatives was optimum in the cultures grown under 3/21 hours light and dark photoperiod cultural regime[22].

The effects of both media optimization and replenishment strategies were adopted to achieve improved production of *E. purpurea* adventitious roots and caffeic acid derivatives. Different media strengths were tested for the culturing of adventitious roots in 5 liter bioreactors. They showed that half strength MS medium was the most suitable medium for the growth of adventitious roots. This medium accumulated optimum biomass (73.6 g/l F.W. and 10.03 g/l D.W.), phenolics (61.14 mg/g D.W.) and flavonoids (38.30 mg/g D.W.).Furthermore, fed batch cultivations (media replenishment) showed high adventitious root biomasses (83.1 g/l F.W. and 15.30 g/l D.W.) with feeding of 0.50 MS medium at the end of 2nd week. This led to slight decreases in the total production of phenolics and flavonoids, however, this feeding was responsible for increases in the accumulation of caftaric acid (5.76 mg/g D.W.) and cichoric acid (26.12 mg/g D.W.)[22].

Adventitious roots of *Echinacea purpurea* were cultured in airlift bioreactors (20 L, 500 L balloon-type, bubble bioreactors and 1,000 L drum type bubble bioreactor) using Murashige and Skoog (MS) medium with 2.0 mg/L indole butyric acid and 50 g/l sucrose for the production of cichoric acid, chlorogenic acid and caftaric acid. In the 20 L bioreactor a maximum yield of 11 g/l dry biomass was achieved after 60 days. However, the amount of total phenolics (57 mg/g D.W.), flavonoids (34 mg/g D.W.) and caffeic acid derivatives (38 mg/g D.W.) were highest after 50 days. Based on these studies, pilot scale cultures were established and 3.6 kg and 5.1 kg dry biomass were achieved in the 500 L and 1,000 L bioreactors respectively. The accumulation of 5.0 mg/g D.W. chlorogenic acid, 22 mg/g D.W, cichoric acid and 4.0 mg/g D.W. caftaric acid were achieved with adventitious roots grown in 1,000 L bioreactors[22].

Also, adventitious roots of *Echinacea purpurea* were cultured in ballon type airlift bioreactors (5 liters capacity) and the investigations were made to optimize inoculum density and aeration rate affecting the accumulation of biomass and secondary metabolites. The greatest increment of biomass as well as metabolite content occurred at the inoculum density of 7.0 g/l fresh weight (F.W.) and the aeration volume of 0.1 vvm (air volume/culture volume per min). Under culture conditions 60.7 mg/g dry weight (D.W.) of phenolics and 38.8 mg/g D.W. of flavonoids were produced in the adventitious roots after five weeks of culture. Cichoric acid content was highest (26.64 mg/g D.W) among the caffeic acid derivatives. The results suggest that the accumulation of biomass and secondary metabolites were maximized by optimizing inoculum density and aeration volume in bioreactor cultures [23].

Some researchers evaluated the feasibility of using balloon type bubble (air-Lift) bioreactor to produce caffeic acid derivatives from the adventitious roots of *Echinacea purpurea*. They found that an approximately 10-fold increase in biomass and secondary compounds was observed after 4 weeks of culture in balloon type bubble bioreactors (5 liter capacity containing 4 liter of half strength MS medium). In addition, a linear relationship was observed between the concentration of biomass, sucrose and ion consumption rate. Furthermore the concentration of biomass in the bioreactor culture was found to increase as the conductivity decreased. An inoculum density 7.0 g/L F.W. and aeration rate 0.1 vvm were found to be suitable for inducing the accumulation of biomass and secondary metabolites. Of the three caffeic acid derivatives evaluated (caftaric acid, chlorogenic acid, and cichoric acid), the concentration of cichoric acid was the highest (26.64 mg/g D.W.)[24].

1.5.2. Effect of elicitors addition

Cell cultures have been established from many plants but they often do not produce sufficient amounts of the required secondary metabolites. However, in many cases the production of secondary metabolites can be enhanced by treatment of the undifferentiated cells with elicitors. An 'elicitor' may be defined as a substance which, when introduced in small concentrations to a living cell system, initiates or improves the biosynthesis of specific compounds. Elicitation is the induced or enhanced biosynthesis of metabolites due to addition of trace amounts of elicitors. Elicitors can be classified on the basis of their 'nature' like abiotic elicitors or biotic elicitors. Abiotic elicitors' are substances of non-biological origin, whereas 'biotic elicitors' are substances with biological origin[25].

Exposure of cell or hairy root cultures to a variety of biotic enzymes, cell wall fragments of microorganisms, polysaccharides from microorganisms (chitin, glucan), glycoproteins, and phytochemicals produced by plants in response to physical damage, fungi or bacteria attack, polysaccharides from plant cell wall (pectin, cellulose), chitosan, salicylic acid, methyl jasmonate (MeJA) that are formed by the action of plants on microbial cell wall and abiotic (inorganic salts, heavy metals and physical factors acting as elicitors like Cu and Cd ions, Ca^{2+} and high pH, UV irradiation, high salinity, high and low osmolarity, extreme temperatures, and high pressure) factors have been shown to improve the yield of secondary metabolites[18].

Although elicitors were first used to increase plant resistance to pathogens, it was found that the mechanism involved increased polyphenol levels. Consequently, elicitors can be regarded as an interesting alternative for obtaining plants with higher polyphenol content[26]. The use of biotic or abiotic elicitors to stimulate product formation has become an important progress strategy and has been very useful in reducing the process time required to attain high product concentrations and increased volumetric productivity[27].

It has been reported that the total phenolic content of sweet basil significantly increased after 0.1 and 0.5 mM MeJA treatments compared with the control not subjected to MeJa. Two phenolic compounds, rosmarinic acid and caffeic acid, were identified as strong antioxidant constituents of the sweet basil. Their amounts also significantly increased after the MeJA treatment. In addition, eugenol and linalool concentrations increased to 56% and 43%, respectively[28].

Different elicitors including yeast, MeJA, glutathione, and manganese ions were used for the production of bioactive compounds of *Echinacea purpurea* cell cultures and compared for their effects on the formation of phenolics in the cell cultures. Glutathione and manganese did not affect phenolic content accumulation. However, in yeast- and MeJA-treated cell suspension cultures, other phenolics were formed and accumulated in the medium as lignans, neolignans and acetophenone derivatives as main elicitor-enhanced products. The cells mainly contained phenolic glycosides including a new compound, alpha O-beta-D-glucopyranosyl-acetovanillone[29].

The production of chlorogenic acid in embryogenic suspension cultures of *Eleutherococcus senticosus* upon exposing them to different concentrations of MeJA were studied during the culture period. An increase in chlorogenic acid yield (3.9 fold) was obtained with 200 µM MeJA treatment[30].

The effect of nitric oxide elicitation in the synthesis of secondary metabolites within the adventitious roots of *Echinacea purpurea* was investigated. They reported that when roots were treated with 100 µM sodium nitroprusside (SNP), an exogenous nitric oxide producer, the accumulation of phenolics, flavonoids, and caffeic acid derivatives was enhanced. Additionally, they found that eliciting *E. purpurea* adventitious roots with a concentration of 100 µM SNP is beneficial to their accumulation of secondary metabolites. This level of SNP also induced an antioxidant defense, as indicated by increases in superoxide dismutase, ascorbate peroxidase and ascorbic acid, along with decreases in hydrogen peroxide, lipid peroxidation and dehydroascorbate/ascorbic acid[18].

The effects of two elicitors; JA and MeJA were studied on rosmarinic acid accumulation in cell suspension cultures of *Mentha piperita* and observed that the highest rosmarinic acid accumulation was measured 24 h after addition of 100 µM MeJA. A similar concentration was detected 48 h after application of 200 µM JA. Those values were nearly 1.5 times higher compared to the control, without elicitation. There was no substantial influence of elicitors on rosmarinic acid secretion into the culture media[31].

The effects of yeast extract and selected polysaccharide elicitors were studied on secondary metabolite production, particularly anthocyanin and phenolic acids, in cell suspension cultures of *Vitis vinifera*. All elicitors either maintained or promoted cell growth in culture. Overall, secondary metabolite production in *Vitis vinifera* cell suspension cultures responded differently to different elicitors. Chitosan, pectin and alginate enhanced production of anthocyanin within 13 days of culture over that of control. Chitosan, alginate, and Arabic gum significantly promoted accumulation of phenolic acids, particularly 3-O-glucosyl-resveratrol, in *Vitis vinifera* cultures, as well as in the culture medium. Intracellular phenolic acid production was significantly enhanced by alginate and chitosan with 1.7- and 1.5-fold levels, respectively, of that of control[27].

1.5.3. Effect of precursor feeding

The shikimate pathway **(Figure 2)** is a major biosynthetic route in higher plants and microorganisms that leads to the production of several secondary metabolites and its regulation is assumed to respond to the requirements for synthesis of secondary metabolites. This pathway can be divided into two parts i.e. pre-chorismate and prephenate. The pre-chorismate pathway consists of seven metabolic steps that convert the primary metabolites phosphoenol pyruvate (PEP) and erythrose-4-phosphate (E-4-P) to chorismate. Chorismate serves as a common precursor for the synthesis of three aromatic amino acids phenylalanine (Phe), tyrosine (Tyr), and tryptophan (Trp). Aromatic amino acids act as a precursor for a wide variety of secondary metabolites including phenolic acids, flavonoids, lignin, coumarins, alkaloids, and cyanogenic glycosides[32].

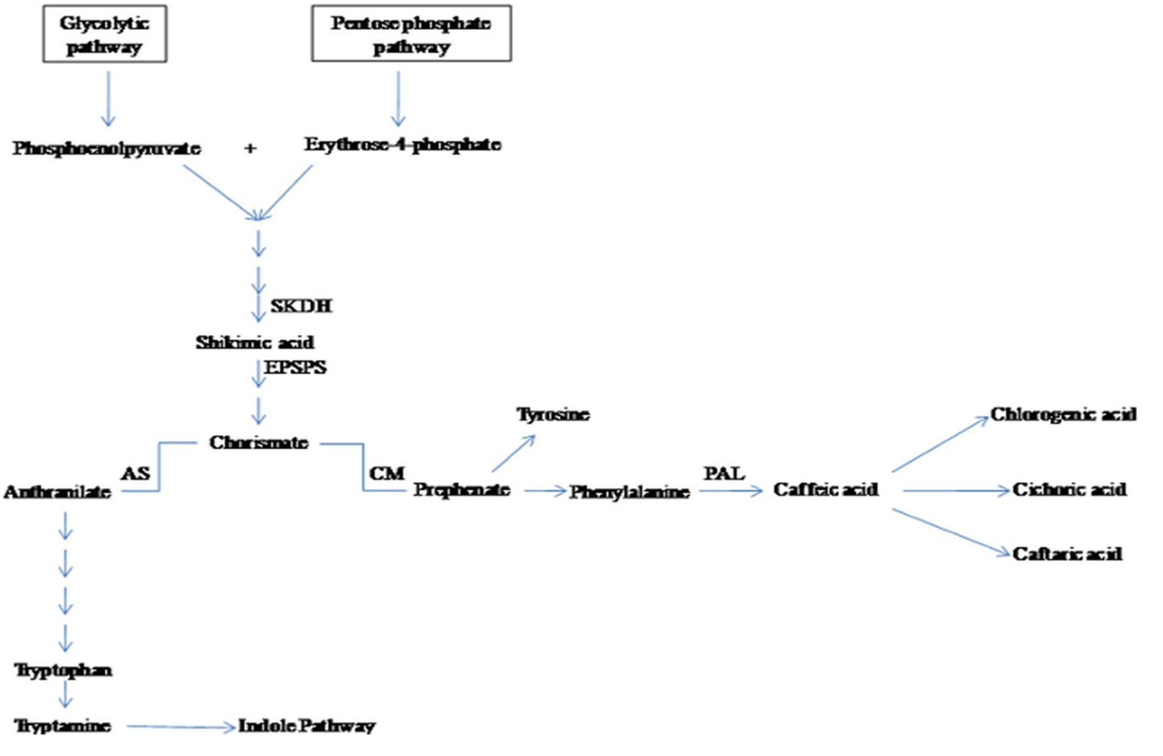


Figure 2: Summary of some of the important reactions of shikimic acid pathway leading to the production of phenol, flavonoids and CADs in adventitious roots of *Echinacea purpurea* (L.) Moench SKDH; shikimic acid dehydrogenase, EPSPS: 5-enolpyruvylshikimate-3-phosphate synthase, AS: anthranilate synthase, CM: chorismate mutase and PAL: phenylalanine ammonia lyase[32].

It has been cited that phenylalanine ammonia-lyase (PAL) activity was enhanced in the callus of rosemary (*Rosmarinus officinalis*) due to exogenous application of L- tyrosine[33].

Other researchers enhanced the production of rosmarinic acid in *Mentha piperita* L. and *Mentha arvensis* L. by adding two precursors are phenylalanine (Phe) and tyrosine (Tyr) in the nutrient media at different levels. Tyrosine was found to be very effective for augmenting rosmarinic acid content in *Mentha piperita* L. However, phenylalanine significantly affected the production of rosmarinic acid in *Mentha arvensis*. No significant increase in biomass was observed after addition of these precursors indicating that the added amino acids acting as precursor's synthesis were readily utilized in producing rosmarinic acid without promoting growth[34].

The response of growth, accumulation of phenols, flavonoids and caffeic acid profiles to feeding of aromatic amino acids (L- phenylalanine and L- tryptophan) were studied in adventitious root cultures of *Echinacea purpurea*. Feeding of L-phenylalanine enhanced the growth but accumulation of phenols, flavanoids and caffeic acid was lower in comparison to the L- tryptophan fed cultures. The addition of L- tryptophan (0.5, 1 and 5 mM) resulted in an increase up to 56, 61, 84 % in phenol, 24, 12, 3 % in flavonoids and 86, 63, 39 % in total caffeic acid[32].

1.5.4. Hairy root cultures

When products are known to be produced in roots, as is the case with *Echinacea* spp., hairy root cultures may be initiated using *Agrobacterium rhizogenes* as the vector. Hairy root cultures offer great potential for the production of valuable plant secondary metabolites. The advantages of using hairy roots are their independence of plant growth regulators, high growth rates and genetic and biosynthetic stability[34].

Studies showed that hairy root cultures offer an excellent biological model to study the biosynthetic pathway of medicinally important caffeic acid derivatives (CADs), where HPLC analyses of methanolic extracts from hairy roots revealed the presence of important caffeic acid derivatives (CADs): cichoric acid (19.21 mg/g dry biomass), caftaric acid (3.56 mg/g dry biomass), and chlorogenic acid (0.93 mg/g dry biomass) [35].

Hairy roots were produced from leaf explants of *Echinacea purpurea* with *Agrobacterium rhizogenes*. The kinetics of growth, the uptake of macronutrients, and the accumulation of caffeic acid derivatives (CADs) were investigated in heterotrophically cultured hairy roots for a 50 days period. The maximum dry biomass (12.2 g/l) was achieved in MS nutrients supplemented with 30 g/l sucrose on day 40. HPLC analyses of methanolic extracts from hairy roots revealed the presence of important CADs: cichoric acid (19.21 mg/g dry biomass), caftaric acid (3.56 mg/g dry biomass), and chiorogenic acid (0.93 mg/g dry biomass)[35].

Some researchers established a method of the transformed hairy roots cultures of *Echinacea purpurea*, infecting different types of explants with three strains of *Agrobacterium rhizogenes*. For transformed hairy roots the contents of polysaccharides and phenolic compounds were 236.0 and 18.9 mg/g D.W., respectively. While the contents of polysaccharides and phenolic compounds in non-transformed roots were 161.5 and 33.3 mg/g D.W. respectively[36].

The effects of light on growth rate and caffeic acid derivative (CADs) biosynthesis in hairy root cultures of *Echinacea purpurea* (Moench) were studied and it has been reported that light-grown hairy roots accumulated increased levels of anthocyanins. The light grown root cultures had thickened morphology compared with the dark-grown controls. The growth rate and cell viability of the hairy root cultures in light did not show obvious difference in comparison with those in dark. However, biosynthesis of CADs including cichoric acid, caftaric acid, chlorogenic acid and caffeic acid was significantly increased in hairy root cultures grown in the light. The enhanced accumulation of CADs and anthocyanìns in *E. purpurea* hairy root cultures was correlated to an observed light-stimulated activity of phenylalanine ammonium lyase (PAL)[37].

The transformation of the three most economically important species of *Echinacea* (*Echinacea purpurea*, *Echinacea pallida* and *Echinacea angustffolia*) was studied with two strains of *Agrobacterium* to produce the hairy root phenotype. Transformed roots of all three species exhibited consistent accelerated growth and increased levels of alkamide production. Optimization of the culture of *Echinacea* hairy roots was implemented to enhance both growth and alkamides production concomitantly. The use of half strength Gamborg's B5 medium supplemented with 3.0% sucrose was twice as effective in maintaining hairy root production than any other media tested, The addition of indolebutyric acid increased the growth rate of roots by as much as 14-fold. Alkamide production increased several fold in response to the addition of jasmonic acid, but did not respond to the addition of indolebutyric acid. Induced accumulation of the important bioactive compounds, alkamides 2 and 8, was observed both in transformed roots and in response to jasmonic acid treatments[38].

Ultrasound stimulation strategy was studied for improving the hairy root growth and caffeic acid derivatives (CADs) biosynthesis in the hairy root cultures of *Echinacea purpurea* L. The l5-day-old hairy roots were stimulated every 5 days by ultrasound for 6 min which resulted in production of high amount of CADs after 30 days of culture. This increase was related to the increase of phenylalanine ammonium lyase (PAL) activity. These results provided a basis for understanding of improving growth and secondary metabolism in the process of hairy root culture stimulated by ultrasound[39].

1.6. Active components of *Echinacea*

The raw materials used for the manufacture of medicinal preparations from *Echinacea* are both blooming herbs and their roots. All three species of *Echinacea* being used in therapy can be characterized by an outstanding diversity of substances, which can affect the parameters of the immune system, however still there is a debate on the relative importance of these groups. It is generally thought that there is no single constituent or group of constituents responsible for the activity of *Echinacea*. Due to the abundance of those substances (various additionally in each species), obtaining the standardized preparations was highly problematic. These active constituents can be divided into three major groups, namely the alkamides, caffeic acid derivatives (phenylpropanoids) and polysaccharides[3].

1.6.1. Alkamides

Alkamides (also known as alkylamides) found in *Echinacea* roots and aerial parts are often identified by a numbering system that is often preserved in more recent literature by giving numbers from 1 through 20, that was identified according to the pioneering studies of both Bohlmann and Hoffmann [40] which revealed the structure, chemistry and biological activities of these alkamides. Alkamides are fatty acid amides containing one or more double bonds that may be accompanied by up to three acetylenic linkages. **Figure 3** shows the alkamide constituents in *Echinacea* extracts. Alkamides have been isolated from *E. angustifolia* and *E. purpurea* roots and aerial parts, but they are largely absent from *E. pallida*, The major constituents of the roots of *E. pallida* are ketone compounds, which can be a marker for *E. pallida*, since it is not found in *E. angustifolia* and *E. pupurea*[41].

E. purpurea contains at least the first 11 of these alkamides, including 9 isobutylamides, identified by some researchers[40,42]. Most of these root alkamides contain a diene in conjugation with the carbonyl (2, 4-diene). The major tetraene alkamides occur as a mixture of dodeca-2E, 4 E, 8 Z, 10E/Z-tetraenoic acid isobuty-lamides 8 and 9. Total alkamide concentrations range were from 0.004% to 0.039% D.W[43,44].

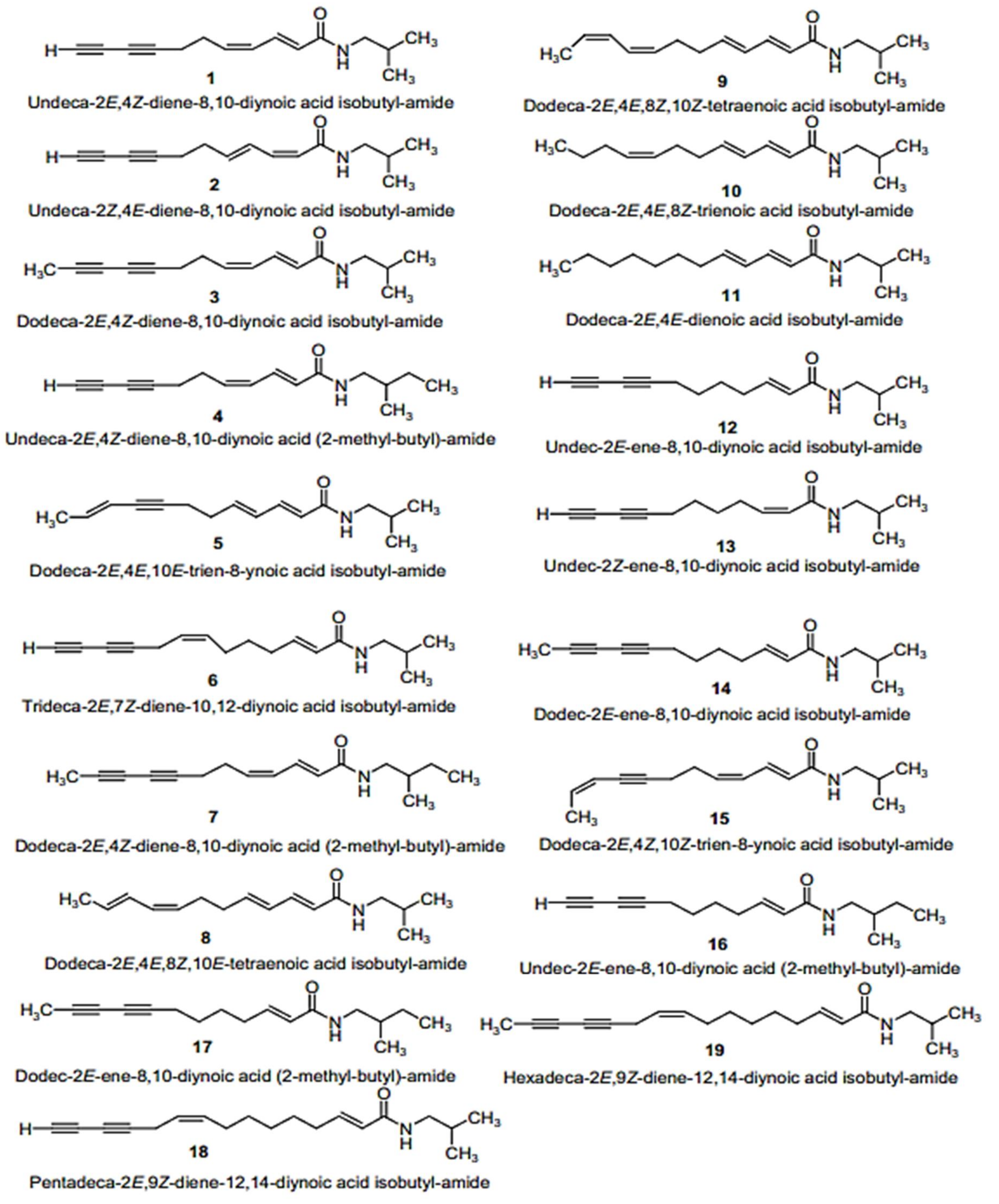


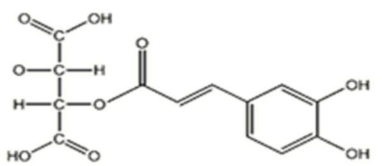
Figure 3: Main alkamides in *Echinacea* species [41]

1.6.2. Caffeic acid derivatives (CADs) (phenylpropanoids)

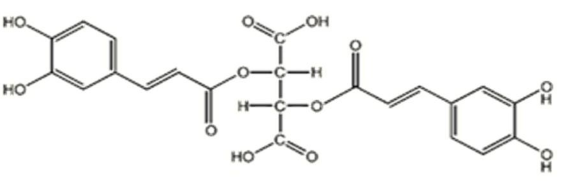
Caffeic acid derivatives are another major constituents of *Echinacea*. The basic structures of caffeic acid derivatives consist of one or two caffeoyl moieties with a linking molecule, such as tartaric acid, quinic acid or a sugar residue. Examples of caffeic acid derivatives are shown in **Figure 4**. Of the common caffeic acid derivatives, cichoric acid appears to have the greatest reported activity. It is found in appreciable amounts in *E. purpurea*[45]

CADs are considered to be among the most valuable and important active components of *Echinacea* spp., the principle CADs in *E. purpurea* root are cichoric acid (2, 3-O-dicaffeoyl tartaric acid) and caftaric acid (2-O-caffeoyltartaric acid) as major constituents and chlorogenic acid (3-O-Caffeoylquinic acid) and caffeic acid (3,4-dihydroxycinnamic acid) as a minor components (**Figure 4**)[46], Root extracts of *E. purpurea* were found to contain (µg/ml) chlorogenic acid (0.0157 µg), caftaric acid (0.1568 µg), caffeic acid (trace), cichoric acid (1.0147 µg). Degradation of cichoric acid during soxhlet extraction was suggested, based on the increased yield of cichoric acid when performing alcoholic, ultrasonic extraction of dried, ground roots. Echinacoside, has been identified in the alcoholic extracts of *E. angustifolia* and *E. pallida* roots, but has never been isolated from *E. purpurea* roots, allowing a distinction to be made between the species[45].

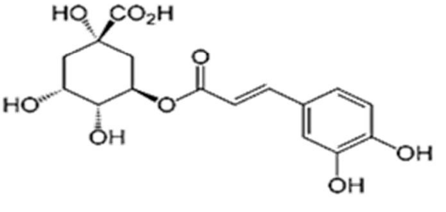
The quinic acid derivative, Cynarin (1, 3-Dicaffeoylquinic Acid), is present in *E. angustifolia* but not *E. pallida*, providing a chromatographic marker to distinguish these species[45].



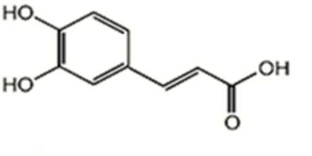
A



B



C



D

Figure 4: Caffeic acid derivatives found in *Echinacea purpurea* roots
A: Caftaric acid **B:** Cichoric acid **C**: Chlorogenic acid **D:** Caffeic acid

1.6.3. Polysaccharides

Two polysaccharides, PS I and PS II, with immunostimulatory properties have been isolated from the aerial parts of *E. purpurea*. Their structures were determined as 4- O-methyl-glucuronoarabi-noxylan (average MW 35,000) and an acidic arabinorhamnogalactan (MW 50,000), respectively, and each of them showed significant activity in both *in vitro* and *in vivo* immunological assays. The expense of obtaining pure polysaccharides from plant extracts and the difficulty of obtaining reproducible activities led to the use of tissue culture for their isolation. Three homogeneous polysaccharides, two neutral fucogalactoxyloglucans with molecular weights of 10,000 and 25,000, and an acidic arabinogalactan (MW 75,000) were isolated from cell cultures of *E. purpurea*[46].

1.7. Uses and activities

1.7.1. Immune functions

Echinacea is well known and used as an immunostimulating agent. It has been proved to increase the number of white blood cells and potentiate the process of phagocytosis by macrophages and granulocytes. *Echinacea* is a wide-spectrum immunomodulator that modulates both innate and adaptive immune responses, but there is still much to understand about the way *Echinacea* root impacts the human immune system. Each *in vitro* study by its nature can provide just a narrow insight into a few specific aspects of immune function[47].

A number of experimental *in vivo* and *in vitro* studies have shown that *Echinacea*-containing remedies increase immunologic activity and may protect experimental animals against systemic viral, bacterial, parasitic and fungal infections. They also exert antioxidant and anti-inflammatory activities; enhance antibody production, phagocytosis, and cellular respiratory activity. The mechanism of immunostimulatory effects is not fully understood, but it is probably connected with synergistic action of polyphenols, polysaccharides, glycoproteins and alkamides[48].

The overall immunostimulant activity of the alcoholic and aqueous extracts appears to depend on the combined effects of several constituents[49,50]. Alcoholic extracts of *Echinacea* roots are more active than aqueous extracts of the aerial parts[51]. All of these extracts caused phagocytosis to increase by 20% to 30% which corresponded well with *in vivo* results[49]. In human polymorphonuclear (PMN) cells, *Echinacea*`s polysaccharides and the fresh juice enhanced spontaneous motility and phagocytosis also increased the ability of these cells to kill *Staphylococci*[52]. *E. purpurea* root extracts have been shown to stimulate the production of natural killer (NK) cells in the bone marrow and precursors cells of monocytes and NK cells of experimental animals[53,54]. Studies reporting that *Echinacea* extracts have the ability to activate human phagocytic function both *in vitro* and *in vivo* [55-59]. Although some studies have failed to find immunostimulatory activity *in vivo*[60], other researchers have noted up-regulation of immune function in *ex vivo* models in human immunodeficiency disorders[61].

1.7.2. Cancer

Due to *Echinacea*`s ability to modulate the immune response, it has been investigated as a treatment for cancer. The anticancer/antitumor effects of *Echinacea* are related to its general immune-potentiating actions[62]. *In vitro* studies have shown various modes of anticancer activity for complex extracts of *Echinacea*[63].

In vitro research has demonstrated inhibition of angiogenesis induced by lung and kidney cancers by an alkylamide containing extract[64]. Animal studies found that dietary administration with *E. purpurea* preparations significantly decreased prostate weight of rats and increased lymphocyte numbers after 8 weeks[65]. *Echinacea purpurea* root hexane extract reduced cell viability of human pancreatic cancer MIA PaCa-2 and colon cancer COLO320 cell lines in a concentration- and time-dependent *in vitro* [66]. *E. purpurea* flower extract and cichoric acid significantly inhibited proliferation in a dose- and time-dependent manner human colon cancer cells Caco-2 and HCT- 116[67]. Cichoric acid treatment decreased telomerase activity in HCT-116 cells. Further, cichoric acid effectively induced apoptosis in colon cancer cells, which were characterized by DNA fragmentation[62].

1.7.3. Inflammatory response and wound healing properties

Anti-inflammatory activities of *Echinacea* extracts have been attributed to direct inhibition of hyaluronidase[49]. A number of *Echinacea* constituents have been shown to exhibit anti-hyaluronidase activity[68]. Cichoric acid, cynarine and other compounds from *E. angustifolia* have anti-hyaluronidase activity, which may reduce inflammatory changes in damaged tissues[68]. Several *Echinacea* constituents have protected collagen from degradation during exposure to free radicals, leading to suggestions that *Echinacea* may be helpful in protecting against sun damage to skin[69]. Topical applications of *Echinacea* extracts have been traditionally used to promote wound healing. The polysaccharide fraction (echinacin B) appears to promote wound healing by forming a hyaluronic acid-polysaccharide complex that indirectly leads to the inhibition of hyaluronidase[70] and stimulating the growth of fibroblasts[49,71]. An *E. pallida* extract and echinacoside alone were shown to exhibit wound healing activity also[72]. In the Croton oil-induced edema model, swelling of the rat ear was significantly reduced by the polysaccharide fraction of. *E angustifolia* roots applied topically. In this test high molecular weight polysaccharides in the fraction were more active than lower weight polysaccharides[73]. *Echinacea* extracts have been shown to inhibit enzymes that involved in the synthesis of pro-inflammatory chemical mediators[45,62,74].

1.7.4. Anti-viral activity

For purified caffeic acid derivatives, antiviral activities have been demonstrated[75], the components may block viral receptors on the cell surface[49]. From the flowers and leaves of *E. pallida*, besides caffeic acid, the caffeic acid derivative echinacoside, and cichoric acid were reported to show *in vitro* virustatic and antiviral activity against vesicular stomatitis virus[76].

Incubation of vesicular stomatitis virus with cichoric acid for 4 hours reduced the number of viral particles in mouse L-929 murine cells by more than 50[74]. In cultures of mouse cells, treatment with aqueous *E. purpurea* extracts for four to six hours prior to exposure to influenza and Herpes viruses caused 50-80% resistance to infection for 24 hours after exposure; by 48 hours, the cells were again sensitive to infection[62,77-79].

1.7.5. Antioxidant activity

There has also been in vitro antioxidant/radical scavenging activity by the phenylpropanoids demonstrated for *E. purpurea* and *E. angustifolia* extracts which is linked to improving immune function[69,80,81]. Phenylpropanoids, especially cichoric acid and caffeic acid, are some of the most efficient antioxidants from natural sources[82]. Cichoric acid is also found in the aerial parts of *E. pallida*. Other caffeic acid derivatives are found in the three main *Echinacea* species, especially in the aerial parts[49,62].

Constituents of *E. purpurea* cover a wide range of polarity, from polar polysaccharides, to moderately polar caffeic acid derivatives, to the rather lipophilic alkamides. Therefore the application of extracts is still reasonable and hence the native extract is regarded as "the active principle" for regulatory purposes by the health authorities. However, standardization of these extracts is a must for a rational therapeutic application of phyto-preparations, also standardization via biological activity is needed, but it is difficult to agree on an appropriate assay and in vivo assays are not acceptable to the public opinion[83].

In spite of all of the aforementioned studies, it is obvious that not a single, but several constituents contribute to the immune-stimulatory activity of *Echinacea* extracts. Besides the polyphenols and the phenylpropanoids (caftaric acid, caffeic acid, chlorogenic acid, cichoric acid) , which are the focus of this dissertation, there are other constituent groups that may have immunomodulatory activity.