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Mini Review

Biosynthesis and pharmacological evaluation of shikonin – A highly valuable metabolite of North-Western Himalayas: Mini Review

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ABSTRACT

Shikonin is one of most important secondary metabolite having vast medicinal properties. The source for shikonin and its derviatives are mainly the roots and rhizomes of Boraginaceae family which include *Lithospermum erythrorhizon*, *Arnebia euchroma, Arnebia benthamii, A. guttata, A. hispidissima, A. nobilis, A. decumbens, A. nandadeiensi, Arnebia griffithi* and *Onosma paniculatum.* Shikonin and its derivatives have high value in the international market attributed to its pharmacological applications. Most of the plant species used for extraction of shikonin have been listed endangered because of their over exploitation and hence there is a need to identify alternative sources to meet the escalating demand of this valuable metabolite. The biosynthetic pathway of shikonin has remained elusive or fragmentary. Poor understanding of biosynthetic pathway limits the large scale shikonin production. A genetic intervention coupled with biosynthetic pathway understanding will be helpful for enhanced production that eventually fulfill the industrial requirements. We therefore, give a brief review on our current understanding and limitations of shikonin biosynthesis along with their pharmacological properties.

Keywords: Secondary metabolite, shikonin, biosynthetic pathway, pharmacoligical properties

INTRODUCTION

Secondary metabolites, mainly produced as a stimuli to survival of plants under stress conditions can be produced through different biosynthetic pathways, which include terpenoids (29,000), alkaloid derivatives (12,000) and phenolics (8,000) (Francisco *et al.*, 2002; Zwenger *et al.*, 2008). These have multifold industrial applications as a food additive, drug, flavor, fragrance, dye, color, pesticide, pharmaceutical, agrochemical, biopesticide and more recently as a nutraceutical. Shikonin is such kind of secondary metabolite having higher value in international markets because of its vast medicinal properties including anti-bacterial, anti-tumor, anti-fungal, anti-topoisomerase-I, anti-HIV-I activity, anti-inflammatory, anti-allergic, anti-hydropic and used as a dyestuff for food, fabric, and cosmetic, curing of ulcers and burnt skin (Sasaki *et al.*, 2002). This valuable metabolite is usually obtained from medicinal plant species belonging to Boraginaceae family including *Alkana tinctoria, Arnebia gutata, A. hispidissima, A. benthamii, A. nobilis, Lithospermum erythrorhizon* etc. Shikonin is the first secondary metabolite being used for a long time at commercial scale. Shikonin is biosynthetically derived from two precursors- p-hydroxybenzoic acid (PHB) (obtained *via* phenylpropanoid pathway/shikimate pathway) and geranyl diphosphate (GPP) (obtained *via* mevalonate pathway) (Newman *et al.*, 1999; Heide *et al.*, 1998). Due to higher economic value in the international market, shikonin producing plant species are overexploited and therefore, placed in the category of critical endangered plant species (Kala *et al.*, 2004; Kumar *et al.*, 2014). Previously, various alternate strategies have been tried for production of shikonin through cell culture systems such as establishment of cell suspension cultures by employing precursor feeding,

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optimisation of media, elicitor treatment, optimisation of physico-chemical factors and so on, but limited success has been achevied (Syklowska-Baranek *et al.*, 2012). Shikonin production can be improved through metabolic engineering of the pathway which is yet to be elucidated.

By keeping this in view, the foccus of present review is on the pharmacological properties of shikonin along with recent advancements of its biosynthetic pathway.

Shikonin biosynthesis

The biosynthesis of shikonin is quite complex. Research is ongoing to describe the steps involved in its biosynthesis throughout the world. It is suggested that shikonin formation takes place in the vesicles derived from the endoplasmic reticulum (ER) and transport through the multi enzymes system located in the plasma membrane towards the cell wall to release outside from the cells (Tsukada *et al.*, 1984; Tabata *et al.*, 1996). Shikonin derivatives are mostly found in the form of esters such as acetyl shikonin, isobutyl shikonin, β, β-dimethyl acryl shikonin, β-hydroxyisovaleryl shikonin, isovaleryl shikonin and α-methyl- n-butyl shikonin except for deoxyshikonin which can not form any esters due to lack of a hydroxyl group at C-1 position of side chain **(Figure 1).**

Shikonin derivatives formation mainly occur through the two basic precursor– Geranylpyrophosphate (GPP) and p-Hydroxybenzoic acid (PHB) derived from the different pathways. GPP derived from the cytosolic mevalonate (MVA) and plastid 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway (Lichtenthaler *et al.*, 1997; Newman *et al.*, 1999). 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) is formed by the three molecules of acetyl Co A, further HMG Co A is converted into MVA. Isopentenyl pyrophosphate (IPP) is formed from MVA by the 5-phosphate kinase, finally IPP is converted into the first precursor GPP through MVA Pathway. On the other hand, pyruvate and glyceraldehyde 3-phosphate condensed to form 1-deoxy-D-xylulose 5-phosphate (DXP), DXP is converted into MEP, further MEP into IPP and IPP, finally GPP is formed through MEP pathway (Eisenreich *et al.*, 2001; Rodriguez-Concepcion *et al.*, 2002). GPP and PHB condensed to form an intermediate compound m-Geranyl-p-Hydroxybenzoic Acid (GBA) with the help of PHB geranyltransferase (Inouye *et al.*, 1979) **(Figure 2).** There are various intermediates such as Deoxyshikonin, Dihydro-shiknofuran, Dihydroechinofuran and Echinofuran participated in the shikonin biosynthesis which is yet to be elucidated. Twelve genes have been reported in shikonin biosynthesis, out of which two genes namely, PHB geranyltransferase and 3-hydroxy-3-methylglutaryl-CoA reductase are the key genes controlling shikonin biosynthesis (Singh *et al.*, 2010).

Pharmacological actions of shikonin

Shikonin has been shown to confer diverse pharmacological activities, including anti-fungal, anti-inflammatory, antibacterial, antioxidant and antitumor activities (Papageorgiou *et al.*, 2006; 1999). Scientists showed the keen interest on highly valuable metabolite- shikonin as the articles were increasing day by day on its pharmacological properties.

Antifungal activity

Antifungal activities of naphthoquinone derivatives that are constitute of shikonin, were investigated against several fungal pathogens. When the biological activity of these compounds was tested against fungi, a wide range of antifungal activity was recorded. Sasaki *et al.* (2002) investigated the effects of these naphthoquinone derivatives on a variety of fungi and recorded stronger activities (four-fold) against *Candida krusei*, *Saccharomyces cerevisiae* and *Candida glabrata*. Moreover, Miao *et al.* (2012) also reported the inhibitory effects of shikonin on *Candida albicans* growth. The results showed that shikonin (MIC(80) value 4 μg/mL) was >16 times effective than Fluconazole (FCZ) (MIC(80) >64 μg/mL) to some FCZ- resistant *Candida albicans*. It has been found that shikonin treatment enhanced the generation of reactive oxygen species (ROS) but antioxidants *viz*. glutathione (GSH) and N-acetylcysteine (NAC) could result in a significant decrease of shikonin antifungal activity in *Candida albicans* (Miao *et al.* 2012). Recently, the induction of reactive oxygen species by antifungal agents was also reported by Delattin *et al.* (2014). The effect of shikonin was also screened on various fungi *viz*. *Pythium aphanidermatum*, *Pythium ultimum*, *Phytophthora parasitica*, *Phytophthora capsicii*, *Nectria hematococca*, *Colletotrichum destructivum*, *Aspergillus niger*, *Rhizoctonia solani*, *Monosporascus cannonballus*, *Fusarium oxysporum* and *Fusarium proliferata* (Brigham *et al.,* 1999). The results showed that *Nectria hematococca* exhibited little inhibition of hyphal growth even at the highest shikonin concentration whereas, *Aspergillus niger* was moderately inhibited by shikonin. On the other hand, *Pythium ultimum* and *Rhizoctonia solani* showed showed increasing sensitivity to increasing amounts of shikonin. The result provides a rational basis for the clinical use of shikonin and shows the possibility of its use in medicinal treatment as an anti-inflammatory agent with the antifungal activity.

Anti-inflammatory activity

Naphthquinone isolated from *Arnebia hispidissima* in hexane extract and its anti-inflammatory activity is checked

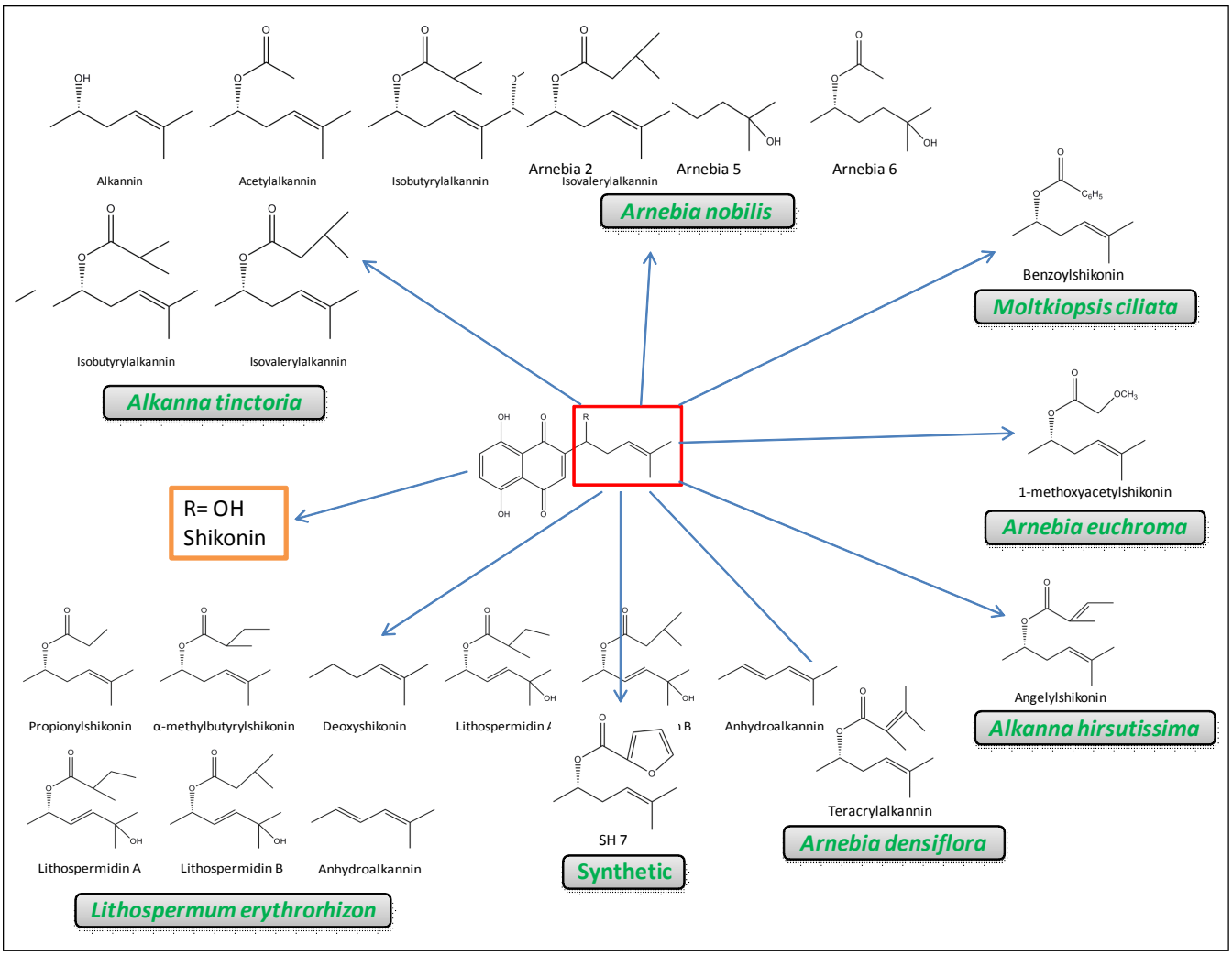

Figure 1: Occurrence and structural variation in selected members of the Boraginaceae family for shikonin production

by the carrageenan-induced acute arthritis and complete Freund's adjuvant (CPA)–induced chronic arthritis in rats as described by Singh *et al.* (2003). Pre-treatment with cycloarnebin-7 significantly inhibited the carrageenan – induced acute arthritis and Arnebin-1, significantly suppressed the development of chronic arthritis induced by CFA. *A.hispidissima* is used to yield a number of shikonin derivatives such as Arnebin-5, Arnebin-6, Teracryl shikonin arnebinone and acetyl shikonin. Root extract (Ethyl root extract) of *A. hispidissima* through column chromatography having anti-inflammatory activity was a first time report. Ping *et al.* (2009) also reported anti inflammatory effect of shikonin through significant inhibition of mice auricular swelling by three different dose groups of shikonin i.e. low, middle and high dose along with the dexamethasone group. Moreover, the different doses of shikonin showed inhibitory effects on rat granuloma formation and at high doses, the effects of shikonin were comparable to that of dexamethasone. This indicated that shikonin has anti-inflammatory effects in the animal models of acute and subacute inflammation. Role of inflammatory damage in cerebral ischemic pathogenesis sets up a new target for treatment of stroke. Recently, the role of shikonin in acute ischemic stroke was deciphered (Wang *et al.*, 2014). The results showed that dosage of shikonin (10 and 25 mg/kg) once a day for 3 days before surgery and another dosage after operation exhibited the inhibition of TLR4, TNF-α, NF-κB, the pro-inflammatory mediators, and phosphorylation of p38MAPK in the ischemic cortex. This indicated that shikonin provides protection to the brain against ischemic damage by regulating inflammatory responses and ameliorating BBB permeability. The anti-inflammatory property of shikonin, extracted from *Lithospermum erythrorhizo* (medicinal Chinese herb) was

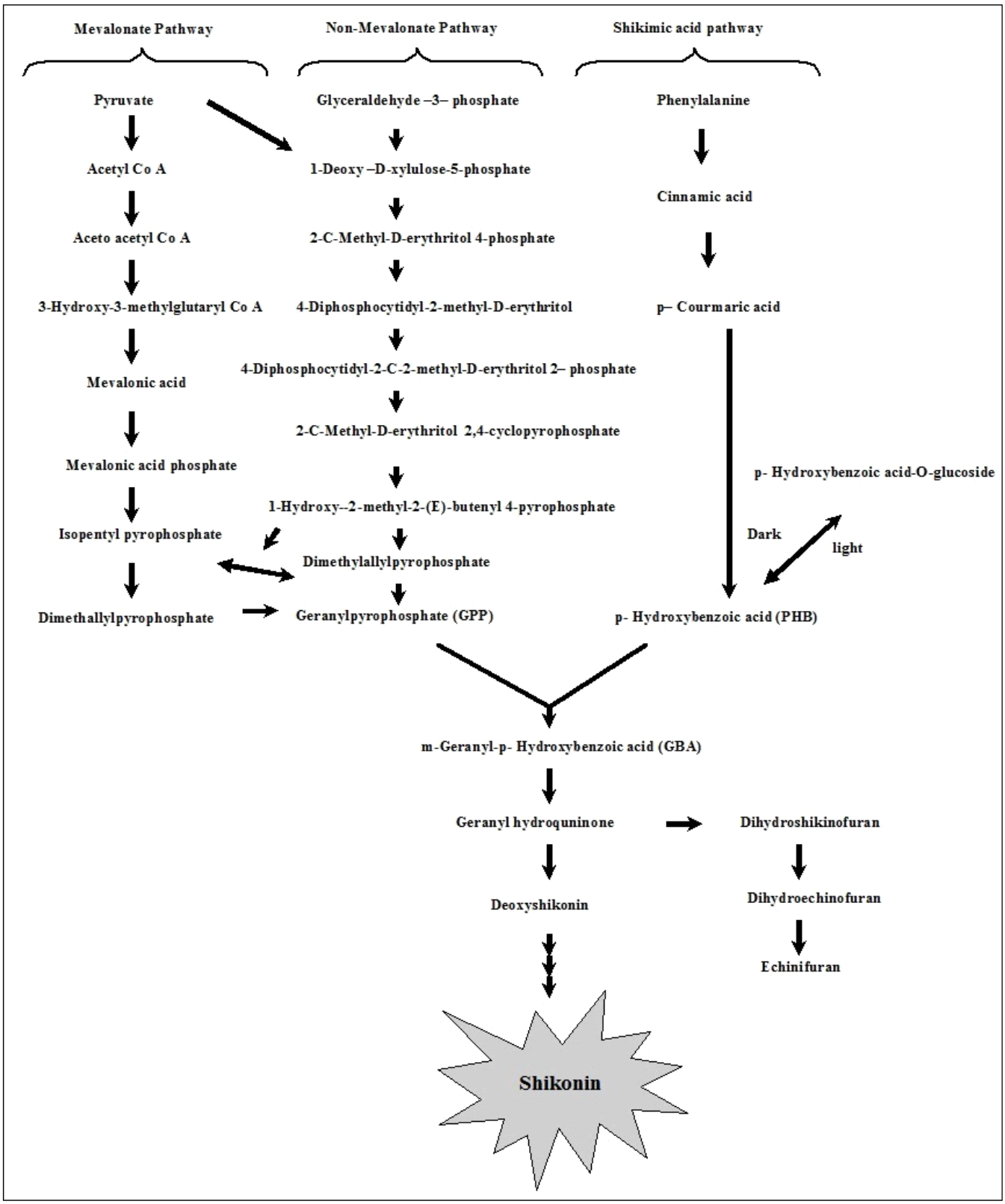

Figure 2: An outline of shikonin biosynthetic pathway as adapted from Inouye *et al.* (1979); Singh *et al.* (2010); Kumar *et al.* (2014)

reported by Lu *et al.* (2011). The study showed decreased proteasomal activity by shikonin under inflammatory conditions. Moreover, Liang *et al.* (2013) also showed the anti-inflammatory activity of shikonin isolated from the root of *Lithospermum erythrorhyzon* in a murine model of lipopolysaccharide (LPS)-induced acute lung injury (ALI). The study reported that shikonin altered the expression of pro-inflammatory cytokines through inhibition of the NF-κB signaling pathway and thus indicated to be a potential agent for the prophylaxis of ALI. The anti-inflammatory effects of shikonin were also reoprted on Astrocytes and experimental Colitis (Hosseini *et al.*, 2012; Andujar *et al.*, 2012) . These findings about shikonin indicated that this medicinal compound has great potential to be developed into an anti-inflammatory agent.

Antibacterial activity

Shikonin and its derivatives posseses antibacterial activities and was evaluated as a multi-functional antibacterial and UV-protective agents on the silk fabric (Dhandapani *et al.*, 2007). Alkannin β, β- dimethylacrylate, a major component of the dye extracted from *Arnebia nobilis* was evaluated as an antibacterial on various textile substrates *viz*. nylon, polyester, silk, wool, cotton and acrylic (Arora *et al.*, 2012). The study showed significant antibacterial activity of dye along with its components against *Staphylococcus aureus* and *Escherichia coli*. The study reported excellent antibacterial activity of shikonin against *Pseudomonas aeruginosa* (50 mm), E. *coli* (45 mm), *S. aureus* (40 mm) and *Klebsiella pneumonia* (38 mm). Naphthoquinone derivatives are known to confer numerous molecules with antibacterial activities. A series of 1,4-naphthoquinones was syntheiszed and tested against several gram-positive and gram-negative bacteria. The compounds inhibited *S. aureus* at concentrations ranging from 30 to 125 μg/ml (Riffel *et al.* 2002). This encouraged further studies of its application in antibiotic therapy. Chung *et al.* (2009) also reported novel naphthoquinone compounds which showed significant activity against methicillin resistant form of pathogenic *S. aureus* and thus have potential to be useful in the treatment of antibiotic resistant bacteria. The effect of shikonin derivatives on pathogenic denal bacteria was reported by Li *et al.* (2012) . The study showed that acetylshikonin, a derivative of shikonin from *Lithospermum erythrorhizon* has potential to be an antimicrobial agent against different species of oral bacteria *viz*. *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Streptococcus mutans* and *Lactobacillus acidophilus*.

Antitumor activity

The antitumor effects of shikonin was studied over the years by investigating its potential mechanisms *in vitro* and *vivo*. Besides the prevalence of literature available on its antitumor potential, recent updates are still to be explored. Han *et al.* (2007) propose an agent i.e. shikonin that induced a cell death in MCF-7 and HEK293 (drug-sensitive cancer cell lines) and their drug-resistant lines overexpressing P-glycoprotein, Bcl-2, or Bcl-x_L. Moreover, shikonin and its analogs *viz.* alkannin also inhibits tumor-specific pyruvate kinase-M2 (PKM2) thereby, inhibited the glycolytic rate by regulating cellular lactate production and glucose consumption in drug-sensitive and resistant cancer cell lines (MCF-7, MCF-7/Adr, MCF-7/Bcl-2, MCF-7/Bcl-x_L and A549) (Chen *et al.*, 2012). Topoisomerase inhibitors are also found to play a crucial role in anti-cancer therapies. Zhang *et al*. (2013) showed that shikonin and topotecan, topoisomerase I inhibitors, repressed the growth and apoptosis of glioma cells, thereby, indicated their potential against targeting gliomas as anticancer agents to provide a novel therapeutic strategy. Shikonin was also exploited as an adjuvant for dendritic cell-based cancer vaccines by Chen *et al.* (2012). The study showed the retardation in tumor growth by shikonin treated tumor cell lysate-loaded dendritic cell vaccines which resulted in increased survival of test mice. Shikonin has been found to induce the expression of RANTES at the skin immunization site which resulted in enhanced anti-tumor potency of a gene based cancer vaccine (Chen *et al.*, 2012). Despite having enhanced antitumor activities of this active naphthoquinone i.e., shikonin against various types of cancers, its role in thyroid cancers was recently deciphered (Yang *et al.*, 2013). The results showed involvement of shikonin in the inhibition of thyroid cancer cell migration and invasion by the downregulating expression of Slug and MMP-2, -9, and -14. Furthermore, shikonin also exerted antitumor activity by targeting tumor proteasome and inhibited its activity (Yang *et al.*, 2009).

Mechanisms of action of shikonin

Shikonin and its derivatives compunds used as a cancer chemopreentive and therapeutic agents in recent times. Shikonin directs the regulation of cell cycle, levels of reactive oxygen species, cytoskeletal formation and mitochondrial function, therby induces the apoptsis (Wiench *et al.*, 2012). Previously studies have been demonstrated that shikonin and its derivatives induces apoptsis and cell cyle arrest in cancer cell lines (Hsu *et al.*, 2004; Wu *et al.*, 2004). However, exact molecular mechanisms underlying apoptsis induced by shikonin remains to be elucidated. Preivously studies have been undertaken to elucidate the plausible role of shikonin in apoptsis, but little success has been achevied. Ahn *et al.* (1995) described that shikonin and its derivatves blocked EGFR (epidermal growth factor receptor) signaling and inhibit topoisomerase activity. Wu

et al. (2004) and Min *et al.* (2008) demonstrated that shikonin activates p53 and caspase-9 pathways follwed by inactivation of NF-κB pathway in Human Oral Squamous Cell Carcinoma Tca-8113 cell lines. Research is ongoing world-wide to decipher the mechanisms of action of shikonin and shikonin dervieates for better understanding the apoptsis process.

In-vitro production systems for shikonin and its derivatives

Plant cell cultre systems including hariy root cultures, callus cultures and cell suspension cultues are being emplyoed for the production of shikonin. Biotechnological interventions provide a promising tool for the production of desired natural products through cell cultures systems. Previously various approaches such as optimizations of culture conditions, using a two-phase culture system, addition of elicitor, precursors and inhibitors, selection of high-producing cell lines and metabolic engineering have been applied to enhance the secondary metabolites in plant cell cultures systems (Yue *et al.*, 2014). Unfortunately, a litte success has been achevied to scale-up secondary metabolites/natural products at commericial scale by using these approaches, therefore, there is urgent need to take intiative to overcome the problems associated for large scale-up production. Therefore, studies focusing on the elucidation of incomplete biosynthetic pathways, gene expression, signal transduction, seleciton of a suitable bioreactors and enzyme activity in the biosynthesis of highly valuable metabolites must be undertaken to decipher the knowledge impart for large scale production. Only a very few number of metabolites such as paclitaxel, shikonin saponins, protoberberines, rosmarinic acid, ginsenoside, echinaceae polysaccharides and scopolamine marked their position at commerical scale (Cai *et al.*, 2012a,b; Georgiev *et al.*, 2009; Wu and Zhong, 1999). In case of shikonin, high producing cell lines or systems are not known till date for large scale production. Hence, there is urgent need to developed such kind of *in-vitro* production systems to fulfil enclasting demands. Understaning of shikonin biosynthetic pathway provides an alternative and effective way for future studies aimed on increasing the shikonin content via *in-vitro* cultures systems.

Future prospects

Metabolic engineering is an alternative way for optimizing genetic and regulatory processes in order to get the desired amount of natural product from the medicinal plants. Many plant species such as *Nicotiana tabacum*, *Atropa belladonna, Artemisia annua, Catharanthus roseus*, *Digitalis lanata, etc.* have been genetically engineered to enhance the metabolite content. Different genetic transformation technologies have been applied for enhanced metabolite production through DNA delivery into the host cells like insertion of genes either indirectly *via* genetic vectors or directly through particle gun, protoplast fusion, electroporation and microinjection approaches (Boyle *et al.*, 2012). Recently, new trends are being used in metabolic engineering like heterologous expression, metabolic flux analysis, RNA interference technologies (RNAi) and overexpression of genes involved in the biosynthetic pathways which aim to achieve highly efficient productive *in-vitro* system. The succesful stories of genetic engineering has been found in case of *Catharanthus roseus and Hyoscymus muticus,* in which strictosidine synthase (Str) and N-methyltransferase (PMT) have been over expressed, respectively to achieve higher metabolite production (Whitmer *et al.*, 1998). Two such stories of metablic engineering of shikonin biosynthesis had been reported by Boehm *et al.* (2004) and Sommer *et al.* (1999) in *Lithospermum erythrorhizon* Sieb. et Zucc. hairy root cultures via introduction of bacterial genes, ubiA and ubiC from *E. coli* encode for 4-hydroxybenzoate-3-polyprenyltransferase that catalysed geranyl diphosphate (GPP) to form 3-geranyl-4-hydroxybenzoate and chorismate pyruvate-lyase (CPL) that convert chorismate into 4-hydroxybenzoate, key intermediates in the shikonin biosynthesis. But, unfortunately, these interventions, did not enhance the shikonin content, highlighting the limitations of *in-vitro* production via heterologous expression of intermediary genes. This alert as for further studies on understanding the pathway regulations in greater details leading towards rational metabolic engineering, therby, limits the *in-vitro* production. More studies are need to be undertaken for elucidation of genes/proteins involved in the regulation of shikonin biosynthesis for increasing everlasting demands of this important moiety. Modern omics technologies such as metabolomics, transcriptomics and proteomics could be useful tools for elucidation of unknown genes/protiens and metabolites in deep understanding the shikonin biosynthesis.

CONCLUSION

Medicinal plants constitutes a source for industrially valuable phytochemical/metabolites. The biosynthetic pathways of metabolites produced in diverse plant species are rudimentary or not fully understood, therby limiting any strategies for enhancement in production of such metabolites. Same thing as follows with the shikonin which could not met industrial needs. By thorough understanding of shikonin pathway, increasing demands may be fullfll through modern highthroughput technolgies. The review enlightens the current available knowledge on the biosynthesis of shikonin and its pharmacological aspects. In future, studies can be undertaken for enhanced shikonin production through cell cultre systems by targeting the rate limiting steps of pathway. By knowing the pharmacolgical

mechanism of this valuable metebolite, helath sector can be formulated a novel drug to enhance the helath standards of individual.

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Conflict of interest

The authors declare that they have no conflict of interest.

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