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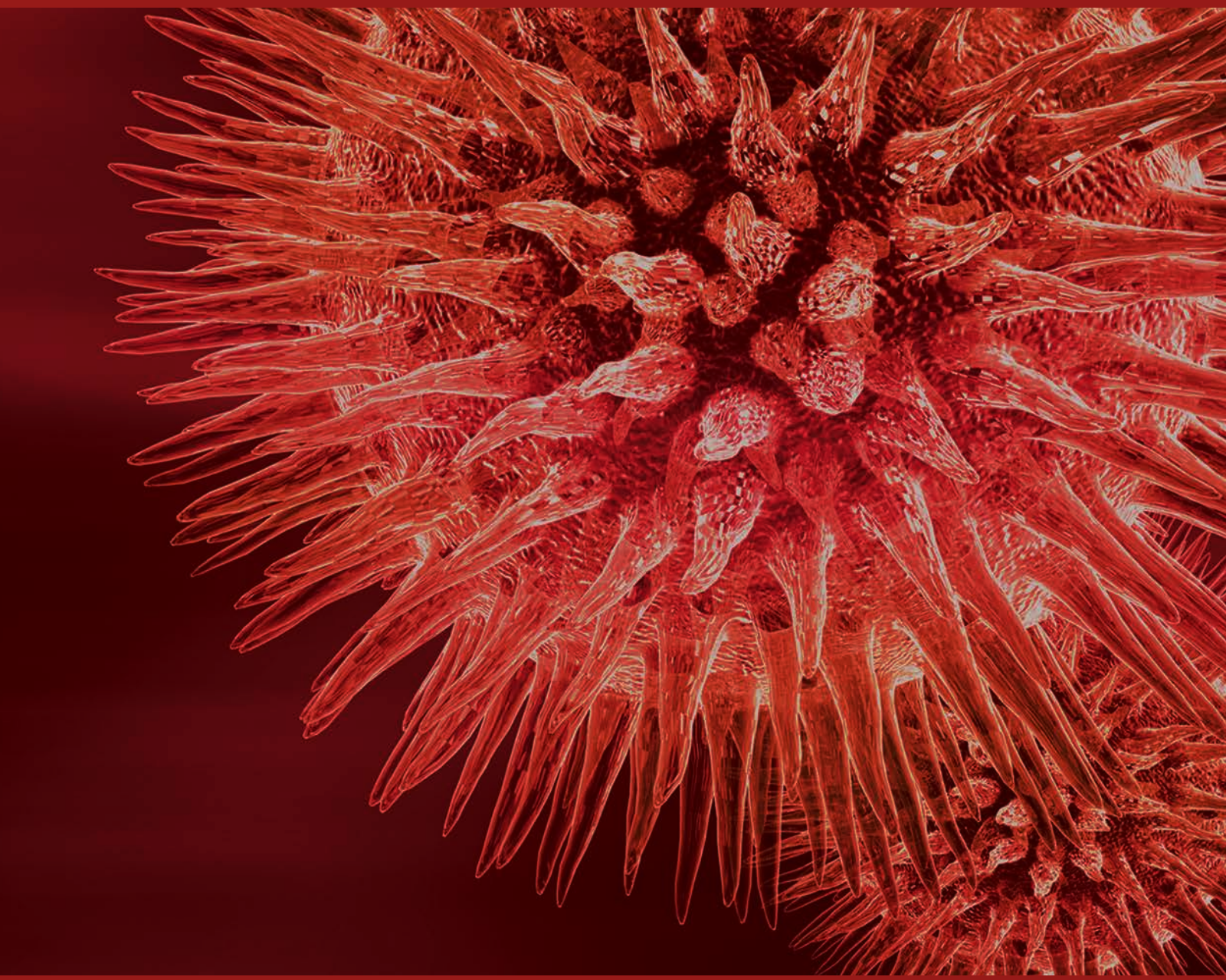
Contaminants of Emerging Concern: From the Detection to Their Effects on Human Health

Guest Editors: Fernando Barbosa Junior, Andres Campiglia, Bruno Rocha, and Daniel Cyr



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Editorial

Contaminants of Emerging Concern: From the Detection to Their Effects on Human Health

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In the past years with the advance of analytical instrumentation/methods, some chemicals are being discovered in water and other environmental matrices that previously had not been noticed or are being detected at levels that may be diverse more than expected. These are often generally referred to as "contaminants of emerging concern" because the potential risk to human health and the environment associated with their presence, frequency of occurrence, or source may not be known. Some of these compounds include household and industrial chemicals such as same retardants, plasticizers, detergent compounds, pharmaceutical and personal care products, fragrances, antimicrobial cleaning agents, nanomaterials, and some toxic elements [1–5].

This special issue affords the opportunity to bring together the results of 5 research articles and 1 review paper covering several aspects related to this topic.

An overview of emerging contaminants and associated human health effects was presented by M. Lei et al. The authors have summarized the conclusions of the comprehensive epidemic literature and representative case reports relevant to emerging contaminants and the human body to address concerns about potential harmful health effects in the general population. They concluded that the current evidence is not conclusive and comprehensive, suggesting prospective cohort studies in the future to evaluate the associations between human health outcomes and emerging environmental contaminants.

W.-H. Yu et al. evaluated the expression and function of Oat1 and Oat3 after administering arsenic and mercury containing traditional Chinese medicine (realgar and cinnabar) *in vivo* in mice. The authors have found that the traditional Chinese medicine investigated is probably related to kidney damage through inhibiting several members of the organic anion transporters (such as OAT1 and OAT3).

Q. Liu et al. evaluated whether there were miRNA and mRNA aberrant expression profiles and potential role in malignant transformation of 16HBE induced by cadmium. Their results provided a link for the miRNA-mRNA integrated network and implied the role of novel miRNAs in malignant transformation of 16HBE induced by cadmium.

N. Sarker et al. determined the levels of various metals in three different floral honeys from Bangladesh. Several physical parameters were also determined. According to the authors, honeys from Bangladesh are of good quality and the tested parameters met the requirements set by the International Honey Commission. The heavy metal concentrations were also within the limits, indicating their purity. Moreover, the higher concentrations of Fe in the investigated honeys signify that these honeys are a good source of these elements, which is very important for the human diet.

Q. Hu et al. concluded that mycotoxins from mycoinsecticides have limited ways to enter environments. Moreover, according to the authors the risks of mycotoxins from mycoinsecticides contaminating foods are likely controllable.

I. Macharia investigates the determinants of pesticide-related cost of illness (COI) and acute symptoms, using a balanced panel of 363 farmers interviewed from seven major vegetable producing districts of Kenya. The author observed

that the personal protective equipment (PPE), education level, record keeping, and geographical location considerably determined health impairments. Moreover, encouraging the proper use of PPE and record keeping of pesticide use could greatly reduce poisoning cases and COI.

Acknowledgments

We would like to thank all the reviewers that have contributed their time and insight to this special issue.

Fernando Barbosa Junior
Andres Campiglia
Bruno Rocha
Daniel Cyr

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Review Article

Risks of Mycotoxins from Mycoinsecticides to Humans

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There are more than thirty mycotoxins produced by fungal entomopathogens. Totally, they belong to two classes, NRP and PK mycotoxins. Most of mycotoxins have not been paid sufficient attention yet. Generally, mycotoxins do not exist in mycoinsecticide and might not be released to environments unless entomogenous fungus proliferates and produces mycotoxins in host insects or probably in plants. Some mycotoxins, destruxins as an example, are decomposed in host insects before they, with the insect's cadavers together, are released to environments. Many species of fungal entomopathogens have the endophytic characteristics. But we do not know if fungal entomopathogens produce mycotoxins in plants and release them to environments. On the contrary, the same mycotoxins produced by phytopathogens such as *Fusarium* spp. and *Aspergillus* spp. have been paid enough concerns. In conclusion, mycotoxins from mycoinsecticides have limited ways to enter environments. The risks of mycotoxins from mycoinsecticides contaminating foods are controllable.

1. Introduction

Entomopathogenic fungi are the important factors to control natural populations of many pest species. Several species have been developed as biological control agents (BCAs) from more than 800 species of fungal entomopathogens in the world. In the BCAs, there are more than 100 mycoinsecticides for commercial use worldwide [1]. And at least 30 mycoinsecticides were registered in China; among them, *Beauveria bassiana* is the most popular species up to 14 products for control of locust, pine moth and diamond back moth, and so forth; *Metarhizium anisopliae* and *Paecilomyces lilacinus* with the 8 and 7 products were registered to application of grubs, corn borer, aphids and whitefly, and so forth (http://www.chinapesticide.gov.cn/hysj/index.jhtml). There is much public interest in the use of fungal biological control agents as alternatives to chemical pesticides. However, there are some concerns about the safety of BCAs to human health. Many researches about the safety of BCAs have been carried on since the 21st century. Through assessing the risks of infections, allergies, and poisoning/toxic effects [2–4], the most used mycoinsecticides such as *B. bassiana* and *M. anisopliae* were verified as safe biocontrol agents [5–7]. However, many entomopathogens produce mycotoxins which pose risks to humans and the environment; how these mycotoxins affect human health and environment are not clear yet.

Numerous mycotoxins were found from fungal entomopathogens. They can be characterized to lots of classes according to the chemical structure [8]. But briefly, they can be classified as two main classes: nonribosomal peptide (NRP) synthetase mycotoxins and polyketide (PK) synthase mycotoxins according to their biosynthetic pathways.

2. NRP Mycotoxins

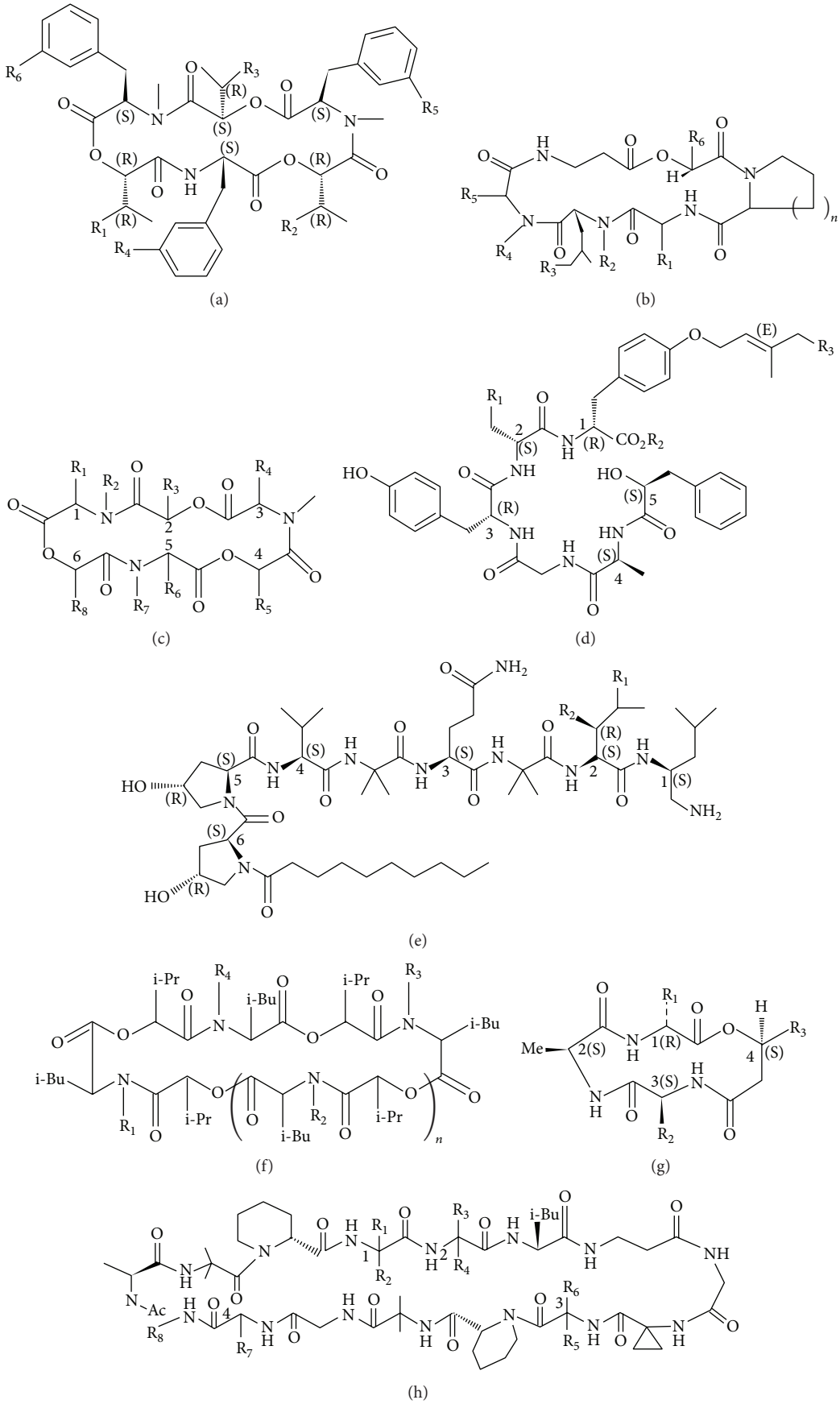
Fungal entomopathogens produce various kinds of NRPs that are usually taken as pathogenic factor of these fungi species. Chemically, NRPs are the secondary metabolic compounds mainly composed of specific or modified amino acids and hydroxyl acids. They are synthesized via thiotemplate multienzyme mechanism of multifunctional enzyme complex system other than on ribosome. NRP synthetase gene of fungi is an open reading frame encoding a peptide chain composed of several modules, which activate amino acids and combined with a specific peptide product. Each module has a number

Table 1: NRP mycotoxins of fugal entomopathogens.

Mycotoxin name	Producing fungal entomopathogen	Bioactivity	References
Cicadapeptins	*Cordyceps heteropoda* *Isaria sinclairii*	Inhibits acetylcholine- (Ach-) evoked secretion Antibacterial activity	[40–42]
Culicinins	*Culicinomyces clavisporus*	Inhibits breast tumor cells	[43, 44]
Efrapeptins	*Tolypocladium* spp.	Insecticidal activity; anti-immunity; antifungal activities; inhibitors of F1F0-ATPase	[45, 46]
Neoefrapeptins	*Geotrichum candidum*	Insecticidal activities	[47]
Bassianolides	*Beauveria bassiana* *Verticillium lecanii*	Inhibit muscle contraction Insecticidal activities	[48]
Beauvericins	*Beauveria* spp. *Paecilomyces* spp.	Insecticidal Fungicidal activity Cytotoxic Antitumor	[28, 49–51]
Beauverolides	*Beauveria* spp. *Paecilomyces* spp.	Inhibits insect immunity	[26]
Beauveriolides	*Beauveria* spp.	Antiatherogenic Antiobesity activities	[26]
Conoideocrellide	*Conoideocrella tenuis* *Paecilomyces militaris*		[52]
Paecilodepsipeptides	*Paecilomyces cinnamomeus*	Antiproliferative activity Antitumor activity	[52–55]
Cordycommunin	*Ophiocordyceps communis*	Inhibits *Mycobacterium tuberculosis*	[56]
Destruxins	*Metarhizium anisopliae* *Aschersonia* sp.	Insecticidal Herbicidal Cytotoxic	[26, 32, 33, 57]
Hirsutellides	*Hirsutella kobayasii*	Antimalarial	[58]
Hirsutides	*Hirsutella* spp.		[59]
Isariins	*Isaria cretacea*	Insecticidal activity	[26, 60]
Isaridins	*Isaria* spp.	Inhibits growth of *Plasmodium falciparum*	[26, 61, 62]
Isarolides	*Isaria* spp.		[26]
Serinocyclins	*Metarhizium anisopliae*	Insecticidal activity	[63]
Verticilides	*Verticillium* spp.	Inhibits acyl-CoA: cholesterol acyltransferase of CHO cells Inhibits ryanodine receptors of cockroach	[64–66]
Cyclosporines	*Beauveria* *Verticillium* *Trichoderma polysporum* *Cylindrocarpon lucidum*	Insecticidal activities Immunosuppressive effect	[26]
Cordyheptapeptides	*Cordyceps* spp.	Antimalarial activity Cytotoxicity to Vero cell lines	[67, 68]

of domains, and a specific reaction is catalyzed by one domain. The main domains include adenylation domains (A domains), thiotion domains (T domains), condensation domains (C domains), epimerization domains (E domains), and methylation domains (M domains) [9].

To date, more than twenty kinds of NRPs were isolated and identified from entomogenous fungi genera: *Beauveria, Conoideocrella, Cordyceps, Culicinomyces, Hirsutella, Isaria, Metarhizium, Paecilomyces, Verticillium*, and so forth. These NRPs include bassianolides, beauvericins, beauverolides, beauveriolides, cicadapeptins, conoideocrellides, cordycommunins, cordyheptapeptides, culicinins, cyclosporin, destruxins, efrapeptins, enniatins, hirsutellides, hirsutides, isariins, isaridins, isarolides, paecilodepsipeptides, and serinocyclins (Table 1, Figures 1 and 2). Every NRP above includes a series of analogues. Based on the molecular structures, the NRPs could be divided into chain peptides (e.g., cicadapeptin and efrapeptin) and cyclic peptides including a subdivision of cyclopeptides and cyclodepsipeptides. Cyclopeptides are cyclic structures built by amino acid residues through peptide bonding (e.g., cyclosporin), while cyclodepsipeptides are lactone compounds consisting of amino acids and hydroxyl acids which are connected by peptide bonds. Most of the NRPs belong to



(a) (b) (c) (d) (e) (f) (g) (h)

Figure 1: Continued.

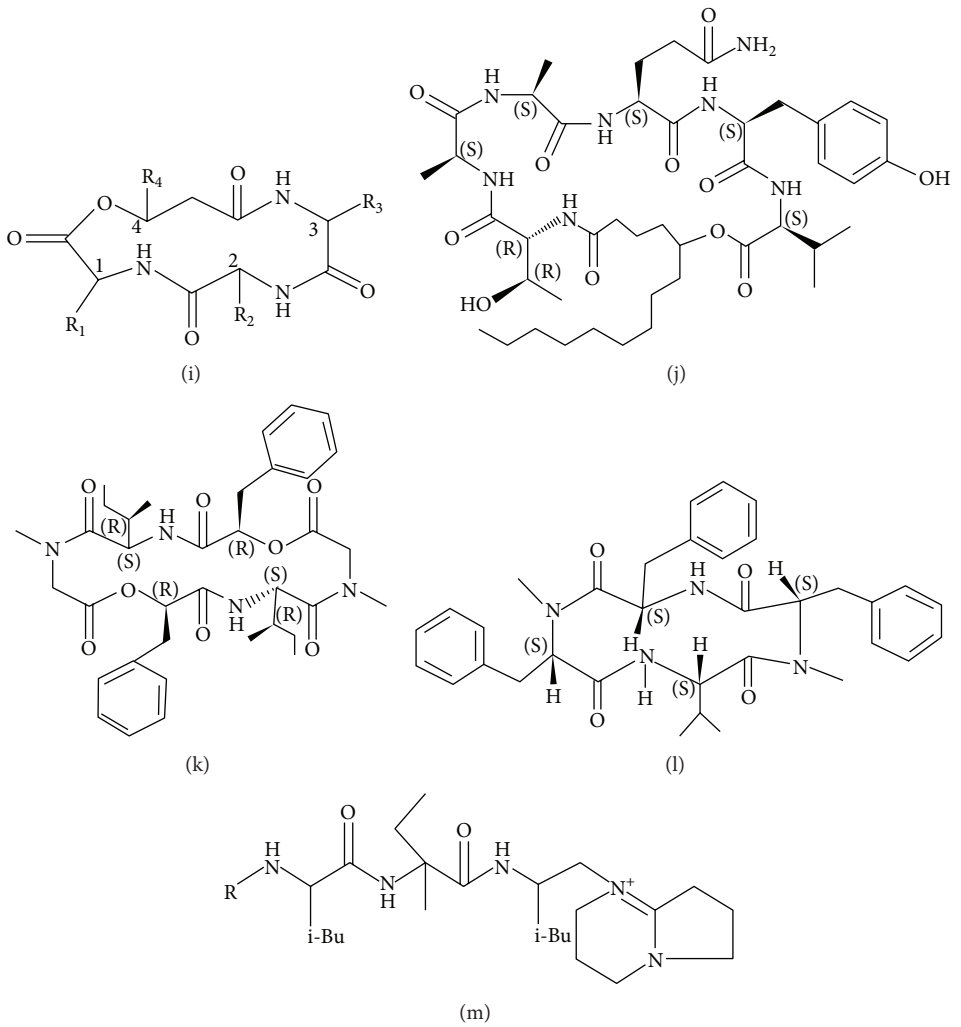


Figure 1: The structure of beauvericins (a), destruxins (b), enniatins (c), conoideocrellide (d), cicadapeptins (e), bassianolides (f), beauveriolides (g), neoefrapeptins (h), beauverolides (i), cordycommunin (j), hirsutellide (k), hirsutide (l), and efrapeptins (m).

the group of cyclodepsipeptides [10]. To date, destruxins, beauvericins, and enniatins are the best researched NRPs. However, their detailed biosynthesis, biotransformation, and behavior and fate in the environments are not clear yet.

In all NRPs of entomogenous fungi, beauvericin is considered as emerging mycotoxins likely contaminating the foods and products including rice, wheat, maize, follow-up infant formula, and Chinese medicinal herbs [11–15]. The fungal entomopathogens of *Beauveria* spp., *Paecilomyces* spp., and *Isaria* spp. produce beauvericins [16–18]. Traces of beauvericins were also detected in animal tissues and eggs [19, 20]. However, the cases of contaminations of beauvericins and enniatins are all from the infection of various *Fusarium* species other than entomogenous fungal species [15, 19, 21–25]. Chemically, beauvericins are a kind of cyclic hexadepsipeptide with alternating methyl-phenylalanyl and hydroxy-iso-valeryl residues (Figure 1(a)). Several documents reviewed beauvericins [15, 19, 21]. Totally 11 analogues of beauvericin were found [26]. The insecticidal effects of beauvericins at a microgram level were reported in several insects [21]. The cytotoxicity of beauvericins on human cells and cancer cells was also discovered [27–29]. Acetyl coenzyme-A (acyl-CoA: cholesterol acyltransferase, ACAT) is probably the target protein of beauvericins, while some research reports indicated that beauvericins might act as ionophores [30, 31].

Destruxins were isolated from culture medium of entomog-enous fungii *M. anisopliae* and *Aschersonia* sp., and the fungal phytopathogen *Alternaria brassicicola* [32] (Figure 1(b)). Among 39 destruxin analogues, destruxins A, B, and E (DA, DB, and DE, resp.) are the most analogues and show substantial bioactivity [33]. However, the linear molecule resulting from the opening of the DA cycle is not toxic and DE would degrade to less toxic DE-diol upon enzymatic action [33]. Destruxins have insecticidal activity against many pests with various mode of action including contact action, gut toxicity, antifeedant effect, and ovicidal and oviposition deterrent activities [34]. Destruxins damage the innate immunity of insects [35–37]. Destruxin maybe acts as a kind of calcium ionophore and an inhibitor of V-H+-ATPase [38]. The antiviral, antitumor, and herbicidal

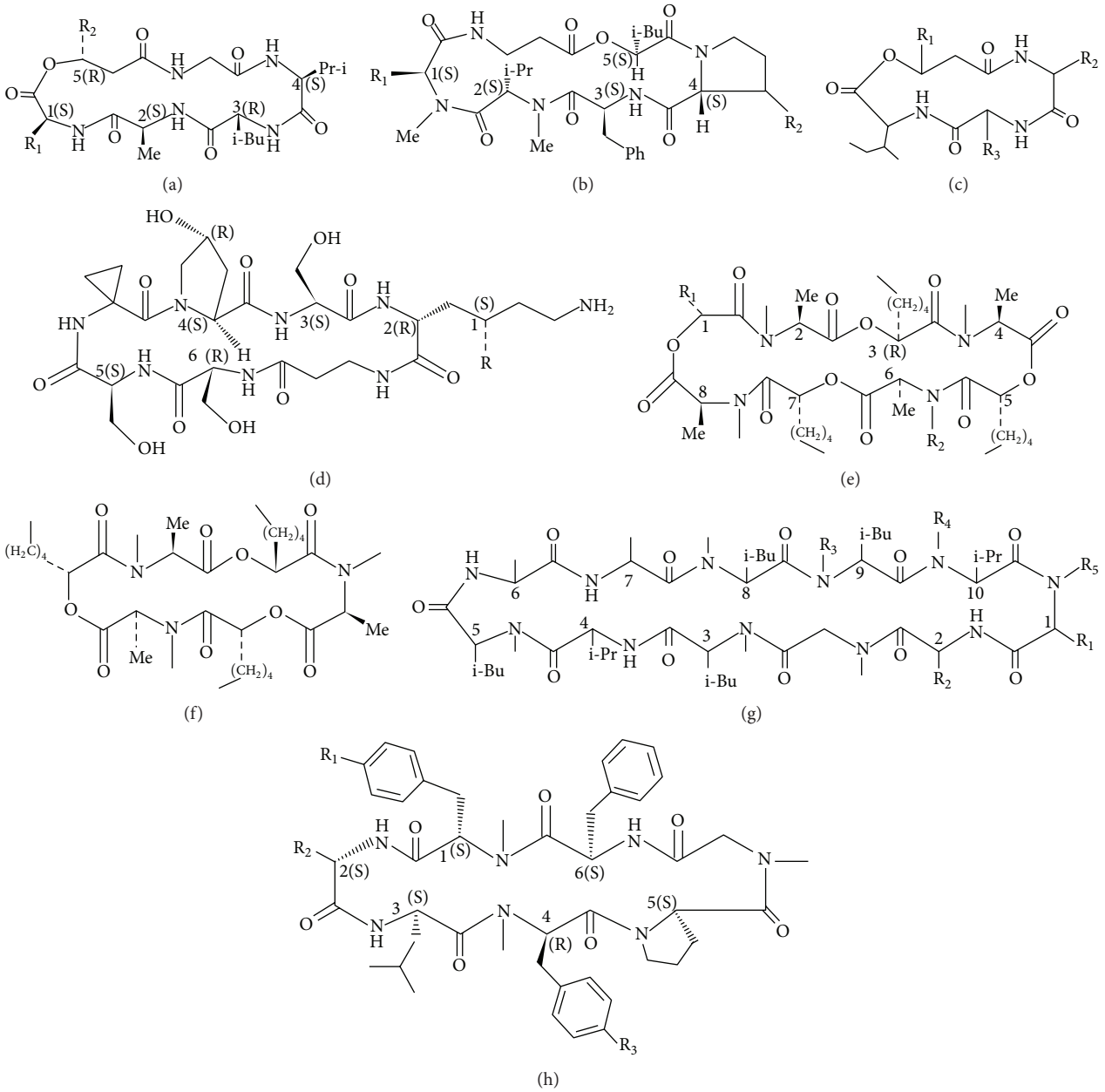


FIGURE 2: The structure of isariins (a), isaridins (b), isarolide (c), serinocyclins (d), verticilides A (e), verticilides B1 (f), cyclosporines (g), and cordyheptapeptide (h).

activities and cytotoxicity were reported as well [33]. Destruxins were decomposed in host insects before they, with the cadaver, were released to environments, so it is unlikely to contaminate the food chains [39]. In fact, there are no records about residues of destruxins in agricultural products and foods.

Enniatins could be produced by the fungal entomopathogen, *Verticillium hemipterigenum* BCC 1449 [69]. Enniatins are N-methylated cyclohexadepsipeptides, composed of three units each of N-methylated branched-chain L-amino acid and D-2-hydroxy acid arranged in an alternate fashion (Figure 1(c)). To date, 29 enniatins have been isolated and characterized, either as a single compounds or as mixtures of inseparable analogues [70]. Enniatins have multiactivities including antifungal, antibiotic, and cytotoxic properties. Fusafungine, one drug developed from a mixture of enniatins, is used as a topical treatment of upper respiratory tract infections by oral and/or nasal inhalation. Enniatins inhibit ABC transporters [71]. Enniatins are also a common contaminant in grain-based foods, but they were produced by the fungal species of *Fusarium* spp. other than entomopathogens [11, 12, 72–74].

There is no information about other NRP mycotoxins influencing environments and human health.

Table 2: PK mycotoxins of fugal entomopathogens.

Mycotoxin name	Producing fungal entomopathogen	Bioactivity	References
Annullatins	*Cordyceps annullata*	Exhibit potent agonistic activity toward the cannabinoid receptors CB1 and CB2.	[87]
Cryptosporioptide A	*Cordyceps gracilioides*	Inhibit the activity of protein tyrosine phosphatases	[88]
Cytochalasins	*Metarhizium anisopliae*	Inhibitor of the actin-cofilin interaction	[84, 85]
Farinosones A, B, and C	*Paecilomyces farinosus*	Cytotoxic	[89]
Fumosorinones	*Isaria fumosorosea*	Inhibits tyrosine phosphatase 1B (PTP1B) to treat type 2 diabetes mellitus (T2DM)	[90]
Indigotides	*Cordyceps indigotica*		[91]
Militarinones	*Paecilomyces militaris*	Cytotoxic	[92]
Opaliferin	*Cordyceps* sp.		[93]
Pinophilin C	*Cordyceps gracilioides*	Inhibit the activity of protein tyrosine phosphatases	[88]
Tenellin	*Beauveria bassiana*		[76, 78]
Terreusinone A	*Cordyceps gracilioides*	Inhibit the activity of protein tyrosine phosphatases	[88]
Tenuipyrone	*Isaria tenuipes*		[94]
Torrubiellones	*Torrubiella* sp.	Antimalarial	[95]
13-Hydroxyindigotide A	*Cordyceps indigotica*		[96]
8-O-Methylindigotide B	*Cordyceps indigotica*		[96]
Oosporein	*Cordyceps cardinalis*	Antibiotic Antifungal Antitumor	[83, 97]
Bassianin	*Beauveria* spp.	Inhibits erythrocyte membrane ATPase and inhibits Ca^{2+}-ATPases	[98]

3. PK Mycotoxins

Many fungal entomopathogen mycotoxins are polyketides and its derivatives (PKs); more than 20 PKs were discovered (Table 2, Figures 3 and 4). Fungal polyketide biosynthesis typically involves multiple enzymatic steps, and the encoding genes are often found in gene clusters. The enzymatic machinery for the formation of the polyketides consists of different modules characteristic of each fungus (e.g., keto synthases, acyl transferases, carboxylases, cyclases, dehydrases, aromatases, reductases, thioesterases, and laccases) [75].

One of the best characterised fungal polyketide synthesis pathways is that of the tenellin (Figure 3(a)) from the insect pathogen *B. bassiana* [76, 77]. Tenellin is not involved in insect pathogenesis [76], but tenellin acts as an iron chelator to prevent iron-generated reactive oxygen species toxicity in *B. bassiana* [78]. This toxin inhibits total erythrocyte membrane ATPase activity probably because of a consequence of membrane disruption, since all pigments caused alterations in erythrocyte morphology and promoted varying degrees of cell lysis [79]. There are no reports about the risk of tenellin as a mycotoxin to contaminate foods.

Oosporein (Figure 4(e)) is the major secondary metabolite excreted by *B. bassiana* [80] and *B. brongniartii* [81]. It had a median oral toxicity to 1-day-old cockerels [82]. Oosporein inhibits total erythrocyte membrane ATPase activity in a dose-dependent manner caused alterations in erythrocyte morphology and promoted varying degrees of cell lysis [79]; meanwhile, the toxin also exhibits broad spectrum of antimicrobial, antioxidant, and cytotoxic activities [83]. However, oosporein is a rather strong organic acid; it can be concluded that oosporein can hardly be adsorbed by organisms, so oosporein is unlikely to enter food chains and influence human health [81].

Bssianin (Figure 4(f)) is a PK pigment isolated from *B. bassiana*. It inhibits total erythrocyte membrane ATPase activity as well [79].

The fungal entomopathogen *M. anisopliae* produces cytochalasins (Figure 3(b)), a famous PK [84, 85]. Cytochalasins belong to a kind of cytochalasans which comprise diverse group of fungal polyketide-amino acid hybrid metabolites with a wide range of distinctive biological functions [86]. To date, more than 80 cytochalasans have been isolated from other fungi such as *Phomosis, Chalara, Hyposylon, Xylaria, Daldinia, Pseudeurotium,* and *Phoma exigua* [75]. Cytochalasans have phytotoxins or virulence factors and exhibit antimicrobial or cytotoxic activities and inhibit cholesterol synthesis or interfere with glucose transport and hormone release. However, the origin of their name is derived from the Greek terms kytos, meaning cell, and chalasis, meaning relaxation, pointing to the best known property of cytochalasans, the capping of actin filaments. As a result,

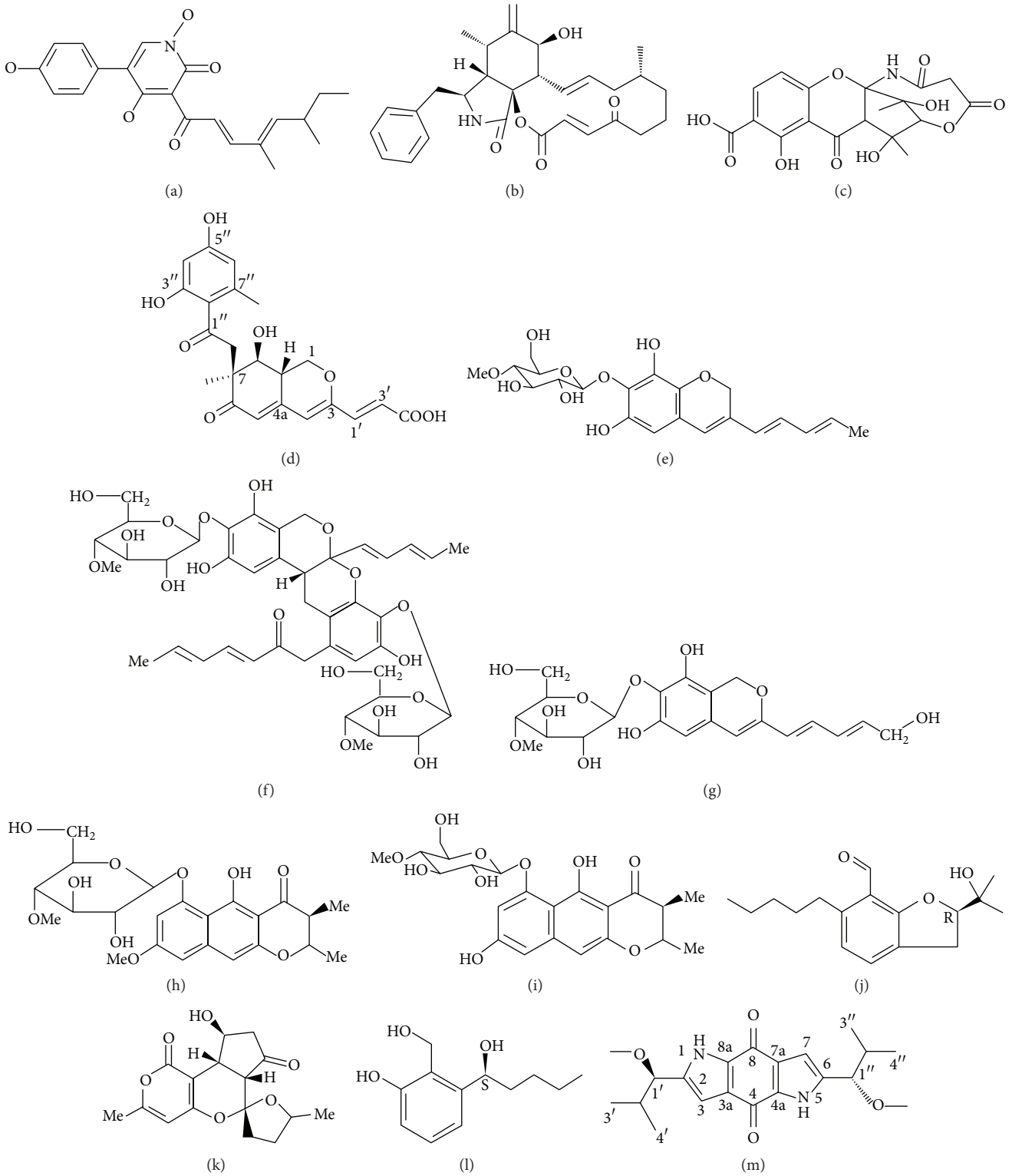


Figure 3: The structure of tenellin (a), cytochalasins (b), cryptosporioptide A (c), pinophilin C (d), indigotide A (e), indigotides C-F (f), 13-hydroxyindigotide A (g), 8-O-methylindigotide B (h), indigotide B (i), annullatin A (j), tenuipyrone (k), annullatin E (l), and terreusinone A (m).

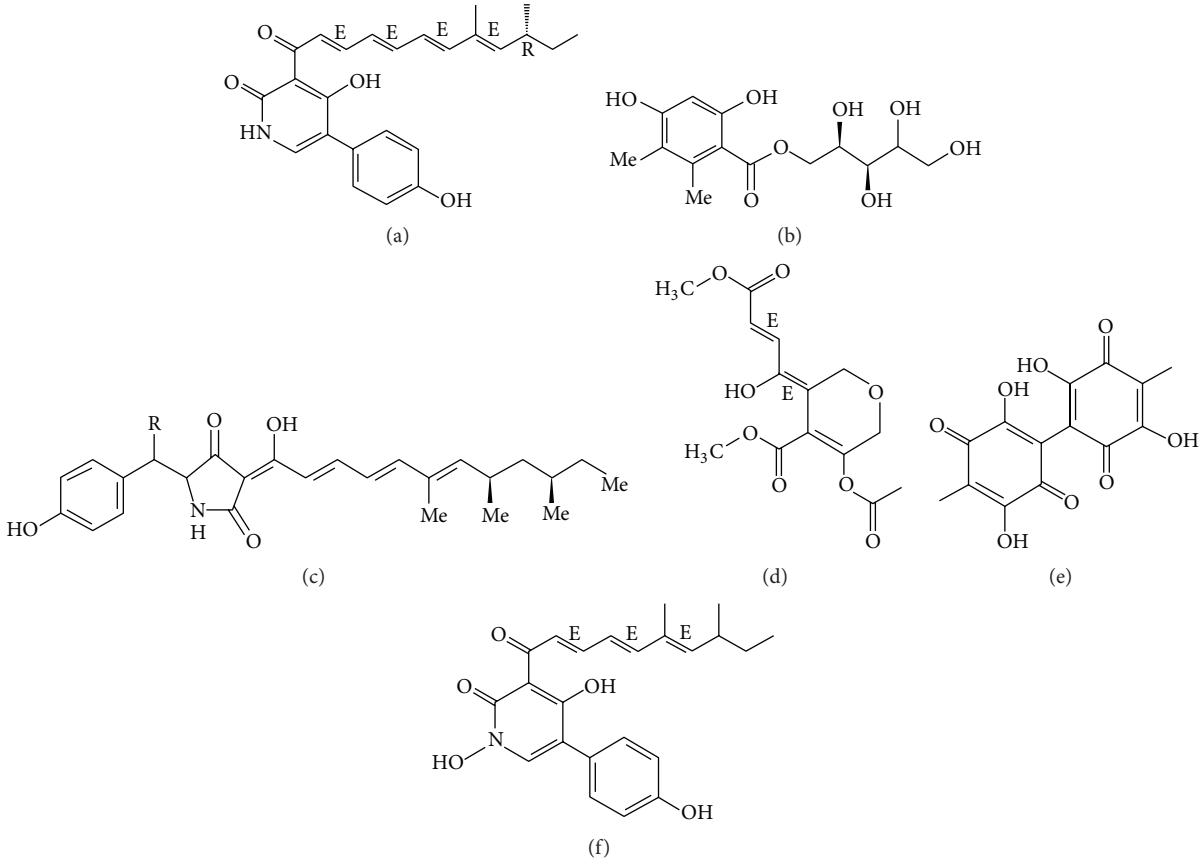


Figure 4: The structure of farinosones A (a), paeciloside A (b), and militarinones B (c), isariketide (d), oosporein (e), and bassianin (f).

cytokinesis is effectively inhibited while mitosis remains unaffected, thereby generating giant multinucleated or even, at higher concentrations, denucleated cells. These properties are exploited in molecular and cell biology research, especially in cell imaging methods, cytoskeleton, and cell cycle studies [86].

In the entomogenous fungal genus, *Cordyceps*, many species produce PKs. For example, *C. indigotica* produces aromatic polyketides, indigotides (Figure 3(f)), 13-hydroxyindigotide A (Figure 3(g)) and 8-O-methylindigotide B (Figure 3(h)) [91, 96]. Terreusinone A (Figure 3(m)), pinophilin C (Figure 3(d)), and cryptosporioptide A (Figure 3(c)) were isolated from *C. gracilioides*; these three compounds inhibit the activity of protein tyrosine phosphatases [88]. Annullatins (Figures 3(j) and 3(l)) were isolated from *C. annullata* [87]. Opaliferin, a polyketide with a unique partial structure in which a cyclopentanone and tetrahydrofuran were connected with an external double bond, was isolated from the insect pathogenic fungus *Cordyceps* sp. NBRC 106954 [93]. However, there is no information about the risks of these PK toxins to human health.

As to *Isaria* genus, *I. tenuipes* produces tenuipyrone (Figure 3(k)) [94]. *I. felina* KMM 4639 produces isariketide Figure 4(d), showing moderate cytotoxicity toward HL-60 cells [99]. Militarinones were isolated from cultures of the *Cordyceps*-colonizing fungus *I. farinosa*. It showed significant cytotoxicity against A549 cells [100]. For the *Paecilomyces* genus, farinosones (Figure 4(a)) were isolated from the strain *Paecilomyces farinosus* RCEF 0101. They induce outgrowth but cytotoxicity in the PC-12 cell line [89]. Paeciloside A (Figure 4(b)) is isolated from *Paecilomyces* sp. CAFT156. Paeciloside A displays inhibitory effects on two gram-positive bacteria, *Bacillus subtilis* and *Staphylococcus aureus,* and moderate cytotoxicity towards brine shrimp larvae (*Artemia salina*) [101]. *P. militaris* produces militarinones (Figure 4(c)) [92]. There is no other information of these PKs.

4. The Fate of Mycoinsecticide and Its Mycotoxins

In mycoinsecticide, the main component is usually the spores of fungal entomopathegen. Some of mycotoxins maybe exist inside of spores other than outside of spores. Mycoinsecticide itself is almost not the resource of mycotoxins. In fact, mycotoxins mainly come from the target pests or host insects infected by fungal entomopathogen of mycoinsecticide. The endophytic entomopathogenic fungus is maybe the other important mycotoxins resources. Of course, if considering the nonmycoinsecticide factors, the crops and products infected by other fungal species such as *Fusarium* spp., *Aspergillus* spp. should be the more important resources of mycotoxins.

Totally, mycoinsecticide in its production and application has six fates (Figure 5). The first fate, humans, may

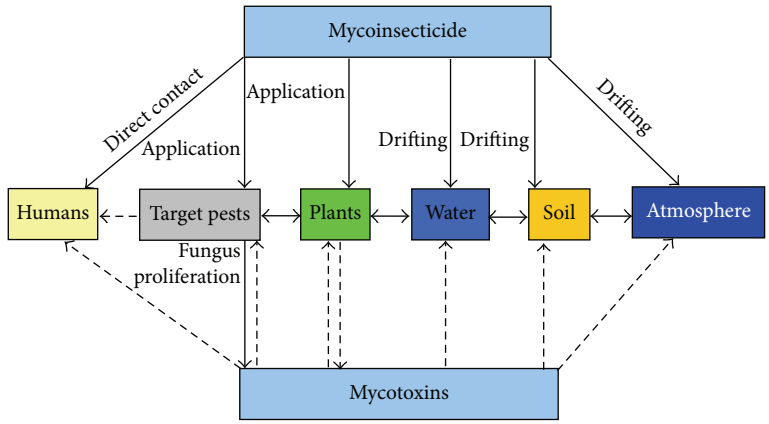


FIGURE 5: The fates of mycoinsecticide and its mycotoxins. → indicating the actually existing pathway, ⇢ indicating the pathway not found to date.

be exposed to the risks of directly contacting the fungal entomopathogens. These humans are mainly the persons who long-timely produce and use the mycoinsecticide. There were several reports about fungal spores allergy of workers producing biocontrol agent of *Beauveria bassiana* and *Metarhizium anisopliae* [5, 6]. But there are no evidences supporting that the allergy is because of mycotoxins.

When mycoinsecticide is used, the important fate is the target insects. The fungal spores of mycoinsecticide adhere insect surface and then start a pathogenic progress. After penetrating the cuticle, the fungus proliferates itself and produces mycotoxins in host insect. At last, the fungal phages and its mycotoxins along with the cadavers of host insects are released to environments. To date, we do not know how many of the mycotoxins enter the environment. However, a few research cases indicate that the mycotoxins from entomopathogens are scarcely released to environments. For example, the amount and type of destruxin produced are dependent upon the fungal strain and insect host and the fact that these compounds decomposed shortly after host death. Destruxin decomposition was presumably due to the activity of hydrolytic enzymes in the cadavers and appeared to be independent of host or soil type and biota. So, destruxins are essentially restricted to the host and pathogen and are unlikely to contaminate the environment or enter the food chain [39].

Plants including target crop and weeds are the important fate of mycoinsecticide (Figure 5). The main fungal resources of plants is from mycoinsecticide application and target pests. Plants maybe hardly receive the fungus from the systems of water, soil, and atmosphere. Fungal entomopathogen is not phytopathogen, and in general, the phages of fungal entomopathogen only deposit the plants surface. However, many species of entomogenous fungi such as *B. bassiana, M. anisopliae*, and *I. fumosorosea* have been found the endophytic characteristics [102–104]. If so, the detection and management of mycotoxins from fungal entomopathogens are becoming more important, especially for those food crops.

Soil is an important storage bank of fungal entomopathogens. Fungal spores in soil can survive for long time. Through drifting from application and dropping from target pests cadavers, fungal phages and mycotoxins maybe enter the soil system. *Beauveria* spp., *Metarhizium* spp., *Paecilomyces* spp., and *Isaria* spp. can be often isolated from soil [105] and the entomopathogens in soil can be detected after mycoinsecticide is used [106]. But there are no reports that mycotoxins of fungal entomopathogen are detected in soil.

Water is another fate of mycoinsecticide. Beauvericins were detected in drainage water after *Fusarium* spp. was inoculated on wheat plants [107]. However, there are no researches indicating mycotoxins from mycoinsecticides entering the water system.

Atmosphere obtains fungal entomopathogens from drifting. Also, fungus might be exchanged between soil, water, and atmosphere systems. But we can not ensure that fungal mycotoxins enter atmosphere.

5. Conclusion

There are more than thirty mycotoxins isolated from fungal entomopathogens. Based on the biosynthesis, they are classified to NRP and PK mycotoxins. Beauvericins, enniatins, destruxins, cytochalasins, and tenellin are given relevantly intensive researches; other mycotoxins have not been paid sufficient attention. Mycotoxins are produced by cells of fungal entomopathogens used as mycoinsecticide. But mycotoxins are generally not in mycoinsecticide. So, mycotoxins might not be released to environments unless fungus proliferates and produces mycotoxins in host insects or probably in plants. To date, we only know little information about if mycotoxins enter environments. For example, destruxins were decomposed in host insects before they, with the cadaver, were released to environments [39]. Although entomopathogenic fungi are generally not the plants pathogens, many of them have the endophytic characteristics. However, we nowadays neither know if fungal entomopathogens produce mycotoxins in plants and release them to environments nor have enough information that the food chains are contaminated by mycotoxins the host insect produced and that human health are influenced by them. On the contrary, the same mycotoxins produced by phytopathogens

such as *Fusarium* spp., *Aspergillus* spp. have been paid more attention.

In conclusion, mycotoxins from mycoinsecticides have limited ways to enter environments. The risks of mycotoxins from mycoinsecticides contaminating foods are likely controllable.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Arsenic and Mercury Containing Traditional Chinese Medicine (Realgar and Cinnabar) Strongly Inhibit Organic Anion Transporters, Oat1 and Oat3, *In Vivo* in Mice

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Toxic heavy metals, including mercury (Hg) and arsenic (As), accumulate preferentially in kidneys and always cause acute renal failure. The aim of this study was to investigate whether these samples affect organic anion transporters, Oat1 and Oat3, *in vivo* in mice kidney. Mice ($n = 10$) were orally treated with investigational samples. After last administration, all mice were i.v. *p*-aminohippuric acid (PAH), and the blood and kidneys samples were collected. The concentrations of PAH were quantified by spectrophotometry. mRNA expressions of Oat1 and Oat3 were assayed by real-time PCR. In comparison with corresponding control, major pharmacokinetic parameters of PAH in sera were significantly changed by investigational samples ($p < 0.05$), PAH accumulations in the kidney tissues were significantly higher ($p < 0.05$), PAH uptake by renal slices was greatly reduced, Oat1 and Oat3 mRNA expression were significantly inhibited in investigational sample groups. Arsenic and mercury containing traditional Chinese medicine (Realgar and Cinnabar) probably induce kidney damage through inhibiting several members of the organic anion transporters (such as OAT1 and OAT3).

1. Introduction

Drug transporters are known to have a significant impact on the absorption, distribution, elimination, and toxicity of a large number of drugs [1]. It is noteworthy that organic anion transporters (OATs) which are the members of Solute Carrier Family 22 (SLC22) play a pivotal role in renal excretion of water-soluble or negatively charged organic compounds (including endogenous waste products, numerous drugs) and their metabolites. However, some of them lead in some cases to nephrotoxicity [1, 2]. A substantial fraction of such compounds carries a net negative charge at physiological pH and hence is referred to as organic anions (OAs). *p*-Aminohippuric acid (PAH) which is known to interact with multiple basolateral transporters in proximal tubule cells is the prototypic substrate for what is frequently referred to as the "classic" process of renal organic anion secretion [3]. More than a decade ago, two PAH-transporters [organic anion transporter (OAT1 and OAT3)] were identified and functionally characterized on the molecular level [4, 5]. In mice, Oat1 (*Slc22a6*) is detected exclusively in the proximal tubules; however Oat3 (*Slc22a8*) is localized in the proximal tubule, cortical, and medullary thick ascending limb of Henle's loop, connecting tubules, and cortical and medullary collecting ducts [6]. The overlapping substrate specificity and localization at the basolateral membrane of proximal tubules from Oat1 and Oat3 support the assumption that both transporters may play a principle role in the absorption of PAH and other OAs [5, 7].

Toxic heavy metals, including mercury (Hg) and arsenic (As), accumulate preferentially in kidneys and always cause acute renal failure [8]. Renal proximal tubular cells represent the major target site where highly reactive mercuric ions are proved to rapidly accumulate and induce cell injury

[9]. However, both Realgar and Cinnabar are included in some prescription of Chinese herbal formulae. The main component of Realgar is As_2S_2 and that of Cinnabar is HgS. Although As_2S_2 and HgS are difficult to be adsorbed by the gastrointestinal tract of mammals, they slightly contain soluble and extremely toxic components, arsenic (As^{3+}) and mercury (Hg^{2+}), such as $HgCl_2$ and As_2O_3 [10]. As^{3+} and Hg^{2+} can easily gain access to proximal tubular cells primarily via Oat1 and Oat3 in the basolateral membrane [11]. To our knowledge, there is some information regarding modifications (inhibition) of these transporters in nephrotoxic acute renal failure [12, 13]. No similar study has conducted the interaction between arsenicand mercury containing traditional Chinese medicine (Realgar, Cinnabar, and $HgCl_2$) with organic anion transporters in mammalian kidney in literature. Therefore, the aim of the present study is to evaluate the expression and function of Oat1 and Oat3 after administering arsenic and mercury containing traditional Chinese medicine (Realgar and Cinnabar) *in vivo* in mice.

2. Materials and Methods

2.1. Chemicals and Reagents. Realgar and Cinnabar were purchased from Guangzhou Pharmaceuticals Corporation (Lot. 120612). $HgCl_2$ was purchased from Guangzhou Chemical Reagent Factory (Lot. 110601). *p*-Aminohippuric acid (98%, Aladdin, Lot. 120816); probenecid (Aladdin, Lot. 130512); sodium carboxymethylcellulose (CMC-Na, Sinopharm Chemical Reagent Beijing Co., Ltd., China, Lot. 111015); Trizol, RT reagent Kit, and SYBR Premix Ex Taq II (Lot. D9108A, Lot. DRR037A, and Lot. DRR081A, Takara, Dalian, China); and the primers for Oat1, Oat3, and GAPDH were synthesized by Sangon Biotech Co., Ltd., (Shanghai, China). Other chemicals used were of analytical grade commercially available.

2.2. Preparation of Investigational Samples. Four investigational samples (Realgar, levigated Realgar, Cinnabars, and $HgCl_2$) were made into suspension by 0.5% CMC-Na. Grinding Realgar was prepared freshly according to the Chinese Pharmacopeia. To assure quality control, the Cinnabar was validated by the method of the Chinese Pharmacopeia (China Pharmacopoeia Committee, 2010). In order to examine tiny amount water soluble mercury (Hg^{2+}) in Cinnabar, the method of Huo et al. [14] was adopted [(artificial gastric juice, containing 0.08 N HCl (pH 1.5) and 1% pepsin (Sino-American Biotechnology Co., Ltd., USA, Lot. 140218)], and its content was detected by flame atomic absorption spectrophotometer (AAS) (Thermo, USA) [15].

2.3. Experimental Animals and Treatment Protocol. This study was carried out using adult NIH mice (certificate number SCXK 2013-0020), weighing 20~25 g sourced from the Experimental Animal Centre of Guangzhou University of Chinese Medicine. The animal experimental procedures were approved by the animal Ethics Committee of Guangzhou University of Chinese Medicine, Guangzhou, China. All mice were given standard mice chows and pure water ad libitum and housed at 20~26°C. Eleven groups of mice (50% for each gender) were orally given pure water (water control group), 0.5% CMC-Na solution (solvent group), and probenecid (50 mg/kg, positive water control group), and investigational samples were orally given at two (high and low) dosages: Realgar (60 mg/kg and 15 mg/kg), levigated Realgar (60 mg/kg and 15 mg/kg), Cinnabar (120 mg/kg and 30 mg/kg), and $HgCl_2$ (0.2 mg/kg and 0.05 mg/kg), which were twice a day for continuous five days.

2.4. Blood Sampling and Kidney Removal. On the experiment day, 60 min after the last administration of investigational samples, all mice were single i.v. PAH (30 mg/kg B.W., aqueous solution), respectively, according to the protocol of Bertani et al. [16] and blood samples were collected from 10 mice/group by euthanization in each time point at 1.0, 2.5, 5.0, 7.5, 10.0, 20.0, and 30.0 min after PAH injection. Blood samples were centrifuged at 2000 ×g for 5 min. The obtained sera were stored at −20°C until measurement. The two kidneys were rapidly removed and stored at −80°C for later assays.

2.5. PAH Pharmacokinetic Studies. These studies were done in a manner similar to the literatures by Brandoni et al. and Cerrutti et al. [17, 18]. The concentration of PAH in sera was determined according to Saikan and Kiguchi [19] method. The sera concentration versus time curve for PAH, for each individual animal, was fitted with PK software DAS 2.0. The data were fitted to a biexponential curve. The choice of the best fit was based on the determination of coefficient values (R^2) and LSD test [20]. All fits should have R^2 values > 0.98. The following equation was used to describe the biexponential concentration-time curves:

$$C_p = A \cdot e^{-\alpha \cdot t} + B \cdot e^{-\beta \cdot t}, \quad (1)$$

where C_p is PAH serum concentration (mg/mL) at time t (min) after administration: constant α presents the distribution from the central compartment, and β presents an equilibrium constant reflecting the dynamics between k_{21} and k_{10}. A and B represent the initial values of the distribution and elimination components, respectively, extrapolated from y-axis intercept. The estimate parameters (α, β, A, and B) were used to solve the first-order rate constants of transfer from the central to peripheral compartments (k_{12}, k_{21}) and the elimination rate constant from the central compartment (k_{10}) with classical equations. Derived parameters, elimination half-life ($t_{1/2\beta}$), total volume of distribution (Vd_T), total clearance (CL_T), and area under the curve ($AUC_{0-30\,min}$), were calculated according to standard procedures for the compartmental analysis. Concentration of PAH in serum was measured using the method described by Di Giusto et al. [21].

2.6. Accumulations of PAH to Kidney. The mice renal homogenate was prepared by Shihana et al. [22] protocol. Each right kidney was cut into small pieces, which were put into a glass homogenizing tube containing PBS (200 mM sucrose, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and

Table 1: Sequences of the primers of mOat1 and mOat3 for RT-PCR.

Genes	Accession number	Sense primers (5′ to 3′)	Antisense primers (5′ to 3′)	Product size (bp)
mOat1	NM_008766	ATGCCTATCCACACCCGTGC	GGCAAAGCTAGTGGCAAACC	417
mOat3	NM_031194	CAGTCTTCATGGCAGGTATACTGG	CTGTAGCCAGCGCCACTGAG	338
GAPDH	M32599	GGTGAAGGTCGGTGTGAACG	CTCGCTCCTGGAAGATGGTG	233

2 mM NaH_2PO_4; pH 7.4) at a ratio of 8 mL/g wet weight and then were homogenized with a motor-driven Teflon pestle (1000 rpm/min) and spun down for 30 min at 20,000 ×g. The supernatant was isolated and then stored at −20°C until determination. The kidney homogenates were prepared from each group which was removed at 1, 5, 10, 20, and 30 min after i.v. PAH. Protein content of kidney homogenates was quantified using the Lowry Folin phenol reagent (Nanjing Jiancheng Bioengineering Institute, Ltd., China, Lot. 130918). The concentrations of PAH in kidney homogenate were determined the same as that in the sera.

2.7. PAH Uptake by Mice Renal Slices. The uptake of PAH by mice renal slices was investigated using the procedures described by Henderson and Lindup [23]. Briefly, mice were executed, and the kidneys were immediately harvested, decapsulated, and placed in an ice-cold oxygenated rinse PBS (pH 7.4). Renal slices (weight 10–20 mg/slice) were cut freehand with Gillette valet strip blades to about 0.2 inches in length (Sabre International Products Ltd., Reading, UK). Slices of one kidney were preincubated for 5 min at 37°C and incubated in a 12-well plate with 1 mL of oxygenated PAH-buffer which consisted of 2 mM PAH, 97 mM NaCl, 40 mM KCl, 0.74 mM $CaCl_2$, and 7.5 mM sodium phosphate-chloride buffer in each well. The uptake study was carried out at 37°C in the shaking bath. After incubating for 20 min, each slice was rapidly removed from the PAH-buffer, immediately inactivated the proteins with trichloroacetic acid, was washed in ice-cold saline, was blotted on filter paper, was weighed, and then was homogenized at a ratio of 3 mL/g wet weight. The following operations were the same as described in Section 2.6.

2.8. RNA Isolation and Real-Time PCR. Total RNA was extracted using Trizol reagent from renal cortical tissues according to the manufacturer's instructions and RNA concentration and purity were evaluated by measuring the ratio of $A_{260\,nm}/A_{280\,nm}$. First-strand cDNA was generated by adding 1 μg total RNA; 2 μL 5x gDNA Eraser Buffer; 1 μL gDNA Eraser; 5 μL RNase Free dH_2O (step 1: 42°C, 2 min for genomic DNA elimination reaction); 2 μL 5x PrimeScript Buffer 2; 1 μL Prime Script RT Enzyme Mix I; 1 μL RT Primer Mix; 4 μL RNase Free dH_2O; and 10 μL reaction solution from step 1, which were used to reach a total reaction volume of 20 μL. The condition of reverse-transcription (RT) reaction was as follows, 15 min at 37°C and 85°C for 5 s, and stored at 4°C.

All the primers set spanned an intron and the information of primer was collected in Table 1. The PCR reaction of components was combined in a master mix composed of 10 μL SYBR *Premix Ex Taq* II (2x); 0.8 μL PCR Forward Primer (0.4 μM); 0.8 μL PCR Reverse Primer (0.4 μM); 0.4 μL ROX Reference Dye II (50x); 6 μL dH_2O; 2 μL cDNA.

The real-time quantitative PCR was conducted in ABI 7500 (Applied Biosystems, USA) and the cycling program was set at 1 cycle of predenaturation at 95°C for 30 s and then 40 cycles at 95°C for 15 s, 56°C for 30 s, and 72°C for 31 s. All the real-time PCR experimentation was conducted strictly according to the rules of the MIQE. Quantification of the target cDNAs in all samples was normalized to GAPDH rRNA ($Ct_{target} - Ct_{GAPDH} = \Delta Ct$) and the difference in expression for each target cDNA in the investigated groups was expressed to the amount in the water control group ($\Delta Ct_{treated} - \Delta Ct_{control} = \Delta\Delta Ct$). Fold changes in target gene expression were determined by taking 2 to the power of this number ($2^{-\Delta\Delta Ct}$).

2.9. Statistical Analysis. All data were shown as means ± standard deviation, and n referred to the number of animals used in each experiment. Pharmacokinetic analysis was done by PK Software DAS 2.0 (Bontz Inc., Beijing, China). All statistical tests were performed using SPSS for windows (SPSS 17.0, Chicago, IL). Comparisons among groups were carried out using one-way analysis of variance (ANOVA) followed by least significant difference (LSD) test for multiple comparisons of observed differences between means. Significance was determined at a probability of $p < 0.05$.

3. Results

3.1. Pharmacokinetic Studies. The major pharmacokinetic parameters of serum PAH in mice were shown in Table 2. Both 0.5% CMC-Na group and water control group showed no significant differences in each examined pharmacokinetic parameter. For all investigational sample groups, the total apparent volume of distribution (Vd_T) was observably decreased, total clearance (CL_T) was remarkably reduced, and the area under the curve ($AUC_{0-30\,min}$) was significantly increased (see Figure 1). Elimination half-life ($t_{1/2\beta}$) however was markedly prolonged only in $HgCl_2$ group.

3.2. PAH Accumulation Studies in Kidney. As shown in Figure 2, comparing with C-1 group, the PAH accumulations were not influenced by 0.5% CMC-Na ($p > 0.05$). However, the PAH accumulations in the kidneys of investigational sample groups (A, B, D, and E) were observably increased at all sampling times after i.v. administration of PAH ($p < 0.01$). The accumulations are evidenced by the $AUC_{0-30\,min}$ of PAH in kidney tissue for each sample. The distribution profiles in kidney tissue were very similar to those in blood.

Table 2: Major pharmacokinetic parameters in mice sera after a single dose of PAH.

Groups	$t_{1/2\beta}$/min	Vd_T/L·kg^{-1}	CL_T/mL·min^{-1}·kg^{-1}	$AUC_{0-30\,min}$/g·L^{-1}·min
C-1	9.88 ± 0.83	0.423 ± 0.05	53.2 ± 5.3	0.464 ± 0.043
C-2	10.06 ± 0.71	0.428 ± 0.03	51.9 ± 3.4	0.474 ± 0.030
C-3	11.57 ± 1.59*	0.302 ± 0.01**	33.9 ± 2.7**	0.721 ± 0.053**
A-I	10.05 ± 1.45	0.331 ± 0.03**	41.5 ± 3.7**	0.599 ± 0.045*
A-II	10.91 ± 0.86*	0.325 ± 0.02**	43.7 ± 2.6**	0.555 ± 0.037**
B-I	11.07 ± 1.50*	0.346 ± 0.03*	40.5 ± 4.1**	0.608 ± 0.049**
B-II	10.13 ± 0.90	0.387 ± 0.04	46.2 ± 5.2	0.539 ± 0.054
D-I	10.83 ± 1.33	0.352 ± 0.03	41.9 ± 3.9**	0.585 ± 0.045**
D-II	10.68 ± 0.91	0.378 ± 0.03	46.7 ± 5.0	0.527 ± 0.054
E-I	12.40 ± 2.08**	0.320 ± 0.02**	36.6 ± 3.2**	0.660 ± 0.040**
E-II	11.89 ± 1.17**	0.329 ± 0.03**	41.8 ± 3.7**	0.579 ± 0.048**

Note: C-1: water control group; C-2: 0.5% CMC-Na group; C-3: probenecid group. Higher dosages were expressed by I, and lower dosages were expressed by II. A: Realgar, B: levigated Realgar, D: Cinnabar, and E: $HgCl_2$ (full text). $t_{1/2\beta}$ elimination half-life, CL_T total clearance, Vd_T total volume of distribution, and AUC area under curve. The results are expressed as the mean ± s.d.; $^{*}p < 0.05$; $^{**}p < 0.01$ in comparison with C-1 (LSD test).

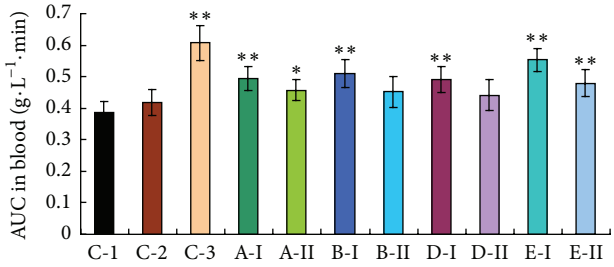

Figure 1: The AUC of PAH in sera after treatment with various investigational samples. C-1: water control group; C-2: 0.5% CMC-Na group; C-3: probenecid group. Higher dosages were expressed by I, and lower dosages were expressed by II. A: Realgar, B: levigated Realgar, D: Cinnabar, and E: $HgCl_2$ (full text). The results are expressed as the mean ± s.d.; $^{*}p < 0.05$; $^{**}p < 0.01$ in comparison with C-1 (LSD test).

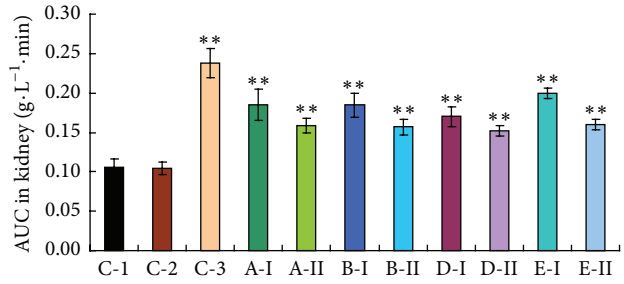

Figure 2: The AUC of PAH in kidney tissue after treatment with various investigational samples. C-1: water control group; C-2: 0.5% CMC-Na group; C-3: probenecid group. Higher dosages were expressed by I, and lower dosages were expressed by II. A: Realgar, B: levigated Realgar, D: Cinnabar, and E: $HgCl_2$ (full text). The results are expressed as the mean ± s.d.; $^{*}p < 0.05$; $^{**}p < 0.01$ in comparison with C-1 (LSD test).

3.3. PAH Uptake by Mice Renal Slices. As shown in Figure 3, the active uptake of PAH by renal slices was observably inhibited by all the investigational compounds ($p < 0.01$). However, the PAH uptake of the renal slices in 0.5% CMC-Na group (C-2) has no influence compared with control one ($p > 0.05$).

3.4. mRNA Expression Levels. As shown in Figure 4, Oat1 and Oat3 mRNA levels were not influenced in 0.5% CMC-Na (C-2) group compared with water control ($p > 0.05$). Both Oat1 and Oat3 mRNA levels were clearly downregulated in kidneys from all investigational groups as compared with C-1/C-2 groups ($p < 0.01$).

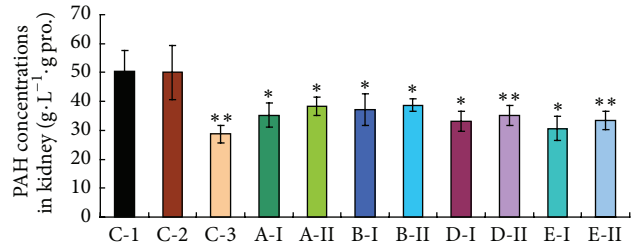

Figure 3: Effect of the investigational samples on PAH uptake by kidney slices of mice after treatment twice a day for continuous five days. C-1: water control group; C-2: 0.5% CMC-Na group; C-3: probenecid group. Higher dosages were expressed by I, and lower dosages were expressed by II. A: Realgar, B: levigated Realgar, D: Cinnabar, and E: $HgCl_2$. Each column represents the mean ± s.d. ($n = 10$). $^{**}p < 0.01$, significantly different from C-1/C-2.

4. Discussion

Arsenic and mercury have been recognized as a hazardous environmental pollutant which is harmful to the plants, animals, and even mammals; people can be easily exposed to them through contaminated water and food [24, 25]. The soluble arsenic (As^{3+}) and mercury (Hg^{2+}) can be simply absorbed in the gastrointestinal tract and distributed throughout the body. It is worth noting that the oral LD_{50} for Realgar (As) and Cinnabar (Hg) in mice is about 3200 mg/kg and 2678 mg/kg, but the oral LD_{50} for arsenic trioxide (As^{3+}),

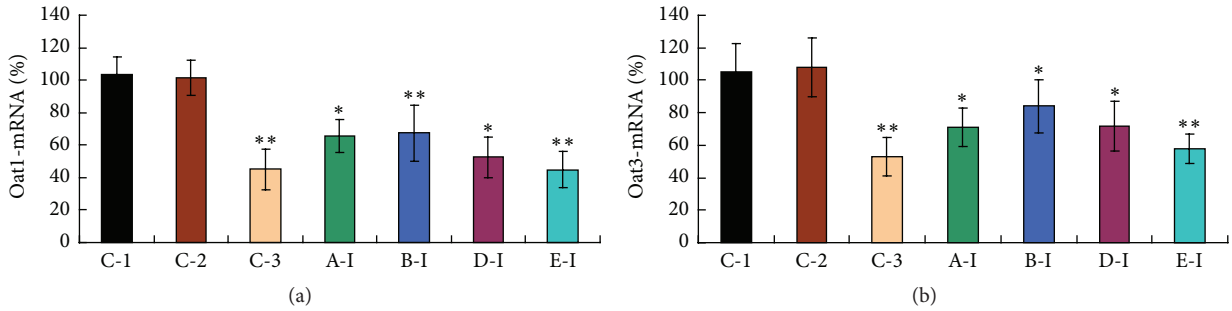

FIGURE 4: Expression levels of (a) Oat1 and (b) Oat3 mRNA in the kidney tissue of mice after treatment with investigational samples. C-1: water control group; C-2: 0.5% CMC-Na group; C-3: probenecid group. Higher dosages were expressed by I, and lower dosages were expressed by II. A: Realgar, B: levigated Realgar, D: Cinnabar, and E: $HgCl_2$. All data were expressed as percentages referred to the corresponding control, and are expressed mean ± s.d. ($n = 12$). Asterisks sign designates significant differences. $^{*}p < 0.05$ versus C-1/C-2; $^{**}p < 0.01$ versus C-1/C-2.

arsenic pentoxide (As^{5+}), and divalent mercury (Hg^{2+}) in mice is as small as 33 to 39 mg/kg, 112 to 175 mg/kg, and 7 to 10 mg/kg, respectively, dozens of times difference compared with Realgar and Cinnabar [26–28]. As_2O_3 and $HgCl_2$ are two established nephrotoxicant compounds in mice and rats which dose-dependently affect the membrane transporters (Oat1 and Oat3) of the proximal tubules [29]. At the basolateral membrane of proximal tubular cells, the organic anion transporters (Oat1 and Oat3) mediate the uptake of a number of As^{3+}- or Hg^{2+}-thiol conjugates from plasma [30]. The ability of these carriers to transport As^{3+} or Hg^{2+} is thought to be dependent upon the conjugation of low molecular weight thiols (such as cysteine and homocysteine) with As^{3+} or Hg^{2+} [31].

According to the literature, divalent mercury is a highly toxic element because of its accumulative and persistent nature in the environment and biota [32]. Cinnabar usually slightly contains about 0.011~2.98% of the divalent mercury [14, 33]. In Cinnabar used in our experiments, however, the content of water soluble mercury was 0.07% and mercuric sulfide was 98.6%, and those are consistent with the reported studies [14, 33].

In the present study, each of two different dosages of Realgar, levigated Realgar (it was processed by levigated courses described in the Chinese Pharmacopoeia), and Cinnabar were designed, respectively, and they were about four times (higher dosage) or equivalent (lower dosage) to the higher dosages of Chinese Pharmacopoeia (according to the calculations of mice and human body surface area at the equivalent to the clinical dosage) [34]. However, the soluble mercury in Cinnabar was almost of equal amounts to the dosage of $HgCl_2$ administered in the present study according to the result of AAS determination.

The current PAH-clearance test shows that mice treated with investigational compounds (Realgar, levigated Realgar, Cinnabars, and $HgCl_2$) exhibited an obvious decrease in the total volume of distribution (Vd_T) and a significant increase in the area under the curve (AUC). Nevertheless, the decrease in the total clearance (CL_T) of PAH might be justified on one hand by the decreased PAH uptake in renal basolateral membranes and on the other hand by the fact that Oat1 and Oat3 mediate the uptake of a number of As^{3+}- or Hg^{2+}-thiol conjugates [31, 35]. Even the constants α, β, k_{12}, k_{21}, and k_{10} (data not shown) were slightly decreased in each group of the tested mice. Based upon this observation, Realgar and Cinnabar may affect pharmacokinetics of PAH by inhibition of renal excretion via Oat1 and Oat3. This leads us to guess that organic anion transporters play an important role not only in the elimination of PAH via the kidney but also in mediating the nephrotoxicity of heavy metals tested in the experiments.

In kidneys, the first step in active secretion is the extraction of organic anions from the peritubular blood plasma by the proximal tubular cells through the basolateral membrane [21]. Probe substrates for, and inhibitors of, specific transporters are desired to evaluate quantitatively *in vivo* functions of transporters in mice. This basolateral uptake of organic anions has been extensively investigated with PAH as the test substrate [36]. However to evaluate the functional activity of Oat1 and Oat3 in renal tubular cells, we measured PAH uptake in mice kidney slices prepared from whole kidney of all investigational groups according to the method of Henderson and Lindup [23]. The results have showed that a significant reduction in PAH uptake via mice renal slices was found, which means the activity of Oat1 and Oat3 was markedly inhibited by the investigational compounds. The differences in PAH uptake indicate that a lower number of functional carrier units exist in renal slices which were made from tested mice; this is also in agreement with the lower activities of Oat1 and Oat3 in renal slices.

AUC is generally considered to be one of the most important PK parameters in the pharmacokinetic study, so the present study majorly concerned the AUC of PAH in blood and in kidney tissue for each tested sample. Compared with the water/CMC control group, both serum $AUC_{0-30\,min}$ and kidney $AUC_{0-30\,min}$ in each tested sample were all increased (44% to 88% for kidney, 14% to 42% for serum), whereas the ratios of $AUC_{0-30\,min}$ for kidney and serum were comparatively constant, around 0.3 (0.27~0.31), which means no significant difference between the groups, indicating that

PAH was almost not influenced by the other transporters or metabolic enzymes except OATs. When the blood PAH concentrations were increased, the renal tissue PAH concentrations were also increased in a similar proportion, but the AUC ratio (kidney/serum) was almost the same in each group, ranging from 0.27 to 0.31.

The mice genes of Oat1 and Oat3 are highly homology with human OATs (OAT1, OAT3) [37, 38], which are also highly expressed in the basolateral membranes of renal proximal tubular cells [11, 39]. In this experiment, Oat1 and Oat3 mRNA expressions significantly decreased in the renal cortex of mice in all tested sample groups. The over 20% reduction of Oat1 mRNA expression may be mediated by the arsenic and mercury toxicity-dependent inhibition of the specific genes for Oat1 [9]. The expression of Oat3 mRNA was significantly reduced in kidneys from all investigational samples, indicating a clear downregulation at the amplification level of mRNA happens. However, obvious decrease in Oat1 and Oat3 mRNA levels (40%) was observed in $HgCl_2$ group mice and also suggests that the inhibiting effect of $HgCl_2$ on OATs may be stronger than Cinnabars in the same dosage. Hence, Oat1 and Oat3 mediate arsenic and mercury compounds' access to the proximal tubule cells; their downregulation mRNA expression might be another defensive mechanism developed by the cell to protect itself against arsenic and mercury injury.

As a conclusion, in consideration of the results mentioned above, we have found that all investigational compounds (Realgar, levigated Realgar, Cinnabars, and $HgCl_2$) interfered with the PAH uptake through Oat1 and Oat3 in renal basolateral membrane of proximal tubule. Although the theory of traditional Chinese medicine says preparation procedures (like water grinding) for Realgar and Cinnabar could always reduce the toxicity and enhance the therapeutic effect for them, unfortunately, the water levigated process could not completely delete the water soluble substances contained in Realgar and Cinnabar and they still remarkably regulate the function of organic anion transporters of Oat1 and Oat3.

Drug-drug interactions take place at OAT1/Oat1 and OAT3/Oat3 level may retard the excretion of endo- or exogenous toxic compounds and then cause serious unwanted side effects. We rediscovered that Realgar and Cinnabar which were known as potential carcinogen [40] are dangerous and even processed before use according to theory of Chinese medicine via the experiment. We still need to take with caution and should discern that preparation procedures (like water grinding) for them are probably ineffective in reducing the toxicity of known toxic substances like Realgar and Cinnabar.

Conflict of Interests

The authors have declared that no competing interests exist.

Authors' Contribution

Wen-Hao Yu and Na Zhang had equal contribution and are designated as co-first authors.

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Research Article

Pesticides and Health in Vegetable Production in Kenya

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This paper investigates the determinants of pesticide-related cost of illness (COI) and acute symptoms, using a balanced panel of 363 farmers interviewed from seven major vegetable producing districts of Kenya. Finding shows that the incidences of pesticide-related health impairments have increased. Variation in number of symptoms and symptom severity significantly explained COI. The personal protective equipment (PPE), education level, record keeping, and geographical location considerably determined health impairments. Encouraging the proper use of PPE and record keeping of pesticide use could greatly reduce poisoning cases and COI.

1. Introduction

The health effects of pesticide use have become one of the major public health problems worldwide. In developing countries, frequent exposure to pesticides by farmers and farm workers is very common [1–3]. The frequent exposures to pesticides result in both short-term (acute) and long-term (chronic) illnesses. Scientifically confirmed pesticide-related acute illnesses include headaches, stomach pains, vomiting, skin rashes, respiratory problems, eye irritations, sneezing, seizures, and coma [4]. The chronic illnesses include cancer, asthma, dermatitis, endocrine disruption, reproductive dysfunctions, immunotoxicity, neurobehavioral disorders, and birth defects [5–13]. Furthermore, deaths resulting from direct exposure to pesticides are also common [14].

The World Health Organization (WHO) and the United Nations Environment Program (UNEP) estimate pesticide poisoning rates at 2-3 per minute [14]. The largest numbers of pesticide poisonings and deaths are said to occur in developing countries [15]. It has been argued that pesticide-related health issues constitute a serious threat to development and can easily reverse or undermine the gains made in agricultural growth [16]. Poor access to health services and the inability of medical professionals to recognize pesticide-related morbidity raise further concerns [17]. Although pesticide-related poisoning is still not as high or more pronounced in Africa as in Asia, it is a growing problem as the increasing intensification of agricultural production with more widespread use of pesticides will result in possible increase in pesticide poisoning [18].

In Kenya, pesticide use and farmers health have been documented by some empirical studies [19–21]. However, these studies were based on a snap shot of cross-sectional surveys and a clear trend of poisoning is not well understood. In addition, only two studies looked at the determinants of pesticide-related acute poisoning symptoms among farmers [19, 20]. However, the problem is that pesticide poisoning effects on human are not random but rather depend on other unobserved characteristics such as genetic characteristic. Such effects cannot be captured with cross-sectional data as utilized in the above studies. Thus the true underlying causal relations may be very different, either larger or smaller, compared with those noted in those researches.

The objective of this paper therefore is to examine the incidences and the determinants of acute pesticide poisoning among vegetable farmers in Kenya controlling for unobserved heterogeneity.

2. Methods

2.1. Surveys and Data. The study was conducted in seven major vegetable producing districts of Central Province (Kiambu, Kirinyaga, Murang'a, Nyandarua, and Nyeri North) and Eastern Province (Makueni and Meru Central) of Kenya (Figure 1) in the year 2005 with follow-up visits in 2008.

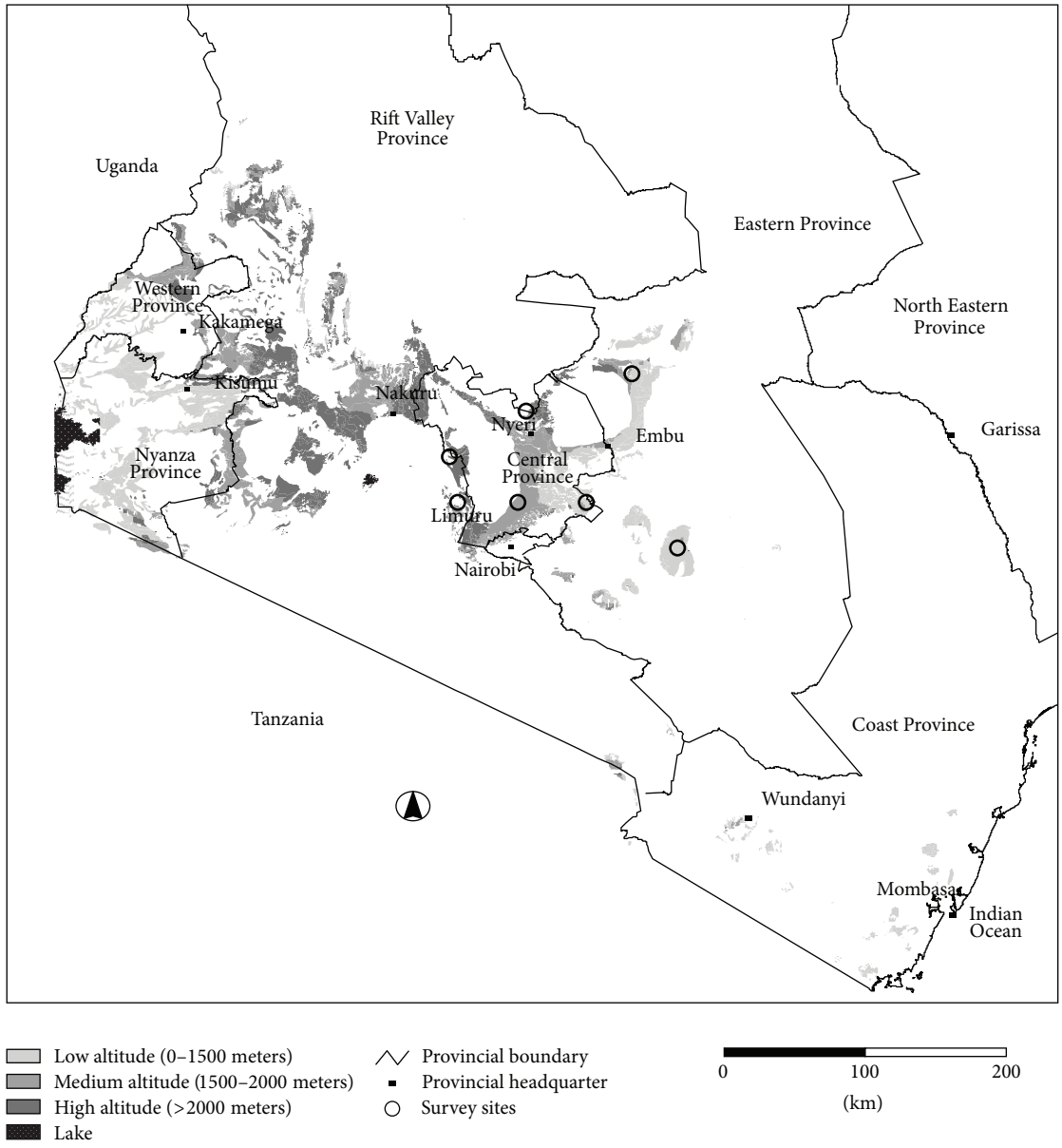

Figure 1: Study sites. Source: this study based on GIS mapping of potential vegetable production areas.

The 2005 survey comprised 839 interviews from the Diamondback moth biological control impact assessment survey ("DBM" with 295 farmers) and the Global Good Agricultural Practices ("GLOBALGAP" with 544 farmers) assessment survey. GLOBALGAP (formerly known as EUREPGAP) is a private sector body that sets voluntary standards for the certification of agricultural products around the globe. In both surveys, a multistage sampling procedure was used to select districts, sublocations, and farmers, respectively. First, districts were purposely sampled according to intensity of vegetable production and agroecological zones. Lists of farmers that were compiled by extension workers at sublocation level served as sampling frame from which 839 farmers were randomly sampled by probability proportional to size (PPS) procedure.

Sampled farmers were then monitored in one cropping season and were trained in record keeping of their production activities by trained enumerators. The trained enumerators under direct supervision of the researcher visited the farmers to check the records and transferred the information to the survey questionnaire.

Due to budget constraints, the 2008 survey was a recall survey of a random subsample of 425 farmers among the 839 farmers. However, we only obtain 363 balanced data set after the 2 years of study. Table 1 displays the distribution of farmers in the sampled districts.

The semistructured questionnaires employed covered a wide range of topics, such as cropping systems, demographics, common farming practices, pesticide use and handling practices, and type and quantities of pesticides sprayed.

Table 1: Regional distribution of survey respondents.

Province	District	Main vegetable crops		Previous surveys (2005)	Number of farmers sampled (2008)	Balanced data (farmers)
		Domestic	Export			
Central	Kiambu	Cabbages, kales, and spinach		48	27	19
	Kirinyaga	Peas, tomatoes	French beans	155	74	66
	Muranga	Tomatoes, kales	French beans	51	24	21
	Nyandarua	Cabbages, potatoes		119	52	49
	Nyeri North	Peas, cabbage, onions, and carrots	French beans	277	116	107
Eastern	Makueni	Cabbages, kales	Asian vegetables[a]	49	22	8
	Meru Central	Peas, tomatoes, cabbage, and onions	French beans	140	110	93

[a]Brinjals, karella, dudhi, okra, turia, valore, and aubergine.
Source: this study.

Health symptoms investigated were specified as those that only began during the spraying operation or within 24 hours after spraying. Additional information collected included the following: number of times the symptom occurred, workdays lost partially or completely due to the health impairment, medication taken by victims, and direct costs due to the symptoms, that is, pharmacy cost, consulting fees, and indirect costs such as travel expenses to and from health centre and dietary expenses resulting from illness like drinking milk or taking honey.

2.2. Analytical Framework. As discussed earlier panel data setup was used to control for the unobserved heterogeneity. In general, panel data model offers some distinct advantages over the cross-sectional data analysis. Greene [22] concluded that the main advantage of panel data is that one can formally model the heterogeneity across groups that are typically present in panel data. Baltagi [23] confirms this in his statement that the first benefit of panel data is "controlling for individual heterogeneity." Additional benefits of using panel data are the ability to capture both cross section and time-series variation in the dependent variable and measure not only the effects that observable variables have on the dependent variable, but also the effects of relevant unobservable or nonmeasurable influences.

A general panel regression model is presented as

$$y_{it} = \alpha_0 + \beta X_{it} + \gamma Z_i + V_{it},$$
$$V_{it} = \varepsilon_i + \mu_{it}, \quad (1)$$

where y_{it} is the response of the dependent variable (in our case this is the cost of illness (COI) or number of acute symptoms) for the ith farmer in the sample at the tth year. α_0 is an intercept that may be different for each point in time, and β and γ are vectors of coefficients. X_{it} is the set of K-vector of time-variant covariates for the ith farmer at the tth year and Z_i is another set of predictor variables that do not vary over time (time-invariant), for example, gender and location. V_{it} is the error term, which is decomposed into ε_i and u_{it}. ε_i is regarded as the combined effect on y of all unobserved variables that are constant over time (time constant unobserved heterogeneity such as cognitive ability and motivation), and μ_{it} represents the idiosyncratic error term (what is unaccounted for in the model) and varies over individual farmers and over time.

The two main methods of dealing with ε_i are to make the random effects (RE) or fixed effects (FE) assumption. Random effects assumed that ε_i are random variables that is, ε_i is i.i.d. $(0, \sigma_\varepsilon^2)$ and that $\mathrm{Cov}(x_{it}, \varepsilon_i) = 0$, while with fixed effects, ε_i are assumed to be potentially correlated with X_{it}. In fixed effect regressions we cannot estimate the effects of time constant covariates as these are normally cancelled out by the within transformation. Thus, classic fixed effects approaches do not produce any estimates of the effects of variables that do not change over time. Moreover, in some cases fixed effects estimates may have substantially larger standard errors than random effects estimates, leading to higher p values and wider confidence intervals. In addition, fixed effects estimates use only within-individual differences, essentially discarding any information about differences between individuals unlike random effects that used information both within and between individuals. Thus, if predictor variables vary greatly across individuals but have little variation over time for each individual, then fixed effects estimates will be rather imprecise. When neither the cross-sectional unit nor times have significant effects, all of the data can be pooled and one can have the constant coefficients model.

The analysis was implemented in two steps. First, the COI model was estimated to evaluate the determinants of health costs among the vegetable farmers. Cost of illness was computed as the sum of farmer-reported medical treatment costs to clinics and private physicians, the opportunity cost of workdays lost to illness, travel costs to and from health facility, time spent in traveling, and the cost of home-based health care. In the second stage, the principal factors associated with the pesticide poisoning symptoms were examined seeking ones that are relevant at policy recommendation.

2.3. The Models

2.3.1. Cost of Illness Model. In previous studies, the health costs of pesticides were modeled using a Logarithmic regression model [19]. In this study the estimation of the determinants of health costs was modeled using a censored random effects Tobit model (Xttobit), since zero costs from respondents who had suffered pesticide-related illnesses but incurred no costs were considered. Using a Logarithmic model would have required adding a small unity value as log of zero is undefined. Estimation of dependent variables results in biased estimators in linear models [24]. The structural equation in the Tobit model is represented as

$$y_{it}^{*} = x_{it}\beta + \varepsilon_i + u_{it}, \tag{2}$$

where $\varepsilon_i \sim N(0, \sigma_\mu^2)$ and y^* is a latent variable that is observed for values greater than T and censored otherwise. The observed y is defined by the following measurement equation:

$$y_{it} = \begin{cases} y^* & \text{if } y^* > T \\ T_y & \text{if } y^* \leq T. \end{cases} \tag{3}$$

In the typical Tobit model, we assume that $T = 0$; that is, the data are censored at 0.

For the empirical model, the explanatory factors for the model explaining health costs incorporate four broad classes of variables, namely, those related to health (number of acute symptoms and symptoms severity), farmer characteristics variables (age, education, and gender), farm management variables (farm size (proxy for wealth), GLOBALGAP certification, and record keeping), and location control (district dummies) (see (4)). Variable definitions and descriptive statistics are presented in Table 2.

It is hypothesized that the number of acute symptoms, symptom severity, age, and farm size are positively associated with the health costs, while a negative association is expected for level of education, GLOBALGAP certification, and record keeping. The direction of the effect of gender on health costs is not clear a priori.

It is anticipated that young farmers may have a higher tendency to protect against pesticides exposure and consequently reduce the pesticide-related acute symptoms and associated health costs. Increased education is also expected to reduce health costs because farmers are more likely to read pesticide labels and follow the recommendation, again reducing the exposure and the acute symptoms. Likewise, GLOBALGAP certification and record keeping can result in a more judicious use of pesticide and higher tendency to protect against pesticide intoxication resulting in reduced acute symptoms:

$$\begin{aligned} \text{HEALTHCOST} = f\,(&\text{TACUTE, SEVERE, AGE,} \\ &\text{AGESQ, EDUCATION, GENDER, FARMSIZE,} \\ &\text{GLOBALGAP, RECORD, District Dummies,} \\ &\text{YEAR 2008 Dummy}). \end{aligned} \tag{4}$$

Acute Symptoms Model. The determinants of the number of acute symptoms were modeled as random effects. A Negative Binomial Regression model (Xtnbreg) was chosen to account for overdispersion, since the equidispersion assumption that has to be met with the Poisson model was violated; that is, the variance was larger than the mean and just over two-thirds of the counts were zero. When there is overdispersion, the Poisson regression is not appropriate because the standard errors estimated are biased downward and the p values are small and spurious [25].

A Negative Binomial Regression model is a count data model and a good facet of the model is that the Poisson model is nested within it [25]. However, the assumption of the standard Poisson model that the variance of the dependent variable is equal to the mean is not binding for the negative binomial model [26]. Negative Binomial Regression model deals with the problem of overdispersion by assuming that y_{it} has a negative binomial distribution, which can be regarded as a generalization of the Poisson distribution with an additional parameter allowing the variance to exceed the mean. The negative binomial function can be presented as

$$\begin{aligned} &f\left(y_{it} \mid \mu_{it}, \varepsilon_i\right) \\ &\quad = \frac{\Gamma\left(\mu_{it} + y_{it}\right)}{\Gamma\left(\mu_{it}\right)\Gamma\left(y_{it} + 1\right)} \left(\frac{\varepsilon_i}{1 + \varepsilon_i}\right)^{y_{it}} \left(\frac{\varepsilon_i}{1 + \varepsilon_i}\right)^{\varepsilon_i}, \end{aligned} \tag{5}$$

where Γ is the gamma function, parameter u_{it} is assumed as both the mean and the variance, and parameter ε_i is assumed constant over time for each individual, while the mean and variance of y_{it} are given by

$$\begin{aligned} E\left(y_{it}\right) &= \varepsilon_i \mu_{it}, \\ \text{var}\left(y_{it}\right) &= \left(1 + \varepsilon_i\right)\varepsilon_i \mu_{it}. \end{aligned} \tag{6}$$

Under this model, the ratio of the variance to the mean is $1 + \varepsilon_i$ that can vary across individuals but is constant over time. The basic idea for this model is that the predictor information is related to the rate of the response to increase or decrease in counts.

For the empirical model, the acute symptom model aggregates skin irritation, diarrhea, sneezing, headache, dizziness, vomiting, stomach poisoning, blurred vision, eye irritation, and backache episodes incurred by the farmer during and/or soon after spraying pesticide as the dependent variable. For the explanatory variables, the medical literature indicates that the type and severity of pesticide poisoning depend on the toxicity of the pesticides, amount of pesticides involved in the exposure, and route of exposure [27]. The model accounted for these factors. In addition, in order to understand farm management variables that can affect pesticide poisoning, GLOBALGAP certification and record keeping were included. Furthermore, following Antle and Pingali [4], Wilson and Tisdell [28], and Asfaw [19], other control variables under farmer characteristics, that is, age, gender, education, and geographical location, were also included (7).

A priori, it is anticipated that WHO class Ia, Ib, and II pesticides are positively correlated with incidences of acute poisoning whereas negative correlation can be expected with

Table 2: Descriptive statistics of variables used in empirical estimations ($N = 726$).

Variables	Definition	Unit	Mean[a]		t or z stat.[b]
			2005	2008	
Dependent variables					
TACUTE[c]	Number of symptoms	Count	1.89 (0.13)	1.09 (0.03)	−7.07***
TACUTE	Number of symptoms	Count	0.38 (0.48)	0.37 (0.03)	−0.15
HEALTHCOST[c]	Cost of illness	US$	4.15 (1.70)	7.98 (1.57)	1.57
HEALTHCOST	Cost of illness	US$	0.84 (0.35)	2.72 (0.58)	2.80**
Farmer characteristics variables					
AGE	Age of the farmer	Years	43.19 (0.66)	46.18 (0.67)	6.30***
AGESQ	Age of the farmer (years squared)	Years	2024.43 (62.80)	2292.64 (66.85)	65.21***
EDUCATION	0 = none; 1 = preprimary; 2 = primary; 3 = secondary; 4 = college	Ordinal	2.45 (0.05)	2.51 (0.04)	1.09
GENDER	Male	1/0	0.70 (0.02)	0.70 (0.02)	0.00
EXPERIENCE	Farming experience	Years	18.42 (0.74)	20.56 (0.07)	2.38**
Health-related and pesticide exposure variables					
HEALTH	Farmer reported a symptom	1/0	0.20 (0.02)	0.34 (0.02)	4.26***
SEVERE	1 = mild; 2 = severe; 3 = very severe	Ordinal	1.11 (0.08)	1.59 (0.36)	1.22
PWHOIab	WHO Ia and WHO Ib (extremely hazardous)	g	8.79 (2.32)	33.55 (12.79)	1.92**
PWHOII	WHO category II (moderately hazardous)	g	129.87 (10.15)	432.63 (25.20)	10.97***
PWHOIII	WHO category III (slightly hazardous)	g	18.95 (3.39)	166.12 (19.23)	7.45***
PWHOU	WHO category U (no hazard)	g	79.87 (7.47)	167.79 (16.86)	4.87***
PESTHA	Total amount applied	g/ha/season	1,473.00 (201.82)	2,124.87 (118.28)	2.97***
NPEST	Pesticide products	Count	2.89 (0.09)	3.32 (0.08)	3.37***
COAT	Wear coat/apron	1/0	0.49 (0.03)	0.71 (0.02)	6.06***
GLOVE	Wear gloves	1/0	0.26 (0.02)	0.35 (0.02)	2.49**
GUMBOOT	Wear boots	1/0	0.26 (0.02)	0.89 (0.02)	17.35***
MASK	Wear facemask	1/0	0.24 (0.02)	0.40 (0.02)	4.36***
TPPE	Protective equipment	Count	2.81 (0.07)	4.00 (0.11)	10.85***
Farm management variables					
FARMSIZE	Total farm size	ha	1.46 (0.08)	1.06 (0.05)	−4.46***
GLOBAL-GAP	GLOBALGAP certified farmers	1/0	0.07 (0.01)	0.19 (0.02)	0.15
RECORD	Records keeping	1/0	0.71 (0.02)	0.32 (0.01)	−10.47***

All monetary variables for example health cost were adjusted (normalized) to US$ of 2008 to take account of inflation. US$ = 72 KSh (2005) and 75 KSh (2008).
[a]Figures in parenthesis are standard errors.
[b]Statistical significance at the 0.01 (***), 0.05 (**), and 0.1 (*) levels of probability. Categorical variables were analyzed using z-test.
[c]With only farmer who reported the health impairment.
Source: this study.

category III and U pesticide. Pesticides in WHO Ia, WHO Ib, and WHO II are very harmful, while WHO III and WHO U are less harmful [29].

Age could increase acute symptoms, as older farmers may be less concerned about health effects of pesticides. As already mentioned in cost of illness model it is expected

that pesticide-related acute symptoms decrease with the increase in level of education, GLOBALGAP certification, record keeping of production activities, and appropriate use of personal protective equipment:

$$\begin{aligned} \text{TACUTE} = f\,(&\text{AGE, AGESQ, EDUCATION,}\\ &\text{GENDER, GLOBALGAP, RECORD, NPEST,}\\ &\text{PWHOIab, PWHOII, PWHOIII, PWHOU, COAT,}\\ &\text{GLOVE, GUMBOOT, MASK, District Dummies,}\\ &\text{YEAR 2008 Dummy}). \end{aligned} \quad (7)$$

The models were estimated using the random effect estimator as the Hausman test showed the fixed effects were not correlated with the regressors. All variables were cross-checked for the problem of multicollinearity, through the simple correlation matrix and variance inflation factor (VIF). The highest correlation coefficient was 0.32 and VIF were by far less than three, indicating that correlation between explaining variables could not affect the estimation of coefficients. Likewise, for endogeneity none of the independent variables was suspected to be explained within the equation in which they appeared. Misspecifications of the models were also checked using a regression specification error test [30]. In respect to the robustness of the Negative Binomial Regression model, a Poisson model was first fitted and the likelihood ratio test together with the statistical evidence of overdispersion indicated that the Negative Binomial Regression model was preferred to the Poisson model. In addition, to check the robustness of all the models other restricted models were estimated in which subsequently insignificant variables were dropped. The statistical quality of the models and the direction of the signs did not change, and the coefficients deviated only marginally.

3. Empirical Results and Discussion

3.1. Descriptive Statistics of Variables Used in Empirical Estimations. Table 2 summarizes the main descriptive statistics comparing 2005 and 2008 with t- and z-tests for the main variables investigated. The results showed that the incidences of pesticide-related acute illness had increased by over 70%. By cross-check although not indicated in the table the analysis showed that only 45% of the farmers consequently reported the effect once more in 2008 showing a high rate of new episodes cases. However, the number of symptoms per farmer dropped by almost half in 2008. In terms of frequency of symptom occurrence, headache and sneezing were reported as the main symptoms in both surveys. Dizziness as one of the major neurological effects of pesticide exposure was also found to have doubled in 2008. These symptoms have been associated with pesticides acute poisoning [27]. They are also consistent with other studies of pesticides exposure on farmers' health elsewhere [31–33].

A total of 62 pesticides products, comprising 36 active ingredients formulated singly or in mixture, were used to control various vegetable pests in 2005. The number increased slightly to 66 products in 2008 with 44 active ingredients in the formulations. However, close analysis showed that 19 new products were applied in 2008, implying that 15 products of those used in 2005 were dropped. The commonly used products included dimethoate (WHO II), used by 48% of farmers, lambda cyhalothrin (WHO II, 27%), cymoxanil (WHO II, 22%), cypermethrin (WHO II, 22%), cyfluthrin (WHO Ib, 20%), mancozeb (WHO U, 18%), and deltamethrin (WHO II, 14%).

For minor poisoning, many farmers used home remedies such as milk, lemon juices, honey, and herbs. The medicines from the local pharmacy shops which were sometimes painkillers were bought in cases where the symptoms of illness were mild and farmers visited the health clinic if the symptoms either persisted or became serious; that is, the victim was unable to talk, walk, or see or vomited continuously. This evidence seems to suggest that many farmers treat acute pesticide effects as minor problems that do not warrant medical attention. Although in only about a quarter of the poisoning cases a physician was consulted, this cost component accounts for the largest share of the total cost of treatment.

The health cost almost doubled in 2008 as compared to 2005. On average, health cost was estimated at US$ 6.55/farmer/season for 28% of the farmers who reported pesticide-related illnesses. These costs equal 47% of mean household pesticides expenditures in 2008. Considering all the farmers this translates to a mean of US$ 1.77/farmer/season and assuming two crop seasons per year the costs amount to US$ 3.54/farmer/year. However, the true health costs are likely to be much higher because the costs arising from chronic diseases resulting from long-term pesticides exposure were not considered, as this would have required more detailed medical assessments. Moreover, only costs directly involving family members were reported; costs occurring to hired farm laborers were not included. Furthermore, other "costs" to restore health status completely and nonmonetary costs like suffering and income lost by family members assisting in seeking treatment were not captured [34, 35]. In addition, preventive costs associated with precautions taken to reduce exposure such as wearing protective equipment were not considered because they were mainly improvised from old clothing or pieces of cloth wrapped around the nose and mouth to reduce inhalation exposure. The cloths were also used for other purposes like spraying on coffee and other farm work and it was difficult to specify those used for spraying pesticides on vegetable crops alone. However, the combined mean of personal protective equipment used increased by 43%, with the largest increment noted for gumboots. Over 20% of farmers also paid wage premiums of up to 32% above the normal wage to hired labor for spraying pesticides, which were normally paid in cash.

Comparison with other studies conducted in developing countries shows similar results. Pingali et al. [36] showed that 58%–99% of the farmers exposed to pesticides had at least one health effect symptom in Indonesia, Philippines, and Vietnam. In Tanzania farmer spending on health due to pesticide and exposure ranges between US$ 0.018 and 116 in

Table 3: Tobit model for cost of illness estimations.

Model	Unrestricted		Restricted	
Variables	(Coefficient)[a]	z-value	(Coefficient)[a]	z-value
TACUTE	7.45 (4.08)**	1.83	6.20 (2.00)***	3.10
SEVERE	9.01 (2.52)***	3.58	11.07 (2.17)***	5.12
AGE	−0.48 (1.09)	−0.44		
AGESQ	0.01 (0.01)	0.61		
EDUCATION	1.46 (2.36)	0.62		
GENDER	−2.84 (4.33)	−0.66		
FARMSIZE	3.25 (2.88)	1.13		
GLOBALGAP	−21.75 (3.40)*	−1.62	−18.71 (7.47)**	−2.50
RECORD	−1.08 (4.89)	−0.22		
KIAMBU	2.50 (10.31)	0.24		
MAKUENI	−15.08 (17.25)	−0.87		
MERU CENTRAL	1.65 (8.71)	0.19		
MURANGA	−5.48 (11.75)	−0.47		
NYANDARUA	−5.61 (8.81)	−0.64		
NYERI NORTH	6.62 (8.47)	0.78		
YEAR 2008	7.93 (9.15)	0.87		
Constant	−23.23 (29.41)	−0.79	−19.54 (4.45)***	−4.39
Log likelihood	−464.10		−549.55	
Wald χ^2/LR χ^2	40.18***		43.22***	

[a]Figures in parenthesis are robust standard errors, statistically significant at the 0.01 (***), 0.05 (**), and 0.1 (*) levels of probability.
Source: this study.

a year [37]. In West Africa, the economic value of pesticide-related health costs equals US$ 3.92/household/season in the case of cotton-rice systems [38]. Zimbabwe cotton growers incur a mean of US$ 4.73 in Sanyati and US$ 8.31 in Chipinge on pesticide-related direct and indirect acute health effects [2]. In Sri Lanka, cost to farmers from pesticide exposure equals 10 weeks' income [39], while in India the average annual welfare loss to an applicator from pesticide exposure amounts to US$ 36 [40]. The immediate costs of a typical intoxication (medical attention, medicines, and days of recuperation) equaled the value of 11 days of lost wages in Ecuador [41].

Pesticide application rate/hectare/season also increased by 47%. Comparison between the years for the specific farmers who participated in the DBM survey showed that many farmers had reduced the pesticides application rate by 8%, while the GLOBALGAP surveyed farmer had increased by 40%. Similar findings in support of the reduction of pesticide use were reported by Jankowski [42] and Löhr et al. [43] where farmers in the study areas with DBM biocontrol (*Diadegma semiclausum*) reduced pesticide applications with others even stopping spraying altogether. The increase in application rate by GLOBALGAP farmers can partially be explained by the low number of farmers who were certified at the time of survey and the failure of the farmers certified in 2005, to maintain their certification status; that is, certified farmers dropped from 18% to 7%, with only 31% of the farmers maintaining their certification for 2008.

3.2. Model Estimations

3.2.1. Cost of Illness Estimation. The estimation results of the Tobit models with the health costs as dependent variable are reported in Table 3. Result shows that health costs are positively associated with number of symptoms and symptoms severity, which implies that an increase in any of these variables spontaneously influences positively the health costs, holding other factors constant.

The finding that the GLOBALGAP certification tends to decrease the health cost could indicate that the certified farmers use adequate safety precautions or use low toxic pesticides, which generally reduce the health impairments and thus decrease costs. It could also be that these farmers are able to use the minimum treatment possibilities.

Among the farmers' characteristics variables, that is, age, education, and gender, none had any discernible effect on health costs. In addition, farm size though considered as an indicator of wealth does not have a direct effect on health costs, though it has the correct sign. Perhaps it could be because farms do not present "liquid cash" that can be accessed immediately in time of need. In addition, no direct association was found between record keeping and the health costs.

District controls are insignificant, so location does not directly affect the health costs. When the model was rees-timated (restricted) by dropping insignificant variables, the estimates of the coefficients were robust.

Table 4: Binomial Regression model for the acute symptoms estimations.

Model	Unrestricted		Restricted	
Variables	(Coefficient)[a]	z-value	(Coefficient)[a]	z-value
AGE	0.04 (0.04)	1.13		
AGESQ	−0.00 (0.00)	−1.21		
EDUCATION	−0.16 (0.07)**	−2.13	−0.14 (0.07)*	−1.94
GENDER	−0.10 (0.16)	−0.67		
GLOBALGAP	−0.33 (0.29)	−1.11		
RECORD	−0.44 (0.17)***	−2.57	−0.55 (0.15)***	−3.77
NPEST	0.09 (0.05)**	1.88	0.10 (0.05)**	2.39
PWHOIab	0.00 (0.00)	1.28		
PWHOII	0.00 (0.00)	0.68		
PWHOIII	−0.00 (0.00)	−0.28		
PWHOU	−0.00 (0.00)	−0.13		
COAT	−0.29 (0.16)*	−1.82	−0.29 (0.15)**	−2.03
GLOVE	−0.26 (0.21)	−1.23		
GUMBOOT	0.32 (0.23)	1.36		
MASK	−0.35 (0.20)*	−1.74	−0.39 (0.17)**	−2.30
KIAMBU	1.69 (0.36)***	4.67	1.63 (0.32)***	5.20
MAKUENI	1.74 (0.49)***	3.55	1.50 (0.46)***	3.35
MERU CENTRAL	1.18 (0.31)***	3.82	0.95 (0.25)***	3.77
MURANGA	0.64 (0.46)	1.40		
NYANDARUA	0.90 (0.34)***	2.66	0.80 (0.28)***	2.81
NYERI NORTH	0.93 (0.30)***	3.07	0.79 (0.24)***	3.26
YEAR 2008	−0.05 (0.21)	−0.23		
Constant	−1.22 (1.06)	−1.15	−0.01 (0.48)	−0.02
Log likelihood	−518.85		−535.52	
Wald χ^2	73.74***		60.96***	

[a]Figures in parenthesis are robust standard errors, statistically significant at the 0.01 (***), 0.05 (**), and 0.1 (*) levels of probability.
Source: this study.

3.2.2. Acute Symptoms Estimation. Given the critical contribution of pesticide-related acute symptoms to the health costs as indicated in Table 2, the principal determinants of these symptoms are reported in Table 4.

The model shows that pesticide-related acute symptoms increase significantly with the number of pesticide products handled. This is not surprising, given that different pesticide products require different application rates and have different levels of toxicity. In addition, handling different pesticide products can increase incidences of symptoms since an interaction between pesticides can lead to unknown toxic chemical reactions [44]. Likewise, although the coefficients for pesticides in WHO Iab and WHO II are insignificant, they are positively correlated with acute symptoms whereas negative correlation is observed with WHO III and WHO U pesticides. The significant negative sign of the variable "record keeping" suggests that the probability of pesticide-related illnesses is less for farmers who keep records. In general, record keeping of pesticide products handled, their application dosage, application techniques, and production activities enabled farmers to be more judicious on pesticides use and higher tendency to protect them. With records, a farmer can also see how well she/he is managing production operations and can identify the strengths and weaknesses in those activities.

The level of education reduces the probability of reported symptoms, which implies that farmers with a higher education level are more knowledgeable and therefore have a better understanding of the dangers posed by pesticides. In previous studies, however, the contrary effect was found because respondents with higher knowledge were more likely to report more health symptoms [2].

The use of personal protective equipment particularly the use of a coat/apron and facemask significantly reduced the number of symptoms. Exposure to pesticides is often attributed to a failure to use protective equipment [34]. The positive sign of the use of boots although insignificant seems perverse and alarming at first glance. However, as the researcher had observed in the field, the improper use, that is, putting the trouser inside the boots, may offer a partial explanation of this apparently perverse result. This finding is analogous to that found by Ohayo-Mitoko et al. [45], where use of gumboots was associated with high *acetyl cholinesterase* inhibition. *Acetyl cholinesterase* is enzyme that breaks down acetylcholine (ACh) into choline and acetic acid. It is released onto the sarcolemma of muscle fibers and destroys ACh after

the ACh has combined with receptors on the muscle fiber. Thus, it prevents continued muscle contraction in the absence of additional nervous stimulation.

Location control for agroecology and differences in institutional settings shows that farmers in the districts of Kiambu, Meru Central, Makueni, Nyandarua, and Nyeri North experience significantly high cases of pesticide ascribed health symptoms as compared to Kirinyaga (base). Perhaps this is due to the use of protective equipment by farmers located in Kirinyaga.

Contrary to the expectations, the analysis does not support the hypothesis of a significant influence of GLOBALGAP certification on the outcome of health, but the variable has the correct signs. Once again, the low number of farmers who were certified and the failure of the certified farmers to maintain their certification may be the cause of the insignificance. The hypothesis that gender and age have a stronger relation to the acute symptoms is also not supported by the results.

The likelihood ratio test used to assess the statistical quality of the model showed that the model was statistically valid, that is, dispersion parameter alpha was greater than zero. The reduced model with only the variables that had a significant effect on the dependent variable shows that the statistical quality of the model does not differ much and the directions of the coefficient are identical, suggesting the robustness of the model.

4. Conclusions and Recommendations

The results of the study give indications of increase of pesticide-related health impairments with over 70% new episodes. The most frequently reported symptoms were sneezing, dizziness, headache and blurred vision, and skin irritations. The result further shows that farmer loses on average US$ 3.54/farmer/year on pesticide-related indirect health costs. These costs are significantly explained by variation in number of symptoms and severity of the symptoms. Pesticide-related acute symptoms increase significantly with the number of pesticide products handled and considerably reduce with level of education, use of PPE, and record keeping. These findings hint at some important points for policies aiming to reduce pesticide poisoning among vegetable farmers. Firstly, the results support the already widely known notion that proper use of PPE (coat/apron and facemask) reduces the pesticide-related impairment. Encouraging of PPE and record keeping of pesticide use activities by farmer is thus recommended.

Future efforts to measure pesticide-related health costs should cover the health costs of all individuals exposed to pesticides, for example, entire public, consumers, and hired workers, and also incorporate pesticide-induced chronic illnesses and deaths.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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Review Article

Overview of Emerging Contaminants and Associated Human Health Effects

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In recent decades, because of significant progress in the analysis and detection of trace pollutants, emerging contaminants have been discovered and quantified in living beings and diverse environmental substances; however, the adverse effects of environmental exposure on the general population are largely unknown. This review summarizes the conclusions of the comprehensive epidemic literature and representative case reports relevant to emerging contaminants and the human body to address concerns about potential harmful health effects in the general population. The most prevalent emerging contaminants include perfluorinated compounds, water disinfection byproducts, gasoline additives, manufactured nanomaterials, human and veterinary pharmaceuticals, and UV-filters. Rare but statistically meaningful connections have been reported for a number of contaminants and cancer and reproductive risks. Because of contradictions in the outcomes of some investigations and the limited number of articles, no significant conclusions regarding the relationship between adverse effects on humans and extents of exposure can be drawn at this time. Here, we report that the current evidence is not conclusive and comprehensive and suggest prospective cohort studies in the future to evaluate the associations between human health outcomes and emerging environmental contaminants.

1. Introduction

Emerging contaminants are chemical substances or compounds characterized by a perceived or veridical threat to the environment or human health with a lack of published health criteria. An "emerging" contaminant may also be identified from an unknown source, a new exposure to humans, or a novel detection approach or technology [1, 2]. Emerging contaminants include an extensive array of synthetic chemicals in global use, such as perfluorinated compounds, water disinfection byproducts, gasoline additives, pharmaceuticals, man-made nanomaterials, and UV-filters, which are significant for the development of modern society [2–5]. Because of their rapidly increasing use in industry, transport, agriculture, and urbanization, these chemicals are entering the environment at increasing levels as hazardous wastes and nonbiodegradable substances [1, 2]. Furthermore, adequate and robust epidemic information on their behavior and fate in the global environment, as well as on human exposure, serum and tissue concentrations, and threats to ecological and human health, have not been well documented [2]. Therefore, this review emphasizes the current consensus and representative studies in the relevant fields. Here, we will discuss some emerging contaminants arousing general concern and summarize the evidence with respect to concepts, classification, and application and, particularly, outline potential human adverse effects based on a number of comprehensive epidemic literature reports and representative case reports.

2. Perfluorinated Compounds

Perfluorinated compounds (PFCs), which have been produced since the late 1940s, are composed of a fully fluorinated hydrophobic alkyl chain attached to a hydrophilic end group [6]. Perfluorooctanesulfonate (PFOS), perfluorooctanoic acid (PFOA), and their salts are the most essential representative PFCs and are widely used in fire-fighting foams, lubricants, metal spray plating and detergent products,

inks, varnishes, coating formulations (for walls, furniture, carpeting, and food packaging), waxes, and water and oil repellents for leather, paper, and textiles [6–8]. PFCs exhibit high heat, light, and chemical stability, and they are not easily degraded by microbial metabolism [8]. Therefore, PFCs are regarded as persistent, bioaccumulative, and potentially hazardous to animals and humans [7, 9]; however, their transport pathways and global fate have not been adequately documented to date [9, 10]. PFCs undergo wide transportation across all environmental media, including direct sources, such as the production, use, and disposal of consumer products containing these compounds, and indirect sources, such as volatile and neutral PFC precursor degradation [9, 11]. Currently, PFOA and PFOS have been detected in the surface water, sea, wildlife and drinking water, human serum, and even breast milk [7]. According to a study on PFCs, the presence of these species in serum, food, indoor substances, and consumer products and occupational exposure contribute to PFC exposure [12]. Nevertheless, inadequate and limited data about the adverse effects on humans exposed to the environment are available. The outcomes of some investigations on the impact of PFCs and their human health effects are summarized below.

2.1. PFCs and Cancer. The potential carcinogenicity of PFOS and PFOA has been investigated in laboratory animals, which demonstrated that these chemicals induce benign liver adenomas, pancreatic adenocarcinoma, and Leydig cell adenomas in rodent models [13–15]; however, the data on PFCs' potential carcinogenicity in the general population are sparse [14, 16]. During 1993 to 2006, Eriksen et al. performed a nested prospective cohort study among 57,053 participants from the general population aged 50–65 with no prior cancer in Denmark to analyze the connection between PFC serum levels and cancer hazard [17]. Considering PFOS and PFOA in the blood plasma as the exposure measurement, the end point was a clinical diagnosis of liver, bladder, prostate, or pancreas cancer during the follow-up duration. The study identified 67, 128, 322, and 713 patients with liver cancer, pancreatic cancer, bladder cancer, and prostate cancer, respectively. Then, a random comparison of 680 men and 92 women without cancer was performed to balance the novel confounded factors for cancer, and the result did not imply a virtual association between PFC concentration and cancer risk, except for prostate cancer in a population in Denmark. That study measured the plasma level of PFCs using only one method. Moreover, the half-life of PFOS varies between 8.7 years and 139 days in humans, and a single measurement of the PFC plasma level may not adequately reflect the plasma levels from previous years or the subject's exposure, which may vary during the observation [16]. Vassiliadou et al. conducted a cross-sectional study in 2010 to measure the serum PFOA and PFOS levels among 40 hospitalized cancer patients at the St. Savas Anticancer Hospital in Athens [18]. Then, the authors compared the results with 56 healthy working employees in an urban area and 86 ambulatory patients and healthy individuals in a rural area; both groups underwent a medical examination in Athens. The outcome showed no significant differences between the three participating groups, except a remarkable difference between the PFOA and PFOS levels of men and women in all groups involved. Because the samples in the study were collected during 2009, the temporal trends of PFOA and PFOS in Greece and any differences between cancer patients and PFCs in the rough cross-sectional data were difficult to assess. Two other case-control studies also investigated PFOA and PFOS levels in the blood of Inuit women with breast cancer (BC) and prostate cancer patients [19, 20]. Bonefeld-Jorgensen et al. used case-control studies to analyze the association between the serum levels of ten types of PFCs in 31 Inuit BC cases and 115 population-based controls without BC [19]. The authors concluded that the serum PFCs might be risk factors for BC in Inuits and that a potential mechanism might be hormone disruption related to xenoestrogenic and xenoandrogenic activities, which increase the risk of developing BC. Eight types of PFC serum levels were measured by Hardell et al. among 201 prostate cancer cases and 186 control subjects without prior cancer [20]. In the cancer group, a higher risk was found for hereditary prostate cancer after adjusting for confounded factors.

These four reports were relatively high-quality studies with long follow-up periods; however, only one study utilized a prospective exposure measurement [17]. Additionally, the cross-sectional study did not balance for confounded factors [18], and the two case-control studies investigated only data collected at a single hospital [19, 20], which could not explain the causal relationship between PFCs and cancer. Above all, the four studies focused on the association between PFC serum levels in the general population and cancer patients, which provided evidence for PFCs' adverse effects on humans, despite various drawbacks in the study designs. Only studies made by Eriksen et al. and Hardell et al. showed statistical significant association between PFC concentration and prostate cancer [17, 20]. The remaining studies did not indicate any causal association between PFC and cancer sites. In future studies, a causal interaction and mechanism between environmental PFC exposures and cancer in a general population would be valuable [14].

2.2. PFCs and Other Health Effects. The association between the serum level of PFCs and reproductive dysfunction in a general population has been widely studied in numerous reports addressing infertility, breastfeeding, and semen quality in humans. Fei et al. investigated whether exposure to PFCs and the potential hormonal disruptors might increase infertility [21]. The PFOS and PFOA concentrations with common exposure at weeks 4–14 of gestation were measured among 1240 women in developed areas based on the Danish National Birth Cohort (DNBC) program (1996~2002). The adjusted fecundity odds ratios (FORs) for the three highest-exposure quartiles compared with the lowest quartile of PFOS were 0.70, 0.67, and 0.74, and those of PFOA were 0.72, 0.73, and 0.60, respectively. These outcomes indicate that, at the plasma levels observed, PFOS and PFOA exposure may reduce fecundity in the general population; however, the underestimated association might be higher because of

the selection bias: only successful pregnancies were included in this study. In addition, Fei et al. studied the association between parental PFC concentrations and the breastfeeding period, also based on the DNBC [22]. The outcome indicated that PFOS might decrease the women's capacity to lactate, except primipara. The association between the PFOS serum level and multiparous women was not as convincing because multiparous women previously breastfed and because the PFOS serum levels could be reduced through excretion. A potential association was investigated between testicular function, semen quality, and perfluoroalkyl acids (PFAAs) by Joensen et al. [23]. The authors classified PFAA levels into 10 degrees and examined the serum concentrations of reproductive hormones under each PFAA degree to evaluate semen quality among 105 Danish men from the general population. The results implied that higher PFAA concentrations were related to fewer-than-normal sperm but not at statistically significant levels. These studies suggest that exposure to PFCs leads to some reproductive dysfunction; however, the association between exposure levels and the degree of dysfunction remains unclear.

Other studies have emphasized the potential associations between the serum PFOA and PFOS concentrations and prevailing thyroid disease. Pirali et al. measured the PFOS and PFOA levels among 28 participants who underwent a thyroid operation for benign diseases (7 for Graves' disease and 15 for multinodular goiters) and malignant thyroid diseases (5 for papillary carcinoma and 1 for follicular carcinoma) to determine whether there was a significant association between the serum and tissue concentrations of PFCs [24]. All thyroid samples from the surgical specimens were examined to determine the PFOS and PFOA levels. PFOS and PFOA were detected in operational and autopsy thyroid tissues. The average PFOA and PFOS levels were 2.0 ng/g and 5.3 ng/g, respectively, in the surgical specimens, similar to the autopsy thyroid from patients with thyroid diseases. In addition, the serum levels of PFOS and PFOA were remarkably higher than those in the relevant surgical specimens. These outcomes do not indicate that PFOS and PFOA are actively condensed in the thyroid. This study did not provide sample recruitment and control information. Melzer et al. analyzed the PFOA and PFOS levels and health conditions based on the National Health and Nutrition Examination Survey (NHANES project), which encompassed 3,974 adults [25]. The authors analyzed the PFC levels during three different periods and the incidence rates of reported thyroid disease and current thyroid dysfunction in women and men. Employing fully adjusted logistic models, the results indicated that higher PFOS and PFOA serum levels were associated with current thyroid disease among a general adult population in the US. No overlap was detected between the NHANES samples, the measured PFC levels, and the samples from people with thyroid hormones measured, which limited the study data. Knox et al. analyzed the data of the C8 Health Project and found that PFOS and PFOA were correlated with considerable increases in serum thyroxine (T4) and decreases in triiodothyronine (T3) uptake in all cases studied [26]. A cross-sectional study conducted by Shrestha et al. investigated the effects of PFCs on thyroid hormone in 87 older adults in New York [27]. The authors concluded that higher serum perfluoroalkyl substances (PFASs) levels were associated with increased fT4 and T4; however, another cohort study performed by Webster et al. showed that PFASs were significantly correlated with TSH and negatively related to fT4 in a population of pregnant women with higher TPOAb in Canada, which occurred in 6 to 10 percent of pregnant women [28]. Considering the results reported by Pirali et al., lower PFC levels in thyroid tissues than in serum exerted harmful effects on the thyroid [24]. And outcomes drawn from 28 participants and deficient statistical power in this study limited their findings. The three studies proposed that higher PFC serum levels might change thyroid hormone levels [26–28]; however, the results of these studies were contradictory in some aspects, such as serum PFC-related increases in T4 and fT4 and decreases in T3 and hypothyroidism. These discrepancies might be caused by population differences in sex, age, region, individual specificity, exposure level, and objectives and the methods used in the study.

Studies have also focused on the potential associations between the serum PFOA and PFOS concentrations and metabolic diseases. Frisbee et al. analyzed data from the C8 Health Program, which allowed the examination of a very large population of 69,030 US residents living near a chemical production facility that released PFOA [29]. The authors advocated that the arithmetic average (SD) serum PFOA and PFOS concentrations among 12476 adolescents and children were 22.7 ng/mL and 69.2 ng/mL, respectively. After adjusting for covariants, PFOA was substantially associated with increased total lipid and low-density lipoprotein cholesterol (LDL-C), and PFOS was considerably related to increased total lipid, LDL-C, and high-density lipoprotein cholesterol (HDL-C). The relationships between PFOA and PFOS and gene modifications during the process of lipid metabolism in humans were investigated for the first time by Fletcher et al. [30]. Employing adjusted linear regression models, the authors concluded that increased copy numbers of lipid mobilization genes were related to PFOS levels and observed decreased copy numbers of lipid-transport genes. The results implied that PFC exposure might lead to a hypercholesterolemic condition, with further adverse influences on human health. Lin et al. detected the effect of PFCs on glucose homeostasis by measuring the perfluorononanoic acid (PFNA) serum levels among 474 teenagers and 969 adults from the NHANES and found that higher serum PFNA levels were related to hyperglycemia and higher β-cell activity [31]. The data from the NHANES were also analyzed by Nelson et al. to investigate the associations between lipid, weight, and PFC serum levels [32]. The authors observed that PFOA, PFOS, and PFNA are significantly related to the total lipid and non-high-density cholesterol (NHD-L) levels and negatively related to insulin resistance and weight. Steenland et al. interrogated the association between uric acid and PFCs and concluded that the PFOA serum concentrations were significantly related to a higher rate of hyperuricemia, which is a potential risk factor for hypertension and other cardiovascular diseases [33]. As a result, based on the current studies, there is inadequate evidence to draw scientific conclusions

regarding the potential causal adverse effects of PFCs on human metabolic diseases. In addition, the PFC levels in the environment must be related to the exposure concentration in the human body and further health outcomes.

3. Disinfection Byproducts

Disinfection chemicals used in swimming pool and drinking water purification are key components shielding humans from water-borne diseases [9, 34]. These chemicals, which are usually oxidizing agents, possess strong chemical activities that not only eliminate pathogenic agents but also react with many deoxidizers [35]. As a result, undesired byproducts are created during disinfection procedures. The widespread and frequent use of these chemicals produces disinfection byproducts (DBPs), particularly chlorinated DBPs (CDBPs) in purified water, and nearly all humans are exposed to these chemicals in developed regions through swimming pools and drinking water [4, 36]. More than six hundred DBPs have been discovered, including iodinated trihalomethanes (THMs), aldehydes, ketones, halomethanes, hydroxy acids, carboxylic acids, alcohols, keto acids, esters, and even nitrosamines (NDMA) [9, 37]. THMs and HAAs are the two major types of halogenated DBPs, accounting for over 80% [34].

3.1. DBPs and Cancer. THMs' carcinogenic effects have been confirmed by numerous studies employing laboratory animals, which show that THMs in the drinking water are associated with colorectal tumors, BC, and bladder tumors as a result of nongenetic toxicity [34]. To date, we have not found adequate, causal evidence to support the relationship between cancer and THMs at typical doses in animals. Unfortunately, sparse findings have been observed on the adverse effects on humans upon THM exposure. Villanueva et al. investigated whether bladder cancer was related to THM exposure by oral and respiratory pathways or dermal absorption of water during bathing and swimming [38]. This study enrolled 1,219 subjects and 1,271 matched participants in a case-control study in Spain during 1998–2001. Long-time THM exposure was related to a twofold-higher incidence of bladder cancer, with an OR of 2.10 and 95% CI of 1.09 to 4.02 for mean household THM concentrations of >49 and ≤8 μg/liter. Compared with participants who did not drink chlorinated water, those with THM exposure of ≥35 μg/day by ingestion exhibited an OR of 1.35 and 95% CI of 0.92 to 1.99. The OR for shower or bath duration by THM concentration was 1.83 with 95% CI of 1.17 to 2.87 for the highest compared with the lowest quartile. Swimming in pools was correlated with an OR of 1.57 (95% CI: 1.18–2.09). Bladder cancer was related to long-duration exposure to THMs from chlorinated water typical of the exposure experienced in developed areas. Nevertheless, a large-scale case-control study conducted by Michaud et al. in Spain indicated that water ingestion was negatively related to bladder cancer without considering THM exposure concentrations [39]. Chang et al. also detected whether DBP exposure was relevant to bladder cancer [40]. The authors designed a matched-pair study to analyze the association between the exposure to total trihalomethanes (TTHM) in the drinking water and the mortality rate of bladder cancer among 65 participants in a Taiwanese province. The adjusted ORs of bladder cancer mortality for the municipality's TTHM levels in the drinking water were 1.8 (95% CI: 1.18–2.74) and 2.11 (95% CI: 1.43–3.11), respectively, in the highest and intermediate groups. The outcome of this investigation indicated that there were positive associations between the levels of TTHM in treated water and bladder cancer morality. Salas et al. conducted a case-control study recruiting 559 hospital controls and 548 incident cases to explore potential mechanism between THM exposure and bladder cancer, and the results indicated that THM exposure might be related to DNA methylation [41]; however, evidence regarding the correlation between DBP exposure and cancer was mixed. A case-control study on DBPs and colorectal cancer was integrated by King et al., who divided participants into two groups (more than 35 years of exposure and not more than 10 years of exposure to chlorinated water) and adjusted for confounders. The OR of colon cancer was 1.63 (1.07–2.48) for ≥75 μg/L and OR of rectal cancer was 0.91 (0.55–1.51) for ≥75 μg/L. For males, long-term exposure to DBP showed an excess risk of colon cancer. And females exposed to DBP were not associated with risk of colon cancer. The association between the risk of rectal cancer and participants exposed to DBP was not observed in this study [42]. Rahman et al. recruited King's study and other 12 studies to investigate on DBP and colorectal cancer by a meta-analysis. The authors suggested that, for colorectal cancer, because of the inconsistencies of the outcomes and poor quality of the relevant investigations, we cannot draw any conclusions [43]. Other cancers, such as breast, pancreas, esophagus, lung, kidney, and brain, were interrogated by sporadic studies, from which no meaningful conclusions can be drawn. Additionally, for melanoma and nonmelanoma, leukemia, and skin cancer, no significant correlations with DBP can be confirmed because of the current inadequate evidence [34]. Overall, a small number of sites have been identified by available evidence on the human body as suspected targets, especially the bladder, but drawing crucial conclusions regarding causality is hindered by intrinsic questions such as methodological drawbacks, exposure assessment limitations [38, 40]. Specifically, which DBPs are the most important compounds and their molecular mechanisms in human beings and dose-response relationship remain to be clarified.

3.2. DBPs and Other Health Effects. Recently, these byproducts have been suspected as risk factors for infertility, fetal loss, long gestational duration and poor fetal growth, and fetal anomalies, many of which have been interrogated in published records or current studies. Some studies investigated on DBPs in tap water and semen quality and reported a negative influence of DBP exposure on normal sperm concentration and sperm morphology, but not on motility percentage [44, 45]. And these studies indicated that poor sperm quality in humans was not associated with exposure

to levels of DBPs near or below regulatory standard and recommended further study [44–46]. Another study analyzed the association between DBPs and menstrual cycle function based on data from a prospective study and suggested that THM exposure might affect ovarian function with decreased cycle length and follicular phase length [47]; Waller et al. conducted a prospective study and concluded an increased risk of spontaneous abortion for women with consumption of five or more glasses per day of cold tap water containing ≥75 μg/liter of total THMs [48]; Hoffman et al. focused on DPBs exposure and fetal growth, which showed no correlation except average residential concentrations above regulatory limits [49]. As for fetal malformation, Agopian et al. analyzed data from the National Birth Defects Prevention Study (NBDPS) delivered during 2000–2007 and found that gastroschisis might be associated with shower length, but not relevant to bath length or shower frequency [50]. Righi et al. did a case-control study in Italy on 1917 different congenital malformations and indicated a higher risk of newborns with renal defects (OR: 3.30; 95% CI: 1.35–8.09), abdominal wall defects (OR: 6.88; 95% CI: 1.67–28.33), and cleft palate (OR: 4.1; 95% CI: 0.98–16.8) when maternal exposure of chlorite level was 700 μg/L. And higher risks of newborns with obstructive urinary defects (OR: 2.88; 95% CI: 1.09–7.63), cleft palate (OR: 9.60; 95% CI: 1.04–88.9), and spinal bifida (OR: 4.94; 95% CI: 1.10–22) were observed at women exposed to chlorate level of 200 μg/L [51]. This outcome may be because most of water disinfectant in Italy is chlorine dioxide.

Despite the large scale of research, no determined evidence exists to support reproductive hazards related to DBP exposure levels, besides slight correlations with some types of congenital malformations [50, 51]. Despite the fact that disinfection produces hundreds of substances in different proportions, in these studies only small numbers of these pertinent pollutants are normally evaluated and measured, which may contribute to the mostly negative outcomes. In addition, relationships with sex, smoking, genetic susceptibility, and other risk factors must be clarified.

4. Gasoline Additives

Gasoline encompasses more than five hundred components, such as the known or suspected carcinogenic substances benzene, 1,3-butadiene, and methyl tert-butyl ether (MTBE) [52]. MTBE, the most widely used oxygenated bunker, is diffusely used as a new unleaded petrol additive, particularly in developing districts [53, 54]. MTBE not only enhances the octane additive used in petrol to improve its burning efficiency and decrease carbon monoxide and other hazardous materials, such as ozone and benzene, in automobile exhaust but may also be used to replace tetraethyl lead as an antiknock species [53, 55]. MTBE is a colorless, smelly liquid with limited water solubility (4 g/100 g in water) and easily infiltrates into soil and spreads to the surrounding environment by volatilization [56]. MTBE can also contaminate surface water and groundwater, seriously threatening drinking water sources. Because of its special structure and properties, MTBE has a long half-life in groundwater and is difficult to degrade. MTBE is rapidly absorbed by inhalation exposures [55]. Human also can be exposed to MTBE by dermal absorption and ingestion of contaminated water [56].

Animal studies have revealed that MTBE can lead to testicular, uterine, and kidney cancer and also harm the kidney, immune system, liver, and central nervous system [53–56]. MTBE, listed as a suspected carcinogen, exhibits a potential toxicity on the human body. Symptoms, including sicchasia, headache, and optical and nasal stimulation, have been suspected to be associated with acute exposure to MTBE. Johanson et al. recruited 10 healthy males to measure the adverse effects of MTBE [57]. Subjective ratings (stimulating symptoms, discomfort, and CNS symptoms) and eye (redness, conjunctival harm, blinking frequency, and break-up time of tear film) and nose (summit expiratory flow, aural rhinometry, and phlogistic markers in rhinal lavage) measurements were analyzed. Solvent smell was the only positive rating noted as the exposure level increased. The blocking index number, an index of nasal swelling, was aggravated with exposure duration, but no exposure-response association was found, showing that MTBE was not the decisive factor for this symptom. Joseph and Weiner performed an analytical study and found that the incidences of cough, headache, throat stimulation, hypersensitive rhinitis, upper respiratory communicable disease, sicchasia, dizziness, wheezing, anxiety, otitis media, insomnia, skin rash, palpitations, malaise, and allergy were associated with the MTBE levels in the air [58]. During the winters of 1994-1995 and 1993-1994, the levels of MTBE and the incidence of these symptoms increased, whereas, during the summer, the MTBE levels and the symptom incidence were both relatively low. Wheezing and asthma were particularly increased. Specific health complaints of MTBE exposure have not been reported in those studies.

Occupational exposure of MTBE among workers such as road tanker drivers, garage workers, and gasoline service station attendants had drawn worldwide concern since 1990s, although such exposure does not take place in general population [59–61]. In order to evaluate neuropsychological adverse effects among 101 road tanker drivers, who were exposed to gasoline that contained 10% MTBE, Hakkola and Saarine compared them with 100 milk delivery drivers working in the same district in Finland [60]. After interviewing based on standardized questionnaires, tanker drivers exhibited a higher fatigue score than milk delivery drivers, and 20% of the tanker drivers complained of nausea or headaches. This study did not illuminate specific exposure MTBE of two groups. Vojdani et al. reported that the proportions of abnormal apoptotic cells lymphocytes were higher (26.4 ± 1.8%) in the group exposed to MTBE and benzene polluted water for five to eight years among 60 people than in the unexposed groups (12 ± 1.3%) and so were lymphocyte DNA adducts [59]. The outcomes indicated that long-term exposure to MTBE and benzene could induce genotoxic damage which might signal initiation of carcinogenicity. However, this study did not measure MTBE and benzene levels over the period in polluted water. Moreover, the outcome observed cannot be attributed to either contaminant for exposure to MTBE and benzene was not characterized.

MTBE was once used to dissolve gallstone or remove residual debris in percutaneous transhepatic removal [61, 62]. Leuschner et al. measured blood concentrations of MTBE in patients with cholelithiasis; the peak concentrations of the special exposure were about 1000-fold higher than concentration in workers who were exposed to inhalation [63]. Although MTBE and its metabolite were measured in the urine and blood of these patients and it took several days for elimination, no adverse effects were reported in this study. In other relevant case reports and reviews, adverse effects such as renal failure, coma, intravascular hemolysis, and bile leak were reported after this therapy [64, 65].

MTBE subchronic adverse effects have been reported by many studies, most of which employed mouse models [53]. These studies found that MTBE was associated with cancer [66, 67], adverse neurotoxic effects [68], increased blood-urea nitrogen (BUN) [69], and others. Some authoritative studies focused on MTBE carcinogenicity, and no carcinogenic risk was confirmed because of insufficient evidence in humans and limited evidence in laboratory animals.

5. Manufactured Nanomaterials

Manufactured nanomaterials by definition have a particle size of approximately 1–100 nm, and examples include amorphous silicon dioxide (SiO_2), carbon nanotubes (CNTs), and titanium dioxide (TiO_2) [70, 71]; these materials are considered to be emerging contaminants [72]. Manufactured nanomaterials are widely used in sunscreen products, agriculture, transport, healthcare, materials, energy, and information technologies [70, 73–75]; however, novel trace methods for examining the relevant residues and nanoscale pollutants have not been established because of their limited production and relatively immature detection techniques [76, 77]. Nanoscale materials will generate physical and chemical properties, such as particular surface effects, small size effects, and quantum effects, which may produce uncertain biohazard effects [70]. The atomic interface of nanomaterials can cover 15% to 50% of the overall surface area, and this structure provides nanomaterials with strong adsorption capacity in the air, water, and soil, which can adsorb toxic gases (NO_2, SO_2, and others), toxic heavy metals (copper, lead, mercury, cadmium, and others), and biologically active substances (polycyclic aromatic hydrocarbons, pesticides, microorganisms, proteins, nucleotides, refractory organics, and others) [78, 79]. Other special properties of nanomaterials, such as their catalytic character and superior toughness and strength, make these materials resistant to degradation using chemical and biological methods [70]. Manufactured nanomaterials undergo long-term migration, conversion processes, and complex chemical reactions in the environment while adsorbing various inorganic and organic molecules on their surfaces. As a result, new pollutants are formed.

In animal tests and in vitro assays, manufactured nanomaterials have shown well-defined carcinogenic potentials, especially reproductive and developmental toxicity at high doses [80]. Some scientists have suggested that nanomaterials may be carcinogenic, regardless of their chemical components [81]. Some epidemiological studies in different periods and regions estimated the association between the exposure levels of TiO_2 and lung cancer in humans, but none have shown statistically significant results [82–84]. Regarding CNTs, no relevant studies have been published in this field to date [85]. A meta-analysis of twenty-eight cohorts and fifteen case-controls by Pelucchi et al. investigated the exposure levels of silica and lung cancer risk and found no significant evidence to support the carcinogenicity of SiO_2 [86]. Nanomaterial biological safety issues have attracted worldwide interest [70]. To date, the mechanisms underlying the toxicity to humans and animals remain unknown [71]. Therefore, more attention should be paid to the environmental safety of manufactured nanomaterials and to strengthening research related to human health effects.

6. Human and Veterinary Pharmaceuticals

Pharmaceuticals are emerging contaminants in the environment because of their increasing applications in humans and animals [87–89]. Some medicines persist in the body after application and exhibit a novel pattern of action [87]. In recent years, because of the continuously increasing amounts of drugs and advanced ultra-trace detection technologies, considerable human and veterinary drugs have been detected in the environment, especially in water [88, 90, 91]. Approximately three thousand different chemicals involved in human medicine, including lipid regulators, anti-inflammatory drugs, analgesics, contraceptives, neuroactive medicine, antibiotics, and beta-blockers, exist [87, 90]. The main pathway through which pharmaceuticals enter the surface water is human intake, followed by subsequent excretion in municipal wastewater, hospitals, pharmaceutical waste, and landfills [90]. The human pharmaceuticals present in sewage are difficult to remove, and, as a result, high levels of medicine are being released in treated effluents. Inputs of pharmaceuticals into the water systems have been reported in rivers, lakes, treated effluents, and groundwater [90, 92]. After long periods of enrichment, high concentrations of drug residues will threaten human health and the ecosystem [91]. Thus, there is increasing widespread concern about the potential influence of pharmaceutical residues in the environment [87]. Additionally, the considerable use of antibiotics has garnered ubiquitous attention regarding the wide and extensive antibiotic resistance of microorganisms [93, 94].

6.1. Veterinary Antibiotics (VAs). VAs are being increasingly used in many regions to protect the health of animals and treat diseases to improve the feed efficiency of livestock, poultry, pets, aquatic animals, silkworms, bees, and so on [92, 95]. VAs are mainly divided into several pharmacological types: antimicrobial, anthelmintic, steroidal and nonsteroidal, anti-inflammatory, antiparasitic, astringent, estrus synchronization, nutritional supplement, and growth promoter [92]. A large number of antibiotics employed in animal food production are inefficiently adsorbed in the animal's gut, and, as a result, almost 30–90% of these drugs are excreted [96].

Moreover, VA additives can be active and converted back to the prototype after excretion [97, 98]. Thus, a considerable percentage of the veterinary antibiotics may spread into the surroundings in bioactive forms, which will cause long-term adverse effects on the soil, water, microorganisms, plants, and animals and finally affect human health through the food chain [92]. The frequent use of VAs has drawn attention about the potential increasing populations of new resistant strains of bacteria. Detected bacterial populations from gut of animals given antibiotics were about five times to be resistant to common antibiotic resistant microbial strains [99]. Esiobu et al. reported a 70% enhancement in resistance to certain antibiotics including streptomycin, penicillin, and tetracycline after using soil manure from animals in a dairy farm [100]. However, no reliable studies have elaborated the relationship between VAs use and human antibiotics resistance and other health outcomes at present.

6.2. Human Pharmaceuticals. Drugs for humans are designed to play an active role in the specific metabolic and molecular pathways [90]; however, some also have side effects in humans. Pharmaceuticals in the environment have ecotoxicological effects on some nontarget species that have the same active sites, such as organs, tissues, cells, and active molecules with target species [91]. Recently, numerous studies focusing on the acute toxic effects to aquatic organisms resulting from drug consumption have been reported. Propranolol exhibits strong acute toxicity on benthos and zooplankton, whose lethal dose of 50% (LC_{50}) is about 1 mg/L, and fluoxetine toxicity on benthos is stronger than propranolol, whose LC_{50} is less than 0.5 mg/L [101, 102]. Often, human pharmaceuticals acute toxicity is nonspecific, for example, unspecific membrane toxicity by oxidative stress. Moreover, because acute effects levels are about 100–1000 times higher than pharmaceuticals residues detected in the aquatic environment, acute toxicity to aquatic organisms hardly occurs at detected environmental levels [91]. Studies on environmental pharmaceuticals acute toxicity to human have not been reported yet. The reason may be also due to pharmaceuticals environmental levels being relatively at low levels which cannot induce human adverse effects.

Studies on the long-term exposure toxic effects with lower drug doses are relatively limited. Fenske et al. reported that 17α-ethinylestradiol (EE2), a widely used oral contraceptive, induced male gonad developmental arrest in zebrafish exposed to man-made EE2 at an environmentally relevant concentration (3 ng/L) in a flow-through system [103]. Previously, researchers suggested that EE2 is present at low levels and is not harmful, but this study found that zebrafish exposed to a typical environmental concentration of EE2 displayed estrogenic effects, such as abnormal phenotype development and reduced reproductive success. Because of the increasing levels of EE2, estradiol, and other estrogenic substances being detected in surface water, animals with chronic exposure to these biological compounds may suffer some potentially toxic effects without obvious signs.

Most human and veterinary pharmaceuticals exist in the environment at low concentrations that do not cause acute toxic effects. However, some organisms are exposed to low doses over long periods during their lifetime, leading to remarkable chronic toxic effects [91]. Studies on long-term and low-dose exposure are more accurate and should directly reflect the ecotoxicological effects of human and veterinary pharmaceuticals. However, current investigations are not sufficient to derive an accurate profile of the possible hazards of pharmaceuticals, and some studies on animals, such as fish, may imply potential mechanisms and influences on human.

7. Sunscreens/Ultraviolet Filters

Sunscreens/ultraviolet filters (UV-filters) are mainly used in personal care products, such as lipsticks, perfumes, hairsprays, hair dye and moisturizers, skin care products, shampoos, and makeup, as well as in noncosmetic products, including furniture, plastics, carpets, and washing powder [104, 105]. Sunscreens are popular protective products against ultraviolet radiation hazards, early skin aging, and skin cancer [106]. UV-filter formulations can be organic (chemicals) or inorganic (minerals) [107]. According to the FDA, inorganic sunscreen scatters UV radiation with wavelengths of 290 to 400 nm [108, 109]. Organic sunscreens absorb novel photons of UV and include 3-(4-methylbenzylidene)camphor (4-MBC), benzophenone-3 (BP-3), 2-ethylhexyl 4-methoxycinnamate (OMC), 2-ethylhexyl 4-dimethylaminobenzoate (OD-PABA), 3-benzylidene camphor (3-BC), homosalate (HMS), and 4-aminobenzoic acid (PABA) [105]. In addition, highly produced lipophilic sunscreens can spread into the aquatic environment by bathing, washing clothes, and swimming [110]. Potential exposure patterns of humans and animals overlap through the food chain.

Many in vitro and in vivo studies based on lab animals have indicated the endocrine-disrupting influences of sunscreen, including disruption of the hypothalamic-pituitary-thyroid axis (HPT) and reproductive and developmental function [105]. Few human investigations have addressed the possible adverse effects of UV-filters, and the study durations are inadequate to be conclusive. Frederiksen et al. investigated BP-3, a UV-filter, and found that it could be detected in 96% of American urine specimens and 85% of Swiss breast milk specimens analyzed [104]; however, UV-filter potential hazards on humans are difficult to assess using exposure data alone. At present, most case reports are related to dermatitis caused by sunscreens [106]. The associations between sunscreens and adverse effects on humans have not been deeply and widely investigated.

8. Conclusions

Humans and the ecosystem as a whole are exposed to various emerging contaminants through different methods, both known and unknown [3, 4]. Our review demonstrates that these contaminants continuously produce emerging and urgent challenges to the soil, water, air, and ecosystems, particularly human health (Figure 1) [2]. Moreover, new chemical outputs spread and usually exceed the abilities

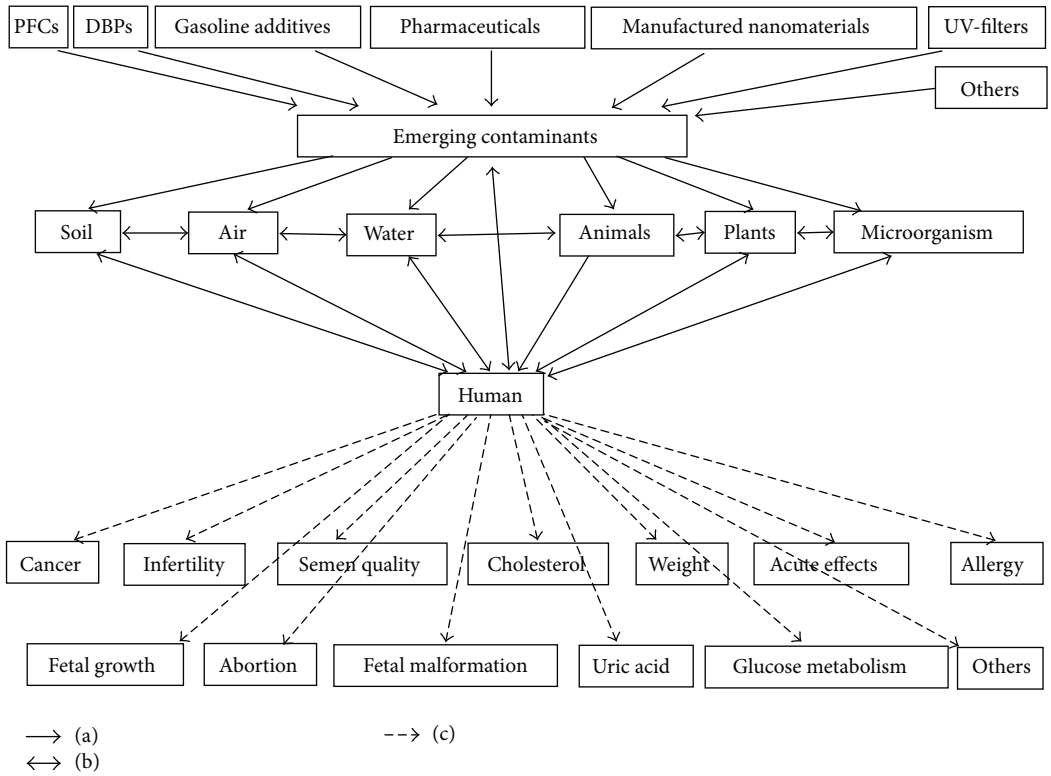

Figure 1: (a) represents that perfluorinated compounds, water disinfection byproducts, gasoline additives, manufactured nanomaterials, human and veterinary pharmaceuticals, UV-filters, and other pollutants are emerging contaminants. The impact of emerging contaminants acts on soil, air, water, animals, plants, microorganisms, and human. (b) represents interactions between soil, air, water, animals, plants, microorganism, human, and emerging contaminants. (c) represents that human in exposure of emerging contaminants may have potential adverse effects.

of safety remediation, risk evaluation methods, monitoring techniques, and current preventative factors [1, 2]. Given the current conditions, the following several interactive factors should be considered to assess human health effects of pollutant exposure: contaminant concentration, category and properties, scale and level of pollutants, and the hazard intensity generated for health and available resources. The elimination of emerging contaminants in the environment and human body depends on future techniques and studies to establish overall remediation theories and methods [1, 3].

Based on current investigations and studies, the emerging contaminants present suspected mutagenicity, teratogenicity, and carcinogenicity to humans and other animals. On the one hand, no sufficient and large-scale evidence has proven causal associations between emerging contaminants and adverse effects on the human body. On the other hand, whereas the exposure levels in animal experiments may not represent human exposure levels in reality and long-term chronic exposure is seldom employed in animal models, we cannot ignore the adverse effects indicated by animal experiments. Further large-scale studies with improved designs are needed to provide more convincing and clear outcomes.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Heavy Metal Contents and Physical Parameters of *Aegiceras corniculatum*, *Brassica juncea*, and *Litchi chinensis* Honeys from Bangladesh

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The present study was undertaken to determine the heavy metal levels and the physicochemical parameters (pH, electrical conductivity (EC), and ash, moisture, and total sugar content) of honeys from Bangladesh. Three different floral honeys were investigated, namely, khalsi (*Aegiceras corniculatum*), mustard (*Brassica juncea*), and litchi (*Litchi chinensis*) honeys. The heavy metals in the honeys were determined by using a High Temperature Dry Oxidation method followed by Atomic Absorption Spectroscopy. The mean pH, EC, and ash, moisture, and total sugar contents of the investigated honeys were 3.6, 0.51 mS/cm, 0.18%, 18.83%, and 68.30%, respectively. Iron was the most abundant among all the investigated heavy metals, ranging from 13.51 to 15.44 mg/kg. The mean concentrations of Mn and Zn in the investigated honeys were 0.28 mg/kg and 2.99 mg/kg, respectively. Cd was below the detection limit, and lead was found in some honey samples, but their contents were below the recommended Maximum Acceptable Level. Cr was also found in all of the samples, but its concentration was within the limit. The physicochemical analysis of the honey samples yielded levels within the limits set by the international honey legislation, indicating that the honey samples were of good quality and had acceptable values for maturity, purity, and freshness.

1. Introduction

Honey is a natural product produced by honey bees, and the composition and properties of any individual honey sample depend highly on the type of flowers visited by the bees as well as on the climatic conditions in which the plants grow [1]. The mineral content of honey depends on the botanical origin and the geographical origin of the honey [2]. Honey is an indicator of environmental pollution [3]. The forage area of bees is very large (more than 7 km^2 from the hive), and during the foraging process bees come into contact not only with air but also with soil and water [4]. The bees can come into contact with air and water that is contaminated with metals, which could be transported to the colony [5]. Moreover, air and water from industry and traffic contain heavy metals, which can also contaminate the bee colony and its products.

In Bangladesh, the possible emission of heavy metals occurs in various industrial sectors and activities, such as textiles and dyeing [6], ship-breaking areas, vehicular emissions, and agricultural runoff [7]. In Bangladesh, honey is produced and consumed on a large scale both commercially and noncommercially. Sundarbans, which is the largest mangrove forest in the world, is an ideal place for honey collectors. In recent years, the annual harvest has been

Table 1: Floral type, source, and collection time of the investigated Bangladeshi honeys.

Honey type	Sample ID	Source location	Time of collection
Khalsi (*Aegiceras corniculatum*)	BH-1	East Satkhira range, Sundarbans	April 2013
	BH-2	East Satkhira range, Sundarbans	April 2013
	BH-3	East Satkhira range, Sundarbans	April 2013
	BH-4	East Satkhira range, Sundarbans	April 2013
	BH-5	East Satkhira range, Sundarbans	April 2013
	BH-6	East Satkhira range, Sundarbans	April 2013
Mustard (*Brassica juncea*)	BH-7	Birganj, Dinajpur	January 2014
	BH-8	Birganj, Dinajpur	January 2014
	BH-9	Singair, Manikganj	January 2014
	BH-10	Singair, Manikganj	January 2014
	BH-11	Madhupur, Tangail	January 2014
	BH-12	Madhupur, Tangail	January 2014
Litchi (*Litchi chinensis*)	BH-13	Dinajpur Sadar, Dinajpur	March 2013
	BH-14	Dinajpur Sadar, Dinajpur	March 2013
	BH-15	Manikganj Sadar, Manikganj	March 2013
	BH-16	Manikganj Sadar, Manikganj	March 2013
	BH-17	Kalaroa, Satkhira	April 2013
	BH-18	Kalaroa, Satkhira	April 2013

established at approximately 200 metric tons of honey, with the honey produced from the Sundarbans area contributing to approximately 50% of the honey produced in Bangladesh. Large amounts of honey are also produced in different regions of the country. The plants and various agricultural crops grown in Bangladesh are excellent for bee foraging because bees are necessary for their pollination. Given their close proximity to urban development, the mangrove ecosystems are exposed to significant direct contaminant input [8].

To date, although honeys are produced in large quantities and are widely consumed in Bangladesh, very few data are available on heavy metal contamination in the honeys originating from different regions. Thus, the present work was aimed at determining the levels of seven metals, namely, cadmium (Cd), chromium (Cr), lead (Pb), copper (Cu), iron (Fe), zinc (Zn), and manganese (Mn), and six physicochemical parameters (pH, electrical conductivity (EC), ash content, moisture content, and sugar content) of various floral honeys that are produced in different areas of Bangladesh. The values were compared with two other honey samples (manuka honey from New Zealand and tualang honey from Malaysia). In addition, the honey was also investigated for correlation among individual constituents.

2. Materials and Methods

2.1. Chemicals and Reagents. Reference standard heavy metals such as Cd, Cr, Pb, Cu, Fe, Zn, and Mn were purchased from Kanto Chemical Co. Inc. (Tokyo, Japan). The chemicals used for digestion analysis were hydrochloric acid (HCl), nitric acid (HNO_3), sulfuric acid (H_2SO_4), and hydrogen peroxide (H_2O_2); they were of analytical grade and they were purchased from Merck (*Darmstadt*, Germany).

2.2. Honey Samples. During this study, eighteen honey samples from three different floral types, namely, khalsi (*Aegiceras corniculatum*), mustard (*Brassica juncea*), and litchi (*Litchi chinensis*), were investigated (Table 1). The samples were collected directly from the comb and local apiary of different regions in Bangladesh between March 2013 and January 2014. At least 100 g of each honey sample was collected under sterile air in a tight glass bottle, labeled properly, and stored at 4-5°C until analysis. Two honey samples (manuka honey from New Zealand and tualang honey from Malaysia) were used as gold standards to compare their values with those from Bangladesh. The honeys were used as standards due to their well established chemical and biological properties. Manuka honey is a monofloral honey produced in New Zealand and Australia from the nectar of the manuka tree (*Leptospermum scoparium*) while tualang honey is a wild multifloral honey produced by the *Apis dorsata* bees. The bees collect nectar from plants and blossoms from a tall *Koompassia excelsa* tree, known locally as the "tualang tree," in which the bees build their hives.

2.3. Physicochemical Parameters

2.3.1. pH. The pH values of the honey samples were determined by using a pH meter (Cole-Parmer, Illinois, USA). A 10% (w/v) honey solution was prepared in fresh Milli-Q water. The pH of each honey was measured on the same day, and the experiments were conducted in duplicate for each sample.

2.3.2. Ash Content. Approximately 1 g of honey sample was transferred into a porcelain crucible and heated for approximately 6 h at 450°C. Following complete ashing (the ash became white and grayish white), the samples were cooled in

a desiccator and weighed [9]. The ash content was calculated by using the following formula:

$$\text{Ash (\%)} = \frac{\text{Weight of sample after ashing}}{\text{Weight of fresh sample}} \times 100. \quad (1)$$

2.3.3. Moisture Content. Approximately 3 g of honey was transferred into a porcelain crucible and heated for 3 h in an oven at 105°C. To ensure the complete removal of moisture, each crucible was reheated and weighed until the weight became constant. The moisture content was calculated by using the following formula:

$$\text{Moisture (\%)} = \frac{\text{Weight of fresh sample} - \text{Weight of dry sample}}{\text{Weight of fresh sample}} \times 100. \quad (2)$$

2.3.4. Electrical Conductivity. The EC was determined according to the method established by the International Honey Commission [4]. The EC was measured at 20°C in a 20% (w/v) solution (dry matter basis) in distilled water by using a Hach conductivity meter. The result was expressed in millisiemens per centimeter ($mS{\cdot}cm^{-1}$).

2.3.5. Total Sugar Content. The total sugar contents of the honeys were determined by using a refractometer (Delta Refractometer, Code 20–70, Bellingham + Stanley Ltd., England). The sugar content is represented by °Brix.

2.3.6. Sample Preparation for Metal Analysis. The collected honey samples were prepared by a High Temperature Dry Oxidation method [9]. Each honey sample (1 g) was dried in a porcelain crucible at 100°C to its dry weight, which was then heated to 445°C for 6 h in an electrical furnace. After complete ashing, 3 mL of HNO_3 was added, followed by acid evaporation on a hot plate at 100°C. Afterwards, 5 mL of HCl was added, and the volume was filled to 10 mL with distilled water (dilution 1 : 2). The solution was filtered and preserved in a refrigerator at 4-5°C until further metal analysis with an Atomic Absorption Spectrophotometer, model AA-6300, Shimadzu (Kyoto, Japan), equipped with a Shimadzu model GFA-EX7i graphite furnace atomizer to determine the heavy metals.

The measurement wavelengths for different heavy metals were as follows: Cd (228.67 nm), Cr (357.65 nm), Cu (324.57 nm), Mn (279.43 nm), Pb (217.35 nm), Zn (213.93 nm), and Fe (248.30 nm). Each sample was analyzed in triplicate. Two blanks were injected for each determination. For the calibration curve, standard solutions of each metal solution were prepared at different concentrations (0.0, 0.1, 1.0, 5.0, 10.0, 20.0, and 40.0 μg/L) (Table 3). The metal analysis method used for the honeys was validated by using a recovery analysis. The percentage recoveries were calculated by using the following equation:

$$\text{Percentage recovery} = \left(\frac{\text{CE}}{\text{CM}} \times 100\right), \quad (3)$$

where CE is the experimental concentration that was determined from the calibration curve and CM is the spiked concentration.

2.4. Statistical Analysis. The assays were performed in triplicate, and the results were expressed as the mean values with standard deviations (SD). Correlations were established by using Pearson's correlation coefficient (r) in bivariate linear correlations ($p < 0.01$). The correlations were calculated with SPSS version 18.0 (IBM Corporation, New York, USA), and the other analyses were performed with Microsoft Excel 2007.

3. Results and Discussion

3.1. Physicochemical Parameters. The mean pH value of the investigated honeys was 3.6, and there were large variations (Table 2). The honey samples collected from Sundarbans (khalsi) have relatively high pH values (3.69) in comparison with mustard (3.49) and litchi honey (3.62). The pH values of the investigated honeys were lower than that of manuka honey but similar to that of Malaysian tualang honey. The variations in the pH of the investigated honey occurred because of the variation in different acids and mineral contents in the honey [10]. In addition, floral differences may also contribute to the variability in the pH values. Our findings (mean pH: 3.60) were similar to the pH values reported for Malaysian acacia honey samples (mean pH: 3.43) [11]. However, our results were slightly lower than the ones reported previously for honey samples from Bangladesh [12].

Overall, the pH values of the investigated honey samples were within the acceptable range established by the Codex Alimentarius Commission [13], indicating the freshness of the honey samples and their potential use as good antibacterial agents [14]. The elevated acidity of honey reportedly occurs because of the fermentation of sugar, resulting in the conversion of sugar into organic acid; this acid is said to be responsible for honey's flavor and stability against microbial spoilage [15]. Thus, the low pH of honey creates unfavorable conditions for bacteria or any other microorganisms to grow.

The EC values of the investigated honeys from Bangladesh ranged from 0.32 to 0.74 mS/cm, which were within the allowable limit (lower than 0.8 mS/cm) set by the Codex Alimentarius and European legislation [16]. These values were also similar to values for previously reported honey samples from Bangladesh (0.2 to 0.8 mS/cm) [12] and India (0.33 to 0.68 mS/cm) [17] but lower than honeys from Portugal (0.63 to 0.65 mS/cm) [18]. In comparison, the EC values of the manuka and tualang honeys were 0.53 and 0.67 mS/cm, respectively. The differences in EC values shown here may be contributed by different amounts of minerals, organic acids, proteins, variability in floral origin, and the amount of plant pollen [19] for each type of honey.

The ash content provides an insight into the honey quality [20]. The investigated honey samples from Bangladesh had ash contents ranging from 0.07 to 0.24%, indicating that these honey samples had different micro and macro mineral contents, possibly because of their different botanical origins. All the investigated honey samples from Bangladesh had ash

Table 2: Physicochemical parameters of the analyzed honeys in the present investigation.

Floral type	pH	Electrical conductivity (mS/cm)	Ash content (% w/w)	Moisture content (% w/w)
Khalsi (Sundarbans)	3.69 ± 0.04 (3.66–3.75)	0.74 ± 0.05 (0.67–0.80)	0.23 ± 0.01 (0.22–0.24)	18.55 ± 0.61 (18.09–19.45)
Mustard	3.49 ± 0.03 (3.46–3.54)	0.32 ± 0.03 (0.28–0.37)	0.10 ± 0.04 (0.07–0.16)	19.31 ± 0.58 (18.57–19.27)
Litchi	3.62 ± 0.09 (3.46–3.74)	0.47 ± 0.10 (0.32–0.55)	0.20 ± 0.03 (0.16–0.21)	18.64 ± 0.78 (17.89–19.58)
Mean ± SD	3.60 ± 0.10	0.51 ± 0.21	0.18 ± 0.07	18.83 ± 0.42
Manuka	3.94 ± 0.01	0.53 ± 0.03	0.27 ± 0.02	10.03 ± 0.15
Tualang	3.67 ± 0.02	0.67 ± 0.01	0.33 ± 0.01	14.81 ± 0.25

Mean ± SD (standard deviation) (min–max).

Table 3: AAS parameters.

Elements	Wavelength (nm)	Lamp intensity (mA)	Slit width (nm)	Linear range	Correlation coefficient (r)
Cd	228.67	8	0.7	80 μg/L	0.998
Cr	357.65	10	0.7	80 μg/L	0.997
Pb	217.35	10	0.7	80 μg/L	0.998
Cu	324.57	6	0.7	40 μg/L	0.999
Fe	248.30	12	0.2	6.0 mg/L	0.999
Zn	213.93	8	0.7	1.0 mg/L	0.994
Mn	279.43	10	0.2	80 μg/L	0.998

contents below 0.60%, which were lower than the contents of both manuka and tualang honeys. However, the mean ash levels were similar to levels in an earlier report on honeys (0.03 to 0.43%) from India [17] and Morocco (0.04 to 0.40%) [21] but lower than the levels in honeys (0.47 to 0.64%) from Portugal [18].

The moisture contents of honeys from Bangladesh varied between 17.89 and 19.58%. These values were within the maximum prescribed limit for honey moisture content (≤20%) in accordance with the Codex Alimentarius [13] and EU Council Directives [16]. Honey with a high water content is more likely to ferment, making its preservation and storage more difficult [22]. The moisture contents of the honeys in our study were similar to the moisture of previously reported honeys that were also from Bangladesh [12]. The moisture content range of pure honeys from Ireland was reportedly 16.10 to 23.36% according to Downey et al. [23], which is higher than our results. The low moisture content (<20%) in the honey samples from Bangladesh indicates that the investigated honey was ripe and suitable for storage.

The total sugar contents of the honey samples from Bangladesh ranged from 65.51 to 71.18% (Figure 1). These values were similar to values for honeys from Estonia (62.88–78.32%) [24], but they were higher than values in honey samples from Bangladesh [12]. A relatively higher total sugar content (78.4–82.4%) was reported for honeys from India [17], which was higher than our results. According to the Codex Alimentarius [13], the total sugar contents of honey should be more than or equal to 60%, as they were in our findings, which clearly indicated the good quality of the investigated honey samples.

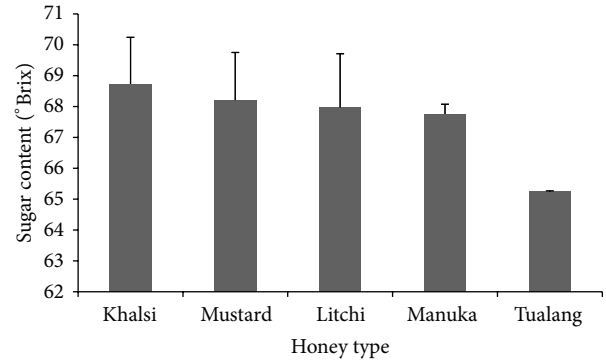

Figure 1: Sugar contents of the analyzed honey samples.

3.2. Metal Contents. Our study is the first extensive report on the metal contents of different types of honeys originating from Bangladesh (Table 4). The percentage recoveries of the investigated heavy metals in the honey samples were as follows: Cd (77.1%), Cr (93.4%), Cu (84.57%), Mn (76.51%), Pb (87.56%), Zn (77.42%), and Fe (87.56%). According to the European Commission [25], a method can be considered accurate and precise when the accuracy of the data is between 70 and 110%, and our result clearly supports the suitability and accuracy of our method for analyzing metals in honey.

Cd is of great concern, and Cd contamination usually has an anthropogenic source. Fredes and Montenegro [26] stated that higher Cd contents occur in honey that comes from beehives that are close to highways and processing equipment. According to the European legislation, the maximum

Table 4: Metals contents in the analyzed honeys samples.

Floral type	Cd (mg/kg)	Cr (mg/kg)	Pb (mg/kg)	Cu (mg/kg)	Mn (mg/kg)
Khalsi (Sundarbans)	BDL	0.76 ± 0.18 (0.47–0.90)	BDL	0.11 ± 0.10 (0.01–0.23)	3.49 ± 0.98 (2.25–4.59)
Mustard	BDL	0.52 ± 0.29 (0.13–0.81)	0.10 ± 0.07 (BDL–0.19)	0.09 ± 0.04 (0.03–0.14)	2.78 ± 1.26 (1.46–5.10)
Litchi	0.01 ± 0.00 (BDL–0.01)	0.39 ± 0.32 (0.10–0.82)	0.16 ± 0.04 (BDL–0.19)	0.15 ± 0.06 (0.06–0.22)	2.69 ± 1.66 (1.13–5.19)
Range	BDL–0.01	0.39–0.52	BDL–0.16	0.09–0.15	2.69–3.49
Manuka	0.06	6.40	BDL	0.30	0.94
Tualang	0.08	6.16	BDL	0.41	1.02

BDL: below detection limit.

limit for Cd content is 0.1 mg/kg [16]. With the exception of a single honey sample from Bangladesh, the Cd levels were generally below the detection limit for all honeys. The Cd contents for the manuka and tualang honeys were 0.06 mg/kg and 0.08 mg/kg, respectively.

Pb was detected in four mustard and three litchi honey samples, ranging from a level below the detection limit to 0.19 mg/kg. However, the levels were still within the recommended Maximum Acceptable Level (MAL) for Pb as suggested by the European Union at 1 mg/kg. The Pb concentration is usually related to environmental pollution [27]. The Pb concentration in the khalsi honey was below the detection limit, and similar results were also found for manuka and tualang honeys. The mean Cr concentrations in khalsi, mustard, and litchi were 0.76, 0.52, and 0.39 mg/kg, respectively.

The Cr concentrations in the honey samples from Bangladesh were lower than the concentrations in manuka (6.40 mg/kg) and tualang honeys (6.16 mg/kg). In addition, all the investigated honey samples in the present study had lower Cr contents than the honey samples from Switzerland as reported by Bogdanov et al. [28], at 0.003 to 0.329 g/kg. Higher Cr levels (8.1 ± 5.3 mg/kg) were also detected in honey samples from Urmia, Iran [29]. The lower Cr concentrations may indicate better quality honey in our samples. The variation in the Cr levels of different honeys from several countries may be related to geographical differences in the origins of the honeys [30]. Cr is usually directly deposited on the nectar via air or added by bees during pollination. Notably, the Cu concentration was higher in litchi honey (0.15 mg/kg) than in khalsi (0.11 mg/kg) and mustard honeys (0.09 mg/kg). However, the levels were lower than previously reported concentrations (0.12 to 0.34 mg/kg) in honey samples from Lithuania [31] and Hungary (0.02 to 0.78 mg/kg) [32]. By comparison, a relatively low Cu content was exhibited by both manuka and tualang honeys.

Fe was the most abundant among all the metals analyzed during the present investigation. The mean Fe concentrations in mustard, khalsi, and litchi honeys were 15.44, 14.76, and 13.51 mg/kg, respectively, which were relatively similar and higher than the concentrations in manuka and tualang honey (Figure 2). Our result is similar to the level found in honeys from France at 11.03 mg/kg [33] but higher than that reported by Bogdanov et al. [28] at concentrations between

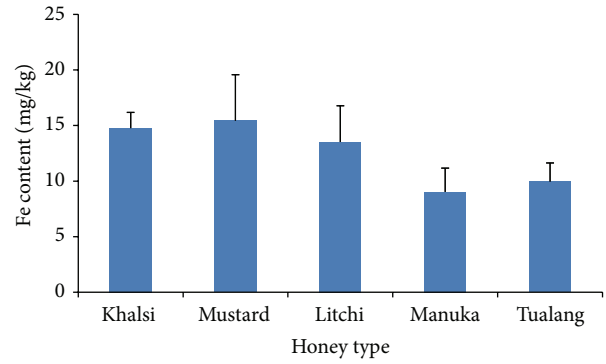

Figure 2: Fe contents of the analyzed honey samples.

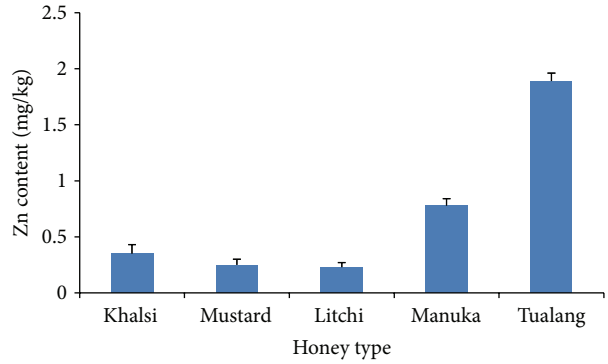

Figure 3: Zn contents of the analyzed honey samples.

0.14 and 9.85 mg/kg and in honey samples from Hungary (0.11 to 2.86 mg/kg) [32]. The higher Fe concentration in the investigated honeys from Bangladesh may be related to the high iron availability in the botanical sources of mustard, litchi, and khalsi plants, which may be transferred to the honey.

The Zn concentrations of the investigated honeys from Bangladesh were between 0.23 and 0.35 mg/kg (Figure 3). However, a higher Zn concentration was exhibited by tualang honey (1.89 mg/kg), which had higher Zn than all the honey types. The Zn levels in the investigated honey samples were lower than the concentrations reported for Chilean honey, which ranged from 0.19 to 4.39 mg/kg [26]. The variation

Table 5: Pearson correlation coefficients between metals in the investigated honeys.

	Cr	Cu	Zn	Fe	Mn
Cr	1.000	0.653	0.001	−0.268	0.908*
Cu	0.653	1.000	0.524	−0.245	0.811
Zn	0.001	0.524	1.000	−0.422	0.099
Fe	−0.268	−0.245	−0.422	1.000	−0.390
Mn	0.908*	0.811	0.099	−0.390	1.000

*Correlation is significant at the 0.05 level (2-tailed).

between the levels may be related to their different botanical and geographical sources. The amount of Zn in honey usually depends on the geographical location, botanical origin, and natural and anthropogenic sources [28].

Generally, Mn is present as a natural ingredient of minerals that are present in soils. The mean concentration of Mn was higher for khalsi honey (3.49 mg/kg) than mustard (2.78 mg/kg) and litchi (2.69 mg/kg) honeys, and it was 0.94 mg/kg for manuka and 1.02 mg/kg for tualang honeys. The Mn concentration was 0.03 mg/kg in honeys from Turkey [34] and 3.00 mg/kg for honeys from Nigeria [35].

Pearson correlation coefficients were determined between the metals within each type of honey originating from Bangladesh (Table 5). A strong positive correlation (r = 0.908) was observed between the Cr and Mn contents of the investigated honeys, indicating the relations between these two elements. However, Cr showed a negative correlation with Cu (r = −0.895). Moreover, statistically significant correlations were also observed between Pb and Cu (r = 0.997) and Pb and Mn (r = 0.997), indicating that the levels of these elements are interlinked with one another, which could be further investigated.

Hydroxymethylfurfural (HMF) is an important physical parameter of honey to determine its quality and freshness. However, HMF content of the currently investigated honeys was not determined due to unavailability of important equipment such as high performance liquid chromatography (HPLC) and gas chromatography mass spectrometry (GCMS) in the present investigation. Future studies are recommended including a higher number of honey samples originating from different parts of Bangladesh to measure the levels of different heavy metals and trace elements to analyze their levels and potential toxicity to the environment. Although the samples used in this investigation included mainly those from nonindustrialised areas, future study should also include analyzing the samples collected from industrialized areas for better comparison.

4. Conclusions

The levels of various metals in three different floral honeys from Bangladesh were reported for the first time in the present study. Several physical parameters were also determined, and our results indicated that all of the investigated honeys from Bangladesh are of good quality and the tested parameters met the requirements set by the International Honey Commission. The heavy metal concentrations were also within the limits, indicating their purity. Moreover, the higher concentrations of Fe in the investigated honeys signify that these honeys are a good source of these elements, which is very important for the human diet.

Conflict of Interests

The authors declare that there is no conflict of interests among them regarding the publication of this paper.

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Research Article

MicroRNAs-mRNAs Expression Profile and Their Potential Role in Malignant Transformation of Human Bronchial Epithelial Cells Induced by Cadmium

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Background. Our study was designed to elucidate whether there were miRNA and mRNA aberrantly expression profiles and potential role in malignant transformation of 16HBE induced by Cd. *Methods*. mRNA and miRNA expression profiles were determined in 35th Cd-induced 16HBE and untreated 16HBE by microarray. A series of bioinformatics analyses such as predicting targets, GO, KEGG were performed to find DEGs, coexpressing networks between miRNAs and mRNAs and its functions. *Results*. 498 DEGs were found. 8 Cd-responsive novel miRNAs predicted previously were identified, and 5 of them were downregulated. 214 target genes were predicted for the Cd-responsive miRNAs, many of which appeared to regulate gene networks. Target gene CCM2 was showed reciprocal effect by miRNAs. According to the combination analysis, hsa-miR-27b-3p regulated most of the mRNAs, especially upregulated expression genes. The differentially expressed miRNAs are involved in the biological processes and channels, and these GO and KEGG enrichment analyses result were significantly enriched in the Cd-responsive. *Discussion*. These results provided a tight link for the miRNA-mRNA integrated network and implied the role of novel miRNAs in malignant transformation of 16HBE induced by Cadmium. It is better to understand the novel molecular mechanism of cadmium-induced tumorigenesis.

1. Introduction

Cadmium (Cd) is a heavy metal that is widely applied in industry. However, its toxicity affects human health through occupational and environmental exposure [1–3]. High levels of Cd can be accumulated in various organs, and toxicological responses to Cd exposure include lung, kidney, and liver damage [4–6]. Several lines of experimental evidence have shown that Cd is carcinogenic to human and animals [7]. Based on epidemiology and laboratory evidences, Cd and its compounds were classified as human carcinogens in 1993 by the International Agency for Research on Cancer (IARC) [8].

The molecular mechanism is well understood in the etiology of heavy metal-associated diseases, including known interactions with epigenetic changes, protein functional groups, DNA damage, increased DNA replication, and cell division [9–11]. Most of these associations have been demonstrated by mRNA expression assays [12, 13] and experimental data also have linked altered miRNAs expression with exposure Cd recently [11, 14].

Small RNAs may be involved in the regulation/signaling of metal toxicity response [14, 15]. miRNAs are a class of endogenous, noncoding, single-stranded, small RNA molecules of 19–22 nucleotides. miRNAs regulate expression of target genes at the posttranscriptional level by binding to 3′ untranslated regions of target mRNAs [16]. Moreover, the functional strand of mature miRNA loads onto the RNA-induced silencing complex (RISC) to suppress the expression of specific mRNA targets by directing mRNA degradation or translational repression [17, 18]. In addition, miRNAs also play a serious role in cell fate decisions; they are involved in the many vital biological events, including proliferation, differentiation, apoptosis, metabolism and viral infection, and many diseases including diseases of various organ systems, metabolic disorders, and cancer at several sites [19–21]. Many

miRNAs have been proved to act as oncogenes and tumor suppressors [22].

miRNAs have widespread impact on expression and evolution of protein-coding genes [23]. To date, several computational methods have been successfully developed for predicting miRNAs and their targets [24, 25]. With regard to the species-specific miRNAs, they can be predicted depending on the intragenomic matching between miRNA candidates and their targets coupled with support vector machine classification of miRNA precursors. Inhibition efficiency of miRNAs to target genes mainly was determined by the 5′ end of a nucleotide sequence and site from 2 to 8.

The microarray analysis revealed that this metal modulated expression of nearly 400 genes in *Arabidopsis* and more than 1,700 in rice [26]. Recent research showed that cadmium stress affects the levels of miRNAs in soybean, rape plants, and rice [27–31]. Martínez-Pacheco et al. investigated the immortalized mouse fibroblast cell line BALB/3T3 A31-1-1 with exposed metal mixture including cadmium, which showed that the metal mixture resulted in miRNA expression profile that might be responsible for the mRNA expression changes observed under experimental conditions in which coding proteins were involved in cellular processes, including cell death, growth, and proliferation related to the metal-associated inflammatory response and cancer [32].

We previously established a model system of morphological cell transformation with cadmium chloride ($CdCl_2$) in 16HBE [12]. This could provide in vitro human model system to examine the molecular events occurring of Cd carcinogenesis [12]. In the current study, Cd-transformed cells at the 35th passage were chosen as the treatment group and untreated normal 16HBE as the control group. In order to identify and study differentially expressed mRNAs and miRNAs after cadmium exposure, miRCURY LNA 7th miRNA chip and the Human mRNA Array v2.0 (8 × 60 K, Arraystar) were simultaneously used for high-throughput screening. And this can also provide theory evidence for further researching. After the related target genes were identified, bioinformatics methods of GO and pathway analysis were used.

2. Materials and Methods

2.1. Cell Culture and Treatments. 16HBE cells were morphologically transformed using $CdCl_2$, as previously described [12]. Untransformed 16HBE cells (controls) and Cd-transformed cells at the 35th (5 μmol L^{-1} Cd for 14 weeks) passage were cultured in RPMI-1640 containing L-glutamine (Sigma, St Louis, USA), 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin (Life, California Carlsbad, USA) at 37°C in a 5% CO_2 humidified atmosphere. The cells were passaged twice weekly and cells in logarithmic growth phase (2–5 × 10^5 cells/mL) were harvested for following experiments.

2.2. RNA Isolation. Total RNA was harvested by using TRIzol (Invitrogen, California Carlsbad, USA) and miRNeasy mini kit (QIAGEN, Dusseldorf, Germany) according to manufacturer's instructions. The RNA quality was assessed on agarose gels and the concentration was determined with a NanoDrop spectrophotometer (ND-1000, Namedrops Technologies, Wilmington, DE, USA).

2.3. mRNA Expression Assays and Analysis. Microarray assays were performed by a service provider, Kangchen Bio-tech (http://www.kangchen.com.cn/). The sample preparation and microarray hybridization were performed based on the manufacturer's standard protocols with minor modifications. Briefly, mRNA was purified from total RNA after removal of rRNA (mRNA-ONLY Eukaryotic mRNA Isolation Kit, Epicentre, Wisconsin Madison, USA). Then, each sample was amplified and transcribed into fluorescent cRNA along the entire length of the transcripts without 3′ bias utilizing a random priming method. The labeled cRNAs were hybridized onto the Human mRNA Array v2.0 (8 × 60 K, Arraystar, Shanghai, China). For our experiments, scanned images were then imported into Agilent Feature Extraction software (version 11.0.1.1) for grid alignment and data extraction. Experiments were conducted in three 16HBE and three 35th 16HBE, and the 35th 16HBE were treated with different concentration of cadmium chloride (low toxicity, medium toxicity, and high toxicity).

Quantity normalization and subsequent data processing were performed by using the GeneSpring GX v11.5.1 software package (Agilent, California, USA). After quantile normalization of the raw data, mRNAs where at least 5 out of 6 samples had flags in present or marginal ("All Targets Value") were chosen for further data analysis. Differentially expressed mRNAs were identified through fold change (fold change ≥ 2.0) and t-test (p values < 0.05) filtering.

2.4. miRNA Expression Assays and Analysis. MiRNA microarray assays were also performed by a service provider, Kangchen Bio-tech (http://www.kangchen.com.cn/). The 3,100 miRNA sequences were predicted by Lindow et al. The 7th generation of miRCURY LNA Array (v.18.0) (Exiqon, Copenhagen, Denmark) was used to identify Cd-responsive miRNAs on microarray chips. Microarray experiments were performed twice using distinct biological samples. Scanned images were then imported into GenePix Pro 6.0 software (Axon, Groningen, Holland) for grid alignment and data extraction. Replicated miRNAs were averaged and miRNAs with intensities ≥30 in all samples were chosen for calculating normalization factor. Expressed data were normalized using the Median normalization. After normalization, differentially detected signals were those with fold change ≥1.3 and p values < 0.05.

2.5. Bioinformatics Analysis of Data. Both mRNA and miRNA expression data were normalized with internal housekeeping sequences. The fluorescent signal intensity and background measurements were obtained on six spots for each mRNA. The results filtering and normalization were processed with the Microsoft Excel software, where the background value was subtracted from the fluorescence of the sample for both the control and experimental condition, and then compared between them to express the result regulation direction as fold change and t-test. Before comparing the differential expression of genes in response to Cd treatment,

Table 1: Sequences of primers used in qRT-PCR detection.

Primer	Sequence	Length
has-miR-27b-3p		
Forward	5′-ACACTCCAGCTGGG TTCACAGTGGCTAAG-3′	98
Reverse	5′-ATCCAGTGCAGGGTCCGAGG-3′	
RT primer	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAG GCAGAACT	
has-miR-944		
Forward	5′-GCGGCGGAAATTATTGTACATC-3′	98
Reverse	5′-ATCCAGTGCAGGGTCCGAGG-3′	
RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTCATC	
CCM2		
Forward	5′-GAGACCATTGGCGTGAAGGA-3′	177
Reverse	5′-GATGTCCGAGATCATGCGGT-3′	

normalized gene expression levels were obtained by normalizing the number of raw clean tags in each library to the number of transcripts per million clean tags (TPM). A rigorous algorithm method was performed for the differential expression detection of genes across samples.

A combination of FDR < 0.001 (using Benjamini and Hochberg method; 1995) and the absolute value of $\log_2$Ratio ≥ 1 was used as the threshold to determine the significance of differentially expressed genes. Online databases like miRanda, TargetScan, and miRBase were chosen and searched to find predicted targets. In order to further identify differential miRNA genes, which may play potential roles in biological processes, the differentially expressed targets of specific miRNA were compared with the differentially expressed mRNA, and the intersection genes were defined as relevantly potential genes and involved in potential pathways. GO and pathway enrichment analysis were based on the gene ontology (http://www.geneontology.org/) and KEGG pathways (http://www.genome.jp/kegg/) [33–35].

2.6. Quantitation Real-Time RT-PCR of miRNAs and Target Gene. Total RNA (1 μg) was isolated from untreated 16HBE cells, 35th Cd-induced 16HBE cells, and tumorigenic cells using TRIzol according to manufacturer's instructions. The purity and integrity of total RNA were analyzed by spectrophotometry and agarose gel electrophoresis, respectively. After purification, the miRNAs were reverse transcribed into cDNA with the specific RT primer by the Prime Script RT reagent Kit (Promega, China). qPCR was performed using PCR reagent Kit (Promega, China) according to manufacturer's instructions. The expression of CCM2 mRNA was detected with reverse transcription-polymerase chain reactions (RT-PCRs) using Reverse Transcription System (Promega: Madison, WI, USA). Gene expression levels were calculated based on the comparative quantitative method (the ΔΔCT method) [36]. The primers used in qRT-PCR were listed in Table 1.

3. Results

3.1. Identification of Cd-Responsive Novel Candidate mRNAs. With abundant and varied probes (30,215 mRNAs) in our microarray, of them, there were 366 mRNAs with upregulated expression and 132 with downregulated expression (≥2.0 fold change, $p \leq 0.05$; see Supplementary Table S1 in the Supplementary Material available online at http://dx.doi.org/10.1155/2015/902025) in Cd-induced 35th cells when compared with untreated 16HBE cells. The Volcano Plot (Figure 1(a)) was applied to identify differentially expressed miRNAs with statistical significance. The heat map diagram (Figure 1(b), Supplementary Table S2) showed the result of the two-way hierarchical clustering of mRNAs and samples.

3.2. Identification of Cd-Responsive Novel Candidate miRNAs. To identify differentially expressed miRNAs with statistical significance, we performed Volcano Plot (Figure 2(a)) filtering between the two groups from the experiment. miRNAs with intensities ≥ 30 in all samples were chosen for calculating Median normalization factor. The threshold we used to screen up- or downregulated miRNAs is fold change ≥1.3 and p value ≤ 0.05. A total of 8 putative miRNAs (Supplementary Table S3) were detected to be Cd-responsive by microarray assays. They were considered to be new miRNAs and named according to the probe ID on the microarray chips as follows: hsa-miR-27b-3p, hsv1-miR-H6-5p, hsa-miR-1265, hsa-miR-944, hsa-miR-3960, hsa-miR-4708-3p, hsa-miR-877-5p, and hsa-miR-1261 (Table 2, Supplementary Table S5). hsa-miR-27b-3p, hsv1-miR-H6-5p, and hsa-miR-1265 were upregulated under Cd stress, whereas the other miRNAs were all downregulated. The heat map diagram (Figure 2(b), Supplementary Table S4) showed hsa-miR-27b-3p, hsv1-miR-H6-5p, and hsa-miR-1265 genes in Cd-induced 35th cells were higher (deeper) than in untreated 16HBE cells, suggesting that these differentially expressed genes in Cd-induced 35th cells were upregulated and this was consistent with the fact that 3 genes out of 8 differentially expressed genes were upregulated by differentially expressed genes analysis (y-axis and x-axis).

3.3. Analysis of MicroRNAs to Target mRNAs. In our further analysis, we focused on the trend of expression changes of miRNA and its target genes. In order to identify correlations between them, we searched three online databases to find predicted targets in the miRanda (http://www.microrna.org/),

Table 2: Predicted targets of Cd-responsive miRNAs in human bronchial epithelial cells

miRNAs	Target genes
Upregulated	
hsa-miR-27b-3p	CCM2, ADAMTS10, B4GALT3, C12orf35, DLGAP3, EML1, FAM108C1, GALNT5, HORMAD2, KIAA0146...
hsa-miR-1265	ABAT, ELAVL2, IKZF4, MAGIX, PPAP2B, XPNPEP2...
Downregulated	
hsa-miR-877-5p	CCM2, ARFIP2, CAP1, FXR2, HSD11B1, PHF8, SMG5, SORBS3, TRIM10...
hsa-miR-944	CCM2, AASS, BRIP1, C18orf34, DDX5, EPC2, FANCE, GAS6, HIVEP1, KCNH2, ZMYM6...
hsa-miR-1261	AQP4, BRCA1, CCDC85C, EIF2A, EIF4E3, EIF4G2, FRMPD4, SUPT7L...
hsa-miR-3960	C14orf43, HOXB8, MARVELD1, PCDHA8, PCDHA12, SLC9A3...
hsa-miR-4708-3p	BAZ2B, ATP2A3, C12orf53, EFNB1, EIF2S2, EIF4EBP2...

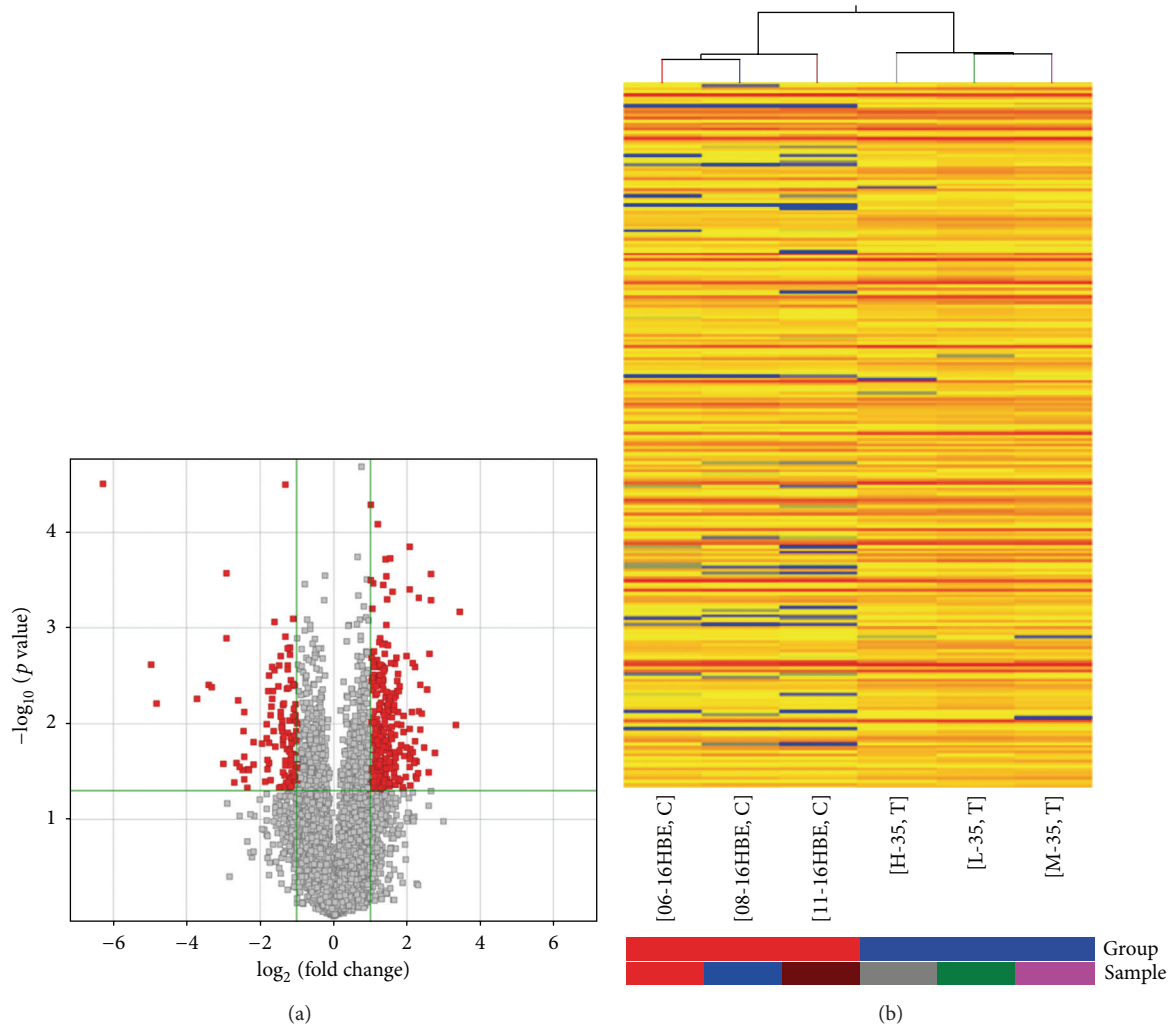


Figure 1: Differentially expressed mRNAs analysis. Volcano Plot (a) shows the relationship between fold change and statistical significance. The red points in the plot represent the differentially expressed mRNAs with statistical significance. The heat map diagram (b) shows cluster analysis of DEGs. The colors represents the expression values of DEGs. Sample names are listed in the horizontal axis, and high, medium, and low are 16HBE treated with different concentration of $CdCl_2$ for 35 passaged. Right vertical axis represents the clustering of mRNAs.

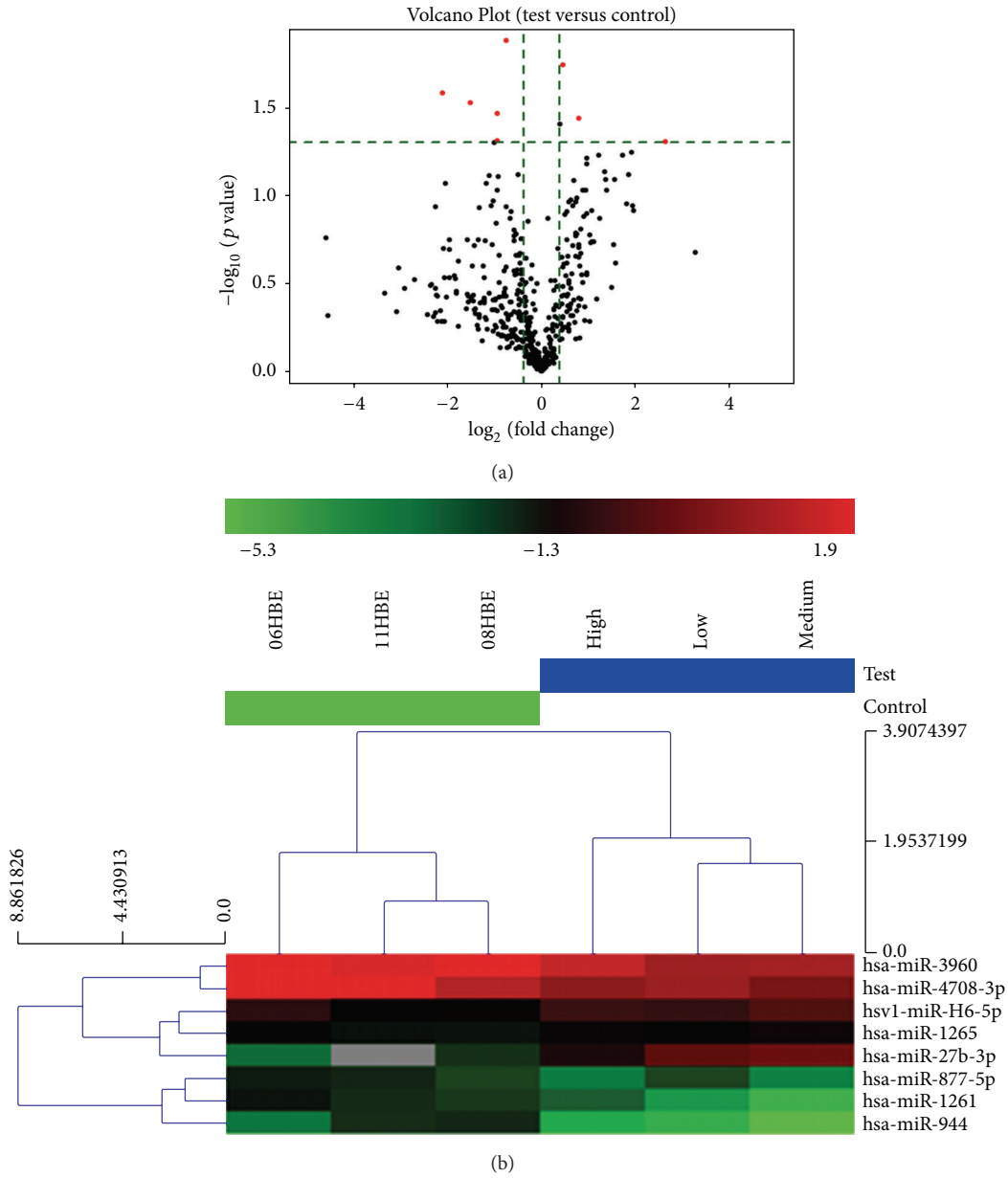
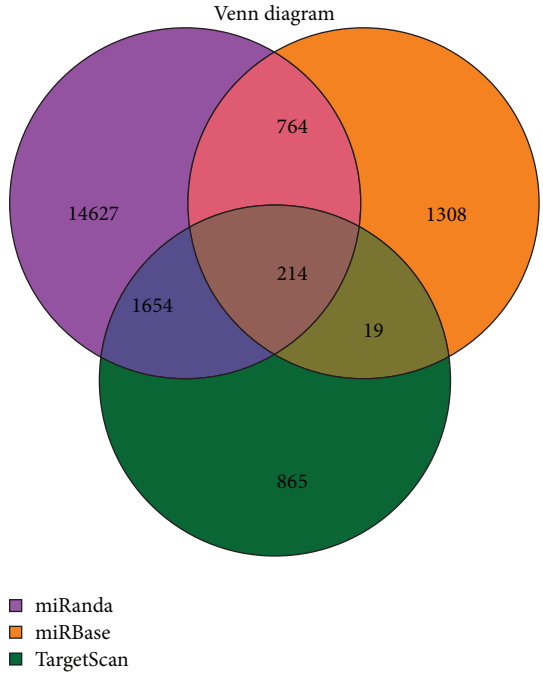


Figure 2: Differentially expressed miRNAs analysis. (a) Showing the relationship between fold change and statistical significance. The red points in the plot represent the differentially expressed miRNAs with statistical significance. (b) Showing cluster analysis of DEGs. The colors from green to red represent the fact that the expression values of DEGs become higher and higher. Sample names are listed in the horizontal axis, and high, medium, and low are 16HBE treated with different concentration of $CdCl_2$ for 35 passaged. Right vertical axis represents the clustering of miRNAs.

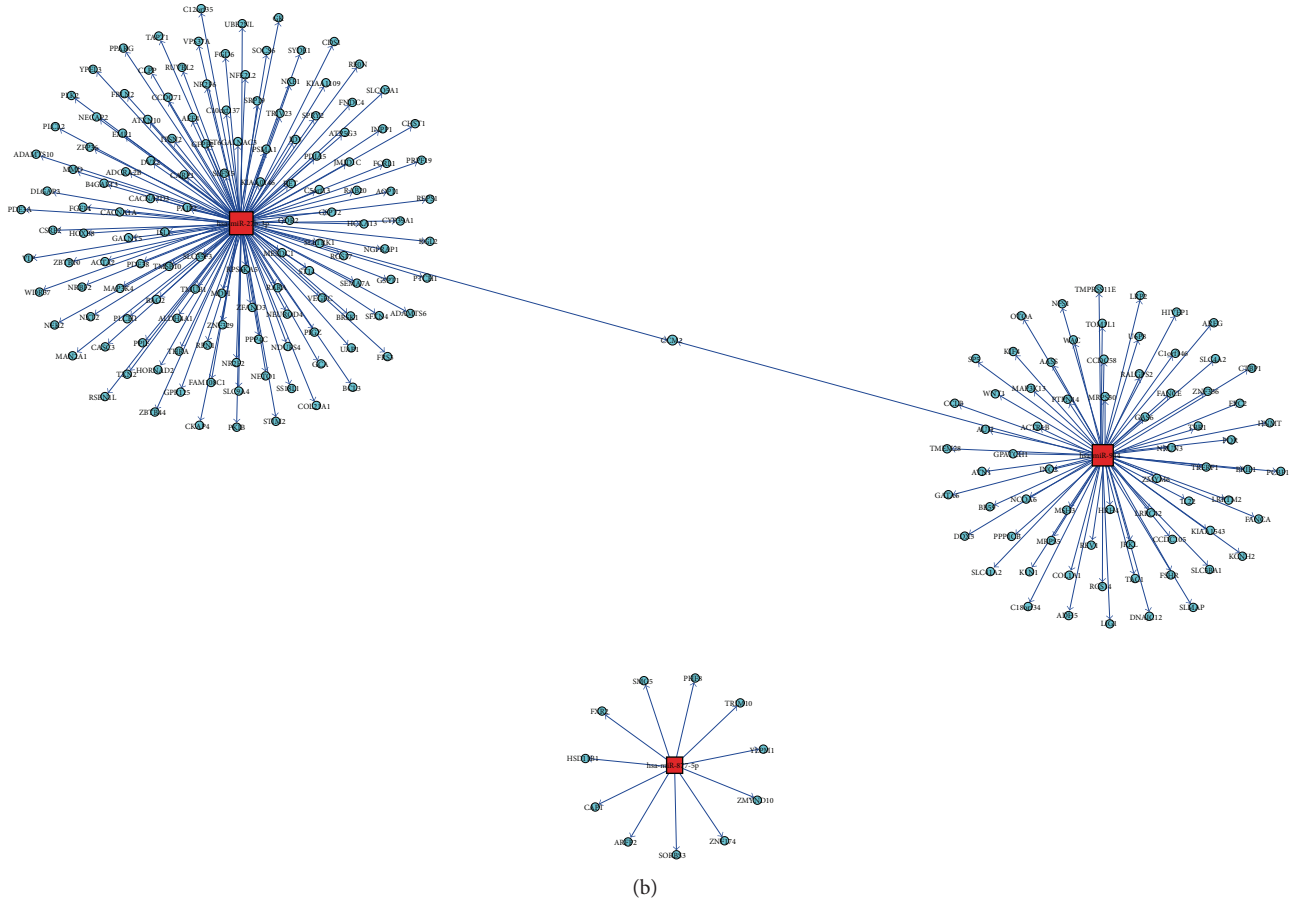
TargetScan (http://www.targetscan.org/), and miRBase (http://www.mirbase.org/). There were 17,259 miRNA-mRNA pairs in miRanda, 2,305 pairs in miRBase, and 2,752 pairs in TargetScan (Supplementary Table S5). A Venn diagram (Figure 3(a), Supplementary Table S5) showed that 214 of the microRNA-mRNA interaction pairs were acquired from three of the databases. The network figure (Figure 3(b)) showed the network relationship between miRNA and mRNA. CCM2 is the common target between hsa-miR-27b-3p and hsa-miR-944.

3.4. Combination Analysis of MicroRNA Microarray and mRNA Microarray. According to the combination analysis, three miRNAs were found in the intersection, including hsa-miR-27b-3p, hsa-miR-944, and has-miR-877-5p. Only the two former miRNAs could regulate same genes in mRNA microarray, especially has-miR-27b-3p. The details of genes were shown in Table 3.

3.5. GO Analysis of DEGs. For GO analysis, the ten most related genes and pathways were used. As shown by Figure 4

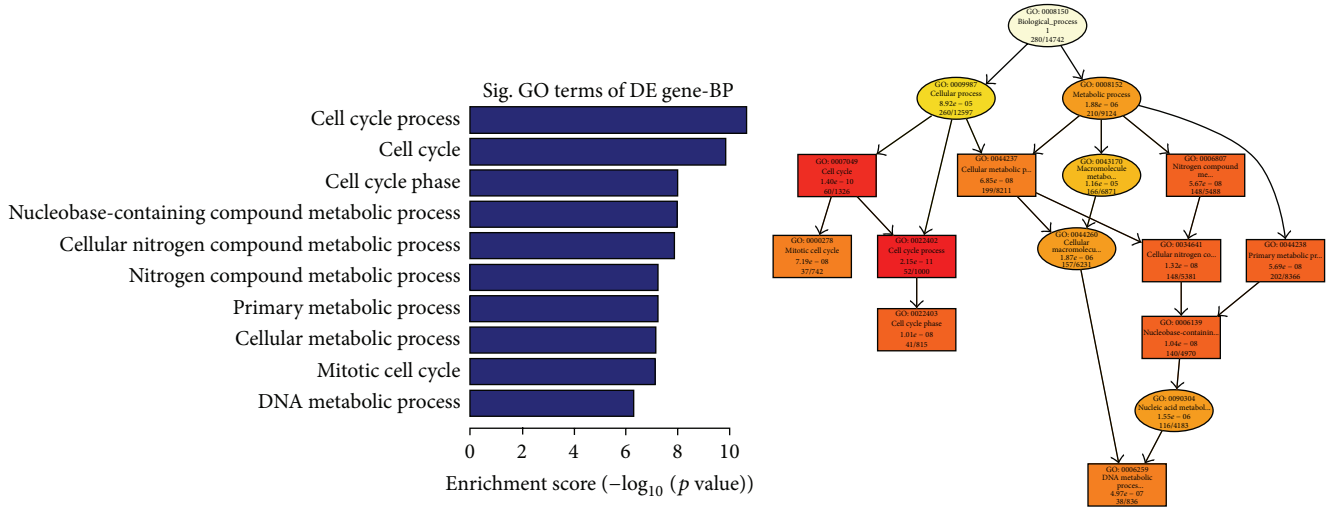


(a)

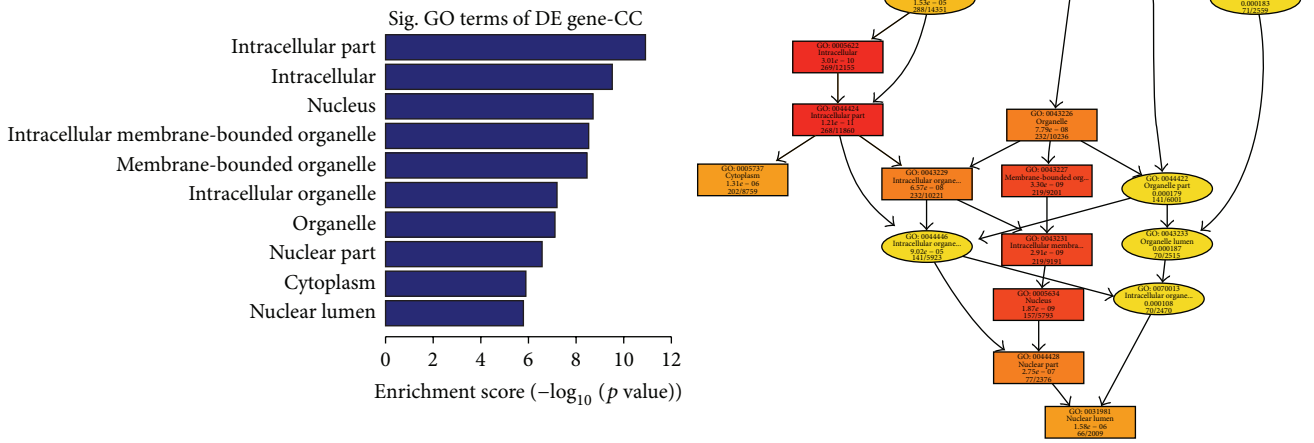


(b)

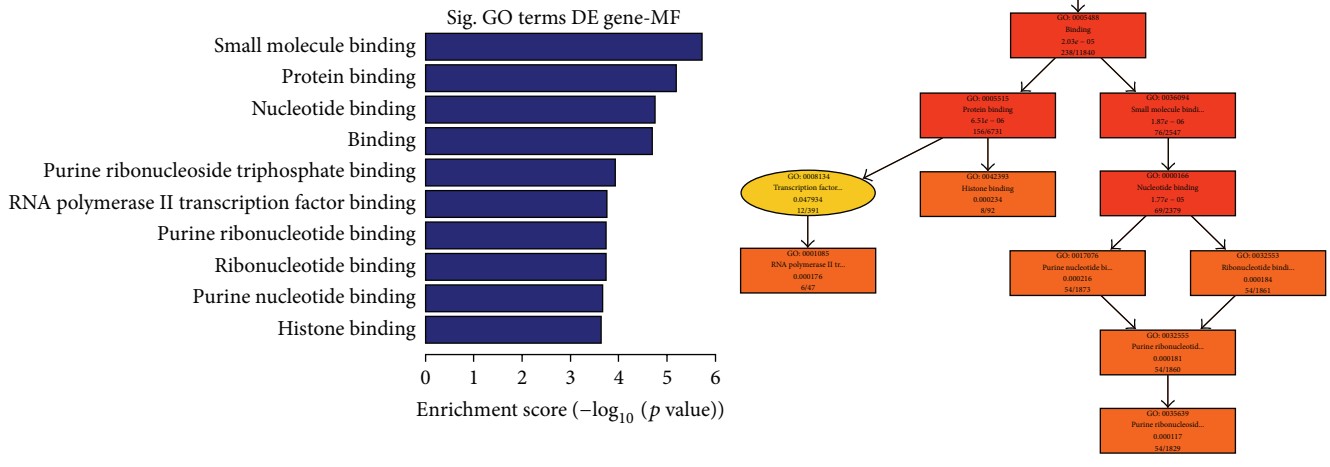
Figure 3: Targets of DEGs. (a) Venn diagrams showing the unique and shared regulated targets in DEGs; (b) showing the networks of 214 microRNA-mRNA interaction pairs of three of the databases.



(a)



(b)



(c)

Figure 4: Continued.

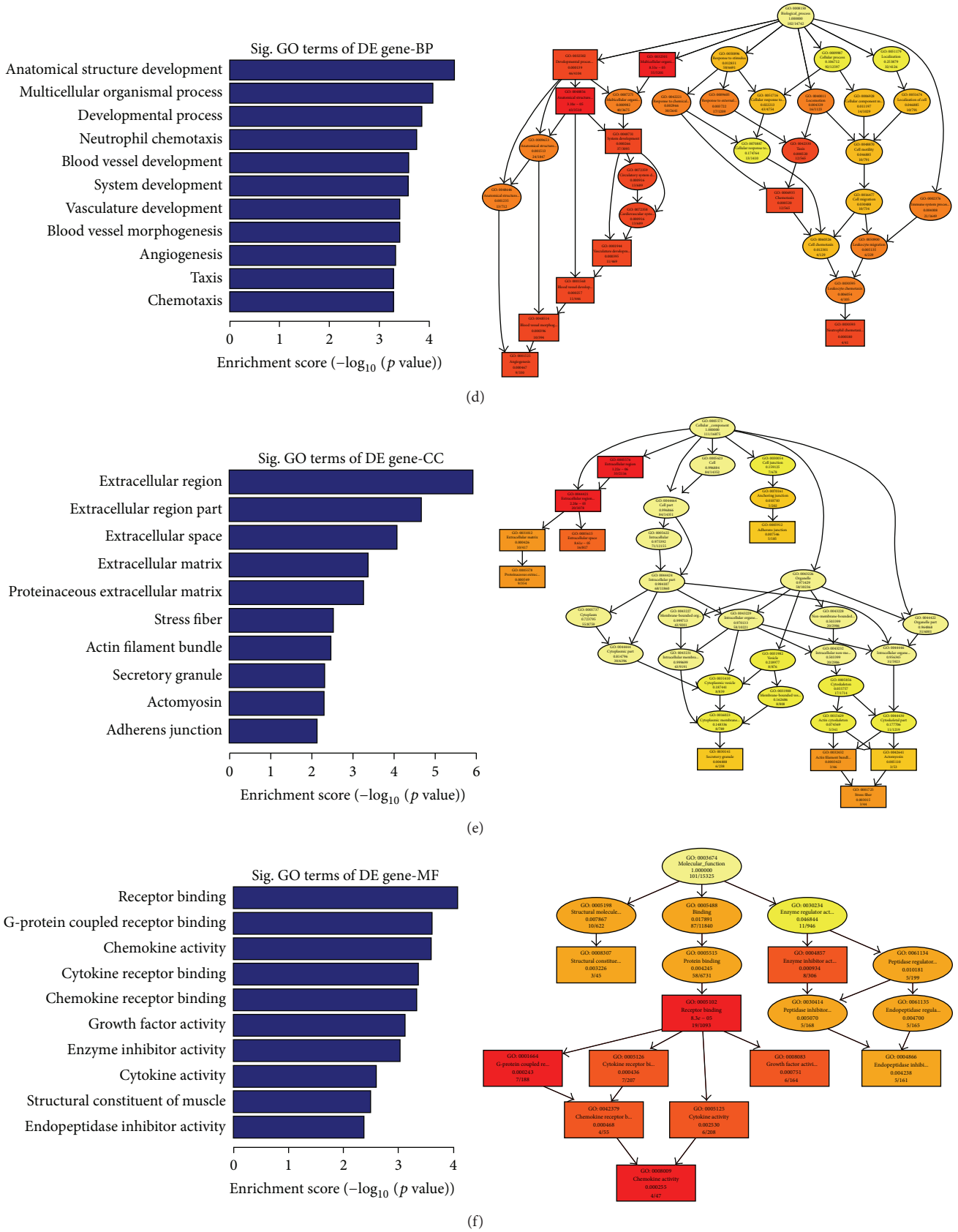


FIGURE 4: GO analysis of differentially expressed mRNAs in response to Cd stress. The top 10 of the most related parts were shown (a–f); (a–c) showed biological process (a), cellular component (b), and molecular function (c) of upregulated mRNAs, respectively. (d–f) showed biological process (d), cellular component (e), and molecular function (f) of downregulated mRNAs.

Table 3: Combinational analysis in the data of microRNA and mRNA microarray.

	hsa-miR-27b-3p	hsa-miR-944
Up/down	Up	Down
mRNAs		
Upregulated	RGS17, ITSN2, PRPF19, and PLK2	PPP1CB
Downregulated	SYDE1	DNAJC12

(Supplementary Table S6), GO analysis showed that the aberrant mRNA expression genes took part in many biological processes: primary metabolism, cell metabolism, cell cycle progression, DNA damage and repair, biological cycle, and so forth. GO analysis showed that the targets of aberrant miRNA took part in many biological processes, such as primary metabolism, cellular response to stress, negative regulation of transcription, DNA-dependent, and regulation of endocrine process (Figure 5, Supplementary Table S7). These results supported the idea that Cd-induced malignant transformation was related to DNA damage and repair and biological cycle.

3.6. Pathway Analysis of DEGs. For the KEGG pathway analysis, pathways with p value less than 0.05 were used. Pathway analysis of differentially expressed mRNAs (Figure 6, Supplementary Table S8) showed that they were involved in the cell cycle, P53 signaling pathway, prostate cancer, thyroid cancer, and endometrial cancer, Wnt signaling pathway, glioma, bladder cancer, pancreatic cancer, and axon guidance.

3.7. Expression Analysis of has-miR-27b-3p, has-miR-944, and Their Common Target Gene in Cd-Induced 16HBE Cells. A significant upregulated expression of has-miR-27b-3p and CCM2 in malignant 16HBE cells (16HBE cells, 35th Cd-induced 16HBE cells, and tumorigenic cells) was found, while has-miR-944 was downregulated in malignant 16HBE cells. The trend was aggravated as the cells passage (Figure 7). The results are consistent with microarrays.

4. Discussion

At present, lots of studies have confirmed molecular mechanisms of cadmium carcinogenesis including inhibit apoptosis, DNA repair and DNA methylation, histone acetylation [9, 11, 37], reactive oxygen species in cadmium response, and cadmium-induced signal transduction pathways [38]. In recent years, microRNAs have become the research focus in the scientific field. It is identified that about 50% of human microRNA genes are located at fragile sites and genomic regions involved in cancers [39], which clarifies the important role of miRNA in the tumorigenesis. Global expression profiling analysis of miRNAs and mRNAs in the same samples may provide a unique opportunity to enhance our understanding of potential miRNA regulatory mechanisms in 16HBE cells which were exposed to Cd. In this study, these altered expression profiles of mRNA and miRNA were screened by mRNA and miRNA microarray after 16HBE cell exposed cadmium. To further validate the regulatory role of miRNAs in Cd-responsive cells, the corresponding target mRNAs of Cd-regulated miRNAs were predicted by intersection of the database of miRanda, TargetScan and miRBase. 214 target mRNAs of 3 miRNAs were identified by bioinformatics prediction (Table 1, Supplementary Table S5). 133 genes were found as the target genes of hsa-miR-27b-3p and 11 genes were found as the target genes of hsa-miR-877-5p; however 70 genes were found as the target genes of hsa-miR-944. CCM2 was the same target gene of hsa-miR-27b-3p and hsa-miR-944. We also found that other miRNAs were related with CCM2 in these databases, miRanda, TargetScan, and miRBase. However the target genes of two miRNAs in TargetScan are CCM2, while more than two miRNAs' target genes are CCM2 in the nother two databases, both of them including hsa-miR-27b-3p, hsa-miR-877-5p, and hsa-miR-944 (Supplementary Table S5). The combination analysis could narrow down the range of miRNA candidates and clearly explicit the biological functions of target genes.

Most of miRNAs are clustered and shared similar expression patterns, implying that they are transcribed as polycistronic transcripts [40, 41]. Further research found the mutations of CCM2 gene lead to cerebral cavernous malformations. CCM2 encodes a scaffold protein that functions in the stress-activated p38 mitogen-activated protein kinase (MAPK) signaling cascade. The protein interacts with SMAD (small mothers against decapentaplegic) specific E3 ubiquitin protein ligase 1 (also known as SMURF1) via a phosphotyrosine binding domain to promote RhoA degradation. The protein is required for normal cytoskeletal structure, cell-cell interactions, and lumen formation in endothelial cells. Multiple transcript variants encoding different isoforms have been found for this gene (provided by RefSeq, Nov 2009). The analysis of GO showed differently expressed miRNAs of Cd-responsive cells were reported to be involved in several biological processes, including negative regulation of transcription from RNA polymerase II promoter, cellular macromolecule metabolic process, and cellular response to stress, involved in several molecular functions transcription factor binding, ligand-activated sequence-specific DNA binding RNA polymerase II transcription factor activity, and protein binding. Those illustrated miRNAs are transcribed by RNA polymerase II as primary miRNAs (pri-miRNAs), which is consistent with the research of Cai et al., 2004 [42], Lee et al., 2004 [43], and so forth.

It is shown that cadmium can upregulate genes of encoding pathogen related proteins, antioxidant enzymes, transporters, TFs, and proteins associated with glutathione metabolism. With the literature supporting and confirmation, we show the importance of miRNAs in key cellular processes by modulating a small gene set in this study. Together, our findings allow us to assert the great importance of miRNAs in the development and/or establishment of cadmium-associated diseases, especially cancer, through key cellular processes modulated by cadmium exposure targets that have also been cited as above. The patterns of mRNA and miRNA expression that predict establishment of cadmium health effects are helpful for understanding the aberrant molecular mechanisms of human cells. However, further study is

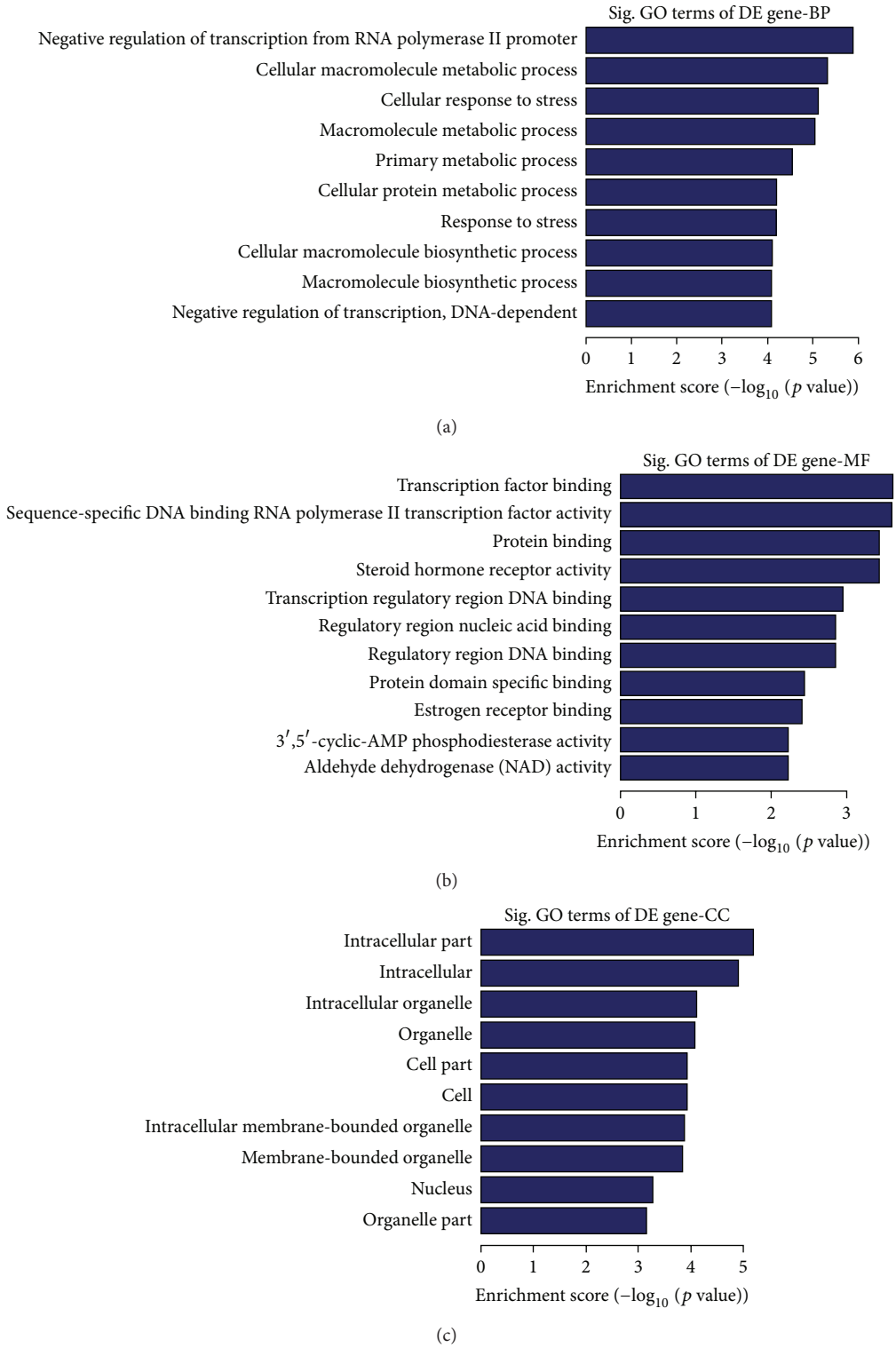


Figure 5: GO analysis of differentially expressed miRNA targets in response to Cd stress. The top 10 of the most related parts were shown (a–c); biological process (a), cellular component (b), and molecular function (c).

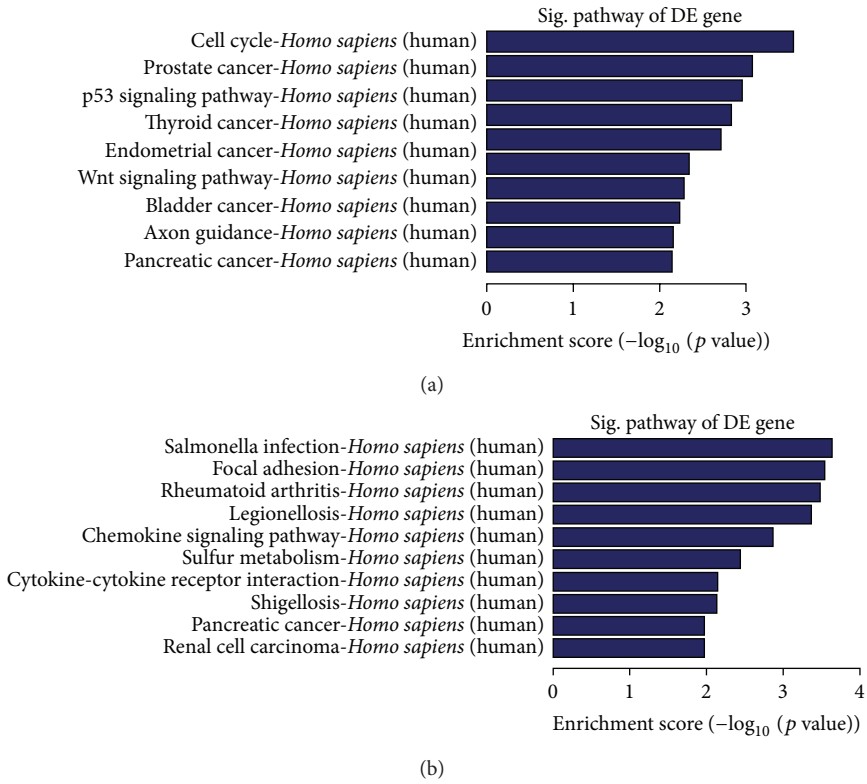


Figure 6: The KEGG pathway analysis of differentially expressed mRNAs. Pathways with p value less than 0.05 were shown in the figure. All the p values were transformed into log p value.

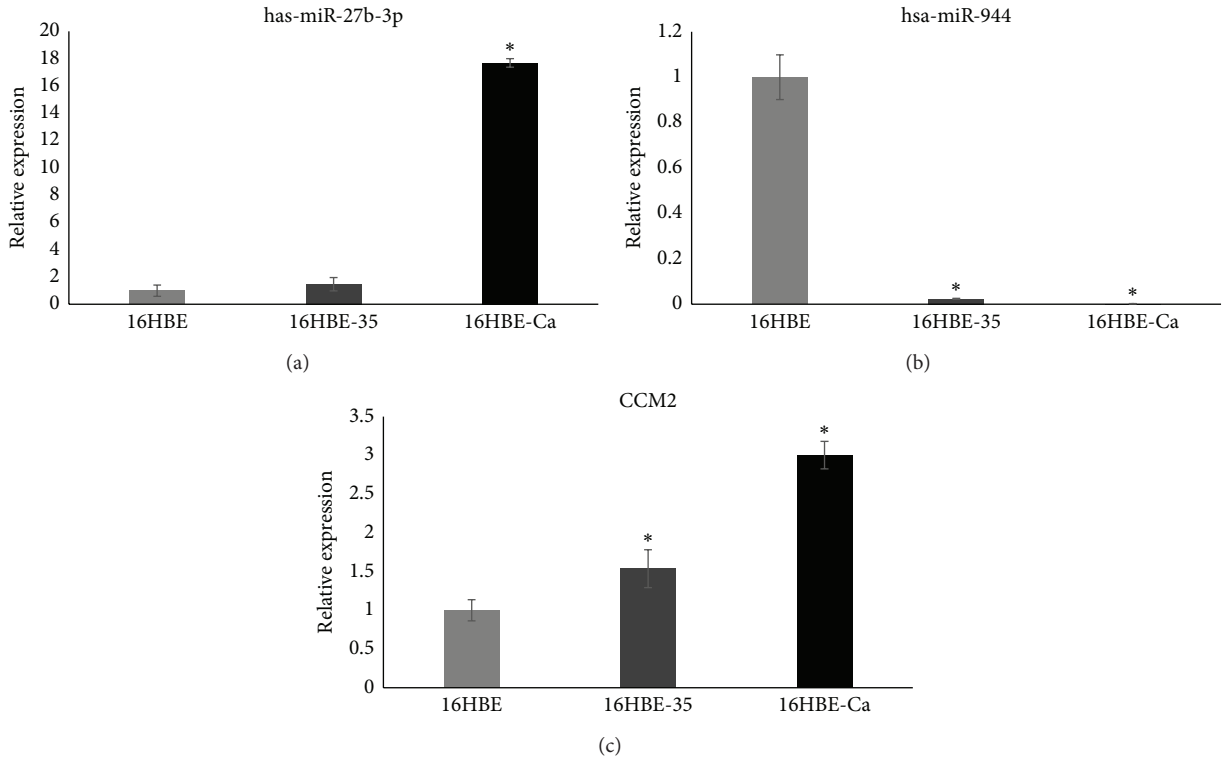


Figure 7: The expression of has-miR-27b-3p, has-miR-944, and CCM2 by qRT-PCR in different groups. $*$ represents $p < 0.05$ as compared to 16HBE, respectively.

needed to understand the relationship between genetic predisposition and epigenetic disruption in the development of cadmium-associated diseases.

In summary, our results provide a tight link for the miRNA-mRNA integrated network after 16HBE exposed Cd. These results provide some new and meaningful candidate miRNAs and genes to allow further investigation of the mechanism after 16HBE differentiation and to provide several important molecular markers for the diagnosis and therapy of related diseases. Further study of the potential function of miRNA-mRNA integration in Cd-response will promote our understanding of the key role of 16HBE in Cd-response, such as further research of differentially expressed genes demonstrated by Western Blot, or various methods about the target gene CCM2 and genes of combination analysis.

Abbreviations

16HBE: Human bronchial epithelial cells
Cd: Cadmium
$CdCl_2$: Cadmium chloride
CO_2: Carbon dioxide
CIP: Calf Intestine Phosphatase
CCM2: Cerebral cavernous malformations 2
DEGs: Differently expression genes
FDR: The false discovery rate
GO: Gene ontology
IARC: The International Agency for Research on Cancer
KEGG: Kyoto Encyclopedia of Genes and Genomes
miRNA: MicroRNA
mRNA: Messenger RNA
MAPK: Mitogen-activated protein kinase
RISC: RNA-induced silencing complex
SMAD: Small mothers against decapentaplegic
TPM: Transcripts per million clean tags.

Conflict of Interests

The authors have declared that no conflict of interests exists.

Authors' Contribution

Qun Liu and Zhiheng Zhou conceived and designed the research. Chanjiao Zheng and Huanyu Shen were responsible for literature searching and data extraction. Zhiheng Zhou was responsible for the quality assessment of studies. Qun Liu wrote the paper. Zhiheng Zhou and Yixiong Lei revised the paper.

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