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Review

Aristolochic acids in herbal medicine: Public health concerns for consumption and poor regulation of botanical products in Nigeria and West Africa

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Aristolochic acids are naturally occurring biomolecules found in plants of the genus Aristolochia and Asarum belonging to the family Aristolochiaceae. They are reported to be carcinogenic and nephrotoxic; and are implicated in kidney diseases, aristolochic acid nephropathy (AAN) which may result in kidney failure, other health complications and possibly death. Aristolochic acids are highly genotoxic and are linked to upper urothelial cancer in animals and humans. Some Aristolochia species are used in traditional medicine practice in Nigeria and other West African countries without regard to safety concerns. Several countries, especially in the Western world, have banned the use and importation of herbal products containing aristolochic acids. There is need for warning and strict regulation on the importation and consumption of aristolochic acids-containing botanical products in Nigeria. This study aims to review the availability of aristolochic acids, their toxicity, circulation, as well as the quantitative analytical techniques and regulations. It analyzes the herbal products containing aristolochic acids, and aristolochiaceae plants grown in Nigeria in respect to public health implications. It highlights the importance of doing an extensive study on indigenous plants producing aristolochic acids and imported herbal products used as weight loss supplements marketed in Nigeria. There is need to emphasize the labeling of herbal products containing aristolochic acids.

Key words: Aristolochic acid, herbal medicine, Nigeria, aristolochiaceae, toxicity, regulation.

INTRODUCTION

Medicinal plants are used in combating multiple and complex disease conditions affecting humans because of their popularity, accessibility, affordability and claimed efficacy (Ayodele et al., 2010). Developing countries depend on ethno-medicines especially at the most basic level of health care due to perceived ease of accessibility...
and affordability. Adulteration and safety of botanical products continue to be of great concern (Mustapha, 2013).

Aristolochic acids are a group of naturally occurring compounds produced by aristolochiaceae family of plants; they are reported to have various physiological effects on living tissues (Bode and Dong, 2015). Aristolochic acids refer to a mixture of structurally related nitrophenanthrene carboxylic acids (Gbadamosii and Egunya, 2012). The most abundant aristolochic acid is aristolochic acid I (1), followed by aristolochic acid II (2) (Shibutani et al., 2007). Aristolochic acids are carcinogens (National Toxicological Program, 2008).

Most plants reported to contain aristolochic acids belong to the genus Aristolochia or Asarum of the family Aristolochiaceae (Arlt et al., 2002a, b). The use of Aristolochia species in traditional medicines and herbal products has been of global concern since the 1990s after a toxic herbal slimming product used in a Belgium clinic was found to contain Aristolochia fangchi instead of Stephaniae tetrandrae. After consuming the product, more than 100 patients were admitted to hospitals with renal failure and severe atrophy of the proximal renal tubules (Ekor, 2014).

Aristolochia fangchi was reported to contain aristolochic acids, whereas Stephaniae tetrandrae did not (Ekor, 2014). This inadvertent exchange of plant species containing aristolochic acids ultimately resulted in many patients suffering from end-stage renal failure and urethral damage (Heinrich et al., 2009). There are over twenty aristolochic acids and analogues known, that are produced by plants (Center for Food Safety and Applied Nutrition, 2001). Many dietary supplements containing aristolochic acids have been reported (AHPA Botanical Identity References Compendium, 2017).

Plants belonging to Aristolochiaceae family known to produce aristolochic acids are widely cultivated in Nigeria and actively used in traditional medicine practice to treat various ailments without recourse to their toxicity and public health hazards. Also, while the regulatory bodies of many countries have made definite laws and regulations concerning aristolochic acids, there are no strict governmental regulations and restrictions on the importation and consumption of aristolochic acids-containing botanical products in Nigeria and other West African countries.

The aim of this review was to show that consuming aristolochiaceae plants and herbal products containing aristolochic acids is dangerous to health and stresses the need for regulatory action to be taken in Nigeria and West Africa.

ARISTOLOCHIC ACIDS AND ANALOGUES

Aristolochic acids I and II

Aristolochic acid I (aristolochic acid A) is a crystalline solid. Its molar extinction coefficient (ε) in ethanol is 6,500 at 390 nm, 12,000 at 318 nm, and 27,000 at 250 nm. Aristolochic acid II is also called Aristolochic acid B (Kumar et al., 2003).

Analogue of aristolochic acid

In addition to aristolochic acids I and II, other chemically related compounds found in Aristolochiaceae family of plants (Figure 1) include aristolochic acid I methyl ester (3), 7-hydroxy aristolochic acid I (aristolochic acid la) (4), aristolochic acid II methyl ester (5), aristolochic acid III (6), aristolochic acid Illa (aristolochic acid C) (7), aristolochic acid III methyl ester (8), aristolochic acid IV (9), aristolochic acid IVa (aristolochic acid D) (10), aristolochic acid IV methyl ester (11), aristolochic acid V (12), aristolochic acid Va (13), aristolochic acid Vla (14), aristolochic acid VII (15), aristolochic acid Vlla (16), aristolochic acid E (17), aristolactams (18) and dioxoaporphines (19) (National Toxicological Program, 2008; Kumar et al., 2003; Cosyns JP. 2003). Kumbiegel et al (1987) identified aristolactam I (20), aristolactam la (21), aristolochic acid la (4), aristolic acid I (22) and 3,4-methylenedioxy-8-hydroxy-1-phenanthrenecarboxylic acid (23) in rodents (Figure 1).

NATURAL SOURCES OF ARISTOLOCHIC ACIDS

Biosynthesis of aristolochic acids

The biosynthetic pathway of aristolochic acids is not clear. However, a biogenetic relationship between aristolochic acids and the aporphine alkaloids has been postulated based on structural similarities. Magnoflorine, an aporphine alkaloid, is associated with aristolochic acids in various Aristolochia species (Comer et al., 1969; Schutteu et al., 1967). An aporphine alkaloid, 4,5-dioxoaporphine (24), is found mostly among the Aristolochiaceae family of plants and regarded as possible intermediates of the precursors of aristolochic acids and aristolactams (Kumar et al., 2003). A total of about seventeen aporphine alkaloids have been characterized from Aristolochia species. Aristolactams were thought to originate from the cyclization condensation reaction of the reduction products of aristolochic acids. Twelve aristolactams have been reported from Aristolochia species (Kuo et al., 2012).

Distribution and occurrence of aristolochic acids

Aristolochic acids are nitro-compounds, non-alkaloidal constituents of different parts of a wide range of species of the family Aristolochiaceae (National Toxicological Program, 2008; Heinrich et al., 2009). Some Aristolochia species are native to Brazil but introduced into West
Figure 1. Chemical structures of aristolochic acids and analogues.

African gardens, hence found in Nigeria and Cameroon. The prominent species widely distributed in West Tropical Africa include Aristolochia abida, Aristolochia bracteolata, Aristolochia clegans, Aristolochia gibbosa, among others (Oladipupo, 2000).

About thirty Aristolochia species are native to the United States; the most widely distributed species include A. serpentaria (Virginia snakeroot), A. tomentosa (woolly Dutchman’s pipe), A. macrophylla (pipevine) and A. clematitis (birthwort) (National Toxicological Program, 2008). Aristolactams have been reported in Aristolochiaceae and related plant families, including the genus Piper (family Piperaceae), Stephania
(Menispermaceae) and Schefteromita (Annonaceae) (National Toxicological Program, 2008). Aristolochic acids are found in several species of butterflies that feed on Aristolochia plants (National Toxicological Program, 2008). Other herbs identified as producing aristolochic acid and botanicals which may be adulterated with aristolochic acid have been reported (U.S FDA/ FDA, 2001; U.S. Food and Drug Administration/Center for Food Safety and Applied Nutrition 2001; National Toxicological Program, 2008).

Out of 16 samples of slimming pills and powders studied, the principal component was aristolochic acid I in Aristolochia fang chi with content ranging from 437 to 668 ppm; whereas aristolochic acid II was the principal component in Aristolochia contorta. Twelve of the samples were reported to contain aristolochic acids I and/or II. The principal component of majority of the slimming products was aristolochic acid II with content ranging from less than 1 to 148 ppm. Aristolochic acids I and II were detected in all the plants from the genus Aristolochia and at trace levels in some plants from the genus Asarum (National Toxicological Program, 2008).

Many herbal products advertised on the internet have been reported to contain aristolochic acids (National Toxicological Program, 2008; Center for Food Safety and Applied Nutrition, 2001). Guang fang ji (Aristolochia fang chi) was one of three types of fang ji (Chinese herbs) sold, but known to contain aristolochic acids. The root of Han fang ji (Stephania tetrandra) and mu fang ji (Cocculus trilobus) can be mistakenly substituted with guang fang ji because of similarities in their appearance (Center for Food Safety and Applied Nutrition, 2001). Despite extensive warnings on the dangers of aristolochic acids, aristolochic acid-containing Chinese herbal products, like Mu Tong, which has been associated with an increased risk of cancer of the bladder in humans, can still be purchased on the internet as an antibiotic and a remedy to improve cardiac function (Bode and Dong, 2015).

**Occurrence of aristolochic acids in foods**

Extracts from Asarum canadense (Canadian snakeroot or wild ginger) and Aristolochia serpentaria (Virginia snakeroot) are used as flavoring agents in foods or alcoholic beverages (National Toxicological Program, 2008).

**Occurrence of aristolochic acids in insects**

Aristolochic acids can be found in larvae of several species of butterflies, particularly those of the genera Atrophaneura, Battus, Pachliopta, and Troides, which feed on Aristolochia plants (Shibutani et al, 2007; Fordyce, 2000; Rothschild et al., 1972).

**Anatomical characteristics of Aristolochia species**

Aristolochia is a large plant genus with over 500 species (Minari and Idris, 2015). Aristolochia species can be perennial shrubs, lianas, or herbs bearing essential oils. The morphology of the whole plant varies from silica bodies to non-silica bodies, and climbing to self-supporting. The leaves could be alternate, spiral or flat and do not usually possess conspicuous aggregations; they are usually dorsiventral or bifacial with hairs present and possessing minor leaf veins without phloem cells. The plants stems are axial, with the presence of cork cambium. The fruits are usually non-fleshy dehiscient fruits while the seeds usually contain alkaloids and flavonoids as secondary metabolites, but lacking cyanogen. The supposed basic chromosome number of the family Aristolochiaceae is seven. Aristolochia species reproduce by either pollination of the flowers or by non-pollination and usually grow more in temperate regions but less in sub-tropical to tropical regions (Watson, 1992).

**Uses of Aristolochia species**

The U.S. Food and Drug Administration’s “Approved Drug Products with Therapeutic Equivalence Evaluations” (“Orange Book”) does not list any prescription or over-the-counter products (current or discontinued) that contain aristolochic acids (National Toxicological Program, 2008). The name Aristolochia means “the best delivery or birth” thus reflects centuries of use in traditional birth (Frei et al., 1985). Plant products containing aristolochic acids have been used extensively in traditional herbal medicine for various illnesses. They have been used as antirheumatics, as diuretics, in the treatment of edema, in wound healing, to facilitate childbirth, and for less common conditions such as hemorrhoids, cough and asthma (Li et al., 2005).

Various Aristolochia and Asarum species have been used in herbal medicines since ancient times in obstetrics and in treatment of snakebite, wounds and tumors, and they are still in use today, particularly in Chinese herbal medicine (Arlt et al., 2002b; Jiménez-Ferrer et al., 2005). Aristolochic acids have been reported to have antibacterial, antiviral, antifungal, and antitumor effects and in more recent times, have been used in conventional pharmaceuticals (Kupchan and Doskotch, 1962; Zhang et al., 2004). Herbal remedies containing aristolochic acids have been reported for different illnesses such as hepatitis, urinary tract infection, vaginitis, oral ulcer, upper respiratory tract infection, eczema, headache, dysmenorrhea, arthralgia, neuralgia, hypertension, cerebrovascular accident, bronchitis, pneumonia, heart failure and edema (Li et al., 2005a).

The leaves and bark of Aristolochia indica were used in gastrointestinal disturbances and intermittent fever in children (Kumar et al., 2015).
In Africa, the use of Aristolochia had been reported. A decocction of Aristolochia ringens with Picralima nitida seed was used as stimulant for men by herb sellers at Adeleye market, Bariga, Lagos State and Oke Aje market, Ijebu Ode, Ogun State, Nigeria (Minari and Idris, 2015; Idu et al., 2010). Aristolochia albida Duch has been used by Zimbabweans to combat malaria (Ngarivhume et al., 2015). A. albida, Aristolochia bracteolate and Aristolochia repens have been used in Nigeria for the management of diabetes and other diseases (Ezuruike and Prieto, 2014). Overall, members of the Aristolochia genus seem to have a long history of medicinal use in Europe, Asia (including China), Africa, and Central America, which was also exemplified in studies on indigenous Mexican medicine (Heinrich et al., 2009).

Aristolochic acids and aristolochiaceae in Nigeria

Aristolochic acid 1 is extracted from the rhizome of A. albida in Nigeria (Haruna and Ilyas 2000). The root bark, stem and root of A. bracteata Lam known as Ga-daukuka (Hausa, Nigeria) are used as spices in Nigeria (Kayode and Ogunleye, 2008). The phytochemical constituents and antimicrobial activity of A. ringens are reported (Fasola et al., 2015; Oladoye et al., 2014). The tuber of A. albida Duch (known as kaucin kasa in Hausa) and the aerial part of Aristolochia spp (commonly known as Madakin kasa or kiwaye tsamiya in Hausa) are used in the management cancer (Ayodele et al., 2010; Ngulde et al., 2015). A. albida, Aristolochia bracteolate and Aristolochia repens are used in Nigeria for the management of diabetes and other diseases (Ezuruike and Prieto, 2014; Soladoye et al., 2012; Gbadamosi and Egunyomi, 2012; Idu et al., 2010; IARC, 2012; Wooltorton, 2004). Harmful effect of A. ringens has been reported by Sulyman et al. (2017). A. repens Mill. (Ako igun in Yoruba) is used by the people of Abeokuta in traditional healthcare for deworming (Idu et al., 2010). A. albida Duch is abundantly available in Nigeria and very much used by the traditional herbalist for a variety of purposes which include treatment of abdominal colic and management of snake bites (Oladipupo, 2000). A. ringens Mills is used for the traditional management of infantile dermatitis in Odeda, South Western Nigeria (Minari and Idris 2015; Erinoso et al., 2016).

LEGISLATIVE AND REGULATORY ACTIONS ON ARISTOLOCHIC ACIDS

FDA documentations on aristolochic acid

Consumption of products containing aristolochic acids is associated with permanent nephropathy, which may result in kidney failure and other complications, hence in May 2000, U.S. Food and Drug Administration (FDA) alerted consumers to discontinue use of botanical products containing or suspected to contain aristolochic acid (U.S FDA/ FDA, 2001). Due to the potent carcinogenicity and nephrotoxicity of aristolochic acids, the agency also issued alerts to manufacturers, distributors, importers and health professionals of dietary supplements urging them to review their manufacturing procedures to ensure that botanicals are free of aristolochic acids (Center for Food Safety and Applied Nutrition, 2001; U.S. Food and Drug Administration/ Center for Food Safety and Applied Nutrition, 2001).

Legislative and regulatory actions taken on aristolochic acids by different countries

Germany 1981: The German Federal Health Office withdrew all preparations containing aristolochic acids from the national market following demonstration of their carcinogenic potential in a three-month toxicity study in rats; banned branded drugs containing aristolochic acid as well as herbal preparations or extracts prepared from plants belonging to the aristolochiaceae family, with the exemption of homeopathic preparations prepared to a dilution of at least 1:100,000,000,000. Aristolochic acid was identified as potent carcinogen even after dosage discontinuation (United Nations Publication 2005).

Austria 1981: The inherent risk associated with the use of preparations containing aristolochic acid led the Australian Federal Ministry of Health and Environmental Protection to instruct pharmacists against its use in Austria (United Nations Publication 2005).


USA 2001: The FDA cautioned consumers against consuming any dietary supplement or traditional medicine containing aristolochic acids (United Nations Publication 2005).

France 2001: All homeopathic preparations containing Aristolochia brasiliensis and homeopathic preparations containing products belonging to Aristolochiaceae or related plant families were withdrawn due to risks of nephrotoxicity and carcinogenicity associated with aristolochic acids (United Nations Publication 2005).

Oman 2001: Oman prohibited importation and marketing of aristolochic acids or products containing plants from aristolochiaceae family, due to kidney toxicity and urinary tract cancer associated with preparations containing aristolochic acids (United Nations Publication 2005).

Canada 2001: Health Canada issued a Customs Alert to ban the sale and import of products containing aristolochic acid. Manufacturers, retailers and importers were requested to withdraw from the market all existing
products containing aristolochia and aristolochic acids (United Nations Publication 2005).

**Australia 2001:** A traditional herbal product named Longdan Qiegan Wan (Wetness Heat Pill) containing aristolochic acids was removed from the Australian Register of Therapeutic Goods (United Nations Publication 2005).

**Venezuela:** Aristolochic acids containing products are not approved for use and/or sale in Venezuela (United Nations Publication, 2005).

**ANALYTICAL METHODS FOR DETERMINATION OF ARISTOLOCHIC ACIDS**

Several methods have been studied in analysing aristolochic acids in botanical samples and human tissues. Detection methods vary over time, with ultraviolet (UV) light absorption being most common, mass spectrometry (MS), electrochemical detection (ED), diode-array detection (DAD), laser-induced fluorescence (LIF) detection, fluorescence detection, and other methods have also been reported in more recent publications (National Toxicological Program, 2008; Chang et al., 2007a; Chang et al., 2007b). The United States Food and Drug Administration (FDA) issued a Laboratory Information Bulletin for the determination of aristolochic acids in traditional Chinese medicines and dietary supplements (National Toxicological Program 2008).

**High performance liquid chromatography (HPLC)**

The concentrations of aristolochic acids in botanical products were determined by high-performance liquid chromatography (HPLC) with UV absorption detection at 390 nm (IARC, 2002, Trujillo et al., 2006). To detect and quantify aristolochic acid in human detection, a hollow fiber liquid-phase microextraction technique in conjunction with high-performance liquid chromatography was used (Heinrich et al., 2009). Aristolochic acids have been determined in medicinal plants and slimming products using HPLC with RP-18 reversed phase column. An average recovery of 97.8% was obtained when aristolochic acids in Aristolochia plant samples were quantified by reversed-phase HPLC method involving extraction with methanol and chromatographic separation with a mobile phase of acetonitrile–water–trifluoroacetic acid–tetrahydrofuran in the ratio of 50:50:1:1, using photodiode array detection. The limit of detection was 0.10 g per injection with a 5 μl injection volume (National Toxicological Program, 2008; Heinrich et al., 2009; Li et al., 2005a).

**Ultra-high-performance liquid chromatography-multistage fragmentation mass spectrometry (UHPLC/MS)**

This is a hyphenated technique applied to determine aristolochic acid I in herbal dietary supplements (Yang et al., 2014). Furthermore, ultraperformance liquid chromatography-triple quadrupole mass spectrometry is a noninvasive and efficient method developed to detect aristolactam-DNA adducts in exfoliated urothelial cells (Yang et al., 2014).

**Liquid chromatography-mass spectroscopy**

This method with limit of quantitation equivalent to 140 mg/ml has been applied to determine aristolochic acids in botanical samples as well as in renal cortex, using either an ion-trap mass spectrometer or a triple quadrupole mass spectrometer (Heinrich et al., 2009; IARC, 2002; Rao et al., 1975).

**Targeted liquid chromatography/serial mass spectrometry (LC/MS/MS)**

This method has been employed in detecting aristolochic acids I and II in multi-component herbal remedies, using a quadrupole ion-trap mass spectrometer. Aristolochic acids were determined to be between 250 pg and 2.5 ng on-column within a matrix containing compounds extracted from 2 mg of herbal remedy (National Toxicological Program, 2008).

**Capillary zone electrophoresis (CZE)**

Capillary zone electrophoresis (CZE) was used for the analysis of aristolochic acids in medicinal plants. The limits of detection for aristolochic acids I and II were 30 and 22.5 mg/kg, respectively (IARC, 2002).

**Enzyme-linked immunosorbent assay (ELISA)**

This has been used and reported to have limit of detection (LOD) for aristolochic acid I of 0.7 ng/ml, or ~ 2 × 10⁻⁹ M, but its LOD for aristolochic acid II was similar to the other methods previously mentioned (18 ng/ml, or ~ 6 × 10⁻⁸ M) (National Toxicological Program, 2008).

**Pressurized liquid extraction method**

Extraction and analysis of aristolochic acids I and II in medicinal plants (Radix aristochiae) using pressurized liquid extraction method reportedly gave better result than ultrasonic and soxhlet extraction methods (IARC, 2002).
P-post-labeling and ultra performance liquid chromatography–electrospray ionization/multistage mass spectrometry (UPLC-ESI/MS
)

P-post-labeling technique was the most widely employed method for detecting putative DNA adducts in humans. It was used to analyze aristolochic acid–DNA adducts in the kidneys of CHN patients (National Toxicological Program, 2008; Art et al., 2001a, b; Schmeiser et al., 1996).

ABSOSRTION, METABOLISM AND EXCRETION OF ARISTOLOCHIC ACIDS

Pharmacokinetics studies

Aristolochic acids are absorbed from the gastrointestinal tract and distributed unchanged and/or in metabolized form throughout the body (IARC, 2012; Lunn et al., 2008). They are metabolized by oxidation and reduction pathways called phase I metabolism. Aristolactam I which is the product of aristolochic acid I (AA-I) reduction was observed in urine. Further metabolism by O-demethylation of aristolactam I resulted in aristolactam IA as the primary metabolite (IARC, 2002). Nitroreduction yielded an N-acetyl nitretrium ion, an important ion in mutagenicity of aristolochic acid I (IARC, 2012; Lunn et al., 2008). AA-I was reportedly metabolized along two major pathways; aerobic demethylation to 8-hydroxyaristolochic acid-I (aristolochic acid la) which, in turn, was metabolized by phase II glucuronide or sulphate conjugation reactions. An alternative pathway was by enzymatic reduction of the nitro group to generate the biologically inactive aristolactam-I (Shibutani et al., 2007; IARC, 2002), which was further subjected to phase II conjugation. Aristolochic acid II (AA-II), which lacks the O-methoxy group, was reduced to aristolactam-II (L-II) and further hydroxylated at C-8 to form 8-hydroxyaristolactam 1a (L-la). Excretion of aristolochic acids and their metabolites is through the urine (Shibutani et al., 2007; Lunn et al., 2008).

Metabolites

Aristolochic acid I and II metabolites such as aristolactam I, aristolactam Ia, aristolochic acid Ia, aristolic acid I (22); and 3,4-methylenedioxy-8-hydroxy-1-phenanthrene carboxylic acid (23) were identified in rodents following the oral administration of aristolochic acid I and aristolochic acid II. In rats, the major metabolite was aristolactam Ia (46% of the dose in urine and 37% in the faeces). In both rats and mice, the metabolites of aristolochic acid II were identified as aristolactam II, aristolactam Ia, and 3,4-methylenedioxy-1-phenanthrene carboxylic acid. Generally, fewer metabolites were observed in beagle dogs, rabbits, guinea-pigs, and humans than in rodents. Among the metabolites of aristolochic acids I and II, only aristolactam I and aristolactam II were identified in human urine samples collected from 6 healthy volunteers to whom a mixture of aristolochic acids I and II were given over several days (National Toxicological Program, 2008; IARC, 2012).

Metabonomic studies

The renal proximal tubule is reported as the principal target of aristolochic acids in rats. Nephrotoxicity has been reported in male rats by identifying elevated serum urea and creatinine levels, and urinary protein and glucose. Furthermore, increased activity of the enzymes; gamma glutamyl transferase (γ-GT) and N-acetyl-β-D-glucosaminidase (NAG) was observed in rats exposed to aristolochic acids, which was interpreted as resulting from a lesion of the renal duct epithelial cells (National Toxicological Program, 2008).

TOXICITY OF ARISTOLOCHIC ACIDS

Nephrotoxicity

Three main terms have been used to designate the renal disease due to consumption of herbs. These are Chinese herb nephropathy (CHN), aristolochic acid nephropathy (AAN), phytotherapy-associated interstitial nephritis (PAIN) and Endemic (Balkan) nephropathy (BEN) (National Toxicological Program, 2008; Heinrich et al., 2009).

The ingestion of herbal remedies containing aristolochic acids is associated with the development of a chronic, progressive renal disease, termed aristolochic acid nephropathy (AAN) (National Toxicological Program, 2008; Ekor, 2014; Debellé et al., 2008).

Nephrotoxicity is reflected by gradual and progressive atrophy of renal proximal tubules and development of a characteristic form of interstitial fibrosis involving the outer renal cortex and progressing toward the medulla, while the glomeruli are spared. This nephropathy is associated with less inflammation than most types of interstitial nephritis. Steady progression of the disease leads to chronic renal failure and a strong association with transitional cell (urothelial) carcinoma of the upper urinary tract (National Toxicological Program, 2008; Heinrich et al., 2009).

Although both aristolochic acid I and II (AA-I and AA-II) are cytotoxic to cells in culture, AA-I is solely responsible for the nephrotoxicity associated with AAN in mice (National Toxicological Program, 2008). Endemic (Balkan) nephropathy affected people in rural areas of Bosnia, Bulgaria, Croatia, Romania, and Serbia, and has been linked to the consumption of aristolochic acids.
containing products (Heinrich et al., 2009).

In 1992, it was reported in Brussels, Belgium that a Chinese herbal product containing Aristolochia fangchi (Guang Fang Ji) was mistakenly labelled as containing Stephania tetrandra (Han Fang Ji), a Chinese slimming regimen. This unintentional substitution was confirmed by phytochemical analysis of 12 different batches of the herb powders. Only one batch was found to contain tetrandrine and not aristolochic acids I and II; one contained both tetrandrine and aristolochic acids and 10 contained aristolochic acids only (National Toxicological Program, 2008). Similar incidence of substitution was also reported in Hong Kong (Liang et al., 2006). Consequently, patients who took the regimen developed interstitial renal fibrosis and subsequently, end-stage renal disease (Comer et al., 1969). It is also documented that about 5% of the exposed population (patients taking the weight-loss regimen from May 1990 to October 1992) developed renal disease. The mean average exposure per patient was about 900 mg of powder per day for 6 to 12 months (National Toxicological Program, 2008). To avoid unintentional substitution involving aristolochic acid-producing plant, nomenclature by pharmaceutical name was recommended (Wu et al., 2007).

The presence of aristolactam-DNA adducts formed by aristolochic acid was confirmed in series of kidney samples obtained from 38 patients with AAN six years after their exposure to the so-called Stephania tetrandra powder (actually, Aristolochia fangchi). These adducts were absent in kidney tissues obtained from eight patients with renal disease of other origin (National Toxicological Program, 2008; Ekor, 2014).

Similar cases of aristolochic acid nephropathy were reported in many other countries: four cases in France resulting from the intake of slimming pills labeled as containing Stephania tetrandra which was, in fact, A. fangchi (Arlt et al., 2004); one case in Spain after chronic consumption of a tea made with a mixture of herbs containing Aristolochia pistolochia, two cases in the United Kingdom after treatment of eczema with Mu Tong containing aristolochic acid (Lord et al., 2001), 12 cases in Taiwan related to the use of various unidentified herbal medications for different purposes (Chang et al., 2001; Yang, 2000), one case in the USA after intake of herbal medicine containing aristolochic acid for low back pain (Stewart et al., 2003), and 12 cases in Japan, five of which were herbal medicines containing aristolochic acid; in the other cases there was confusion of Mokutsu (Akebia quinata) with Kan-Mokutsu (Aristolochia manshuriensis) and Boui (Sinomenium acutum) with Kou Boui (Aristolochia fangchi) or Kanchu-Bou (Aristolochia heterophylla) (National Toxicological Program, 2008; Ekor, 2014).

In Japan, the cases of Chinese herb nephropathy often presented with adult-onset Fanconi syndrome. A similar case was reported in Germany after intake of a purported Akebia preparation containing aristolochic acid (National Toxicological Program, 2008; Ekor, 2014).

Recently, it was estimated that about 100,000 individuals were at risk of Endemic (Balkan) nephropathy (BEN), while about 25,000 have developed the disease with the highest prevalence rates in Serbia, Bulgaria, Romania, Bosnia and Herzegovina and Croatia (Jadot et al., 2017).

Natural food chain contamination by root uptake

Another possible pathway of aristolochic acid exposure to human bodies has recently been proposed and is known as natural food chain contamination by root uptake. When Aristolochia spp grows, degenerates and decomposes, it may leave deposits of aristolochic acid in the soil which is accumulated by root uptake in other crops grown successively. Some authors proved that the roots of maize plant and cucumber were capable of absorbing aristolochic acids from the soil. To strengthen this proposed intoxication pathway, AAs were subsequently identified in corn, wheat grain and soil samples collected from the endemic village of Kutles in Serbia (Jadot et al., 2017).

Carcinogenicity

Aristolochic acid is considered a potent human carcinogen (IARC, 2012) and listed among the most potent 2% of carcinogens known (Woollorton, 2004). It was classified as a human carcinogen class I by the World Health Organization International Agency for Research on Cancer (Jadot et al., 2017). Aristolochic acid nephropathy was associated with a high prevalence of urothelial cell carcinoma (National Toxicological Program, 2008; Kuo et al. 2012), which often occurs years after the onset of chronic renal disease and tend to develop in the upper urinary tract unlike most other urothelial cell tumors (National Toxicological Program, 2008). The carcinogenic effects of aristolochic acids are thought to be a result of mutation of the tumor suppressor gene TP53, which is unique to aristolochic acid-associated carcinogenesis (Go’kmen et al., 2013). Aristolochic acid-associated cases of urothelial cancer were reported in many countries including China (Li et al., 2005).

Genotoxicity

Following metabolic activation, aristolochic acid reacts with DNA to form aristolactam (AL)-DNA adducts lesions which concentrate in the renal cortex as a sensitive and specific biomarker of exposure (Kuo et al. 2012). Several mammalian enzymes have been shown to be capable of activating both AAI and AAIL in vitro and in cells (Arlt et al., 2002a). Aristolochic acid I (AAI) and aristolochic acid
II (AAII) have genotoxic and carcinogenic effects as deduced by their ability to form AA-DNA adducts in target organs and tissues of intoxicated mice (Jadot et al., 2017). 7-(deoxyadenosin-N6-yl)-aristolactam I, a distinct DNA adduct, was an established biomarker of AA exposure that can be persistent decades after AA exposure, thus attesting to the role of AA in human urothelial malignancy (Schmeiser et al., 2014).

Aristolochic acids structure-activity relationships

Studies conducted in mice regarding structure-activity relationships revealed that the carboxyl group aristolochic acid 1 is an absolute structural requirement for its transport and high affinity interaction with organic anion transporter (OAT) 1, 2 and 3, while the nitro group is only required by OAT1; the O-methoxy group present at the 8-position may be a functional key determinant for AA-induced toxicity in mice but was not involved in transport (Jadot et al., 2017). Consequently, it was demonstrated that only AAII was capable of inducing nephrotoxicity as evidenced by tubular damage and development of interstitial fibrosis in AAII-treated mice (Jadot et al., 2017).

FUTURE SCOPE

Future scope of research and regulatory concerns on exposure to aristolochic acid include: Are there incidences of unreported cases aristolochic acid nephropathy in regions of Nigeria where Aristolochia species are used in treating ailments? Could more Aristolochia species than already studied be growing in Nigeria but yet to be identified due to poor coverage and inadequate taxonomy? Is there a possible correlation between the recent upsurge in incidences of chronic kidney disease (CKD) and bladder carcinoma in Nigeria, and the consumption of aristolochic acid producing plants, herbal supplements adulterated with Aristolochia spp and imported botanical products? What regulations are put in place for the production and use of products containing Aristolochia spp in Nigeria and West African sub-region? This is need for proper identification and comprehensive study of Nigerian local herbs belonging to aristolochaceae family, particularly Aristolochia spp and related plants for possible aristolochic acids content. Appropriate regulatory bodies should conduct extensive investigation of imported herbal products and weight loss supplements marketed in Nigeria for possible adulteration with aristolochic acids. Local and imported herbal products label should contain information on aristolochic acid content.

CONCLUSION

Aristolochia species belonging to Aristolochiaceae family of plants known to produce aristolochic acid are not native to Nigeria and other African countries; they are widely cultivated in Nigeria and are actively used in traditional medicine practice to treat various ailments without recourse to their toxicity. This review exposed the fact that not many studies have been done on aristolochic acids and aristolochic acids-producing plants grown in Nigeria with respect to their toxicity profiles. In the course of this review, it was observed that while the regulatory bodies of many countries have made definite laws and regulations concerning aristolochic acids, there are no strict governmental regulations and restrictions on the importation and consumption of aristolochic acid-containing botanical products in Nigeria. As obtainable in other countries, the Nigerian Federal Ministry of Health should issue a warning on the danger of consuming aristolochic acids, botanical products containing aristolochic acids or herbal products containing plants belonging to aristolochiaceae or related families.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

In vitro effect and scanning electron microscopic changes of Nigella sativa loaded chitosan nanoparticles on Schistosoma mansoni adult

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Nanoparticles can act as drug carriers that can modulate pharmacokinetics, increase bioavailability and target release with minimal toxic effects. The present work aimed to assess the therapeutic effect and electron microscopic changes of Nigella sativa loaded Chitosan Nanoparticles (NSLCN) on adult Schistosoma mansoni in vitro. Adult worms were removed from the portal and mesenteric veins of infected mice after 90 days, and then three to five mature worms including both sexes were cultured. Schistosoma adult was exposed to NSLCN at concentrations of (10, 20, 40, 60, 80, and 100 μg/ml) for 24, and 48 h. Examination for worm viability was done after 24, and 48 h using a stereomicroscope comparing with control negative and control positive groups. The mortality rate in worms reached 88.9% in the group treated with 100 μg and 80 and 84.6% in groups treated with 80 and 60 μg respectively (p-value <0.001). After 48 h of incubation with the same concentration, there were variable effects on motility and death of worms, the death rate reached 100% in all groups treated with nanoparticles. After 24 h incubation, the live worms have sluggish motility and reached dead score at 48 h of incubation. By (SEM) there were tegumental changes of both dead male and female in the form of loss of spines, swollen suckers and swollen inter tubercular ridges in male and loss of smooth architecture of female tegument with multiple pores. In conclusion, NSLCN appears as a new potential candidate drug against schistosomiasis. We successfully applied nanoemulsion preparation against the adult stage of S. mansoni in vitro.

Key words: Schistosoma mansoni, Nigella sativa, chitosan nanoparticles, scanning electron microscope.

INTRODUCTION

Schistosomiasis has been estimated to infect more than 207 million people with 779 million people at risk of infection (Steinmann et al., 2006). Schistosomiasis represents a major public health problem in about 78 tropical and subtropical countries with the majority (up to 90%) of the cases are located mainly in sub-Saharan Africa (WHO, 2013).

Praziquantel is effective against all species of
Schistosoma infecting humans and has been used for the last decades. It is well tolerated, easily administered in tablet form, and cheap (Cioli and Pica-Mattoccia, 2003). However, the development of resistant strains has been reported, leading to schistosomiasis treatment failure that highlighted the importance of developing new and more effective drugs for this disease (De Moraes et al., 2013). As a consequence, in the last years, important efforts have been made in the search for new active compounds against Schistosoma, mainly those obtained from plants (Allegritti et al., 2012). Recently promising studies have been developed for the use of natural compounds derived from plant extracts as drugs against Schistosoma spp., being safe and with less medical side effects (Parreira et al., 2010).

Abaza (2013) reviewed all herpes that were used in the treatment of schistosomiasis including Chinese medicine, Carvacrol (essential oil of Origanum vulgare obtained from pepperwort), Myrrh (oleo-gum-resin from Commiphora molmol), artemisinin derivatives isolated from Artemisia annua, curcumin (C. longa), quinine lack seeds and quindine (Cinchona officinalis), garlic extract (Allium sativum), black seed (Nigella sativa) and other several native plants from Brazil. The essential oil of N. sativa is one of the promising alternative drugs of plant origin that have antischistosomal effects (Mostafa and Soliman, 2002; Mohamed et al., 2005).

Nanoparticles can act as drug carriers that can modulate pharmacokinetics, increase bioavailability and target release with minimal toxic effects (Khalil et al., 2013). In this study, we used chitosan nanoparticles (CS NPs) as it is biodegradable and nontoxic (Yien et al., 2013). Several studies used scanning electron microscopy (SEM) to determine the alterations in the surface topography of Schistosoma for the evaluation of several drugs/compounds since the tegument of Schistosoma is an important target for such drugs (Jirauungkoorskul et al., 2005). Our study aimed to assess the therapeutic effect and electron microscopic changes of NSLCN on adult S. mansoni in vitro.

**MATERIALS AND METHODS**

The present study was carried out at the Schistosoma Biological Supply Center (SBSC), Theodor Bilharz Research Institute (TBDI), Giza, Egypt and at electron microscope unit, Faculty of Science, Ain Shams University during the period from June 2018 to August 2018.

**Preparation of NSLCN**

93% degree of deacetylation, sodium tripolyphosphate, Phosphate buffered saline (PBS) and an acetic acid were purchased from Sigma-Aldrich, USA. N. sativa (Baraka) was obtained from Pharco, Egypt. Chitosan nanoparticles (CS NPs) were synthesized via the ionotropic gelation of chitosan with Tripolyphosphate (TPP) anions. TPP has been used to prepare (CS NPs) as it is nontoxic, multivalent and its ability for gel formation via ionic interactions. The charge density of TPP and chitosan can control this interaction, under the influence of the solution pH. Chitosan was dissolved at various concentrations of an acetic aqueous solution; 1, 2 and 3 mg/ml. The chitosan concentration was 1.5 times lower than that of the acetic acid in aqueous solution. The TPP solution (1 mg/ml) was prepared with double-distilled water. CS NPs were made up with the drop wise adding about 5 ml of the chitosan solution to 2 ml of TPP solution under 1000 rpm magnetic stirring for 1 h at room temperature. The suspension was performed under the same above-mentioned conditions. Separations of the nanoparticles were done by centrifugation at 20000 g at 14°C for 30 min, and then they were freeze-dried and stored at 4°C. NSLCN was made by adding a chitosan solution to TPP solution (containing N. sativa a concentration of 500 mg/2 ml). NSLCN were separated from the suspension by centrifugation (20000 g at 14°C) for 30 min. Then, sediment was collected and weighed. The total protein content/mg of chitosan encapsulating powder was calculated by dividing the protein concentration of the loaded N. sativa by the nanoparticles weight (Danesh-Bahreinini et al., 2011). The loading capacity efficiency of the nanoparticles was determined:

\[ \%LC = \frac{[(A-B)/C] \times 100}{A} \]

A is the total amount of N. sativa, B is the free amount of N. sativa and C is the weight of nanoparticles.

**Characterization of NSLCN**

Their weights were measured, and they were characterized using the transmission electron microscope (TEM) (JEOL 100 CX) at the electron microscope unit, Faculty of Science, Ain Shams University.

**Parasites and culture media**

Adult of S. mansoni Swiss albino mice CD-1, weighing 18-22 g each, were obtained from SBSC, kept under environmentally-controlled conditions (temperature 25°C; humidity 70%; 12 h light and 12 h dark cycle) and acclimatized for one week before infection. The maintenance and care during the experimentation of animals were compliant with international guidelines for the human use of laboratory animals. Adult worms were removed from the portal and mesenteric veins of infected mice after 90 days (Duvall and Dewitt, 1967) sexed and counted (Xiao et al., 2009). Three to five mature worms including both sexes were obtained from SBSC, kept un sexed and counted (Xiao et al., 2009). Three to five mature worms including both sexes were cultured per well in 24-well plates containing RPMI medium at 37°C and 5% CO₂ immediately after animal perfusion to ensure their vitality.

**Evaluation of drug effect on S. mansoni adult worms**

After Schistosoma adult was exposed to NSLCN at concentrations of (10, 20, 40, 60, 80, and 100 μg/ml) for 24, and 48 h. Examination for worm viability was done after 24, and 48 h using a stereomicroscope comparing with control negative (adults incubated with 0.5% DMSO plus culture media) and control positive (worms incubated with 1 μg/ml PZQ plus the culture media) groups. Worms showing no signs of motility for one minute, associated with worm deformity such as blackening, twisting, and contracting were considered dead. The activity of the drug was measured by calculating the number of dead worms relative to the total number of worms. In the case of any doubt about the viability of worms, they were allowed to recover in clean medium and re-examined.

**SEM study**

To observe the morphological changes in the suckers and
Figure 1a, b. TEM of NSLCN showing regular, rounded shape with a smooth surface. Their mean size was 40 nm.

tegument of the adult parasites, *Schistosoma* was monitored using SEM following the standard procedure as described by Hassan et al. (2003). Adult male worms of *S. mansoni* were collected in glutaraldehyde-buffer solution (25%) as a fixative overnight at 4°C, then washed out of any of the fixative by keeping them overnight at 4°C in phosphate-buffered saline, then passed into rising concentrations of alcohol (30, 40 and 50%) each for 15 min and kept in 70% alcohol until the time of examination. Before the examination, they have washed twice for 30 min in 80 and 90% alcohol respectively. The last wash was for one hour in 100% alcohol. Worms were then mounted on stainless steel holders and put in a drier for about 30 min and then subjected to sputter coat of gold, the different parts of worms were examined using Joel JEM-1200 scanning electron microscope, provided with a camera fitted to it. Areas in the worms that showed specific changes were examined and photographed mainly, suckers and the tubercles on the tegument.

Statistical analysis

The collected data were analyzed using SPSS version 16 software, data were presented as number and percentage. Fissure extract test was used to detect the P-value. P<0.05 was considered significant.

RESULTS

Nanoparticles characterization by the Transmission electron microscope (TEM), NSLCN were regular, rounded and have a smooth surface. Their mean size was 40 nm (Figure 1a, b). After 24 h incubation, the live worms have sluggish motility and reached dead score at 48 h of incubation (Table 1). There was no statistically significant difference (P> 0.05) in its effect on males and females (Table 2). The death rate in worms reached 88.9% in the group treated with 100 μg and 80 and 76.6 in groups treated with 80 and 60 μg respectively (P-value <0.001) after 24 h of incubation (Table 1) and there were variable effects on motility. After 48 h of incubation with the same concentration and there was the death of all worms to reach 100% in all groups treated with NSLCN. Morphological alterations on the surface of male *Schistosoma* were in the form of worm deformity and swollen suckers. The tegument was swollen in some parts and flattened in other parts with shrinking and furrowing with edematous interpapillary ridges. *Schistosom* from negative control groups showed an intact tegument. The female tegument treated with NSLCN showed marked deformity in the form of wrinkles and furrowing and shrinking as shown in (Figure 2).

DISCUSSION

The in vitro test with *Schistosoma* is one of the useful tools to explore the antischistosomal properties of a known effective drug and also helps to analyze the mode of action against *Schistosoma* (Doenhoff et al., 2009). The seeds of *N. sativa* were subjected to a range of pharmacological investigations in recent years. These studies have shown a wide spectrum of activities such as antibacterial (Sasikumar et al., 2011), antitumor (David et al., 1998), anti-inflammatory (Mutabagani and El-Mahdy, 1997), CNS depressant and analgesic (Ramadhan et al., 2011), hypoglycemic (Boseila and Messalam, 2011), smooth muscles relaxant (Aqel and Shaheen, 1996), cytotoxic and immunostimulant (Swamy and Tan, 2000). Besides, the essential oil was shown to have...
Table 1. Effect of *N. sativa* loaded chitosan nanoparticles on motor activity of adult *S. mansoni* in vitro.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Time of incubation (h)</th>
<th>Total exam</th>
<th>Normal Score=3</th>
<th>Slow Score=2</th>
<th>Sluggish Score=1</th>
<th>Dead Score=0</th>
<th>FET</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control -ve</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control +ve (treated with pzq)</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Nano-treated</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 μg</td>
<td>24</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>8(88.9)</td>
<td>11.92</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>9</td>
<td>15.2</td>
<td>&lt;0.001**</td>
<td></td>
</tr>
<tr>
<td>80 μg</td>
<td>24</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>8(80)</td>
<td>10.21</td>
<td>0.001**</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>16.2</td>
<td>&lt;0.001**</td>
<td></td>
</tr>
<tr>
<td>60 μg</td>
<td>24</td>
<td>13</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>10(76.6)</td>
<td>13.0</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>13</td>
<td>19.11</td>
<td>&lt;0.001**</td>
<td></td>
</tr>
<tr>
<td>40 μg</td>
<td>24</td>
<td>11</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>4(36.4)</td>
<td>2.44</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>11</td>
<td>17.18</td>
<td>&lt;0.001**</td>
<td></td>
</tr>
<tr>
<td>20 μg</td>
<td>24</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>9</td>
<td>3(25)</td>
<td>1.16</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>12</td>
<td>18.15</td>
<td>&lt;0.001**</td>
<td></td>
</tr>
<tr>
<td>10 μg</td>
<td>24</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>9</td>
<td>3(25)</td>
<td>1.16</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>12</td>
<td>18.15</td>
<td>&lt;0.001**</td>
<td></td>
</tr>
</tbody>
</table>

P-value between total control –ve and treated with nano at different concentrations and different incubation times. FET used to estimate P-value.

Table 2. Effect of *N. sativa* loaded chitosan nanoparticles on male and female.

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. &amp;% of dead males in relation to total males no.</th>
<th>No. &amp;% of dead females in relation to total females no.</th>
<th>FET</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total      Death Death%</td>
<td>total        death   Death%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control -ve</td>
<td>6          0        0</td>
<td>4          0        0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control +ve (treated with pzq)</td>
<td>6          6        100</td>
<td>6          6        100</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Nano treated</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 μg</td>
<td>6          6        100</td>
<td>3          2        66.7</td>
<td>0.14</td>
<td>0.33</td>
</tr>
<tr>
<td>80 μg</td>
<td>6          6        100</td>
<td>4          2        50</td>
<td>1.28</td>
<td>0.13</td>
</tr>
<tr>
<td>60 μg</td>
<td>7          7        100</td>
<td>6          3        50</td>
<td>1.28</td>
<td>0.13</td>
</tr>
<tr>
<td>40 μg</td>
<td>6          2        33.3</td>
<td>5          2        40</td>
<td>0.0</td>
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</tr>
<tr>
<td>20 μg</td>
<td>6          1        16.7</td>
<td>6          2        33.3</td>
<td>0.0</td>
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<tr>
<td>10 μg</td>
<td>6          1        16.7</td>
<td>6          2        33.3</td>
<td>0.0</td>
<td>1.0</td>
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</table>

antihelmenthic activity (Agarwal et al., 1979) and the seeds were effective against cestodes and nematodes (Akhtar and Rifaat, 1991). In the last decades, plant extracts were widely used for the treatment of *Schistosoma* infection (Sparg et al., 2000). However, *N. sativa* seeds essential oil was recently found to have antihelmenthic activity against *S. mansoni* infection (Mahmoud et al., 2002).

Many studies used nanoparticles as vehicles to deliver drugs for the improvement of their therapeutic efficacy (El-Temsahy et al., 2016). In this study, we used chitosan as a drug carrier for *N. sativa* to improve its efficacy. Chitosan is a natural polymer used in nanomedicines, for its attractive characteristics for drug delivery and its
formulated nanoparticulate form proved to be effective. Its cationic character and its solubility in aqueous medium have been reported as important properties for the success of this polysaccharide (Grenha et al., 2010). This study aimed to assess the therapeutic effect and electron microscopic changes of NSLCN on adult S. mansoni in vitro.

Our results showed that both male and female

Figure 2. Tegumental changes of adult (male and female) S. mansoni after NSLCN. SEM showing normal suckers of normal control group (a) tegument of normal control male showing normal tubercles with intact spines (d) smooth tegument of normal control female (g). The group treated with nanoparticles showing partial loss of spines and swelling of the inter-tubercular ridges (e) and swelling of the ventral sucker (b) and marked changes of female tegument (pores, furrows and shrunken tegument (h). The group treated with PZQ showing complete loss of spines and marked deformity of tubercles in male (f) and edema of female tegument (i) and suckers deformity (c).
parasites are susceptible to NSLCN. We observed that the *Schistosoma* exposed to NSLCN showed motility changes in the form of sluggish contractions after 24 h of incubation. Furthermore, it caused 100% mortality of parasites at all concentration after 48 h of incubation, and affect male and female but the difference was a statically insignificant difference. These results are in harmony with Mahmoud et al. (2002) who stated that, administration of the black seed essential oil to *S. mansoni* infected mice showed high activity against adult worms.

On the other hand, De Araújo et al. (2007) using nanoemulsion of a new schistosomicidal drug (BphEA) showed that male worms moved slowly at the end of 48 h whereas all the female worms died. The recorded decreased worm motility produced by NSLCN has been described to be as a result of smooth muscle relaxation effect of *N. sativa* (Khazdair, 2015). This agrees with Jahromy et al. (2014) who observed that, *N. sativa* (100 mg/kg) significantly improved the muscle rigidity score starting at the 40th minute, while animals treated with extract (50 mg/kg) had no significant difference with the control group (received water). Moreover, *N. sativa* (200 mg/kg) significantly improved the muscle rigidity score starting at all times measured in comparison with the control group. This is attributed to the improvement of penetration of NSLCN through parasite tegument, a result of increase passage of hydrophilic pits in *Schistosoma* tegument and enhanced diffusion of nanoparticles. This occurs as a result of the increased solubility of the tested product in biological media of the parasite.

Alterations in the surface ultrastructure of *Schistosoma* worms were used by several investigators for the evaluation of antischistosomal drugs (Mostafa, 2005). Drug-induced tegumental changes have been described in *S. mansoni* worms after treatment with a variety of schistosomicidal drugs (Mohamed et al., 2005). It seems likely that the tegumental changes in the worms may be an important aspect of drug activity leading to the death and elimination of worms with the stopping of their egg production (Nosseir et al., 2000).

In this study, there were tegumental changes of both dead male and female in the form of loss of spines, swollen suckers and swollen inter tubercular ridges in male and loss of smooth architecture of female tegument with multiple pores. These results are in agreeing with Ali et al. (2016) who reported that there was edema of tubercles and sever dilatation and swelling of suckers of adult male *Schistosoma* treated with *N. sativa*.

The obtained results are in harmony with that observed by Mostafa and Soliman (2002) in their study of the surface topography of adult worms of *S. mansoni* harbored in albino mice treated with black-seed essential oil; they reported that the tubercles on the dorsal surface of the mature males developed in mice treated with black-seed essential oil from 0 days of infection showed extensive loss of spines. Spines may be partially or completely disappeared in some worms. Moreover, the size of the tubercles was greatly reduced. The inter-tubercle tegumental regions showed extensive swelling (edema) while the erosion of the surface was observed. Also, Mostafa (2005) found that the surface topography of male worms obtained from mice treated with Sidr honey alone showed extensive loss of spines. The tegument of worms that developed in mice treated with black-seed essential oil showed moderate structural changes, since the tubercles on the dorsal surface of the male showed partial loss of spines. However, the worms developed in mice treated with Sidr honey and black-seed oil together showed the greatest changes they lost their normal surface architecture and erosion of the tegument and spines loss was noted.

Previous studies have identified that when *Schistosoma* exposed to an immune system containing anti-schistosomal antibody, neutrophils, complement and praziquantel (1 µg/ml), the damage to the worm tegument induced by the drug and attachment of neutrophils on the worm surface aggravated the tegument injury which resulted in worm death within 24 h (Xiao et al., 2009). This agrees with Gonçalves et al. (2013) by using SEM, demonstrated the peeling and erosion of the tegumental surface and swelling of spines, both in the collar of spines region and the eventual erosion of the oral sucker after PZQ treatment. These morphological alterations on the surface of the worm are similar to those found in other trematodes, however, surface blebs were not seen. To summarize, we successfully applied NSLCN preparation against the adult stage of *S. mansoni in vitro*. Further study is needed to highlighting its effect in vivo. The application of nanotechnology may offer a safe, effective, and cheap treatment.

**CONFLICT OF INTERESTS**

The authors have not declared any conflict of interests.

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Full Length Research Paper

Effect of hormones on the seed germination of Bupleurum species

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In Asia, Radix Bupleuri (Bupleurum spp. root) is an important medicine used for treating many diseases over the past 2000 years. However, its cultivation is difficult due to poor germination rates and loss of seedling viability shortly after seeding. In the present study, the effect of 6-benzyl aminopurine (6-BA), dimoxinole, gibberellin (GA3), paclobutrazol, uniconazole, and mepipquat chloride (MC) on the germination of seeds of ‘Zhongchai No. 3’, which is the newest released commercial variety of Bupleurum chinense DC in China was analyzed. Only GA3 was found to promote seed germination significantly, and its optimum concentration was 223.84 to 238.78 mg·L⁻¹. In addition, incubation with GA3 on the first day of germination was extremely important for seedling survival. This protocol could also be applied to ‘Zhonghongchái No. 1’ (B. scorzonerifolium Willd.) and ‘Hubei chái hé’ (B. chinense DC.). The combination of GA3 treatment and centralized incubation could be an efficient way to industrialize the production of secondary metabolites from Bupleurum species.

Key words: Hormone, Bupleurum spp., germination, gibberellin (GA3).

INTRODUCTION

Radix Bupleuri (root of Bupleurum spp.) is one of the most important medicinal herbs in Eurasia and North Africa for treating fever, chronic hepatitis, kidney syndrome, inflammatory diseases, menstrual disorder, and digestive system ulcers (Pistelli et al., 1996; Guo et al., 2000; Ikegami et al., 2006; Mabberley, 2008). Utilization of Bupleurum species in preparations was firstly recorded for more than 2000 years in Shen-Nong’s Herbal of China (Xie et al., 2009).

Nearly 250 bioactive compounds from this genus have been identified through phytochemical investigations; in particular, saikosaponin a and saikosaponin d are known for their pharmacological activities (Ashour and Wink, 2011). Bupleurum species are officially listed in the Chinese and Japanese Pharmacopoeias in addition to the WHO monographs of the commonly used medicinal plants of China and Korea (WHO, 1997, 1998). Due to its medicinal importance, the demand for R. Bupleuri has increased steadily in recent years. Nearly eight million kilograms of R. Bupleuri are required each year in China, and gross sales of manufactured prescription medicines containing...
R. Bupleuri in Japan amounted to 27 billion yen in 2002 (Pan, 2006; Zhu et al., 2009).

Due to the annual increased market requirement and considerable exploitation of Bupleurum species, natural wild resources have decreased sharply. Wild R. Bupleuri shows high variability in its pharmacological active components. Therefore, the cultivated Bupleurum has become economically important. In the last 10 years, three commercial varieties (Zhongchai No. 1, Zhongchai No. 2, and Zhongchai No. 3) of B. chinense DC and one commercial variety (Zhonghongchai No. 1) of B. scorzonerifolium Willd. were released to meet the market requirement in China (Yao et al., 2013). All these varieties showed uniform performance in their agronomic traits and high saikosaponin content. However, due to the short domestication history of Bupleurum species, there are still challenging traits to deal with, specifically poor seed germination and viability loss in a short period.

The aim of this work was to develop a protocol to promote Bupleurum seed germination, thus making the cultivation of Bupleurum easier. The study involved the initial screening of hormones to identify a candidate that could promote the germination of Zhongchai No. 3 seeds, which is the newest released commercial variety. The effect of this optimized protocol on other varieties of Bupleurum species was also validated.

MATERIALS AND METHODS

Plant materials

Seeds of ‘Zhongchai No. 3’ were used in all studies, except in validation of the optimal protocol. The seeds used in the validation of the optimal protocol were those of the commercial varieties ‘Zhongchai No. 1’, ‘Zhongchai No. 2’ and the landrace ‘Heilongjiang hongchahutu’ of B. scorzonerifolium Willd; the landrace ‘Rongxianzhuye’ of B. marginatum; and the landrace ‘B1’ of B. fruticosum. All materials were kindly provided by Professor Jianhe Wei from the Institute of Medicinal Plant Development (IMPLAD), Chinese Academy of Medical Sciences & Peking Union Medical College. All varieties were planted in the Sichuan province in 2011–2013, and the seeding rate was 0.6–0.75 million plants per hectare. The planting area of each variety was over 0.1 hectares. All seeds were harvested in November 2013.

Incubation condition and germination evaluation

One hundred seeds were placed on moist filter paper at the bottom of covered culture dishes (diameter, 10 cm). The filter in every culture dish was initially moistened with 6 mL of hormonal solution at different concentrations. Distilled water was added to a few dishes to avoid drying during seed incubation. All culture dishes were placed in an incubator. Temperature was maintained at 20°C. Emergence of seeds greater than 0.5 cm was considered as successful germination, at which point the germinated seed was removed from the culture dish. The cumulative germination percent was calculated for each dish. Germination index was calculated as:

\[ GI = \sum (Gt/l) \]

Where, Gt indicates the germinated seed at the tth day.

Experiment 1: Candidate hormone screening

The hormones in this screening study were 6-benzyl aminopurine (6-BA), daminozide, giberellin (GA3), paclobutrazol, uniconazole, and mepiquat chloride (MC). For each hormone, six concentrations (i.e., 0, 20, 40, 60, 80 and 100 mg L\(^{-1}\)) were utilized. Each treatment consisted of two replicates.

Experiment 2: Critical GA3 concentration and duration of treatment

The promotion of germination of Zhongchai No. 3 seeds was insignificant in a preliminary experiment when the GA3 concentration was above 500 mg L\(^{-1}\). Two sets of Zhongchai No. 3 seeds in 11 dishes were cultured in the presence of GA3 at concentrations of 0, 50, 100, 150, 200, 250, 300, 400, 450, 500 mg L\(^{-1}\). The results from the experimental trials varied significantly and, consequently, different quadratic and cubic regression models were fit to each experimental trial. Critical GA3 concentrations were calculated from the different models.

The optimal concentration of 230 mg L\(^{-1}\) was used for the analysis of the duration of treatment for GA3. The seeds were incubated with GA3 for 0, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 d. All seeds were incubated with distilled water following GA3 treatment for 10 d. The converse treatment sequence consisted of incubating the seeds with distilled water for 0, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 d followed by GA3 treatment for 10 d. Then, all seeds were incubated with distilled water for 30 days.

Experiment 3: Validation of the optimal protocol

Seeds of ‘Zhongchai No. 1’, ‘Zhongchai No. 2’, ‘Zhonghongchai No. 1’, ‘Hubei chaihu’, ‘Rongxianzhuye’, ‘Fengshun landrace’, ‘Heilongjiang hong chahutu’ and ‘B1’ were incubated with 230 mg L\(^{-1}\) GA3. Distilled water was utilized as the control. Three replicate experiments were conducted.

Statistical analysis

All statistical analyses were performed using the SPSS 16.0 software (Norusis, 2008). Quadratic and cubic regression models were used to identify the critical incubation concentration of GA3. Duncan’s multiple range test (p < 0.05) was used to compare the differences.

RESULTS AND DISCUSSION

To obtain the highest number of plants for the production of secondary metabolites, several studies based on micropropagation cultures of B. fruticosum, B. chinense DC, B. scorzonerifolium Willd, and B. smithii Wolff have been conducted (Fraternale et al., 2002; Li et al., 2008; Hao and Guan, 2012).

However, since the plants of Bupleurum species are small, the use of micropropagation cultures to industrialize the production of secondary metabolites is costly and has high labor requirements. Since plants of Bupleurum species can produce an abundance of seed, propagation from seed might be a more efficient method once the problem of poor germination is solved.

Seed germination represents the irreversible developmental-phase transitions from seed dormancy to germination in plants. These transitions involved
The germination of cubic regression model was more than 9 days. These formula-calculated concentrations used. In the case of 6-BA, the concentration of 20 mg L⁻¹ 6-BA, we chose GA3 as the candidate hormone to further study germination promotion in Bupleurum species (Figure 1).

Gibberellins are diterpenoid, promoting germination and regulating plant growth. In previous studies, this plant hormone have been reported to stimulate the synthesis and accumulation of α-amylase, and regulate the expression of the Osem gene to control the production of one of the embryogenesis abundant proteins, resulting in the germination of seeds (Yamaguchi, 2008; Miransari and Smith, 2014).

In the present study, the Zhongchai No. 3 seeds showed significantly different germination when treated with the different hormones (Table 1). Decreased germination was observed in the presence of daminozide, paclobutrazol, uniconazole, and MC. GA3 significantly promoted both cumulative germination percent and germination index (Table 2). Critical concentrations for promoting ‘Zhongchai No. 3’ seed germination were calculated from each model (Table 2, Figure 2). The predicted critical concentrations for increasing the germination index were in the range of 223.84–237.67 mg L⁻¹, and the predicted critical concentrations for increasing the cumulative germination percentage were in the range of 230.21–238.78 mg L⁻¹. Therefore, the best GA3 concentration for promoting ‘Zhongchai No. 3’ seed germination might be 223.84–238.78 mg L⁻¹. When the ‘Zhongchai No. 3’ seeds were incubated with 230 mg L⁻¹ GA3, both the cumulative germination percentage and germination index were significantly higher than those of the control seeds when the incubation period was longer than 1 days (Table 3). However, a significant increase in cumulative germination percentage was observed when the converse treatment sequence was applied for more than 7 days, and a significantly increased germination index was observed when the duration of converse treatment was more than 9 days. These results indicated that incubation with an appropriate GA3 concentration on day 1 might be the most important factor in promoting ‘Zhongchai No. 3’ seed germination.

Significant germination variation was observed for other seed varieties. The commercial varieties showed a higher cumulative germination percentage and germination index than the landraces (Table 4). Only ‘Zhonghongchai No. 1’ and ‘Hubei chaihu’ seeds showed significantly increased cumulative germination percentages when the seeds were incubated with 230 mg L⁻¹ GA3. However, the increase in the germination index of ‘Hubei chaihu’ was insignificant. This finding indicated that different germination mechanisms exist for promoting

<table>
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<tr>
<th>Hormones</th>
<th>Cumulative germination percentage under different concentrations</th>
<th>Germination index under different concentrations</th>
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<tr>
<td></td>
<td>CK 20 mg L⁻¹ 40 mg L⁻¹ 60 mg L⁻¹ 80 mg L⁻¹ 100 mg L⁻¹</td>
<td>CK 20 mg L⁻¹ 40 mg L⁻¹ 60 mg L⁻¹ 80 mg L⁻¹ 100 mg L⁻¹</td>
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<td>6-BA</td>
<td>25.00 38.50 18.00 8.00 4.00 3.00 1.62 2.23 0.99 0.39 0.22 0.16</td>
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<td>GA3</td>
<td>25.00 52.50 55.50 57.50 51.50 51.50 1.61 2.86 3.83 3.55 3.72 3.31</td>
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<tr>
<td>daminozide</td>
<td>25.00 16.00 17.00 22.00 13.00 11.00 1.60 0.95 1.05 1.28 0.87 0.67</td>
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</tr>
<tr>
<td>MC</td>
<td>25.00 5.00 3.50 7.00 7.50 9.00 1.59 0.30 0.26 0.44 0.43 0.57</td>
<td></td>
</tr>
<tr>
<td>paclobutrazol</td>
<td>25.00 2.00 0.00 0.50 0.00 0.00 1.58 0.10 0.00 0.00 0.00 0.03</td>
<td></td>
</tr>
<tr>
<td>uniconazole</td>
<td>25.00 0.00 0.00 0.00 0.00 0.00 1.57 0.10 0.00 0.00 0.00 0.03</td>
<td></td>
</tr>
</tbody>
</table>

aHormones are 6-benzylaminopurine (6-BA), gibberellic (GA3), daminozide, mepiquat chloride (MC), paclobutrazol, and uniconazol
bCK is the concentration of 0 mg L⁻¹.
among different varieties of Bupleurum species. This could explain why several reported protocols for promoting Bupleurum seed germination were ineffective in other studies (Yao et al., 2011).

Therefore, in the case of commercial varieties other than ‘Zhonghongchai No. 1’ and ‘Hubei chaishu’, protocols using different hormones or varying incubation temperatures should be explored. However, in our study, a change in temperature for germinating Zhongchai No. 2 seeds was ineffective (data not shown).

Since Bupleurum species seeds are sensitive to water and temperature and have a long germination period, direct seeding in the field is difficult. In addition, the plants of Bupleurum species grow slowly at the early stage, leading to considerable competition in the field between small plants and weeds. Such competition can be potentially avoided by centralized incubation in a greenhouse to increase seedling survival, followed by
transplanting of these seedlings into the field. Hence, the combination of GA3 treatment and centralized incubation might be a more efficient strategy for large-scale cultivation rather than direct seeding.

Conclusion

We found that treatment with 223.84–238.78 mg L⁻¹ GA3 during the first day best promotes Bupleurum ‘Zhongchai No. 3’ seed germination. This hormone treatment is only useful in the case of ‘Zhonghongchai No. 1’ and ‘Hubei chaihu’ seeds. The combination of GA3 treatment and centralized incubation could be an efficient way to industrialize the production of secondary metabolites from Bupleurum species. The future work should focus on the contents variation of secondary metabolites for Bupleurum plants under transplanting and direct seeding.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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