Review Article

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Synthesis and extraction routes of allelochemicals from plants and microbes: A review

Abstract: Allelopathy, a complex phenomenon has unveiled both stimulatory and inhibitory effects in plant processes that are mediated by the release of certain chemical compounds commonly known as allelochemicals. Allelochemicals, a form of bioactive secondary metabolites are produced by a diverse group of plants and microbes in response to biotic and abiotic stress. It ranges from a simple hydrocarbon to complex polycyclic aromatic compounds like phenol, flavonoids, tannins, steroids, amino acids, alkaloids and quinones. These plant bioactive compounds are released into the environment via decomposition, exudation, leaching and volatilization that play a significant role in regulating the intra-specific or inter-specific relations with counterparts. A wide variety of methods has been proposed for analyzing the basic mechanism and overall effect of allelochemicals. However, the lack of a reliable and effective method to identify their molecular mode of action and their modulation in the metabolic pathway still remains as a great challenge. From a commercial perspective, these allelochemicals are deemed to be better candidates for green natural herbicides and weedicides that are proven to be environment friendly, unlike synthetic chemicals. In order to pave a way for the economic viability of these chemicals, a basic understanding of their chemistry is inevitable. This review article is focused to give an in-depth understanding of metabolic pathways genes responsible for the elicitation/secretion and the adoption of a suitable downstream process and analytical techniques that can intensify the process.

Keywords: allelochemicals, allelopathy, secondary metabolites, defense mechanism, extraction, downstream processing

1 Introduction

Secondary metabolites are produced by plants, animals and microbes that do not have a direct role toward the primary functions such as growth and metabolism. These chemicals vary from one organism to another and in fact differ between the species of the same genus. This metabolite profiling has helped the taxonomists to classify the subgroups among the same genera. Even though many functional roles of secondary metabolites have been elucidated, allelopathy is a notable characteristic that aids in several ways for both plants and microbes to overcome biotic and abiotic stress [1]. Abiotic stress factors include extreme temperature changes, drought, flood, excess or deficit of radiations like infrared, ionization, etc. It also includes wounding, external pressure, wind and magnetic fields. However, biotic stresses are influenced by microbes, animals and other plants. These stresses lead to signal perception and transduction, to elicit metabolic responses and gene expressions [2]. The exposure of biotic stress on plants potentially creates higher allelopathic activity further increasing the concentration of the allelochemicals produced. It is also stated that allelopathic on a geographic region can appear and disappear according to the environmental conditions and thus the production of these allelochemicals is influenced by the stress created on these plants [3]. An increase in the overall allelopathic potential was observed in a *Quercus rubra* L. and *Acer rubrum* L., as a result of high temperature and drought eventually leading to an increased production of tannins [4]. Pedro et al. [5] also reported that the synthesis of

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allelochemicals varies on the type and magnitude of the stress caused over the plants. Allelopathy is described as a positive or negative effect exerted due to any biochemical constituents produced by either plants or microbes that affect the same or other species (plant–plant; plant–microbes and microbes–microbes). Most of the allelochemicals are derived from either acetate or amino acids participating in the shikimic acid pathway [6]. The effect of allelochemicals is a concentration-dependent phenomenon and introduced into the environment together with a vast number of other secondary metabolites. The kinds of effects include interruptions in growth, reproduction, germination and distribution, etc. [1]. For example, ethylene exudation from the root of *Striga lutea* Lour., has shown an inhibitory effect on weed germination thereby, acting as a natural biocontrol agent [6,7]. A few bacterial species are capable of producing the allelochemicals that in turn affect the plant growth. Rhizobacteria is one such root colonizing bacteria that act as a plant growth-promoting microbe by establishing a symbiotic behavior through chemotaxis [8]. On the other hand, the allelochemical producing bacteria compete with the plants for some of the macro- and micronutrients. The plant allelochemicals are excreted from different parts such as roots, flowers, seeds, stems, leaves and pollen grains [7]. These low molecular weight allelochemicals have a high medicinal and agricultural value that could be used as growth regulators, herbicides, insecticides and anti-microbial crop-protective agents. On a commercial scale, the allelochemicals were extracted from the plants by leaching, volatilization, exudation and decomposition of plant residues [9].

Conventionally, the extraction of metabolites is carried out through solid–liquid extraction process followed by distillation and refining. The major compounds obtained from this type of extraction include phenolic acids, quinones, flavones, flavonoids, tannins and coumarins which are documented with allelopathic activities (anti-microbial and anti-oxidative) [7,8]. Most of these allelopathic compounds exhibited a positive effect (stimulation/germination) only at a lower concentration and beyond certain critical limits they could induce toxicity. For the solubilization of bioactive compounds, different types of liquid or gaseous solvents (one or more) were used depending on the nature of the compound [9]. In due course of time, with the advancement in metabolic engineering, some of these plant/microbe-derived compounds are explored as better candidates for industrial production of medicines, flavorings, anti-oxidants, plant growth promoting (PGP) and pesticides [7]. However, the bioprocess should be designed in such a way to obtain low-volume high-value-added products by adopting a suitable strategy for downstream processing (i.e., extraction and purification) [10]. This article is mainly focused on the chemical nature of different allelopathic compounds, metabolic pathways along with the genes responsible for the elicitation/secretion and their suitable extraction methodologies that could pave the economically and technically viable routes for industrial commercialization.

2 Allelopathic compounds: Characteristics and routes of release

2.1 Characteristics

Plants synthesize and release the secondary metabolites via different routes that exhibit the allelopathic effect. The strength or the intensity of these chemicals would differ among the various parts of a single plant [11]. It is known to be active in both natural and manipulated habitats and plays a significant role in the evolution of crop populations and replanting failures [12]. The plant biomass is crushed to a powder form followed by the extraction using solvents such as ethanol, methanol, acetone, ether and chloroform in order to obtain a concoction of chemicals that may comprise the allelopathic compounds [13]. The main purpose of an extraction technique is to maximize the recovery of metabolites derived from the plant matrix while retaining the integrity of the target molecules along with the reduction of some undesired compounds [14,15]. The microbial community in the soil is complex and primarily depends on the type of vegetation and the organic content of the soil. These microbes are either directly involved in the allelochemical production or indirectly act by the transformation of parent chemical substances released from the plant that exhibit the allelopathic effect [16]. It should be noted that the plant chemicals hardly show any effect until it undergoes the chemical modification by means of the microbial action. Figure 1 represents the mode of release of allelopathic compounds, effect on the neighboring plant and its interaction with soil microorganisms.

The negative effect of allelopathy is mainly concerned with the seed inefficiency or the plant growth reduction by disrupting the normal functionalities such as pollen germination, photosynthesis, cell division, nutrient uptake, ion imbalance and vital enzyme functions [17]. This makes the allelochemicals a better alternative for synthetic herbicides and weedicides.
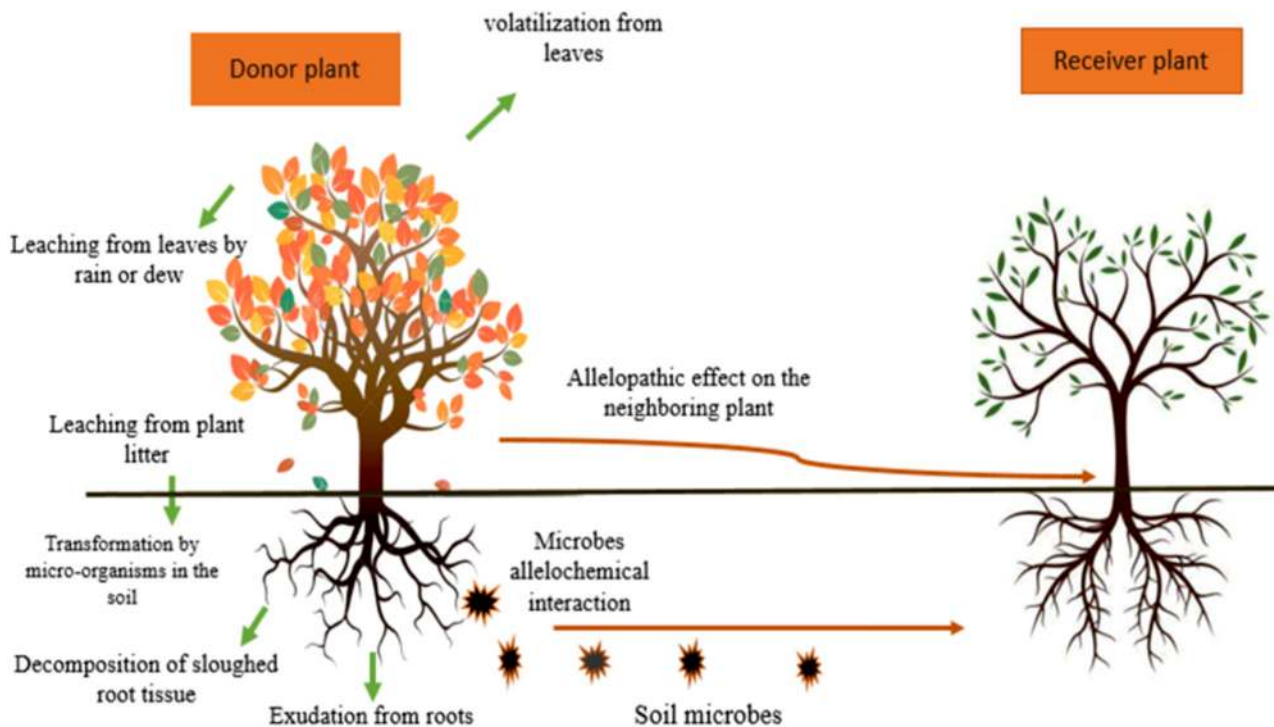
Figure 1: Routes for the release of allelochemicals, elicitation by microbes, and its effect on other plants.

For example, some of the major compounds like phenols, terpenoids, steroids, amino acids, flavonoids, alkaloids and carbohydrates can act either as a sole candidate or a mixture to exert significant allelopathic efficacy. Furthermore, additional factors such as physiological biotic and abiotic stresses, sufficient solar radiation, optimal nutrient, temperature and moisture levels could also affect allelopathic weed destruction [18,19].

2.2 Route of allelochemicals released in plants

A wide range of plant-derived allelochemicals is responsible for the various type of interactions such as plant–plant, microbe–microbe and plants–microbes. The bound form of the bioactive compounds is present in the leaves, stems and roots which do not pose any harmful effects on the producer plant [20]. It has been reported that almost all the plant species would produce the allelochemicals in the form of secondary metabolites; however, only a few have shown the allelopathic effects [21]. The route of the allelochemicals released in the producer plant is based on the following four ways: decomposition, exudation, leaching and volatilization.

2.2.1 Decomposition

The plant litters found on the ground upon decomposition by the microbial action could release allelochemicals. The microorganisms either stimulate or neutralize/deactivate the allelopathic compounds depending on the type of interaction and further transformation of the parent compound. At times, the microbial stimulation could happen based on the availability of suitable physiological conditions, especially the moisture content. Therefore, decomposition is considered an indirect way of allelopathy [22]. It has been reported that the application of alfalfa pellet in the paddy field has caused the destruction of weeds without any alteration in the crop growth parameters. This indicates that the allelochemical effect from a plant is independent of the producer plant viability [23]. Chemical constituents such as *p*-hydroxybenzoic acid, saponins, salicylic acid, syringic acid, gallic acid, vanillin, vanillic acid, *p*-coumaric acid, catechin, protocatechuic acid and ferulic acid were identified as weed growth inhibitors which are released by the alfalfa pellets [3].

2.2.2 Exudation

Generally, in plants, roots have the characteristic feature of secreting chemicals that are mostly carbon-based

compounds, around the rhizosphere in response to physiological stress [24]. The most important functions of these exudates include nutrient uptake and cellular respiration. With regard to nutrient uptake, organic ligands play a significant role in the metal chelation that is termed as "phytometallophores" [7] and are widely found in Poaceae grasses, which allow the uptake of Fe^{2+} from calcareous soils. Such a type of allelopathy is a positive effect that does not reduce or harm other plants [25].

2.2.3 Leaching

Rain causes the leaching of chemicals from different parts of the plant that either fall on the ground or on other adjacent plants. Although in principle, it is considered as an indirect method it depends on the fate of the leached water deposition. The leachate from *Leucaena leucocephala* (Lam.) contained a significant concentration of phenolic compounds (>1 mM) and a non-protein amino acid, named mimosine. These compounds increased the membrane permeability in the leaf tissue of water hyacinth (*Hyacinthus orientalis* L.), thereby destabilizing the plasma membrane and are responsible for the cell death [26]. Thus, it could act as an efficient bio-weedicide to eradicate the aquatic weeds present in the water bodies in a safe and sustainable manner [27].

2.2.4 Volatilization

Commonly, the plants found in temperate and warm climates use this direct volatilization route for the release of allelochemicals [17]. Here, the chemical substances are volatilized through the stomata that are then solubilized in the water that falls on the ground to exert its effect on other plants or taken up as a volatile compound by the other plant's stomata. As an indirect route, dead plant litters and the residues might release the volatile organic compounds (VOCs) [28]. In a controlled experiment, VOCs from *Adenophora* litter have caused higher mortality of the species that are native to India and China, thereby establishing the fact that the allelopathic effect depends on the geographical origin of the receptive plants. On the other hand, the effects of *Adenophora* VOCs on seedling germination and growth do not differ between native and non-native species. Litters from the non-native *Adenophora* plants also possess the ability to produce VOCs that are quantitatively different in terms of concentrations of some chemicals as compared to the native populations, but there are no chemicals that are unique to one region [29]. The specific role of plant-derived allelopathic compounds is highly dependent on the basic chemical characteristics that could alter the metabolism of the counterpart. The subsequent section deals with the basic chemistry behind the allelopathic compounds and their metabolic pathway that enables a better understanding of their mode of action and its modulation. Figure 2 shows the role of VOCs in various plant signal transduction applications.

3 Basic chemistry of allelochemicals

3.1 In plants

The natural plant-derived products that are exploited for commercial application can be classified into two groups, namely primary and secondary metabolites. Primary metabolites comprise standard proteins, amino acids, sugars, nucleic acids and chlorophyll [16]. The secondary metabolites include alkaloids, flavonoids, cyanogenic glycosides, terpenoids, tannins, waxes and resins that induce allelopathic effects on other plants as part of their defense mechanism. The allelochemicals can be categorized into the following types based on the structural similarity: low molecular weight water-soluble organic acids, linear chain aldehydes, ketones and alcohols; lactones; fatty acids (long-chain); the polymeric form of acetylenes; cinnamic acid its derivatives; quinone derivatives (anthraquinone/benzoquinone); simple phenols; benzoic acid; tannins; coumarin; flavonoids, steroids and terpenoids; non-protein amino acids and oligopeptides; alkaloids and cyanohydrins; sulfide and glycosylates; and purines and nucleosides. On the other hand, allelopathic compounds with positive effects include some plant growth regulators (PGRs) such as salicylic acid, ethylene and gibberellic acid. In plants, the Shikimate pathway is considered as a prime locus for the synthesis of important allelochemicals and their derivatives as depicted in Figure 3. Recent advancements in analytical techniques such as GC-MS/MS, NMR, MALDI-TOF and radioisotope labeling have paved the way for the identification of novel allelochemicals from plants. There are many other unexplored plant-derived chemicals that could play a significant role as biocontrol agents.

Phenolic compounds contain hydroxyl groups attached to an aromatic ring. These groups of compounds include sweet-smelling phenol, flavonoids, tannins and quinones [17]. Figure 4 depicts some of the phenolic acid derivatives
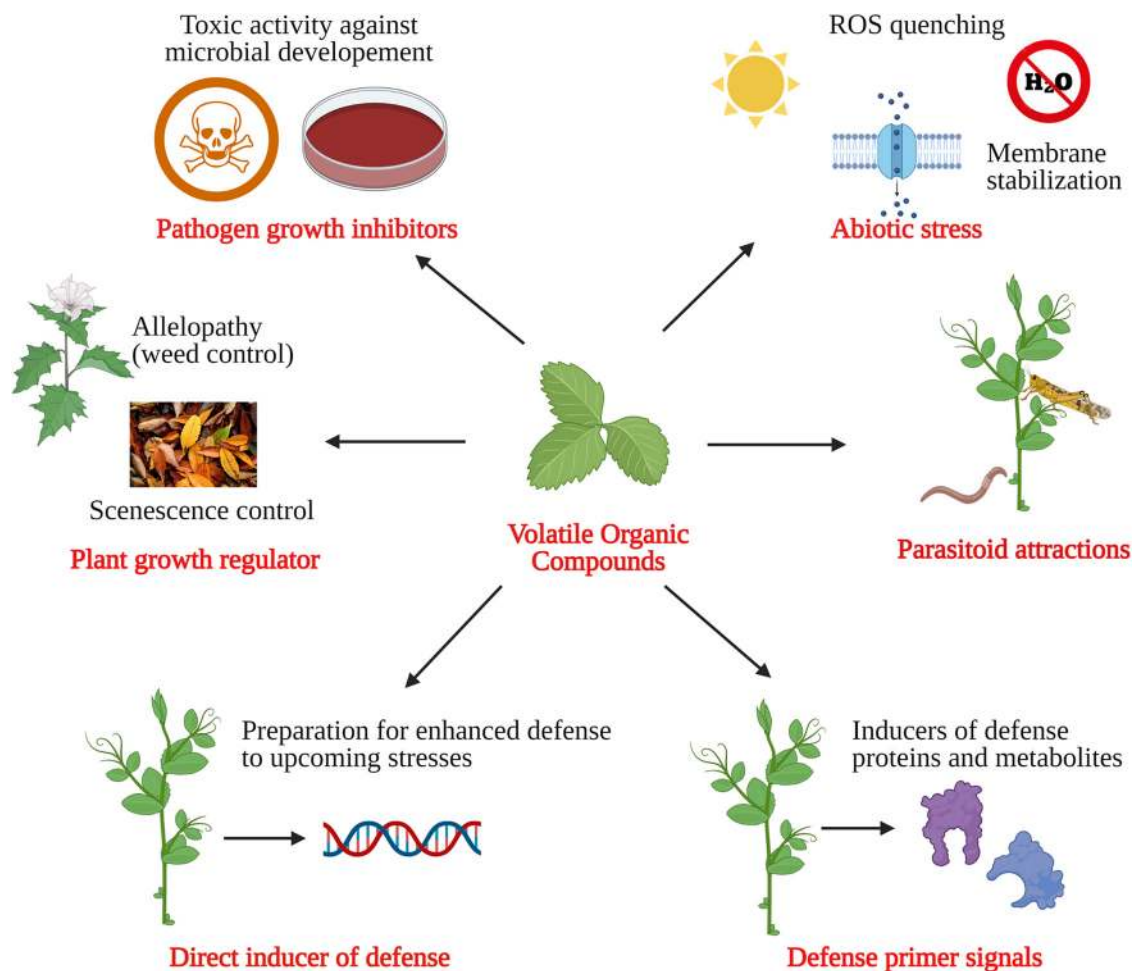
Figure 2: Role of VOCs for different plant signal transduction.

that play a significant role in allelopathy. Some scientific reports have revealed that the alkaloids are known for their effective allelopathic impact. Nicotine, gramine and caffeine are few among the alkaloid-based allelopathic products [18]. Caffeine is responsible for autotoxicity in tea and coffee cultivation through nicotine influences seed proliferation at an elevated concentration. Phytotoxicity of gramine on oat, wheat, rye and weed *Lolium rigidum* was examined; likewise, *Rhazya stricta* contains alkaloids with an allelopathic action [19]. Terpenoids were utilized as a folklore medicine in old times; nevertheless, there are a few allelochemicals compounds such as 1,8-cineole and camphor that are unpredictable monoterpenes and articulate the inhibitory results on the plant development [19]. Upon hydrolysis, glycosylates with a sulfur-rich structure are converted into isothiocyanates that were assumed to play an essential role in safeguarding against assault by bugs [20]. These allelochemicals are highly unstable and removed from the soil in due course of time. A study examined

that hirsutin, arabin and camelinin are allelopathic substances acquired from *Rorippa indica* (L.) hiern roots [21]. Flavonoids are a wide group of compounds with a central (flavone) structure. Some examples of common flavonoids are catechin and kaempferol. Only a limited number of flavonoids are found to be involved in allelopathy so far.

In flavonoid ring structure, the formation of a stable free radical is readily adopted that can easily contribute to the dimers and oligomers where few phytotoxic compounds are also identified [21]. In the plant kingdom, natural quinones are found to be widespread, among them only a very few have been tested for their involvement in allelopathy. There are some plant quinones that are significantly associated with allelopathy, for example, phytotoxic quinones and juglone which have the longest history of study [18]. It occurs as a colorless, non-toxic, and reduced form inside the living tissue. High phytotoxicity at micromolar levels has been reported for juglone against a large number of plant species [25].
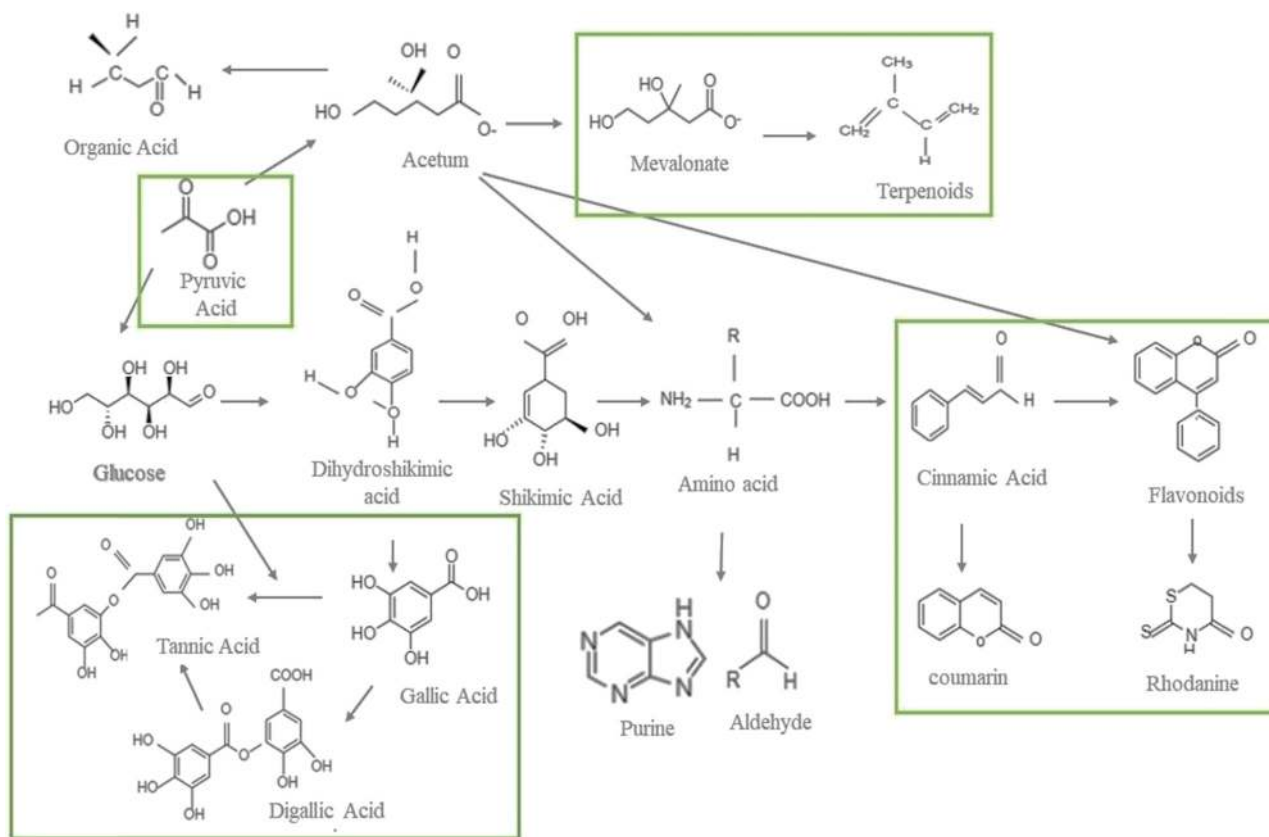
Figure 3: Biosynthetic pathways of major allelopathic substances [6].
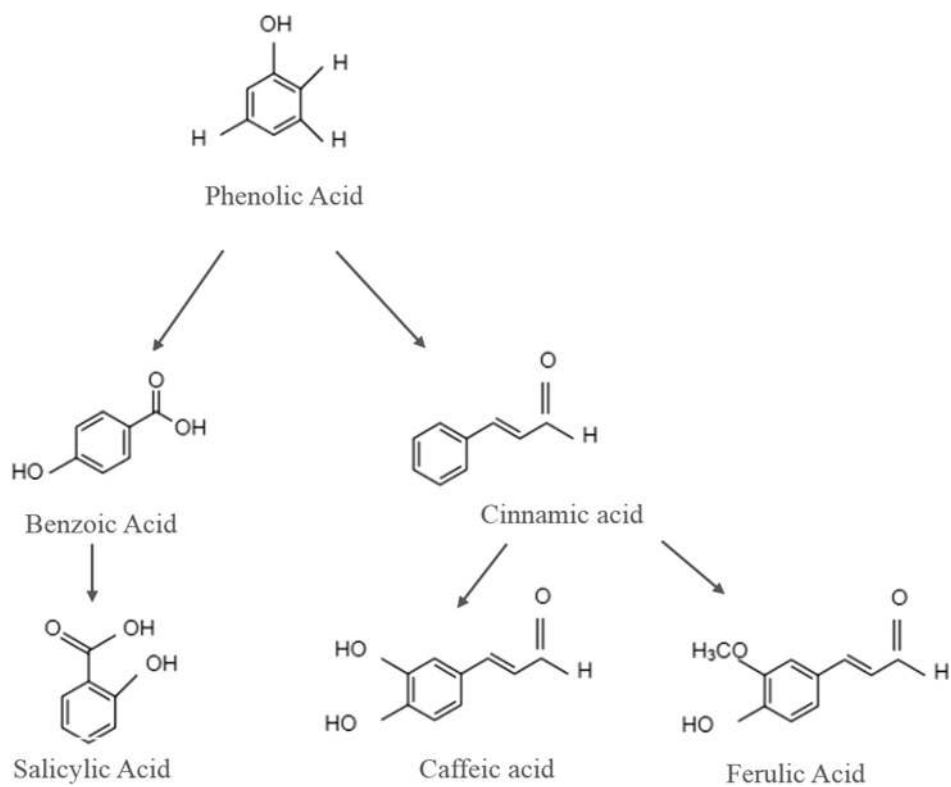

Figure 4: Phenolic acid derivatives that exhibit allelopathy.

3.2 In microbes

Microorganism produces secondary metabolites with both positive and negative effects, which include a change in the bioactive compound and metabolic activity of plants and suppresses or induces the growth of the plants. For example, *Bacillus subtilis* B-916 has caused growth promotion in rice and induced the disease resistance by producing beneficial allelochemicals [22]. Soil bacteria could interact with the plants in an expanded manner like nutrients mobilization, growth enhancement and inducing the diseases. Root-colonizing bacteria are usually characterized as plant growth-promoting rhizobacteria (PGPR) or deleterious rhizobacteria (DRB) [23]. Allelochemicals are also known for their contribution toward the unique chemical patterns that have been adapted for the synthesis of novel herbicides and their molecular modes of action. Some non-pathogenic bacteria with allelopathic properties can affect plant growth in natural and agricultural systems. There is an accumulation of phytotoxic bacteria or other allelopathic microorganisms around the root zones of the plants. The most successful microbial products that have led to the development of commercial herbicides are bialaphos (commercially available in Japan) and glufosinate (marketed worldwide). Glufosinate is the ammonium salt of phosphinothricin, which is the active ingredient of bialaphos derived from a non-phytopathogenic *Streptomyces* species [23]. Utilization of phytotoxic allelochemicals produced by pathogenic or non-pathogenic microorganisms as bioherbicides is one of the approaches that use pathogens toward weed control. Pathogens infect and injure the plants by disrupting the metabolic competence of the host via saprophytic action, nutrient competition, production of tissue digestive enzymes and/or by the production of effective phytotoxins that have various molecular modes of action [3].

Many microbes belonging to pathogens and non-pathogens groups are able to produce multiple phytotoxins. Thus, the action of several allelochemicals perhaps responsible for the injury or mortality when a plant is being attacked by a pathogen. On the other hand, genetic manipulation of pathogens has been studied to improve the bioherbicide efficacy. The molecular genetics of pathogens has been investigated in order to clarify their role in pathogenicity and thus the information obtained could be used in developing the bio-based herbicides [24].

In bacterial genera (*Erwinia*, *Pseudomonas* and *Xanthomona*), various types of gene clusters are found to be involved in phytotoxicity, virulence, hypersensitivity and host range [23]. Studies on microalgal allelopathy have revealed the negative allelopathic effect like growth inhibition. For example, the dinoflagellate *P. aciculiferum* negatively impacts *Synura petersenii* (Chrysophyceae), *Peridiniumin conspicuum* (Dinophyceae), *Cyclotella* sp. (Bacillariophyceae), *Cryptomona* sp. and *Rhodomonas lacustris* (Cryptophyceae) through lysis [24].

E. coli and *S. cerevisiae* are capable of producing diverse terpenoids as their synthesis is based on MEP and MVA pathways, respectively. The synthesis of terpenoids from microbes is observed to be more efficient as it takes a long time for plant growth. Microorganisms with their faster growth and minimum requirement of water and land resources could be effectively used for mass production of terpenoids over plants [30]. Further, metabolic engineering of these microbes could lead to advancements in the future of microbial terpenoid production from microbes [31].

3.3 Plant-derived signaling chemicals

The plant allelochemical-based defense mechanism occurs through inducible and constitutive systems. Inducible protective consequences in plants are known to be locally and systemically triggered by signaling chemicals. The release and production of these signal molecules by the plant are either driven by natural or artificial routes [23]. Studies have discovered that competing neighboring plant influences the defensive biochemistry and modifies the allelochemical production [27]. In plant–plant interactions, a plant primarily notices and identifies its neighboring plant, followed by the initiation of allelopathic action to regulate the intra-specific or inter-specific relations [28]. Air-borne signaling allelochemicals derived from the plant and microbes are found to pose signaling interactions both below and above the ground [29]. The soil-borne signaling allelochemicals are used as parasitic plants for determining the host location. The role of pre-forming signaling chemical in below ground host location by parasitic plants is determined [32].

4 Metabolic engineering and molecular aspects of allelochemicals

Most of the research is being focused on the molecular mechanism of allelopathy in some specific allelochemical compounds, for example, the molecular mechanism of

phenolics or benzoxazolinon (BOA). This model demonstrates the internal reaction of plant and allelochemicals along with the tactics of different microbes to cope up with the particular compound. BOA and DIBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one) are the secondary metabolites of the numerous cereals such as rye or maize and some other species. All the bioactive compounds could be released into the environment by means of root exudation. Volatiles from the plants are proven to be involved in signaling and thus the plant also uses chemical signals in order to regulate their own responses to the external stimuli [33].

The biosynthesis of allelopathy and the genetic differentiation of allelochemical activity have paved the way for developing the natural species with allelopathic potential. Even though the breeding target has not yet been achieved, research studies are being conducted for studying the gene regulator of plant allelopathy [21]. Some studies have identified the gene regulator of wheat allelochemical with the help of isogenic wheat lines (NILs) to produce Hartog (weakly allelopathic) into Janz (strongly allelopathic) [34]. The activity of allelochemical of Janz lines had strong allelopathic activity, whereas BC-Hartog lines (backcrossed to Hartog) were found to be weak. All these consequences revealed that a concentrated chemical is being involved in the inhibition that is triggered by root exudation [33]. The biochemical pathways of allelopathic compounds demonstrate that mostly different genes are included in the formation of allelochemicals [35]. Quantitative trait loci (QTLs) in genetic mapping have shed light on the inheritance characteristic of allelopathy. Allelopathic activity has been recognized in the wheat plant with the help of QTL reflection [36]. Weak and strong allelopathic was identified to investigate the gene control of allelopathic plants [37]. The identification of OTLs in allelopathy-related chromosomes was performed with the help of amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP) and microsatellites (SSRs) [33]. Biochemical and analytical methods are frequently applied for allelopathic research. DNA microarray is widely used in the identification of genotyping (polymorphism and gene analysis), gene expression and gene profiling [37]. All these techniques involve the instantaneous recognition of the gene expressions. Similarly, in terms of metabolomics, quantitation and unbiased identification for all the metabolomics in allelochemical have emerged as a feasible way to transcriptome [38]. The method involved in gene expression profiling and metabolite analysis are used to design the structure of regulatory genes encompassed in the biochemical pathways of primary and secondary metabolites of plants and their compounds, such as flavonoids, alkaloids and isoprenoids [38]. Gene expression analysis by DNA microarray offers possibilities in gene transfer function based on cellular dynamics. To date, the crops enhancing the bioanalysis of weed control by allelochemicals have not been identified yet, even though they were made unaffected to herbicides with transgenes insects and pathogens. In *Bacillus thuringiensis* (Bt), a gene has been successfully engineered with cotton to produce an insect toxin [39].

4.1 Gene responsible for the allelochemical production

Various genes are responsible for plant secondary metabolites and allelochemicals; the classification of genes and their fixed enzymes involved in the biogenesis of artemisinin in *A. annua* plants covered the engineering and the identification of this valuable metabolite in cells of both plants and microbes [33]. Several methods have been formulated for the analysis of the overall effect of allelochemicals in higher PGR as well as to assess the basic mechanism of action. In allelochemical target sites, action is not evident by graphic observation of the negative effect of plant secondary metabolites. The Clm1 (terpene cyclase) gene is responsible for the cyclization of farnesyl diphosphate into the intermediate longiborneol and has the protein longiborneol synthase (required for culmorin production) required for culmorin biosynthesis in *F. graminearum* [40].

The FgOs1 gene contains the osmosensor histidine kinase protein and it detects the water stress and transmits the stress signal to a downstream MAPK (mitogen activated protein kinase) cascade and a putative component of the osmotic stress signal. FgOs1 and an osmoregulatory MAPK pathway (consists of FgOs4, FgOs5 and FgOs2) have been shown to regulate the secondary metabolism associated with AUR and TRIs in *F. graminearum* [41–44]. The FgVe1 gene contains the velvet protein (important in the production of allelochemicals), and modulates the production of the AUR (aurofusarin) pigment and is essential for the expression of TRI (trichothecenes) genes and the production of TRIs. It is a positive regulator of virulence and affects hyphal development and reproduction. FgVe1 A and B positively regulate Tris and ZEA production. The studies have shown that loss of the FgVe1 gene from *Fusarium graminearum* strongly reduces the production of AUR and prevents TRI production by suppressing the expression of *Tri* genes [43]. Velvet

protein plays a significant role in the regulation of allelochemicals throughout the Fusarium genus, and they have the ability to regulate positive and negative effects of each allelochemical and it varies between different plants [45]. The Hep1 gene heterochromatin protein has a repressive role on the AUR gene cluster and a positive function for DON (deoxynivalenol) biosynthesis [36]. Another role of HEP1 is the production of secondary metabolites. Deletion of Hep1 in a PH-1 background strongly influences the expression of genes required for the production of AUR and it does not show growth defects but causes an altered secondary metabolite profile [36]. The Map1 gene and MAPK protein, the Map1 signaling protein, controls multiple events in disease establishment and propagation, including root colonization, wheat ear colonization, DON synthesis and perithecia formation [35,43]. Pks12 gene is responsible for the biosynthesis of AUR and is involved in the ZEA (Zearalenone) production [43,46].

Controlled transcription of biosynthetic genes is one major mechanism regulating the secondary metabolite production in plant cells. Several transcription factors involved in the regulation of metabolic pathway genes have been isolated and studied. As the genetic variability of the allelopathy is widespread in the collected rice accessions, it is essential to investigate their underlying genetic diversity. The analysis of genetic variability showed that the rice accessions with higher allelopathic potential could be clustered into different groups, and some of the higher allelopathic rice accessions introduced from the same geographical locations can be grouped into the same sort, such as rice accessions IAC25, IAC47 and IAC120 from Brazil [25]. However, the others from different geographical locations were also clustered in the same group, such as PI312777 and Taichung Native 1 introduced from America and Taiwan, implying that the genes conferring allelopathy in those rice accessions might be allelic or non-allelic [25].

Characterization of allelochemicals and their allelopathic mechanism is necessary to understand the allelochemical biosynthesis, physiological parameter and the reaction conditions influencing the effect at a molecular scale. In allelopathy research, a typical bioassay is primarily concerned for the cultural organism, whereas for uncultivable organisms denaturing gradient gel electrophoresis (DGGE) of 16S rDNA or 18S rDNA would provide a comprehensive analysis of the allelochemical-sensitive uncultivable microbes. Uncovering the potential interaction of allelopathic is followed by performing other characterization techniques like 2D polyacrylamide gel electrophoresis coupled with mass spectrometry (MS) for analyzing the expression levels of the proteins. On the other hand, a comparison of gene expression patterns

in allelopathic microbes or their response to the target microbes has been performed by DNA microarrays. It is a developing DNA chip technology, where thousands of genes were hybridized onto the microarray of expressed sequence, for instance, identification of genes involved in root-derived allelochemicals synthesis. Isobaric tags (isotope coded-affinity tagging) is a technology adopted for the quantitation of gel-free protein expression in cyanobacteria [47,48]. Similarly, real-time (RT) PCR was also employed for the quantitation of specific gene expression involved in allelochemical biosynthesis [49].

Considering the phytotoxic compounds mode of action, analytical tools in metabolomics such as HPLC, GC-MS, FTIR and ^{1}H-NMR could serve as a promising tool in order to explore the molecular target site, i.e., mechanism and its complete process sequence (mode of action) [50,51]. In addition, they also provide information about the biological status of the microbes when exposed to the bioactive compounds. Thus, the potential of metabolomics is well reinforced in allelopathy where plants respond to biotic and abiotic stress.

5 Extraction process

Extraction is the main stage to isolate the allelochemicals from the crude materials or from the plant part. It incorporates different strategies, for example, solvent extraction (SE), refining strategy, squeezing and sublimation. For the most part, the technique for extraction should be comprehensive for the segments to be improved, economical, fast and clear. The various methods of allelochemical extraction along with the yield and advantages/disadvantages are provided in Table 1.

A growing interest in secondary metabolites outgrowths scientists to support and alter the conventional extraction system. It includes maceration, decoction, mixture, permeation and soxhlet extraction. These techniques have certain drawbacks such as the prerequisite of huge amounts of natural solvents, longer extraction time, debasement of labile components and unsuitable extraction proficiency [52].

The modern extraction methods mainly include ultrasonication-assisted extraction (UAE), microwave-assisted extraction (MAE), solid-phase microextraction (SPME), pressurized liquid extraction (PLE), supercritical fluid extraction (SFE), with supercritical or subcritical water and sound waves. Among all these, SEs are the most widely used method [39]. Figure 5 represents the process flow involved in the extraction of allelopathic compounds from plant samples.

Table 1: Allelochemical extraction methods from various plants and microbial sources and their corresponding yield extract

Methods	Chemicals used	Source	Extraction yield	Advantages/disadvantages	Reference
Solvent extraction	Ethyl acetate	Alkaline peroxide mechanical pulp (APMP)	510 (mg/L)	Enhanced yield but the high cost	[92]
	Dichloromethane		330 (mg/L)		
	Methanolic extract	*S. officinalis*	207.48–251.73 (mg/g DW)		[93]
	Ethanol	*Chlorella* sp.	8.1 ± 0.16 (mg/g DW)		[94]
	Hexane	*N. ellipsosporum*	60.35 ± 2.27 (mg GAE/g)		[54]
	80% methanol	*C. pyrenoidosa*	16.8 ± 0.1 (mg GAE/g)		[95]
Maceration	–	*T. serpyllum*	13.1–18.845 (TP mg/L)	Suitable for large scale production but the overall application is limited by solubility issues	[59]
	Sequential alkaline extraction	*A. aureum*	1129.52 ± 10.70 (μg GAE/g DW)		[96]
Ultrasound-assisted extraction	–	*R. apiculata*	977.90 ± 2.13 (μg GAE/g DW)	Provides high cell disruption but a tedious separation process	[97]
	–	*T. serpyllum*	18.58–27.50 (mg/L)		[59]
	–	*C. sinensis*	275.8 (mg of GAE/100 g DW)		[97]
	Ethanol	*K. vitifolia*	85.25–0.69 (mg GAE/g DW)		[98]
	70% MeOH	*C. glomerata*	1.22 ± 0.03 (mg GAE/g DW)		[99]
Microwave-assisted extraction	Methanol/water (60:40, v/v)	*R. officinalis*	21.2 ± 0.4 (mg GAE/g DW)		[100]
	Acetone/water (60:40, v/v)	*O. majorana*	18.37 ± 0.2 (mg GAE/g DW)		
	–	*P. lentiscus*	144.94–150.14 (TP mg GAE/g DW)		[101]
Soxhlet extraction	Ethanol/water	*S. melongena*	1.18 ± 0.08 (mg GAE/g)		[102]
	Methanol	*C. glomerata*	1.57 ± 0.052 (mg GAE/g DW)		[103]
	Ethanol	*V. cinerea*	7.04–8.03 (% w/w)		[104]
	Ethanol/water (60:40, v/v)	*O. europaea*	247.9–337.7 (TFC mmol RE/kg)		[98]
Infusion	Methanol/water	*T. vulgaris*	12.11 ± 1.62 (%)		[105]
Pressurized liquid extraction	Methanol/water	*Z. aquatica*	313.62 ± 27.60 (μg/g)	Enhanced yield but the complex operation	[106]
		C. racemosa	6.43 (mg GAE/g)		[107]

*GAE – gallic acid extract; TP – total phenol content; TFC – total flavonoid content; DW – dry weight; RE – rutin equivalent.
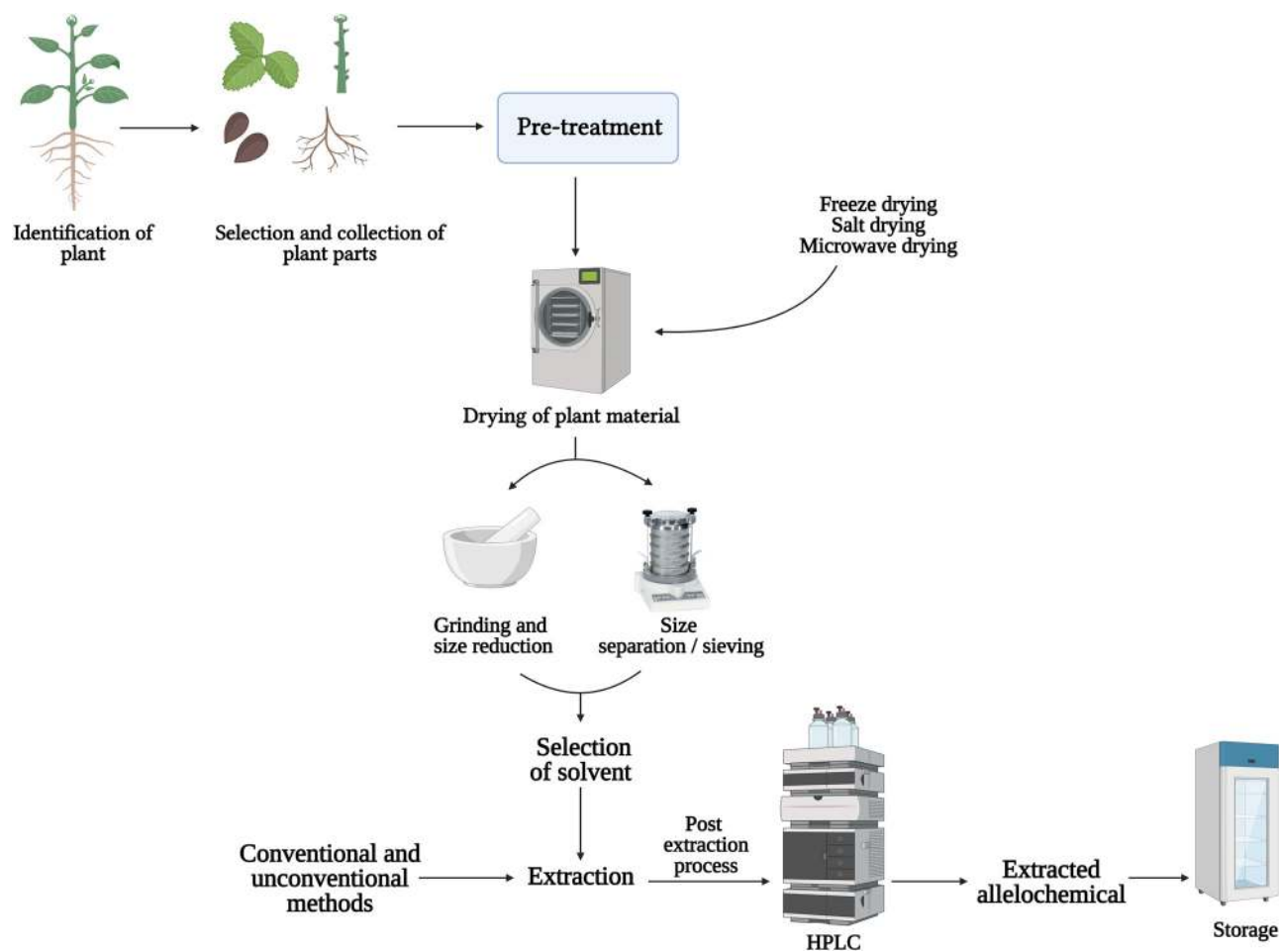
Figure 5: Flow chart of the extraction of allelopathic compounds from plants and analysis.

5.1 Conventional extraction methods

Conventional extraction techniques are being used at a small-scale level to extract natural products from numerous plant materials. These techniques are based on the extraction efficiency of different solvents that are being used for this purpose.

5.1.1 Maceration

Maceration, the simplest traditional extraction technique, used for extracting volatile and thermolabile compounds, where the plant materials are mixed with the extracting solvent and subsequently permitted to attain equilibrium under ambient temperature. In particular, phenolics, antioxidants and cyclotides are found to be macerated using ethanol, methanol, dichloromethane, methanol: water mixture and hydroalcoholic solutions as solvents [53–55]. Similarly, a high yield of anthocyanins and phenols was

attained under optimized conditions (1:20 solid/solvent ratio, 0.75 mm particle size and 50% ethanol) from chokeberry fruit, which shows that the maceration was effective in the phenolic compound extraction [56]. At times, over the extraction phase, recurrent agitation along with heating could be provided, which in turn facilitates the extraction process as observed by Kolberg et al. [57] and Hinneburg and Neubert [58] in extracting the paraquat, diquat and phenolics compounds from cereals and buckwheat herb respectively. On the other hand, Jovanović et al. [59] compared the extraction efficacy of polyphenols using different extraction techniques like maceration, ultrasonic- and heat-assisted extraction. The ultrasonic-assisted extraction showed the highest yield of flavonoids when compared with heat-assisted extraction and maceration. Yet, this method of extraction requires a longer extraction time and shows low efficiency in extracting tightly bound cell-matrix compounds or tissue cell constituents. Further, centrifugation or filtration is required for separating the sample matrix and the extract.

5.1.2 Percolation

In percolation, a conical flask known as a percolator contains the sample matrix, where the extracting solvent is percolated via the matrix bed [60]. Since percolation is a constant process in which the saturated solvent is continuously replaced by the new extracting solvent, thereby the aforementioned technique was found to be more effective than the other extraction methods. For example, Fu et al. [61] investigated the total alkaloid content via optimizing the percolation method using 55% of ethanol for 24 h followed by 12 times percolation using acid–base titration as an index. While using extracting rates of ephedrine hydrochloride and sinomenine compounds as an index, an alternate optimized percolation technique was developed by Gao et al. [62], where 20 times percolation with 70% ethanol has resulted in 78.23% and 76.92% transfer rates of sinomenine and ephedrine hydrochloride respectively. Nevertheless, a system clogging has been observed as a result of swelling up of plant materials and fine powders that were used in the percolation process.

5.1.3 Decoction or infusion

In infusion, hot/cold water is being added to the plant constituents, whereas in decoction the plant material is boiled for 15–30 min in water. Thus, based on the implementation extraction of the sample with hot water, it is called infusion or decoction. For example, Li et al. [63] stated that the decoction method of extraction was more effective in the case of extracting antioxidants from various medicinal plants. However, extracts from this technique have a vast number of water-soluble impurities. Thus, the decoction is not suitable for the extraction of thermolabile or volatile compounds. Zhang et al. [64] reported that the hydrolysis of two flavonoid glycosides (ononin and calycosin-7-O-β-D-glucoside) from Astragali Radix into formononetin and calycosin through the decoction method has revealed that the hydrolysis efficacy is strongly influenced by temperature, pH and the herb amount. The decoction perhaps enhances the dissolution of the bioactive compounds as compared with maceration on monitoring 17 active constituents in Sanhuang Xiexin Tang and Fuzi Xiexin Tang.

5.1.4 Soxhlet extraction

Soxhlet exaction is a classical automatic continuous system integrating the percolation and reflux principle for the constant extraction of herbs with fresh solvents. High efficiency of extraction was attained with less solvent consumption and time as compared to maceration and percolation. A batch extraction procedure with intermittent solvent infusion, i.e., a static extraction was observed after the liquids fill the soxhlet thimble accompanied with the solvent recirculation throughout the extraction process. However, the requirement of high temperature during soxhlet extraction perhaps increases the thermal degradation rate [52,60]. For instance, catechins degradation in tea was observed when the high temperature was applied in soxhlet extraction. In this study, the total polyphenols and alkaloids concentration were found to decrease at 70°C when compared with the maceration technique performed under 40°C [65,66]. Certainly, the prime feature of soxhlet extraction is that the extracting solvent could be recycled over time resulting in high extraction yield, and thus circumvents the solvent saturation upon solvent and the plant matrix interaction followed by equilibrium transfer as mentioned in the maceration section. On the other hand, soxhlet is the recurrently used technique for the natural antioxidant extraction from plant materials in spite of its major drawbacks such as lack of selectivity, large solvent volumes and solute degradation [60].

5.2 Non-conventional method

The main disadvantages of the conventional extraction method include high-purity solvent, longer extraction time, cost-intensive, evaporation of the huge amount of solvent, low extraction selectivity and thermal decomposition of thermolabile compounds [67]. To overcome these restrictions, new and promising extraction techniques are introduced. These techniques are referred to as non-conventional or modern extraction techniques.

5.2.1 Ultrasonication-assisted extraction (UAE)

In this technique, extraction is being assisted by sonication/ultrasound known as ultrasonic-assisted extraction (UAE) where ultrasonic waves with a frequency >20 kHz were used. In UAE, the ultrasound produces the cavitation in the extracting solvent, which accelerates the solute dissolution and diffusion together with heat transfer, thereby enhancing the extraction efficiency. In addition, reduced extraction time and temperature, low energy and solvent consumption aids in extracting many compounds like antioxidants, phenolic compounds, oils, fats, dyes,

pigments, proteins, polysaccharides medicines and saponins that are unstable and thermolabile in nature [68,69]. In practice, maceration is primarily executed under an energetic system, which utilizes either ultrasonic probe or bath to enhance the matrix–solute diffusion and disruption. A study by Jovanovic et al. [59] reported that UAE resulted in a high amount of polyphenol from *Thymus serpyllum* L. under optimum conditions such as 1:30 solid/solvent ratio, 50% ethanol, 15 min and 0.3 mm particle size as compared with heat-assisted and maceration type of extraction. The microscopic examination of plant cell walls after UAE revealed that either UAE disrupts the external glands facilitating the release of essential oil or causes the plant cell wall enlargement by swelling and hydration, thereby improving the mass transfer [70–73]. Furthermore, UAE showed good efficacy in the phenolic compound extraction from *Cratoxylum formosum* under optimum reaction conditions such as 45 kHz, 15 min, 65°C and 50.33% (v/v) ethanol [67]. The total phenolic and antioxidant capacities of 23.12 ± 1.01 (mg GAE/g) and 85.64 ± 2.07 (mg TE/g) were extracted as a result of UAE of alga *Hormosira banksii* using 70% ethanol as the solvent [74].

5.2.2 Microwave-assisted extraction (MAE)

MAE is an emerging extraction technique for the soluble compounds derived from plant materials using microwave energy. The heat is generated by microwaves and interacts with organic and polar compounds of the plant matrix followed by dipole rotation and ionic conduction. Thus, in MAE, the heat and mass transfers are in the same direction that provokes a synergistic effect in improving the extraction process and its yield. MAE can be performed in two modes like MAE-based SE for nonvolatile compounds and solvent-free extraction for volatile compounds where the latter enhances the extraction yield by affording selective heating and reduces thermal degradation [70,75]. For instance, Benmoussa et al. [76] investigated the solvent-free MAE for enhancing the essential oil extraction from *Foeniculum vulgare* seeds under atmospheric pressure. In this study, the aromatic profile and the yield were similar to those obtained from hydrodistillation extraction. Li et al. [77] reported that the MAE of phenolic compounds from tomatoes was more effective with a shorter MAE extraction time and high temperature. Chen [78] optimized the MAE extraction of resveratrol from the rhizome and radix of *Polygonum cuspidatum*. A maximum of about 1.765 of resveratrol yield was achieved in the orthogonal experiment under optimum conditions as follows: solid/liquid ratio, 1:25, 7 min, 80% ethanol and 1.5 kW

of microwave power. Similarly, the central composite design (optimum conditions: 200 W, 260 s and 65% methanol) for MAE was developed by Xiong et al. [79] in order to extract bioactive alkaloids, namely nuciferin, dauricine, liensinine, isoliensinine and neferine from *Nelumbo nucifera* seeds. Nevertheless, in classical MAE, the oxygen/thermosensitive plant compounds might undergo oxidation/degradation. Therefore, vacuum MAE has been recently established for extraction of sensitive analytes as reported by Hu et al. [80] in extracting auxin from the plant matrix. Thus, the utilization of MAE for extracting plant active secondary metabolites (terpenoid, quinones and flavonoids) could be relevant for a pilot-scale, yet it remains limited on a commercial scale. The MAE of alga *S. vestitum* using the mixture of acetone/ water (7:3 v/v) extracted a higher total polyphenol content of 74.05 mg GAE/g DW when compared to other solvents used. Ethanol or methanol and water mixtures are also found to be the potential solvents for this extraction [81].

5.2.3 Solid-phase microextraction (SPME)

A simple, real non-solvent technique was used for the analyte partition between the extraction medium and the plant matrix. Stashenko and Martínez [82] stated that SPME is operated under the headspace mode as the plant materials are solid in nature, and thus is being applied for several medicinal plants. Extraction of biogenic volatile components, odor-active compounds (fenugreek), and essential oils from plants have been achieved through SPME. Piccirillo et al. [83] employed the SPME/GC-MS for identifying the antibacterial properties and composition of leaves and stem extract of *Prunus cerasus* L., Rosaceae. In this study, 36 volatile compounds have been identified from the plant extract where terpenes are the major class of compounds. Further, ethyl acetate extract of the stem was rich in cedrene, 4-terpineol, linalool and α-pinene with >10 times higher concentration than in other types of extracts. Thus, SPME serves as a proximate sample extraction technique resulting in high sample throughput via a single step comprising sampling, followed by extraction and concentration. However, sample preparation using liquid–liquid extraction possesses multistep operations that are laborious and time-consuming. Hence, an ideal extraction technique should be simple, efficient, solvent-free, inexpensive and selective for a wide variety of applications.

5.2.4 Pressurized liquid extraction (PLE)

PLE is also known as accelerated/enhanced SE, where high pressure is being applied during extraction. In PLE,

high solubility, high diffusion and high penetration rate of solute and solvent are achieved under high pressure. In addition, PLE has less consumption of solvent and extraction time and yet has better repeatability than the other extraction techniques. PLE has been successfully applied in extracting flavonoids, saponins and essential oils [66,84–86]. For instance, PLE was employed for extracting anthocyanin from black carrots where the short duration of PLE overcomes the high temperature condition and the anthocyanins degradation rate is time-dependent [87]. In comparison with other traditional techniques, this type of extraction in medical plants affords a similar/higher yield with less amount of solvent and reaction time (generally 5–15 min). Further, in case of light-sensitive and oxygenated plant compounds, the PLE equipment setup offers protection, whereas in some cases like thermolabile compounds, it would be affected by the elevated PLE temperature. PLE has been employed in extracting phenolic and anti-oxidant content from macroalgal species, like *Fucus serratus*, *Laminaria digitata*, *Gracilaria gracilis* and *Codium fragile* in order to overcome the drawbacks of the other methods like a high solvent requirement and low efficiency seen during extraction. Thus, PLE is expected to reduce the extraction time as well as increase the extraction yield [88].

5.2.5 Supercritical fluid extraction (SFE)

In SFE, supercritical fluids are the alternative extraction solvents that possess similar diffusivity and solubility to gas and liquids and thus could dissolve a wide range of natural compounds. Near the critical point of the supercritical fluids, the solvating properties of supercritical fluids are intensely changed owing to their small change in extraction temperature and pressure. On the other hand, supercritical CO_2 in SFE is capable of extracting thermolabile plant compounds, nonpolar compounds such as volatile oil and lipids due to its low critical temperature, low polarity, nontoxicity, selectivity and inertness, thus making it an ideal extraction technique [50]. SFE is generally performed in the batch mode, where an initial static phase will be followed by the dynamic phase and depressurization of supercritical fluids is done to isolate the extract solutes [58]. Conde-Hernández [89] compared the extraction efficiency of essential rosemary oil by supercritical CO_2, steam distillation and hydrodistillation, where the antioxidant activity and the oil yield were observed to be higher in the case of supercritical CO_2 as compared to other methods. Furthermore, a modifier in SFE could significantly enhance the solvating properties. For instance, supercritical CO_2 with

2% ethanol as a modifier at 40°C and 300 bar pressure resulted in the increased selectivity of *Catharanthus roseus* vinblastine with 92% efficiency than the other traditional methods [90]. Thus, the application of supercritical CO_2 in extraction could reduce or completely avoid some of the deleterious effects associated with the organic solvents (toxicity, cost and environmental concerns). Moreover, SFE is involved in both qualitative and quantitative identification of various natural plant constituents that include the heat-sensitive compounds, where some degree of lipophilicity is being exhibited by esters, ethers and lactones [91].

5.2.6 Solvent extraction (SE)

SE is the most extensively used extraction technique where the properties of the solvent, solvent/solid ratio, the particle size of the raw materials, extraction time and the extraction temperature would affect the efficacy of extraction. In this method, screening of solvent is an essential step, and the solvent solubility, selectivity, safety and expenditure should be considered [52]. The effectiveness of extraction is improved by the minute particle size owing to the better diffusion of solvents and solutes.

6 Application of allelochemicals

Allelopathy has both positive and negative effects. The harmful consequence of allelopathy is autotoxicity, soil affliction or biological invasion, while the valuable impacts are weed control, plant protection and plant growth. These days numerous research studies are being focused on the application of allelochemicals in terms of development controllers, herbicides, bug sprays and antimicrobial plant protection agents. Weed management is one of the applications of allelochemical, intercropping and mulching and has become a reason for losses in crop production and soil sickness. Continuous utilization of bug spray and chemical pesticides has challenged agricultural practices. Recently, in forestry sectors, the application of allelochemicals for plant growth has been widely adopted instead of the conventional chemical pesticides and herbicides that are toxic to wild animals and plants. On the other hand, several allelochemicals with herbicidal activity have been identified from different plants species. Some of the allelochemicals are water-soluble that control the excess weed growth, for example, water extract of sorghum has shown an inhibiting effect on weed growth. In the context of

economic benefits, there are a lot of bioactive compounds present in the plants, and therefore utilizing an allelochemical provides a conservative method to change the horticulture practice. Nowadays, an instrument-based recognition of bioactive compounds has been established that could add economic value to the industry [25]. The biochemical aspect in relation to the interaction occurring in the rhizosphere and other hosts is predominantly unknown. Recent technologically advanced methods in identifying and detecting the metabolite as well as studying the complex interaction and regulations have been proven efficient to enhance the production system [108]. Metabolic profiling is an important tool to support the previously mentioned study. Extracting and evaluating a plants metabolome could help in identifying and analyzing the functional state of a plant system at a specific time. This tool can be used in combination with proteomics and transcriptomics to entirely construct the biosynthetic pathways to obtain an increased production of specific targets. The advancements in developing different ranges of analytical methods including chromatography combined with high-resolution mass spectrometry (MS) have made the metabolic profiling of targeted and non-targeted metabolites facilitate enhanced allelochemical production [109]. The replacement of conventional methods of extracting like maceration, soxhlet, etc., with non-conventional methods like MAE, UAE and PLE could significantly avoid the bottlenecks associated with the conventional methods [110].

7 Conclusions

Extraction of the natural compounds can be performed by using conventional and non-conventional methods. The efficiency of any conventional method mainly depends on the choice of solvents. The conventional extraction methods using organic solvents for biomaterials will remain, as they build upon many years of experience and have a strong scientific basis. In the past decades, new solvents have been discovered and developed for different applications, such as supercritical fluids, and each of these solvents has its own advantages and drawbacks. This review summarized the several extraction processes of allelochemical and the route of the release of an allelochemical from the plants. Current innovations and green extraction help in the determination of allelochemical substances from the production cycle. The work process starts with the characteristics of allelopathy and various kinds of the allelochemical present in the plant and is trailed by the plant natural product extraction and

various sorts of a course to deliver the allelochemicals in the soil and influence the soil and various plants. In addition, offering a reproducible quality of the extract is a prerequisite and there is no doubt that hyphenation or combination of techniques will increase in the near future as such an approach can afford beneficial aspects of the different techniques.

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