In Vitro Production of Steroids

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Abstract

Steroids are a cluster of secondary metabolites owning a range in their structure and biological activities. Natural products are often linked with harmful effects on health and have many medicinal applications. Secondary metabolites can potentially drive drug design or discovery. The prime information about such steroids has risen systematically. Advancement in biotechnological approaches affects the disciplines of biochemistry, botany, conservation biology, and toxicology. The genetic markers are a beneficial tool that provides more insight into pathways. Genetic markers accompanied by PTC can supply more insight into the path of steroid products. We will here highlight the biological effects of steroids and the biosynthesis pathways. Studies on steroid extraction and in vitro production will also be included. Besides, the bioreactor of steroids will be scaled up.

Keywords

Steroid · Biological effects · Gonane · Biosynthesis pathway · HPLC · Ecdysterone

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11.1 Introduction

Steroids exist as a class of cholesterol-derived lipophilic, low-molecular-weight constituents, small molecules, and are soluble in organic solvents. The basic unit contains gonane (perhydro-1,2-cyclopentanoperhydrophenanthrene) structure and a minor diverse in this structure or having functional groups results in various groups. Steroid group is composed of different natural and synthetic sources (Aav et al. 2005; Stanczyk 2009; Sultan and Rauf Raza 2015). Steroids play a prime role in the living organisms' biochemical and physiological characteristics (Aav et al. 2005; Sultan and Rauf Raza 2015). The family of steroids contains hormones, sterols, and bile acids (Balandrin and Klocke 1998; Pattenden et al. 2004; De Boeck et al. 2005; Marion et al. 2006; Lopez et al. 2006; Jovanović-Šanta et al. 2015). A divergence of synthetic steroids is employed for a broad sense of medicine and pharmacology (Lopez et al. 2006; Emmanuel et al. 2011; Thao et al. 2015; Jovanović-Šanta et al. 2015). These natural compounds can be convened based on their chemical structure, biochemical effects, biological function, molecular actions, and biosynthesis tissues (Morgan and Melinda 1997; Sultan and Rauf Raza 2015). Plant steroids are of two broad groups: phytosterols (sterols) and brassinosteroids.

11.1.1 Chemical Structure of Steroids

Steroid compounds contain gonane structure (perhydro-1,2-cyclopentanoperhydrophenanthrene; Fig. 11.1), and a minor diverse in this structure or owing functional groups causes various classes of steroids. Skeleton of steroids varies in the number of carbons; C17 in gonanes, C18 in estranes, C19 in androstanes, C21 in pregnanes and cortisol, and C24 of testosterone. Except for vitamin D, steroids have a skeleton of cyclopenta (α) phenanthrene or one/more bond scissions, ring expansions, or contractions. Methyl-side is normal at C10 and C13, and mostly an alkyl-side chain is at C17 (Abd El-Bahaman 1991).

Modifications in steroid rings are expressed by a prefix: inclusion of methylene group (e.g., 4a-*Homo*-7-nor-5α-androstane), addition of a hydrogen atom at terminal group (e.g., 2,3-*seco*-5a-cholestane), loss of an unsubstituted methylene group (e.g., 4-*nor*-5a-androstane), or addition of a hydrogen atom at junction atom with the adjacent ring (e.g., *des*-A-androstane).

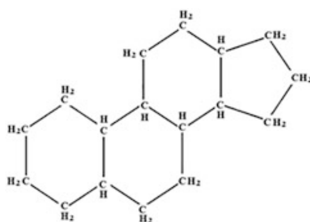

Fig. 11.1 The structure of gonane (cyclopentanoperhydrophenanthrene)

Cardiac glycoside is classified according to the five-membered lactone ring or six-membered lactone ring. (1) Cardenolides (so-called digitalis, Lanoxin, Digitek, Lanoxicaps) have a five-membered lactone ring at C17. Cardenolides have C23 and consist of steroids with methyl groups at C10 and C13. (2) Bufadienolides have the same assembly except for a six-membered lactone ring and two double bonds (Laurie et al. 2012). Stereochemistry shows steroids to be a chair form rather than a boat form through hexagonal C rings. These are oriented in equatorial or axial spot and bonded in trans-configuration. Steroids are identified generally via their trivial names (e.g., cortisol, testosterone), which often leads to confusion in some cases, and through the use of IUPAC, systematic nomenclature is recommended. IUPAC listed a complete description of steroid nomenclature, including (pregnane, androstane or estrange, cortisol, testosterone) number, position, and orientation (α or ß) of all functional groups.

We will here focus on the biological effects of steroids and the pathways of biosynthesis. Studies on steroid extraction and in vitro production will also be included. Also, the bioreactor of steroids will be scaled up.

11.2 Biosynthesis Pathway

Steroids are a class of derived lipophilic, low molecular weight, small molecules, and are soluble in organic solvents. The basic unit is gonane structure (perhydro-1,2-cyclopentanoperhydrophenanthrene), and a minor diverse in this structure or having functional groups results in various classes of steroids. Steroidal skeleton varies based on the number of carbons; C17 in gonanes, C18 in estranes, C19 in androstanes, C21 in pregnanes and cortisol, and C24 of testosterone. Steroids that have C17-C29 skeletons are not triterpenes. Nevertheless, steroid compounds come from the same C30. Squalene comes from the triterpenes. Squalene was isolated first from shark liver oil and found in almost all organisms (Melcangi et al. 2003). The squalene skeleton forms the union of two trans-farnesyl units joined in a head-to-head fashion (St-Onge et al. 2003). The pathway of squalene was mostly found by (Bucourt et al. 1969). The transform of the squalene sequence to the steroid nucleus is with an acid-catalyzed ring-opening of the mono-epoxide derivative. These then involve the formation of carbocationic-mediates series (Abd El-Bahaman 1991; Aav et al. 2005). The steric structures of steroids could streamline from the folding of polyprenyl-chain on the enzyme surface (St-Onge et al. 2003; Melcangi et al. 2003).

We will here highlight the synthesis pathway of ecdysterone (Fig. 11.2), a class of ecdysteroids. Ecdysteroids were discovered first in *Podocarpus nakaii* Hayata called ponasterones A, B, and C, and found later in *Podocarpus elatus* R.Br., *Polypodium vulgare* L. and *Achyranthes faurieri* H. Lev. and Vaniot (Baltaev 2000). Ecdysteroids exist in a broad sense of angiosperms belonging to the families Fabaceae, Caryophyllaceae, Amaranthaceae, Chenopodiaceae, Asteraceae, Lamiaceae Polypodiaceae, Pteridaceae, and Blechneaceae (Cocquyt et al. 2011; Wu et al. 2010; Vanyolos et al. 2012; Nowak et al. 2012, 2013). Ecdysteroids are a sort of sterols chemically associated with triterpenoids and have a direct precursor

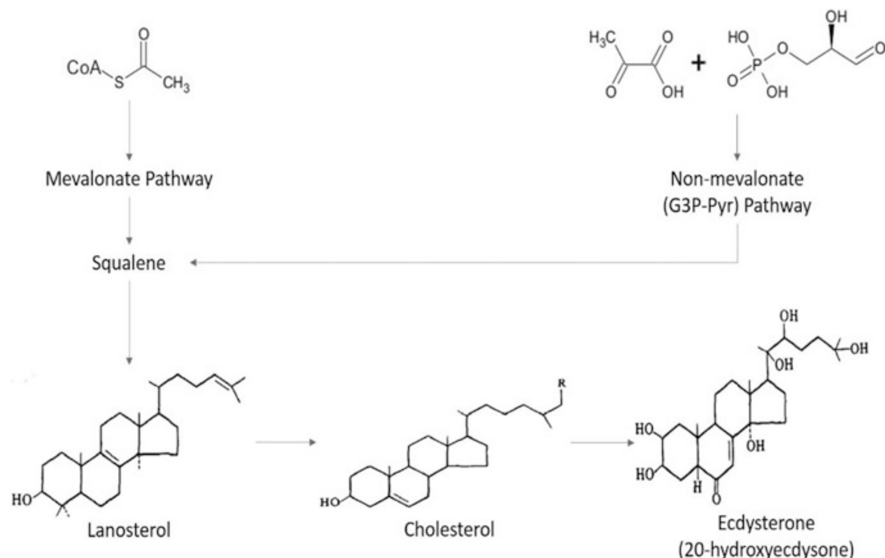

Fig. 11.2 The biosynthesis pathway of ecdysterone (20-E)

in most pathway stages of cholesterol biosynthesis. Ecdysteroids biosynthesized (Fig. 11.2) starting from the acetyl-CoA, through the mevalonate pathway, isoprenoids from pyruvate, and glycerylaldehyde-3-phosphate (via G3P-Pyr or non-mevalonate pathways).

The pathway passes isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). Ecdysteroids depend on C27 cholest-7-en-6-one backbone, follow steps of their biosynthesis including conversion to cholesterol, dehydrogenation to 7-dehydrocholesterol, and more molecular modifications, and, most importantly, hydroxylation at various carbon atoms (Festucci-Buselli et al. 2008; Ikekawa et al. 2013). The aliphatic side chain, attached to the D ring, as in rubrosterone, is cleaved. 20-hydroxyecdysone (20E) and polypodine B (polB) are typical to ecdysteroids. The biosynthesis of ecdysteroids is performed via PTCs (Fujimoto et al. 2000; Okuzumi et al. 2003).

11.3 In Vitro Production Methodology

Due to the importance of steroid derivatives and profitable biosynthesis pathway, in vitro cultures offer rich biomass production. Generally, plant tissue culture (PTC) is highly a biotechnological technique in concern to its utilization of homogeneity explants, clonal propagation, the possibility of rapid, and the regardless of environmental factors (Rout et al. 2000; Karuppusamy 2009; Thiem et al. 2017). Homogeneity of explants also guarantees the integrity of their biometabolite composition. Plant tissue cultures have become an attractive methodology in the production of

biometabolites of importance. Plant tissue culture is a potentially novel approach to obtaining various substances, especially those with complicated structures, of relatively high efficiency, and of low cost (Filova 2014). Therefore, it is predominantly to get a constant source of chemicals with biological activity. Less known biometabolites produced by rare or endangered species can be obtained from plant tissue cultures, as well as their effects can be scanned (Karuppusamy 2009).

Various PTC approaches could be utilized increasingly to produce in vitro bioactive steroids in a plant. The composition of media could be adjusted using variable ratios of plant growth regulators. Besides, all biotechnological strategies, including temperature, use of various biotic/abiotic elicitors, precursor feeding, and photoperiod, must be optimized due to their efficacy having a significant effect on bioactive metabolites yield. Induction of interest constituent production through elicitation is one of the few approaches which have been commercially applied (Smetanska 2008).

Various strategies may enhance steroid production through PCTs. Production of steroids such as ecdysteroids via PTCs has become of increased interest, though it makes available a stable source of these derivatives. Steroids may be extremely variable based upon the origin of tissue, time of excise, and various environmental factors that cannot be controlled. These methods permit in vitro cultures to yield safely bioactive metabolites. In vitro proliferation method can be utilized to overcome natural plant heterogeneity to improve and optimize yielding secondary metabolites (Smetanska 2008; Karuppusamy 2009; Filova 2014). Nowadays, the supply of steroids is based commercially on their extraction. It goes without the obvious obstacles regarding the agriculture, unstable environment, resource consumption, and less in the yield. Various factors, including environmental agents (e.g., temperature, seasonal variation, and geographic location) and biological agents (e.g., source of explants, uniformity of explant), may reflect the steroid-rich plant yields. It makes sure that the steroid-rich plants are an ecological source of biomass for research and supply commercially on steroids (Filova 2014; Thiem et al. 2017).

In vitro proliferation has been applied (Table 11.1) for various important steroid-rich species (Ahmad and Anis 2007; Smetanska 2008; Cheng et al. 2008; Parveen et al. 2008; Flores et al. 2010; Thanonkeo et al. 2011; Ahmad and Anis 2011; Chamnipa et al. 2012; Gnanaraj et al. 2012; Duan et al. 2012; Wang et al. 2013; Kaul et al. 2013; Thiem et al. 2013; Ahmad et al. 2013; Maliński et al. 2014; Vasconcelos et al. 2014; Zand et al. 2014; Wang et al. 2014; Sen et al. 2014; Skała et al. 2015). This procedure may be an alternative for biomass production. Various physicochemical agents may reflect on the in vitro production of bioactive steroids. This starts successfully from (1) the choice of a proper explant with a suitable technique, (2) optimizing tissue culture conditions with the high biomass growth and bioactive constituents, and (3) the selection of a high-performance line. Some modifications of the nutrition media composition or application of biotechnological strategies (e.g., elicitation or precursor feeding) may accumulate steroids (Karuppusamy 2009; Thanonkeo et al. 2011; Thiem et al. 2013; Skała et al. 2015; Thiem et al. 2017).

Some reviews showed that the in vitro production of steroids has a significant effect on future commercial utilization. However, it should recommend that the

Table 11.1 Best methods used for in vitro steroid-produced species

Species	Treatment	Method	Reference
Amaranthaceae			
Achyranthes bidentate Blume			
	MS + 1.5 μM NAA + 1.5 μM BAP	Cell suspension	Wang et al. 2013
	MS + 5.0 mg l^{-1} BAP + 1.0 mg l^{-1} IBA	Shoot tips	Gnanaraj et al. 2012
	MS + 0.5 mg l^{-1} 2,4-D + 1.0 mg l^{-1} NAA + 0.1 mg l^{-1}IBA + 0.1 mg l^{-1} ZT	Callus	Duan et al. 2012
A. aspera L.			
	MS + 5.0 mg l^{-1} BAP + 1.0 mg l^{-1} IBA	Shoot tips	Gnanaraj et al. 2012
	MS + 2.0 mg l^{-1} BAP + 1.0 mg l^{-1} IAA	Shoot tips	Parveen et al. 2008
	MS + BAP 2.0 mg l^{-1} + 0.5 mg l^{-1} NAA/ 3.0 mg l^{-1}IBA	Callus	Sen et al. 2014
Lamiaceae			
Ajuga bracteosa wall ex Benth			
	MS + 5.0 mg l^{-1} BAP + 2.0 mg l^{-1} IAA/0.5 mg l^{-1} IBA	Callus	Kaul et al. 2013
A. multiflora Bunge			
	MS only	Hairy roots	Kim et al. 2005
A. turkestanica (regel) Briq.			
	B_5 + 2.3 μM 2,4-D	Cell suspension	Cheng et al. 2008
	MS + 2.2 μM BAP + 2.5 μM IBA	Cell suspension	Cheng et al. 2008
Caryophyllaceae			
Lychnis flos-cuculi L.			
	MS + 100% BAP + 100% IAA/NAA	Shoot tips	Thiem et al. 2013; Maliński et al. 2014
Commelinaceae			
Cyanotis arachnoidea C.B. Clarke.			
	MS + 3 mg l^{-1} BAP + 0.2 mg l^{-1} NAA	Cell suspension	Wang et al. 2014
Asteraceae			
Rhaponticum carthamoides (Willd.) Iljin.			
	SH only	Hairy roots	Skała et al. 2015
	MS + 0.25 mg l^{-1} 2,4-D + 1.5 mg l^{-1} BAP	Callus	Zand et al. 2014
Lamiaceae			
Vitex glabrata R.Br.			
	B_5; 2.0 mg l^{-1} BA + 1.0 mg l^{-1} 2,4-D	Cell suspension	Sinlaparaya et al. 2007; Thanonkeo et al. 2011
	50:100% MS; 2.0 mg l^{-1} BA + 1.0 mg l^{-1} 2,4-D	Cell suspension	Chamnipa et al. 2012

(continued)

Table 11.1 (continued)

Species	Treatment	Method	Reference
Vitex negundo L.			
	MS + BAP 5.0 lM + 0.5 lM MS? IBA 10 lM	Shoot tips	Ahmad and Anis 2011
Amaranthaceae			
Pfaffia glomerate (Spreng.) Pedersen			
	MS + BAP 2.22 lM + NAA 2.68 lM + 0.1 lM glucose	Shoot tips	Vasconcelos et al. 2014
	MS only	Shoot tips	Flores et al. 2010
P. tuberosa (Spreng.) Hicken			
	MS + TDZ 1.0 lM (10.3 shoots)	Nodal segments	Flores et al. 2010

2,4-D 2,4-dichlorophenoxyacetic acid, *B5* Gamborg's medium, *BAP* 6-benzyloaminopurine, *IAA* indole-3-acetic acid, *IBA* indole-3-butyric acid, *MS* Murashige and Skoog medium, *NAA* a-naphthalene acetic acid

potential for biotechnological strategies of PTCs has not been examined due to their limitations (Filova 2014). Some biometabolites produced on scale-up production despite the PTCs have been highlighted in the first half of the twentieth century (Verpoorte et al. 2002; Karuppusamy 2009; Filova 2014).

11.4 Scale-up Techniques and Bioreactors

A bioreactor-based plant cell suspension process has the economic-potential advantages for large-scale production in plant pharmaceutical industry (Isah et al. 2018; Xu and Zhang 2014).

Bioreactor-based and scaling-up of desired SMs production is the final stage for the in vitro biosynthesis strategy. The plant in vitro system has the potential to provide a low-cost production of various plant-derived products. However, the scale-up production across bioreactors may take on many biologic obstacles, including low metabolite yield, cell size, heterogeneity, and genetic instability. Also, cell aggregation, shear stress sensitivity, aeration, and intensive mixing, besides adhesion of the cells and foaming, are considered the other technological obstacles of the bioreactors system. The bioreactor design and cultivation parameters must be optimized by maintaining temperature, light irradiation, adequate agitation, and ensuring gas exchange and homogeneity to enhance the biosynthetic capacity of secondary metabolites derived by plant cells (Georgiev 2020). There are many difficulties in obtaining plant steroid compounds since the amount of these compounds in raw materials is low; their chemical synthetics are not economically gainful for commercial investment. Therefore, industrial biotechnology is an alternative strategy for cost-effective large-scale production by using microbial (Shao et al. 2015; Kreit 2017; Mancilla et al. 2018) or plant cell cultures (Miras-Moreno et al. 2016). There are earlier successful attempts to produce well-known steroidal

bioactive compounds in relatively large quantities by cell suspension systems (Kaul and Staba 1968; Kaul et al. 1969; Khanna et al. 1975; Marshall and Staba 1976). Kaul et al. (1969) have reported that diosgenin, a prime raw material in the commercial production of corticosteroids and steroid contraceptives, can be produced by suspension cultures of *Dioscorea* with a 1.5% dry weight content (Kaul et al. 1969). Kreis and Reinhard (1990) succeeded in the production of deacetyllanatoside C in 20-liter airlift bioreactors from two-stage cultivation of *Digitalis lanata* cells (Kreis and Reinhard 1990).

Monitoring the physiological case of cells is an important task. When scaling apparatus cultivation of cell cultures to industrial volumes according to this consideration, it reported that bubble bioreactors (20 and 630 L) with the annular aerator (K La 7.1–8.0 h − 1) showed higher biosynthetic rates (7.7–13.9%) of dry cell weight of steroidal glycosides production from *Dioscorea deltoidea* cell cultures (Titova et al. 2015). The biotic elicitors, *E. coli* (1.5%), proved best with a 9.1-fold increase in diosgenin content of *Helicteres isora*. The scaling-up across the suspension culture was performed for the production of diosgenin. It is worth mentioning that, in the scaling-up stages, the diosgenin yield obtained was in the range between 7.91 and 8.64 mg /l, where diosgenin content increased as the volume of the medium increased. The quantitative real-time PCR (qRT-PCR) analysis exhibited that biotic elicitors increased the expression levels of regulatory genes in the diosgenin biosynthetic pathway, which correlate with elicited diosgenin (Shaikh et al. 2020). Three configurations of bioreactors are optimized for extraction conditions of the steroidal glycosides of *Dioscorea deltoidea* wall cell suspension culture for LC-MS determination (Sarvin et al. 2018). Also, withanolides production during cell/organ culture, as important medicinal steroidal derivatives compounds have been cultured through bioreactors system. Sivanandhan et al. (2014) cultured *W. somnifera* on a medium consisting of 1 mg/l picloram, 0.5 mg/l kN, 200 mg/l glutamine, and 5% sucrose along with chitosan (100 mg/l) and squalene (6 mM) for 4–48 h exposure time, respectively. Withanolides compounds detected, after 28 days of culture, 7606.75 mg of withanolide-A, 4826.05 mg of withanolide B, 3732.81 mg of withaferin-A, 6538.65 mg of withanone, 3176.63 mg of 12-deoxy withanstramonolide, 2623.21 mg of withanoside IV, and 2861.18 mg of withanoside V (Sivanandhan et al. 2014). Ahlawat et al. (2017) elicited the cell suspension culture of Withania somnifera with cell homogenate of *P. indica* fungal elicitor at 3.0% (v/v)/7 days of exposure time to check the gene expression of withanolides biosynthesis pathways via quantitative PCR. Withanolides production was analyzed as withanolide A, withaferin A, and withanone. The bioreactor system enhanced the biomass to (1.13 folds), which is the upregulation of withanolide genes of biosynthetic pathways (Ahlawat et al. 2017). Steroids biosynthesis during date palm cell suspensions induced by adding pyruvic acid as a precursor and the bioreactor system scale-up their production 14 times. The date palm steroids could commercially be produced by using bioreactor systems (El-Sharabasy 2004; El-Sharabasy and El-Dawayati 2017). The evidence that the cell biomass grew by bioreactors enhances the production of steroid compounds and encourages the pharmaceutical industry of steroid derivatives. The cell culture is considered a promised goal for the advancement of innovative biotechnologies.

11.5 Extraction and Detection Techniques

Several detectors are now available to run through different apparatuses for isolating metabolites, including anion and cation exchange, ligand exchange, reverse phase, and size exclusion. Water is not often the best for extraction of lipophilic compounds (Colegate and Molyneux 2008). However, most extraction methods preferably used MeOH or ethanol after powdering the biomass using liquid nitrogen (LN). Treatment with LN, and then storing at low temperatures, should be directly done after the collection to lessen or inhibit the possibility of enzyme-reaction of the interest; Fig. 11.3 illustrates the general steps to the isolation of steroids.

Modern technologies play a significant role in the different bioactive progress of natural products. Several techniques have separated the mixtures of natural products (Colegate and Molyneux 2008). The extracts are very complex compounds in most cases and have mixtures of neutral, acidic, basic, lipophilic, hydrophilic, or amphiphilic compounds, and consequently, there will not be one method that uses all eventualities. The characterization of bioproducts necessitates sophisticated methodologies. These methodologies provide the best selective, sensitive, and structural information and determination on the interested product (Hostettmann and Wolfender 2004; Colegate and Molyneux 2008; Shakhmurova et al. 2012; Vanyolos et al. 2012).

High-throughput screening programs are in crucial need to be more sensitive methodologies. High-performance liquid chromatography (HPLC) is utilized habitually in preparative isolation and purification of natural products (Hostettmann et al.

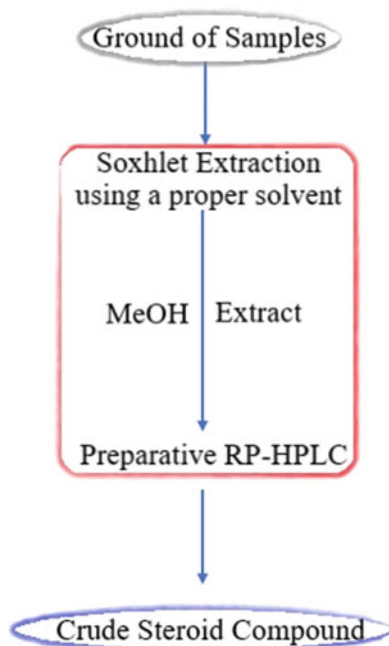

Fig. 11.3 Illustration of general step for isolation of steroids

1998). This detector developed new powerful tools including LC/mass spectrometry (LC/MS; Niessen and Tinke 1995; Niessen 1999), LC/nuclear magnetic resonance (LC/NMR; Albert 1995; Sudmeier et al. 1996; Lindon et al. 1997; Wolfender et al. 1998a; Spraul et al. 1993), and LC/UV-photodiode array detection (LC/UV-DAD; Huber and George 1993). Combination with these detectors has achieved complementary spectroscopic data on an LC-peak of a target, which has often permitted their unambiguous structure determination. The LC-detectors integrated swiftly for the scan of crude extracts (Wolfender et al. 1998b). Other techniques, including IR or X-ray crystallography, have been utilized less when the other spectroscopic methods failed to give complete structure determination. The combination of different LC-techniques to determine metabolites in an extract ought to empower the full structure description at the corresponding LC-peak. The inherent sensitivity of used spectroscopic techniques is the core-one of the identification problems for LC (Hostettmann and Wolfender 2004). The LC/NMR allows structural data more than LC/MS, but it has much order of the magnitudes with minimal sensitivity. Several limitations have elucidated the use of potential LC-techniques.

LC/UV-DAD (Huber and George 1993) was utilized, in several labs, detecting metabolites (Bramley 1992; Yoshimura et al. 1994). LC/UV-DAD gives complementary information for different metabolites owing to strong chromophores. The selection of the proper LC-solvents should perform through their inherent UV limit to avoid interfering. Nonetheless, the only limitation of its application in plant extract analysis is that. New apparatuses allow the record of UV-spectral libraries, and automatic computer search can be achieved provided that UV metabolite-databases have built up. The UV-spectra measurements depend on the structure of solvent systems used, and matching with spectra measured in other solvents shows differences.

Mass spectrometry is right now one of the most sensitive molecular analyses. Moreover, it has probability to build up data on the molecular weight and structure of the test. Numerous LC/MS has been utilized and are available nowadays. These LC/MS crossing-points necessarily achieve nebulization and vaporization of a liquid, ionization of sample, removal of the excess solvent vapor, and extraction of the ions into the mass analyzer. Nowadays, the most commonly used LC/MS interfaces including electrospray (ES; Whitehouse et al. 1985), atmospheric pressure chemical ionization (APCI; Bruins et al. 1987), thermospray (TSP; Blakley and Vestal 1983), and continuous flow fast atom bombardment (CFFAB; Caprioli 1990) are very efficient for metabolites ionization (Wolfender et al. 1995).

Several analyzers, including the time of flight (TOF), ion traps (IT), and quadrupole (Q), could be utilized, which have, in terms, resolution, mass accuracy, and MS/MS possibilities (Mosi and Eigendorf 1998). The prime problems of LC/MS are the response for buffer and solvent used, nature of constituents, flow rate, and type of interfaces. Natural extracts have various physicochemical properties, which make it difficult to find optimum LC/MS conditions (Wolfender et al. 1994). Nevertheless, LC/MS/MS are extra reproducible, and the use of MS/MS databases of natural products can be considered for dereplication. Therefore, the LC/MS configuration can enormously be an influential system for screening crude extracts, but the precise

Table 11.2 Solvent used for steroid using TLC

Solvent	References
Benzene/benzene: ehyl acetate	Hunyadi et al. 2007); Nowak et al. 2013
Chloroform: ethanol	Lafont et al. 2000
Ethyl acetate: cyclohexane	Hunyadi et al. 2007); Nowak et al. 2013
Methanol: water	Lafont et al. 2000
Cyclohexane: heptane	Hunyadi et al. 2007); Nowak et al. 2013
Cyclohexane: ethyl acetate	Hunyadi et al. 2007); Nowak et al. 2013
Benzene: Chloroform	Hunyadi et al. 2007); Nowak et al. 2013
Chloroform: methanol: water	Cheng et al. 2008
Ethanol: water	Lafont et al. 2000
Acetonitrile: water	Lafont et al. 2000

conditions of ionization will have to be sensibly optimized. With the LC/MS appropriate configuration, the analysis of nonpolar to polar constituents, including the possibility of proteins will mostly determine the optimal ionization method and polarity used (Niessen 1999).

NMR provides the most useful evidence for the identification of natural products (Albert 1995). The combination of NMR and HPLC gives a piece of important information for direct metabolite identification. The rapid progress, including pulse field gradients and probe technology, has been given an impulse to LC/NMR (Spraul et al. 1993). Difficulty in detecting resonances existing in large resonances of the mobile phase is the prime problem in LC/NMR (Spraul et al. 1993; Albert 1995). Water suppression enhanced through T1 (WET) produces high-quality spectra in the procedure of on-flow and stop-flow (Albert 1995; Smallcombe et al. 1995). On-flow LC/NMR has been limited directly to the direct measurement of interest constituents and frequently loaded LC-conditions. One way to improve the exposure limit is to work in the stop-flow mode, which requires the retention times of interest or a sensitive method of the scan including LC/NMR, LC/UV, and LC/MS to activate the scan (Holt et al. 1998).

Thin-layer chromatography (TLC) is a widely used method for the isolation of natural and synthetic products. Norm- and reversed-phase TLC on silica plates with a solvent system (Table 11.2), such as chloroform: methanol: water (Cheng et al. 2008), chloroform: ethanol, methanol: water, ethanol: water, acetonitrile: water (Lafont et al. 2000) and others (Hunyadi et al. 2007); Nowak et al. 2013), has been described being an efficient way to separate steroids. The TLC plates are observed by UV lamps (254 nm) and performed visualization by nonspecific color reactions with anisaldehyde spray reagent (Cheng et al. 2008; Nowak et al. 2013), vanillin/sulfuric acid spray reagent, sulfuric (VI) acid or "specific" reactions with ammonium carbonate (fluorescence induction), 2,4-dinitrophenylhydrazine, triphenyltetrazolium chloride, and Folin-Ciocialteu reagent (color reactions) (Dinan 2001).

The TLC with mass spectrometry (TLC/MS; TLC/MS/MS) is a potential technique for identifying steroids from the crude plant extracts (Wilson et al. 1990).

Likewise, many TLC can permit more accurate quantitative measurements such as automatic multiple developments of TLC (AMDTLC), over-pressurized TLC (OPTLC), and high-performance TLC (HPTLC) (Read et al. 1990; Wilson et al. 1990; Lafont et al. 2000).

11.6 Biological Activities (Major)

Steroids play a prominent part in plant development and growth. Many steroids are isolated from plants, and the literature concerning this matter has been surveyed (Sultan and Rauf Raza 2015). Characteristics of the plant sterol biosynthesis and their role in cells are reviewed. Steroids play prime roles in several biochemical and physiological properties in all living organisms in which these are found (Abd El-Bahaman 1991; Sultan and Rauf Raza 2015). A broad sense of synthetic steroids is used as antihormones (Jovanović-Šanta et al. 2015), contraceptive drugs (Lopez et al. 2014), anticancer agents (Thao et al. 2015), cardiovascular agents (Rattanasopa et al. 2015), osteoporosis drugs (Emmanuel et al. 2011), antibiotics, anesthetics, anti-inflammatories, antidiabetic (Graf et al. 2014), and antiasthmatics (Aav et al. 2005).

Steroids in plants are of two main groups: brassinosteroids and phytosterols. Brassinosteroids, which are applied in several plants, tend to respond to biotic and abiotic stresses (Xia et al. 2009). Brassinosteroids show a broad sense of biological responses such as the inhibition of wilt, stimulating cell division, suppression of stress-regulated genes, and light-regulated genes in the darkness (Schwarz et al. 2003). Brassinosteroids have shown remarkable benefits for various responses through application in several plants. Previous reports pointed to an increase in metabolism and the elimination of pesticides. Therefore, it lessens the human ingestion of residual pesticides (Sondhi et al. 2008). The 24-epibranssinolide (EBL), isolated from *Aegle marmelos* Correa (family: Rutaceae), has pointedly diminished the maleic hydrazide-induced genotoxicity in chromosomal aberrations of *Allium cepa* (Howell and Buzdar 2005) and protected neuronal PC12 cells from 1-methyl-4-phenylpyridinium-(MPP+-)-induced oxidative stress and apoptosis in dopaminergic neurons (Julie et al. 2011). Analogs of branssinosteroid plant growth regulators such as 5α-hydroxy-6-ketopregnanes have been surveyed (Biggadike and Morton 2003). The 17-substituted pregnadienes have been made (Mellon et al. 2004) as potential inhibitors of testosterone-5α-reductase. An unusual Δ20-pregnene got from an octacoral has been inhibited by the mitochondrial respiratory chain (Meggers 2007).

Plant sterols are called phytosterols, which are a cluster of steroid alcohol that biosynthesize in plants. Phytosterols, which are white powders with mild and odor, solubilize in alcohol. Phytosterols are used in medicine, cosmetics, and food additive and fight cancer (Delvin 2002). Plant sterols sell as a dietary supplement (Delvin 2002). Ergosterol (Fig. 11.4) lowers the cholesterol level in human up to 15% (Banthorpe 1994; Vieira et al. 2005). Dietary guidelines for health call for lessening the cholesterol intake. For doing that, one strategy takes in the plant sterols, e.g., sitosterol, stigmasterol, stigmastanol, and campesterol. Despite their structural

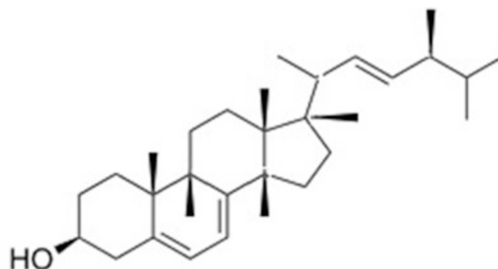

Fig. 11.4 Structure of ergosterol

similarity to cholesterol, minor isomeric modifications in the present/absence of methyl/ethyl groups in the side chains cause their less absorption by intestinal mucosal cells. Plant sterols block the absorption of cholesterol very high-efficient through intestinal cells despite not their absorbed by the body. Plant sterol drugs progressed to subject to the structures of sterols and the form of administered factor. For example, sitosterol is unsaturated and absorbed poorly in the human intestine, while sitostanol is a saturated analog and practically fully unabsorbable. The evidence of the plant sterols running in a soluble, micellar procedure effect in the inhibition of cholesterol absorption than those in a solid-crystalline form (Sharma et al. 2008).

Some miscellaneous steroids have the same vital roles. Derivatives of androstanes are separated for the treatment and inhibition of allergy (Sanjiv et al. 2010); others are effective against cardiovascular disease, cerebral-degenerative disorder, cartilage degeneration, periodontal disease, osteoporosis, metastatic bone disease, Paget's disease, endometriosis, uterine fibroid disease, restenosis, vascular smooth muscle cell proliferation, obesity, inflammatory bowel disease, hypertension, retinal degeneration, and cancer especially against breast cancer, uterus, and prostate (Chermnykh et al. 1988). The treatment by steroid multiple-course can increasingly and effectively get a better chance against preterm and Respiratory Distress Syndrome (RDS) babies and help the fragile babies to survive (Chohan et al. 2006). Antenatal steroids lessen the chances of hyaline membrane disease and intraventricular hemorrhage and chronic lung disease or death in very low-birth-weight infants (Singh et al. 2006). Steroids, e.g., stigmasterol, show antimutagenic, anxiolytic, analgesic, anticonvulsant, sedative, hypnotic, and anesthetic properties (Paschke et al. 2003; Chohan et al. 2004). By enhancing GABA receptor function in a non-genomic manner, some steroids produce an opponent for treatment of CNS abnormalities such as stress, PMS seizures, the anxiety produced by epilepsy and block muscle tension, depression, and induce anesthesia (Hollman 1996).

Cardiac glycosides (Fig. 11.5) are classified into cardenolide and bufadienolides (Laurie et al. 2012). Those have high potent cardiotoxins. Cardiac glycosides are used in the treatment of cardiac arrhythmia and congestive heart failure. Also, those have anticancer properties (Laurie et al. 2012).

Digoxin isolated from *Digitalis lanata*, ouabain (g-strophanthin) isolated from ripe seeds of *Strophanthus gratus,* and the bark of *Acokanthera ouabaio* are cardenolides. Cardenolides have a vital role in the treatment of various heart

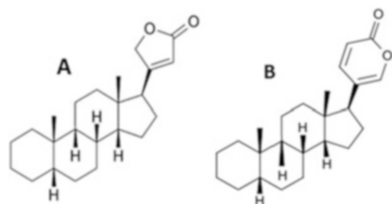

Fig. 11.5 Structures of cardiac glycosides: (**a**) cardenolide and (**b**) bufadienolide

conditions and various health risks such as increased blood pressure (BP) and tissue perfusion, anorexia, nausea, hallucinations, disorientation, insomnia, impaired yellow color perception, and gynecomastia in males (Gao et al. 2002; Pierog et al. 2009). In this trend, ouabains (g-strophanthin) are used by Somali tribesmen to poison hunting arrows due to their toxic effect, thus blocking the Na + pump in a fashion similar to one of steroidal alkaloid batrachotoxins isolated from poison arrow frogs (Wang and Sun 1994; Schneider et al. 1998).

Bufodienodiles, e.g., cinobufagin isolated from Chusan island toad (*Bufo gargarizans*) and Bufagins (a constituent of bufotoxins) from the secretion of the Cane toad (*Bufo marinus*)—so-called Toad's milk, have been utilized. Treatment with low doses of cinobufagin used traditional Chinese medicines against atrial fibrillation (Cardarelli and Kanakkanatt 1983). Bufagins have a characteristic of cardiotoxins. Bufagins derivatives have anesthetic action, anticancer properties against leukemia, melanoma, and prostate cancer cells (Jiun et al. 2002; Bick et al. 2002) nuclei.

Steroidal alkaloids, e.g., a perhydro-1,2-cyclopentanophenanthrene nucleus, are biosynthesized in the various plants as a glycosidic combination with carbohydrate moieties. Dihydroplakinamine K from a marine sponge (*Corocium niger*) is used for cytotoxic activity (Enev et al. 1998). Batrachotoxins isolated from *Phyllobates* spp., *Pitohui* spp., and *Iflita* spp. are very efficient neurotoxins. Samandarin isolated from *Salamandra salamandra* causes muscle convulsions, high blood pressure, and hyperventilation in vertebrates (Philomin et al. 1993).

Some steroidal derivatives have been used as fluorescent detectors for polycyclic aromatic hydrocarbon (PAH), like that results from cholic acid, which has a tweezer-like structure (Simon et al. 1999). The cholesteryl benzoate forms cholesteric liquid crystals with helical structures. Cholesteryl benzoate is used in thermochromic liquid crystals, hair colors, and some cosmetic preparations (Sevillano et al. 2002).

11.7 Commercial Utilization and Prospects

Steroids could be employed in several disciplines, e.g., multidrug resistance. The problematic obstacle of steroid application is the lipophilic compounds acting as inhibitors of vital bioactive macroconstituents (Martins et al. 2013). Several reports (Martins et al. 2013; Sultan and Rauf Raza 2015; Thiem et al. 2017) on multidrug resistance reported that dioxolane may lead reasonably to the invention of an effective MDR inhibitor (Martins et al. 2013). Antioxidant, free radical scavenging,

and effects of 20-hydroxyecdysone neuroprotective were evidenced in in vitro oxidative damage and in vivo ischemic injury models. 20-hydroxyecdysone showed a protective effect in the adrenal gland and pheochromocytoma of rats against cobalt chloride-induced cell damage (Hu et al. 2010).

The effect of 20E on NF-κB and JNK signaling pathways and inhibition of the caspase-3 activity responsible for apoptosis reported that all destructive intracellular characteristics, e.g., the disruption of the mitochondrial membrane and inducing calcium level, have decreased via 20-hydroxyecdysone (Hu et al. 2010, 2012).

11.8 Conclusions and Recommendations

Despite the progression in enhancing bioactive metabolites production, the automation of production is still restricted. The most natural products biosynthesize in small quantities and disordered tissues. Economic production is the central bottleneck. Only a small number of plant kingdom around the globe has been economically and practically submitted to the various screening of bioactivities. There is currently a renaissance of interest as steroid-rich sources for introduction into different bioactivities programs. The rapid rate of loss of plant species has meant that it needs to report new methods leading to the rapid isolation and identification of bioproducts. The approach assumed to acquire utilizable pure constituent is interdisciplinary in botany, pharmacognosy, pharmacology, chemistry, toxicology, conservation biology, and genetics. The risk of pointless isolation with known activity is by following the guide of fractional processes. Besides, it needs to accompany constituent isolation of interest with its activities. Avoiding the time length in extraction, the automation of hyphenated techniques is used at the earliest stage of extraction. It is valuable to detect bioproducts with interest structures and to aim at their segregation.

Many studies on steroids reported raising interest in having beneficially interdisciplinary activities. The prime problems with steroids' commercial utilization are that the extraction processed hardly with a lower yield and is mostly based on numerous environmental factors. So, PTCs are a valuable alternative approach because plant biomass proliferated under the control of the whole conditions. The automation of production procedures may be improved using a broad sense of recent biotechnological methods. Progression in biotechnological tools may affect the advancement of other disciplines, including biochemistry, botany, conservation biology, and toxicology. The approach of genetic markers is a very beneficial tool that provides more insight into pathways. Till now, mini reviews of genetic markers are carried out. It should combine genetic markers with other methods. Besides, more research using genetic markers must investigate more into the pathway systems of steroids.

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