



Drug discovery from natural products – Old problems and novel solutions for the treatment of neurodegenerative diseases



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ABSTRACT

The use of natural products has been shown to be a fruitful approach in the discovery of novel pharmaceuticals. In fact, many currently approved drugs originated from compounds that were first identified in nature. Chemical diversity of natural compounds cannot be matched by man-made libraries of chemically synthesized molecules. Many natural compounds interact with and modulate regulatory protein targets and can be considered evolutionarily-optimized drug-like molecules. Despite this, many pharmaceutical companies have reduced or eliminated their natural product discovery programs in the last two decades. Screening natural products for pharmacologically active compounds is a challenging task that requires high resource commitment. Novel approaches at the early stage of the drug discovery pipeline are needed to allow for rapid screening and identification of the most promising molecules. Here, we review the possible evolutionary roots for drug-like characteristics of numerous natural compounds. Since many of these compounds target evolutionarily conserved cellular signaling pathways, we propose novel, early-stage drug discovery approaches to identify drug candidates that can be used for the potential prevention and treatment of neurodegenerative diseases. Invertebrate *in vivo* animal models of neurodegenerative diseases and innovative tools used within these models are proposed here as a screening funnel to identify new drug candidates and to shuttle these hits into further stages of the drug discovery pipeline.

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1. Introduction

Plants, bacteria, and fungi have historically been important and rich sources of biologically active compounds [1–4]. The following are just a few of the numerous natural compounds that have been incorporated into the repertoire of widely-used and FDA approved medicines: aspirin (acetylsalicylic acid), statins, morphine, codeine, paclitaxel, penicillin, artemisinin, reserpine, colchicine, and galantamine, among many others [4–7]. Plant-derived molecules have also been extensively utilized as templates for the development of medicines currently used for the management of neurodegenerative diseases, such as Alzheimer's (AD) or Parkinson's (PD) disease [4,6,7]. Galantamine and rivastigmine (the semisynthetic derivative of physostigmine) are natural alkaloids approved for the management of AD and are classified as acetylcholinesterase inhibitors, based on the outdated cholinergic hypothesis of AD [4,8]. Other possible mechanisms of action for these two compounds include interactions with evolutionarily conserved cellular signaling pathways [4,9]. Levodopa (L-DOPA), the number one drug used in the management of PD, was first identified in broad beans (*Vicia faba*) [10]. Current therapies for patients with PD attempt to both preserve and restore dopaminergic neurotransmission [6,7]. Following enzymatically catalyzed decarboxylation, L-DOPA is converted to the neurotransmitter dopamine, the levels of which are known to decrease in PD patients. Using compounds that bind to and activate postsynaptic dopaminergic receptors is another approach aimed at increasing dopaminergic neurotransmission. Lisuride, pergolide, and bromocryptine, known dopamine-receptor agonists, are derivatives of alkaloids first isolated from rye ergot fungus (*Claviceps purpurea*) [11]. Apomorphine, a non-selective dopamine receptor agonist derived from morphine, is another example of a naturally-derived compound used in the management of PD [12].

Indisputably, natural compounds have a great potential to be developed into potent pharmaceuticals. However, in the era of automated high-throughput screening techniques, the pharmaceutical industry seems to favor the screening of large libraries of synthetic molecules [13]. Yet, the number of new drugs entering the market has been still on the decline. This is especially evident in the case of drugs used to prevent or treat neurodegenerative diseases [14]. One of the most significant challenges of drug discovery from natural products is the complexity of natural samples [5]. Extensive time and effort are needed to identify and isolate specialized metabolites that can be further tested for pharmacological activity. High-throughput screening (HTS) techniques designed to test extensive libraries of individual molecules are less suitable for analyzing complex natural matrices. Additional steps, following HTS, are needed to identify the molecule(s) responsible for the observed biological effects.

This complexity of natural samples has also resulted in an abundance of studies that focus only on assessing the activity of the entire extract without the further identification of the individual compounds responsible for the observed biological effects. The specialized metabolite profile of complex natural mixtures varies from batch to batch; therefore, it is important to either identify the molecules responsible for the biological effect or to assess the complete specialized metabolite profile that yielded the observed pharmacological activity.

The simplicity of certain preliminary screening assays that can be used to evaluate selected biological activities has also resulted in an overabundance of studies that focus on the antioxidant or enzyme-inhibitory activity of the entire extract. For many years, the predominant hypothesis in the field of natural product discovery has promoted the idea that direct antioxidant activity of certain specialized metabolites is majorly responsible for the health-benefits that arise from certain physical activities and dietary choices [4,9]. However, rigorous clinical trials have disproved the direct antioxidant activity hypothesis of natural products [4]. Here, we review early pipeline strategies that may speed up the drug discovery process and that are based on the idea that natural products are evolutionarily-optimized drug-like molecules.

One of the other serious bottlenecks in drug discovery from natural products is the lack of suitable tools allowing for the identification of biologically active specialized metabolites that are present in the full extract [5]. There is a dire need to develop novel approaches to speed up the discovery of pharmacologically active compounds present in complex natural samples.

One area of disease with a desperate need for new treatment options is age-associated neurodegeneration. Interestingly, numerous natural compounds have been found to extend lifespan, to improve healthspan, and to prevent the development of neurodegenerative diseases across multiple model organisms and are currently considered to be new drug leads in the field [1–4,9]. The following are just a few examples of natural or nature-derived compounds that have been proven to extend and improve lifespan in numerous model organisms: rapamycin (produced by bacterium *Streptomyces hygroscopicus*), metformin (a derivative of a natural compound galegine, isolated from *Galega officinalis*), and several polyphenolic compounds: resveratrol, curcumin, fisetin or quercetin [6].

Epidemiological data strongly suggests that a diet rich in fruits, vegetables and herbs is associated with a lowered risk of developing chronic, age-associated diseases, including neurodegenerative disorders [15–25]. Most of these studies associate the beneficial effects of plant-rich diets with a certain class of abundant specialized plant metabolites called polyphenols, and, more specifically, the family of polyphenols called flavonoids [26,27]. In drug discovery literature, these plant-derived metabolites have been often considered as invalid metabolic panaceas (IMPS) that lack the essential characteristics typically associated with valuable drug leads (e.g.: lack of well-defined targets, stability, bioavailability etc.) [28–30]. Additionally, most of these natural compounds have been observed to give false-positive results in high-throughput screening assays, and therefore have been classified as PAINS (pan-assay interference compounds) [31]. Therefore, polyphenols are usually excluded at the early stages of drug discovery programs from natural samples. Although polyphenols do not possess the typical characteristics associated with many drug leads, these abundant molecules may play an important role in biological systems, as is the case with many vitamins [30].

Many non-communicable diseases are associated with chronic inflammation, that has been involved in the pathogenesis of numerous diseases, including neurodegenerative disorders [32]. Interestingly, a diet rich in plants and omega-3 fatty acids has been shown to decrease the risk of developing chronic inflammatory diseases [32]. Omega-3 derived electrophilic molecules have been

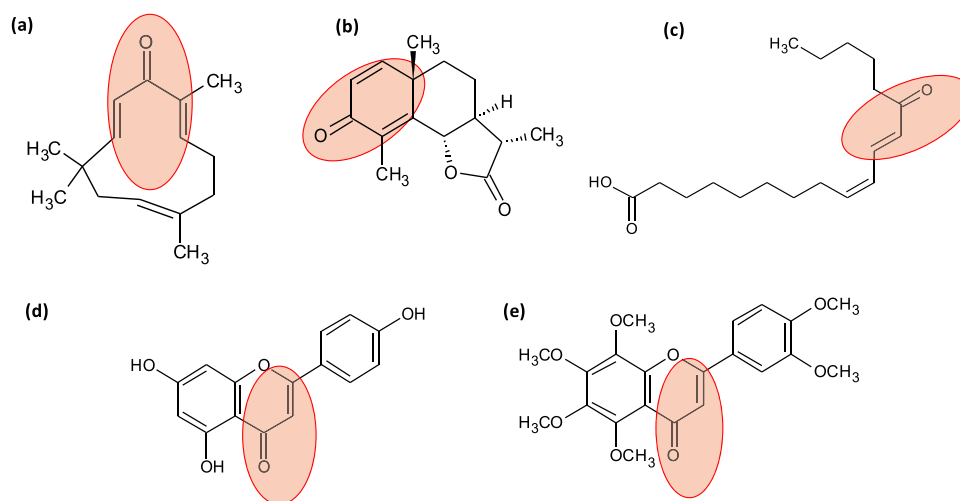


Fig. 1. Examples of natural compounds possessing electrophilic centers (α,β -unsaturated carbonyl groups) that can react as Michael acceptors: (a) zerumbone (terpenoid), (b) α -santonin (terpenoid), (c) 13-EFOX-L₂ (linoleic acid electrophilic oxo-derivative), (d) apigenin (flavone), and (e) nobiletin (flavone).

shown to be important and specialized pro-resolving mediators involved in the resolution of inflammation (catabasis) [32–37]. Numerous flavonoids and their metabolites, similarly to electrophilic lipids, contain structural elements that allow them to behave like soft electrophiles: namely, α,β -unsaturated carbonyl groups that allow them to undergo Michael additions with nucleophiles, such as cysteine thiols (Fig. 1) [35,38,39]. We propose that certain flavonoids, together with some essential fatty acids, may fulfill the role of essential soft electrophilic molecules that are required by biological systems to resolve inflammation [40]. The presence of a reactive electrophilic center is one of the factors associated with the high reactivity of these molecules in high-throughput screening assays. Therefore, it should be encouraged to test the biological effects of these molecules in the context of living organisms. This matter of investigation may serve to verify signal selectivity and reversibility of the reaction between plant-derived soft electrophiles and thiols.

This review focuses on presenting the pharmacological potential of evolutionarily-optimized drug-like natural compounds. Frequent problems in the drug discovery process from natural samples are considered and novel solutions are proposed. In the following subsections, we consider why plants, bacteria, and fungi have evolved to produce such complex molecules. Further, we review modern screening and early drug discovery pipeline approaches used in the identification of new cures derived from nature. Finally, we present the use of simple *in vivo* invertebrate models for the identification of neuroprotective natural compounds. Modern screening approaches, together with *in vivo* phenotypic assays, may serve as a screening funnel at the beginning of the drug discovery pipeline from natural products. Compounds identified in these steps may then progress to the next stages of the pipeline: validation in mammalian models.

This article focuses on the characteristics of drug discovery for the prevention and/or treatment of neurodegenerative diseases.

2. Natural products as a source of new drug hits and leads – an evolutionary perspective

Biochemical communication evolved as a simple method of information exchange between different organisms that occupied the same or overlapping ecological niches. Immobile organisms, such as

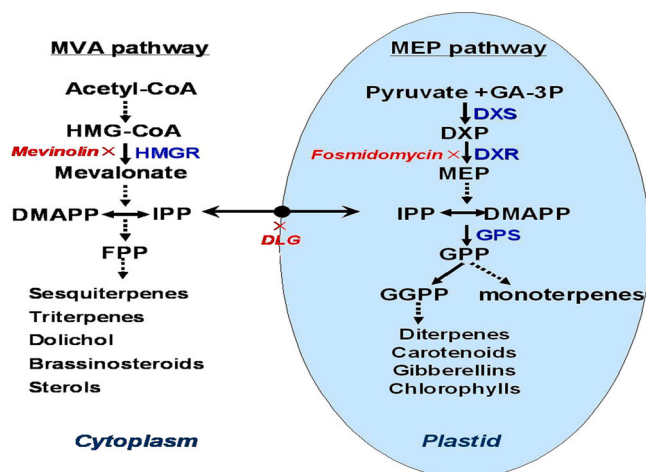


Fig. 2. Biosynthesis of terpenoids via mevalonate (MVA) and non-mevalonate pathway (MEP) in plants. Reused from Yang et al. [43] in accordance with the Creative Commons Attribution (CC BY) license.

plants, synthesize a great variety of specialized metabolites in response to numerous abiotic and biotic signals from the environment as an effective way to defend themselves from environmental insults [1–4,6,9,41]. Many specialized metabolites produced by microorganisms and plants evolved to interact with other organisms and animals such as humans. Interestingly, the chemical structures of certain signaling molecules are conserved between microorganisms, plants, and animals [42]. This is particularly evident for terpenoids, which are synthesized by all known organisms on Earth through the mevalonate pathway (Fig. 2) [43]. It has been hypothesized that terpenoids evolved as signaling molecules before life became multicellular. The terpenoid theory of the origin of life postulates that the first cellular membranes were terpenoid-based structures, rather than phospholipid bilayers [44]. Numerous terpenoid signaling molecules might have evolved from compounds that initially played structural roles as a form of communication between unicellular

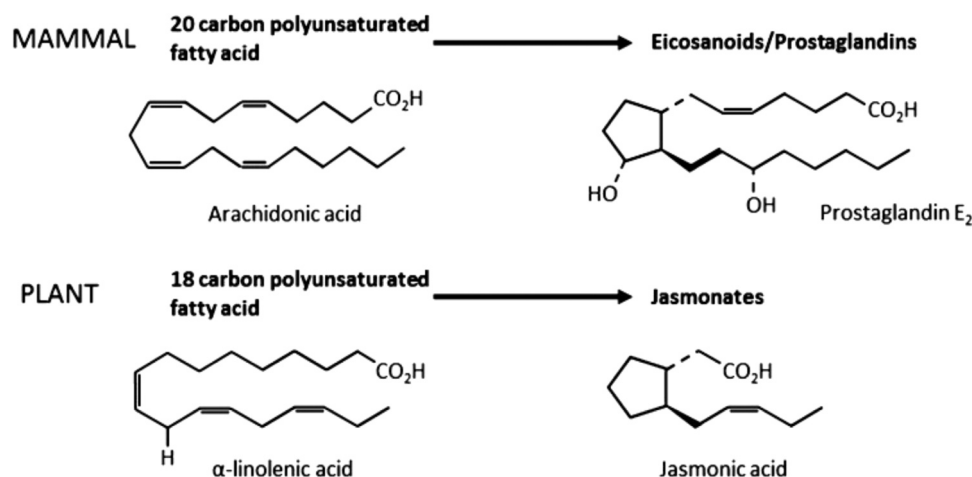


Fig. 3. The comparison between the plant jasmonate hormones and orthologous mammalian eicosanoids. Reproduced with permission from [48].

forms and the surrounding environment [44]. Mevinolin, more commonly known as lovastatin, is an example of a natural compound produced by the fungus *Aspergillus terreus* that inhibits one of the enzymes within the mevalonate pathway, 3-hydroxy-3-methylglutaryl-coenzyme A reductase [45]. High evolutionary conservation of the mevalonate pathway resulted in the successful use of lovastatin as a cholesterol-reducing medication in humans. Similarly to certain polyphenols, numerous terpenoids have also been shown to possess anti-inflammatory activity [35]. This activity has been observed in compounds that possess electrophilic centers (e.g., α,β -unsaturated carbonyl groups) that can react as Michael acceptors. Examples of these compounds include zerumbone, helenalin, α -santonin, and citral, among many others (Fig. 1) [35,36,38].

Polyphenolic compounds are, without a doubt, the most intensely studied group of specialized plant metabolites. Polyphenols have been proposed to be responsible for the health-promoting effects of a diet rich in vegetables, fruits, and herbs [26,27,46]. Studies focused on the direct antioxidant activity of polyphenols have dominated scientific literature for many years. However, multiple rigorous clinical trials have disproved the direct antioxidant activity hypothesis of polyphenolic compounds [4].

Emerging data suggest that plant polyphenols may interact with and modulate evolutionarily conserved cellular signaling pathways [2,4]. Howitz and Sinclair proposed that polyphenols evolved as signaling molecules in a common unicellular ancestor of animals and plants [2]. Polyphenols are ubiquitous molecules in plants and have the ability to play different roles, such as antimicrobial or anti-feedant activity [47]. Some polyphenols, such as salicylates, play an important role in controlling the plant's response to numerous biotic and abiotic stressors [48]. Interestingly, the same molecules may regulate the synthesis of proinflammatory substances in animals [33]. A comparison of oxylipin pathways illuminates the evolutionary conservation of biochemical molecules between plants and animals (Fig. 3) [48]. Therefore, polyphenols may have evolved as chemical cues that play a crucial role in the stress response in both plants and animals [47,48]. It has been recently shown in a *Drosophila* model that salicylates increase the synthesis of electrophilic pro-resolving lipids involved in the resolution of inflammation [33]. Future research should focus on better understanding whether natural polyphenols act directly as electrophilic pro-resolving molecules or if they upregulate the synthesis of these physiological electrophilic pro-resolving mediators.

During evolution, the animal phyla might have lost the ability to synthesize polyphenolic compounds in the same way that humans have lost their ability to synthesize ascorbic acid or certain amino acids [2,47]. However, animal cells still possess protein targets that respond to polyphenolic compounds. It has, furthermore, been shown that numerous polyphenols can interact with and modify the activity of various animal enzymes and transmembrane proteins *in vivo* [2,4,49]. The ability of polyphenols to interact with multiple endogenous targets has resulted in their classification as pan-assay interference compounds (PAIS) and their removal from the serious consideration as potential drug leads [31]. However, what is currently considered to be a weakness of these polyphenolic compounds may in fact be a potential stress response mechanism, *via* the evolutionary conservation of biochemical molecules between organisms [2].

Throughout evolution, plants acquired the ability to synthesize molecules to protect themselves from herbivory or omnivorous predation [4,41]. Some plant species developed biosynthetic pathways that allow them to transform compounds that might have primarily evolved as signaling molecules (terpenoids and polyphenols) into noxious phytochemicals [4,9,41,50,51]. However, another class of bitter-tasting molecules called alkaloids evolved as the most effective natural pesticide, deterring insects, as well as other herbivores and omnivores [4,9]. Alkaloids evolved to target very specific groups of proteins, which allow them to confer biological activity at low concentrations. The majority of currently approved drugs first identified in nature are alkaloids. Examples of these include morphine, codeine, ephedrine, galantamine, quinine, atropine, scopolamine, and papaverine, among many others [4].

Animals feeding on plants evolved warning systems to protect them from the poisonous effects of alkaloids [9]. Alkaloids evoke a bitter taste that serves as a warning to limit the intake of plants containing these chemicals. By trial and error and the observation of other animals, humans learned which plants were safe to eat and which provide health benefits. Traditional medicine approaches are oftentimes based on this knowledge acquired through generations by close observations of the surrounding environment.

Biologically active specialized metabolites are present in very complex matrices that makes the identification of these molecules challenging. The following section discusses the most common problems encountered when working with natural samples.

3. Common obstacles in drug discovery from natural matrices

One of the most challenging obstacles in drug discovery from natural products stems from the complexity of natural samples [5,52]. The drug discovery workflow from natural matrices usually starts with testing extracts or extract fractions to identify those characterized with biological activity. The phytochemical profile of natural samples varies from batch to batch; therefore, to assure study reproducibility, a phytochemical profile of the tested samples should be provided. Alternatively, the individual compounds responsible for biological effects should be identified, isolated, and their activity confirmed. One of the major challenges in screening complex mixtures for drug leads is the lack of efficient tools to identify and isolate biologically active compounds [5]. Traditional screening methods often require the isolation of individual compounds from mixtures through chemical separation, followed by dereplication and analysis. These processes are typically time consuming, labor intensive, and costly [53]. High throughput screening techniques mainly focus on synthetic libraries of individual compounds [13]. For mixtures, laborious isolation and characterization are needed to identify the pharmacologically active molecules. The most commonly practiced approach focuses on the isolation of individual compounds followed by testing them in their purified forms. This usually leads to isolation of the most abundant novel compounds or already well-characterized molecules.

There is a critical need to develop novel drug discovery approaches to speed up the process of identification of pharmacologically active compounds present in complex samples. Modern isolation techniques, including high speed countercurrent chromatography, have been introduced to speed up the process of the isolation of targeted metabolites [54–56]. Fractions obtained with the use of these methods increases the chance for the identification of biologically active natural compounds. Several other screening approaches that may be used at the beginning of the drug discovery pipeline are reviewed in Section 4.

The biological activity of isolated compounds does not always match the activity of the entire extract [57]. This discrepancy can often be attributed to the complexity of extracts where numerous compounds exert synergistic and/or antagonistic effects. If both the extracts and individual compounds are tested in *in vitro* assays, the difference may stem from the fact that compounds other than the isolated one might interact with the target (*i.e.*, an enzyme). In the case of phenotypic assays, multiple compounds may interact with numerous regulatory proteins, resulting in biological activity different from that observed for purified molecules. Another important issue related to the assessment of the biological activity of natural compounds is the purity of the isolated compounds. Oftentimes, the observed biological effects are the result of other compounds present in the isolated sample [58,59].

Natural compounds have also been characterized by pleiotropic effects, because of their ability to interact with multiple regulatory molecules in biological systems [2,30,49]. This has led to the classification of many of these molecules as invalid/improbable/interfering metabolic panaceas (IMPS) and pan-assay interference compounds (PAINS) [28–31]. The predominant paradigm in drug discovery focuses on designing exquisitely selective ligands in an attempt to reduce off-target effects [30]. However, multiple diseases are associated with the dysfunction of various biochemical processes that are controlled by networks of regulatory molecules [7].

Although considered negative, pleiotropic effects exerted by natural compounds may stem from their evolutionary potential to

act as signaling and regulatory molecules [2,4,49]. For example, many studies that have attempted to develop polyphenolic compounds into drugs have been unsuccessful, due to the inability of the polyphenols to act as selective ligands at desired biological targets [30]. This behavior may be compared to the biological activity of certain essential nutrients, such as vitamins, that interact with and modify multiple targets. Flavonoids, for example, have been previously called vitamin P; however, their vitamin-like effects have not been proven [30]. As previously mentioned, certain flavonoids may potentially play roles similar to that of essential fatty acids (previously referred to as vitamin F) in the regulation of inflammation by acting as essential electrophilic molecules. Future research should explore whether certain natural polyphenols, together with omega-3 fatty acid derivatives, act as essential electrophilic molecules involved in important nucleophilic addition reactions. Dietary phytochemicals have been considered to fall into the category of nutritional dark matter, an underrecognized class of small molecules that may play an important role in human nutrition beyond traditional nutrients [60]. Future research should consider the possibility that ubiquitous specialized metabolites may possibly play important roles as essential nutrients.

It must also be noted that, in many cases, the reported biological activity of natural molecules is only tested in *in vitro* assays, without further verification *in vivo*. This may be very problematic for molecules such as polyphenols which contain highly reactive groups, such as α,β -unsaturated carbonyls or hydrogen-bond forming hydroxyl groups. These molecules may be tagged as reactive when tested in *in vitro* assays that contain isolated proteins or cells. Additionally, polyphenols may react with other molecules present in the assay mixture, resulting in false positive results. To better understand the biological potential of these molecules, it is important to have them tested in the context of a living organism [6,7]. Additionally, *in vitro* assays often use high concentrations of the investigated molecules that may activate stress response pathways in the cells used in these assays. Activation of these pathways may mask the actual pharmacological potential that the investigated compounds may exert *in vivo*.

There is a need to use simple and high-throughput invertebrate drug screening assays at the beginning of the drug discovery pipeline in order to verify the activity of natural compounds in the context of a living organism at physiologically relevant doses. The use of simple, efficient, and high-throughput *Drosophila* and *C. elegans* assays in the identification of natural neuroprotective compounds is reviewed in Section 5.

4. Novel screening solutions to speed up the identification of bioactive compounds interacting with regulatory proteins

Different screening approaches have been proposed to speed up the identification of biologically active specialized metabolites present in complex matrices [5,52]. Previously published papers have thoroughly reviewed techniques most commonly used in the drug discovery process [5,52]. Here, we discuss the general principles of several screening techniques that can be used in the facilitation of the identification of biologically active compounds present in complex natural mixtures. Since numerous drugs used in the treatment of neurological disorders target transmembrane proteins, special emphasis is placed on approaches utilizing immobilized transmembrane proteins.

One of the first screening approaches used in drug discovery from complex natural samples involve either the use of filtration

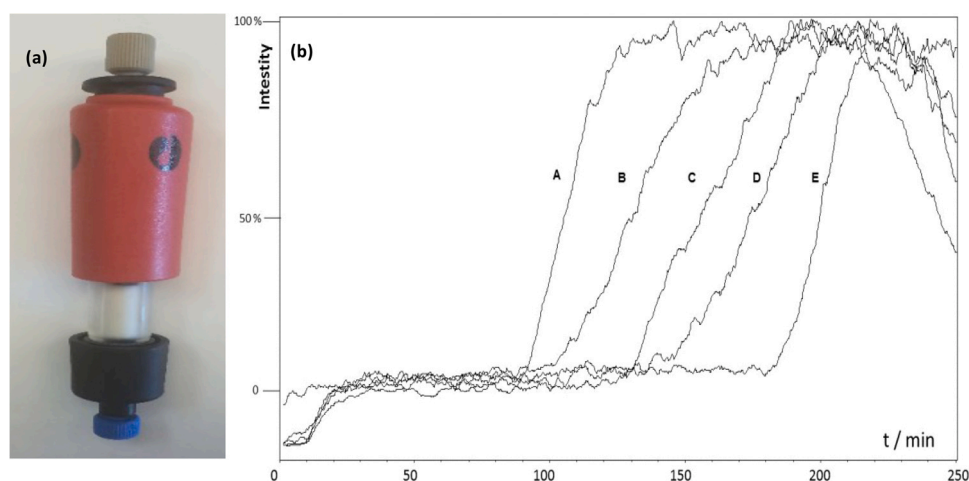


Fig. 4. Figure (a) Fully assembled cell membrane affinity chromatography column after packing IAM particles with the immobilized cell membrane fragments into a glass column; (b) Frontal chromatograms of (a) increasing concentrations of [3H]-epibatidine (A = 240 pM, B = 150 pM, C = 100 pM, D = 80 pM and E = 60 pM) on the $\alpha 3\beta 4\alpha 5$ nicotinic receptor column.

Panel (b) reproduced with permission from [76].

techniques or cytoplasmic proteins [5,52]. Detailed descriptions of these techniques, along with several examples of their use, can be found elsewhere [5,52,61–65].

In the ultrafiltration approach, semi-permeable membranes are applied to identify small molecules interacting with cytoplasmic proteins [63]. The targeted protein is incubated with the studied mixture, then followed by the ultrafiltration step. The complex formed between the cytosolic protein and the biologically active compounds does not permeate through the membrane, allowing for the identification of compounds interacting with the protein. Ultrafiltration requires relatively high concentrations of the targeted protein, as well as the use of approaches that allow for the discrimination between specific and nonspecific interactions between protein target and small molecules. Despite the aforementioned difficulties, several successful applications of ultrafiltration have been reported. For more details regarding this technique and for relevant examples, please refer to other review articles [5,52].

The immobilization of cytosolic proteins is another example of an approach developed to speed up the identification of pharmacologically active metabolites [5,52,66–75]. First, the cytosolic protein is attached to the surface of a column adsorbent (bioaffinity chromatography) or magnetic bead surface (ligand fishing). In this approach, the targeted protein is immobilized through chemical bonds and the protein C or N terminus. Since the immobilization process involves the use of covalent bonds, one of the crucial elements of the experimental procedure is to determine the activity of the protein after the immobilization process. This procedure is not useful for proteins whose activity is substantially altered by the immobilization procedure. Several successful applications of the immobilized cytosolic proteins have been reported in the identification of natural products modifying protein activity [5,66–75]. To avoid the alteration of cytosolic protein activity, several approaches that do not involve the immobilization step have been proposed. One of the approaches involves the incubation of the targeted protein in a reaction coil

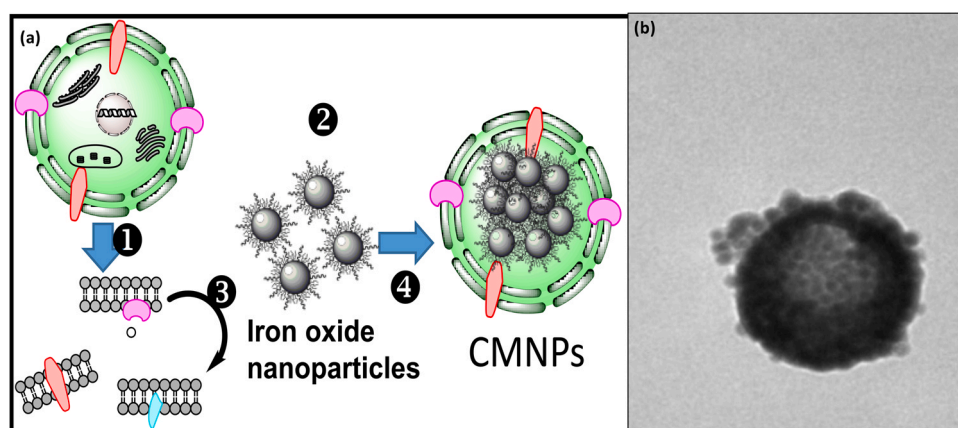


Fig. 5. (a) Overview of cell membrane encapsulated nanoparticles formation: (1) cells expressing targeted proteins, (2) iron oxide nanoparticles, (3) cell membrane fragments obtained after cell lysis, and (4) cell membrane encapsulated nanoparticles; (b) typical transmission electron microscope image of cell membrane encapsulated nanoparticles. Panel (a) reproduced with permission from [77].

positioned past the chromatographic column [65]. Following the separation step on a chromatographic column, the compounds are then directed into the reaction coil, where they are allowed to interact with the investigated protein. The interaction between cytosolic proteins and small molecules is detected based on the formation of a new product, inhibition of the enzymatic reaction, or decreased fluorescence.

While the development of novel screening techniques has been relatively successful for cytosolic proteins, more than half of the drug discovery programs target transmembrane proteins. However, the immobilization of transmembrane proteins can be even more challenging, as the activity of these targets depends also on preserving subtle interactions between these proteins and lipid bilayer. Several analytical approaches have been proposed, including cellular membrane affinity chromatography (CMAC) (Fig. 4), cell membrane coated (encapsulated) magnetic nanoparticles (Fig. 5), and carbon nanotubes modified with magnetic nanoparticles, which allow for the preservation of proteins in their natural bilayer environment [5,52,76,77].

Cell membrane fragments can be immobilized on a solid surface and subsequently used to screen complex matrices for compounds interacting specifically with the transmembrane targets. In all cases, the first step involves acquiring cell membrane fragments with the studied transmembrane protein. Cell lines stably overexpressing the proteins are usually used, although the application of human and animal tissues have also been reported. Cell membranes are harvested after cell lysis and the removal of cellular organelles using centrifugation at different speeds. Cell membrane fragments are subsequently immobilized on the surface of artificial membrane stationary phase particles (CMAC preparation) or used to encapsulate iron oxide nanoparticles or carbon nanotubes. Transmembrane receptors are immobilized without the formation of covalent bonds and still submerge into the lipid bilayer. The adsorption of cell membrane fragments without using covalent bonds excludes any unwanted modification of the functionality of the transmembrane proteins.

Different classes of transmembrane proteins have been reported to be successfully immobilized using CMAC technology, including ligand-gated ion channels, GPCRs, and protein transporters [5,78–84]. Recently developed nanoparticle encapsulation technology has been proven useful in the immobilization of ligand-gated ion channels and tyrosine kinase receptors and applied in the screening of complex mixtures for new ligands binding to nicotinic receptors, tropomyosin receptor kinase B, and epidermal growth factor receptors [52,77,85]. Discerning between specific and non-specific interactions is of crucial importance when using both CMAC and encapsulated nanoparticle technology. To rule out nonspecific interactions, negative control CMAC columns and cell membrane encapsulated nanoparticles are prepared using parental cell lines without the targeted protein. Small molecules that interact with the columns or the cell membrane coated nanoparticles that express the targeted protein, but that don't interact with the negative control columns/nanoparticles, are the molecules specifically interacting with the targeted protein. The identification of compounds that specifically interact with the studied target allows researchers to focus the isolation process only on those compounds and, therefore, speeds up the drug discovery process.

The activation of neurotrophic and neurogenic factors, as well as signaling pathways, such as brain-derived neurotrophic factor (BDNF) and neurotrophic tyrosine kinase receptor 2 (TrkB), has been gaining much attention in the possible prevention and treatment of

numerous neurological disorders, including major depressive disorder and neurodegenerative diseases [4,86]. It has been recently shown that major antidepressant drugs act by directly binding to TrkB neurotrophin receptors [86]. Certain natural compounds have also been previously shown to bind to TrkB, to mimic the effects of BDNF, and to activate the BDNF and TrkB signaling pathway [85,87]. TrkB neurotrophin receptors can be immobilized on the surface of chemically modified nanoparticles or artificial cell membrane fragments and used to screen natural samples, as was recently shown for gotu kola plant extracts [85]. Additionally, these immobilization techniques can also be utilized to study the interactions between the isolated compounds and the immobilized receptor [5,76]. It has been shown that TrkB signaling is linked to cholesterol levels and that cholesterol regulates TrkB signaling [86]. Our group has also recently been exploring the use of CMAC technology in assessing different levels of cholesterol on the interaction between immobilized TrkB neurotrophin receptors and natural compound ligands (unpublished data).

For more details regarding the preparation of CMAC columns and cell membrane encapsulated nanoparticles, please refer to previously published literature [5,52].

One of the critical decisions to make before using any of the presented screening techniques is to choose the plant species to be tested [88]. Some approaches commonly used in ethnopharmacology is either consulting historical documents that report the use of plant species as therapeutics or interviewing informants that may possess generational knowledge of a plant's medicinal abilities [89]. This approach lies at the foundation of one of the most recent and successful discoveries in natural product history: artemisinin, a potent antimalarial drug discovered by Dr. Youyou Tu. Dr. Tu consulted historical Traditional Chinese Medicine texts to identify plants that were used in the treatment of malaria and fever. This led to her eventual isolation of artemisinin, a potent antiprotozoal sesquiterpene lactone [89].

Although effective in the identification of compounds interacting with regulatory proteins, there is a chance that the screening assays may not be translatable or congruent with biological activity *in vivo*. Therefore, in order to develop and maintain a sustainable drug discovery pipeline, we argue that it is important to combine these approaches with *in vivo* screening. We propose the use of simple model organisms, such as *D. melanogaster* or *C. elegans*, at the beginning of the drug discovery pipeline combined with the use of natural product mixtures. Below, we present examples of high-throughput animal models of neurodegenerative diseases used to identify extracts with potential neuroprotective effects. Complex natural mixtures that prevent and/or treat neurodegenerative disorders can then be further screened for the compounds responsible for observed effects.

5. High-throughput invertebrate animal models for the identification of neuroprotective compounds

As discussed, working your way down the drug discovery pipeline can be a long and arduous process. Screening thousands of potential drug candidates can be time-consuming and costly, and often leads to uncertain success. There are many ways in which one can speed up the process of drug discovery while still ensuring valuable data is being collected, but choosing the right model organism to begin this process is of the utmost importance. The use of mammalian models tends to be extremely financially straining; rodents also have a long lifespan and relatively slow reproductive

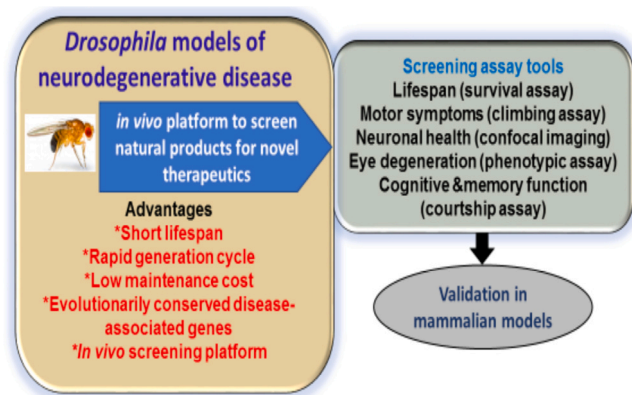


Fig. 6. *Drosophila* models of neurodegenerative diseases in the identification of neuroprotective compounds from natural products.

rates, making data collection take much longer than would be necessary in small invertebrate models, such as *D. melanogaster* or *C. elegans*. Therefore, choosing a small invertebrate model organism for large-scale screening of potential drug leads is an indisputable way to accelerate the discovery of novel pharmaceuticals.

5.1. *Drosophila* models of neurodegenerative diseases

Drosophila melanogaster, commonly known as the fruit fly, has been a powerful tool in genetics research and modeling human disease [90–93]. The completion of the *Drosophila* genome sequencing in 2000 revealed a high degree of conserved molecular pathways between the fly and human, with an estimated of 75% of known human disease-linked genes being conserved in the fly [94,95]. The annotated genome sequence of *Drosophila* is readily accessible through the online database “Flybase”. The presence of evolutionarily conserved genes with similar molecular functions makes *Drosophila* an ideal model to unravel the pathogenic mechanisms of human disease, including neurodegenerative diseases.

5.1.1. Advantages

Neurodegenerative diseases are mostly progressive disorders of the nervous system. These diseases pose a massive economic burden, due to the rise in the aging population. In recent years, several disease-associated genes have been identified in familial cases of Alzheimer’s (AD), Parkinson’s (PD), Huntington’s disease (HD), leading to the development of animal models to study the disease pathogenesis for therapeutic intervention [93]. The availability of these animal models helps to gain insight into disease mechanisms, since most human studies involve analysis of post-mortem tissues that fail to reflect early molecular changes during the pre-symptomatic stage. However, the use of rodent models during the early stages of the drug discovery pipeline is relatively costly, time-consuming, and poses ethical issues. Furthermore, *in vitro* screening in cell culture models often fails to mirror pathogenic events in the context of the whole organism. *Drosophila* offers several advantages over traditional rodent models, due to the rapid generation cycle, shorter life span, and lower maintenance cost [96]. The shorter lifespan allows for faster mating and generation of a larger number of progeny in a shorter period of time, thereby

reducing the overall experimental cost. Although there are differences in terms of the gross anatomical structure of the brain between humans and flies, several disease-associated genes and pathways are conserved. In addition, the simplicity of the *Drosophila* nervous system allows researchers to dissect the molecular function of target genes involved in the pathogenic mechanisms underlying neurodegeneration (Fig. 6).

5.1.2. Screening platform for therapeutics

The availability of different genetic tools for targeting *in vivo* gene manipulation makes *Drosophila* a lucrative model for screening natural products against neurodegenerative diseases like AD, PD and HD for therapeutic intervention. Several gene manipulation approaches have been successfully applied to generate transgenic fly models of neurodegenerative diseases that are readily available at the Bloomington Stock Center (<http://fly.bio.indiana.edu/>). For example, the GAL4-UAS binary system was first described by Brand and Perrimon, which allows for the tissue or neuronal subtype-specific expression of dominant mutants of genes, including α -synuclein-A30P or LRRK2-G2019S to model disease phenotype in transgenic flies [97,98]. RNA interference (RNAi) constructs can also be used with GAL4 drivers for targeted gene deletions in specific cells using the GAL4-UAS system [99]. Furthermore, specific neurons can also be labeled using fluorescent markers like green fluorescent proteins (GFP) that can be visualized using microscopy techniques [100]. Both forward and reverse genetic screens have been applied in fly models to unravel gene targets and molecular mechanisms underlying disease pathology in several neurodegenerative diseases [96]. In AD, excessive accumulation of amyloid-beta ($A\beta$) peptides is thought to induce neuronal dysfunction, and transgenic fly models of AD are generated that encode human amyloid precursor protein (APP) and mimic human disease pathology [101]. *Drosophila* models of AD provide a powerful toolkit to investigate not only the underlying pathogenic events during disease progression but also to screen and identify pharmacological modifiers of the disease phenotype [102]. Similarly, fly models of PD exhibit similar disease phenotypes, including loss of dopaminergic (DA) neurons, mobility defects, oxidative stress, mitochondrial dysfunction, and protein aggregation [103,104]. Moreover, *Drosophila* models have linked α -synuclein with tau, thereby suggesting highly conserved pathways between human and fly models in PD pathogenesis [105]. Transgenic flies over-expressing the human mutant Huntingtin (HTT) gene has been shown to recapitulate human disease phenotypes, including protein aggregation, neurodegeneration, and a reduced lifespan [106]. Aging remains the primary risk factor for the majority of neurodegenerative disorders, including AD and PD [107]. Interestingly, *Drosophila* models serve as an excellent platform for age-related studies due to short lifespan, which makes it attractive for both life and health span studies [108,109]. *Drosophila* models have been routinely used to screen and identify secondary plant metabolites that confer life span extension and improve overall health status. For example, several recent studies have employed *Drosophila* models to study the effects of both single compounds, such as the flavanol epicatechin and the flavonol fisetin, as well as complex extracts like cocoa powder, green tea extract, *Ludwigia octovalvis* extract, apple polyphenols, and black tea extract on lifespan and healthy aging. Another recent study identified the geroprotective effects of certain flavonoids like naringin, luteolin, chrysin in both *Drosophila* and *C.elegans* models along with underlying molecular mechanisms [46,110]. Our lab has recently used the environmental toxin-induced PD model in flies to

identify a polymethoxyflavonoid, Gardenin A, that confers neuroprotection by regulating the neuroinflammatory and cellular death responses [7]. Collectively, these neurodegenerative models provide an ideal *in vivo* platform for evaluating the efficacy and safety profile of drug candidates in the context of whole live animals [111].

5.1.3. Phenotypic screening tools

Drosophila models of neurodegenerative disease also offer several advantages as a screening platform for new drug discovery, due to the easily observable phenotypes associated with a specific disease condition [112,113]. For example, most neurodegenerative diseases generally exhibit age-dependent slowness or loss of locomotor ability. PD is a movement disorder characterized by rigidity, tremor, mobility defects, and postural instability. The negative geotaxis climbing assay is a fast and reliable way to monitor locomotion defects in flies based on their natural tendency to climb up against gravity [114]. Simple feeding assays can be employed to monitor the therapeutic effect of natural products by either mixing in the food or filter paper saturated with sucrose [115]. The toxicity levels can be assessed by survival assays to obtain optimal drug concentrations that can rescue the disease phenotype. Survival assays can also be used to examine the effect of potential drug candidates on lifespan and developmental defects. The loss of a specific subset of neurons is one of the most prominent features of neurodegenerative diseases, which can be easily monitored by microscopy techniques using fluorescent markers to tag specific neuronal subsets. For example, the loss of dopaminergic neurons and neuronal degeneration in the hippocampus have been observed in transgenic fly models of PD and AD, respectively, and can be used as a screening tool to monitor neuronal health for novel therapeutics [101,103]. Eye degeneration in transgenic fly models can also be used as a tool for pharmacological screening [92]. For example, the GAL4-UAS system-driven overexpression of the A β and tau or the α -syn transgene involved in AD and PD, respectively, induces apoptotic eye degeneration, leading to reduced eye size and ocular deformity [116]. Dementia and learning impairment are the characteristic symptoms of AD, which can be assessed in transgenic fly models by courtship behavior and olfaction tests [117]. The availability of powerful genetics tools and relatively simple phenotypic assays, along with their low maintenance and experimental costs, make *Drosophila* a versatile *in vivo* platform for screening the therapeutic potential of natural products [118].

5.1.4. Limitations

There are certain drawbacks of using *Drosophila* models to study neurodegenerative disease. Fly models don't allow the researcher to

accurately predict the dosage of drugs that is directly applicable to humans and should, therefore, be validated in mammalian models. In terms of drug testing, it is important to be aware of the differences in the physiology of the blood-brain barrier between mammals and invertebrates that might have differential effects on permeability and toxicity. However, the basis of neuronal transmission appears to be regulated by similar mechanisms in flies and humans [119]. Although fruit flies lack an adaptive immune system which might lead to differential immune responses in the context of certain neurological disorders [120], it serves as an ideal model to study innate immune responses associated with neurodegenerative diseases, due to the highly conserved mechanistic pathways between flies and humans. Flies also do not express certain pathogenic factors (e.g., α -Syn) that are important in human disorders. However, the vast availability of genetic tools, including the Gal4/UAS system and evolutionarily conserved molecular pathways, make it easier to model and study human diseases in flies effectively. Despite these limitations, the incorporation of invertebrate models such as *Drosophila* during the initial stages of drug screening have the ability to accelerate the discovery of positive hits in the context of a whole organism at a reasonable cost and leading to subsequent validation in mammalian models.

5.2. *C. elegans* models of neurodegenerative diseases

Caenorhabditis elegans is a small roundworm—about 1 mm in length at maturity. The legacy of *C. elegans* began when Dr. Sydney Brenner published a paper on its genetics. He was determined to establish this nematode as a model organism in research within cell and molecular biology by publishing a paper on its genetics [121]. He was also particularly interested in neuronal development, and his efforts eventually earned him the Nobel Prize in Physiology or Medicine in 2002. Since that time, *C. elegans* has become the only organism in history to have a completed diagram of neuronal circuitry [122], as well as the first multicellular organism to have its genome fully sequenced. This genomic sequencing, as well as other screenings, have led to the discovery that approximately 38% of genes in worms have a functional human ortholog—a necessary attribute in studying neurodegenerative disease [123]. Furthermore, visualization of the expression patterns of specific genes in live animals, such as worms, was made possible in 1994 with Dr. Martin Chalfie's introduction of green fluorescent protein (GFP) reporter technology [124]. In recent history, *C. elegans* as a model organism has grown to become an essential tool in many research

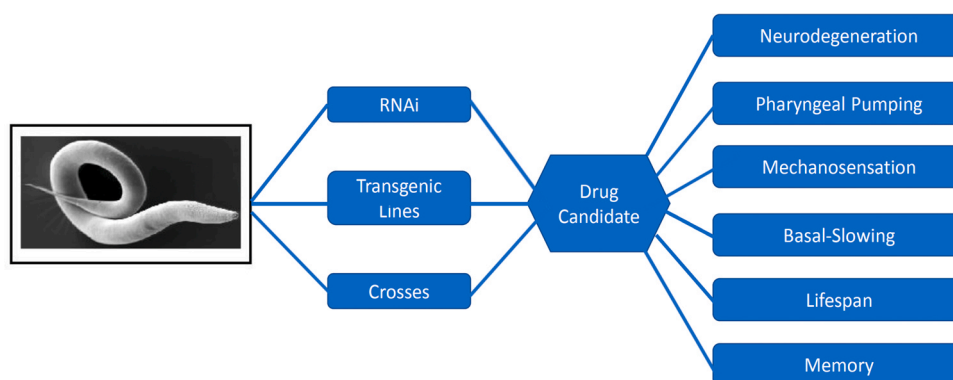


Fig. 7. *C. elegans* models of neurodegenerative diseases in the identification of neuroprotective compounds from natural products.

laboratories—used to gain a greater understanding of the molecular mechanisms of many diseases.

5.2.1. Advantages

C. elegans is easily cultured in the lab because of its minute size, short lifespan, and rapid reproductive rates. It is non-parasitic, and its transparent anatomy makes it a very useful organism for genetic screening. The majority of the animals within the species are hermaphroditic [125], with about one in every thousand being male. These males can be induced to become more abundant in subsequent generations by methods such as heat shock, and they possess a specialized projection on their tails which is used strategically for mating [126]. This trait makes crossing strains in this organism a straightforward task. *C. elegans* is also one of the simplest organisms with a well characterized nervous system. In the hermaphrodite, this system is composed of a total of 302 neurons [127]. These animals are also capable of being frozen for long-term storage and are viable when thawed, making it possible to create a “worm library” containing a multitude of different strains which is useable for years [128].

5.2.2. Screening platform for therapeutics

Genetic manipulation is relatively simple in *C. elegans*. This makes studying the effects of differential gene expression in regards to neurodegeneration and the application of potential drug candidates a valuable tool in these worms. One of the ways in which this process is done is by a gene knock-down technology called RNA interference (RNAi)—a process first discovered in *C. elegans* itself. This strategy involves first delivering double-stranded RNA (dsRNA) to the worms by methods such as soaking in a concentrated buffer, feeding of bacteria expressing dsRNA, or injection [129]. It has also been found that worms neuronally expressing SID-1, a transmembrane protein, have an increased response to being fed dsRNA [130]. This dsRNA delivery leads to the specific degradation of the corresponding mRNA, effectively knocking down the gene of interest and its expression with the nematode [129]. Fortunately, RNAi feeding libraries are commercially available—two of which being the Ahinger and the Vidal library. These libraries contain clones of the vast majority of all *C. elegans* genes, making the likelihood that one's gene of interest is available to be knocked down with RNAi quite probable. The availability of this technology and its use in any larval stage of development in *C. elegans* makes it possible to strategically knock down a variety of genes in the worms, as well as to study their effects in regards to neurodegenerative diseases, such as AD, PD, or HD, as well as any potential compounds being tested to treat them.

Another advantageous tool used when screening a variety of drug candidates to combat neurodegenerative disease within *C. elegans* is the use of transgenic animals that either over-express or ectopically express certain genes. The gene of interest is generally associated with a certain fluorescent marker, such as GFP or mCherry (a monomeric red fluorescent protein) [131]. These animals can be synthesized using DNA transformation by microinjection. This involves injecting manipulated genetic material into the gonads of hermaphrodites at the L4 stage of development. This genetic material then enters the developing oocytes, where it will be expressed with variable probability in the F1 generation. After successful microinjection, the use of a co-injection marker allows for worms expressing the mutation to be specifically picked under a fluorescent dissecting scope. Furthermore, the modulation of gene expression within these transgenic animals is typically achieved by promoter-

driven expression. It is possible to direct the selected transgene to be expressed within the neuronal subtype associated with specific neurodegenerative disease pathology [132]. For example, Alzheimer's disease can be characterized by the presence of amyloid-beta ($A\beta$) plaques and hyperphosphorylated tau aggregates called neurofibrillary tangles [133]. It has also been shown that the glutamatergic neurons within the hippocampus are the subtype primarily affected by these $A\beta$ -aggregates [134]. Therefore, when studying Alzheimer's disease in *C. elegans*, one might use a transgenic strain which expresses $A\beta$ strictly within the glutamatergic neurons. Using this combination of both amyloid-beta and GFP being expressed within the glutamatergic neurons, one can rapidly and effectively study the effects of Alzheimer's disease within live animals, as well as screen a multitude of compounds for their potential efficacy in fighting this disease. This method is also translatable to a wide variety of neurodegenerative diseases.

5.2.3. Phenotypic screening tools

There are a wide variety of assays that can be used in order to study the effects of various compounds on neurodegenerative disease and aging in *C. elegans* (Fig. 7). Neuronal subtypes expressing a fluorescent marker allow for the scoring of neuronal health within the worms at various days post-hatching. Pharyngeal pumping assays can be used to measure either the frequency or amplitude of pharynx contractions. These parameters can be used as indicators of neuronal health. Using an eyelash pick, soft-touch assays can be performed on the worms in order to assay mechanosensation. This is done by gently stroking the worm at specific areas of both the head and tail, in order to stimulate six precise sensory neurons and assay their health. A healthy worm should respond to the touch by moving either forward or backward in response to it [135]. Furthermore, dopaminergic-mediated basal slowing responses within the worms can also be used to study diseases like Parkinson's. This mechanosensory response is described as the natural slowing in locomotion that occurs whenever a healthy worm encounters food. This response is dopamine-dependent and therefore can be used to study the neurodegeneration that occurs within Parkinson's Disease [136]. Lifespan assays, as a method of detecting changes in the aging process, have also been extensively used in *C. elegans* to assay various gene knockdowns and potential drug candidates [137]. In fact, both the natural products baicalein [138] and quercetin [46] have been shown to increase both stress resistance and lifespan in *C. elegans* using an assay such as this. Even memory, by the use of habituation assays, can be analyzed within these extremely flexible animals. These assays include a variety of both visual and touch stimuli [139]. These are just a handful of the assays out of the very large toolbox that *C. elegans* researchers have to draw from. As evidenced above, *C. elegans* is an incomparable resource to be used within the first steps of the drug discovery pipeline.

5.2.4. Limitations

As with any disease model, there are certain drawbacks to the use of *C. elegans* as a model organism in the drug discovery pipeline. While the minute size of the worm leads to ease of handling and storage, this small stature can also be a limitation when it comes to biochemical analysis. Traditionally, this means that whole worm extracts must be analyzed rather than singular animals. This can cause uncertainty when attempting to isolate a tissue-specific signal. As with *Drosophila*, compound testing in worms also cannot give the researcher an accurate prediction of the concentration or dosage of

the compound that will be beneficial in humans. Therefore, both the safety and efficacy of these compounds must be further verified by the use of mammalian models. *C. elegans* also lacks the complex body plan and organ structure seen in higher mammals, including a lack of most internal organs, a circulatory system, skeletal elements, and defined fat cells. This can cause concern over the translatability of pharmacological data from worms to humans. Despite these limitations, the utilization of a *C. elegans* model of disease within the introductory phases of the drug discovery process is a distinguished way to facilitate the screening of potential drug leads with rapid turnaround rates and at a low cost to the researcher.

6. Conclusions

Specialized metabolites synthesized by plants, bacteria, and fungi play an important role in the process of discovery of novel drug hits and leads. To overcome the common problems encountered when working with natural products, novel approaches are proposed to be introduced at the beginning of the drug discovery pipeline. The combination of bioaffinity based screening assays along with high-throughput *in vivo* invertebrate animal models is recommended as a screening funnel in both building and maintaining a sustainable drug discovery pipeline for neurodegenerative disease. This concept can be easily optimized with other disease models by using properly designed bioaffinity assays and appropriate invertebrate animal models. This screening funnel facilitates the identification of biologically active molecules that interact with regulatory proteins and provides the opportunity to verify the activity of the identified compounds in the context of a living organism. While mostly pursued as potential drug candidates, certain specialized metabolites characterized with pleiotropic effects may better fall into the category of essential nutrients. Future research should consider the possibility that certain abundant dietary phytochemicals lacking drug-lead characteristics may play an essential role in biological systems, as is the case with other essential nutrients such as vitamins or fatty acids. Discovery of pharmacologically active compounds *via* the use of natural products is both a historical and promising source of successful drug leads.

CRedit authorship contribution statement

Urmila Maitra: Conceptualization, Writing – original draft, Writing – review & editing. **Cayman Stephen:** Conceptualization, Writing – original draft, Writing – review & editing. **Lukasz M. Ciesla:** Conceptualization, Writing – original draft, Writing – review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Lukasz Ciesla collaborates with Regis Technologies, the provider of the IAM.PC.DD2 particles utilized in the development of CMAC technology.

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