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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

Pelczar JR, Harley JP, Klein DA (1993). Microbiology: Concepts and Applications. McGraw-Hill Inc., New York, pp. 591-603.

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Review

Transgenic plants as green factories for vaccine production

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Edible vaccine technology represents an alternative to fermentation based vaccine production system. Transgenic plants are used for the production of plant derived specific vaccines with native immunogenic properties stimulating both humoral and mucosal immune responses. Keeping in view the practical need of new technology for production and delivery of inexpensive vaccines, especially in developing world, plant derived edible vaccines is the best option in hand to combat infectious diseases. Plant derived vaccine is easy to administer, cost effective, readily acceptable, have increased safety, stability, versatility and efficacy. Several plant derived vaccines are under research, some are under clinical trials for commercial use. Like most biotechnology products, the IP situation for edible vaccines is complex as IP rights influence every stage of vaccine development.

Keywords: Transgenic plants, edible vaccines, chimeric viruses, bacterial diseases, viral diseases.

INTRODUCTION

Transgenic plants are the plants in which foreign genes of desired characters have to be inserted. Transgenic plant have been found to have many advantages like, development of high yielding varieties of crop plants and disease resistant, and are plants with improved tolerance to biotic and abiotic stress (Ahmad et al., 2008; 2010a; b; 2011; Ahmad and Umar, 2011; Ahmad and Prasad, 2012a; b; Sarwat et al., 2012). Apart from the above, transgenic plants have been employed for the production of vaccines for the treatment of various infectious diseases (Kant et al., 2011; Vianna et al., 2011; Yoshida et al., 2011; Sharma and Sood, 2011; Twyman et al., 2012). Infectious diseases are major cause of mortality and morbidity worldwide (Goldblatt and Ramsay, 2003)

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Abbreviations: IP, Intellectual Property; **HBsAg,** hepatitis B surface antigen**;HIVgag,** HIV Gag protein ; **LT-B,** heat labile enterotoxin B subunit; **CT-B,** cholera toxin B subunit; **ETEC,** entero toxigenic *Escherichia coli*; **M cells,** microfold cells; **CaMV,** *Cauliflower mosaic virus*; **CpMV,** *Cow pea mosaic virus*; **TMV,** *Tobacco mosaic virus*; **CTB,** Eholera toxin B subunit; **PA,** protective antigen; **LF,** pethal factor; **HBV,** hepatitis B virus; **JEV,** *Japanese encephalitis* virus

and one-third of the deaths are caused by the infectious agents. Vaccine is an immuno-biological substance, used for specific protection against both infectious and non-infectious diseases (reviewed by Ahmad et al., 2012; Twyman et al., 2012). Vaccine is responsible for the stimulation of protective antibody and other immune mechanisms. The vaccines can be made from live or killed inactivated organisms, extracted cellular fractions, toxoid or combination of these. Recent preparations are sub-unit vaccines and recombinant vaccines. The main limitation with vaccines is their dependence on cold chain system, which is used to store and transport the vaccine under strict controlled conditions (Park, 2005). Other limitations are risk of adverse reactions such as reactions inherent to inoculation, reactions due to faulty techniques etc (Goldblatt and Ramsay, 2003). Thus, for the implementation of a successful global vaccination strategy, a well designed subunit oral vaccine system should satisfy the following criteria (Chargelegue et al., 2005; Levine et al., 2006; Nochi et al., 2007): (a) Produce sufficient quantities of desired antigen; (b) preserve the expressed antigen for a long time at room temperature; (c) induce protective immunity; (d) be protected from enzymatic digestion in the gastrointestinal tract.

Therefore, in the 1990s, an International campaign was initiated to immunize all the world's children against six devastating diseases. The target was to reach 80% of infants and reduce the annual death toll from these infections by roughly three million. Still, 20% of infants are un-immunized by six vaccines against polio, measles, diphtheria, pertusis, tetanus and tuberculosis. In many developing countries, millions of children still die from infectious diseases due to immunizations being non-existent, unreliable or too costly (Ramsay et al., 1999). None will be entirely safe until every child has routine access to vaccines. Hence, there is an urgent need to search for vaccines which are easy to administer, easy to store, cost effective, easy to transport and possess readily acceptable delivery system. Hence, there is a lot of scope in developing plant derived vaccine (Streatfield et al., 2001; Ahmad et al., 2012). Now the question arises what is plant derived vaccine? Advances in transgenic research have made use of crop plants to serve as bioreactor for the production of recombinant molecules (Raskin et al., 2002; Kant et al., 2011; Vianna et al., 2011; Yoshida et al., 2011; Sharma and Sood, 2011). This means that transgenic plants are used to express antigen proteins induced by plant transgenic vectors and to produce certain special vaccines with high anti-disease ability (reviewed by Mei et al., 2006; Malabadi et al., 2012) (Figure 1). Plant derived vaccines significantly increase availability of vaccines in places where maintenance of cold chain system is difficult (Webster et al., 2002; Kant et al., 2011; Vianna et al., 2011; Yoshida et al., 2011; Sharma and Sood, 2011; Twyman et al., 2012). Important examples on the development of plant bioreactors are shown in Table 1.

The immunogenicity and safety of plant derived vaccines was declared in phase I clinical studies (Tacket, 2009). During the last decade, different types of efficient plant-based expression systems have been studied and more than 100 different types of recombinant proteins including plant-derived vaccine antigens have been successfully expressed in different types of plant tissues (Tiwari et al., 2009; Rybicki, 2010; reviewed by Ahmad et al., 2012). Positive effects of edible vaccines include decrease in potential hazards such as toxic compounds, responses to allergy and risk of attenuated strains reverting to pathogenic strains associated with established production technologies that use bacteria, yeast and mammalian cells (Pelosi et al., 2012).

TRANSGENIC PLANTS FOR THE PRODUCTION OF PLANT DERIVED VACCINES

Through recombinant DNA technology, different level of antigen expression for each independent line has been observed in plants (Karg and Kallio, 2009; Shih and Doran, 2009; Wilken and Nikolov, 2012). In 1990 first edible vaccine, surface protein A from *Streptococcus mutans* was expressed in tobacco (Curtis and Cardineray, 1990). Plant derived vaccine in the form of seed or fruit can be easily stored and transported from one place to another without the worry of its degradation or damage. A large amount of plant derived vaccine can be easily produced by cultivation in fields with relatively few inputs. Autoimmune disorders like Type I diabetes, multiple sclerosis, rheumatoid arthritis etc., can also be suppressed by using plant derived vaccines (Prakash, 1996).

Plants are selected which expresses highest level of antigen and least number of adverse effects. Till date various types of antigens are successfully expressed in different plants (Mason and Arntzen, 1995; reviewed by Ahmad et al., 2012). With the development of plant genetic engineering, the expression system for transgenic plants are no longer limited to model plants, but extended to some orally or high protein content plants. Various plant plateforms have been demonstrated for production of recombinant proteins in plants, including leafy crops, cereals and legume seeds, oil seeds, fruits, vegetables, higher plant tissue and cell cultures, hydroponic systems, algae and halobios (reviewed by Mei et al., 2006). Co-expression of adjuvant along with antigen has also been done in the same plant (Lal et al., 2007). The use of rice storage protein gene promoters to express transgenes in rice grain is well documented (Nicholson et al., 2005).

Furtado et al. (2008) compared use of storage protein gene promoter and non-storage gene promoter with regard to spatial and temporal control of expression from barely, wheat and rice. Storage protein promoter from barley and wheat directed the expression in endosperm but not in embryo; expression was leaky, as it was observed in seed maternal tissues, leaf and root tissues;

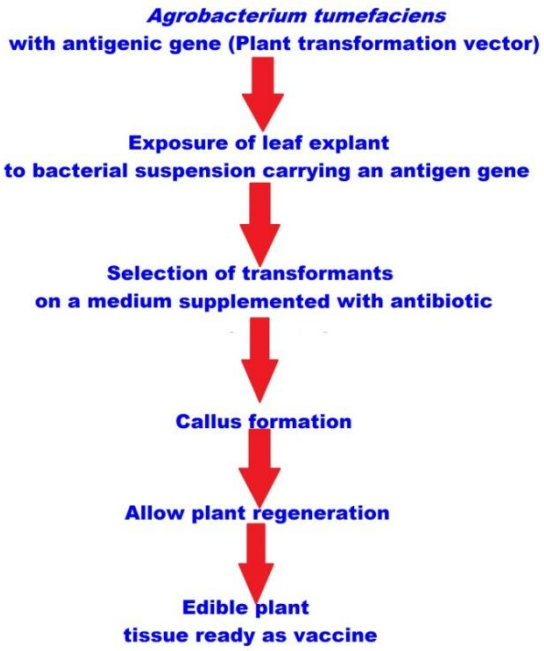

Figure 1. Strategy for the production of candidate vaccine antigen in plants

Table 1. Representative plant-based vaccines: under clinical development or in market.

S/N	Product	Plant Host	Expression system	Indication	Route of administration	Product stage development	Reference
1	*E. coli* LT-B	Potato	Transgenic	Diarrhea	Oral	Phase 1	Tacket et al. (2007)
		Maize kernels	Transgenic			Phase 1	Tacket et al. (2009) Chikwamba et al. (2003)
2	Norwalk virus	Potato , Tobacco	Transgenic	Diarrhea	Oral	N A	Tacket et al. (2000) Santi et al. (2008)
		Tobacco (VLP's)					Zhong et al. (2005)
		Tomato fruit (Capsid protein)					
3	HBsAg IgG (hepatitis B virus)	Potato Banana Tobacco Cherry, tomato Tobacco	Transgenic	Hepatitis B	Oral	Phase 1	Kong et al. (2001) Kumar et al. (2005) Kostrzak et al. (2009) Gao et al. (2003); Valdes et al. (2003)
4	Rabies virus GP/NP	Spinach Tobacco	Transient (viral vector)	Rabies	Oral	Phase 1	Modelska et al. (1998) Roy et al. (2010)
5	Newcastle disease virus HN	Tobacco Cell Suspension Potato	Transgenic	Newcastle disease	Subcutaneous	USDA approved (not marketed)	Yusibov et al. (2011) Gómez et al. (2008)
6	Personalized anti-idiotype scFVs	*Nicotiana benthamiana*	Transient (viral vectors)	Non-Hodgkin's lymphoma	Subcutaneous	Phase 1	Yusibov et al. (2011)

Table 1. Contd.

7	Personalized anti-idiotype dcFVs	*Nicotiana benthamiana*	Transient (magnICON vectors)	Non-Hodgkin's lymphoma	Subcutaneous	Phase (ongoing)	1	Yusibov et al. (2011)
8	H5N1 influenza HA VLP	*Nicotiana benthamiana*	Transient (agrobacterial binary vector)	H5N1 "avian" influenza	Intramuscular	Phase (ongoing) Phase (Health Canada approved)	1 2	Yusibov et al. (2011)
9	H5N1 influenza HAI1	*Nicotiana benthamiana*	Transient (launch vector)	H5N1 "avian" influenza	Intramuscular	Phase 1		Yusibov et al. (2011)
10	H1N1 influenza HAC1	*Nicotiana benthamiana*	Transient (launch vector)	H1N1 "swine" influenza	Intramuscular	Phase (ongoing)	1	Yusibov et al. (2011)
11	L1 capsid protein	Potato	Transient	Human Papilloma virus	Subcutaneous	NA		Biemelt et al. (2003)
		Tobacco	Transient (biolistic delivery system)		Oral Subcutaneous Intramuscular	NA		Warzecha et al. (2003) Kohl et al. (2006)
12	Protective antigen (PA)	Tobacco	Transient	Anthrax	Subcutaneous	NA		Aziz et al. (2002)
		Tobacco	Transgenic (Microprojectiles)	Anthrax	Subcutaneous	NA		Koya et al. (2005) Watson et al. (2004)
13	S protein	Tomato Tobacco	Transient Transient	SARS	Oral Oral	NA		Pogrebnyak et al. (2005)
14	MV-H protein	Tobacco Lettuce	Transient NA NA	Measles Virus	Oral Intraperitoneal intranasal	NA NA NA		Webster et al. (2002) Webster et al. (2005) Webster et al. (2005)
15	Spike protein	Maize	NA	Swine transmissible gastroenteritis virus	Oral	NA		Lamphear et al. (2004)
16	D2 peptide of fibronectin-binding protein (FnBP)	Cowpea	Transient	*Staphylo-coccus aureus*	Intranasal oral	NA		Brennan et al. (1999)
17	Intimin protein	Tobacco	Transient	E. coli 0157:H7	Oral	NA		Judge et al. (2004)
18	FaeG of K88 fimbrial antigen	Tobacco	Transient	Entero-toxigenic *E. coli* (Strain K88)	Intraperitoneal	NA		Huang et al. (2003)
19	Cry jI, Cry jII	Rice	NA	Japanese Cedar pollen allergens	**Oral**	NA		Takagi et al. (2005)
20	VP1	Alfalfa Tobacco chloroplasts	NA	Foot and Mouth Disease Virus	Parenterally oral	NA		Wigdorovitz et al. (1999) Li et al. (2006)

Table 1. Contd.

21	F protein	Tomato	NA	Respiratory Syncytial Virus	Oral	NA	Sandhu et al. (2000)
22	SSA	Narrow Leaf Lupin	NA	Sunflower seed albumin	oral	NA	Smart et al. (2003)
23	B5	Tobacco	NA	Influenza Virus	NA	NA	Shoji et al. (2008)
24	F1-V fusion protein	Tomato	Transient	Plague	Oral	NA	Alvarez et al. (2006)
25	2L2I peptide	Tobacco Chloroplast	NA	Canine Parvovirus	Parenteral route with Oral booster	NA	Molina et al. (2005)
26	Envelope protein (E)	Tobacco Rice		Japanese encephalitis virus			Appaiahgari et al. (2009) Wang et al. (2009)
27	ESAT-6 antigen	Arabidopsis	NA	Tuberculosis	Oral	NA	Rigano et al. (2005)
28	VP6 HRV-VP7	Alfalfa Potato	NA	Rotavirus	Oral	NA	Yuan and Saif (2002) Yu-Zhang et al. (2003)

whereas, rice promoters directed the endosperm-specific expression in transgenic rice (Furtado et al., 2008). Alfalfa (*Medicago sativa*) is considered as a good bioreactor for production of recombinant proteins as it contains high levels of protein content and low levels of secondary metabolites (Dus Santos et al., 2002). Cereal crops can be the most suitable candidate and can be used to enhance the antigen concentration and help to reduce oral dose as they have ample amount of soluble protein in endosperm (Ahmad et al., 2012). Potato, tomato and carrot have been successfully reported to express vaccine candidates (Walmsley and Arntzen, 2000). Antigen genes encoding HBsAg, HIVgag and Rabies Capsid Proteins have been successfully transformed to tomato (Sala et al., 2003). High levels of recombinant protein expression were observed in proplastids of cultured carrot cells (Daniell et al., 2005). Oral delivery of the therapeutic proteins via edible carrot preserved the structural integrity of their target proteins as no cooking is needed (Muller et al., 2003). Other vegetable crops like lettuce (*Lactuca sativa*), celery cabbage (*Brassica rapa* var. pekinensis), cauliflower (*Brassica oleracea* var. botrytis) are under study for the production of vaccines. The only problem in these vegetables is low expression levels (Koprowski, 2005; Tacket and Mason, 1999). The earliest fruit used for the plant transgenic programme is banana (*Musa acuminate*) (Mason et al., 2002).

According to Trivedi and Nath (2004) papaya (*Carica papaya*) is another ideal plant species for vaccine production. Apart from fruit, vegetable and cereal crops scientists have used algae to produce metabolites and heterologous proteins for pharmaceuticals applications (Mayfield and Franklin, 2005). The species under study are: *Chlamydomonas reinhardii* (Sun et al., 2003), *Phaeodactylum tricornutum* (Zaslavskaia et al., 2000), *Amphidinium carterae*, *Symbiodinium microadriaticum* (ten Lohuis and Miller, 1998) and *Cylindrotheca fusiformis* (Fischer et al., 1999). Exciting progress has been made with the chloroplast based production of two particularly important classes of pharmaceuticals, vaccines and antibodies (Bock and Warzecha, 2010; Scotti et al., 2012). Extraordinarily high expression levels and the prospects of developing edible pharmaceuticals make transgenic chloroplasts a promising platform for the production of next-generation vaccines and antimicrobials (Waheed et al., 2012). During the past few years, several vaccine candidates have been produced successfully via plastid transformation, which emphasizes that transplastomic plants, as a second generation expression system, have great potential to fill gaps in conventional production platforms. A salient feature of plastids is that they combine characteristics of prokaryotic and eukaryotic expression systems, which is exemplified by the production of virus like particles and of bacterial antigens (reviewed by Bock and Warzecha, 2010). Successful expression of antigens in plants was carried out for *Escherichia coli,* heat labile enterotoxin B subunit (LT-B) in tobacco and potato (Hirst and Holmgren, 1987), *Rabies virus* G protein in tomato (Mc Garvery et al., 1995), *Hepatitis B virus* surface antigen in tobacco and potato (Thanavala et al., 1995), *Norwalk virus* capsid protein in tobacco and potato (Mason et al., 1996) and cholera toxin B subunit (CT-B) in potato (Arakawa, 1997).

Antigen expressed in plant or plant products can be administered orally or by intramuscular or by intravenous injection. Homogenized leaves, fruits or vegetables are used through oral route. Purified antigen containing plant

tissue can be delivered in a capsule or powder (pill) form. Capsule may be suitable because capsule coating can be modified in such a way that coating material dissolves in particular area of stomach, and vaccine can be released in a specific area of the body. Purified component can also be used by intramuscular and intravenous administration. Oral administration of plant derived vaccine induces both mucosal and systemic immunity. When antigen is administered orally, it induces more mucosal response than intramuscular or intravenous injections. So, more importance has been given to those antigens, which induce mucosal immune response to produce secretory Ig A at mucosal surfaces. Mucosal immunity is very effective in diarrhoeal diseases caused by rotavirus, *Norwalk virus*, *Vibrio cholerae*, entero toxigenic *E. coli* (ETEC) and also in respiratory diseases such as pneumonia.

Second generation plant derived vaccines are known as multi component vaccines, provides protection against several pathogens. Both Enterotoxigenic *Escherichia coli* (ETEC) heat-labile enterotoxin (LT-B) and the capsid protein of *Norwalk virus* were successfully expressed in plants and induced immune response against both *E. coli* and *Norwalk virus* in mice (Huang et al., 2001).

ADVANTAGES OF EDIBLE VACCINES OVER INJECTED VACCINES

Edible vaccines have many advantages over the injected vaccines like:

1. Edible vaccines are cost effective, have low risk of contamination and no cost for transportation. Pharmaceutical companies spend million dollars for the production of vaccines and to preserve vaccines. Transgenic plants does not need cold chain storages.
2. Pharmaceutical companies need the hitech machines for the production of vaccines. In the case of edible vaccines production we need soil rich land instead of machines.
3. Long distance transportation is not required in the case of edible vaccines.
4. The cost of materials needed for field grown plants is lower compared to cell culture grown in bioreactors (Xu et al., 2011).
5. Edible vaccines have a low cost for medical equipment as well, because needles and syringes are not needed for delivery (Streatfield, 2006; Xu et al., 2011).
6. Medical professionals are not needed for oral delivery (Streatfield, 2006).
7. Transgenic plants have low contamination risks as compared to injected vaccines
8. Needles and syringes are responsible for spreading of second hand diseases (Nochi et al., 2007).
9. Oral delivery has efficiency to provoke a mucosal immune response, which produces cell mediated responses (Streatfield, 2006).

Edible vaccines have multi-component ability that is possible due to the crossing of 2 plant lines (Lal et al., 2007). These vaccines with multi-component abilities are known as second generation edible vaccines as they allow for several antigens to approach M cells (microfold cells) simultaneously (Lal et al., 2007). The multi-component edible vaccines can prevent multiple diseases for example ETEC, chlorea and ratovirus (Lal et al., 2007). Injected vaccines do not have this property, so there are less effective than edible vaccines (Ramessar et al., 2008a; b; Naqvi et al., 2011).

Chimeric viruses

Over-coat and epi-coat technology is used to produce chimeric viruses. Over-coat technology provides expression of entire protein, whereas epi-coat technology permits the plant to produce only the foreign proteins (http://www.geocities.com/plantvaccines/transgenicplants .html). Plant viruses redesigned to carry the desired genes and used to infect differently in different parts of the plant. *Alfalfa mosaic virus*, CaMV (*Cauliflower mosaic virus*), CpMV (*Cow pea mosaic virus*), TMV (*Tobacco mosaic virus*), *Tomato bushy stunt virus* and *Potato virus* are redesigned to express fragments of antigens on their surface. There are reports that they produce plant based chimeric virus such as foot and mouth disease virus; mint enteritis virus. Fragment of gp41 surface protein of HIV virus put into CpMV could evoke a strong neutralizing antibody response in mice (Moffat, 1995; Wang et al., 2012).

APPROACHES TO PRODUCE PLANT DERIVED VACCINES

Plants serve as an important source to produce cost-effective vaccine derivatives. Plant based production of vaccine candidates can help to reduce the economic burden on the developing countries and can be made easily available to every individual. Various models to produce vaccine candidates are described below.

Bacterial

Enterotoxigenic Escherichia coli (ETEC)

Enterotoxigenic *Escherichia coli* strains are a major cause of enteric diseases in live stock and humans. ETEC is attached to specific receptors on the surface of enterocytes in the intestinal lumen by fimbriae. ETEC produces a heat-stable enterotoxin (ST) which consists of five B subunits and one A subunit. B subunit binds to sugar residues of ganglioside Gm1 on the cells lining the

villi and crypts of the small intestine. Insertion of the B subunit into the host cell membrane forms a hydrophilic transmembrane channel through which the toxic A subunit can pass into the cytoplasm (Roy et al., 2010). Raw transgenic potato expressing LT-B were fed to 11 volunteers, out of which 10(91%) developed neutralizing antibodies and 6(55%) of individuals also showed mucosal response (Tacket et al., 1998). Different reports are there on synthetic heat-labile enterotoxin (LT-B) gene and their expression in plants such as potato, banana, tobacco and tomato; and all were tested in mice (Mason et al., 1998). Expression of *E. coli* fimbrial subunit protein in transgenic plants can be used to vaccinate against these diseases. Joensuu et al. (2006) evaluated transgenic plants to produce Fae G protein and adhesion of F4 fimbriae. Oakes et al. (2007) reported the edible transgenic soyabean plant producing *E. coli* fimbrial subunit proteins. Tacket (2009) discussed early human studies of oral transgenic plant-derived vaccines against enterotoxigenic *Escherichia coli*. Genetic combination of gene coding for an LTB:ST protein in tobacco by *Agrobacterium* mediated transformation displays antigenic determinants from both LTB and ST. Presence of mucosal and systemic humoral responses in mice when dosed orally with transgenic tobacco leaves also confirmed that plant-derived LTB:ST can lead to immunogenicity development via oral route (Rosales-Mendoza et al., 2011).

Vibrio cholera

Cholera is due to contaminated food or water which triggers an acute intestinal infection by *V. cholera* (López-Gigosos et al., 2011). Enterotoxin such as cholera toxin (CT) was expressed in tobacco plant (Arakawa et al., 1998). Nochi et al. (2007), showed oral immunization with transgenic rice encoding the cholera toxin B subunit (CTB) which stimulates secretory Ig A, shows resistant to gastrointestinal digestion. Karaman et al. (2012) introduced synthetic gene encoding for CT-B by the control of a γ-zein promoter in maize seeds. CT-B levels were checked via ganglioside dependent ELISA. Anti-CTB IgG and anti-CTB IgA were found in the sera and fecal samples of the orally immunized mice protected against holotoxin challenge with CT.

Anthrax

Anthrax is a disease most commonly occur by inoculation of *B. anthracis* through the skin of infected animals, their products and inhalation of spores in dust or wool fibers. Virulence factors is a toxin complex, which consists of three proteins. The protective antigen (PA) binds the complex receptors on the macrophage surface. After proteolysis, oedema factor and lethal factor are released which after endocytosis, blocks the adenyl cyclase pathway within the cell. The main effect of this toxin complex is to increase vascular permeability, which leads to a shock. Protective antigen was expressed in transgenic tobacco chloroplasts by inserting the pag A gene into the chloroplast genome. Cytotoxicity measurements in macrophage lysis assays showed that chloroplast-derived PA was equal in potency to PA produced in *B. anthracis*. Chloroplast-derived protective antigen provides cleaner and safer anthrax plant-derived-vaccine at a lower production cost (Koya et al., 2005). Koya et al. (2005) published for the first time the PA expression in plants from stable nuclear-transgenic tobacco. Aziz et al. (2002) also reported the expression of PA in leaves of stable nuclear-transgenic tomato plants. Expression of PA in tobacco or tomato was enhanced in combination with a second *B. anthracis* protein, lethal factor (LF), and showing cytolytic activity when applied to macrophage-like cell lines. Also, when tomato leaf material was injected into mice, antisera could be recovered with neutralizing activity to anthrax lethal toxin (LT), which is a combination of PA and LF.

Porphyromonas gingivalis

Periodontal diseases are caused by oral anaerobic bacterium *Porphyromanas gingivalis*. It is thought to be initiated by the binding of *P. gingivalis* fimbrial protein to saliva coated oral surfaces. Shin et al. (2009) has successfully transferred FIM A protein producing gene into potato tuber tissues and produced native FIM A protein in edible plant cells.

Viral

Norwalk virus

Calci viruses are a major cause of food and water associated outbreaks of diarrhoea and vomiting, affecting individuals of all age groups. A capsid protein of *Norwalk virus* was expressed in transgenic tobacco and potato plants. Potato tubers expressing *Norwalk virus* antigen were fed to mice, it developed serum IgG specific for *Norwalk virus* (Mason et al., 1996). According to Tacket et al. (2000) volunteers fed with transgenic potato expressing *Norwalk virus* antigen showed seroconversion.

Hepatitis B virus

It is estimated that 3 to 6% of the world population has been infected with Hepatitis B virus (HBV) and there are 300 to 400 million carriers in the world. India alone has over 40 million carriers. In the acute stage there are signs of inflammation in the portal triads: the infiltrate is mainly lymphocytic. In the liver parenchyma, single cells show

ballooning and form acidophilic (councilman) bodies as they die. In chronic hepatitis, damage extends out from the portal tracts, giving the piecemeal necrosis appearance. Some lobular inflammation is also seen. As the disease progresses fibrosis develops and eventually, cirrhosis. Hepatitis B virus replicates in the hepatocytes, reflected in the detection of viral DNA and HBs Ag in the nucleus and HBs Ag in the cytoplasm and at the hepatocyto-membrane (Simmonds and Peutherer, 2003). Hepatitis B virus is carried in the blood and blood derived bodily fluids of infected persons and can be transferred through contact with a carrier's blood caused by unsafe injections or transfusions, sexual contact and tattooing. Long term protection against Hepatitis B virus is possible with vaccine. HBs Ag was expressed in transgenic potato plant and tested in mice for production of antibodies (Richter et al., 2000).

Pniewski et al. (2011) has shown the production of small surface antigen for HBV (S-HBsAg) in genetically modified glufosinate-resistant lettuce. They orally immunised mice by using lyophilised form of plant material and showed the presence of secretory IgA (S-IgA) and total serum antibodies. Li et al. (2011) also demonstrated the transformation of HBsAg (hepatitis B surface antigen) gene in to tomato mediated by *Agrobacterium tumifaciens*.

Lou (2007) has experimentally expressed hepatitis B virus large surface antigen in transgenic tomato plant. Transgenic lettuce plant carrying recombinant hepatitis B virus antigen HBs Ag was demonstrated in Brazil (Marcondes and Hansen, 2008). Tacket (2009) has discussed early human studies of oral transgenic plant-derived vaccines against hepatitis B virus. A phase I clinical trial with plant derived hepatitis B vaccine has boosted antigen-specific serum antibodies titer (Tacket, 2009).

Measles

Millions of people live in areas where measles are endemic and resources are scare. Measles are transmitted from person to person by respiratory droplets. Measles in an acute febrile illness, the onset is flu-like with high fever, cough and conjunctivitis, red spots with a bluish-white centre on the buccal mucosa called Koplik's spots. Measles antigens expressed in plants have been shown to be antigenic and immunogenic both after invasive and oral vaccination (Marcondes and Hansen, 2008). Crude *Quillaja* saponin extracts stimulates measles' virus specific immune responses in mice, following oral immunization with plant based measles virus haema-gglutinin protein (Pickering et al., 2006).

Webster et al. (2002) confirmed that the transgenic tobacco plants-derived MV-H protein vaccine, which when, modified to MV-H DNA vaccine, to prime-boost vaccination strategy demonstrated the MV hemagglutinin protein (MV-H) expression. Orally immunized mice with plant-derived MV-H showed MV-specific IgG.

Japanese encephalitis

JE virus is a single stranded positive sense RNA virus belonging to family flaviviridae transmitted through a zoonotic cycle between mosquitoes, pigs and water birds. It causes encephalitis all over the world especially in Eastern and South-eastern Asia. JE affects some primary organs like thalamus, corpus striatum, brainstem and spinal cord. With the absence of specific antiviral therapy, it is managed mainly by its symptom and by supportive therapies along with preventive measurements (Misra and Kalita, 2010). Transgenic rice expressing the envelope protein of *Japanese encephalitis* virus (JEV), under control of a dual cauliflower mosaic virus (CaMV 35s) promoter, was generated. JEV specific neutralizing antibody was detected in mice after immunization of mice with protein extracts from transgenic rice plant by intraperitoneal or oral immunization (Wang et al., 2009). Appaiahgari et al. (2009) showed the expression of Japanese encephalitis viral envelope protein (E) in transgenic tobacco can produce immunogenic response in mammalian system.

Influenza virus H5N1

Shoji et al. (2009) described the production of hema-gglutinin from A/Indonesia/05/05 strain of H5N1 influenza virus by transient expression in plants. The results indicate that immunization of ferrets with plant-derived hema-gglutinin elicited serum hemagglutinin-inhibiting antibodies and protected the ferrets against challenge infection with a homologous virus. Plant derived vaccine may be the solution in the rapid, large scale production of influenza vaccine in the face of pandemic.

Kalthoff et al. (2010) showed the expression of full-length recombinant hemagglutinin (rHA0) of H5N1 in *Nicotiana benthamiana* with optimize expression levels. Their results showed to provide an immunogenic protection protect chicken against lethal challenge infection with heterologous HPAIV H5N1 of 96% homology to rHA0 by plant-expressed hemagglutinin. Jul-Larsen et al. (2012) demonstrated recombinant influenza haemagglutinin antigen (HAC1) that was derived from the 2009 pandemic H1N1 virus and expressed in tobacco plants. They showed that the tobacco derived recombinant HAC1 antigen is a promising vaccine candidate recognized by both B- and T cells.

Shoji et al. (2011) showed the advantages provided by the plant system for influenza vaccine antigen production is their independence from pathogenic viruses, and cost and time efficiency. They produced large-scale of recombinant hemagglutinin proteins from A/California/04/09 (H1N1) and A/Indonesia/05/05 (H5N1) strains of influenza virus in *N. benthamiana* plants, and

tested their immunogenicity (serum hemagglutination inhibition and virus neutralizing antibodies), and safety in animal models.

Madhun et al. (2011) produced influenza subunit antigen in transient plant expression systems as an alternative. A needle-free intranasal influenza vaccine is an attractive approach to be followed. Plant-derived influenza H5N1 (A/Anhui/1/05) antigen, alone or formulated with bis-(3', 5')-cyclic dimeric guanosine monophosphate (c-di-GMP) as adjuvant induces strong mucosal and systemic humoral immune responses. Search for safe and effective adjuvant to enhance H5N1 intranasal vaccine with extracts of mushroom mycelia was found to be good (Ichinolhe et al., 2010).

LIMITATIONS

Before the commercial production of plant derived vaccines, there is urgent need to consider the following points;

1. Searching for suitable plant which will give ideal antigen expression.
2. Identification of proper dosage (whether plant parts, plant products, pill, intramuscular or intravenous injection of purified antigen) can produce proper dose.
3. Verification of allergens in the plant and plant products.
4. Study the impact of plant derived vaccines on the environment and human health.
5. Genetically altered crops producing plant derived vaccine could get mixed with human food supply or animal feed, causing potential threat to public health.
6. Cross pollination and their problems.
7. Effects on insects and soil microbes.
8. Regulation of plant derived vaccines in the form of food, drug or agricultural product.
9. Cultivation of plant derived vaccines and their delivery in capsule or pill form.

Risks of plant derived vaccines

Plant derived vaccines pose serious risks to the public if they are not handled with care. Safety of transgenic plants includes many aspects like ecology, agronomy and molecular biology which focus on food and environmental safety (Ahmad et al., 2012). Environmental issues and biodiversity concern are raised because of the transgenic seeds or plants that escape into the wild. Moreover, plant derived vaccines cannot be distinguished from non-plant derived vaccines of the same plant. Plant derived vaccine tomato plant looks like a traditional tomato. There is always a risk of mis-administration.

Although, plant derived vaccine technology can save many lives in developing countries. At the same time, there is an urgent need to address proper commercialization of plant derived vaccine technology and to prevent misuse of technology because it possesses great risk on environment and human health. Development of vaccine into a stable seed form or production in leaf is mostly favoured but its to spoilage to prevent loss/leaking out of antigen into environment is to be checked. The amount of plant which can be taken up as raw food is to be strictly monitored as over dose may cause toxic/allergic reactions. Most of the edible crops are destroyed by attack of insects and hence their effect on vaccine producing plant has to be evaluated. Even though plant derived vaccines have shown promising results but evaluation of their tolerance needs in-depth study **(**Ahmad et al., 2012).

CONCLUSION AND FUTURE PERSPECTIVE

Edible plant derived vaccine may lead to a future of safer and more effective immunization. They would overcome some of the difficulties associated with traditional vaccines like production, distribution and delivery and they can be incorporated in to the immunization plans. Edible vaccines have lot of advantages over injected vaccines like, well established cultivation, low cost of production, no need for “cold chain” delivery, rapid scale-up, simple distribution by seeds, ease of genetic manipulation, oral delivery and low health risks from human pathogen and toxin contamination, etc. Significant progress has been achieved in employing plants as vaccine expression system, for example vegetables, fruits, cereal crops, etc. Tobacco, tomato, maize, rice are leading production plateforms for recombinant protein production. The basic advantage of using plants as vaccine production system is that plants being higher eukaryotes provide opportunities for unlimited production, the range and diversity of recombinant molecules namely peptides, polypeptides and complex multimeric proteins that cannot be made in microbial systems. Plant production system provides a wider flexibility in designing of new pharmaceutical proteins. Days are not too far when we eat delicious vegetables, fruits etc, to prevent ourselves from infectious diseases. Developing and under-developed countries will be benefited more by this edible vaccine production system because the methods in production are reasonably affordable and the vaccine products would be more openly accessible to the population.

One of the most important bottlenecks in edible vaccine technology is yield improvement, as this factor has a major impact on economic feasibility. Different strategies in hand which can lead to improved production of edible vaccines include the development of novel promoters, improvement in protein stability by protein engineering approach, targeted expression of protein of interest and last but not least the improvement in downstream processsing. The potential concern of edible vaccine technology

is differential glycosylation of proteins in *in vitro* systems or in non-native species. Strategies should be devised to humanize the plant glycosylation machinery by inhibiting glycosylation enzymes. The use of plastids as vaccine production platform is quite promising to prevent transgene escape through pollens or seed dispersal and it needs an extensive research to improve expression levels and prevention of proteolysis in plastids.

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Anastomosis grouping and genetic diversity analysis of *Rhizoctonia solani* isolates causing wet root rot in chickpea

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Rhizoctonia solani **is considered as one of the most destructive soil-and-seed borne plant pathogens infecting various agricultural crops including chickpea. The 50 chickpea isolates of *R. solani* representing 10 different states of India were variable in hyphal anastomosis reactions and they were grouped into seven AGs as AG1, AG2-2, AG2-2LP, AG2-3, AG3, AG4 and AG5. Genetic diversity of the pathogen was determined by using molecular markers namely, simple sequence repeats (SSR) and rDNA internal transcribed spacer (ITS). A neighbor-joining tree constructed based on the profiles generated by SSR markers grouped the isolates into eight categories. This revealed 90% of genetic similarity among the isolates and partial correlation with reference to their geographical origin and AGs. The isolates were amplified with a set of primers ITS 1 and ITS 4 and they produced a specific band ≈650 bp. Low level of (7%) variability was observed in the nucleotide sequences of the ITS regions of these isolates. The phylogenic tree generated from bootstrap neighboring joint analysis grouped the Indian populations of *R. solani* into two categories.**

Key words: Chickpea**,** wet root rot, genetic diversity, simple sequence repeats (SSR), internal transcribed spacer (ITS).

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is an important winter legume crop of India and cultivated on an area of 8.21 m ha with an average annual production of 7.48 m tones along with productivity of 911 kg ha^{-1} (Anonymous, 2011). Its production and productivity is affected by numerous diseases. Among the diseases, wet root rot (WRR) caused by *Rhizoctonia solani* Kühn (Teleomorph: *Thanatephorus cucumeris* (Frank) Donk. is the most destructive in nature (Dubey et al., 2012). The disease is most commonly observed at early in the season when the soil moisture content is often high. Characteristic symptoms include root rotting, often originating at the distal tip of the young root and gradual yellowing and wilting of foliage (Dubey and Dwivedi, 2000).

R. solani affects wide range of food crops because of its polyphagous nature and high saprophytic ability (Nelson et al., 1996). Generally, the fungi have been identify and classified mainly on the basis of characteristics of their sexual and asexual methods of reproduction (Hibbett et al., 2007); but, identification, grouping and taxonomy of *R. solani* was always challenging because of hetero-geneous group of filamentous fungi that share similarities

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Abbreviations: WRR, Wet root rot; **AGs,** anastomosis groups; **RAPD,** randomly amplified polymorphic DNA; **ISSR,** inter-simple sequence repeats; **SSR,** simple sequence repeats; **ITS,** internal transcribed spacer; **PDA,** potato dextrose agar; **URPs,** universal rice primers.

in their anamorphic,sterile state. They do not produce asexual spores and the teleomorphic (sexual state) occurs only rarely in nature. Earlier studies on researches studied about pathogen variability were based on morphology and pathogenicity on various plant species to classify *Rhizoctonia* spp (Sneh et al., 1991). Hyphal anastomosis concept was introduced by Parmeter (1970) for identification and characterization of *Rhizoctonia* isolates. This method implies that genetically similar isolates of *Rhizoctonia* recognize and fused with each other, whereas genetically dissimilar isolates do not fuse (Carling, 1996). Ogoshi (1987), classified *R. solani* primarily based on anastomosis behaviour; at present, 14 anastomosis groups (AGs) are recognised (Carling et al*.,* 2002).

Different molecular markers are being used by researchers for fingerprinting, genetic diversity and taxonomy of plant pathogens. Earlier, many potential molecular markers as randomly amplified polymorphic DNA (RAPD) (Dubey et al., 2012), inter-simple sequence repeats (ISSR) (Sharma et al., 2005; Dubey et al., 2012), simple sequence repeats (SSR) (Mwang' ombe et al., 2007; Dubey et al., 2012) and internal transcribed spacer (ITS) (Godoy-lutz et al., 2008; Pannecoucque and Hofte, 2008) were used for addressing genetic diversity of *R. solani*. Several informative regions are present in the fungal genome sequence for molecular level detection and taxonomy (Rakeman et al., 2005).

Among the regions, ribosomal DNA is the most conserved site in the all eukaryotic genome, with high variability at the species and sub species level. The ITS regions are non-coding sequences interspaced among highly conserved fungal rDNA and have been shown to have a high heterogeneity among different fungal genera and species (Iwen et al., 2002). Sharon et al. (2008) used rDNA-ITS sequence analysis for identification and classification of *Rhizoctonia* spp. Several other workers also used ITS regions as a target in molecular-based assays for the characterization and identification of *R. solani* (Salazar et al., 1999; Pannecoucque and Hofte, 2008).

So far, no attempt has been made for AG grouping, molecular diversity analysis and ITS region sequencing of *R. solani* causing wet root rot in chickpea. Keeping these points in view, the present study was aimed to find out the anastomosis grouping and genetic diversity of *R. solani* associated with chickpea of India. In the present study, first time microsatellite markers have been designed from the whole genome sequence of *R. solani* available in the National Center for Biotechnology Information (NCBI) database genbank and used for genetic diversity analysis.

MATERIALS AND METHODS

Fungal cultures

Fifty (50) isolates of *R. solani* representing major chickpea growing areas of India were collected from the Pulse laboratory, Division of Plant Pathology, IARI, New Delhi (Table 1) for the present study. The isolates were purified by single hyphal tip culture on 1.5% water agar and were transferred to potato dextrose agar (PDA) medium (Himedia, India). Pure cultures of different isolates of *R. solani* were maintained at 25±1°C on PDA slants for further studies.

Identification of the anastomosis group

The identification of the anastomosis group of each *R. solani* isolate was carried out on sterilized glass slides coated with 2% water agar medium placed on Petri dishes and mycelium of an AG tester and an unknown isolate were placed on either side of a slide. After 24 h of incubation at 25±1°C, the slide was removed and mycelium was stained with lactophenol cotton blue solution. Sites of hyphal interaction were observed under optical microscope (100X, Olympus BX41 TF, Japan) and occurrence of anastomosis was determined when hyphae were fused and exchanged cytoplasm. The identification of AGs was performed twice. Anastomosis reactions were grouped into four categories namely, C0: No reaction (different AG); C1: hyphae contact only (same/different AG); C2: killing reaction which represents a vegetative incompatibility response between genetically different individuals (same AG) and C3: perfect fusion (same AG) of vegetative hyphal cells between two isolates that suggestive of genetically similar (Carling, 1996). The tester isolates for AG1 (BBA 62990), AG2-2 (BBA 69670), AG2-2LP (BBA 71917), AG2-3 (BBA 71921), AG3 (BBA 63008), AG4 (63002) and AG5 (BBA 62999) obtained from culture collection of JK Institute (BBA), Germany were used.

Extraction of DNA

For DNA extraction, mycelial cultures of the isolates of *R. solani* and pathogenic fungi of chickpea used in the present study were grown in potato dextrose broth (Himedia, India) for 5 days in incubator shaker (120 rpm) at 25±1°C. Mycelium was harvested and DNA was extracted according to standard protocols (Murray and Thompson, 1980). The mycelium (1 g) was collected with a pre-cooled mortar and pestle and mixed with pre-warmed (65°C) 2% cetyl trimethyl ammonium bromide (CTAB) DNA extraction buffer. The tubes were incubated in a water bath at 65°C for 1 h with gentle shaking at every 10 min intervals. After incubation and cooling at room temperature, an equal volume of phenol/ chloroform/isoamyl alcohol (25:24:1) was added and mixed gently to denature proteins and centrifuged at 12000 rpm at room temperature for 20 min.

The aqueous phase was transferred to a new sterile tube and equal volume of chloroform/isoamyl alcohol (24:1 v/v) was added and mixed gently and centrifuged at 10,000 rpm for 10 min. The aqueous phase was transferred to a new sterile tube and last step was repeated once again to get pure DNA. The aqueous phase was transferred to a new tube and DNA was precipitated with 0.6 volume of ice cold isopropanol and 0.1 volume of 3 mol/L sodium acetate and allowed to precipitate at -20°C for 3 to 4 h, followed by centrifuging at 10,000 rpm for 10 min at 4°C. The supernatant was discarded and the pellet was washed twice with 70% ethanol and dried at either room temperature or 37°C. The DNA pellet was resuspended in 100-200 µl TE buffer and stored at -20°C for further use.

Simple sequence repeats-polymerase chain reaction (SSR-PCR) analysis

The SSR primers were designed using Primer 3 (v. 0.4.0) software (Rozen and Skaletsky, 2000) from the whole genome sequence available at NCBI Genbank database of *R. solani*. Sixteen SSR markers (microsatellite) were synthesized from Sigma, Bangalore, out of which nine primers were selected for further use based on

Table 1. Different isolates of *R. solani* isolated from the roots of chickpea collected from different parts of India and their anastomosis groups.

S/N	State	District	Accession No	AG Group
1	Tamil Nadu	Coimbatore	RTNG 4	AG 5
2		Thiruppur	RTNG 5	Unknown
3		Coimbatore	RTNG 6	Unknown
4		Dharmapuri	RTNG 7	AG 1
5		Dharmapuri	RTNG 8	AG 1
6	Karnataka	Dharwad	RKNG 9	AG 1
7		Bangaluru	RKNG 10	AG 2-2 LP
8		Bangaluru	RKNG 11	AG 4
9	Andhra Pradesh	Kurnool	RAPG 9	AG 2-2
10		Kurnool	RAPG 11	AG 3
11		Kurnool	RAPG 13	AG 2-3
12		Kurnool	RAPG 14	AG 2-3
13		Hyderabad	RAPG 15	AG 4
14		Hyderabad	RAPG 16	AG 2-2 LP
15	Maharashtra	Pune	RMHG 23	AG 3
16		Pune	RMHG 24	AG 1
17		Pune	RMHG 25	AG 4
18		Ahmadnagar	RMHG 28	AG 5
19		Jalgaon	RMHG 31	AG 1
20		Jalgaon	RMHG 32	AG 3
21		Jalgaon	RMHG 33	AG 3
22		Jalgaon	RMHG 35	Unknown
23	Rajasthan	Sriganganagar	RRJG 1	AG 5
24		Sriganganagar	RRJG 3	Unknown
25		Hanumangarh	RRJG 4	AG 2-3
26	Gujarat	Ahmedabad	RGJG 1	AG 3
27		Ahmedabad	RGJG 2	AG 5
28		Ahmedabad	RGJG 4	Unknown
29		Kheda	RGJG 5	AG 3
30		Dahod	RGJG 6	AG 5
31		Dahod	RGJG 7	AG 5
32	Uttar Pradesh	Mirzapur	RUPG 96	Unknown
33		Mirzapur	RUPG 97	AG 4
34		Sonebhadra	RUPG 98	AG 3
35		Sonebhadra	RUPG 99	AG 2-3
36		Sonebhadra	RUPG 100	AG 3
37		Jhansi	RUPG 103	AG 5
38		Jhansi	RUPG 106	AG 3
39		Jhansi	RUPG 107	AG 2-2
40	Madhya Pradesh	Damoh	RMPG 28	AG 2-3
41		Chattarpur	RMPG 31	AG 5
42	Haryana	Mahendragarh	RHRG 5	AG 2-3
43		Mahendragarh	RHRG 7	AG 3
44		Bhiwani	RHRG 8	AG 5
45		Bhiwani	RHRG 9	AG 2-2 LP
46		Bhiwani	RHRG 11	AG 3
47		Bhiwani	RHRG 13	AG 5
48		Bhiwani	RHRG 14	AG 3
49		Bhiwani	RHRG 15	AG 3
50	Delhi	New Delhi	RDLG 3	AG 3

Table 2. Primer sequence, number of polymorphic bands, percentage of polymorphism and range of amplicons size obtained from SSR markers.

Primer	Sequence (5' -3')	Annealing temperature (°C)	Total bands (No)	Polymorphism (%)	Range of amplicons size (kb)
SSR G1 F & R	CAAGTCGATGCAGCAAATGT CCGAGAGTGGGATCGAGTT	59	3	100	0.1-1.0
SSR G2 F & R	CAGCGGGGCCTAAAAATAAT GGGCAAGCAAAGTAGTCTCG	58	4	100	0.2-1.0
SSR G3 F & R	CATCCTTTGCAGAGTTGCTG AGAGCACGAACACCTGGACT	58	4	100	0.2-1.0
SSR G4 F & R	CGCATTTTCGCTTTCTTGAT AGTGGCGGATATTACCGAGA	59	5	100	0.5-1.0
SSR G5 F & R	ACAAGGCGCAATGACAAGAT ATTGTCGCACCGCTTCTTAC	62	4	100	0.2-1.0
SSR G6 F & R	TGGGACATCAAACTATGCTCTC TACGCGCAAAGTTGTTGTTC	58	3	100	0.5-1.0
SSR G8 F & R	CATCCTTTGCAGAGTTGCTG ACGAACACCTGGACTTACCG	58	3	100	0.1-1.0
SSR G9 F & R	CTGTACTCGGACGCAAACTG CGCGAACTAATAGGCATGGT	62	4	100	0.2-1.0
SSR G10 F & R	CTCACCAAGAGTCCGAAAGC TCTATGTGCGCGTAACAGGA	58	4	100	0.2-1.0
Total			34	100	

good amplification products (Table 2). PCR was performed in a total volume of 25 µl reaction mixture containing 50 ng template DNA, 1.5 U Taq DNA polymerase (Bangalore Genei, India), 1.5 mM of $MgCl_2$, 0.2 mM of each dNTPs (Bangalore Genei, India), and 15pmol of primer in 10× reaction buffer. Amplification was performed as follows: Initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 5 min, annealing at 58 - 62°C for2 min (depending on the primer used), and extension at 72°C for 2 min and a final step at 72°C for 5 min. The amplified products were analyzed by electrophoresis in a 1.0% agarose horizontal gel in 1x TAE buffer. A 100 bp ladder (Bangalore Genei, India) was used as a marker. Gels were stained with ethidium bromide (1 µg/ml) and observed under UV light in gel documentation system (Bio-Rad™, USA).

Data analysis

DNA fingerprint data generated by SSR primers were converted into a binary matrix. The presence (1) and absence (0) of each DNA band of a specific molecular weight was recorded for each gel. A neighbor- joining tree was constructed based on the simple matching dissimilarity matrix of a nine SSR markers and 50 isolates of *R. solani* were genotyped in chickpea by using DARwin 5.0.156 program (Perrier et al., 2003).

ITS amplification and sequencing

The ITS1, ITS2 and 5.8S rDNA of 50 isolates of *R. solani* was amplified with a set of markers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) as described by White et al. (1990). Amplification were done in a 25 µL reaction mixture containing 25 ng template DNA, 1.5 U Taq DNA polymerase (Bangalore Genei, India), 1.5 mM of $MgCl_2$, 0.2 mM of each dNTPs (Bangalore Genei, India), and 5 pmol of each primer in 10x reaction buffer. PCR was performed by using an Eppendorf gradient thermal cycler at 94°C for 5 min for initial denaturation followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min with a final elongation at 72°C for 5 min. Amplification products were analyzed by electrophoresis in a 1.0% agarose gel in 1x TAE buffer. Gels were stained with ethidium bromide (1 µg/ml) and observed under UV light of Bio Rad™ gel documentation system. A 1 kb (Bangalore Genei, India) ladder was used as a marker.

The amplified ITS fragments (≈650bp) of 12 isolates (RKNG9 AG 1, RAPG11 AG 3, RKNG10 AG 2-2L, RAPG14 AG 2-3, RAPG9 AG 2-2, RMHG23 AG 3, RKNG11 AG 4, RTNG4 AG 5, RUPG107 AG 2-2, RAPG13 AG 2-3, RAPG15 AG 4 and RTNG7 AG 1) representing different AG groups and area of origin were eluted and purified using Qiagen gel extraction and purification kit (Qiagen, USA) as per manufacturers instructions. The eluted and purified DNA samples were sequenced by Bangalore Genei, India. The nucleotide sequences were subjected to Basic Alignment Search Tool (BLAST) analysis (http://www. Ncbi.nih.gov/index.html). Sequences were submitted to *GenBank* at NCBI and accession numbers were obtained. The multiple sequence and pairwise alignments were made using CLUSTAL W BioEdit version 7.0.5 (Hall, 1999). A neighbour joining phylogenetic tree was reconstructed using the MEGA 5.1 software with 1000 bootstrap replicates.

RESULTS

Anastomosis groups

Fifty (50) chickpea isolates of *R. solani* collected from different states of India were characterized into seven

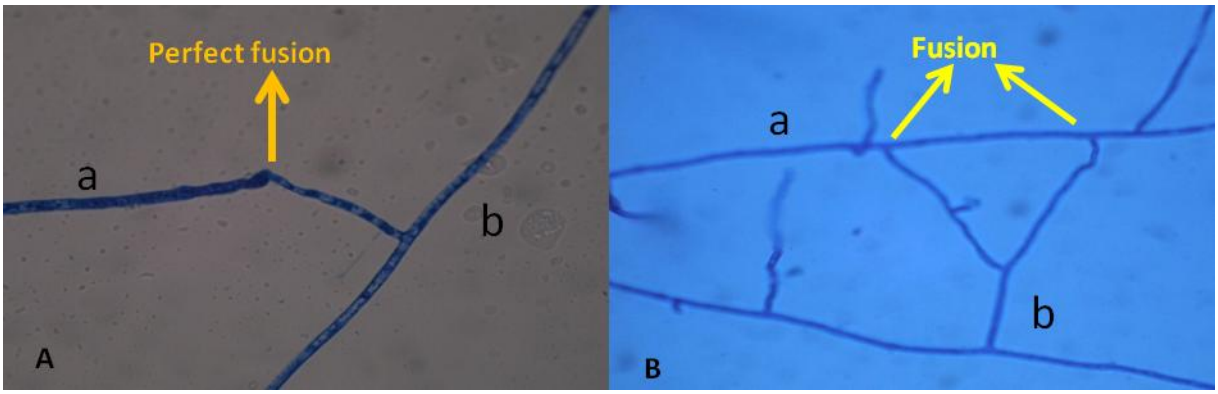

Figure 1. The hyphae of *R. solani* showed perfect fusion between (A) tester of AG1 (a) and RKNG 9 (b) and fusion between (B) tester of AG3 (a) and RHRG7.

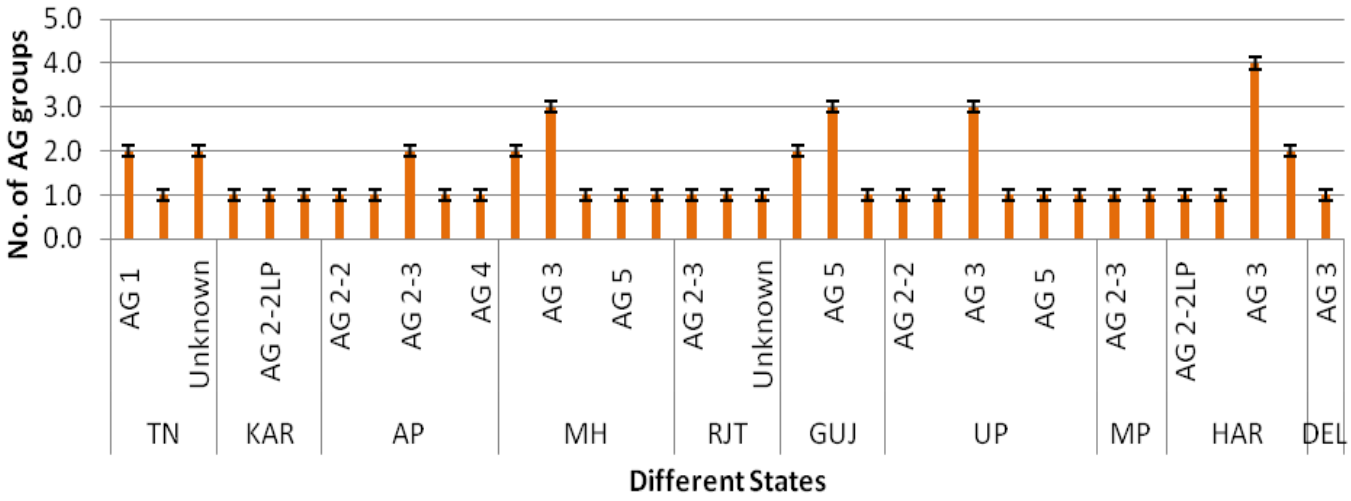

Figure 2. Distribution of different AGs of chickpea isolates of *R. solani* in different states of India (TN- Tamilndu, KAR- Karnataka, AP- Andhra Pradesh, MH- Maharashtra, RJT- Rajasthan, GUJ- Gujarat, UP-Uttar Pradesh, MP- Madhya Pradesh, HAR- Haryana and DEL- Delhi).

anastomosis groups namely, AG1, AG2-2, AG2-2LP, AG2-3, AG3, AG4 and AG5 with the help of international testers (Table 1, Figure 1). Out of 50 isolates, majority of the isolates belonging to AG3 (14 isolates) followed by AG5 (10 isolates), AG2-3 (6 isolates), AG1 (5 isolates), AG4 (4 isolates), AG2-2LP (3 isolates), AG2-2 (2 isolates) and for remaining 6 isolates did not determine AG. Each state of India had different AGs of the pathogen. Uttar Pradesh, Andhra Pradesh, Haryana and Madhya Pradesh had maximum number of AGs, whereas only one AG (AG 3) was present in Delhi (Figure 2).

SSR analysis

Nine SSR primers used for amplification of DNA of 50 isolates of *R. solani* produced reproducible and scorable bands ranging from 3 to 5 in numbers with 0.1-1 kb size. A total 34 bands were obtained and all bands (100%) were polymorphic (Table 2). The fingerprint generated from markers SSRG2 F& R to SSRG10 F& R are given Figure 3.

The phylogenetic neighbour joining tree constructed using Darwin 5 (Version 5.0.156) software clearly demonstrated that the isolates were grouped into 8 distinct clusters at 90% of similarity (Figure 4). First cluster had five isolates from Gujarat namely, RGJG2 (AG5), RGJG4 (unknown AG), RGJG5 (AG3), RGJG6 (AG5) and RGJG7 (AG5) and one isolate from Delhi RDLG3 (AG3). Second cluster consisted of 8 isolates from three different states namely, Tamil Nadu (4 isolates; RTNG 5 (unknown AG), RTNG 6 (unknown AG), RTNG7 (AG1), RTNG8 (AG1), Maharashtra (2 isolates; RMHG23 (AG3), RMHG24 (AG1)) and Andhra Pradesh

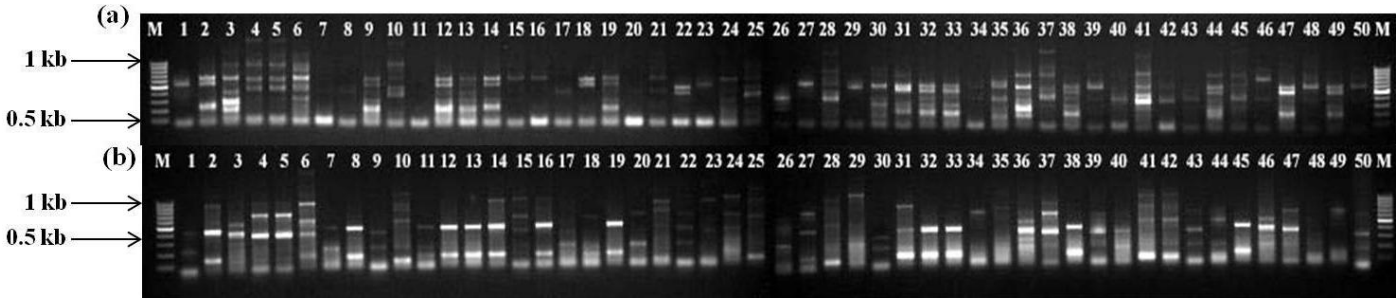

Figure 3. Fingerprint patterns for 50 chickpea isolates of *R. solani* generated with SSR G2 F & R primer (a) and SSR G10 F & R (b). Lanes 1-5, AG 1; 6-7, AG 2-2; 8-10, AG 2-2LP; 11-16, AG 2-3; 17-30, AG 3; 31-34, AG 4; 35-44, AG 5; AG 45-50, undetermined AG; M, 100 bp ladder.

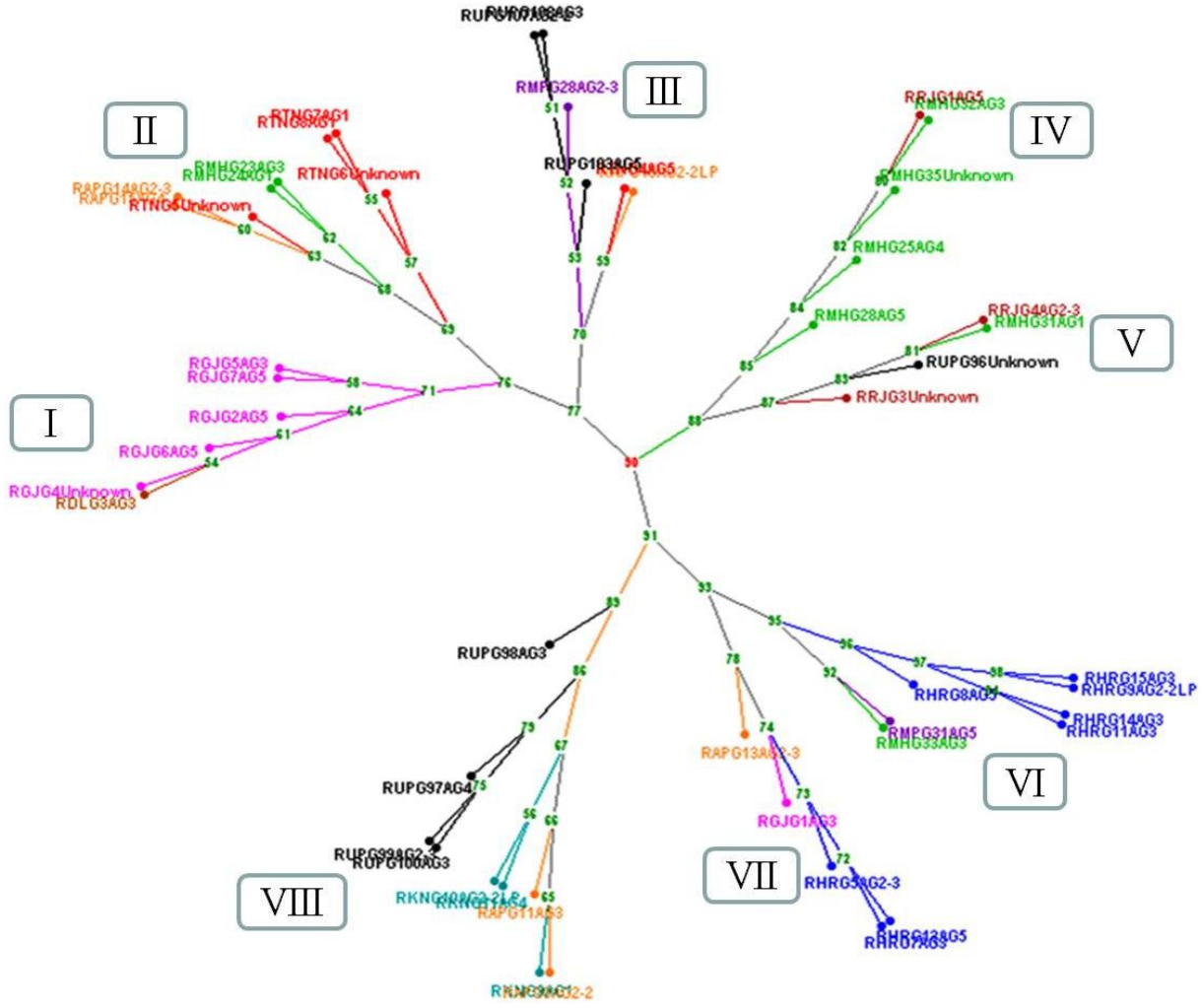

Figure 4. Neighbor- joining tree illustrating the clustering of 50 isolates of *R. solani* isolated from chickpea based on fingerprint patterns using SSR primers.

(2 isolate; RAPG14 (AG2-3), RAPG15 (AG4)). Three isolates namely, RUPG103 (AG5), RUPG106 (AG2-2) and RUPG107 (AG2-2) from Uttar Pradesh and each one isolate from Madhya Pradesh (RMPG28 AG2-3) and Andhra Pradesh RAPG16 (AG2-2LP) placed into third cluster. Four isolates from Maharashtra namely, RMHG

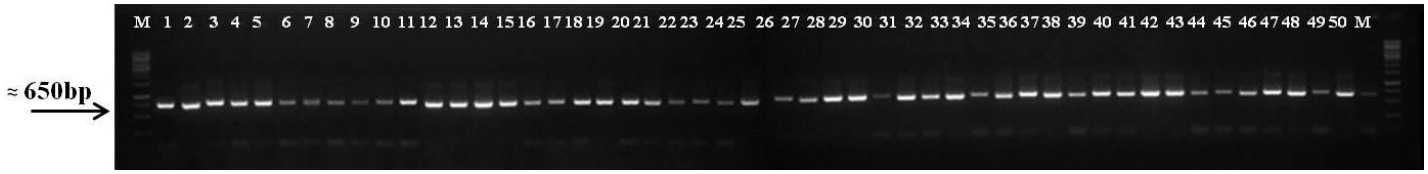

Figure 5. Agarose gel showing PCR products amplified ≈650 bp using ITS1 and ITS 4 primer for 50 chickpea isolates *R. solani*. Lanes 1-5, AG1; 6-7, AG2-2; 8-10, AG2-2LP; 11-16, AG2-3; 17-30, AG3; 31-34, AG4; 35-44, AG5; 45-50, undetermined AG and M-1Kb ladder.

Table 3. Genbank accession numbers of *Rhizoctonia solani* with type of AG and length of nucleotide sequence.

Genbank Accession No.	Accession number of isolates with type of AG	Total no of base pairs (bp)	Geographic origin
JX454669	RKNG9, AG 1	572	Dharwad, India
JX454670	RAPG11, AG 3	636	Kurnool,India
JX454671	RKNG10, AG 2-2L	632	Bengaluru, India
JX454672	RAPG14, AG 2-3	633	Kurnool, India
JX454673	RAPG9, AG 2-2	636	Kurnool, India
JX454674	RMHG23, AG 3	659	Pune, India
JX454675	RKNG11, AG 4	627	Bengaluru, India
JX454676	RTNG4, AG 5	676	Coimbatore, India
JX454677	RUPG107, AG 2-2	715	Jhansi, India
JX454678	RAPG13, AG 2-3	683	Kurnool, India
JX454679	RAPG15, AG 4	638	Hyderabad, India
JX454680	RTNG7, AG 1	646	Dharmapuri, India

25 (AG4), RMHG28 (AG5), RMHG31 (AG1) and RMHG35 (unknown AG), one isolate from Rajasthan namely, RRJG1 (AG5) grouped into fourth cluster. The isolate RRJG3 (unknown AG), RRJG4 (AG2-3) from Rajasthan and one each isolate from Maharashtra (RMHG31 - AG1) and Uttar Pradesh (RUPG96 - unknown AG) placed into fifth cluster. Out of 7 isolates of sixth cluster, 5 isolates namely, RHRG9 (AG2-2LP), RHRG8 (AG5), RHRG11 (AG3), RHRG14 (AG3) and RHRG15 (AG3) from Haryana and one isolate each from Madhya Pradesh (RMPG31-AG5) and Maharashtra (RMHG33-AG3). Out of five isolates in seventh cluster, 3 isolates (RHRG5-AG2-3, RHRG7-AG3 and RHRG13-AG5) were from Haryana, one isolate (RAPG13-AG2-3) from Andhra Pradesh and one isolate (RGJG1-AG3) from Gujarat. Four isolates from Uttar Pradesh namely, RUPG97 (AG4), RUPG98 (AG3), RUPG99 (AG2-3) and RUPG100 (AG3) placed into eighth cluster. Three isolates (RKNG9-AG1, RKNG10-AG2-2LP and RKNG11-AG4) from Karnataka and also 2 isolates (RAPG11-AG4, RAPG9-AG2-2) from Andhra Pradesh were also included into eighth cluster.

ITS amplification and sequencing

All the isolates of *R. solani* produced ≈650 bp amplicons during amplification with ITS1 and ITS4 primers (Figure 5). The nucleotide sequences of ITS I, 5.8s rDNA and ITS II regions of 12 isolates representing different AGs of the pathogen were varied from 572-715 bp. The sequences of the isolates were deposited at the NCBI GenBank nucleotide database (Table 3). It was largest (715 bp) in RUPG107 (AG2-2) (Uttar Pradesh) isolate whereas, smallest (572 bp) in RKNG9 (AG1) (Karnataka) isolate. The phylogeny tree constructed from bootstrap neighboring joint analysis of nucleotide sequences of these isolates grouped them into 2 clusters. Eight isolates, namely, RKNG9 (AG1), RKNG11 (AG4), RKNG10 (AG2-2LP), RAPG14 (AG2-3), RAPG11 (AG3), RAPG9 (AG2-2), RTNG4 (AG5) and RMHG23 (AG3) were grouped in one cluster and the rest 4 isolates, namely, RUPG107 (AG2-2), RAPG15 (AG4), RAPG13 (AG2-3) and RTNG7 (AG1) were placed in to second cluster (Figure 6).

The phylogeny tree constructed from the nucleotide sequence similarity of these 12 isolates along with 17 other ITS sequences of *R. solani* which showed 95-100% sequence similarity during BLAST analysis grouped them into two major clusters (Figure 7). Three Indian isolates included in the present study namely, RAPG15 (AG4), RAPG13 (AG2-3) and RTNG7 (AG1) grouped separately in one cluster whereas, the rest of 9 Indian isolates, namely, RKNG9 (AG1), RKNG11 (AG4), RKNG10 (AG2-

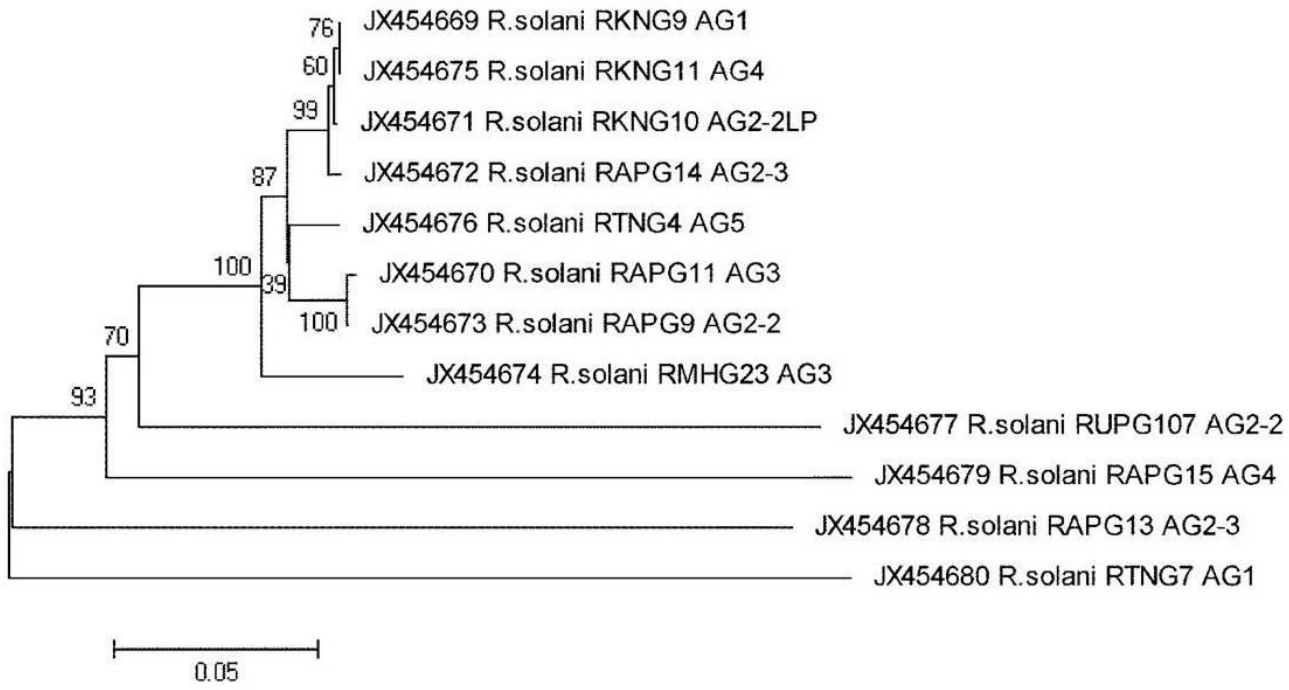

Figure 6. Neighboring joint tree showing the phylogenetic relationship among the 12 chickpea isolates of *R. solani* based on their ITS sequences.

2LP), RAPG14 (AG2-3), RAPG11 (AG3), RAPG9 (AG2-2), RTNG4 (AG5) and RMHG23 (AG3) and RUPG107 (AG2-2) were grouped into major cluster along with 17 other ITS sequences of *R. solani* taken from NCBI GenBank database.

DISCUSSION

The 50 isolates of *R. solani* causing WRR in chickpea collected from 10 different states of India were characterized and grouped into 7 AGs namely, AG1, AG2-2, AG2-2LP, AG2-3, AG3, AG4 and AG5. The distribution pattern of AGs in different states of India is random and the presence of number of AGs varied in different states. This random distribution may be because of pulse crops grow in India under different agro-ecological conditions and cropping patterns. Interestingly, the numbers of AGs were more in the states where the diversity of pulse crops cultivation is more.

The present findings are in accor-dance with the observation of Dubey et al. (2012). They reported that 90 isolates of *R. solani* representing 7 AGs isolated from pulse crops of 16 agro-ecological regions of India, showing variable atmospheric temperature and relative humidity and low to medium levels of soil organic matter and nutrients. Different types of AGs were ran-domly distributed in different states of India because the geographical distribution of AGs of *R. solani* has been associated with such factors as soil type (Parmeter et al., 1969), altitude (Galindo et al., 1983), and cropping pattern (Ogoshi and Ui, 1983). Each state of India had variable soil types and cropping pattern. AGs 1, 2, 3, 4 and 5 are the biggest pathogen groups, which infect mostly rice, soybean, potato, cotton and wheat, respectively (Watanabe and Matsuda, 1966; Carling, 1996). Many subgroups have been identified in AGs 1, 2, 3, 4, 6, 8 and 9 (Carling et al., 2002; Godoy-lutz et al., 2008) and their members can have different hosts or climatic conditions. In the present study, the anastomosis subgroups AG2-2, AG2-2LP, AG2-3 in *R. solani* were reported.

In India, different kinds of cropping patterns (around 150) are based on major crops such as, rice, other cereals, maize, sorghum, pearl millet, groundnut, cotton, wheat, chickpea and commercial crops like sugar-cane, tobacco, potato, tea, coffee (Anonymous, 2012). The variable cropping pattern may be the reason for the presence of different AGs of *R. solani* in chickpea. Sometimes, during cropping pattern, first crop infected by *R. solani* represent any AGs, which may infect subsequent crops (You et al., 2008). The wind-borne basidiospores of some *R. solani* AGs may serve as a primary inoculum for root rot and foliar diseases of various cultivated plant species under favorable environmental conditions (Naito, 1996). Therefore, basidiospore dispersal, immigration, spread of sclerotia and mycelium during agricultural practices may leads to the temporal and spatial distribution of *R. solani* AGs in natural populations.

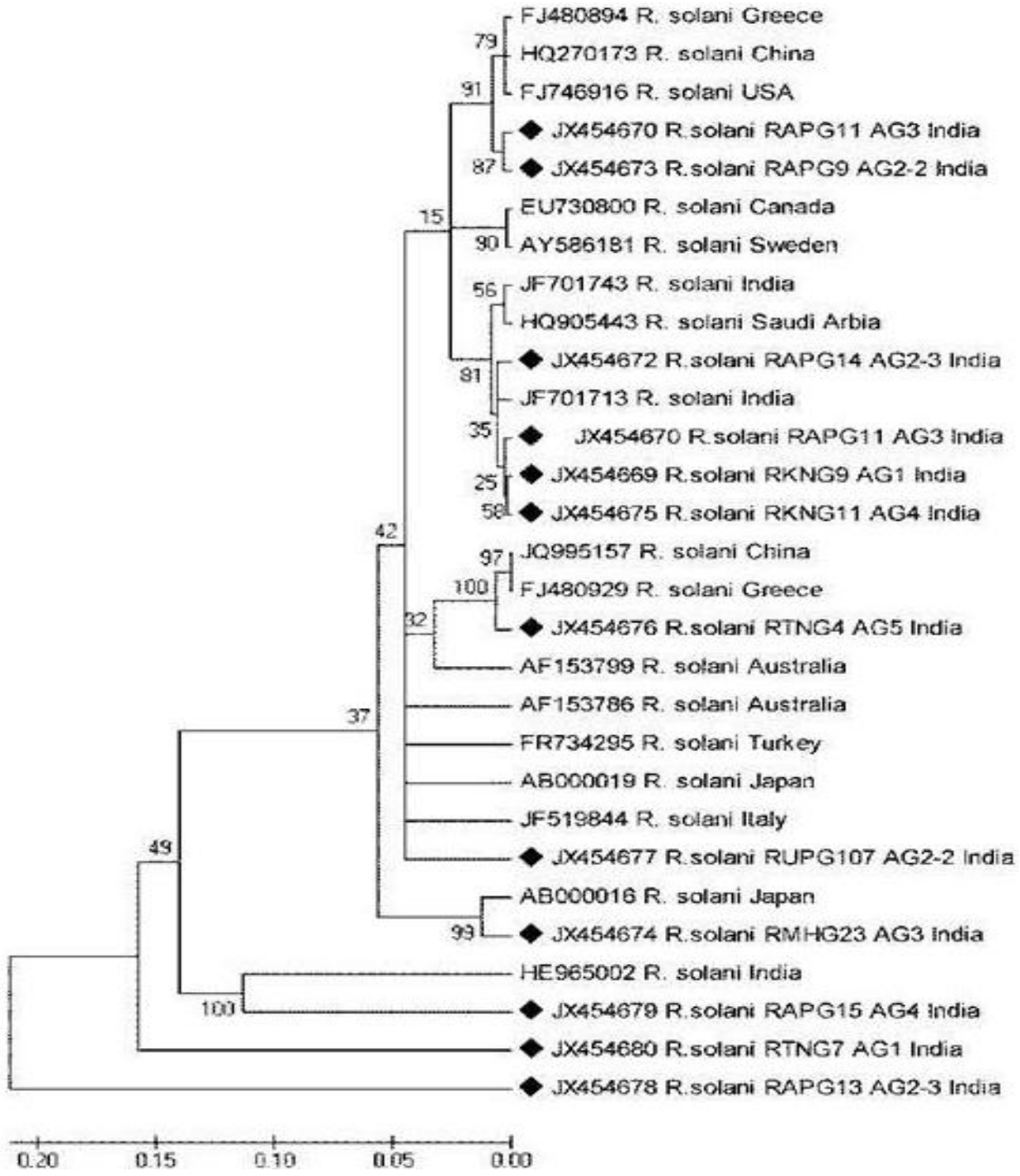

Figure 7. Neighboring joint tree showing the phylogenetic relationship among the chickpea isolates of *R. solani* based on their ITS sequences. The sequences generated in present study were labelled with diamond (◆).

DNA markers such as SSR and ITS sequence analyses performed on genomic DNA of 50 isolates of *R. solani* revealed the presence of considerable level of genetic relatedness. The phylogenetic neighbor joining tree, which was constructed based on the genetic distance matrix, showed genetic similarity among the 50 isolates ranging from 51-90% as well as the majority of the isolates showed more than 80% genetic similarity during analysis of microsatellite markers. The high degree of genetic similarity among the Indian population of *R. solani* may be evolved from a common ancestor. After that, some degree of variation observed among the *R. solani* isolates that may be due to mutation, migration mating compatibility and rarely sexual hybridization may

provide an opportunity for developing genetic variability in a population even if the primary mode of reproduction is asexual (Mcdonald et al., 1995; Cubeta et al., 1993).

The SSR analysis was also indicated that *R. solani* isolate groups partially associated with geographical origin. For instance, first group consists of 5 isolates belonging to Gujarat and fourth group had 4 isolates belonging to Maharashtra. Earlier, Sharma et al. (2005) also reported that *R. solani* isolates collected from same hosts and same geographical regions showed similarity in molecular DNA fingerprint analysis barring few exceptions. Some groups had isolates from neighboring states, for example third group had isolates from Uttar Pradesh (three isolates), Madhya Pradesh (one isolate) and Andhra Pradesh (one isolate). Rajasthan (2 isolates) and Uttar Pradesh (one isolate) as well as Maharashtra (one isolate) belonged to the fifth group.

The present results did not correlate with AGs but partially corresponded to geographical origin. These results are in accordance with Yang et al. (1996) who have observed significant varia-tions within *R. solani* AG-9 anastomosis group based on RAPD markers. Genetic variation was more in the isolates obtained from different geographical regions (Duncan et al., 1993). The isolates originating from different geographical areas belonged to second (Haryana, Delhi and Gujarat) and third (Madhya Pradesh, Uttar Pradesh, Maharashtra and Tamil Nadu) groups.

Dubey et al. (2012) also used various molecular markers such as universal rice primers (URPs), RAPD and ISSR for study of diversity of *R. solani* isolates infecting various pulse crops from 16 different agro-ecological region of India. They reported that the isolates did not correspond to their region of origin, and AGs. Mwang' ombe et al. (2007) used SSR primer to analyze diversity among the Kenyan isolates of *R. solani* from common bean. They also reported that there was no relationship between molecular groups and geographical origin of the isolates. The molecular analysis was useful in assessing the intra and inter species specific diversity. The clusters formed in the present study partially correspond to their geographical origin. So this study helps in the development of area specific markers and identification of the pathogen. In future it will be useful for integrated disease management and to understand the evolution of WRR pathogen.

The ITS sequences of *R. solani* obtained from representing of AGs showed 93 - 100% of sequence homology. Further, the sequences of 12 isolates included in the present study showed 49 to 100% sequence similarity with the isolates of *R. solani*. The phylogenetic analysis clearly indicated that some of the Indian isolates of *R. solani* had variable ITS sequences, therefore, grouped separately in one major cluster although they were from different AGs and place of origin. The present results also in agreement with Kuninga et al. (1997). They found that 5.8s rDNA sequence was completely conserved across all the AGs examined, whereas the ITS rDNA sequence was found to be highly variable among 45 isolates of *R. solani*. The sequence homology in the ITS regions was above 96% for the isolates of the same subgroup, 66 - 100% for the isolates of different subgroups within an AG, and 55 - 96% for isolates of different AGs. Boysen et al. (1996) also observed sequence variations in ITS region of 9 *R. solani* isolates of AG4. The present study highlights the genetic variation of the pathogen at the species level, especially ITS region was very much useful in intra specific diversity of the pathogen and helps in development of species level diagnostic molecular markers. The present findings clearly indicated that the chickpea populations of *R. solani* are highly variable in respect of AGs and genetic levels, but they are less variable in respect of ITS region.

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Full Length Research Paper

Studies on morpho-physiological characters of different *Avena* species under stress conditions

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Seven species of oat (*Avena*) were evaluated for their relative drought tolerance under soil moisture stress. The plant height, leaf area production and biomass yield reduced under soil moisture stress. Among the species tested, minimum reduction in height was recorded in *Avena vaviloviana*, *Avena abyssinica* and *Avena sterilis* at vegetative and flowering stages. Significant decrease in leaf area production was recorded at vegetative stage, whereas at flowering stage, the decrease in leaf area production was marginal in *A. sterilis* followed by *A. abyssinica* predicting their more adaption to stress environment. The increase in specific leaf weight (SLW) of all the species of *Avena* showed increase in leaf thickness, exhibiting high water retention capacity under soil moisture stress condition which is a requisite trait for drought tolerance. Soil moisture stress imposed at vegetative and flowering stages reduced fresh biomass yield in all the species. Minimum reduction in dry biomass accumulation under stress environment at vegetative stage was recorded in *A. sterilis* followed by *A. strigosa* and *A. sativa*, exhibiting their tolerance to drought at early stages of growth. However, at flowering stage, minimum decrease in dry biomass production was recorded in *A. sterilis* (3.47%) followed by *A. marocana* (12.56%) indicating their relative drought tolerance at flowering stage of crop growth. A significant positive correlation between total leaf area and dry biomass (r^2=0.738) under stress environment indicates that dry biomass accumulation was governed by total leaf area production. *A. sterilis* accumulated maximum fresh and dry biomass under soil moisture stress with minimum reduction over the non stress environment, indicating its drought tolerance potential as compared to other genotypes tested.

Key words: *Avena,* biomass, flowering stage, leaf area, soil moisture stress, vegetative stage.

INTRODUCTION

Water is expected to be one of the most critical inputs to maintain the agricultural production in the twenty-first century. The identification of crop species and varieties capable to optimize productivity under stress environment will get more and more attention in the present and future research. Drought stress is the major causes for crop loss worldwide, reducing average yields with 50% and above (Wang et al., 2003). A number of physiological and biochemical attributes have been employed to screen crop plants for their drought tolerance (Singh et al., 1972; Jones and Rawson, 1979; Yadav and Bhatt, 1989). The genus *Avena* comprises several hexaploid, tetraploid and diploid species of which the widely cultivated hexaploid species is *Avena sativa*. Several other species have

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Table 1. Plant height and tiller production in *Avena* species under water stress and non stress at vegetative and flowering stage (data in parentheses indicates percent decrease over control).

Species	Plant height (cm)				Tiller number			
	Vegetative stage		Flowering stage		Vegetative stage		Flowering stage	
	Control	Stress	Control	Stress	Control	Stress	Control	Stress
A. strigosa	58.67	45.33 (22.73)	135.33	114.67 (15.27)	11	10 (9.09)	21	9 (57.14)
A. brevis	52.33	36.33 (30.57)	144.67	138.67 (4.33)	17	17 (0.0)	15	11 (26.67)
A. vaviloviana	46.67	43.67 (6.43)	130.33	129.67 (0.51)	18	14 (22.22)	18	13 (27.78)
A. abyssinica	50.00	42.67 (14.66)	93.33	93.00 (0.35)	12	10 (16.67)	22	15 (31.82)
A. sativa	52.33	40.00 (23.56)	120.67	114.67 (4.97)	10	10 (0.0)	20	14 (30.00)
A. marocana	34.33	25.67 (25.23)	69.67	67.33 (3.35)	40	27 (32.5)	26	19 (26.92)
A. sterilis	31.67	26.33 (16.86)	70.67	70.33 (0.481)	24	24 (0.00)	26	26 (0.0)
Mean	46.57	37.14	109.24	104.05	18.85	16	21.14	15.28
CD at 5%	T : 2.303 S : 6.410 T x S : 9.065		T : 1.836 S : 2.797 T x S : 3.955		T : 3.916 S : 4.578 T x S : 6.474		T : 0.547 S : 2.595 T x S : 3.670	

T, Treatment; S, species.

the potential to grow under wide range of edaphic and climatic variability and lend themselves for economic exploitation as a grain/pasture/cultivated forage crop. Oat is one of the major cultivated fodder crops in Indian sub continent. In a study by Pandey et al. (2012), at vegetative stage, all the seven species of *Avena* were grouped under three categories in response to the extent of moisture stress. The group one comprised of three species viz. *Avena brevis*, *Avena marocana* and *Avena sterilis* showing the maximum stress up to the 16th day water holding. The second group comprised of three species (*Avena strigosa*, *Avena vaviloviana* and *Avena abyssinica*) showing tolerance up to the 12th day and in the third group, the stress tolerance went up to 9th day with only one species (*A. sativa*). The present investigation was undertaken to evaluate the promising species of oats (*Avena*) under soil moisture stress for their relative growth and biomass production potential and also to select the drought tolerant species for further multiplication and improvement programme.

MATERIALS AND METHODS

Seed of seven oat species (*viz*. *Avena strigosa*, *Avena brevis*, *A. vaviloviana*, *A. abyssinica*, *Avena sativa*, *A. marocana* and *A. sterilis*) were sown in porcelain pots (30" x 20") containing garden soil at pot culture experimental site of IGFRI, Jhansi (25 °N and 78 °E, 275 amsl) during 2006 to 2007. After uniform germination, three plants from each species in every pot was maintained. All species were planted in three pots. After 15 days of germination, one set of pot was kept under stress and another set of pots was watered regularly. The water stress was created with holding of irrigation at vegetative and flowering stage of crop growth. After extreme stress, plants were re-watered. The crop was grown as per recommended agronomical practices. The observations on plant height, the number of tiller, fresh and dry biomass and leaf area per plant were recorded at 35 days after germination at vegetative stage and at 50% flowering stage. For recording of morphological and growth parameters, three plants of each species were uprooted under each treatment and the data presented is the mean of three pots. The leaf area of fresh leaves was measured by using automatic portable leaf area meter (LI 3000, LICOR, USA). Specific leaf weight (SLW) was expressed as the dry weight of leaf per unit leaf area in mg/cm^2 (Yoshida et al., 1976). The specific leaf area was calculated as the leaf area/leaf dry weight and expressed in $cm^2\ g^{-1}$. The plant samples were harvested and separated into leaves, stem and root and fresh weight of different parts were recorded. The plant samples were dried at 80°C for 48 h to record the dry weight. The dry weight of the individual portions was recorded and the sum of their weight was taken as total dry weight.

RESULTS AND DISCUSSION

Although no definite trend was observed in the reduction of plant height under soil moisture stress as imposed at vegetative and flowering stage of crop growth (Table 1), minimum reduction was recorded in *A. vaviloviana* (6.43%) followed by *A. abyssinica* (14.66%) and *A. sterilis* (16.86%) at vegetative stage. At flowering stage, minimum reduction in plant height was observed in *A. abyssinica* (0.35%) followed by *A. sterilis* (0.48%). The primary effect of soil moisture stress is to decrease water content and water potential in the plant cells. The decrease in water potential affects water movement into growing regions and hence cell elongation rate decreases (Bradford and Hsiao, 1982). It is also reported that internal moisture stress in plant causes reduction in cell division and cell elongation which causes stunted plant growth (Steinberg et al., 1990). At vegetative stage, there was absolutely no effect of the moisture stress on tiller production in *A. sterilis, A. brevis* and *A. sativa.* However, at flowering stage, the stress had no effect on

Table 2. Effect of soil moisture stress on leaf area (cm^2/plant) in *Avena* species at vegetative and flowering stage (data in parentheses indicates percent decrease over control).

Species	Vegetative stage		Flowering stage	
	Control	Stress	Control	Stress
A. strigosa	162.52	80.10 (50.71)	1825.48	1070.03 (41.38)
A. brevis	200.84	58.57 (70.84)	466.13	321.34 (31.06)
A. vaviloviana	248.70	119.16 (52.08)	1467.41	512.74 (65.06)
A. abyssinica	391.46	80.91 (79.33)	747.30	615.98 (17.57)
A. sativa	131.28	63.26 (51.81)	2215.62	1182.98 (46.61)
A. marocana	390.41	105.73 (72.92)	1882.97	721.11 (61.70)
A. sterilis	252.40	96.20 (61.88)	2361.89	2348.89 (0.55)
Mean	253.94	86.27 (66.02)	1566.69	967.58 (38.24)
CD at 5%	T : 21.35 S : 14.56 T x S : 31.21		T : 19.34 S : 16.43 T x S : 29.12	

tiller production of *A. marocana* and *A. sterilis* (Table 1). Hence, *A. sterilis* showed tolerance to moisture stress with respect to growth in plant height and tiller production.

Total leaf area production was recorded at the time of harvest at vegetative and flowering stage of the crop growth and presented on per plant basis (Table 2). On average of all the species, the leaf area production decreased to 66 and 38% at vegetative and flowering stage, respectively. However, the percentage of decrease in leaf area varied from species to species. The minimum decrease in leaf area was recorded in *A. sterilis* (0.55%) followed by *A. abyssinica* (17.57%) as recorded at 50% flowering stage. Leaf extension has been shown to be very sensitive to water stress (Boyer and Younis, 1984). Garg et al. (2001) also found that increasing water stress progressively decreased water potential and leaf area production in moth bean genotypes. The specific leaf area (SLA) decreased under water stress in all the species (Table 3). At vegetative stage, minimum decrease in SLA was recorded in *A. sterilis* (5.8%) followed by *A. brevis* (9.67%) and *A. strigosa* (12.29%) over the control. However, at flowering stage, minimum decrease was recorded in *A. strigosa* (5.24%) followed by *A. sterilis* (13.36%) over the control. The SLW showed an increasing trend under moisture stress environment over the well watered plant. The increase in SLW indicates increase in leaf thickness, showing high water retention capacity under moisture stress situation which is a requisite parameter under stress environment. On the other hand, the decrease in SLA indicated the slow leaf expansion with less leaf surface area to control transpiration and evaporation from the leaf surface during the moisture stress period. *A. sterilis* showed the drought tolerant characters in terms of leaf expansion and the specific leaf weight may be because of the fundamental differences in species characteristics like thicker leaves than other species by which the water retention capacity of the leaf increased to combat the moisture stress environment.

Total fresh and dry biomass production as per plant basis was recorded at vegetative and flowering stages (Table 4). Water stress imposed at vegetative stage reduced total fresh biomass in all the species of *Avena* and the reduction was 50% or even more than the control but at flowering stage, the reduction ranged from 25.21 (*A. abyssinica*) to 67.52% (*A. strigosa*). The minimum decrease in fresh biomass production at vegetative stage was recorded in *A. sterilis* and *A. sativa* followed by *A. strigosa* over the non stress. However, at flowering stage, minimum decrease in the fresh biomass production was in *A. abyssinica* followed by *A. marocana* and *A. sterilis* over the control. All the species showed significant differences in dry biomass production under stress and non stress environment (Table 4). The decrease in dry biomass accumulation under soil moisture stress varied from species to species.

In vegetative stage, minimum decrease in dry biomass production was recorded in *A. sterilis* (30.38%) whereas *A. strigosa* (34.58%) and *A. sativa* (35.87%) were at par. However, at flowering stage, minimum decrease in dry biomass production was recorded in *A. sterilis* (3.47%) followed by *A. marocana* (12.56%) over the plants grown under well watered environment as compared to other species tested. Minimum reduction in dry matter accumulation in *A. sterilis* indicates its drought tolerance potential. It has been reported that in restricted water supply, high moisture stress caused rapid respiration (Kramer and Kozlowski, 1960) which lead to reduction in dry matter accumulation of plant components (Steinberg et al., 1990). There was a significant positive correlation between total leaf area production and dry biomass (r^2 = 0.738) at flowering stage (Figure 1). It seems that biomass accumulation is completely governed by leaf area production. Partitioning of biomass in root, stem and

Table 3. Change in specific leaf area (SLA) and specific leaf weight (SLW) under water stress in *Avena* species at vegetative and flowering stage.

Species	SLA (cm^2/g) (Data in parentheses: percentage decrease over control)				SLW (mg/cm^2) (Data in parentheses: percentage increase over control)			
	Vegetative stage		Flowering stage		Vegetative stage		Flowering stage	
	Control	Stress	Control	Stress	Control	Stress	Control	Stress
A. strigosa	171.58	150.49 (12.292)	225.59	213.76 (5.244)	5.828	6.645 (12.295)	4.433	4.678 (5.419)
A. brevis	151.62	136.96 (9.669)	182.82	137.56 (24.757)	6.595	7.302 (9.682)	5.470	7.270 (24.759)
A. vaviloviana	219.31	183.43 (16.360)	254.17	180.06 (29.158)	4.560	5.452 (16.361)	3.934	5.554 (29.168)
A. abyssinica	246.31	188.33 (23.539)	222.65	155.99 (29.939)	4.060	5.310 (23.540)	4.491	6.411 (29.949)
A. sativa	127.67	103.89 (18.626)	226.86	162.95 (28.172)	7.833	9.626 (18.627)	4.408	6.137 (28.173)
A. marocana	231.96	171.01 (26.276)	286.23	146.84 (48.699)	4.311	5.848 (26.282)	3.494	6.810 (48.693)
A. sterilis	151.53	142.74 (5.801)	164.41	142.45 (13.357)	6.599	7.006 (5.809)	6.082	7.020 (13.362)
Mean	185.71	153.83 (16.080)	223.25	162.80 (25.618)	5.684	6.741 (16.085)	4.616	6.268 (25.646)
CD at 5%	T : 7.923 S : 44.149 T x S : 62.436		T : 27.021 S : 48.305 T x S : 68.314		T : 0.152 S : 1.053 T x S : 1.490		T : 0.653 S : 1.360 T x S : 1.924	

Table 4. Effect of soil moisture stress on fresh and dry biomass production in *Avena* species at vegetative and flowering stage (data in parentheses indicates percentage decrease over control).

Species	Fresh weight (g/plant)				Dry weight (g/plant)			
	Vegetative stage		Flowering stage		Vegetative stage		Flowering stage	
	Control	Stress	Control	Stress	Control	Stress	Control	Stress
A. strigosa	22.41	11.00 (50.91)	147.32	47.85 (67.52)	4.51	2.95 (34.58)	41.31	22.85 (47.81)
A. brevis	31.63	11.48 (63.70)	80.12	49.96 (37.64)	7.29	2.93 (59.80)	23.85	17.90 (24.95)
A. vaviloviana	22.44	9.42 (58.02)	89.88	36.31 (59.60)	4.32	2.33 (46.06)	22.29	14.85 (33.38)
A. abyssinica	22.72	8.25 (63.69)	55.65	41.62 (25.21)	4.73	2.39 (49.47)	17.79	11.82 (33.56)
A. sativa	21.06	10.71 (49.15)	163.22	80.45 (50.71)	4.21	2.70 (35.87)	38.31	28.22 (26.34)
A. marocana	23.67	8.09 (65.82)	72.27	50.85 (29.63)	4.33	2.37 (45.27)	25.08	21.93 (12.56)
A. sterilis	21.41	10.50 (49.00)	185.30	123.27 (33.27)	4.18	2.91 (30.38)	44.91	43.35 (3.47)
Mean	23.62	9.92	113.39	62.90	4.79	2.65	30.86	24.021
CD at 5%	T : 3.463 S : 5.598 T x S : 7.916		T : 11.285 S : 20.148 T x S : 28.493		T : 0.678 S : 1.301 T x S : 1.840		T : 4.365 S : 6.424 T x S : 9.085	

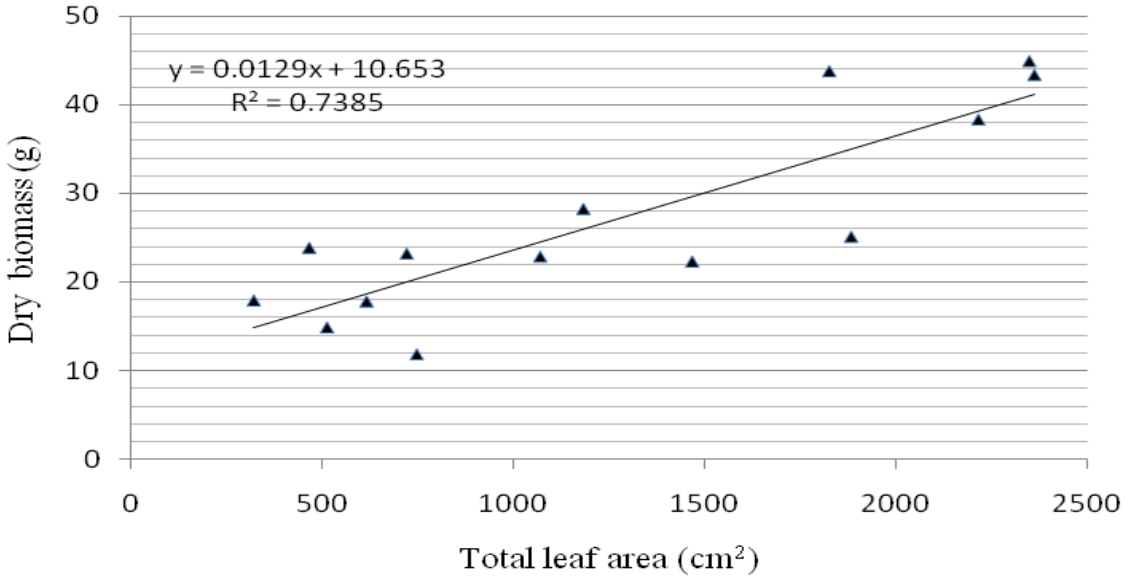

Figure 1. Linear relationship between dry biomass yield vs. total leaf area.

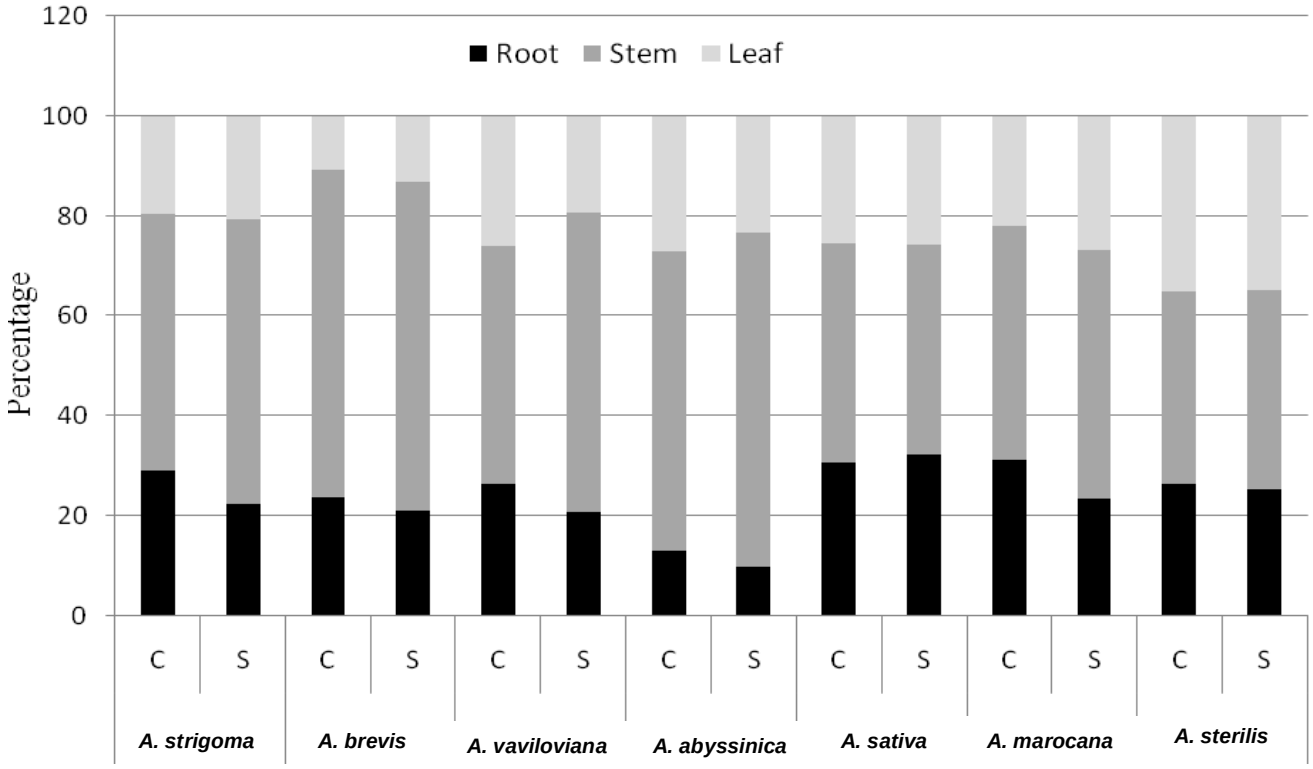

Figure 2. Partitioning of biomass in root, stem and leaves of different species of oat under control (C) and stress (S) environment at 50% flowering stage.

leaves under stress and non stress environment at 50% flowering stage was calculated and presented in Figure 2. Maximum biomass partitioning was observed in stem in all the species under soil moisture stress. No definite pattern was observed in biomass partitioning in leaves and roots but in *A. sterilis*, partitioning of biomass was

almost equal in all the parts under soil moisture stress and non stress indicating that there was no adverse effect of drought on the allocation of biomass in different parts even under stress environment.

Thus, on the basis of the above results, it is concluded that *A. sterilis* maintained leaf area production under stress environment leading to the higher biomass accumulations which indicates its suitability for drought situations and can be further taken for improvement programme for the development of drought resistant varieties in oats.

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Full Length Research Paper

Studies on cytotoxic, phytotoxic and volatile profile of the bark extract of the medicinal plant, *Mallotus tetracoccus* (Roxb.) Kurz.

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This study was aimed at analysing the compounds present in the bark extract of *Mallotus tetracoccus* (Roxb.) Kurz. by GC-MS analysis and also to investigate the cytotoxic and phytotoxic activity of *Mallotus tetracoccus* (Roxb.) Kurz. bark extract. The major constituents in *M. tetracoccus* (Roxb.) Kurz. bark extract are thiocyanic acid and 2-propynyl ester (52.04%). It possesses biocidal, antioxidative, antimutagenic and anticancer activity. The cytotoxic activity of bark extract was evaluated by brine shrimp lethality bioassay method and the LC_{50} value was found to be 84.72 µg/ml compared to taxol 0.85 µg/ml. Phytotoxicity assay showed significant root length inhibition by the extract at the concentrations of 100, 1000 and 10000 ppm. Similarly, seed germination studies shows that the bark extract possess significant inhibition at concentrations of 1000 and 7500 ppm.

Key words: *Mallotus tetracoccus*, GC-MS analysis, thiocyanic acid, furfural, 4H- Pyran-4 -one, 2, 3- Dihydro-3, 5- dihydroxy-6- methyl, cytotoxicity, phytotoxicity, radish seed, artemia salina.

INTRODUCTION

Interactions between higher plants take place either by competition or by chemical inhibition (Mancini et al., 2009). When the effect is due to the release of an effective phytotoxin, it is called allelopathy. Small quantities of toxins are responsible for massive reductions in plant growth. Plants generally have inhibitory effects on neighbouring plants by releasing allelopathic chemicals into the soil (Harborne, 1988; Inderjit, 1996; Seigler, 1996). Allelochemicals inhibit germination and seedling growth probably by affecting cell division and elongation, processes that are very important at this stage, or by interfering with enzymes involved in the mobilization of nutrients necessary for germination (Batlang and Shushu, 2007). Thus, the phytotoxicity of the bark extract of *Mallotus tetracoccus* was studied using radish seed for root length and seed germination determination.

M. tetracoccus (Roxb.) Kurz. is found in Western Ghats of India. *M. tetracoccus* is one of the medicinally important plants belonging to the family Euphorbiaceae, commonly known as “vatta kanni” in Tamil. Several species of the genus *Mallotus* are a rich source of biologically active compounds such as phloroglucinols, tannins, terpenoids, coumarins, benzopyrans and chalcones (Amakura and Toshida, 1996; Tanaka et al., 1998; Huang et al., 1999; Cheng and Chen, 1999; Wei et al., 2004; Ma et al., 2004; Likhitwitayawuid and Supudompol, 2005). *M. tetracoccus* (Roxb.) Kurz, are found in evergreen forests up to 1600 m. The common names include *Mullu polavu,* Vatta (Tamil), *Thavatta,*

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Vatta, Vatta kumbil, Vetta kumbil (Malayalam) and *Uppale mara* (Kannada). The trees grow up to 5 to15 m tall, leaf blades are triangular-ovate or ovate, sometimes 1- or 2-lobate, 10-25 × 9 to 20 cm, leathery, abaxially brownish tomentose, adaxially glabrous, base obtuse or truncate. The reported bioactivities of the extracts or the individual chemical constituents isolated from this genus include antipyretic (Chattopadhyay et al., 2002), anti-inflammatory, hepatoprotective (Kim et al., 2000), antioxidant and radical scavenging activities (Arfan et al., 2007).

The active compounds present in the *M. tetracoccus* ethanolic leaf extract showed the presence of various chemical constituents such as Bis (2-ethyl hexyl) phthalate (46.78%), 3-methyl-2-(2-oxypropyl) furan (13.31%), E-8-methyl-9-tetradecen-1-ol acetate (6.63%), Octadecanoic acid, 2-oxo (4.46%) and Longiborneol (2.39%) (Ramalakshmi and Muthuchelian, 2011b).

The study report of the *M. tetracoccus* bark (MTB) extract in our laboratory showed to have significant antioxidant, antimicrobial and radical scavenging activities (Ramalakshmi and Muthuchelian, 2012). Thus, the objective was to analyse the cytotoxicity, phytotoxicity and volatile profile of the MTB extract.

MATERIALS AND METHODS

Collection of plant material

The fresh bark of *M. tetracoccus* (Roxb.) Kurz. were collected from the Agasthiar Malai reserved forest, Western Ghats, South India, authenticated by Prof. Dr. K. Muthuchelian, Director, Centre for Biodiversity and Forest Studies, Madurai Kamaraj University, and voucher specimens were deposited in the herbarium of Centre for Biodiversity and Forest Studies of our university (No.AM-07).

Preparation of extract

Fresh barks were shade dried, powdered and extracted with ethanol for 6 to 8 h using soxhlet apparatus. The extract was then filtered through muslin, evaporated under reduced pressure and vacuum dried to get the viscous residue. The ethanolic extract of the bark was used for cytotoxic, phytotoxic studies and GC-MS analysis.

GC-MS analysis

GC-MS analysis was carried out on a GC clarus 500 Perkin Elmer system comprising a AOC-20i auto sampler and gas chromatograph interfaced to a mass spectrometer (GC-MS) instrument employing the following conditions: column Elite-1 fused silica capillary column (30 × 0.25 mm ID ×1EM df, composed of 100% Dimethyl poly siloxane), operating in electron impact mode at 70 eV; helium (99.999%) was used as carrier gas at a constant flow of 1 ml/min and an injection volume of 0.5 EI was employed (split ratio of 10:1), injector temperature was 250°C and ion-source temperature 280°C. The oven temperature was programmed from 110°C (isothermal for 2 min), with an increase of 10 to 200°C/min, then 5 to 280°C/min, ending with a 9 min isothermal at 280°C. Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 40 to 550 Da.

Identification of components

Interpretation on mass spectrum of GC-MS was done using the database of National Institute Standard and Technology (NIST) having more than 62 000 patterns.

Cytotoxicity bioassay

Brine shrimps (*Artemia salina*) were hatched using brine shrimp eggs in a conical shaped vessel (1L), filled with sterile artificial seawater (prepared using sea salt 38 g/L and adjusted to pH 8.5 using 1N NaOH) under constant aeration for 48 h. After hatching, active nauplii free from egg shells were collected from brighter portion of the hatching chamber and used for the assay. Twenty nauplii were drawn through a glass capillary and placed in each vial containing 4.5 ml of brine solution. In each experiment, 0.5 ml of the extract was added to 4.5 ml of brine solution and maintained at room temperature for 24 h under the light and surviving larvae were counted with a hand lens. Experiments were conducted along with control (vehicle treated), different concentrations (10 to 1000 µg/ml) of the test substances in a set of three tubes per dose. Based on the percent mortality, the LD_{50} of the test compound was determined using probit scale (Wardlaw, 1985).

Radish seed phytotoxicity assay

The phytotoxic properties of MTB extract was evaluated using radish seed phytotoxicity assay (Turker and Camper, 2002; Islam et al., 2009). Two type of determination were done for this purpose:

Root length determination

Radish seed was washed with distilled water and with 1% mercuric chloride. Whatman No. 1 filter paper kept on Petri dish and 5 ml extracts (100, 1000 and 10000 ppm) were added separately. Filter paper was dried at room temperature for reducing extra solvent. 5 ml double distilled water was added and then 20 radish seeds were placed on Petri dishes followed by tight sealing and incubation at 23 ± 2°C. Root length was measured after 1, 3 and 5 days of interval. Only double distilled water containing Petri dish was used as control. Each assay was carried out in three times.

Seed germination determination

This part of the determination is similar to that of earlier determination except for the extract concentrations and number of seeds. Here, two different concentrations (1000 and 7500 ppm) and 100 radish seeds were used. Germinated seeds were counted after every day up to 5 days. Each experiment was carried out three times.

Statistical analysis

Results were expressed as the means of three replicates ± the standard deviation of triplicate analysis.

RESULTS AND DISCUSSION

GC-MS analysis

On comparison of the mass spectra of the constituents with the NIST library, five peaks were obtained; all the phytoconstituents were characterized and identified

Table 1. Phytocomponents identified in the ethanolic bark extract of Mallotus tetracoccus (Roxb.) Kurz. by GC-MS.

Number	RT	Name of the compound	Peak area (%)
1	12.45	Furfural	28.31
2	19.48	4H- Pyran-4 -one, 2,3-dihydro-3,5-dihydroxy-6-methyl	8.70
3	21.02	Thiocyanic acid, 2-propynyl ester	52.04
4	24.15	Benzofuran, 7(2,4-dinitrophenoxy)- 3- ethoxy- 2,3- dihydro-2-dimethyl	6.38
5	24.52	Benzaldehyde, 3-hydroxy-4-methoxy	4.57

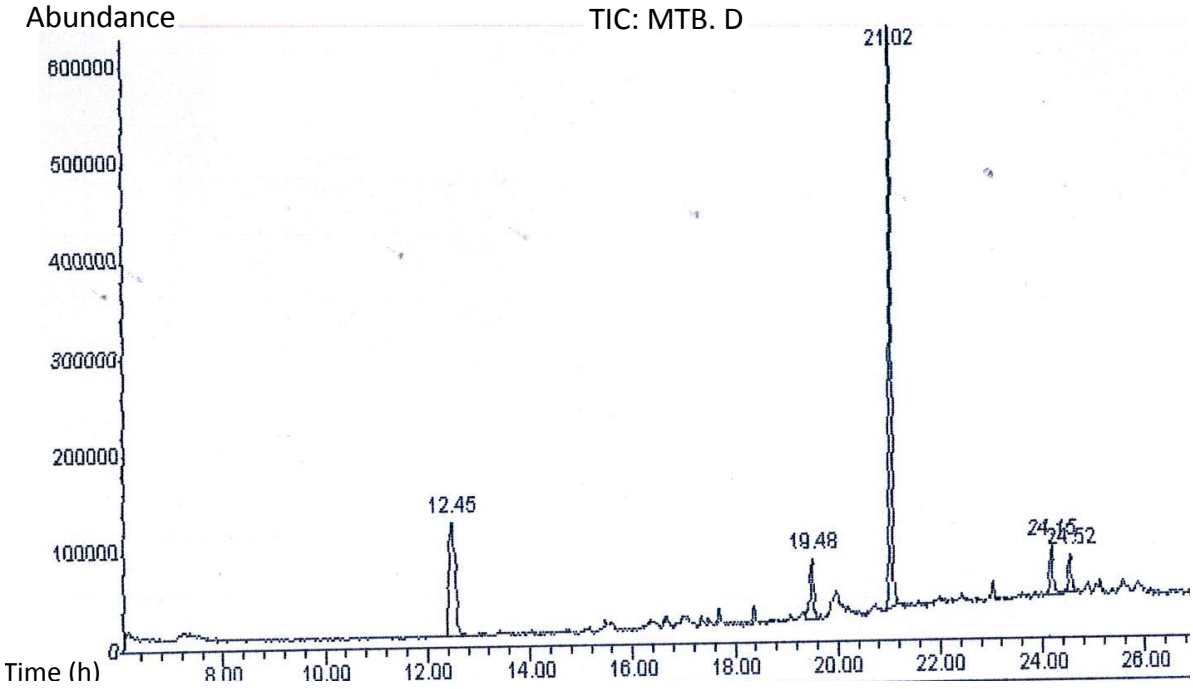

Figure 1. GC-MS Chromatogram of the ethanolic bark extracts of *M. tetracoccus* (Roxb.) Kurz.

(Table 1). GC-MS chromatogram of the MTB is given in Figure 1. The retention times (RT) are in minutes. The major chemical constituents in ethanolic bark extract studied through GC-MS are thiocyanic acid-2-propynyl ester (52.04%), furfural (28.31%), 4H-pyran-4 -one-2,3-dihydro-3,5-dihydroxy-6-methyl (8.70%), benzofuran-7-(2,4-dinitrophenoxy)-3-ethoxy-2,3-dihydro-2-dimethyl (6.38%) and benzaldehyde-3-hydroxy-4-methoxy (4.57%).

The major constituents, thiocyanic acid, and 2-propynyl ester were found at retention time of 21.02 min. Glucosinolates are organic anionic compounds containing sulphur, nitrogen and a group derived from glucose (Kjaer, 1960; Ettlinger and Kjaer, 1968). Glucosinolates are found in all parts of the plant (Kjaer, 1976) and up to 15 different types of glucosinolates have been found in the same plant. Glucosinolates and myrosinase enzyme come in contact when plant tissue is damaged leading to formation of hydrolytic products of glucosinolates (Kaur et al., 2011). The breakdown products of glucosinolates when exposed to myrosinase enzyme include isothiocyanates, nitriles, epithionitriles, and thiocyanates, which are known to possess wide array of biological activities such as biocidal (Vig et al., 2009), antioxidative (Barillari et al., 2005), antimutagenic (Rampal et al., 2010) and anticancer activities (Rosea et al., 2005).

The second main active constituent, furfural (28.31%) was found at retention time of 12.46 min. The antifungal activities of furfural and its derivative have been reported discussing their feasibilities for antifungal treatment (Jouad et al., 2001; Moon et al., 1993). The pine needle extract contained four chemical compounds of which furfural are the main constituent. The extracts were reported to possess significant antifungal activity against plant pathogen fungus, *Alternaria mali* (Jung et al., 2007). The 4H- Pyran-4 -one, 2, 3- Dihydro-3, 5- dihydroxy-6-methyl, a flavonoid compound found at retention time of 19.47 min is said to possess antimicrobial and anti-inflammatory activities (Praveen Kumar et al., 2010; Ramalakshmi and Muthuchelian, 2011a). Benzofuran,

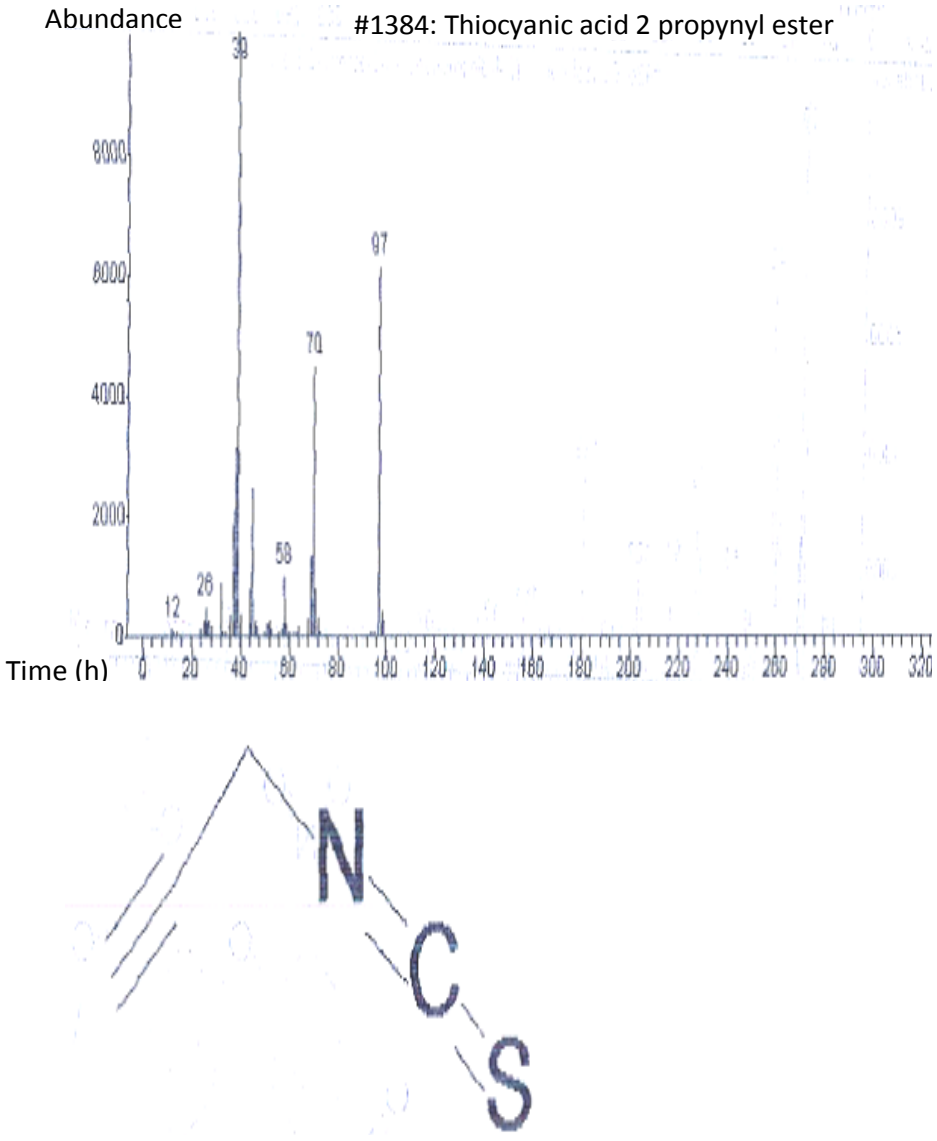

Figure 2. The mass spectrum analysis and structure of thiocyanic acid, 2-propynyl ester.

7(2, 4-dinitrophenoxy)- 3- ethoxy- 2, 3- dihydro-2-dimethyl, a coumaran, is said to possess activities such as antihelminthic, anti-inflammatory, and anti-diarrhoeal activities (Ramalakshmi and Muthuchelian, 2011a). The major phytochemical constituents, thiocyanic acid, 2-propynyl ester, furfural, 4H- Pyran-4 -one, 2, 3- Dihydro-3, 5- dihydroxy-6- methyl present in ethanolic extract of MTB is presented as mass spectra and compound structures in Figures 2, 3 and 4.

Cytotoxicity bioassay

A general bioassay capable of detecting a broad spectrum of bioactivity present in crude extracts is the brine shrimp lethality bioassay (BSLT) (Hamid et al., 2011). The cytotoxicity bioassay against *Artemia salina* is a simple and inexpensive method to test cytotoxicity, to biodirect fractionation of natural products and as a predictor of antitumor and pesticidal activity (Sanchez et al., 1993). The ethanolic MTB extract shows significant cytotoxic activity against brine shrimp and the LC_{50} value was found to be 84.72 µg/ml compared to taxol 0.85 µg/ml (Figure 5).

The inhibitory effect of the MTB extract might be due to the presence of toxic compounds such as thiocyanic acid, 2-propynyl ester, furfural and 4H- pyran-4 -one, 2, 3-dihydro-3, 5- dihydroxy-6- methyl present in the extract possessing antitumor, antimicrobial, antioxidant and anti-inflammatory activity. So the cytotoxic effects of the bark extract enunciate that it can be selected for further cell line assay because there is a correlation between cytoto-

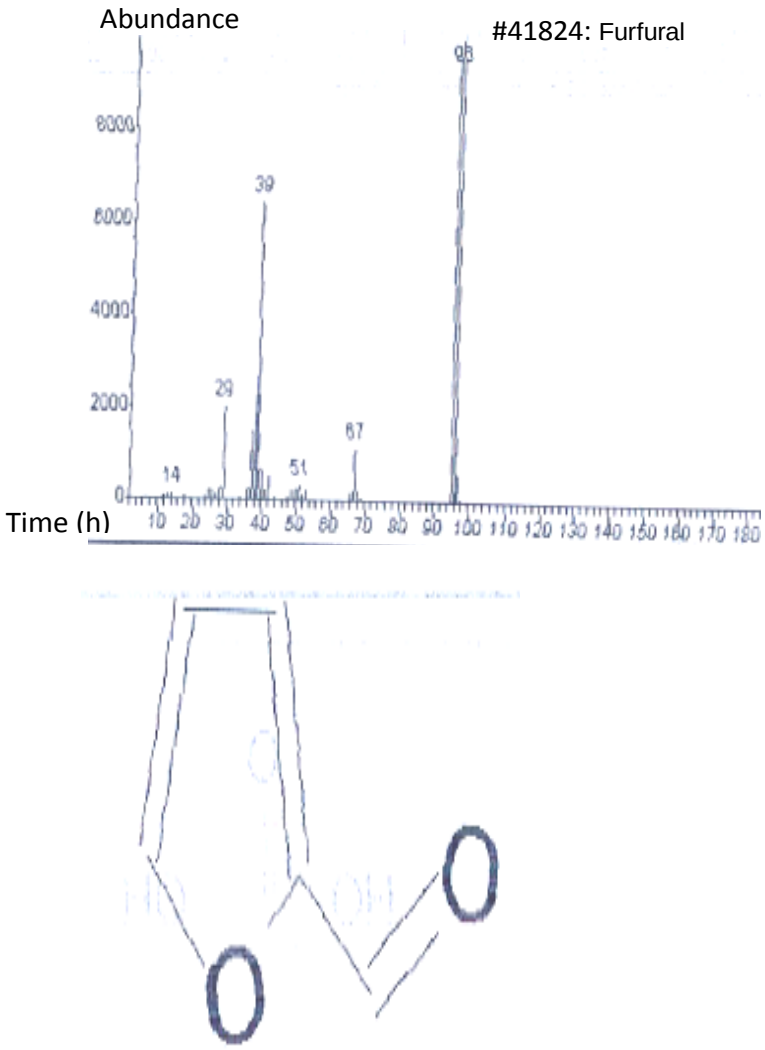

Figure 3. The mass spectrum analysis and structure of furfural.

Xicity and activity against the brine shrimp nauplii using extracts (Manilal et al., 2009; Haque et al., 2009). The results on brine shrimps assay indicate that the extract has LC_{50} value greater than 20 µg/ml; the recommended cut-off point for detecting cytotoxic activity (Geran et al., 1972).

On comparison of our study results with other research work, our extract possessed significant cytotoxic activity. The cytotoxic potential (ED_{50}) of different fractions [crude methanolic extract (CME), n-Hexane fraction (NHF) and aqueous fraction (AQF)] of *Aster thomsonii*, the AQF values were found to possess maximum activity of 154.69 µg/ml (Bibi et al., 2011). Several other cytotoxicity studies show that the results of *Thymus serpyllum*, 466 µg/ml (Rehman et al., 2009), and out of 60 medicinal plants from brazil screened for activity showed that only 10% plants showed ED_{50} < 1000 µg/ml (Maria et al., 2000). Brine shrimp lethality bioassay of petroleum ether and methanol extracts of the seeds of *Khaya senegalensis* possessed significant cytotoxicity LC_{50} values of 827.39 and 51.79 µg/ml, respectively (Juss et al., 2007). The LC_{50} values of standard Vincristin sulphate, petroleum ether, chloroform and ethyl acetate extracts of *Marsilea quadrifolia* were 6.628, 9.543, 7.820 and 8.589 µg/ml respectively (Ripa et al., 2009). The cytotoxic potential of aqueous extract of *Ficus racemosa* seed showed an LC_{50} value of 4.04 µg/ml (Hamid et al., 2011).

Radish seed phytotoxicity assay

Phytotoxicity is an important attribute in determination of allelopathic potential of a plant species (Khan et al., 2011). It is a common tradition that easily grown, sensitive, reliable species like *Lemna minor*, Lettuce (*Lactuca sativa*) and radish (*Raphanus sativa*) seeds are used as test plants in allelopathic studies (Putnam et al., 1983; Einhelling et al., 1985; Leather and Einhelling, 1985).

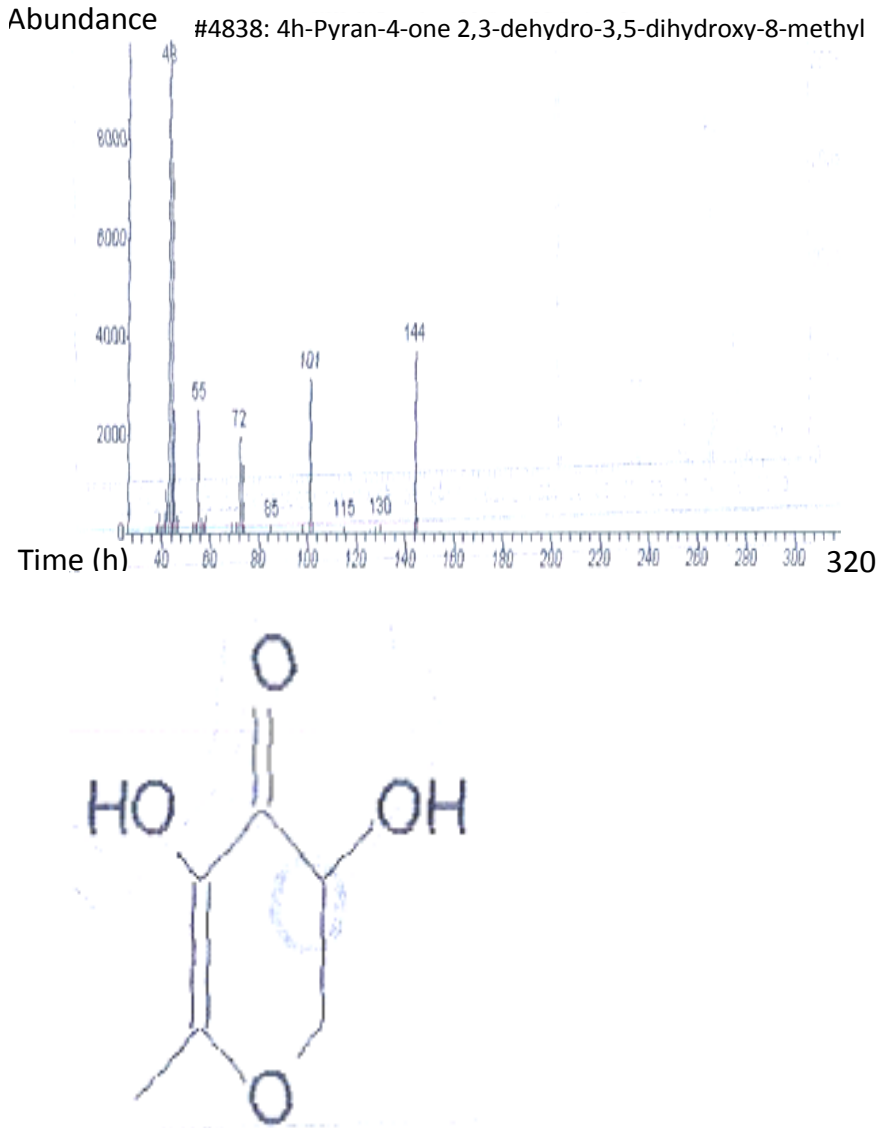

Figure 4. The mass spectrum analysis and structure of 4H- Pyran-4 -one, 2, 3- Dihydro-3, 5- dihydroxy-6- methyl.

This radish seed phytotoxicity assay has a wide range of application in research towards the discovery of active principles in plants (Arzu et al., 2002). The root lengths of radish seeds germinated were significantly inhibited by the bark extracts at concentrations of 100, 1000 and 10000 ppm (Figure 6). Similarly, the seed germination inhibition was said to be significantly high when compared to control (Figure 7). The MTB extract exhibited significant phytotoxicity on radish seeds due to the presence of phytochemicals such as thiocyanic acid, furfural and 4H- pyran-4 -one, 2, 3- dihydro-3, 5- dihydroxy-6- methyl. Similarly, the allyl isothiocyanates (ITC) isolated from black mustard (*Brassica nigra* L.) residues inhibited establishment of grass species. Benzyl-ITC, a break down product of white mustard (Josefsson, 1968; Tollsten, 1988) was phytotoxic to velvet leaf, sicklepod (*Senna obtusifolia*) and sorghum. Other break down products of glucosinate like ionic thiocyanate (SCN-) inhibited the root or shoot growth of many crop species (Brown et al., 1991).

Aqueous extracts of *Nicotiana glauca* Graham (stems, roots and fruits) was evaluated for phytotoxicity on two crops (lettuce and radish), where percentage inhibition was between 15 and 100%, due to the presence of phenolics (Rinez et al., 2011). Root length inhibition was more obvious than shoot length, as root length is a more sensitive indicator of phytotoxic activity (Rinez et al., 2011). The study by Turk et al. (2005) investigated the allelopathic effects of various black mustard (*Brassica nigra* L.) plant parts (leaf, stem, flower and root), where

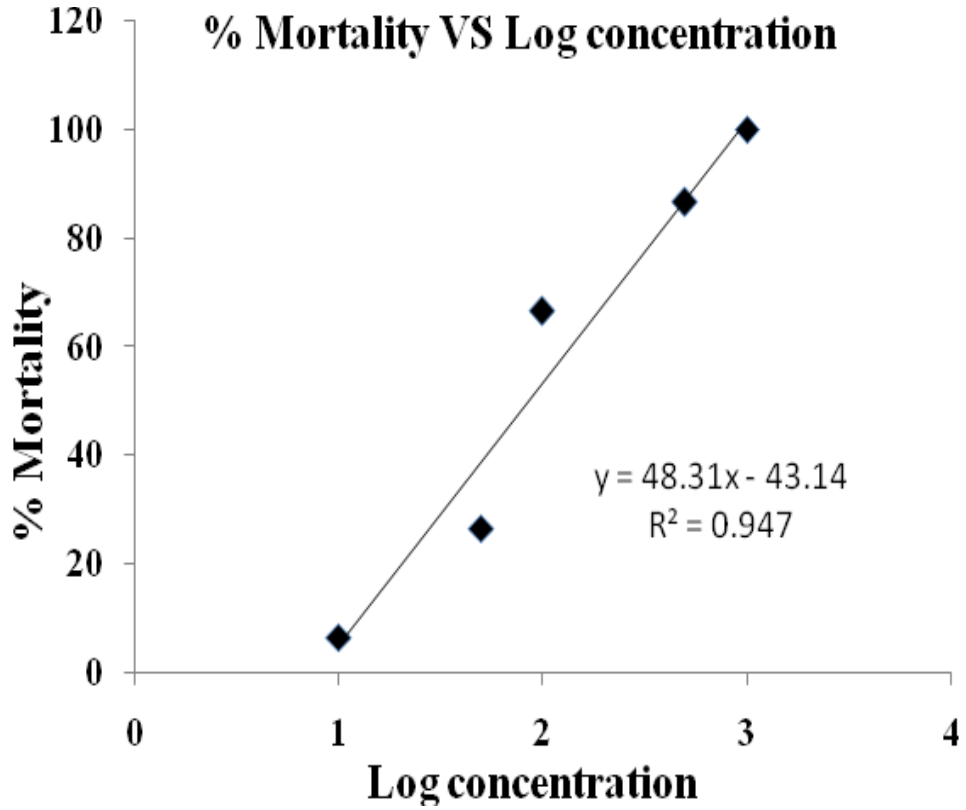

Figure 5. The toxicity effects of the *M. tetracoccus* (Roxb.) Kurz. bark extract using brine shrimp lethality assay after 24 h.

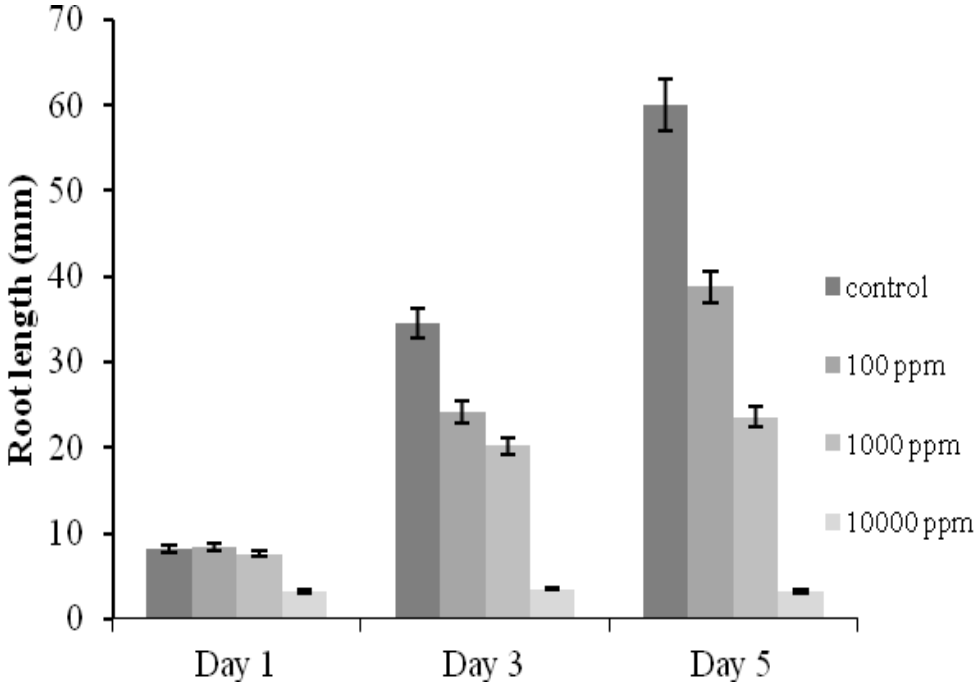

Figure 6. Histogram shows regular root length inhibition by the bark ethanol extract of *M. tetracoccus* at different concentrations (100, 1000 and 10000 ppm). Data was compared with the control.

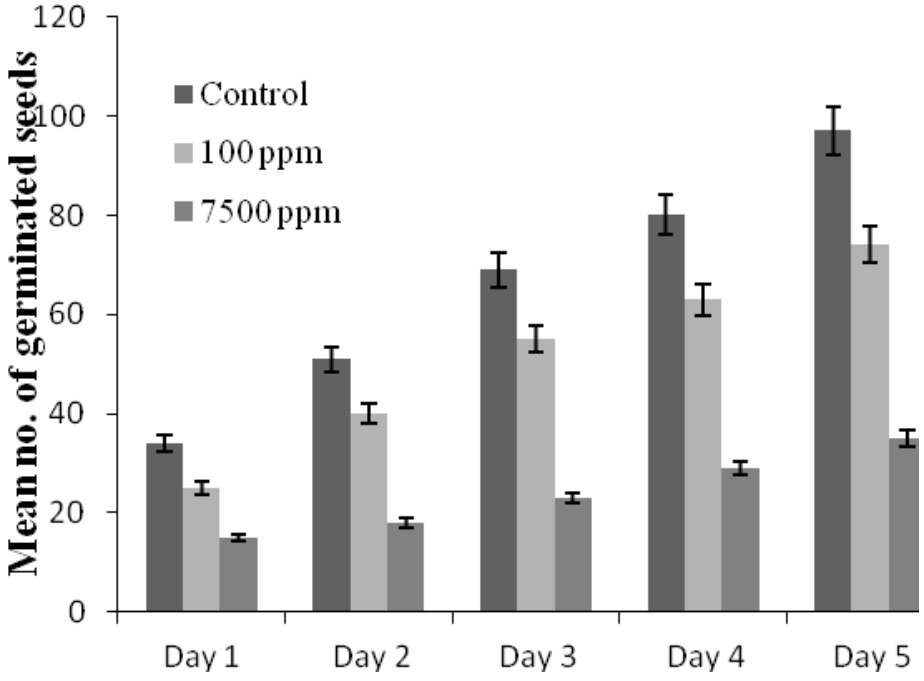

Figure 7. Graph showing phytotoxicity assay on radish seed germination percentage at two different concentrations (1000 and 7500 ppm) of ethanolic bark extract of Mallotus tetracoccus. Data was compared with the control.

the aqueous extracts significantly inhibited radish seed germination and seedling growth when compared with distilled water control. The aqueous root extract of *Ailanthus altissima* was purified to give active compounds such as ailanthone, ailanthinone, chaparrine, and ailanthinol B (quassinoid derivatives), where the alkaloid 1-methoxycanthin-6-one is not active. Then, the compounds where studied for the allelopathic activity using radish, garden cress and purslane seeds, where ailanthone showed greatest inhibitory activity (Feo et al., 2003). Feo et al. (2003) through his studies has reported that out of three seeds studied for phytotoxicity, radish seeds was the most sensitive to allelochemicals.

The essential oils of *S. hierosolymitana* Boiss. and *S. multicaulis* Vahl. var. simplicifolia Boiss. was studied for the phytotoxic effects on *Raphanus sativus* L. (radish) and *Lepidium sativum* L. (garden cress), where the extract inhibited and promoted radish seed germination at doses of 0.625 and 0.24 µg/ml, respectively (Mancini et al., 2009).

Conclusion

GC-MS analysis was found useful in the identification of several constituents such as thiocyanic acid, 2-propynyl ester (52.04%), furfural (28.31%), 4H- Pyran-4 -one, 2, 3-Dihydro-3, 5- dihydroxy-6- methyl (8.70%), benzo-furan, 7 (2, 4-dinitrophenoxy)- 3- ethoxy- 2, 3- dihydro-2- dimethyl (6.38%) and benzaldehyde, 3-hydroxy-4-methoxy (4.57%) present in the ethanolic extract of MTB. The cytotoxic activity of ethanolic extract of MTB was assessed by using brine shrimp, *Artemia salina*, where the bark (84.72 µg/ml) was said to possess significant activity compared to taxol (0.85 µg/ml). The presence of major bioactive compound, thiocyanic acid and furfural justifies the use of the whole plant for various ailments by traditional practitioners. The phytotoxic activity of *MTB* is probably due to the presence of a substantial amount of thiocyanic acid, furfural, 4H- Pyran-4 -one, 2, 3- Dihydro-3, 5- dihydroxy-6- methyl, benzofuran and benzaldehyde. The result obtained from the brine shrimp lethality bioassay of MTB can be used as a guide for the isolation of cytotoxic compounds from the aqueous extract of the bark of this plant.

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Full Length Research Paper

Phytoremediation of heavy metal contaminated soil using different plant species

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A pot experiment was conducted to compare the plant biomass accumulation and heavy metal (HM) uptake by plant species grown in HM contaminated soils. The shoot dry weights of *Eucalyptus camaldeulensis*, *Medicago sativum*, and *Brassica juncea* grown in contaminated soils were reduced by 8, 5, and 3-fold, respectively, compared to the same plants grown in control soil. The Pb concentration in the shoots of *M. sativum, E. camaldeulensis* and *B. juncea* grown in contaminated soil was 8.7, 11.0, and 8.8-fold, respectively, higher than Pb concentration in plants grown in control soils. *M. sativum and E. camaldeulensis* accumulated higher Zn concentrations in roots (71 and 86 mg kg^{-1}) and shoots (49 and 47 mg kg^{-1}), respectively. Zn concentrations in the roots of *M. sativum, E. camaldeulensis* and *B. juncea* were higher than in the shoots by a factor of 1.4, 1.8, and 1.3-fold, respectively. The highest Cu concentration (81 and 37 mg kg^{-1} dwt) was obtained in root and shoot of *M. sativum* grown in contaminated soil, while the highest Cr concentration (133.9 mg/kg dwt) was determined in the root of *E. camaldeulensis*. This suggests that *E. camaldeulensis* was the best candidate species for phytoremediation of HM contaminated soils.

Key words: Keywords: Phytoextraction, Roadside soil, Heavy metal, *Brassica juncea*, *Medicago sativum*, *Eucalyptus camaldeulensis*

INTRODUCTION

Heavy metals (HMs) in roadside soils may come from various human activities, such as industrial and energy production, construction, vehicle exhaust, waste disposal, as well as coal and fuel combustion (Li et al., 2001; Bai et al., 2008). A large number of sites worldwide are contaminated by HMs as a result of human activities. HM contamination of soil requires effective and affordable remediation technologies. Unlike organic pollutants, metals cannot be degraded to harmless products, such as carbon dioxide, but instead persist indefinitely in the environment complicating their remediation (Lasat, 2002). Present technologies rely upon metal extraction or immobilization processes, although both are expensive and result in the removal of all biological activity in the soil during decontamination. Other, metal-extraction processes use stringent physicochemical agents that can dramatically inhibit soil fertility with subsequent negative impacts on the ecosystem (Wenzel et al., 1999).

The use of plants for rehabilitation of polluted environments is known as phytoremediation. This technology was developed after the identification of certain plants, metal "hyperaccumulators", that are able to accumulate and tolerate extremely high concentrations of metals in their shoots (Chaney, 1983; Baker et al., 2000). A few plant species are able to survive and reproduce on soils heavily contaminated with Zinc (Zn), Copper (Cu), Lead (Pb), cadmium (Cd), Nickel (Ni), Chromium (Cr), and Arsenic (As) (Baker, 1987). Such species are divided into two main groups: the so-called pseudometallophytes that grow on both contaminated and non-contaminated soils, and the absolute metallophytes that grow only on metal-contaminated and

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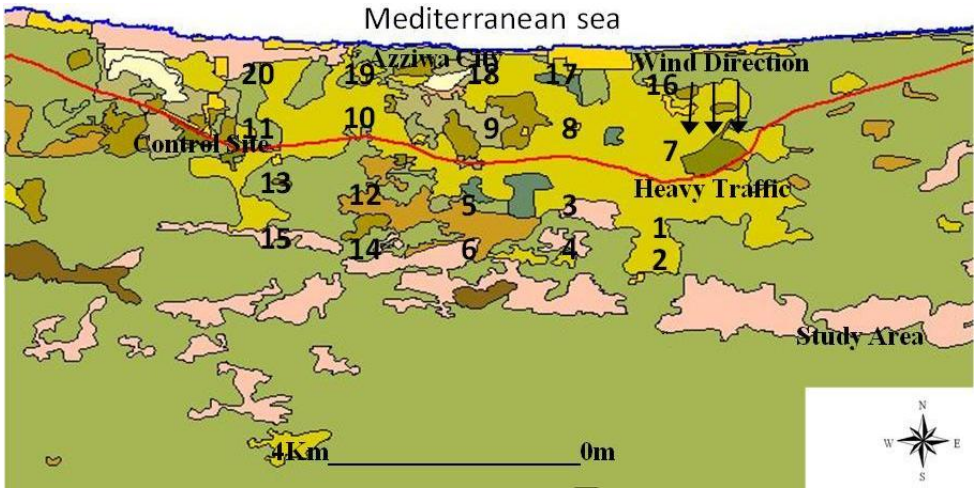

Figure 1. Location of soil sampling sites.

naturally metal-rich soil (Baker, 1987). Phytoextraction technologies are based on the use of hyperaccumulator plants with exceptional metal-accumulating capacity. These plants have several beneficial characteristics such as the ability to accumulate metals in their shoots and an exceptionally high tolerance to HMs (Baker et al., 2000). Baker and Brooks (1989) defined hyperaccumulator plant species as plants which accumulate >1000 mg/kg of Cu, Cobalt (Co), Cr, Ni or Pb, or >10,000 mg/kg of Manganese (Mn) or Zn. Hyperaccumulation of metals has been found in temperate as well as in tropical regions throughout the plant kingdom, but is generally restricted to endemic species growing on mineralised soil and related rock types (Baker and Brooks, 1989).

The capacity to specifically accumulate high amounts of metals in shoots makes hyperaccumulators suitable for phytoremediation purposes. However, various practical drawbacks can reduce the applicability of phytoremediation (Ernst, 1996). Crops with both a high metal uptake capacity and a high biomass production are needed to extract metals from soils within a reasonable time frame (Ebbs and Kochian, 1997; Abou-Shanab et al., 2008). Therefore, the objective of this study was to compare the uptake of Pb, Zn, Cr, and Cu by *E. camaldeulensis*, *B. juncea* and *M. sativum* grown in HM contaminated and uncontaminated soils. The potential use of these species in the phytoremediation of Pb, Zn, Cu and Cr polluted road side soils was also assessed.

MATERIALS AND METHODS

Sampling sites

Twenty sites were selected for the study along the length of the road connecting Triopli, with the southern parts of Libya. These city was chosen based on the high traffic density. Soil samples were collected at different distances from the edge of the main road (3 and 10 m) on both sides, north and south of the road. The distances between each site were about 1 km along the road (Figure 1).

Soil sampling and preparation

Samples were collected in summer to avoid rain washing out the HMs. Five samples of topsoil (0 to 10 cm) depth were collected from each site at the above mentioned distances, with a stainless steel trowel. Soils were mixed in a large container and dried at room temperature, then crushed by hand, sieved through a 4 mm stainless steel sieve to remove rocks and un-decomposed organic materials. Soil mechanical analysis was carried out by the pipette method according to Balck et al. (1982). The percentage of water-holding capacity (WHC) was determined according to the study of Alef and Nannipieri (1995). A glass electrode pH meter and conductivity were used for the determination of soil pH and conductivity. The electrode was immersed in the soil paste with a soil : water ratio of 1:2.5, to avoid errors due to higher dilution (Black et al., 1982). Organic carbon content was measured by the rapid titration method (Nelson and Sommers, 1986).

Cation exchange capacity (CEC) was determined by the method of Thomes (1982). Total nitrogen in soil samples was determined using the Kjeldahl method (Nelson and Sommers, 1980; Jones et al., 1991). Total metals in soil were determined by digesting 0.5 g soil in a mixture of concentrated $HNO_3/HClO_4$ (10: 7, V: V) (McGrath and Cunliffe, 1985), and the water extractable soil metals done by using $CaCl_2$. The total and extractable metal in soil samples were determined by inductively coupled plasma atomic emission spectrometry (ICP-AES) and flame atomic absorption spectrophotometry (AAS).

Pot experiment

To initiate the experiments, about 2 kg of air-dried soils were collected from site number 1 and 2 (Fig. 1) placed into plastic pots (18 cm in diameter and 13 cm in length). Seeds of *B. juncea, M. sativum* and *E. camaldeulensis* were sown in plastic pots which contains heavy metals contaminated soil (site number 1 and uncontaminated soil (site number 13) with four replicates for each treatment. The experiment was carried out in a greenhouse illuminated with natural light. The moisture content of each pot was maintained at 70% WHC by weighing the pots two times per week.

Table 1. Total metal contents and $CaCl_2$ extractable metals (mg/kg) of the studied soils.

Sampling site	Profile (cm)	Co		Cr		Cu		Ni		Cd		Pb		Zn	
		T	E	T	E	T	E	T	E	T	E	T	E	T	E
		Mg/kg dry soil													
Site No. 1 (3 m S)[a]	0 - 10	2	0.02	11	0.12	26	0.30	3	0.03	0.6	0.01	840	8.8	67	0.79
Site No. 13 (3 m S)[b]	0 - 10	0.5	0.01	2.9	0.03	25	0.29	2.1	0.02	0.08	ND	24	0.2	15	0.2

[a]Collected from 3 m south away from roadside; [b]collected from 3 m south away from roadside, T, Total metal; E, extractable metal.

After germination, the seedlings were thinned to two plants per pot and grown for eight weeks.

Plant harvest and analysis

After 8 weeks, plants were gently removed from the pots. Shoot and roots were separated and the lengths of both were measured. Plant shoots and roots were washed with deionized water, rinsed, and dried at 70°C, and the dry matter (DM) measured. Plant materials were ground and 2 g or less of milled plant matter was digested with 3 a mixture of HCl/HNO (4:1, v/v) (McGrath and Cunliffe, 1985), and the HMs (Zn, Cu, Cr and Pb) in the digests were determined using flame atomic absorption spectrophotometry (AAS).. Data were analyzed using Minitab (Version 16 English), significant between variables and were tested by analysis of variance (ANOVA) and means separated by using a Tukey test $P \leq 0.05$.

RESULTS AND DISCUSSION

Physical and chemical characteristics of soil samples

All soils were found to be granular with a sandy texture. Sandy soils are generally poor in nutrient reserves and have low water holding capacity (WHC). Soil moisture influences the chemistry of contaminated soil. Drainage moisture control can influence micronutrient solubility in soils. The moisture content and water holding capacities in soil samples were generally low and ranged from 0.1 to 0.5% and from 7.40 to 9.41%, respectively. Soil pH is one of the most influential variable controlling the conversion of metals from immobile solid-phase to more the mobile and/or bioavailable solution phase .Soils site had a pH of from the (7.5-8.5).

The solubility of heavy metals is generally greater in the pH range of normal agriculture soils. The organic matter (OM) content varied between 0.05% and 0.09%, and cation exchange capacity (CEC) in most of the samples ranged from 7.63 to 9.10 meq 100 g/soil. The behaviour of trace metals in soils depends not only on the level of contamination as expressed by the total metal content, but also on the form and origin of the total metal and the properties of the soils themselves (Tessier and Campbell, 1988; Chlopecka et al., 1996). The physical and chemical properties of the soil influence the form of the metal contaminant, its mobility, and the technology selected for remediation (Gerber et al., 1991). OM and pH are important factors that control the availability of HMs in the soil (Karaca, 2004). In general, soils with high CEC can adsorb larger amounts of HMs than soils with low CEC (Singh et al., 2001).

The total metal content is important because it determines the size of the metal pool in the soil and is thus available for metal uptake (Ibekwe et al., 1995). Therefore the soil samples were analyzed for total and $CaCl_2$ extractable concentrations of Co, Cr, Cu, Mn, Molybdenum (Mo), Ni, Fe, Cd, Pb, and Zn. Results showed that each site exhibited a high concentration of one or two metals. Variation was also recorded in the extractable metal content, that is, biologically available metals in comparison to the total metal content in the same soil. This can be attributed to the behaviour of trace metals in soils that depends not only on the level of contamination, as expressed by the total content, but also on the form and origin of the metal and the properties of the soils themselves (Chlopecka et al., 1996). The bioavailable concentrations of metals extracted by 0.5 M $CaCl_2$ extraction were found to be lower than the total concentration, because there are many factors affecting trace element availability in soils including physical and chemical properties (Alloway, 1995; Pueyo et al., 2003).

The highest values of metal (Pb) were recorded in soil samples collected from 3 m south of the roadside (Table 1). Pb, the element of most concern in environmental HM pollution in Libya, exhibited high levels of contamination closer to highway. Since the fuel used by automobiles in Libya is mostly leaded, the most probable source of such contamination is the Pb particulate matter emitted from gasoline which settles not far from the highway (Harrison and Laxen, 1981). Similar results were found by other investigators (Ho and Tai, 1988; Culbard et al., 1988). As the distance from the road increased, the Pb level decreases. The maximum Pb concentration (840 mg/kg) was detected in a soil sample collected at 3 m south of the road. Therefore, the soil collected from this site was selected to phytoremediate using different plant species.

Effect of heavy metal contaminated soil on plant biomass

The plant species (*B. juncea, M. sativum,* and *E. camaldeulensis*) were chosen for this study based on their high biomass, and their ability to remove heavy

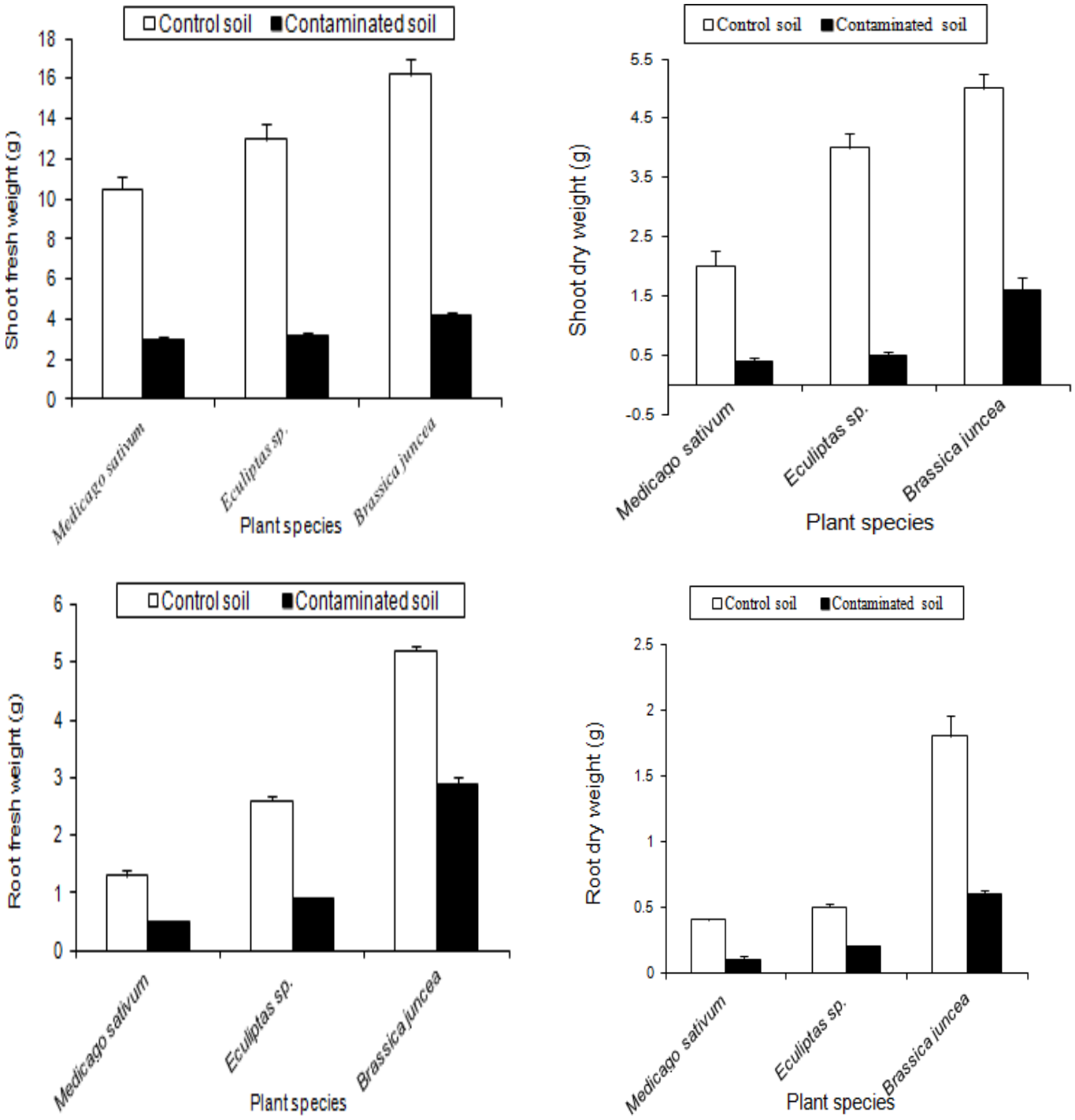

Figure 2. Shoot and root dry and fresh weights of plant species grown in a greenhouse using soil collected from two different sites. Mean values marked with the same letter are not significantly

metals from contaminated sites (Beladi et al., 2011; Turan and Esringu, 2007; Waranusantigul et al., 2011). All three plant species appeared healthy in the low (control) and high Pb contaminated soils. In this experiment, there were higher differences between the three plant species in shoot, root dry and fresh weights (Figure 2). The root dry weights were reduced in all plants grown on Pb contaminated soils compared with the same plants grown on control soils. The root dry weight in *M. sativum, B. juncea* and *E. camaldeulensis* grown in Pb contaminated soils, were reduced by 4, 3 and 2.5-fold lower than the root dry weight of the same

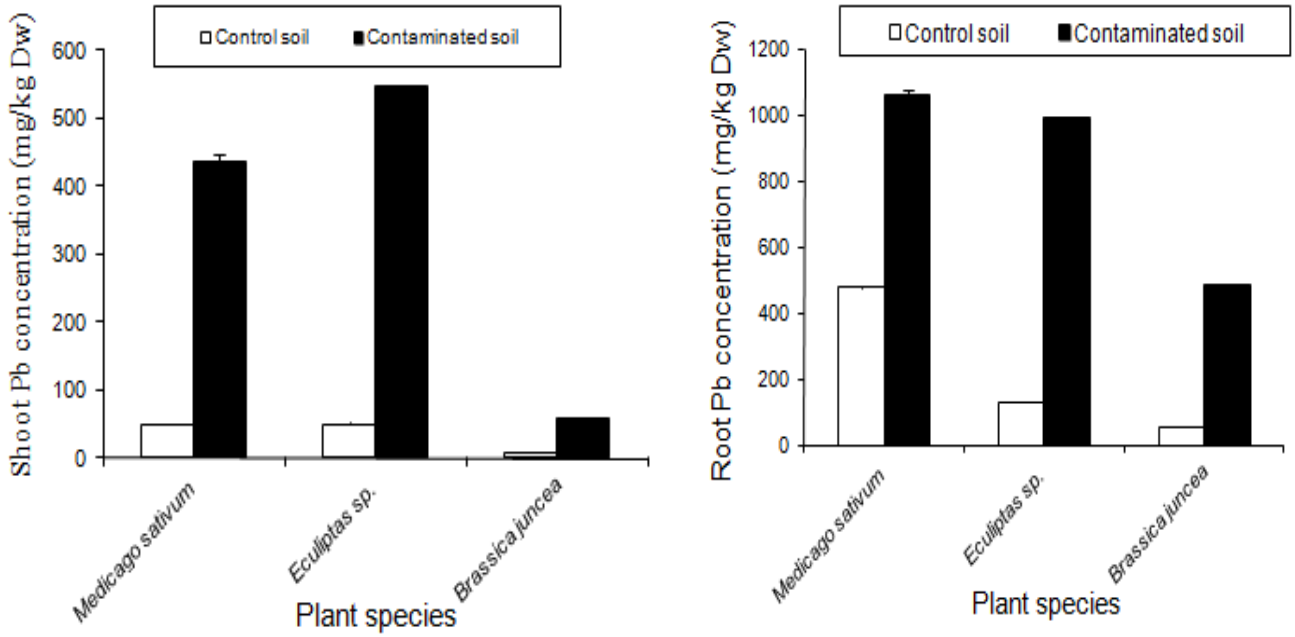

Figure 3. Concentration of Pb in shoot and root of different plant species grown in a greenhouse using soil collected from two different sites. Mean values marked with the same letter are not significantly different $P < 0.05$.

plants grown on the control soil, respectively (Figure 2). The shoot dry weights of *E. camaldeulensis, M. sativum* and *B. juncea* grown on Pb contaminated soils were also reduced by 8, 5 and 3-fold, respectively compared to the same plants grown in the control soil.

The shoot and root fresh weights were also reduced by 3.4, 4 and 3.8-fold and 2.6, 2.8 and 1.8-fold, in *B. juncea*, *M. sativum,* and *E. camaldeulensis*, respectively grown in Pb contaminated soils compared with the same plant species grown in the control soils (Figure 2). Turan and Esringu (2007) reported that the total dry weight of *B. juncea* and canola were affected by the contaminated soil; on average, the metals caused a reduction of about 75% in root and shoot DM of both plants. Similar results in other hyper-accumulator plants were reported by Chen and Cutright (2001), Hajiboland (2005) and Tlustos et al. (2006).

Heavy metal uptake by plant species

Metal concentrations in plant tissues also differed among the three plant species grown on the same soils, indicating their different capacities for metal uptake. Pb concentrations in roots of plants were elevated and varied from 55.8 to 1058 mg/kg dry weight (Dw). The highest concentrations of Pb (548 mg/kg) were found in the shoots of *E. camaldeulensis* grown in Pb contaminated soil. Pb concentrations in the root and shoot of *E. camaldeulensis* grown in contaminated soil was about 11 and 7.5-fold, respectively higher than Pb concentration in the root and shoot of the same plant grown on the control soil. However, the Pb concentration in the roots of *M. sativum, E. camaldeulensis*, *B. juncea,* grown in the Pb contaminated soil was 2.2, 7.5 and 8.6-fold higher than Pb concentrations in the roots of the same plant species grown on control soils (Figure 3), while, the Pb concentration in the shoots of *M. sativum* and *E. camaldeulensis*. *B. juncea* grown on the Pb contaminated soil was 8.7, 11.0 and 8.8-fold higher than Pb concentration in the same plant species grown on control soils. Our results indicated that the highest Pb concentration (1058 and 548 mg/kg) was found in the root of *M. sativum* and shoot of *E. camaldeulensis*, respectively grown in Pb contaminated soil (Figure 3). *M. sativum* and *E. camaldeulensis* accumulated higher Zn concentrations in roots (71 and 86 mg/kg) and shoots (49 and 47 mg/kg), respectively than Indian mustard grown in the same soil. Zn concentrations in the roots of *M. sativum*, *E. camaldeulensis* and *B. juncea* were higher than in the shoots by a factor of 1.4, 1.8 and 1.3-fold, respectively (Figure 4). As shown in Figure 5, the highest Cu concentration (81 and 37 mg/kg Dw) was obtained in root and shoot of *M. sativum* grown on Pb contaminated soil. The highest Cr concentration (133.9 mg/kg Dw) was determined in the root of *E. camaldeulensis* grown on contaminated soil (Figure 6).

Accumulation and translocation of metals in plants

The mobility of the HMs from the polluted substrate into the roots of the plants and the ability to translocate the metals from roots to the harvestable part of the plant

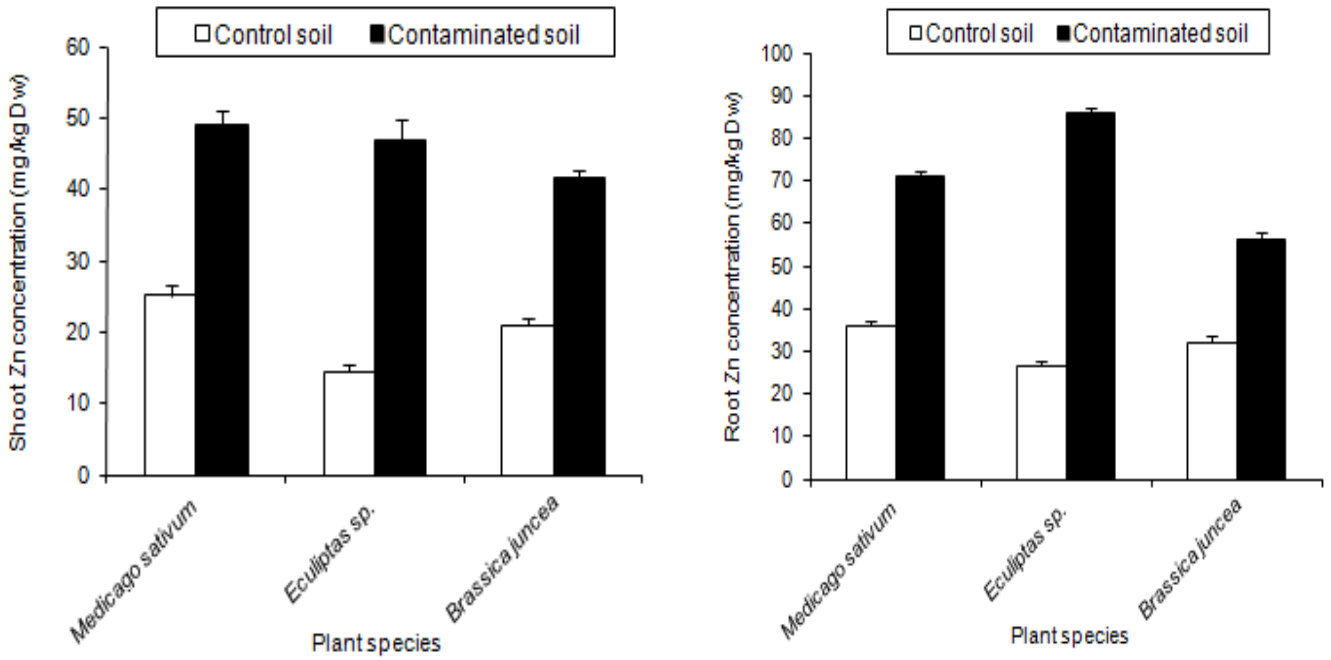

Figure 4. Concentration of Zn in shoot and root of different plant species grown in a greenhouse using soil collected from two different sites. Mean values marked with the same letter are not significantly different $P < 0.05$.

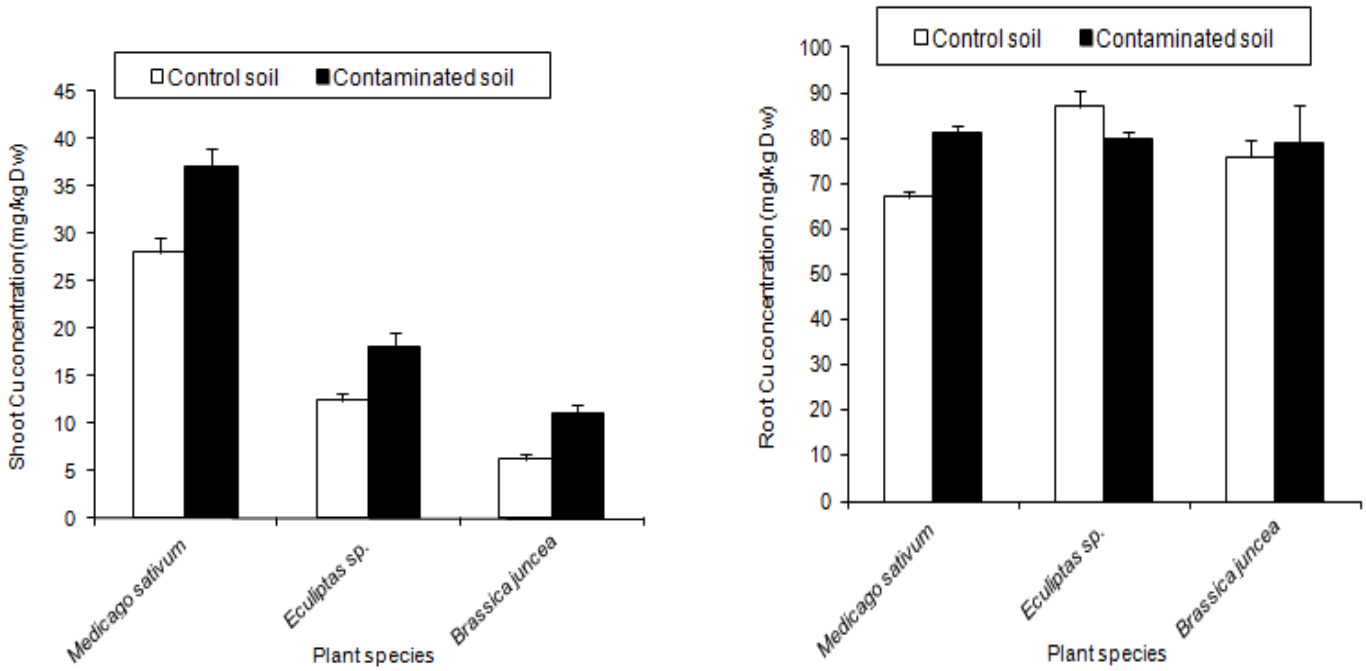

Figure 5. Concentration of Cu in shoot and root of different plant species grown in a greenhouse using soil collected from two different sites. Mean values marked with the same letter are not significantly different $P < 0.05$.

were evaluated, respectively by means of the bioconcentration factor (BCF) and the translocation factor (TF). BCF is defined as the ratio of metal concentration in the roots to that in soil ([metal]root/ [metal]soil). TF is the ratio of metal concentration in the shoots to the roots ([Metal]Shoot/[Metal]Root). The ability of plants to tolerate and accumulate HMs is useful for phytoextraction and phytostabilization purposes (Yoon et al., 2006). Plants with both BCFs and TFs greater than one (TF and BCF> 1) have the potential to be used in phytoextraction.

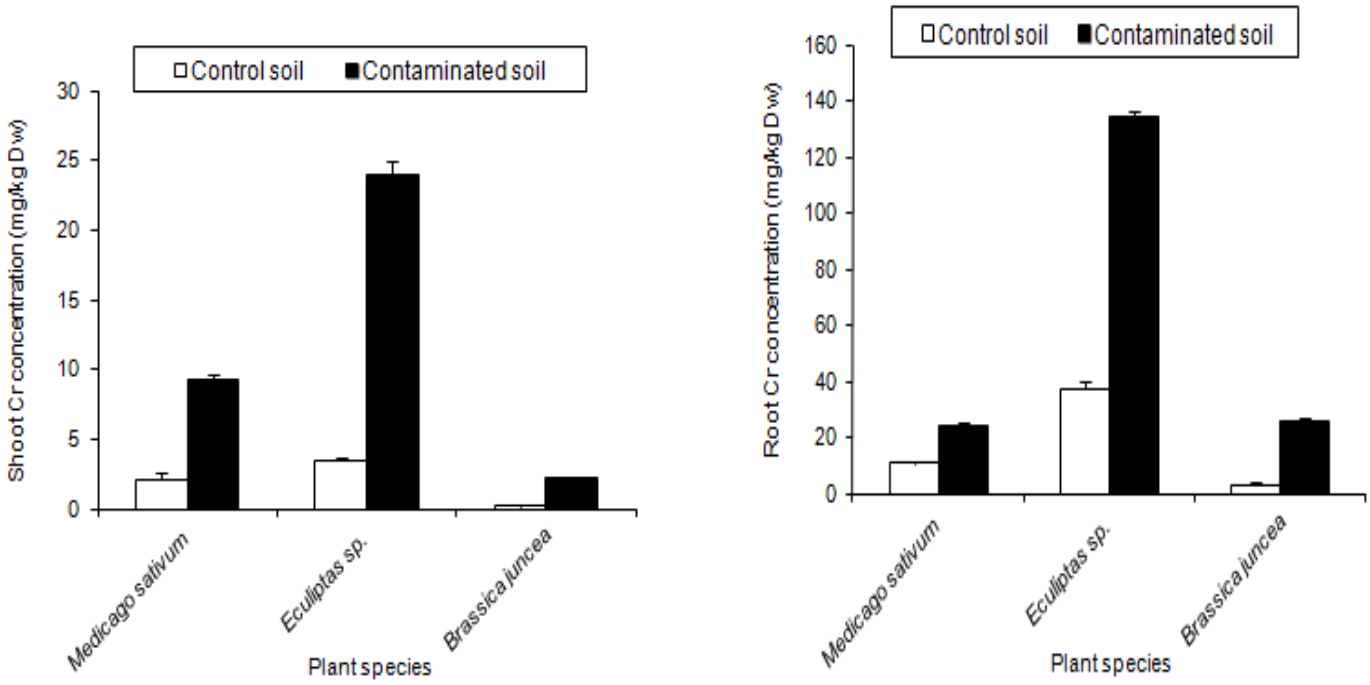

Figure 6. Concentration of Cr in shoot and root of different plant species grown in a greenhouse using soil collected from two different sites. Mean values marked with the same letter are not significantly different P < 0.05.

Table 2. Accumulation and translocation of Pb, Zn, Cu and Cr in plant species grown in metal contaminated soil.

Plant species	Bioconcentration factor (BCF)				Translocation factor (TF)				Enrichment factor (EF)			
	Pb	Zn	Cu	Cr	Pb	Zn	Cu	Cr	Pb	Zn	Cu	Cr
M. sativum	1.4	1	2.7	2	0.9	1.3	0.4	0.4	0.5	0.7	1.1	0.8
E. camaldeulensis	1.1	1.2	3	11	4.1	1.7	0.2	0.1	0.6	0.7	0.7	2.1
B. juncea	0.5	0.8	2.8	2.2	0.9	1.3	0.1	0.08	0.1	0.6	0.1	0.2

*BCF is calculated by relation:- ratio of metal concentration in the roots to that in soil ([Metal]Root/ [Metal]Soil), TF is given by relation:- the ratio of metal concentration in the shoots to the concentration of metal in the roots ([Metal]Shoot/[Metal]Root) and EF is calculated by relation:- the ratio of the concentration of metal in the shoots to the concentration of metal in the soil (Yoon et al., 2006).

Besides, plants with BCF greater than one and TF less than one (BCF> 1 and TF< 1) have the potential for phytostabilization (Yoon et al., 2006). A plant's ability to accumulate metals from soils can be estimated using the BCF and a plant's ability to translocate metals from the roots to the shoots can be measured using the TF. The process of phytoextraction generally requires the translocation of HMs to the easily harvestable plant parts, that is, shoots (Yoon et al., 2006), while phytostabilization process requires the strong ability to reduce metal translocation from roots to shoots (Deng et al., 2004).

By comparing BCF and TF, the ability of different plants in taking up metals from soils and translocating them to the shoots can be compared (Yoon et al., 2006). Among tested plant species, *E. camadulensis* was suitable for phytoextraction of Pb and Zn, while *M. sativum* and *B. juncea* were suitable for phytostabilization of Cu and Cr (Table 2).

Conclusion

Phytoremediation is an emerging technology for the remediation of metal contaminated soils requiring more of hyperaccumulator species, especially for specific metals. The result of this study generally revealed the metal concentrations in the roadside soils at this site, and are in the order of Pb>Zn>Cu>Cr. The level of Pb was high compared with other studies. Accumulation of metal in the soil and subsequent transfer to plants growing along the edge of the road could occur as a result of continual usage of the road by automobiles research in to the patterns of Pb, Zn, Cu and Cr accumulation in *B. juncea*, *M. sativum* and *E. camaldeulensis* growing in Pb

contaminated soils revealed accumulation of Pb, Zn, Cu, and Cr in significantly different quantities in their shoots and roots. Considering the TF and BCF, *E. camadulensis* was suitable for phytoextraction of Pb and Zn, while *M. sativum* and *B. juncea* were suitable for phytostabilization of Cu and Cr.

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Full Length Research Paper

Nutrient enrichment of pineapple waste using *Aspergillus niger* and *Trichoderma viride* by solid state fermentation

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The enrichment by microbial fermentation of agro industrial waste to alleviate their nutritional problems has been proposed but the nutritional value of the subsequent feed for animal consumption has not been fully elucidated. This study investigates whether solid state fermentation of pineapple waste using the fungi *Aspergillus niger* and *Trichoderma viride* could improve its nutrient content. Results show that fermentation of pineapple waste by solid state fermentation using the fungi *A. niger* and *T. viride* significantly ($P < 0.05$) enriches the nutrient content of the waste, particularly increasing the crude protein and ash content while lowering the crude fiber content. The most significant nutrient enrichment was recorded at 72 h of fermentation using *A. niger* and at 96 h of fermentation using *T. viride*. Indiscernible changes were noted in the mineral content of pineapple waste (PW). Dry matter increased significantly ($P < 0.05$) as fermentation progressed with the highest values recorded at 96 h. This study establishes no significant differences ($P > 0.05$) in the fermentation abilities of the two fungi, *A. niger* and *T. viride*. Fermented pineapple waste may be a potential supplement in compounding animal feed provided that it is acceptable and highly digestible.

Key words: Agro industrial waste, crude fiber, crude protein.

INTRODUCTION

Waste disposal represents a serious problem to many agro industries since it is usually prone to microbial spoilage and causes major environmental problems. The utilization of agro industrial waste by conversion into value added products such as animal feed or manure may be an innovative solution to the environmental waste problem. Agro-industrial wastes in recent times have been the focus of research in animal nutrition especially for monogastric animals (Iyayi and Aderolu, 2004; Iyayi and Fayoyin, 2005). In fact, many feeds that can be fed alternatively at cheaper cost to monogastric livestock are based on the use of agro-industrial waste that are of no food value to humans (Iyayi and Fayoyin, 2005).

The use of microorganisms through fermentation to improve nutritional value of agro-industrial wastes, thereby offering the potential to make dramatic contribu-

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tions to sustainable livestock production has been well documented (Iyayi and Aderolu, 2004; Fasuyi, 2005; Iyayi and Fayoyin, 2005). The conventional commercial feeds are becoming expensive to afford, therefore substitutes for conventional feeds such as crop residues and agro-industrial wastes have been proposed as a sustainable means of livestock production (Fasuyi, 2005; Iyayi and Fayoyin, 2005).

When the pineapple fruits are canned or consumed the crown, the outer peel and the central core are discarded as pineapple waste which accounts for about 50% of the total pineapple fruit weight corresponding to about ten tons of fresh pineapple or one ton of dry pineapple waste per hectare (http://www.fao.org). Pineapple wastes are recommended as tremendous sources of organic raw materials and are potentially available for conversion into useful products such as animal feeds (Hemalatha and Anbuselvi, 2013). Pineapple waste contains high amounts of crude fiber and suitable sugars for growth of microorganisms (Hemalatha and Anbuselvi, 2013).

The utilization of fungi for nutrient enhancement in agro-industrial waste by fermentation has been studied for years and their efficiency shown in substrates such as lignin, cellulose and hemi cellulose polymers found in agro-industrial waste (Howard et al., 2003) A*spergillus niger* and *Trichoderma viride* have been successfully used in a number of fermentation studies towards solid waste management, biomass energy conservation and production of secondary metabolites in various agro-industrial wastes (Omojasola et al., 2008; Femi-Ola et al., 2009; Kareem et al., 2010).

In recognition of this potential, this study was conducted to investigate the use of fungi; *A. niger* and *T. viride* for nutrient enrichment of pineapple waste by solid state fermentation for possible use as animal feed supplement. It is hoped that the results shall provide more knowledge on the application of microorganisms in recovering pineapple waste through fermentation for environmental sustainability and utilization in animal feeds.

MATERIALS AND METHODS

Fungi

The fungi used were isolated pure strains of *A. niger* and *T. viride* grown on SARBORAUD agar medium. Slants of the microbes were obtained from the culture bank of the Department of Veterinary Pathology, Parasitology and Microbiology, University of Nairobi, Kenya. The fungi were then sub cultured in CZAPEK dox broth media, and incubated at 37°C for four days. The grown fungal spores were then maintained in a refrigerator set at 5°C.

Solid state fermentation

Pineapple waste (peelings and core) of 90 g portions were weighed into twenty one 500 ml beakers, covered with aluminum foil and autoclaved at 121°C for 15 min. After sterilization, the samples were then spread on separate foil paper trays in uniform layers of 1 cm thick on open racks allowing sufficient aeration. A set of nine beakers were aseptically inoculated with 15 ml of 2% fungal spore suspension of *A. niger,* another set of nine with *T. viride* and three uninoculated. Sets of three samples were withdrawn after 48, 72 and 96 h, dried at 60°C in an oven for 72 h. The samples were then milled and stored in tightly sealed 100 g plastic bottles. Samples from the three control beakers were treated alike.

Analysis of samples

The nutrient content of the samples were determined quantitatively by proximate analysis and specific mineral analysis. Analysis was done in triplicate for each sample. Proximate analysis was conducted using the methods of AOAC (1998). The specific minerals were determined by the methods outlined by Okalebo, Gathua and Woomer (1993). All data were presented as the mean standard values of three replicates and subjected to a one way analysis of variance (ANOVA) and post ANOVA. Significance was accepted at $P \leq 0.05$.

RESULTS AND DISCUSSION

Results in Table 1 show significantly ($P < 0.05$) higher crude protein content in pineapple waste fermented using the fungi *A. niger* and *T. viride* than in the unfermented pineapple waste for the 48, 72 and 96 h fermentation periods. The post fermentation increase in crude protein content could be attributed to the possible secretion of some extra cellular enzymes (protein) such as amylases, xylanases and cellulases into the pineapple waste mash by the fermenting fungi in an attempt to make use of the carbohydrates in the mash as a carbon and energy source (Raimbault, 1998). *A. niger* has been reported to have high specific activity for cellulases and hemi-cellulases (Howard et al., 2003). Additionally, *T. viride* and *A. niger* have found use in the production of extra cellular enzymes including cellulase, amylase and xylanase (Nair et al., 2008).

Fungi colonize substrates for utilization of available nutrients. They synthesize and excrete high quantities of hydrolytic extra cellular enzymes, which catalyze the breakdown of nutrients to products that enter the fungal mycelia across cell membrane to promote biosynthesis and fungal metabolic activities leading to growth (Raimbault, 1998). Therefore, increase in the growth and proliferation of fungal biomass in the form of single cell protein (SCP) or microbial protein accounts for part of the increase in the protein content after fermentation (Raimbault, 1998).

Earlier findings by Guerra et al. (1986) reported similar significant ($P < 0.05$) increase in crude protein content in pineapple waste fermented for 72 and 96 h by liquid state fermentation method using fungi, *A. niger*, *T. viride* and *Myrothecium verrucaria*. Similar findings have been reported with the same methods and fungi using

Table 1. Proximate composition of fermented pineapple waste samples (g / 100 g).

Nutrient content	Unfermented	Fungi	Fermented samples		
			48 h	72 h	96 h
Crude protein	3.69 ± 0.05	*A. niger*	4.53 ± 0.19[a]	10.28 ± 0.14[bd]	8.89 ± 0.28[cef]
		T. viride	4.44 ± 0.14[a]	8.32 ± 0.01[bd]	9.04 ± 0.16[cef]
Ash	2.61 ± 0.23	*A. niger*	5.14 ± 0.03[a]	4.18 ± 0.23[b]	4.79 ± 0.08[c]
		T. viride	4.95 ± 0.06[a]	5.64 ± 0.55[b]	6.48 ± 0.12[c]
Crude fiber	10.80 ± 0.10	*A. niger*	14.09 ± 0.01[a]	5.78 ± 0.01[bd]	2.49 ± 0.01[cef]
		T. viride	9.09 ± 0.01[a]	8.04 ± 0.04[bd]	7.59 ± 0.08[cef]
Dry matter	82.13 ± 0.46	*A. niger*	89.74 ± 0.22[a]	88.81 ± 0.41[b]	90.39 ± 0.27[c]
		T. viride	87.24 ± 0.13[a]	88.16 ± 0.05[b]	90.84 ± 0.41[cef]
Carbohydrate	75.83 ± 1.12	*A. niger*	80.07 ± 0.22[a]	74.36 ± 0.09[d]	76.71 ± 0.05[e]
		T. viride	77.85 ± 0.08	74.20 ± 0.85	75.32 ± 0.33

Values are expressed as mean ± SEM (n = 3) for different determinations. [a]Significant difference of values between 0 and 48 h; [b]significant difference of values between 0 and 72 h; [c]significant difference of values between 0 and 96 h; [d]significant difference of values between 48 and 72 h; [e]significant difference of values between 48 and 96 h; [f]significant difference of values between 72 and 96 h. Significant difference determined at $P < 0.05$.

substrates such as cassava waste (Pothiraj et al., 2006), wheat offal (Iyayi, 2004), maize offal, palm kernel meal (Iyayi and Aderolu, 2004) and rice bran (Oshoma and Ikenebomeh, 2005).

The results in Table 1 indicate significant changes in crude protein content of fermented pineapple wastes at the three fermentation periods in the order 48 < 72 < 96 h using *T. viride* and 48 < 96 < 72 hours using *A. niger*. This is attributed to the increased hydrolytic enzyme activity with prolonged fermentation and increased fungal biosynthesis resulting in increased fungal biomass hence crude protein (Raimbault, 1998). Significantly ($P < 0.05$) lower crude fiber content was recorded in the fermented pineapple waste using both fungi compared to the unfermented pineapple waste for the three fermentation periods (Table 1). The ability of fungi to degrade crude fiber has been reported by several workers (Iyayi and Aderolu, 2004; Iyayi, 2004).

The fermentation process in addition to enriching the substrate with protein also releases oligosaccharides and simple sugars into the medium as a result of microbial degradation of otherwise unavailable polysaccharides (Duru and Uma, 2003). This suggests the production of hydrolytic enzymes in the fermentation culture by fungi that enable them to metabolize complex carbohydrate polymers (Duru and Uma, 2003; Oboh, 2006). This could explain the decrease in crude fiber content. The significant decrease in crude fiber was noted to coincide with significant increase in crude protein. Other researchers have reported similar findings (Duru and Uma, 2003; Oboh, 2006).

The carbohydrate content of which crude fiber is a constituent, acts as the carbon source for the growing microbes hence its depletion results from its utilization to produce fungal biomass, which is microbial protein or SCP (Raimbault, 1998). Despite the degradation of crude fiber by the fermenting fungi, no significant change in carbohydrate content in pineapple waste (Table 1) was recorded at 72 and 96 h of fermentation using A. *niger* and 48, 72 and 96 h using *T. viride,* as expected since fungi metabolize complex sugars to simple sugars. This is attributed to the ability of fungi to further hydrolyze the simple sugars for use as a carbon source to synthesize fungal biomass rich in protein (Oboh, 2006).

Dry matter content (Table 1) was significantly ($P < 0.05$) high in all the fermented samples compared to the unfermented samples. This is in line with the significant ($P < 0.05$) increase in protein content in all the fermented samples compared to the unfermented. This is attributed to the increased fungal biomass in the fermented samples because of inoculation and growth of the fungi in the fermented compared to the unfermented samples. The insignificant changes in dry matter levels at the 48, 72 and 96 h fermentation periods may be explained by the equal utilization and biosynthesis of nutrient during fermentation hence the lack of significant changes on summation.

There were no discernible trends (Table 2) reported in the specific mineral content (calcium, phosphorous and magnesium) in the fermented samples compared with the unfermented. Despite this, a significant increase in ash content was recorded in all fermented samples using both fungi when compared to the unfermented. This may suggest the introduction of specific mineral(s) by the inoculums that were not investigated. There were no significant differences ($P > 0.05$) in the fermentation abilities of the fungi, *A. niger* and *T. viride* with regard to pineapple waste fermentation and fermentation of pine-

Table 2. Minerals composition of fermented pineapple waste (mg/g).

Nutrient	Unfermented sample	Fungi	Fermented samples		
			48 h	72 h	96 h
Calcium	12.83 ± 0.03	*A. niger*	21.54 ± 0.01[a]	10.32 ± 0.04[d]	11.99 ± 0.22[e]
		T. viride	12.50 ± 0.14	9.82 ± 0.03	2.63 ± 0.01[cef]
Phosphorous	1.2 2 ± 0.02	*A. niger*	0.70 ± 0.02	1.92 ± 0.12	2.55 ± 0.02
		T. viride	1.69 ± 0.03	1.99 ± 0.05	1.87 ± 0.05
Magnesium	1.15 ± 0.00	*A. niger*	1.20 ± 0.00[a]	1.05 ± 0.00[bd]	1.47 ± 0.00[cef]
		T. viride	1.17 ± 0.00[a]	1.13 ± 0.00[bd]	1.51 ± 0.00[cef]

Values are expressed as mean ± SEM (n = 3) for different determinations. [a]Significant difference of values between 0 and 48 h; [b]significant difference of values between 0 and 72 h; [c]significant difference of values between 0 and 96 h; [d]significant difference of values between 48 and 72 h; [e]significant difference of values between 48 and 96 h; [f]significant difference of values between 72 and 96 h. Significant difference determined at $P < 0.05$.

apple waste for periods of 72 and 96 h are equally viable as no significant differences ($P > 0.05$) were established when assessing the amount of yield obtained at these two times. The significant ($P < 0.05$) increase in protein content of the pineapple waste after fermentation with *A. niger* and *T. viride* and the significant decrease ($P < 0.05$) in crude fiber concludes that fermentation of pineapple waste by solid state fermentation using the fungi *A. niger* and *T. viride* enriches the nutrient content of the waste and this by product could be good supplement in compounding animal feed provided that it is acceptable and highly digestible. Future fermentation studies using pineapple waste as substrate should be planned with improvements in the fermentation technique by including substrate pretreatment to convert the raw substrate into a more suitable form to increase its utilization by the fermenting microorganism and control of parameters such as pH which have been suggested to slow down the fermentation process (Iyayi, 2006). Further research is also proposed to determine appropriate incubation periods for optimal fermentation results.

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Effects of ethanol extract of *Cissus quadrangularis* on induced gastric ulcer in rats

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Antiulcer activities of the ethanol extract of *Cissus quadrangularis* roots on indomethacin and ethanol-induced gastric ulcers were investigated. The results obtained show that the ulceration in gastric linings of the stomach of rats pre-treated with the *C. quadrangularis* extract before induction with ethanol and indomethacin decreased significantly when compared to the control. The protective effect of the extract increased in a dose-dependent manner in both ulcer models. There were significant decreases ($p < 0.05$) in the number of ulcer lesions when rats were administered with the graded doses of the extract and ranitidine (100 mg/kg body weights) compared with the control groups in both models. Results from this study suggest that the extract of *C, quadrangularis* roots possesses antiulcer activities.

Key words: *Cissus quadrangularis,* ranitidine, indomethacin, ethanol and ulcer lesions.

INTRODUCTION

The use of plants and its extracts have aided mankind in the treatment of different ailments including infectious and non-infectious diseases for many years. Over the decades, researchers have been exploring the biodiversity of the plant kingdom to find new and better drugs that could cure many diseases that are afflicting human population (Abdul et al*.,* 2009 ; Omale and Okafor, 2008; Shanthi et al., 2010; Anoop and Jagdeesan, 2006; Jainu and Devis, 2004; Jainu and Devis, 2003).

Several natural products, mostly of plant origin have been shown to possess promising activities that could assist in the prevention and/or amelioration of diseases such as human immunodeficiency virus / acquired immunodeficiency syndrome (HIV/AIDS), malaria, tuberculosis,among others. Many of these agents have other medicinal values as well, which afford them further prospective as novel, which leads to the development of new drugs that could deal with both viruses and other diseases (Adebayo and Kretti, 2011).

According to the World Health Organization (2004), about three-quarters of the world population rely on plants for the treatment of many illnesses and useful drugs have been developed from plants used in traditional medicine which aspects of toxicity and efficacy may be known from the long history of usage.

In the traditional system of medicine, various plants parts such as stem back, root back, aerial root, vegetative bud, leaves, fruits and latex are used in the treatment of variety of ailments (Davies and Evans, 2008).

Most of the clinical drugs that are currently in use were derived from plants and developed because of their usage in traditional medicine. Aspirin (anti-pyretic), atropine, digoxin, morphine (pain killer), quinine and so on, were discovered through the study of ethno-botany.

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Medicinal plants are of great importance to the health of individuals and communities.

The medicinal value of these plants lies in some chemical substances that produce a definite physiological action on the human body. The most important of these bioactive constituents of plants are used as spices and food plants. They are also sometimes added to foods meant for pregnant and nursing mothers for medicinal purposes, and some of these medicinal plants used in herbal medicine in South Eastern Nigeria include Cleome *rutidosperma, Emilia coccinea, Euphrobia heterophylla, Physcalis bransilensis, Sida acuta, Spigelia anthelmia, Stachytarpheta caynnensis and Tridxa procumbens* (Okwu, 2001).

Cissus quadrangularis is a low growing shrub with a characteristic, four sided stem. It is a climbing plant, often found growing over the lower growing vegetation. *C. quadrangularis* has a slender stem with varying length. It is dichotomously branched sub-angular, glabrous, brown fleshy, fibrous, smooth with four-winged internodes constricted at nodes. *C. quadrangularis* reaches a height of 15 cm and has quadrangular-sectioned branches with internodes of 8 to 10 cm long and 1.2 to 1.5 cm wide. Along each angle is a leathery edge, toothed leaves 2 to 5 cm wide appear at the nodes.

Each has a tendril emerging from the opposite side of the node (Attawish et al., 2002; Jainu and Devi, 2004; Jainu et al., 2006; Shanthi et al., 2010; Anoop and Jagdeesan, 2006; Jainu and Devis, 2003). Extracts of *C. quadrangularis* roots have been used traditionally in the treatment of inflammation and pain in which the plant functions as an anti-inflammatory agent and analgesic (Shanthi et al., 2010; Anoop and Jagdeesan, 2006; Jainu and Devis, 2004; Jainu and Chennam, 2006; Jainu and Chennam, 2005; Jainu and Devis, 2003; Evans, 2001; Attawish et al., 2002). The aim of this study was to investigate the therapeutic effects of ethanol root extract of *C. quadrangularis* against indomethacin and ethanol-induced ulcer in rats.

MATERIALS AND METHODS

Animals

Adult albino rats (120-200 g) of either sex were used for the experiment. They were procured from the animal house of the Faculty of Agricultural Sciences, University of Nigeria, Nsukka. The animals were acclimatized in metal cages for one week in the animal house of Department of Biochemistry, University of Nigeria, Nsukka, prior to analysis.

Plants

Roots of *C. quadrangularis* were obtained from Mr. A. Ani of BDCP (Bioresource Development and Conservation Program) Nsukka. The leaves were identified and authenticated by the officer in-charge of the *hebarium* in the Botany Department, University of Nigeria, Nsukka. The roots were dried under the sun for two weeks until they were completely dried and then milled to a coarse powder with an electric milling machine.

Instruments/apparatus

The following instruments/apparatus were used in the current study: Chemical balance (Gallenkamp England), plastic container (Gallenkamp England), filter paper (Whatman), electric blender machine, (Gunagztion, China), measuring cylinder (Pyrex, England), beakers (Pyrex, England), refrigerator (Kelvinator, Germany), metal cages, Petri-dish, dissecting kit (Stainless, Japan), cotton wool (Neomedic Ltd, Thailand), hand gloves (DANA JET, Nigeria), syringes, rotary evaporator, razor (Czech Republic), scissors (steel, China), magnifying glass and plastic funnel

Analytical chemicals/reagents

Analytical chemicals/reagents used included: ethanol (absolute) (Sigma-Aldrich, Switzerland), distilled water (Energy Research Center, UNN), ethanol (analytical) (Sigma-Aldrich, Switzerland), chloroform (Sigma-Aldrich, Switzerland), sodium chloride (BDH Chemicals, England), inodmethacin (Emzor Pharmaceuticals, Nigeria) and ranitidine (Emzor Pharmaceuticals, Nigeria).

Extraction procedure

The fresh roots of *C. quadrangularis* were dried under the sun for two weeks and the dried roots were reduced to powder with an electric blending machine. 1.25 kg quantity of the pulverized roots was obtained and then this stock quantity was macerated with ethanol and allowed to stay for 24 h after which filtration was done using Whatman filter paper. The filtrate was concentrated in beakers with the aid of a rotary evaporator and water bath at reduced temperature. After concentration, a crude brownish semi-solid substance weighing 52.6 g was obtained. The substance was then preserved in a small container (film container), covered in a water-proof and then kept inside a cupboard at stable room temperature until when needed.

Determination of the weight of the extract

After concentration, the weight of the beaker with the extracted material inside it was measured using a sensitive weighting balance. The actual weight of the extract was obtained by subtracting the original weight of the beaker from that of the weight of beaker plus extracted material.

Determination of the percentage yield of the extract

The percentage yield of the extract is calculated as follows:

$$Percentage\ (\%)\ yield\ of\ the\ extract = \frac{Weight\ of\ extract\ X\ 100}{Weight\ of\ pulverized\ leaf}$$

Determination of the acute toxicity of ethanol extract of *C. quadrangularis*

The acute toxicity studies were investigated in mice following Pihan et al. (1987) method with slight modification. Sixteen (16) albino mice of either sex were used. They were divided into four groups of

four mice each. Group one received 100 mg/kg body weight of the extract. Group two mice were treated with 1500 mg/kg body weight of the extract. Group three received 2500 mg/kg body weight of the extract and Group four mice were treated with 5000 mg/kg body weight of the extract using the dosage control formula given as:

$$Dosage\ control\ formula = \frac{mg\ of\ drugs/kg\ B.W}{1000}\ X\ \frac{Weight\ of\ animal\ (g)}{conc.\ of\ drug\ (mg/ml)}$$

After the administration, the animals were left for 24 h after which the number of death were observed and recorded in each group.

Determination of the effect of the extract on indomethacin-induced ulcer in rat stomach

Twenty (20) albino rats of either sex were fasted for 24 h with access to water following the method of Agrawal and Dajani (1993) with slight modification. At the end of the fasting period, the animals were weighed using weighing balance (triple beam) and their weights (120-194 g) determined. They were grouped into five (5) main groups with four rats each and were treated orally as follows: Group 1 (control) were administered with 0.9% normal saline (50 ml/kg) and indomethacin (50 m/kg). Group 2 were treated with 100 mg/kg body weight of the extract and indomethacin (50 mg/kg). Group 3 received 200 mg/kg body weight of the extract followed by inodmethacin (50 mg/kg). Group 4 received 400 mg/kg body weight followed by indomethacin (50 mg/kg). Group 5 received standard drug (ranitidine) 100 mg/kg and indomethacin (50 mg/kg) using the dosage control formula described above.

After the administration, the animals were left for 8 h after which they were sacrificed by chloroform anesthesia in an air-eight plastic container and then dissected. There stomach were removed by cutting from the oesophagoal and pyrolic ends and were operated by cutting along the greater curvature. The contents were removed and the stomach washed with distilled water. The stomach was spread over a dissecting board and the ulcer lesions in the glandular potion were determined with the aid of a hand lens (x10). The lesions were coded as n_1, n_2 and n_3 representing n≤1mm, 1<n≤2mm and 2<n≤3 mm as described by Trease and Evans(1983).

Ulcer indices (mm)

Individual ulcer index (mm)

This can be defined as the total number of ulcerations found in each rat. It is denoted by $N=n_1, + n_2 + n_3 + n_4$.

Group ulcer index (GUI) (mm)

This is the sum of all the individual ulcer indices in a group. It is denoted by $TN = N_1 + N_2 + N_3$. Where, N_1, All n_1 in the group; N_2, all n_2 in the group; N_3, all n_3 in the group; N_4, all n_4 in the group.

Mean ulcer index (MUI) (mm)

This is simply the average of the ulcerations found in a group. It is obtained by dividing the total number of individual ulcerations in a group (GUI) with number of rats in that group.

Percentage inhibition (PI)

With respect to this study, the percentage inhibition can be calculated using the formula:

% Ulcer inhibition (%UI) = (1-Ut/Uc) X 100

Where, Ut represents the ulcer index of the treated group and Uc represents the ulcer index of the control group.

Effect of the extract on ethanol-induced ulcer in rats stomach

This determination was carried out using the method of Pihan et al. (1987). 15 adult rats were randomly divided into five groups of three rats each. The rats were deprived of food for 2 h and treated orally with normal saline and varying doses of the plant extracts. The extracts and drugs used were freshly prepared as a suspension in normal saline and administered orally (PO) to the animals in 5 ml/kg doses. Group I (normal control) was administered normal saline (5 ml/kg). Group II, II and IV were treated with 100, 200 and 400 mg/kg of the plant extracts, respectively. Group 5 (positive control) was administered 100 mg/kg of ranitidine, a standard anti-ulcer drug. Each animal received 1 ml of absolute ethanol orally 30 min later, the animals were sacrificed 4 h after administration of the ethanol, and their stomach removed and opened along the greater curvature. The stomach was rinsed with water, pinned flat on a board, examined with a hand lens (X10) and scored for ulcer. The total ulcer scores and ulcer indices for the groups were obtained and used to calculate the percentage ulcer inhibition as shown above using Trease and Evans (1983) method.

RESULTS

Extraction

After the extraction and concentration, the weight of the extract was 52.6 g from the 1.25 kg of the powdered roots of *C. quadrangularis* and the percentage yield was calculated using the formula shown below:

$$Percentage\ (\%)\ yield = \frac{Weight\ of\ extract\ X\ 100}{Weight\ of\ pulverized\ leaf}\quad \frac{52.6g}{1025g} = 5.13\%$$

Acute toxicity of the extract

From the results shown in Table 1, no mortality was observed in all the groups of mice that were given *C. quadrangularis* orally after 24 h of treatment. Therefore, the LD_{50} value of *C. quadrangularis* was estimated to be above 5000 mg/kg body weight. This results show that ethanol extract of *C. quadrangularis* is relatively safe.

Protective effect of the extract on indomethacin-induced gastric ulceration in rats

The results presented in the Table 2 and Figure 1 below indicate that pretreatments with test extracts reduced the ulceration markedly. The percentage inhibition of ulceration by the text extracts were 70.15, 82.00 and 83.10% at 100, 200 and 400 mg/kg doses, respectively which is comparable to that of standard anti-ulcer

Table 1. The acute toxicity (LD_{50}) of the extract.

Group	Mice used	Dose (mg/kg)	Dead (%)
1	4	100	0
2	4	1500	0
3	4	2500	0
4	4	5000	0

Table 2. The protective effect of the extract on indomethacin-induced ulcer.

Group	Number of rats	Mean ulcer index (MUI) (mm)	Percentage inhibition (%)
1 (Normal control)	4	2.50±0.5439	-
2 (100 mg/kg)	4	0.75±0.3862	70
3 (200 mg/kg)	4	0.45±0.0577	82
4 (400 mg/kg)	4	0.4±0.0816	85

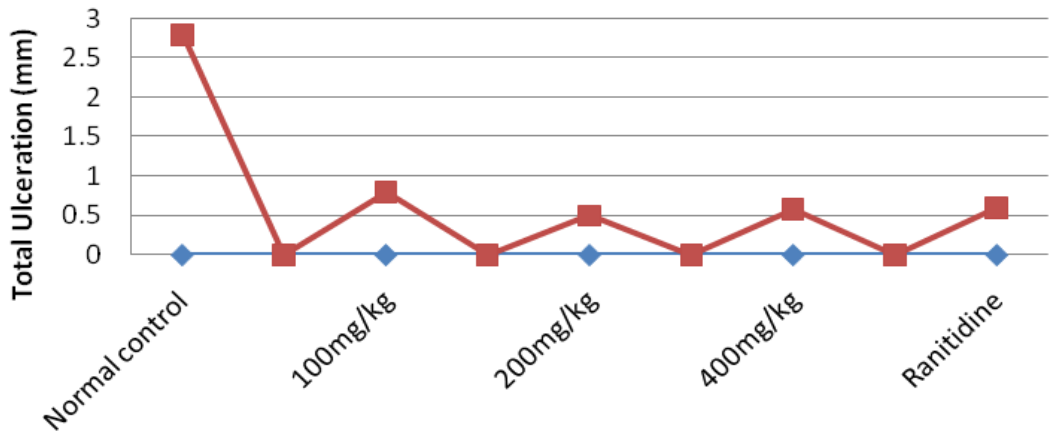

Figure 1. The effect of extract on indomethacin induced ulcer in rats.

drug ranitidine (100 mg/kg) as shown in Table 2. Results in Figure 1 and Table 2 show a significant increase ($p < 0.05$) in the ulceration level of the control group administered with normal saline as compared with the ulceration level of groups [3(200 mg/kg), 4(400 mg/kg) and 5 (ranitidine)]. But there was no significant difference in the ulceration level of group 2 (100 mg/kg) when compared with groups [3(200 mg/kg), 4(400 mg/kg) and 5 (ranitidine)].

Protective effect of the extract on ethanol induced gastric ulceration in rats

The results obtained in ethanol induced gastric ulcer as shown were comparable to that obtained in indomethacin-induced ulcer. The extract proved to be more efficient on indomethacin-induced ulcer than in the ethanol-induced ulcer. This was observed in ulcer lesions inside the rat stomach where the dark spot-like lesions induced by indomethacin reduced more readily than the reddish lesions that were induced by ethanol. From Table 3, the percentage (%) inhibition of the ulceration induced by ethanol by the test extracts are 30, 53, and 57% for 100, 200 and 400 mg/kg B.W., respectively in dose dependent manner. Also, from Figure 2 and Table 3, the result obtained shows that ulceration in groups 3 (200 mg/kg), 4 (400 mg/kg), and 5 (ranitidine) significantly decreased ($p < 0.05$) compared to group 1 (normal control). There was no significant difference ($p < 0.05$) in the level of ulceration in group 2 when compared with group 1 (normal control).

DISCUSSION

Ulceration of body tissue lining such as the gastric lining of the stomach by the non-steroidal anti-inflammatory

Table 3. The protective effect of the extract on ethanol-induced gastric ulcer in rat.

Group	Number of rats	Mean ulcer index (MUI) (mm)	Percentage inhibition (%)
1 (Normal control)	3	2.86±2.76666	-
2 (100mg/kg)	3	2.33±2.3333	30
3 (200mg/kg)	3	1.33±1.3333	53
4 (400mg/kg)	3	1.20±1.20000	57
5 (ranitidine)	3	1.23±1.3333	60

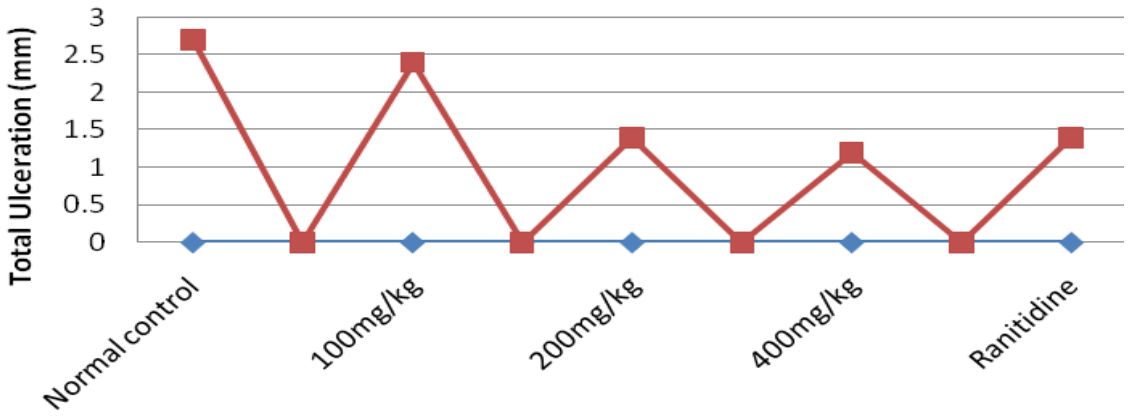

Figure 2. The effect of extract on ethanol-induced ulcer in rats.

drugs (NSAID) which includes indomethacin, ibuprofen could be as a result of inhibition of the cyclooxygenase I (COX-I) enzyme by their metabolic action in order to suppress inflammatory diseases (Agrawal and Dajani, 1993), but the concern over severe side effects of these drugs has led to the search for new antiulcerogenic agents from plants with low toxicity and minimal side effects (Curtis and Griffin, 1991). Previous study on *C. quadrangularis* root extracts showed that the root contains carbohydrates and minerals like sodium, potassium, iron and calcium and also some bioactive components like saponin, flavonoids, glycosides, tannins and phenolic compounds (Enechi and Odonwodo, 2003) and these bioactive compounds could be responsible for its anti-ulcer healing.

The present research has provided first hand information on acute toxicity and the protective effects of the plant extract on indomethacin and ethanol-induced gastric ulceration in rats. The acute toxicity study revealed that the plant is relatively not toxic to the experimental animals and could be used in medical treatments. The results from Table 2 and Figure 1, show that indomethacin (50 mg/kg) that induced ulcer in the stomach of the rats was qualitatively antagonized in a dose-dependent fashion as observed in groups 2, 3 and 5 that were administered with 100, 200 and 400 mg/kg body weight of the extract, respectively.

Also, the results show that the ulceration rate was significantly higher ($p < 0.05$) in the group administered with normal saline (control) compared with the positive control group and test groups. This indicates that the level of ulceration in negative control group is high due to the absence of antiulcerogenic agents and this agrees with the findings of Kabe and Kutimu (1994), who observed the same effect on the negative control group. Also, from Table 3 and Figure 2, the level of ulceration induced by ethanol was also antagonized dose-dependently by the extracts but the level of inhibition was higher in indomethacin induced ulceration compared to that of the ethanol induced ulcer, though both are comparable.

Conclusion

The results shown in this work suggest that ethanol root extract of *C. quadrangularis* has the potential efficacy of protecting the stomach linings against ulceration induced by both indomethacin and ethanol in rats.

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Measurement of β-glucan and other nutritional characteristics in distinct strains of *Agaricus subrufescens* mushrooms

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β-Glucan is a polysaccharide with anticancer properties, and it is present in the mushroom *Agaricus subrufescens*. The aim of this study was to compare two analytical methods to quantify β-glucan in mushrooms and analyze the nutritional characteristics, the concentration of phenolic compounds and the mineral composition, for *A. subrufescens* strains. An enzymatic extraction was performed, and β-glucan was quantified by spectrophotometry and high performance liquid chromatography (HPLC). No significant differences were found between the two methods of quantification of β-glucan. The CS7 and CS10 strains were found to have greater amounts of β-glucan, similar to the levels found in *Pleurotus eryngii* and *Pleurotus-sajor-caju*. *Pleurotus ostreatus* and *Lentinula edodes* samples contained even higher amounts of β-glucan. Significant differences were found in the nutritional characteristics for all parameters assessed, except for fat content. There was no difference between the strains with regard to phenolic compounds or certain mineral components. Fresh *A. subrufescens* mushrooms are not considered to be a high-protein food. However, they are a good source of fiber and minerals, in addition to being low in fat. Spectrophotometry presents the advantages of being quicker and lower in cost; this technique may be suggested as the standard for measurement of the β-glucan.

Key words: β-Glucan, medicinal mushroom, royal sun mushroom, almond portobello.

INTRODUCTION

The nutritional quality of edible and/or medicinal mushrooms may vary according to species, cultivation substrate, environmental conditions and strain (Bonatti et al., 2004; Fan et al., 2007; Guardia et al., 2005; Liu et al., 2005; Ragunathan and Swaminathan, 2003; Toro et al., 2006). The mushroom *Agaricus subrufescens* has drawn the attention of the scientific community, due mainly to its medicinal properties; these properties include anticancer and antioxidant effects, which are characteristics that are associated with the cell wall components (1→6)-(1→3)-β-D-glucan and (1→4)-α-glucan (Bellini et al., 2006; Dong et al., 2002; Firenzuoli et al., 2008; Huang and Mau, 2006; Machado et al., 2005). These polysaccharides have also been associated with the medicinal characteristics of other mushroom species, such as *Pleurotus sp.*, *Lentinula edodes* and *A. bisporus* (Adams et al., 2008; Carbonero et al., 2006; Pramanik et al., 2007).

Studies of genetic diversity (Colauto et al., 2002; Tomizawa et al., 2007) have demonstrated that there are few differences between the various isolates of *A. subrufescens* cultivated in different regions of Brazil; however, some isolates are genetically distinct and are

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Table 1. *Agaricus subrufescens* strains used in the study and its origin.

Strain	Origin (City/State)
CS1	Vitória/ES*
CS5	Araçatuba/SP*
CS7	Porto Alegre/RS*
CS9	Eloi Mendes/MG*
CS10	Belo Horizonte/MG*

*ES, Espírito Santo State; SP, São Paulo State; MG, Minas Gerais State; RS, Rio Grande do Sul State.

considered to be different strains of *A. subrufescens*. These facts imply that distinct strains may have differences in their nutritional characteristics and in their β-glucan content. Investigating the amount of β-glucan and the nutritional and chemical constitution of these strains is of great importance for selecting strains for mushroom production and breeding and for obtaining new strains with desirable characteristics.

Several methods have been used for the extraction and quantification of β-glucans, and the results may vary according to the methods used (Dallies et al., 1998; Manzi and Pizzoferrato, 2001; Park et al., 2003; Rhee et al., 2008). Previously, there was no standard method that fulfilled the requirements of being both quick and highly reproducible for use in ordinal analysis in laboratories. Therefore, in addition to nutritional analysis of the chemical composition, we have tested two methods of β-glucan quantification in the *A. subrufescens* strains cultivated in Brazil.

MATERIALS AND METHODS

Materials

The phosphoric and sulfuric acids, sodium hydroxide, ethanol (95%) and ether were obtained from Sigma-Aldrich (St. Louis, MO). The enzymes α-amylase (Termamyl120L, New Nordisk), protease (New Nordisk)andamyloglicosidase(AMG300,NewNordisk) were obtained from Novozymes Latin America (Araucaria, Parana, Brazil). The kit used for glucose quantification, containing 4-(dimethylamino)-1,5dimethyl-2-phenylpyrazol-3-one (0.025 M), phenol (0.055 M), glycosidase (1U/ ml), peroxidase (0.15 U / ml) and the standard glucose, was obtained from Laborlab Ltd. (Guarulhos, São Paulo, Brazil).

Mushroom strains and cultivation

To measure the β-glucan content and the nutritional characteristics of *A. subrufescens,* five strains were used (CS1, CS5, CS7, CS9 and CS10) (Table 1). The amount of β-glucan obtained from *A. subrufescens* strains was compared with that of the following mushrooms: *Pleurotus ostreatus* (PO), *Pleurotus eryngii* (PE), *Pleurotus-sajor-caju* (PC), *Lentinula edodes* (LE) and *Agaricus bisporus* (AB). All species were obtained from the fungi collection in the Laboratory of Edible Mushrooms in the Department of Biology at the Federal University of Lavras (UFLA).

All strains, including *A. subrufescens*, were maintained on Potato dextrose agar (PDA) medium. Spawn was prepared on paddy rice grains supplemented with 10% wheat bran, 2% gypsum and 2% limestone, using the methods of Siqueira et al. (2009). All mushrooms were produced from strains belonging to the fungi collection of the Laboratory of Edible Mushrooms, except for *L. edodes*, which was obtained from the local market. *Pleurotus* mushrooms were produced in sterilized substrate, using chopped bean straw that was autoclaved twice at 121°C, according to the methods described by Dias et al. (2003). *A. bisporus* and *A. subrufescens* were cultivated according to the standard procedures for this mushroom, as described by Chang and Miles (2004) and Siqueira et al. (2011), using coastcross hay and sugar cane bagasse-based compost, supplemented with superphosphate (1%), limestone (2%), gypsum (2%) and urea (2%).

Mushrooms were cultivated in pots (*Agaricus*) or polypropylene bags (*Pleurotus*), in four replicates. Fruiting bodies were picked and dried for 24 h at approximately 60°C and were subsequently ground in a Wiley mill and homogenized.

β-Glucan extraction

Insoluble fiber, which is mainly composed of β-glucan (Manzi and Pizzoferrato, 2000) was extracted according to the method originally described by Prosky et al. (1988) with the modifications developed by the Japanese Food Analysis Center, as described by Park et al. (2003). 1 g of ground mushroom was added to a 500 mL Erlenmeyer flask containing 50 mL phosphate buffer (80 mmol L^{-1}, pH 6.0). The sample was then subjected to three enzymatic treatment stages: A. 0.1 mL of thermostable α-amylase (Termamyl 120L, New Nordisk) was added, and the flask was incubated in a boiling water bath for 30 min; B, 0.1 mL of neutral bacterial protease (New Nordisk) was added, and the pH was adjusted to 7.5 with 25 mmol L^{-1} NaOH, followed by incubation for 30 min at 60°C; C, 0.3 mL of amyloglucosidase (AMG 300, New Nordisk) was added, and the pH was adjusted to 4.5, followed by incubation for 30 min at 60°C.

After the three enzymatic stages, 200 mL of 95% ethyl alcohol was added to the solution and incubated at 60°C for 01h00. The insoluble fiber that was precipitated from the ethanol was filtered with Whatman number 5B filter paper (samples were weighed before filtering and after fiber removal) and washed three times into a solution of 80% ethyl alcohol and 20% acetone. The fiber precipitate was subsequently dried at room temperature and carefully removed from the filter paper.

For the measurement of β-glucan concentrations in insoluble fiber, each sample was transferred to a 300 mL Erlenmeyer flask and hydrolyzed by adding 10 mL of 72% H_2SO_4 and incubating at room temperature overnight. Distilled water (140 mL) was added, and the solution was incubated for 02h00 in a boiling water bath. The pH of the solution was adjusted to 7.0 using 5 mol L^{-1} NaOH, and the final volume was adjusted to 250 mL. The solution was then filtered with Whatman number 5B filter paper, followed by filtration with Durapore membrane with 0.22 µm pores (Millipore).

Quantification of β-glucan content by the enzymatic method

Quantification of β-glucan based on the enzymatic release of glucose was performed according to McClear and Glennie-Holmes (1985). The kit has the following composition: Standard reactive (glucose solution 100 mg dL^{-1}), enzyme reactive (glucose oxidase (EC 1.1.3.4) ≥ 0.001 L K U^{-1}, peroxidase (EC 1.11.1.7) ≥ 15.10^{-5} L K U^{-1}), color reagent 1 (4-aminophenazone 25 mol m^{-3}, 920 mol m^{-3} Tris) and color reactive 2 (55 mol m^{-3} phenol). The working reagent was prepared by adding in a 250 mL volumetric flask containing

225 mL of distilled water, 12.5 mL of color reagent 1, 12.5 mL of color reagent 2 and 0.75 mL enzyme reactive. The reaction was made of 2.0 mL of working reagent with 20 µL of each sample after extraction for 15 min at 37°C. Absorbance (505 nm) was measured with a UV-VISIBLE spectrophotometer, model UV-1601PC (Shimadzu, Corp., Japan). For standardization, the reaction was made of 2.0 mL of working reagent and 20 µL of standard reactive in the same conditions described above. The results were analyzed using the following Equation 1:

$$\beta - glucan\ \left(\frac{g}{kg}\right) = A\ x f\ x\ 0.9\ x\ 2.5 \quad (1)$$

Where, A is the Reaction absorbance of each sample after acid treatment; $f = 100\ mg\ dL^{-1}/P$, where P is the standard absorbance [20 µL standard reactive (glucose solution 100 mg dL^{-1}) + 2.0 mL reactive work (glycosidase ≥ 3000U; peroxidase ≥ 400U; 4-aminofenazone 0.0015 mol; TRIS buffer 0.0015 mol; phenol 0.00275 mol; pH 7.4 ± 0.1)]. Factor 0.9: 162/180, the factor of free glucose conversion that was determined for anhydrous glucose, which occurs in β-glucan (McClear and Glennie-Holmes (1985)); Factor 2.5: The conversion factor to convert from mg dL^{-1} to g Kg^{-1}, once the final volume of the extraction is 250 mL.

Quantification of β-glucan content by HPLC

For chromatographic analysis, samples were obtained using the previously described procedures. The samples were transferred from storage at -20 to 10°C and were kept at 10°C for approximately 12h00. The samples were then left at room temperature for approximately 04h00 before being analyzed. After temperature stabilization, 25 µL of each sample was diluted by a factor of 20 with ultrapure water, which had been filtered through an ultrafilter Durapore membrane with a 0.20 µm pore size (Millipore). A 20 µL volume of the diluted sample was injected into the chromatograph for analysis.

Values were determined using the AOAC (1992) technique, as modified by Schwan et al. (2001) and Shimadzu (1998). A high performance liquid chromatograph (HPLC), model LC-10Ai (Shimadzu Corp., Japan) was used. It was equipped with refraction index detectors (model RID-10A). For measurement of glucose, a cationic exchange column was used (Shim-Pack SCR-101H) (7.9 mm in diameter x 30 cm in length). For carbohydrate measurement, the column was operated at room temperature, with a mobile phase of ultrapure water adjusted to pH 2.1 and a flux of 0.6 mL min^{-1}. Quantification was done by comparison with a glucose standard curve, which was made using certified standards (Supelco-Sigma-Aldrich, St. Louis, MO, USA). The results read in the HPLC were analyzed using the following Equation 2:

$$\beta - glucan\ \left(\frac{g}{100\,g}\right) = G\ x\ 0.9\ x\ 250 \quad (2)$$

Where, G is the Glucose concentration in mg/mL, determined using a standard curve; Factor 0.9: factor that accounts for the proportion of glucose coming from β-glucan; Factor 250: e conversion factor to convert from mg mL^{-1} to g Kg^{-1}, once the final extraction volume is 250 mL.

Chemical analysis

Samples from different strains of *A. subrufescens* mushrooms were dried, ground in a Wiley Mill and homogenized. They were then conditioned in glass flasks (closed and kept under refrigeration at approximately 5°C). Chemical analyses were performed with four replicates for each treatment.

The following procedures were carried out for chemical analysis, according to the methods adopted by AOAC (2000): Measurement of moisture by drying at 105°C for 06h00, determination of fat content by ether extraction and gravimetry in a Soxhlet extractor, ash measurement by sample incineration and crude fiber measurement by acid digestion. The protein content was determined using the Kjeldahl method, with the amount of crude protein in the mushroom determined based on nitrogen content using a conversion factor (N) of 4.38 (Silva et al., 2007; Tsai et al., 2008). Glucose was measured using the method developed by Somogyi (1945) and Nelson (1944), and total sugar was calculated using the antrona method.

For the measurement of minerals, analyses were performed in triplicate with 0.5 g of dry mushroom for each strain or species. Calcium, magnesium, copper, zinc and iron were measured in Atomic Absorption Spectrometer AA-50 model GTA-110 (Agilent Technologies, Santa Clara, CA, United States). Phosphorus and sulfur were measured by spectrophotometry at 420 nm and boron at 540 nm in Spectrophotometer LAMBDA 25 (PerkinElmer, Waltham, MA, United States) while potassium was measured using a Flame Spectrophotometer model B-262 (Micronal, São Paulo, SP, Brazil) (Malavolta et al., 1997).

Statistical analysis

The means of the results were evaluated using analysis of variance (ANOVA), and the Scott-Knott test was used to compare differences ($p < 0.05$) among the β-glucan content, methods of quantitation of β-glucan and chemical composition. The SISVAR software (Ferreira, 2011) was used for the statistical analysis.

RESULTS AND DISCUSSION

β-Glucan content

β-Glucan extraction from the fungal cell wall can be done using several methods. The method described by Prosky et al. (1988) has been used, with some variations, to extract this polysaccharide for later quantification from soluble and insoluble fiber contained in the fungal cell wall (Dallies et al., 1998; Manzi and Pizzoferrato, 2001; Park et al., 2003; Brauer et al., 2008; Manzi et al., 2004).

In this work, a modified version of the method described by Prosky et al. (1988) was used to extract β-glucan, which was later quantified by spectrophotometry and HPLC. In addition to the *A. subrufescens* strains, other mushroom species were analyzed for comparison. The results of the β-glucan measurements are shown in Table 2. No significant differences were found when results obtained using the different quantification methods (spectrophotometry and HPLC) were compared. The samples quantified by the two tested methods were prepared using the same methods of extract preparation and filtration through filter paper as well as a 0.20 µm ultrafilter membrane. Filtration through an ultrafilter membrane is of extreme importance for avoiding overestimation of the amount of β-glucan and is also necessary prior to HPLC analysis to avoid column block-

Table 2. β-Glucan concentration (g Kg^{-1}) of edible and medicinal mushroom samples determined by enzimatic method and by HPLC.

Sample*	HPLC	Enzimatic method
LE	81.2± 1.29A	90.0± 1.43A
PO	89.2± 0.68A	96.3± 2.04A
PE	60.0± 0.29B	67.2± 1.95B
CS10	57.5± 0.81B	60.3± 0.43B
PC	48.7± 0.85C	70.3± 0.83B
CS7	42.0± 0.65C	50.0± 1.00C
CS9	40.0± 0.29C	42.7± 0.71C
CS5	38.4± 0.38C	41.0± 1.10C
CS2	30.5± 0.50D	32.6± 1.22C
AB	29.9± 0.84D	33.6± 1.02C
CS1	27.0± 1.25D	21.1± 0.67C

Values followed by same letters in the column do not differ among themselves, by Scott-Knott test ($p < 0.05$). * CS1 a CS10: *A. subrufescens* strains; AB, *A. bisporus*; LE: *L. edodes*; PE, *P. eryngii*; PC, *P. sajor-caju*; PO, *P. ostreatus*.

age. For spectrophotometric analysis, ultrafiltration or ultracentrifugation of the sample is important because at the end of the extraction process, small particles present in the sample may affect the absorbance reading, resulting in an overestimation of the β-glucan content. Park et al. (2003) extracted β-glucan from *A. subrufescens* mushrooms using the method of Prosky et al. (1988); however, these authors did not report a second filtration of the samples (through an ultrafilter membrane) before submitting them to spectrophotometric analysis.

The authors (Park et al., 2003) found that the concentration of β-glucan varied from 76.0 to 101.0 g Kg^{-1} of dehydrated mushroom. These values are higher than the ones obtained in the present study, in which the highest β-glucan concentrations were 50.0 and 60.3 g Kg^{-1} for strains CS7 and CS10, respectively. Different strains may have different amounts of β-glucan, but because ultrafiltration was not used by Park et al. (2003), it is possible that their results were an overestimation.

Among *A. subrufescens* strains, the highest β-glucan content was seen in the CS10 strain (60.3 g Kg^{-1}), followed by the CS7 strain (50.0 g Kg^{-1}); the lowest β -glucan content was seen in the CS1 strain (21.1 g Kg^{-1}). Therefore, the CS7 and CS10 strains of *A. subrufescens*, which contained higher concentrations of β-glucan than the others, may have a greater potential for commercial exploitation.

In the comparison between species, the highest β-glucan contents were seen in *L. edodes* (90.0 g Kg^{-1}) and *P. ostreatus* (96.3 g Kg^{-1}), followed by *P. sajor-caju* (70.3 g Kg^{-1}) and *P. eryngii* (67.2 g Kg^{-1}). Several studies have highlighted the medicinal potential of *A. subrufescens* mushrooms (Bellini et al., 2006; Machado et al., 2005; Menoli et al., 2001; Oliveira et al., 2002), which is usually correlated with β-glucan content (Firenzuoli et al., 2008). However, in this work, *P. ostreatus* and *L. edodes* mushrooms were the species that contained the highest amounts of this polysaccharide. It is known that the medicinal properties of mushrooms are related to the type of linkages in their polysaccharide branches as well as the total quantity of polysaccharides (Siqueira et al., 2011; Kawagishi et al., 1989).

A comparison of the two β-glucan quantification methods (spectrophotometry and HPLC) used in this study shows that the spectrophotometric method may be the better choice for this type of analysis because it is a quicker and easier method to execute. It is important to observe that, except for *P. sajor-caju*, all other species and strains kept the same relative positions with regard to β-glucan content when measured by either method. In other words, the species with higher β-glucan concentrations when measured by HPLC also had higher concentrations under the spectrophotometric analysis (Table 2).

Shimizu et al. (2003) reported using the same extraction method described in this work for the β-glucan analysis of two strains of *Pholiota adiposa* mushrooms and observed meaningful differences between the strains. These results corroborate the idea that different strains of the same species may contain different amounts of β-glucan, as was observed in the present study.

Rhee et al. (2008) reported β-glucan extraction from *Inonotus obliquus* mushrooms using two methods. The first method was by enzymatic extraction, using Termamyl and amyloglucosidase enzymes, a method similar to the one used in the present study. The second method consisted of alkaline extraction in sodium carbonate–bicarbonate buffer (pH 10), followed by treatment with HCl. According to the authors, no significant differences were observed between the two extraction methods; this finding demonstrates that different methods may be used to extract β-glucan without compromising the validity of the results.

However, Manzi and Pizzoferrato (2000) reported β-glucan measurements in different *Pleurotus* and *L. edodes* species that were much lower than the values reported in other studies. According to those authors, β-glucan concentrations of 5.3 g Kg^{-1} for *Pleurotus pulmonarius*, 3.8 g Kg^{-1} for *P. ostreatus* and 2.2 g Kg^{-1} for *L. edodes* were observed. However, in that study, the samples were subjected to lichenase hydrolysis and further degradation by β-glucosidase, instead of acid hydrolysis, prior to β-glucan analysis.

Enzymatic hydro-lysis ensures that glucose is released only from β-glucan; however, the hydrolysis may be incomplete. Acid hydroly-sis is normally complete, although it is nonspecific. Given these facts, acid hydrolysis may be a good method for β-glucan analysis in mushrooms, considering that the insoluble fraction of mushrooms contains mainly β-glucan, in addition to some

Table 3. Chemical and nutritional composition (g Kg^{-1}) of dry mushrooms from distinct *Agaricus subrufescens* strains.

Strain	Protein	Lipid	Nifest	Fiber	Ash	Moisture
CS1	245.0± 2.01B	18.6± 0.08A	383.7± 1.6C	165.0± 0.17C	63.6± 0.25C	124.1± 0.28A
CS5	270.7± 0.50C	17.0±0.10A	349.1± 1.5D	175.0± 0.17B	71.7± 0.11A	116.5± 0.92B
CS7	220.8± 0.46A	17.0± 0.10A	431.5± 0.49A	170.0± 0.15C	63.7± 0.19C	97.0± 0.43C
CS9	233.7± 0.46A	18.4± 0.09A	404.8± 0.96B	183.0± 0.66A	67.2± 0.07B	92.9± 0.09C
CS10	248.4± 1.11B	17.9±0.09A	416.1± 1.25B	163.0± 0.19C	67.5± 0.06B	87.1± 0.15D

Values followed by same letters, in columns, do not differ among themselves by Scott-Knott test ($p < 0.05$). Values overwritten represent average pattern deviation.

Table 4. Average values of phenolic compounds and sugars (g Kg^{-1}) in *A. subrufescens* mushrooms in dehydrated matter.

Strain	Reducing sugar	Non reducing sugar	Polyphenols
CS1	0.54± 0.01A	6.1± 0.05B	18.2± 0.42A
CS5	0.45± 0.01C	4.5± 0.01C	22.0± 0.11A
CS7	0.48± 0.02B	7.6± 0.10A	19.6± 0.26A
CS9	0.44± 0.02C	6.3± 0.02B	17.8± 0.08A
CS10	0.39± 0.01D	5.2± 0.01C	16.5± 0.12A

Values followed by same letters, in columns, do not differ among themselves by Scott-Knott test ($p < 0.05$). Values overwritten represent average pattern deviation.

chitin and hemicellulose. The sugars produced by the hydrolysis of chitin and hemicellulose are different from the glucose released from glucan hydrolysis; thus, acid hydrolysis, followed by spectrophotometric measurement of the free glucose released, allows for an efficient quantification of β-glucan. According to Park et al. (2003), the method described by Prosky et al. (1988) and modified by the Japanese Food Analysis Center is the official method in Japan for the analysis of β-glucan.

Rhee et al. (2008) attempted unsuccessfully to standardize a method of β-glucan measurement in *I. obliquus* mushrooms by testing two quantification methods (a gravimetric method and HPLC analysis). Their results were not statistically distinct (101.0 to 107.0 g Kg^{-1} dehydrated matter by the gravimetric method and 81.0 to 83.0 g Kg^{-1} dehydrated matter by HPLC). The authors suggested that HPLC measurement would be more appropriate for quantifying β-glucan when it is present at high levels.

The method of extracting β-glucan used in this work has proven to be efficient for *A. subrufescens* mushrooms and for other mushroom species. Both of the quantification methods (HPLC and spectrophotometry) were found to be equally efficient for measuring the amount of β-glucan in the mushroom samples. The amount of β-glucan in the mushroom samples was the same for both methods. Methods of β-glucan by HPLC are more laborious and more expensive than spectrophotometric methods.

Chemical analysis

With the aim of better understanding the nutritional properties of *A. subrufescens*, nutritional characteristics, concentration of phenolic compounds and mineral composition of five strains were evaluated. Significant differences ($p < 0.05$) were observed for all of the nutritional variables assessed, except for fat content (Table 3). Among the five strains, the CS5 strain had the highest concentrations of protein (270.7 g Kg^{-1}) and ash (71.7 g Kg^{-1}). However, the CS9 strain had the highest crude fiber concentration (183.0 g Kg^{-1}), followed by the CS5 strain (175.0 g Kg^{-1}). These data are very similar to those reported by Tsai et al. (2008) for samples of an *A. subrufescens* strain purchased in a market in Taiwan. Crude fiber is composed of multiple polysaccharides, including β-glucan, which is a major target for studies of compounds with antitumor activity (Bellini et al., 2006; Firenzuoli et al., 2008; Machado et al., 2005; Park et al., 2003). Therefore, fiber content may be related to the concentration of β-glucan in mushrooms. There were no significant differences with regard to the percentage of fat, and all of the strains contained less than 20 g Kg^{-1} fat. A low concentration of lipids is generally considered a common feature of mushrooms (Liu et al., 2005). The various *A. subrufescens* strains showed statistically significant differences in the concentrations of reducing and non-reducing sugars (Table 4). The CS7 strain had the highest concentration of non-reducing sugars (7.6 g

Table 5. Average mineral composition (g Kg^{-1}) of mushrooms from different *A. subrufescens* strains in dried matter.

Strain	P	K	Ca	Mg	S	B	Cu	Mn	Zn	Fe
CS1	11.2± 0.03C	19.4± 0.05A	3.8± 0.02A	1.3± 0.01A	3.7± 0.01B	32.0± 2.11A	0.0286±0.38C	0.0085±0.55B	0.0620±4.70B	0.0703± 7.77B
CS5	12.3± 0.07A	19.5± 0.02A	3.4± 0.02A	1.3± 0.01A	4.1± 0.01A	40.0± 1.50A	0.0539±0.65A	0.0082± 0.29B	0.0515± 0.57D	0.0912± 6.53A
CS7	10.8± 0.01D	19.4± 0.03A	1.8± 0.01B	1.3± 0.01A	3.5± 0.01C	34.0± 1.76A	0.0306± 0.15B	0.0072± 0.10C	0.0463± 0.87E	0.0678± 0.49B
CS9	10.8± 0.02D	19.5± 0.02A	3.6± 0.03A	1.4± 0.01A	3.4± 0.01C	21.7± 0.06A	0.0237± 0.25D	0.0094± 0.40A	0.0560± 0.68C	0.0670± 0.83B
CS10	11.6± 0.02B	19.4± 0.01A	3.7± 0.01A	1.5± 0.01A	3.7± 0.01B	20.0± 0.60A	0.0274± 0.93C	0.0080± 0.12B	0.0679± 0.84A	0.0690± 0.60B

Values followed by same letters, in columns, do not differ among themselves by Scott-Knott test ($p < 0.05$). Values overwritten represent average pattern deviation.

Kg^{-1}) among the five strains; however, the values obtained were very low overall, and the concentration of reducing sugars was negligible. *A. subrufescens* mushrooms are known to possess antioxidant properties, and this characteristic in mushrooms is associated with the presence of phenolic compounds (Barros et al., 2008; Elmastas et al., 2007). The measurement of phenolic compounds from different strains of *A. subrufescens* mushrooms reported in the present study did not show any significant differences between strains (Table 4). The values are similar to those found for *Agaricus arvensis* (28.3 g Kg^{-1}) and are lower than those reported for species from other genera, such as *L. edodes* (47.9 g Kg^{-1}), *Volvariella volvacea* (150.0 g Kg^{-1}), *Leucopaxillus giganteus* (62.9 g Kg^{-1}) and *Sarcodon imbricatus* (37.6 g Kg^{-1}) (Barros et al., 2007; Cheung et al., 2003).

The mineral composition of *A. subrufescens* mushrooms is represented in Table 5, with significant differences ($p < 0.05$) between strains treatments. No significant differences were found between strains for potassium, magnesium, boron and iron. The CS5 strain contained the highest concentrations of phosphorus, sulfur and copper of all of the strains. The highest concentration of manganese was found in the CS9 strain, and the highest concentration of zinc in the CS1 strain. The CS7 strain contained significantly the lowest concentrations of calcium and manganese. Besides Besides, CS7 was among the strains with lowest phosphorus, sulfur and iron concentrations.

It is worth noting that the results presented here refer to dry, rather than fresh, mushrooms. Considering that the amount of moisture in the fresh mushrooms varied from 832.5 to 917.5 g Kg^{-1} depending on the strain, the dry components account for only 82.5 to 167.5 g Kg^{-1} of the total composition of the mushrooms. However, fresh mushrooms should be considered an important source of minerals and fiber and a better source of protein than other vegetables. Furthermore, it is important to emphasize that the results presented in this work demonstrate that the genetic differences observed by Tomizawa et al. (2007) result in different chemical and nutritional properties for different strains. Experiments with different strains of *Pleurotus* spp., *Flammulina velutipes, Lentinula edodes* and *Agaricus bisporus* also demonstrated differences in chemical composition among strains of the same species (Toro et al., 2006; Calonje et al., 1995; Yang et al., 2001).

Conclusions

Fresh *A. subrufescens* mushrooms are not considered to be a high-protein food. However, they are a good source of fiber and minerals, in addition to being low in fat. Therefore, these mushrooms can be considered a beneficial source of nutrients in the diet. Two different methods of quantification (HPLC and spectrophotometry) may be used to efficiently measure the amount of β-glucan in *A. subrufescens*. Spectrophotometry presents the advantages of being quicker and lower in cost, allowing for routine use in the laboratory. Based on these characteristics, this technique may be suggested as the standard for measurement of the β-glucan concentration in edible and medicinal mushrooms.

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Full Length Research Paper

Antioxidant properties of methanol extract of a new commercial gelatinous mushrooms (white variety of *Auricularia fuscosuccinea*) of Taiwan

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White variety of *Auricularia fuscosuccinea* is a newly cultivated gelatinous mushrooms which is found only in Taiwan. In this study, total phenolic and total flavonoid content of methanol extract of white variety of *A. fuscosuccinea* was estimated, and *in vitro* antioxidant properties and antioxidant enzyme activities were also evaluated. When compared with two other common gelatinous mushrooms, *A. polytricha* and *Tremella fuciformis*, white variety of *A. fuscosuccinea* had the highest total phenolic [7.88 mg gallic acid equivalents (GAE)/g] and total flavonoid [1.60 mg quercetin equivalents (QE)/g]. Among all methanol extracts analyzed, white variety of *A. fuscosuccinea* had the lowest EC_{50} value on reducing g power (0.305 mg/ml) and scavenging effect on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical (0.150 mg/ml) had the highest total sugars [44.73 mg dextrose equivalents (DEX)/g] and the lowest EC_{50} value on chelating effect on ferrous ions (0.427 mg/ml). The methanol extracts from white variety of *A. fuscosuccinea* possessed the highest superoxide dismutase activity (2.10 U/mg) and total antioxidant capacity (2.26 mM/g). The glutathione reductase activity (7.97 U/g) of *A. polytricha* was the highest. The analyses of the antioxidant contents phenolic compounds are mainly responsible for the antioxidant effect of gelatinous mushrooms.

Key words: *Auricularia fuscosuccinea*, antioxidant activity, reducing power, scavenging effect, chelating effect.

INTRODUCTION

Butylated hydroxyanisole (BHA) and butylated hydroxyltoluene (BHT) are the two most commonly used synthetic antioxidants. The processing costs of synthetic antioxidants used in the food industry are high, while selected synthetic antioxidants may be harmful to human life. Their toxicity and ability to induce DNA damage led them to be restricted when applied in food industry (Sasaki et al., 2002). Nevertheless, mushroom species could provide the antioxidant capacity in *in vitro* systems (Ribeiro et al., 2006, 2007). Therefore, natural antioxidants from mushroom extracts have attracted increasing interest due to their safety (Mau et al., 2004). Mushrooms have been used as a sort of food ingredient for centuries. The unique and subtle flavor of mushrooms is responsible for their use in seasoning and flavoring.

Research conducted during the last decades has indicated that mushrooms exert a number of nutritional and nutraceutical properties and they are source of beneficial bioactive compounds (Ferreira et al., 2009; Yaltirak et al., 2009; Jayakumar et al., 2009). Mushrooms contain significant amounts of bioactive substances such as vitamins and vitamin precursors, minerals and trace

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Figure 1. Appearance of white variety of *A. fuscosuccinea*.

elements (Kalač, 2009), specific β-glucans, and exert antioxidant properties which are mainly attributed to their phenolic content (Ferreira et al., 2009; Yaltirak et al., 2009). A large body of evidence supports the implication of oxidative stress in the pathogenesis of several chronic and degenerative diseases such as atherosclerosis, diabetes mellitus, chronic inflammation, cancer and aging (Halliwell, 1996; Valko et al., 2007). Therefore, the enhancement of the antioxidant systems for the prevention of cellular oxidative damage via the consumption of antioxidant rich foods is of great interest.

Gelatinous mushrooms are believed to be of high nutritional value since it has the high content of carbohydrates, amino acids, trace elements and vitamins, thus to be processed into a variety of foods (Fan et al., 2006). In previous studies, polysaccharides of gelatinous mushroom were found to have the potent antioxidant activity in both *in vitro* (Fan et al., 2006; Kho et al., 2009) and *in vivo* assay (Chen et al., 2008a, 2008b, 2008c; Wu et al., 2010).

A current research also reported that polysaccharides had the potential application as a new antioxidant agent in food industry (Fan et al., 2006). Other antioxidant compounds in mushrooms such as phenolics (Bendini et al., 2006; Quezada et al., 2004), flavonoids and organic acids (Ribeiro et al., 2007) are used in dietary supplement and by the pharmaceutical industry. White variety of *A. fuscosuccinea* is a white mutant of *A. fuscosuccinea*, originally selected and generated in Taiwan Agricultural Research Institute.

After populari-zation, it could be successfully cultivated in organic farm. The fresh fruit body of white variety of *A. fuscosuccinea* has a shape of dancing skirt and a glossy velvety coat outer surface (Figure 1). After boiled into a sweet soup, the smooth texture tasted like edible nest of cliff swallows and was called “bird’s nest soup for vegetarian”. However, in Taiwan, the rareness and the expensiveness keeps consumers from purchase. On the other hand, the similar appearance of dry fruit body between white variety of *A. fuscosuccinea* and *T. fuciformis* makes it hard for identification by consumers. We investigated the white variety of *A. fuscosuccinea* because its hot water extract has lot of benefits for the proliferation of probiotics. Therefore, it is of great interest to explore other beneficial effects of this mushroom, and the analysis results might be the basis for its application as in functional food.

Mau et al (2001) had examined the antioxidant properties of methanol extracts from several oven-dried ear mushrooms (Mau et al., 2001). However, the investigation about the influence of freeze-drying to the antioxidant contents and corresponding antioxidant activity of gelatinous mushrooms is rather limited. To better interpret the data obtained from white variety of *A. fuscosuccinea*, the antioxidant values were compared with that of two commonly used gelatinous mushrooms, *T. fuciformis* and *A. polytricha*. Because of their beneficial properties like antiinflammatory and antitumor properties, both of these two mushrooms have been widely used in many countries for years in therapeutics for blood pressure regulation, hypercholesterolemia, hyperlipide-mia, cardiovascular disorders and chronic bronchitis (Yang et al., 2002; Zhang et al., 2007).

Since food composition in bioactive or potentially bioactive com-pounds is recognized as critical for throwing light upon the association between diet and health, this study will shed lights on the correlations between the antioxidant contents and antioxidant activity. We further compared these results with commercial antioxidants (BHA, BHT and EDTA) to assess the dietary supplements potential of white variety of *A. fuscosuccinea*. Therefore, these results will help us know the utility of white variety of *A. fuscosuccinea* in functional food industry.

In the present work, we also demonstrate if white variety of *A. fuscosuccinea* contains enzymatic antioxi-dant defense systems (SOD, GPx and GRd) to remove the reactive oxygen stress (ROS).

MATERIALS AND METHODS

Mushroom fruit bodies

Fresh fruit bodies of white variety of *A. fuscosuccinea* and *A. polytricha* were harvested from organic mushroom farm (Wufeng, Taiwan and Caotun, Taiwan). Fresh *T. fuciformis* fruit bodies (approximately 1 kg each) were purchased from supermarkets. Fresh mushrooms were freeze-dried at -50± 2°C.

Methanol extract of mushrooms

A coarse powder was obtained using a mill (20 mesh, Retsch ultracentrifugal mill and sieving machine, Haan, Germany). These powders of dried fruit bodies were placed in Tupperware® boxes and stored in refrigerator at 4°C for further use. For the methanol extraction of mushrooms, a subsample (10 g) was extracted using 100 ml of methanol in a glass conical flask using a shaker at 25°C

for 24 h and then the mixture was filtered through filter paper. The residue was then extracted with an additional 100 ml of methanol as described for the first extraction. the two methanol extracts were combined; 10 ml of the mixture were evaporated at 50°C by oven drying. Total dry matter of methanol extracts was determined gravimetrically as residue remaining after drying. Each methanol extract was adjusted by methanol to 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 mg/ml. All diluted solutions were maintained in the dark until tested.

Determinations of total phenolic, flavonoid and sugars contents

Total phenolic content was determined using the Folin-Ciocalteu reagent method described by Lai et al. (2001). Each methanol extract (100 mg) was dissolved in 5 ml of 0.3% HCl in methanol/water (60:40, v/v), and the resulting mixture (100 µl) was added to 2 ml of aqueous sodium carbonate solution. After 2 min, 100 µl of 50% Folin-Ciocalteau reagent (Sigma) was added to the mixture, which was then left for 30 min. Absorbance was measured at 750 nm against a blank. The content of total phenolic was calculated on the basis of the calibration curve of gallic acid (Sigma).

Total flavonoid content was determined using a method described by Jia et al. (1999). 0.5 ml of each methanol extract was mixed with 1.5 ml of deionized water, 0.1 ml of 1 mg/ml $Al(NO_3)_3 \cdot 9H_2O$ (Wako), and 0.1 ml of 1 M CH_3COOK (Wako). After 40 min at room temperature in the dark, the absorbance of the mixture was determined at 415 nm against a blank. A higher absorbance indicates higher flavonoid content. Content of total flavonoid was calculated on the basis of the calibration curve of quercetin (Sigma). Total sugar content was determined by the modified phenol-sulfuric acid method described by Dubois et al. (1956).

0.2 ml of each methanol extract was mixed with 0.2 ml 5% w/v phenol solution. Added 1 ml concentrated sulfuric acid rapidly and directly on the sample and left for 10 min. The contents were stirred and incubated at 25°C for 30 min and the absorbance of the mixture was determined at 490 nm against a blank. Content of total sugars was calculated on the basis of the calibration curve of D-glucose (Merck).

Determination of reducing power

The reducing power was determined by a method described by Oyaizu (1986), which measured the ability of sample to reduce ferricyanide to ferrocyanide. Each methanol extract (0.4 ml) was mixed with 0.4 ml of a 0.2 M phosphate buffer (pH 6.6; Merck) and 1% $K_3Fe(CN)_6$ (Sigma). The mixture was incubated at 50°C for 20 min, followed by addition of 0.4 ml of 10% trichloroacetic acid (Merck) and then centrifuged for 5 min at 10,000 rpm. The upper layer of solution (1.5 ml) was mixed with 1.5 ml of deionized water and 0.3 ml of 1 mg/ml $FeCl_3$ (Sigma). The mixture was incubated at room temperature for 10 min, and the absorbance was measured at 700 nm against a blank. A higher absorbance indicates a higher reducing power. Methanol was used as a control. BHA was used for comparison, and the concentration used was the same as the sample.

Determination of DPPH radicals scavenging activity

The DPPH scavenging activity was determined using the method described by Shimada et al. (1992). Each methanol extract (4 ml) was mixed with 1 ml of a 10 mM DPPH (Sigma) methanol solution. After 30 min incubation at room temperature in the dark, the absorbance of the mixture was determined at 517 nm against a blank. A lower absorbance indicates a higher scavenging activity. Methanol was used as a control. BHA and BHT were used for comparison, and the concentrations used were the same as with the sample. The following equation was used to determine the scavenging effect:

$$\text{Scavenging effect (\%)} = \frac{\triangle A_{517} \text{ of control} - \triangle A_{517} \text{ of sample}}{\triangle A_{517} \text{ of control}} \times 100$$

Ability of chelating ferrous ions

The chelating effects were determined using a method by Dinis et al. (1994). Each methanol extract (0.1 ml) was mixed with 3.7 ml of methanol and 0.1 ml of 2 mM $FeCl_2 \cdot 4H_2O$ (Merck). The reaction was initiated by adding in 0.2 ml of 5 mM ferrozine (Sigma). After 10 min at room temperature, the absorbance of the mixture was determined at 562 nm against a blank. A lower absorbance indicated a higher chelating power. Methanol was used as a control. EDTA was used for comparison, and the concentration used was the same with sample. The following equation was used to determine the chelating effect:

$$\text{Chelating effect (\%)} = \frac{\triangle A_{562} \text{ of control} - \triangle A_{562} \text{ of sample}}{\triangle A_{562} \text{ of control}} \times 100$$

Determination of total antioxidant capacity

The 2,2'-azinobis-(3-ethylbenzo-thiazoline-6-sulfonic acid) (ABTS) radical scavenging activity was determined at 600 nm by using a Randox total antioxidant status kit (Randox Laboratories Ltd., Crumilin Co. Antrim, UK) according to the method of Miller et al. (1993). In the TAS assay, ABTS is incubated with metmyoglobin and hydrogen peroxide to produce the ABTS· radical cation with a relative stable blue-green color. Antioxidants present in the sample cause a reduction in absorption proportional to their concentration. The total antioxidant capacity (TAC) value was expressed as an equivalent of the concentration (mM) of Trolox solution.

Determination of antioxidant enzyme activity

SOD activity was determined at 505 nm using a Ransod kit (Randox Laboratories Ltd., UK) according to the method of Delmas-Beauvieux et al. (1995). GPx activity was determined at 340 nm using a Ransel kit (Randox Laboratories Ltd., UK) according to the method of Paglia and Valentine (1967). SOD and GPx activities were expressed as U/mg extract. GRd activity was determined at 340 nm using a Rangrd kit ((Randox Laboratories Ltd., UK) according to the method of Goldberg and Spooner (1983) and was expressed as U/g extract.

Statistical analysis

All data were showed as mean ± standard deviation (SD) of three replicated determinations. Data were analyzed using the statistical analysis system (Ver. 9.1 for Windows, 2010) software package. Analysis of variance was performed using one-way ANOVA procedures, analysis of variance. Significant differences ($\rho<0.05$) between means were determined using the Duncan's new multiple

Table 1. Antioxidant contents from methanol extracts of three gelatinous mushrooms.

Content	Content concentrations (mg/g of dry weight)		
	White variety of *A. fuscosuccina*	*A. polytricha*	*T. fuciformis*
Total flavonoid	2.18 ± 0.12^{x}	0.49 ± 0.14^{z}	0.78 ± 0.15^{y}
Total phenolic	10.85 ± 1.00^{x}	2.98 ± 0.44^{y}	1.12 ± 0.28^{z}
Total sugars	22.40 ± 3.13^{x}	18.64 ± 1.36^{y}	24.42 ± 1.33^{x}

Each value is expressed as means ± standard deviation of three replicates. $^{x-z}$Different superscripts with the same row indicate significantly different ($\rho < 0.05$).

range test.

RESULTS

Extraction yield

Methanol was used to extract the dry mushroom material, white variety of *A. fuscosuccinea* showed the highest yield (13.77%), whereas *T. fuciformis* (7.28%) and *A. polytricha* (5.46%) showed less yield.

Determination of total phenolic, flavonoid and sugars contents

Naturally occurring antioxidant components such as phenolics, flavonoids and sugars were found in the methanol extracts from the gelatinous mushrooms (Table 1). Total phenolic contents (per gram of dry weight) in the methanol extracts from the three gelatinous mushrooms ranged from 1.12 to 10.85 mg GAE/g. The descending order of total phenolic was: white variety of *A. fuscosuccinea* (10.82 mg GAE/g)> *A. polytricha* (2.98 mg GAE/g) > *T. fuciformis* (1.12 mg GAE/g). The flavonoid values were in the following descending order: white variety of *A. fuscosuccinea* (2.18 mg QE/g)> *T. fuciformis* (0.78 mg QE/g) > *A. polytricha* (0.49 mg QE/g). The contents of total sugars from the methanol extracts were in the following order: *T. fuciformis* (24.42 mg DEX/g)> white variety of *A. fuscosuccinea* (22.40 mg DEX/g)> *A. polytricha* (18.64 mg DEX/g).

Determination of reducing power

For measurement of the reductive ability, the Fe^{3+} to Fe^{2+} transformation in the three gelatinous mushrooms was investigated. The reducing power of the methanol extracts was concentration dependent. As the concentration increased from 0.1 to 3.5 mg/ml, there was an increase in absorbance.

Amongst the methanol extracts from the three gelatinous mushrooms, the reducing power of white variety of *A. fuscosuccinea* dramatically increased until it reached a plateau status as follows: 1.87 at 1.0 mg/ml, 2.16 at 1.5 mg/ml, 2.19 at 2.0 mg/ml, and 2.21 at 3.5 mg/ml (Figure 2). On the contrary, reducing power of methanol extracts from *A. polytricha* and *T. fuciformis* increased slowly along with increasing concentrations as follows: 0.77 and 0.16 at 1.0 mg/ml, 1.21 and 0.39 at 2.0 mg/ml; 1.99 and 0.42 at 3.5 mg/ml, respectively. These results imply that the reducing power is increasing with increasing concentration of the methanol extracts from gelatinous mushrooms.

Determination of DPPH radical scavenging activity

The hydrogen atom or electron donation abilities of the corresponding extracts were measured from the bleaching of the purple-colored methanol solution of DPPH. The methanol extract from white variety of *A. fuscosuccinea* had a robust scavenging effect on DPPH radicals resulting in a final plateau as follows: 90.65% at 0.4 mg/ml, 91.14% at 0.5 mg/ml and 92.52% at 3.5 mg/ml (Figure 3).

The scavenging effects on DPPH radicals of *A. polytricha* slowly increased along with concentrations until it reached a final plateau and were as follows: 34.84% at 0.4 mg/ml, 72.66% at 1.0 mg/ml, 90.30% at 1.5 mg/ml and 92.74% at 3.5 mg/ml.

The scavenging activities of white variety of *A. fuscosuccinea* and *A. polytricha* increased along with concentrations. However, an exception was observed with *T. fuciformis*, which did not level off with concentration and had lower scavenging activity than white variety of *A. fuscosuccinea* and *A. polytricha*. The *T. fuciformis* scavenging effect was 6.92 at 1.0 mg/ml and 17.63 at 3.5 mg/ml; whereas, the scavenging activities of positive controls, BHA and BHT, at 0.1 mg/ml were 95.18 and 91.07%, respectively.

These results indicate that the antioxidants in the methanol extract of *T. fuciformis* are weak DPPH radical scavengers and therefore required high concentration to have a significant effect.

Ability of chelating ferrous ions

The absorbance of the Fe^{2+}-ferrozine complex linearly decreased in a dose dependent manner, and the

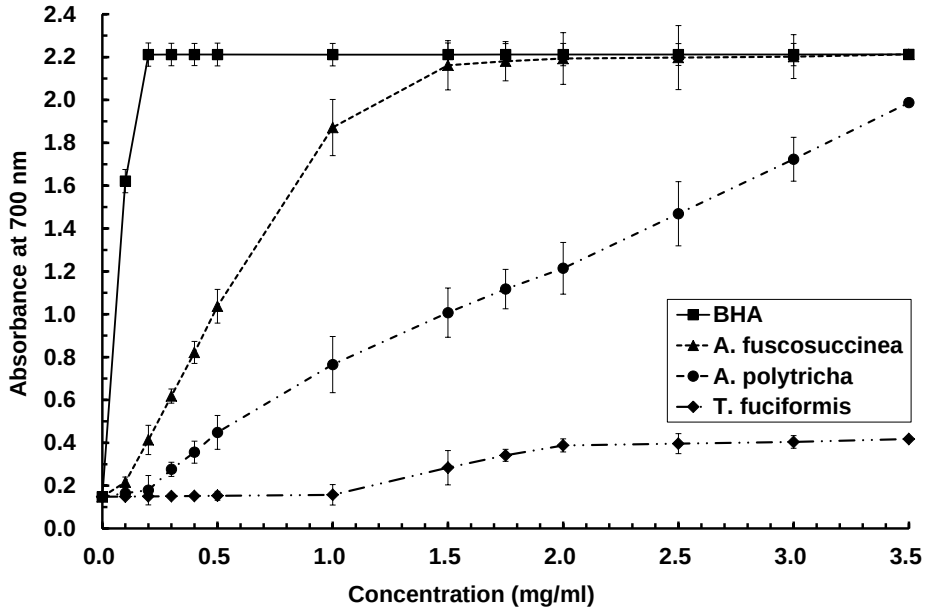

Figure 2. The reducing power of methanol extracts from three gelatinous mushrooms. Each symbol with vertical bars represents the mean ± standard deviation of three replicates. ■, BHA; ▲, methanol extract from white variety of *A. fuscosuccinea;* ●, methanol extract from *A. polytricha*; ◆, methanol extract from *T. fuciformis*.

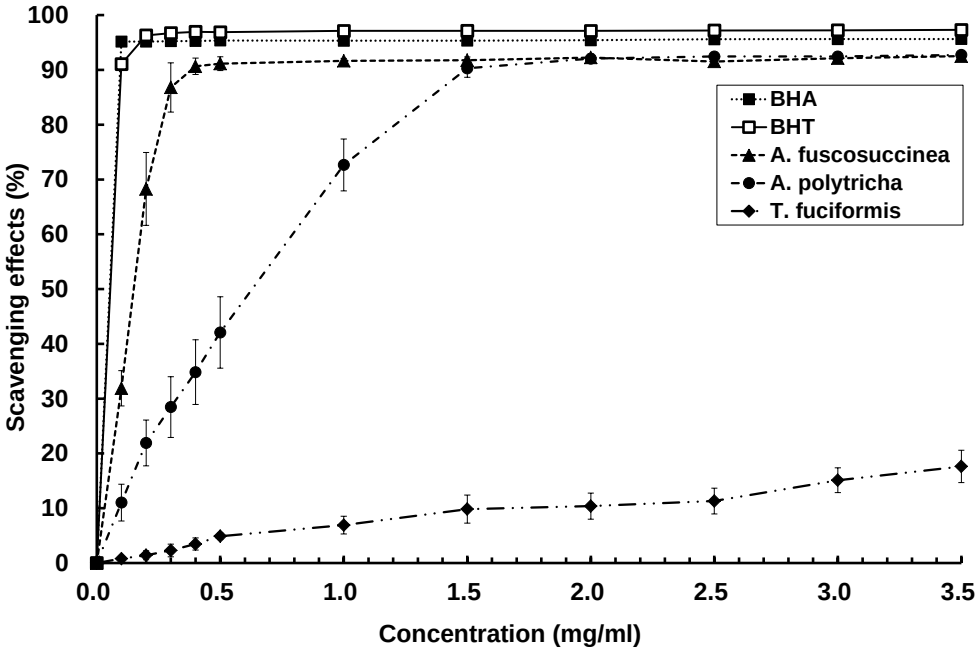

Figure 3. Scavenging effects of methanol extracts from three gelatinous mushrooms on α,α-diphenyl-2-picryl hydrazyl (DPPH) radicals. Each symbol with vertical bars represents the mean ± standard deviation of three replicates. ■, BHA; □, BHT; ▲, methanol extract from white variety of *A. fuscosuccinea*; ●, methanol extract from *A. polytricha*; ◆, methanol extract from *T. fuciformis*.

chelating abilities on ferrous ions of the methanol extracts from the three gelatinous mushrooms increased with concentrations (Figure 4). The chelating effects for white variety of *A. fuscosuccinea*, *A. polytricha* and *T. fuciformis*

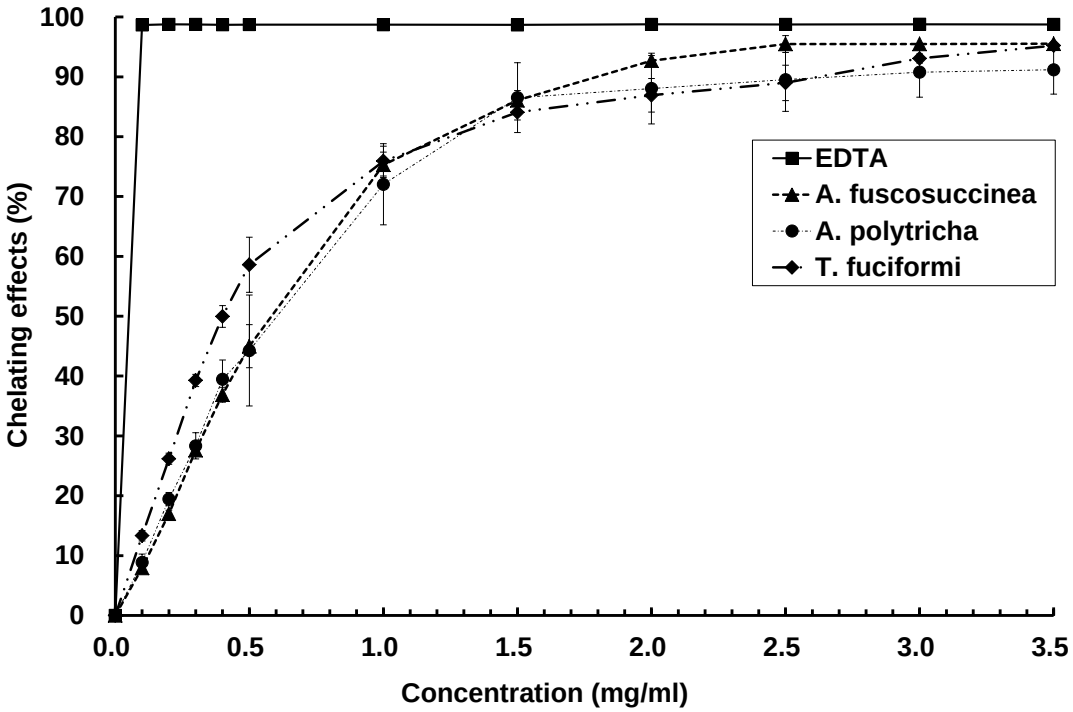

Figure 4. Chelating effects of methanol extracts from three gelatinous mushrooms against ferrous ions. Each symbol with vertical bars represents the mean ± standard deviation of three replicates. ■, BHA; ▲, methanol extract from white variety of *A. fuscosuccinea*; ●, methanol extract from *A. polytricha*; ◆, methanol extract from *T. fuciformis*.

Table 2. EC_{50} values of methanol extracts from three gelatinous mushrooms in antioxidant properties.

Analysis method	EC_{50} values[a,b] (mg extract/ml)		
	White variety of *A. fuscosuccinea*	*A. polytricha*	*T. fuciformis*
Reducing power	0.305 ± 0.085^{x}	0.576 ± 0.018^{y}	N. E.[c,z]
Scavenging effects on DPPH radicals	0.150 ± 0.012^{x}	0.630 ± 0.021^{y}	N. E.[c,z]
Chelating effects on ferrous ions	0.582 ± 0.008^{y}	0.602 ± 0.015^{z}	0.427 ± 0.014^{x}

[a]EC_{50} values, the effective concentration at which the antioxidant activity was 50%; the absorbance was 1.0 for reducing power; the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals were scavenged by 50%, and the ferrous ions were chelated by 50%. EC_{50} values were obtained by interpolation from linear regression analysis. [b]Each value is expressed as means ± standard deviation of three replicates. [c]N. E. = No effect, means not achieve EC_{50} value in determined concentration. [x-z]Different superscripts with the same row indicate significantly different ($p < 0.05$).

were as follows: 44.99, 44.27 and 58.61% at 0.5 mg/ml; 91.28, 88.04 and 86.92% at 2.0 mg/ml; 95.53, 91.19 and 95.22% at 3.5 mg/ml, respectively. However, EDTA was an excellent chelating agent for ferrous ions and it showed a chelating effect was 98.66% at 0.1 mg/ml.

EC_{50} values in antioxidant properties

The antioxidant properties that were assayed from the three gelatinous mushrooms are summarized in Table 2, and the results were normalized and expressed as EC_{50} value (mg dry weight of extracts per ml) for comparison. The EC_{50} value is the effective concentration at which the antioxidant activity is 50%. The lower EC_{50} values of reducing power, scavenging of DPPH radicals and chelating effects on ferrous ions indicated that the methanol extracts of gelatinous mushrooms were more effective. With regard to the reducing power, the EC_{50} value of methanol extracts from white variety of *A. fuscosuccinea* and *A. polytricha* were 0.305 and 0.576 mg/ml, respectively, except for the value of *T. fuciformis* in which we fail to achieve an EC_{50} value with a determined concentration. With regard to the scavenging effect on DPPH radicals, the EC_{50} value of methanol extracts from white variety of *A. fuscosuccinea* and *A.*

Table 3. Enzyme activity from methanol extracts from three gelatinous mushrooms in antioxidant properties.

Enzyme	Enzyme activity[a]		
	White variety of *A. fuscosuccinea*	*A. polytricha*	*T. fuciformis*
SOD[b]	2.10 ± 0.25[x]	1.27 ± 0.15[y]	0.98 ± 0.04[y]
GPx[b]	19.33 ± 0.16	19.50 ± 0.39	19.57 ± 0.28
GRd[c]	5.13 ± 1.10[y]	7.97 ± 0.80[x]	5.37 ± 0.51[y]
TAC[d]	2.26 ± 0.02[x]	1.82 ± 0.07[y]	1.62 ± 0.01[z]

[a]Each value is expressed as means ± standard deviation of three replicates. [b]The activities of SOD and GPx were expressed as U/mg extract. [c]The activity of GRD was expressed as U/g extract. [d]The activity of TAC was expressed as mM/g extract. [x-z]Different superscripts with the same row indicate significantly different (ρ< 0.05).

polytricha were 0.150 and 0.630 mg/ml, respectively, except for the value of *T. fuciformis* in which we failed to achieve an EC_{50} value with a determined concentration. The chelating effect on ferrous ions from *T. fuciformis* was 0.427 mg/ml, which was better than the effects from white variety of *A. fuscosuccinea* (0.582 mg/ml) and *A. polytricha* (0.602 mg/ml).

Determination of antioxidant enzyme activity

SOD activity

The methanol extract of white variety of *A. fuscosuccinea* (2.10 U/mg) contained the highest SOD activity when compared to the SOD activities of *A. polytricha* (1.27 U/mg) and *T. fuciformis* (0.98 U/mg) (Table 3).

GPx activity

With regard to GPx activities (Table 3), there were no significant differences among the methanol extracts from the three gelatinous mushrooms (ranging from 19.33 to19.57 U/mg, ρ>0.05).

GRd activity

The methanol extract of *A. polytricha* (7.97 U/g) had the highest GRd activity whereas *T. fuciformis* (5.37 U/g) and white variety of *A. fuscosuccinea* (5.13 U/g) were significantly lower (ρ<0.05).

Determination of total antioxidant capacity (TAC)

The antioxidant efficiency of the three gelatinous mushrooms was expressed with a TAC value [as an equivalent of Trolox concentration (mM)] according to Miller et al. (1993). The TAC value for the methanol extract from white variety of *A. fuscosuccinea* (2.26 mM/g) was higher than *A. polytricha* (1.82 mM/g) and *T. fuciformis* (1.62 mM/g).

DISCUSSION

Correlation between antioxidant contents and antioxidant activity

Phenolic and flavonoid compounds attract food and medical scientists' attention because of their strong *in vitro* and *in vivo* antioxidant activities and the ability to scavenge free radicals, break radical chain reaction and chelate metals. It is usually speculated that in plant extracts that there is a direct correlation between total phenolic contents, total flavonoid contents and antioxidant activity of the extracts.

According to Orhan and Üstün (2011), polar or phenolic compounds cause higher antioxidant activity (Orhan and Üstün, 2011). A number of studies showed that antioxidant activity of plant extracts is correlated with total phenolic rather than with any individual phenolic compound (Frankel et al., 1995; Meyer et al., 1997; Prior et al., 1998). In addition to, it is rather difficult to characterize every phenolic compound and assess or compare their antioxidant activities (Su and Silva, 2006; Wong et al., 2006).

Therefore, in this study, total phenolic and total flavonoid were measured instead of the individual compounds. Total phenolics of *Lentinula edodes* (0.55 mg GAE/g) (Choi et al., 2006) was significantly lower when compared to ear mushrooms like *A. fuscosuccinea* white strain (8.72 mg GAE/g), *A. auricular-judae* (5.37 to 14.90 mg GAE/g), *A. mesenterica* (4.61 mg GAE/g), *A. fuscosuccinea* brown strain (3.90 mg GAE/g), *A. polytricha* (3.20 mg GAE/g) and *T. fuciformis* (1.04 mg GAE/g) (Mau et al., 2001; Kho et al., 2009).

In contrast, *Dictophora indusiata* (Mau et al., 2002b), *Agrocybe aegerita* var. *alba* (Lo and Cheung, 2005) and *Lactarius deliciosus* (L.) gray (Ferreira et al., 2007) contained 16.28, 15.3 and 17.25 mg GAE/g, respectively. Natural plant sources that contain lower total phenolic content than in gelatinous musrooms include fresh corn (Asami et al., 2003), different colors of fresh peppers (Zhang and Hamauzu, 2003b), and dried Greek aromatic plants (Proestos et al., 2006). These natural sources possessed only 0.25, 0.55 to 0.65, and 0.03 to 0.28 mg GAE/g, respectively. In contrast, total phenolic content in

seeds (Soong and Barlow, 2004) and medicinal plants (Djeridane et al., 2006; Matkowski and Piotrowska, 2006) range between 3.1 to 117 mg GAE/g of dry sample. Salah et al. (1995) demonstrated that the plant phenolic extracts act as agents of other mechanisms contributing to anticarcinogenic actions. High phenolic compounds consumption has been connected with a reduced risk of cardiovascular diseases and some cancers (Marja et al., 1999; Tapiero et al., 2000).

The amount of flavonoids content of *Macrolepiota mastoidea* was observed as 2.84 mg QE/g of extract (Shirmila and Radhamany, 2013). The flavonoid contents were found in the methanol extract of *Boletus aestivalis* [1.53 mg rutin equivalents (RE)/g], *Leccinum carpini* (1.48 mg RE/g) and *B. edulis* (1.46 mg RE/g) (Marijana et al., 2012). Total flavonoid compound was higher in *Pleurotus platypus* [4.46 to 4.73 mg tannic acid equivalents (TAE)/g] when compared to *P. eous* (3.75 to 3.97 mg TAE/g) (Sathyaprabha et al., 2011). The potential benefits of flavonoids on human health include antiviral, antiallergic, antiplatelet, antiinflammatory, antitumor and antioxidant activities (Jia et al., 1999).

Total flavonoids showed significant correlation with antioxidant action through scavenging or chelating process (Ribeiro et al., 2006). Numerous techniques are available to evaluate the antioxidant activity of a sample because there are different types of antioxidants with different antioxidant mechanisms. Besides, this is also due to the following reasons: 1) an antioxidant will turn into a pro- oxidant at critical condition, and 2) an antioxidant will be saturated and become a free radical source when it is not able to step into the chain of electron transport. Therefore, it can be considered that “A single antioxidant is not an antioxidant!” (Truscott, 1996). In this study, reducing power, DPPH free radical scavenging activity and ferrous ions chelating activity were chosen in order to evaluate the antioxidant capacities of white variety of *A. fuscosuccinea*.

Since the reducing power assay can only determine total nonenzymatic antioxidant activity of an extract, it is not able to detect free radical scavenging activity. Hence, the scavenging activity of mushroom extracts in the DPPH free radical assay were assessed to complement the determination of the scavenging effect on free radicals by the extract (Kanatt et al., 2007). Since synergistic action may occur among the different antioxidants in an extract, the relationships among the three different types of assay plus the determination of total phenolic, total flavonoid, and total sugar contents may be useful for the elucidation of the antioxidant properties of white variety of *A. fuscosuccinea*. According to Mau’s (2001) results, the reducing power of methanol extracts from five ear mushrooms was dose-dependent, and the descending order was: white variety of *A. fuscosuccinea* > *A. mesenterica* > *A. polytricha* > *A. fuscosuccinea* (0.67 to 0.74 at 5 mg/ml) > *T. fuciformis* (0.32 at 5 mg/ml). Huang (2000) reported that the methanol extract from *Taiwanofungus camphoratus* showed an excellent reducing power of 0.92 to 0.94 at 5 mg/ml, whereas, that from *Agaricus blazei* showed a reducing power of 0.79 at 5 mg/ml. Methanol extracts from other medicinal mushrooms including *Ganoderma lucidum*, *G. lucidum* antler and *G. tsugae* exhibited a strong reducing power of 1.62, 2.28 and 2.38 at 4.0 mg/ml, respectively (Mau et al., 2002a). However, a good reducing power 0.79 was observed with the methanol extract from another medicinal mushroom, *Coriolus versicolor*, at 4.0 mg/ml.

Reducing powers of methanolic extracts from *Grifola frondosa*, *Hericium erinaceus* and *Tricholoma giganteum* were 1.18, 1.01 and 0.63 at 9 mg/ml, respectively. Among methanol extracts from commercial mushrooms, *P. cystidiosus* and *P. ostreatus* exhibited excellent reducing powers of 1.00 and 1.19 at 10 mg/ml (Mau et al., 2002b). The reducing power of three gelatinous mushrooms in our study agreed with Mau’s results. Mau et al. (2001) also demonstrated that the high reducing power of white variety of *A. fuscosuccina* makes it maintained its white color and faded the original brown color.

Reports suggested that the reducing power is generally asso-ciated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Lillian et al., 2009). Shimada et al. (1992) reported that the antioxidant activity and reducing power are related, and the reducing power may result from their hydrogen donating ability. Duh (1998) reported that the reducing properties are associated with reductones, which may inhibit lipid peroxidation products (LPO) by donating a hydrogen atom and then terminating the free radical chain reaction (Gordon, 1990). Hence, the high reducing power of white variety of *A. fuscosuccinea* may be associated with high amount of reductones like phenolics and flavonoids.

At 1 mg/ml, the methanol extracts from *A. mesenterica* and *A. polytricha* scavenged DPPH radical completely (100%), whereas, those from *A. fuscosuccinea* white strain and *A. fuscosuccinea* brown strain scavenged DPPH radical by 94.5% at 0.4 mg/ml and 95.4% at 3 mg/ml, respectively. However, *T. fuciformis* was not effective in scavenging DPPH radical (71.5% at 5 mg/ml) (Mau et al., 2001). Excellent scavenging effects (96.3 to 99.1 and 97.1%) were observed with methanol extracts from *T. camphoratus* and *A. blazei* at 2.5 mg/ml, respectively (Huang, 2000).

At 6.4 mg/ml, the methanol extract from *Dictyophora indusiata* savenged DPPH radical by 92.1%, whereas scavenging effects of methanol extracts from other specialty mushrooms were 63.3 to 67.8% (Mau et al., 2002b). The methanol extract from *P. ostreatus* at 6.4 mg/ml scavenged DPPH radical by 81.8%, whereas scavenging effects of extracts from other commercial mushrooms were 42.9 to 69.9% (Mau et al., 2002a). Scavenging effects of methanolic extracts from other me-

dicinal mushrooms were measured at up to 0.64 mg/ml and were 24.6, 67.6, 74.4 and 73.5% for *Trametes versicolor*, *G. lucidum*, *G. lucidum antler* and *G. tsugae* (Mau et al., 2002a). These results revealed that gelatinous mushrooms were free radical inhibitor or scavengers, acting possibly as primary antioxidants.

Methanol extracts from gelatinous mushrooms react with free radicals, particularly peroxy radicals, which are the major propagators of the antioxidation of fat, thereby terminating the chain reaction (Shahidi and Wanasundara, 1992). Some researchers referred a high correlation between DPPH radical-scavenging activities and total phenolics (r = 0.971) (Liu and Ng, 2000; Siriwardhana et al., 2003). Kumar et al. (2008) reported that the linear regression analysis of DPPH scavenging with the total phenolic content (GAE) gave an r value of 0.937 which indicated a statistically significant correlation. The data presented in this study indicated that the methanol extracts of white variety of *A. fuscosuccinea* had the highest amount of phenolics, which may explain the high scavenging ability on DPPH.

Mau et al. (2001) reported that the methanol extracts of ear mushrooms were decent chelators for ferrous ions. The chelating effects of methanol extracts from white variety of *A. fuscosuccinea*, *A. mesenterica*, *A. polytricha*, *A. fuscosuccinea* and *T. fuciformis* were 89.16, 92.05, 96.53, 85.13 and 93.64%, respectively at 5 mg/ml. Methanol extracts from *T. camphoratus* chelated ferrous ions by 64.4 to 74.5% at 5 mg/m, whereas that from *A. blazei* showed an excellent chelating effect of 98.6% at 2.5 mg/ml (Huang, 2000). The methanolic extract from *T. versicolor* was not a good ferrous chelator (13.2% at 2.4 mg/ml), whereas, other medicinal mushrooms including *G. lucidum*, *G. lucidum antler*, *G. tsugae* and *C. versicolor* chelated 55.5, 67.7, 44.8 and 13.2% of ferrous ions at 2.4 mg/ml (Mau et al., 2002a).

The methanol extract from *G. frondosa* chelated 70.3% of ferrous ion at 6 mg/ml, whereas at 24 mg/ml, methanol extracts from *D. indusiata*, *H. erinaceus* and *T. giganteum* chelated ferrous ion by 46.4 to 52.0% (Mau et al., 2002b). For commercial mushrooms including *Flammulina velutipes*, *P. cystidiosus*, *P. ostreatus* and *L. edodes* chelated ferrous ion at 1.6 mg/ml (Mau et al., 2002a). Since ferrous ions are the most effective pro-oxidants in the food system (Yamaguchi et al., 1988), the higher chelating effect of methanol extracts from gelatinous mushrooms would be beneficial. The metal chelating abilities of three gelatinous mushrooms and standard antioxidants were determined by evaluating their capacity to compete against ferrozine for the ferrous ions. Therefore, measurement of color reduction rate allows estimation of the chelating efficiency of the coexisting chelator (Yamauchi et al., 1988). It has been reported that chelating agents act as effective secondary antioxidants through reducing redox potential to stabilize oxidized form of metal ions (Gardner et al., 2000). Antioxidant properties of mushrooms were usually related to low-molecular-weight compounds, in particular to the phenolic fractions (Nickavar et al., 2007; Pan et al., 2008).

Past studies indicated that correlations have been found among total phenolic contents, TAC values and nitric oxide (NO) scavenging effects (Tsushida et al., 1994). In this study, compared to the other gelatinous mushrooms, white variety of *A. fuscosuccinea* had significant higher total phenolic (approximately twice than *A. polytricha* and almost four times than *T. fuciformis*) and relatively high total flavonoid content, which is comparable to that detected in *T. fuciformis*. These results might provide a possible explanation why white variety of *A. fuscosuccinea* had highest reducing power, scavenging effect on DPPH radicals. The DPPH and ABTS radicals had been used widely to investigate the scavenging activities of several natural compounds such as phenolic compounds or crude extracts of plants.

The model of scavenging the stable DPPH radical is widely used to evaluate antioxidant activities over a relatively short time compared to other methods. The antioxidant properties and antioxidant enzyme activities of *T. fuciformis* were less effective when compared to the activities of white variety of *A. fuscosuccinea* and *A. polytricha*. This may be explained by *T. fuciformis* having the lowest content of total phenolics. Similar results were reported by Mau et al. (2001). As for the better chelating effects on ferrous ions of the methanol extract of *T. fuciformis*, this study referred that *T. fuciformis* has significant higher total sugar (approximately 1.6 times that of *A. polytricha* and almost 2.5 times that of white variety of *A. fuscosuccinea*); and there is a high correlation between ferrous ions chelating effects and total sugars (r = 0.987).

In this assay, the methanol extracts of the three gelatinous mushrooms and standard antioxidant compounds interfer the formation of the ferrous and ferrozine complex. These results suggest that they have chelating activities and capable to capture ferrous ions before ferrozine. Ferrous ions are the most effective pro-oxidants and are commonly found in foods (Yamaguchi et al., 1988). Therefore, the high ferrous ion chelating abilities of methanol extracts from these mushrooms are beneficial. Among the methanol extracts, white variety of *A. fuscosuccinea* was more effective than *A. polytricha* and *T. fuciformis* in reducing power and scavenging effect on DPPH radicals.

In contrast, *T. fuciformis* was more effective than white variety of *A. fuscosuccinea* and *A. polytricha* in chelating effects on ferrous ions. The methanol extracts had high antioxidant activities, indicating that the methanol extracts may contain some potential natural antioxidant components that are relatively effective. BHA and BHT as a pure/concentrated synthetic phenolic antioxidant can scavenge reactive oxygen species such as DPPH free radicals by donating labile hydrogen and leaving an oxidized phenolic ion stabilized by the inherent resonance of the benzene ring. This may be the reason why BHA

demonstrated a relatively higher free radical scavenging activity than the extracts of *A. auricula judae*. In addition, the extracts tested in this study were crude.

Generally, EC_{50} values lower than 1 mg/ml indicate that the methanol extracts have effective antioxidant properties. Although, BHA and BHT had inhibitory effects on reducing powers and scavenging effects of DPPH radicals and EDTA was able to chelate ferrous ions, they are additives and are present in low levels (mg) in foods. The three gelatinous mushrooms in this study may be used of higher levels (g) in food or food ingredients. Therefore, these mushrooms may serve as possible protective agents in human diet to help humans in reducing oxidative damage.

The mechanism of SOD is to accelerate the dismutation of the toxic superoxide radical ($O_2^{\bullet}$), that is produced during oxidative energy processes to hydrogen peroxide and molecular oxygen. This method employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye.

The SOD activity is measured by the degree of inhibition of this reaction. One unit of SOD is the amount that causes a 50% inhibition of the rate of INT reduction. The high SOD activities that were found in the methanol extracts of white variety of *A. fuscosuccinea* indicated that the extract contained compounds to inhibit the reduction of INT.

GPx catalyses the oxidation of reduced glutathione (GSH) by cumene hydroperoxide. In the presence of glutathione reductase (GR) and NADPH, the oxidized glutathione (GSSG) is immediately converted to the reduced form (GSH) with a concomitant oxidation of NADPH to $NADP^+$. Hydrogen peroxide is accumulated during dopamine metabolism. GPx activity plays an important role to scavenge the elevated levels of hydrogen peroxide.

This result suggests that the three gelatinous mushrooms all have good scavenging activities of hydrogen peroxide. GRd catalyses the reduction of GSSG in the presence of NADPH, which is oxidized to $NADP^+$. Increasing GRd activity may occur to maintain GSH in a reduced form helps.

The methanol extracts of *A. polytricha* had the highest GRd-like activity to remove the toxic GSSG. There are less research shed lights on the correlations between enzyme activities and the antioxi-dant activities of mushrooms.

The present study shows that the methanol extract form white variety of *A. fuscosuccinea* had highest SOD activity and TAC activity compared to *A. polytricha* and *T. fuciformis*. *T. fuciformis* did not show good activities on reducing power and scavenging effect on DPPH radicals. All antioxidant enzymes are essential catalysts which stimulate chemical reactions without becoming consumed or integrated in the reaction. Antioxidant enzymes may also stop the free radical from forming in the first place. In addition, they may interrupt an oxidizing chain reaction to minimize the damage caused by free radicals. Significant differences were detected in antioxidant activities and antioxidant enzyme activities among three gelatinous mushrooms.

Conclusion

According to this study, the consumption of white variety of *A. fuscosuccinea* may be beneficial to the antioxidant protection system of the human body against oxidative damage. Therefore, in addition to these antioxidant components, other components may contributeto the antioxidant properties of white variety of *A. fuscosuccinea*. To study the mechanisms of other potential antioxidant components, the fractionation of methanol extracts and further identification should be in progress.

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Full Length Research Paper

Antimycotoxigenic and antifungal activities of *Citrullus colocynthis* seeds against *Aspergillus flavus* and *Aspergillus ochraceus* contaminating wheat stored

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Plant extracts and their constituents have a long history as antifungal agents, but their use in biotechnology as preservatives, due to the increasing resistance of fungi to fungicides, has been rarely reported. The aim of this study was to assess *in vitro* antifungal and antimycotoxigenic power of methanolic and aqueous extracts of *Citrullus colocynthis* seeds, an aromatic and medicinal plant, of Algerian flora, against two toxigenic species of the genera *Aspergillus* responsible of contamination of wheat stored. The antifungal and antimycotoxigenic activity of methanolic and aqueous extracts were screened against *Aspergillus ochraceus* and *Aspergillus flavus.* Dillution method was used to investigate the antimicrobial and antimycotoxigenic activity. These bioassays are preceded by a phytochemical screening. The phytochemical analysis of seeds extracts revealed the presence of some chemical groups (polyphenols, steroids and alkaloids) which can express the desired activities. The results suggest that the extracts showed a very good antifungal activity against *A. ochraceus*, but for *A. flavus* any antifungal activity was recorded. The extracts have good antiochratoxigenic power in liquid medium. This evaluation confirms that the extracts of *C. colocynthis* seeds used at low concentration may have significant potential for biological control of fungi and theirs toxins.

Key words: *Citrullus colocynthis,* methanolic extract, aqueous extract, phytochemical screening, antifungal activity, antimycotoxigenic activity, antiochratoxigenic activity.

INTRODUCTION

Fungi are the main infectious agents in plants, causing alterations during developmental stages including post-harvest. In fruit and vegetables, there is a wide variety of fungal genera causing quality problems related to aspect,

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Abbreviations: **AFs,** Aflatoxins; **OTA,** ochratoxin A; **TLC,** thin-layer chromatography; **DRBC,** Dichloran Rose-Bengal chloramphenicol agar; **CDA,** Czapek dextrose agar; **PDA,** potatoes dextrose agar; **YES,** yeast extract sucrose; **MIC,** minimum inhibitory concentration; **MFC,** minimum fungicidal concentration.

nutritional value, organoleptic characteristics and limited shelf life (Yanes et al., 2012). In addition, fungi are responsible for allergic or toxic disorders among consumers because of the production of spores or mycotoxins (Dellavalle et al., 2011).

Mycotoxins are secondary metabolites produced by five fungal genera namely *Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria* and *Claviceps*, they are synthesized under favorable conditions of temperature and humidity. They may be developed in several stages, in the field before harvest, during storage and even in the production chain (Petzinger and Weindenbach, 2002). These toxic substances are carcinogenic, nephrotoxic, hepatotoxic and immunosuppressive (Dellavalle et al., 2011; Korhonen et al., 2012). They are found in many food products such as coffee, cereals, wine and fermented products (Cynthia et al., 2012). Aflatoxins (AFs) are the most dangerous mycotoxins.

Five types of aflatoxins are known; AFB1, AFB2, AFG1, AFG2 and AFM, these toxins are produced by *Aspergillus flavus, Aspergillus parasiticus, Aspergillus nomius, Aspergillus pseudotamarii, Aspergillus bombycis, Aspergillus toxicarius, Aspergillus minisclerotigenes, Aspergillus parvisclerotigenus* and *Aspergillus arachidicola* (Samson et al., 2006; Pildain el al., 2008). *Aspergillus flavus* and *Aspergillus parasiticus* are the major producers of AFB1 (Gourama and Bullerman, 1995). Ochratoxin A (OTA) is the second important mycotoxin with fumonisin, zearalenone and trichothecene. OTA is produced by *Penicillium verrucosum, Aspergillus ochraceus, Aspergillus alliaceus, Aspergillus carbonarius, Aspergillus niger* and *Aspergillus melleus* (Da Rocha Rosa et al., 2002; Accensi et al., 2004; Bau et al, 2005; Bayman and Baker, 2006).

Fungi are generally controlled by synthetic fungicides; however, the use of these is increasingly restricted due to the harmful effects of pesticides on human health and the environment (Hermiche et al., 2012). The increased risk of high-level toxic residues in the products and the emergence of pathogens resistant to the products employed, justifies the search for novel actives molecules and new control strategies. Thus, there are a growing interest on the research of possible use of the plant extracts for control of the pest and diseases in agriculture which is less harmful to the health and environment (Nwosu and Okafor, 2000; Logardia et al., 2012).

Several works have demonstrated in laboratory trials that plants tissues, such as roots, leaves, seeds and flowers posses inhibitory properties against bacteria, fungi and insects (Thembo et al., 2010; Benariba et al., 2013). In front these very serious health problems, use of medicinal plants in biomedical research received great interest. This is because herbs are an inexhaustible source of bioactive natural compounds and fewer side effects than drugs (Dramane et al., 2010; Satyavani et al., 2012). Medicinal plants are now an endless source of interesting molecules for scientists and industry, which occur as secondary metabolites (Lozoya and Lozoya, 1989; Karthikeyan et al., 2009). They are grouped as alkaloids, glycosides, flavonoids, saponins, tannins, carbohydrate and essential oils. Molecules from these plants have similar active ingredients which have specific properties giving them an intrinsic behavior (Evon, 2008). A wide spectrum of biological substances extracted from medicinal plants, including oils were tested to replace some of the ways to fight against fungi. In this section, several authors have confirmed the effectiveness of the oils on toxigenic fungi (Ziyada and El Hussein, 2008; Yingying et al., 2008).

Citrullus colocynthis (Schrad), belonging to the family of *Cucurbitaceae*, is an endemic in the south of Algeria. This medicinal plant popularly known as *Handhal, Hdaj* or *Dellaa El-Wad*, is widely used in Algerian folk medicine for treating many diseases such as rheumatism, hypertension hyperglycemia and various contagious diseases, including dermatological problems and gynaecological, urinary and pulmonary infections (Le Flock, 1983; Boukef, 1986; Marzouk et al., 2009; Gurudeeban et al., 2010). .

The objective of this work is to demonstrate the antifungal, antiaflatoxigenic and antiochratoxigenic effect of methanol and aqueous extracts of *C. colocynthis* seeds, after determining their chemical composition, against two toxigenic fungal strains namely: *A. flavus* and *A. ochraceus* isolated from wheat stored.

MATERIALS AND METHODS

Plant

C. colocynthis Schrad. fruits were collected in December (2010) near Ouargla, Algeria in the area of Oued N'sa. The identification was performed according to the flora of Tunisia (Pottier-Alapetite, 1981) and the botanists of Faculty of Biology of Saida University (Algeria).

Extraction protocol

The extractions were performed on the seeds of *C. colocynthis*. Plant materials were washed with tap water, disinfected by immersion in 2% sodium hypochlorite solution for 30 min, rinsed with sterile distilled water to eliminate residual hypochlorite. Afterwards, the seeds are ready for extraction (Jasso de Rodriguez et al., 2005). In this study, water and methanol are the two solvent used for extraction. These two solvent are polar and they can extract the maximum of bioactive substances.

Methanol extract

Twenty grams of seeds were ground with a mixer and added to 100 ml of methanol. After 3 h of maceration with continuous stirring at 200 rev/min, the mixture was then filtered using filter paper (Whatman No 1). This operation is repeated four times after each filtration with renewal of the solvent in order to exhaust the marc and increase the yield. At the end of extraction, the fractions obtained were collected in a vial and then were evaporated by rotavapor at a specific temperature to the solvent (Senhaji et al., 2005).

Aqueous extract

The aqueous extract is prepared by soaking 20 g of the ground seeds in 100 ml of cold distilled water for 3 h with continuous agitation. The mixture was then centrifuged at 3600 g for 30 min. The supernatant was recovered and then filtered through Whatman filter No. 1. This operation was repeated four times after each filtration with renewal of the solvent. At the end of extraction, the fractions obtained were collected in a vial, then, lyophilized or dried in the drying oven, giving the dry aqueous extract (Senhaji et al., 2005).

Determination of extraction yield

The yield is determined by the ratio of the weight of the dry extract after evaporation on the weight of the plant material used for extraction, multiplied by 100%.

$Rd\ \% = (m_1 \times 100) / m_0$

Where, m_1 is the Mass in grams of the dry extract; m_0 is the mass in grams of dry plant material; Rd is the yield.

Qualitative phytochemical screening

Tannins

One milliliter of extract was mixed with 10 ml of distilled water and filtered. Three drops of ferric chloride ($FeCl_3$) reagent (1% prepared in methanol) was added to the filtrate. A blue-black or green precipitate confirmed the presence of gallic tannins or catechol tannins, respectively (Karumi et al., 2004).

Saponins

Ten milliliters of extract were placed in a test tube shaken for 15 s and then deposited for 15 min. A persistent foam height greater than 1 cm indicates the presence of saponins (N'Guessan et al., 2009).

Steroids

After addition of 5 ml of acetic anhydride to 5 ml of hot extract, the mixture was added to 0.5 ml of concentrated sulfuric acid. After stirring, the appearance of a purple or violet ring turning blue to green indicates the presence of steroids (Edeoga et al., 2005).

Flavonoids

Flavonoids were detected by reaction with cyaniding. 2 ml of each extract were evaporated and the residue was taken in 5 ml of alcohol hydrochloric dilute 2 times. By adding 2 to 3 magnesium chips, there is a heat release and an orange-pink coloration or purplish. The addition of 3 drops of isoamyl alcohol has intensified this color which confirmed the presence of flavonoids (N'Guessan et al., 2009).

Alkaloids

Alkaloids have been characterized using reagents of Mayer. 10 milliliters of extract were evaporated until a volume of 0.2 ml was obtained on which, 1.5 ml of HCl (2%) was added. After stirring the acid solution, 1 to 2 drops of reagent were added, and the appearance of a yellowish white precipitate indicates the presence of alkaloids (Mojab et al., 2003).

Anthraquinones

The method cited by Trease and Evans (1996) was used for the detection of anthraquinones. The presence of violet colour in the ammoniacal (lower) phase indicated the presence of free hydroxy anthraquinones (Trease and Evans, 1996).

Coumarins

Coumarins were found from 5 ml of extract placed in a tube brought to boiling until obtaining a volume of 1 ml, this volume is added to 1 ml of hot water. After stirring, the total volume is divided into two volumes, one as a control and the other is added to 0.5 ml of NH_4OH (10%) and examined under a UV lamp. The fluorescence emission indicates the presence of coumarins (Rizk, 1982).

Antifungal activity of plant extracts

Fungal isolation

Dilution plating was used as isolation technique (Pitt and Hocking, 2009). 10 g of the sample were added to 90 ml of 0.1% peptone water. This mixture was then shaken on a rotary shaker for approximately 15 min and diluted 10^{-2}, 10^{-3} and 10^{-4} fold. Aliquots composing of 0.1 ml of each dilution were spread (in triplicate) on the surface of the dichloran Rose-Bengal chloramphenicol agar (DRBC), Czapek dextrose agar (CDA) and potatoes dextrose agar (PDA). All plates were incubated for 5 to 7 days at 28°C in the dark and under normal atmosphere. The identification of fungal strain is realized on the basis of morphological characteristics, under the microscope (Barnett and Hunter, 1972; Pitt and Hocking, 2009), and single spore method by colony characteristics after their culture on different culture media (Pitt, 1973; Pitt and Hocking, 2009).

Identification of strains producing mycotoxins

The strains of *A. flavus* and *A. ochraceus* identified were reseeded separately on 50 ml of yeast extract sucrose (YES) medium. After 14 days of incubation at 27 ± 2°C, the biomass formed is removed by filtering the medium through Whatman filter paper No. 01. The 50 ml of the filtrate are added to 100 ml of chloroform, the mixture is thoroughly stirred for 10 min and then allowed to settle by using a separating funnel. This operation is repeated by adding successively 50 and 30 ml of solvent to the aqueous phase recovered at each separation. The chloroform phase thus obtained is filtered through Whatman paper No. 01 and then concentrated by evaporation under vacuum using a rotary evaporator type (Heidolph efficient Laborota 4000) until a volume of 2 to 3 ml.

Thin-layer chromatography (TLC) is performed on a silica gel plate (silica gel 60 F254). The plate is then placed in a chromatographic tank dipped in a mixture of elution solvent consisting of toluene, ethyl acetate and formic acid with volume (5: 4: 1), respectively. After migration and evaporation of the elution product dry, the plate is examined under a UV lamp at a wavelength of 365 nm (Frayssinet and Cahagnier, 1982).

Evaluation of antifungal and antimycotoxigenic activity of organic extracts

The study of the antifungal and antimycotoxigenic activity of methanol and aqueous extracts were tested against two species *A ochraceus* and *A. flavus* on YES medium in order to be able to extract the mycotoxins produced. On an individual basis, each of

Table 1. Extraction yields (%) and phytochemical screening of *C. colocynthis* seeds.

Extract	Extraction yields (%)	Phytochemical substance						
		Flavonoid	Steroid	Alkaloid	Anthraquinon	Coumarin	Saponosid	Tannin
Methanol	4.89	+	+	+	-	-	+	+
Aqueous	2.72	+	+	+	-	-	-	+

+, Presence; -, absence.

Table 2. Identification of *Aspergillus ochraceus* and *Aspergillus flavus* by single spore method.

Genera species	Medium	Reading (Color)
Aspergillus flavus	MEA 25°C	Pistachio green
	CYA 37°C	Dark brown
	G25N 25°C	Greenish yellow
	AFAP	Orange back
Aspergillus ochraceus	MEA 25°C	Yellow gold
	CYA 37°C	Yellow
	G25N 25°C	Yellow

the two extracts (*C. colocynthis* seeds) was added to 50 ml of YES medium but to varying final concentrations are in the order of 1 to 25 mg/ml. After rigorous agitation, different media are inoculated with discs of 0.6 cm of diameter containing youth cultures of 3 to 7 days of *A. ochraceus* and *A. flavus*. Control tests are made for strains and for each test series (Ezzat and Sarhan, 1991; Al-Rahmah et al., 2011). After an incubation period of 14 days at 27 ± 2°C, the same steps mentioned above for the extraction and the revelation of mycotoxins have been followed. The biomass of the filtered mycelium was determined after drying at 70°C for 4 days till their weights remains constant. The percentage inhibition is calculated by the following formula:

Percentage of mycelial inhibition = [C - T / C] × 100

Where, C and T are the mycelial dry weight (mg) in control and treatment, respectively.

RESULTS

Extraction yield and phytochemical screening

The calculation of the chemical extractions yields relative to the total weight of the dry powder used displayed in Table 1 shows that the *C. colocynthis* seeds gave dry extracts masses greater than 1 g/100 g seed powder. From the point of view profitability by weight, methanolic extract gave the highest proportions by comparing it with the aqueous extract. On same Table 1, the qualitative chemical analysis tests that are designed to demonstrate the different phytochemical families existing in both extracts revealed a slight difference in the composition of the extracts. This difference is noticed by the lack of saponins which are absent in the aqueous extract while they are present in the methanol extract. Both extract react negatively with tests revealing anthraquinons and coumarins, whereas for other photochemical tests, the two types of extracts reacted positively.

Identification of fungal strains

The different microscopic and macroscopic aspects of both fungal strains searched are demonstrated in Figures 1 and 2. The aspects of fungal colonies of the same strains by single spore method on different culture media are shown in Table 2. The results revealed strains producing mycotoxins on TLC which showed that the strain *A. flavus* is producing AFB1 and strain *A. ochraceus* is producing the OTA.

Antifungal and antimycotoxigenic activity of organic extracts

The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) were employed by poisoned food technique to assess fungistatic and fungicidal properties of the effective plant extract. As illustrated in Figure 3, the inhibitory plant extracts showed that the fungal strain *A. ochraceus* is very sensitive to both types of extracts. Beyond 15 mg/ml of methanol extract and 20 mg/ml of aqueous extract, the latter did not develop biomass in YES medium. Transplanting these mycelial discs that could not grow in the presence of extracts on other PDA medium (without extracts) did not provide any radial growth after 14 days of incubation at 25 ± 2°C, which explains that CMF is 15 mg/ml for

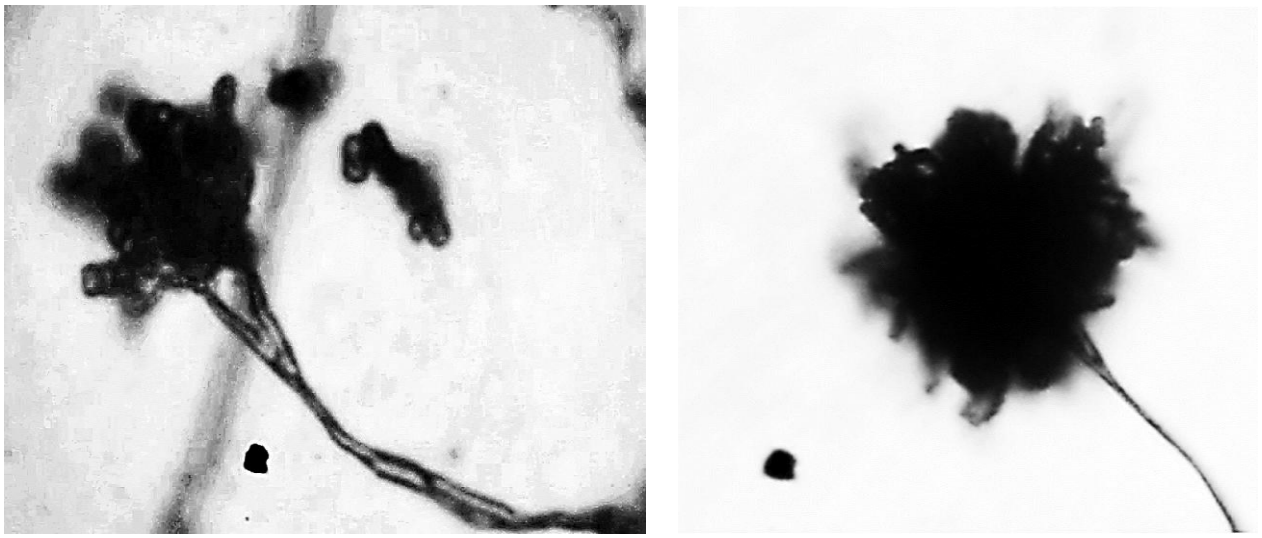

Figure 1. Identification of the genus *Aspergillus* by micro-culture method.

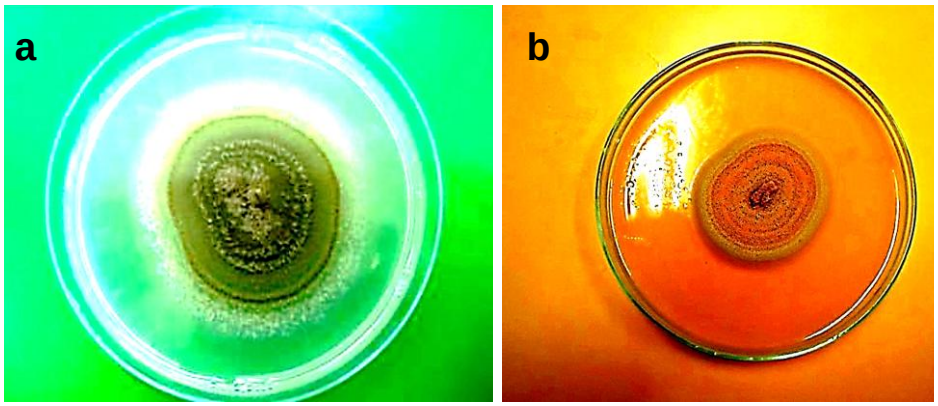

Figure 2. Identification of fungal species by Single Spore method. **a,** Colonies of *Aspergillus flavus* on PDA medium; **b,** colonies of *Aspergillus ochraceus* on PDA medium.

methanolic extract and 20 mg/ml for the aqueous extract. MICs are 10 mg/ml and 15 mg/ml for methanolic and aqueous extract, respectively. Below these two concentrations, the antifungal activity begins to decrease. Figure 4 showed that the strain *A. flavus* has proved highly resistant to two extracts of *C. colocynthis* seeds and no antifungal activity was recorded.

The results displayed in Table 3 achieve the last objective of this study by demonstrating that methanol and aqueous extracts tested against *A. flavus* to determine the power synthesis inhibitor of AFB1 showed no inhibitory activity against this toxin and TLC revealed the presence of a similar spot to the standard of pure AFB1 (Figure 5). For antiochratoxigenic activity (Table 3), methanol extract was able to reduce the synthesis of OTA produced by *A. ochraceus* from 10 mg/ml of extract in the YES medium explained by the reduction of the size of the spot toxin on the TLC plate. For the aqueous extract, the TLC detected a reduction of the synthesis of the toxin produced from 15 mg/ml of extract in the medium. At 15 mg/ml of methanol extract and 20 mg/ml aqueous extract, OTA was not detected on the TLC plate (Table 3).

DISCUSSION

Fungi are ubiquitous in the environment, and infection due to fungal pathogens which has become more common. The genus *Aspergillus* is widely distributed in nature and its species are among the most common destroyers of foodstuffs and grains during storage. It includes species that may damage crops in the field or cause post-harvest decay (Sun et al., 2012). In addition, the

Table 3. Antimycotoxigenic activities of methanol and aqueous extracts of *C. colocynthis* seeds.

Parameter	Concentrations (mg/ml)						
	0	1	5	10	15	20	25
Production of AFB_1 in the presence of Me. E	+	+	+	+	+	+	+
Production of AFB_1 in the presence of Aq. E	+	+	+	+	+	+	+
Production of OTA in the presence of Me. E	+	+	+	-/+	-	-	-
Production of OTA in the presence of Aq. E	+	+	+	+	-/+	-	-

AFB_1, Aflatoxine B_1, OTA, ochratoxine A; Me. E, methanol extract; Aq. E, aqueous extract; +, presence; -, absence.

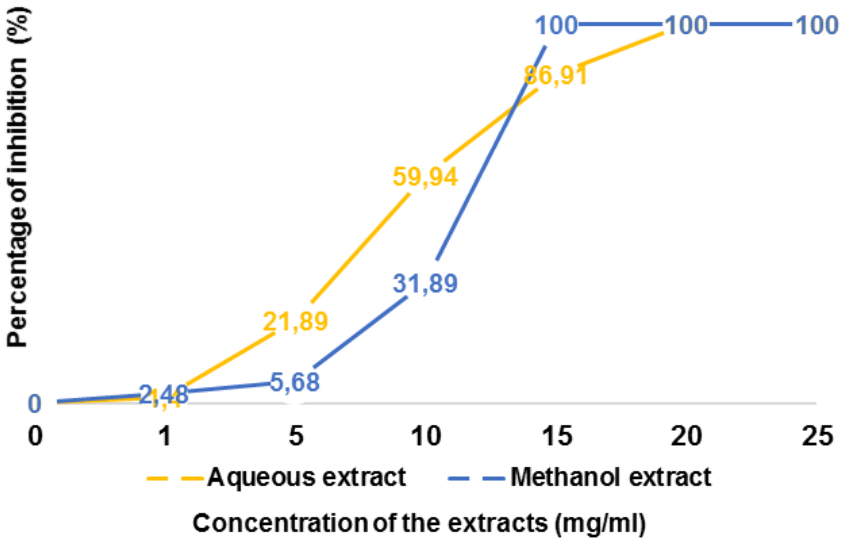

Figure 3. Antifungal activity of methanol and aqueous extracts against *Aspergillus ochraceus* on YES medium.

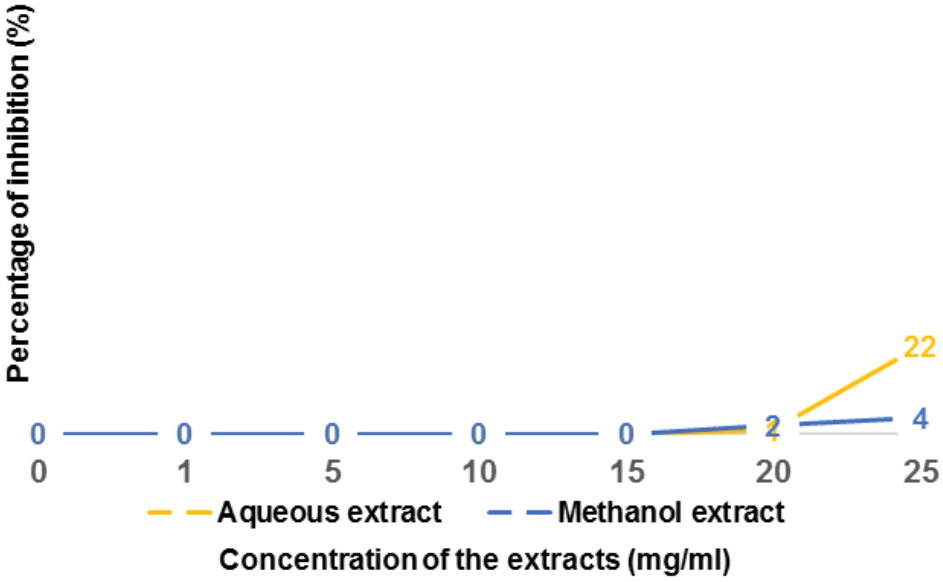

Figure 4. Antifungal activity of methanol and aqueous extracts against *Aspergillus flavus* on YES medium.

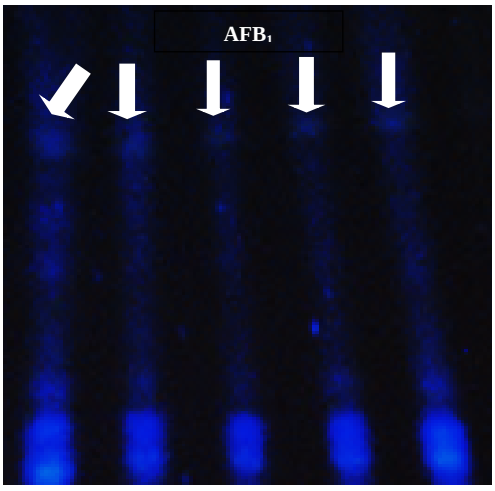

Figure 5. Antiaflatoxigenic activity of methanol extracts against *Aspergillus flavus*.

genus products mycotoxins and studies in the last decade have emphasized its toxicogenic properties. Indeed, the palette adverse effects of mycotoxins on the human and animal health is very extensive and sometimes unknown (Brochard and Le Bacle, 2009; Cynthia et al., 2012). Besides, acute toxic effects or chronic hemorrhagic, immunotoxic, hepatotoxic, nephrotoxic, neurotoxic and teratogenic, some mycotoxins have shown mutagenic and carcinogenic effects in laboratory animals and humans (Korhonen et al., 2012).

Over the last decades, concerns were expressed about the increasing prevalence of pathogenic fungi that are resistant and more precisely those producing mycotoxins. But the problem posed by the high cost and the increased toxic side effects of some synthetic substances coupled with their failure to be treated cannot be underestimated. For this reason, this last decade witnessed increased intensive studies of extracts and biologically active compounds isolated from natural plants (Mabrouk, 2012; Elamathi et al., 2012).

Despite numerous studies on the use of colocynth in the culinary field and that of traditional medicine, little work has been done on the antifungal effect of *C. colocynthis* seeds against fungal strains spoilage of the physicochemical and mycological quality of stored wheat (Gacem et al., 2013). Facing this situation, the aim of this work was to evaluate *in vitro* the antifungal and antimyctoxigenic potential of *C. colocynthis* seeds against toxigenic fungi producing mycotoxins namely *A. flavus* and *A. ochraceus* in order to check possible inhibition activity.

The extraction method performed on the powder of *C. colocynthis* seeds conducted at an ambient temperature can extract maximum compounds and prevent their denaturation or probable modification (Yagoub, 2008). The phytochemical screening of methanol and aqueous extracts reveal the richness of *C. colocynthis* seeds from a qualitative point by secondary metabolites such as steroids, flavonoids, alkaloids and tannins. These compounds have been reported in the *C. colocynthis* by several studies (Gurudeeban et al., 2010; Adebayo-Tayo et al., 2010).

Outcomes related to phytochemical screening of the last class of secondary metabolites show that the alkaloids are present in both extracts from seeds of *C. colocynthis*. This result is confirmed by Sultan et al. (2010) and Benariba et al. (2013) who detected the alkaloids in seeds of this species and Marzouk et al. (2010) who showed that the seeds of *C. colcoynthis* contain 1.64 mg of alkaloid per 100g of material dry. The absence of antraquinons is cited in the study of Suman (2010).

Ethanolic and aqueous extracts from *C. colocynthis* leaves and fruits contain alkaloids, flavonoids, glycosides and saponosides (Najafi et al., 2010). Likewise, the entire *C. colocynthis* plants contain 1.39 mg flavonoids, 0.52 mg saponosides, 1.64 mg alkaloids, 1.64 mg phenolic compounds and 30.12 mg ascorbic acid per 100 g (Sultan et al., 2010). Another study by Gill et al. (2011) documented the presence of alkaloids, steroids, terpenoids, flavonoids, as well as coumarins, glycosides in methanolic and hydromethanolic extracts of *C. colocynthis* seeds. The polyphenol compounds, represented in majority by tannins and flavonoids, are presently a major axis of research, because they are considered as potent antioxidants, anti-inflammatory, anti-bacterial, antiviral and anti-cancer agents (Oliver Chen and Blumberg, 2008).

For instance, Afifi et al. (1973) reported the presence in the whole plant of three alkaloids ($C_{10}H_{15}NO_3$, $C_{20}H_{32}NO$ and $C_{16}H_{24}NO_7$. Hatam et al. (1990) documented the presence of two sterols ($C_{29}H_{48}O$ and $C_{29}H_{50}O$) in a *C. colocynthis* fruits collected in Basra area in Iraq. Thus, *C. colocynthis* contain flavonoids such as quercetin, myricetin and kaemoferol (Oliver Chen and Blumberg, 2008).

Several study identified cucurbitacins in a methanol extract of *C. colocynthis* fruits (Sonja and Hermann, 2000; Seger et al., 2005; Nayab et al., 2006). Such cucurbitacins are relevant to the bitterness and toxicity of the plants, as well as their anti-inflammatory, purgative and anti-cancer activities, such as the inhibition of cell adhesion resulting from the cytoskeleton destabilizing in cancer cells exposed to cucurbitacin E (Jian et al., 2005).

Incidentally, minor differences between the results of distinct studies could be related to differences in local climate and soil composition. The distribution of phytoconstituents such as saponins, tannins, flavonoids and alkaloids, may also vary in distinct parts of *C. colocynthis*, in leaves, fruits, roots and seeds. The study of Gacem et al. (2013) revealed a good activity against strains of *Aspergillus*. The tests of antifungal activities of colocynth in YES medium against strains *A. flavus* and *A. ochraceus* isolated from wheat stored revealed effective-

ness of methanol extract of the seeds of this plant against *A. ochraceus*. The experiment revealed that the methanol extract has a more antagonistic effect than the aqueous extract. This effect is explained by the high yield of methanolic extraction, which is due to the presence of bioactive substances with high quantity. This strong antifungal activity of methanol extract was also reported by several authors (Hadizadeh et al., 2009; Gurudeeban et al., 2010; Gacem et al., 2013).

Chang et al. (2008) and Abdel Ghani et al. (2008) join the antifungal activity of extracts from *C. colocynthis* seeds with bioactive substances of the plant. The power of these phytochemicals compounds to exert higher activity is depending to their concentrations in the extracts (Yan et al., 2008). Among the phytochemicals compounds with antifungal activity, mainly cites alkaloids, polyphenols and steroids (Yan et al., 2008; Oliver Chen and Blumberg, 2008). The antifungal activity of the extracts of the plant depends on its composition, the plant organ to be tested, the nature of the extract and the fungal strains selected (Veldhuizen et al., 2006; Dan et al., 1998).

Several studies have been conducted to understand the mechanism action of plant extracts. Many researchers attribute this feature to phenolic compounds. These compounds can interfere with bio-membranes causing cell damage and causing leakage of cellular materials and finally the death of microorganisms (Veldhuizen et al., 2006; Abdel Ghani et al., 2008). This is a possible mechanism by which the mycelial growth can be reduced or completely inhibited by the effect of extracts acting on the function and structure of the cell membrane. Saponins are a special class of glycosides with a soapy characteristic and very good antifungal activity (Sikkema et al., 1995).

Flavonoids are also responsible for the inhibition of resistant microbes. They are responsible for the scavenging process or chelators and may disrupt microbial membranes. Furthermore, alkaloids contain a detoxifying effect and have a very good antifungal activity (Kessler et al., 2003). Terpens (steroids) affect not only the permeability but also other functions in cell membranes. These compounds can penetrate cell membranes, enter the interior of the cell, and interact with critical sites such as intracellular enzymes and proteins, leading to cell death (Omidbeygi et al., 2007).

The extracts obtained from the upper parts of plants have the ability to suppress the growth of toxigenic fungi and therefore toxin production (Thanaboripat et al., 1997). They can also completely block the biosynthesis of mycotoxins while fungal growth is not affected (Bhatnagar and Mccormick, 1988). These seed extracts of *C. colocynthis* are less important relative to the extracts of *Eucalyptus globolus*, *Olea europea* and *Thymus vulgaris* described in the study of Al-Rahmah et al. (2011), which proved a complete inhibition of AFB1 synthesis and the study of El-Nagerabi et al. (2012) who demonstrated the effect of *Hibiscus sabdariffa* extract and *Nigella sativa* oil for inhibiting the synthesis of AFB1. The phenolic compounds of *C. colocynthis* seeds cannot inhibit the biosynthesis steps of AFB1, explained by their absence in the lipids of the fungal cell wall membrane and mitochondria, disturbing their structure and rendering them more permeable. Leaking of ions and other cell contents can then occur (Cox et al., 2000; Burt, 2004).

Contrariwise, the methanolic extract of *C. colocynthis* seeds showed a very good inhibition of OTA and this extract is ranked higher than other extract as *Ferronia eluhantum, Lawsona innermis* and *Azadirachta indica* causing a reduction of the synthesis only (Warke et al., 2006). The use of this extract is best looked for other substances that have the same effect such as 4-hydroxy-3-(p-toluoyl)-6-(p-tolil)-2H-pyrane-2-one (DHT) and 5-bromo-4-hydroxy-3-(p-toluoyl)-6-(p-tolil)-2H-pyrane-2-one (BrDHT) (Durakovic et al., 1989). The advantage of herbal extracts is their bioactivity, a feature that makes them attractive for the protection of stored products such as cereals against fungal attack.

Conclusion

The results obtained are encouraging and confirm the value of the use of *C. colocynthis* seeds as an antifungal agent and in biotechnology as a preservative for the fight against toxigenic fungi and their mycotoxins. It is therefore interesting to continue this study in order to determine the mode of action of extracts on mold.

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The beneficial effect of combined administration of vitamins C and E on renal function and selected parameters of antioxidant system in diabetic rats fed zinc-deficient diet

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The aim of this study was to examine the progression of kidney damage induced by zinc deficiency in diabetic rats and to evaluate the effect of combined treatment of vitamin E and vitamin C in renal injury by providing protection against deleterious action of zinc deficiency. Female diabetic albino Wistar rats were randomly assigned into five groups. The first group received a diet containing a 54 mg zinc/kg diet (adequate zinc, AZ), the second group received a diet containing 1 mg zinc/kg diet (zinc deficient group, ZD), and the three other groups received ZD diet and treated orally with vitamin E (500 mg/kg body wt) (ZD + Vit E), vitamin C (500 mg/kg body wt) (ZD + Vit C), and combined vitamins C and E (250 + 250 mg/kg body wt) (ZD+VitC+VitE), respectively. Body weight was recorded regularly (twice weekly). After four weeks of dietary manipulation, kidney zinc level, serum albumin and total protein concentration of ZD group were significantly lower than those of AZ group. Dietary zinc deficiency also increased proteinuria excretion, serum and urinary urea and uric acid levels, serum creatinine and kidney malondialdehyde concentration. In contrast, the catalase activity and reduced glutathione level in the kidney were reduced. In conclusion, vitamins E and C act as beneficial antioxidants protect renal function against the noticed oxidative stress due to zinc deficiency and experimental diabetes.

Key words: Experimental diabetes, zinc, vitamin E, vitamin C, oxidative stress, kidney damage.

INTRODUCTION

Zinc is one of the most important essential metals for human nutrition. It is important for cellular processes, like genetic expression, cell division, and growth. This trace element is crucial for the function of more than 300 enzymes (Salgueiro et al., 2000; Jansen et al., 2009) and plays an important role in insulin action, carbohydrate and protein metabolism (Chausmer, 1998). Zinc deficiency has been reported to impact pancreatic function (Banavara et al., 2011) and appears to decrease the ability of the pancreas to respond to glucose, eventually

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Abbreviations: GSH, Reduced glutathione; **CAT,** catalase; **BSA,** bovine serum albumin; **AZ,** adequate zinc; **ZD,** zinc deficient group; **MDA,** malondialdehyde.

leading to islet cell damage (Chiara et al., 2007). Zinc status was proposed to play a key role in the onset and/or progression of diabetes (Miao et al., 2013), as supported by several examples in both man and rodent models (Taylor, 2005). According to the study of Faurea et al. (2007), several complications of diabetes may be related to increased intracellular oxidants and free radicals associated with decreases in intracellular zinc and zinc-dependent antioxidant enzymes. Therefore, abnormal zinc metabolism could play a role in the pathogenesis of diabetes mellitus, wich is accom-panied by sever oxidative stress as a result of an increase oxygen free radical production.

Increased oxidative stress in diabetes is postulated to promote the development of myocardial injury, neuro-pathy, retinopathy and nephropathy (Kowluru et al., 2007). Diabetic nephropathy is a leading cause of end stage renal disease. It is characterized functionally by proteinuria and albuminuria and pathologically by glome-rular hypertrophy, mesangial expansion and tubulointer-stitial fibrosis; these findings are closely related to the loss of renal function (Eun et al., 2007). Because this damage occurs as a result of increased reactive oxygen species production, extensive investigation has evalua-ted the ability of antioxidants like vitamin C and vitamin E to ameliorate complications of diabetes (Robert et al., 2010).

Vitamins C and E can be used as antioxidants separately or in combination. Both vitamins act synergisti-cally to decreased renal oxidative stress, and kidney damage, and increased renal hemodynamics. In addition, vitamins C and E improve vascular function and structure, and prevent progression of diabetic complica-tions. Thus, the aim of this study was to examine the combined effects of vitamins C and E on kidney damage due to zinc defficiency in diabetic rats. We measured markers of oxidative stress and antioxidant state, serum and urinary parameters of renal function.

MATERIALS AND METHODS

Chemicals

Alloxan, 5, 5′dithiobis-(2-nitrobenzoic acid (DTNB), vitamin E (α-tocopherol), and vitamin C were purchased from Sigma Chemical Co (St Louis, France). All other chemicals used in the experiment were of analytical grade.

Animals

Female albino (Wistar) rats of 10 weeks of age, weighing 200 -250 g, were obtained from Pasteur Institute (Algiers, Algeria). Animals were acclimated for one week under the same laboratory conditions of photoperiod (12 h light/12 h dark) with a relative humidity of 40% and room temperature of 22 ± 2°C. Standard rat food and deionized water were available *ad-libitum.*

Induction of diabetes and diet

Diabetes was induced with fresh alloxan monohydrate solution using a previously described method (Pathak et al., 2011). Alloxan was intraperitoneally administered at a dose of 150 mg/kg body weight dissolved in citrate buffer (0.01 M, pH 4.5). Blood glucose was measured seven days after induction of diabetes on samples taken from tail vein. The diabetic state was confirmed by a glucose-meter (ACCU-CHEK, Roche Diagnostics, Paris, France) when the glucose concentration exceeded 14 mmol/l.

The diet for rats consisted of (in g/kg diet): Cornstarch 326, sucrose 326, protein 168 (egg white solids), lipids 80 (corn oil), fiber 40 (cellulose), vitamin mix (sigma) and mineral mix 40. The latter was formulated to contain either adequate (54 mg/kg) or deficient (1.2 mg/kg) quantities of Zn, as determined by atomic absorption spectroscopy. The mineral mix supplied (in g /kg diet) calcium hydrogen orthophosphate 13; disodium hydrogen orthophosphate 7.4; calcium carbonate 8.2; potassium chloride 7.03; magnesium sulphate 4; ferrous sulphate 0.144; copper sulphate 0.023; potassium iodide 0.001, manganese sulphate0.180 and zinc carbonate 0.1. The low Zn diet contained no additional zinc carbonate.

Groups

The rats were randomly assigned into five groups of 8 animals each. The first group received a diet containing a 54 mg zinc/kg diet (adequate zinc, AZ) (Southon et al., 1988), the second group received a diet containing 1 mg zinc/kg diet (zinc deficient group, ZD) for 28 days. The third and the fourth groups received ZD diet and treated orally with vitamin E (500 mg/kg) (ZD + VE) (Demiralay et al., 2007) and vitamin C (500mg/kg) (ZD+VC) (Kaida et al, 2010) respectively. The fifth group received ZD diet and in combination vitamins C and E (250 + 250 mg/kg body weight) (ZD+VC+VE) (Yanardag et al., 2007). The experimental procedures were carried out according to the National Institute of Health Guide-lines for Animal Care and approved by the Ethics Committee of our Institution.

Urine collection

At the end of the experiment, the rats were placed in metabolic cages and 24-h urine was collected for the measurement of urinary protein, urea and uric acid.

Blood collection and preparation of tissue samples

At the end of the experimental period (28 days) after overnight fast, rats were decapitated and blood samples were transferred into ice cold centrifuge tubes. The serum was prepared by centrifugation, for 10 min at 3000 revolutions/min and utilized for total protein, albumin, creatinine, urea and uric acid assays. Absolute kidney weight was determined, one fragment of kidney was rapidly excised, weighed, freeze-clamped at -196°C, ground under liquid nitrogen and stored at -20°C for oxidative stress parameters analysis, the second fragment was washed with isotonic saline (9 g sodium chloride/l distilled water) and blotted to dry at 80°C for 16 h and zinc concentration was determined.

Measurement of biochemical parameters

In serum

Total protein and albumin level in serum were determined with commercial kits from Spinreact, Spain, refs; total protein- 100129 and albumin- 1001020. Serum creatinine, urea and uric acid concentration were also measured utilizing commercial kits (Spinreact, Spain, refs; creatinine-1001113, urea-1001333 and uric acid 1001032).

In urine

Urinary urea, uric acid concentration and proteinuria excretion were measured utilizing commercial kits (Spinreact, Spain, refs; urea-1001333, uric acid 1001032 and proteinury- 100129).

Kidney zinc analyses

Dried kidney was heated in silica crucibles at 480°C for 48 h and the ash taken up in hot hydrochloric acid (11.7 M) for Zn analyses. Kidney zinc concentration was determined by atomic absorption spectrophotometer (Pye Unicam SP 9000 Hitchin, UK) after twenty-fold dilution with doubly distilled water. The accuracy of zinc recovery was checked using standard reference materials; bovine liver and wheat flour. These standards were prepared and analysed in similar conditions to the test items to assess recovery. The recovery of zinc in the standard reference material exceeded 96%.

Antioxidant parameters estimations

Preparation of homogenates

About 0.5 g of kidney was homogenized in 2 ml of buffer solution of phosphate buffer saline 1:2 (w/v; 1g tissue 2ml TBS, pH=7.4). Homogenates were centrifuged at 10000xg for 15 min at 4°C, and the obtained supernatant was used for the determination of antioxidant enzyme activity.

Determination of malondialdehyde (MDA)

Kidney homogenates were prepared at 10% (w/v) in 0.1 mol/L Tris-HCl buffer, pH 7.4, and MDA steady-state level was determined. MDA was measured according to the method described by Sastre et al. (2000). Thiobarbituric acid 0.67% (w/v) was added to aliquots of the homogenate previously precipitated with 10% trichloroacetic acid (w/v). Then the mixture was centrifuged, and the supernatant was heated (100°C) for 15 min in a boiling water bath. After cooling, n-butanol was added to neutralize the mixture, and the absorbance was measured at 532 nm. The results were expressed as nmol of MDA/g tissue.

Estimation of reduced glutathione (GSH) level

The GSH content of kidney homogenates was measured by the method of Ellman (1959). 0.5 g of fresh kidney was homogenized in 3 volumes of 5% TCA using Dounce homogenizer. The samples were centrifuged at 2000 rpm for 15 min. The supernatant (50µl) was diluted in 10 ml phosphate buffer (0.1 *M*, *p*H 8). Consequently, 20 µl of DTNB 0.01 M was added to 3 ml of the dilution mixture. The measurement was performed at 412 nm against a control prepared in the same conditions using 5% TCA. The concentration is expressed in mmoles of GSH / g of kidney. They are deducted from a range of glutathione (GSH), which was prepared with the same conditions as dosage did.

Assay of catalase (CAT) activity

The activity of catalase was estimated according to the method of Clairborne (1985). Determination of CAT activity depends on changes in absorbance result from the decomposition of H_2O_2 by CAT. This change is measured at 240 nm every min for 2 min. Enzyme activity was expressed as unit per mg protein.

Protein determination

Protein concentration in the kidney homogenates was determined by Bradford method, using bovine serum albumin (BSA) as a standard (Bradford, 1976).

Statistical analysis

One-way analysis of variance (ANOVA) followed by post hoc Tukey-HSD test were used for data analysis. Results are presented as mean ± SEM. Values were considered statistically significant if $p<0.05$.

RESULTS

Blood glucose

Figure 1 shows that blood glucose ($p < 0.001$) values were higher in ZD group than in AZ group. Blood glucose ($p < 0.001$) values were significantly lower in ZD + VE and ZD + VC groups in comparison with ZD group. Combined vitamin E and vitamin C treatements significantly reduced blood glucose ($p < 0.001$) when we compared to ZD group and ($p < 0.05$) when compared with ZD + VE.

Body and kidney weights

Figure 2 shows that there was marked reduction in the body weight of diabetic animals fed low zinc diet compared to that of AZ group ($p < 0.05$). In addition, absolute kidney weight in ZD group was significantly increased ($p < 0.01$). Oral administration of vitamin E and C increased the body weight compared to ZD group. Moreover, vitamins treatment led to significant reduction in the kidney weight ($p < 0.001$and $p < 0.01$).

Kidney zinc

The concentration of zinc in kidney is shown in Figure 3 for all studied groups. The concentration was significantly lower in the ZD group than in the AZ group ($p < 0.001$) and significantly higher in ZD + VE ($p < 0.05$), ZD + VC ($p < 0.001$) groups compared to ZD group.

Combined vitamin E and vitamin C treatements significantly elevated kidney zinc status ($p < 0.001$) compared to ZD group and a significant rise in kidney zinc concentrations compared to ZD + VE ($p < 0.05$) and ZD + VC ($p < 0.01$) groups.

Blood biochemical values

Serum albumin, total protein, creatinine, urea and uric acid values are shown in Table 1. Serum albumin ($p < 0.05$) and total protein ($p < 0.01$) values were lower in ZD group than in AZ group. In contrast, creatinine ($p < 0.001$),

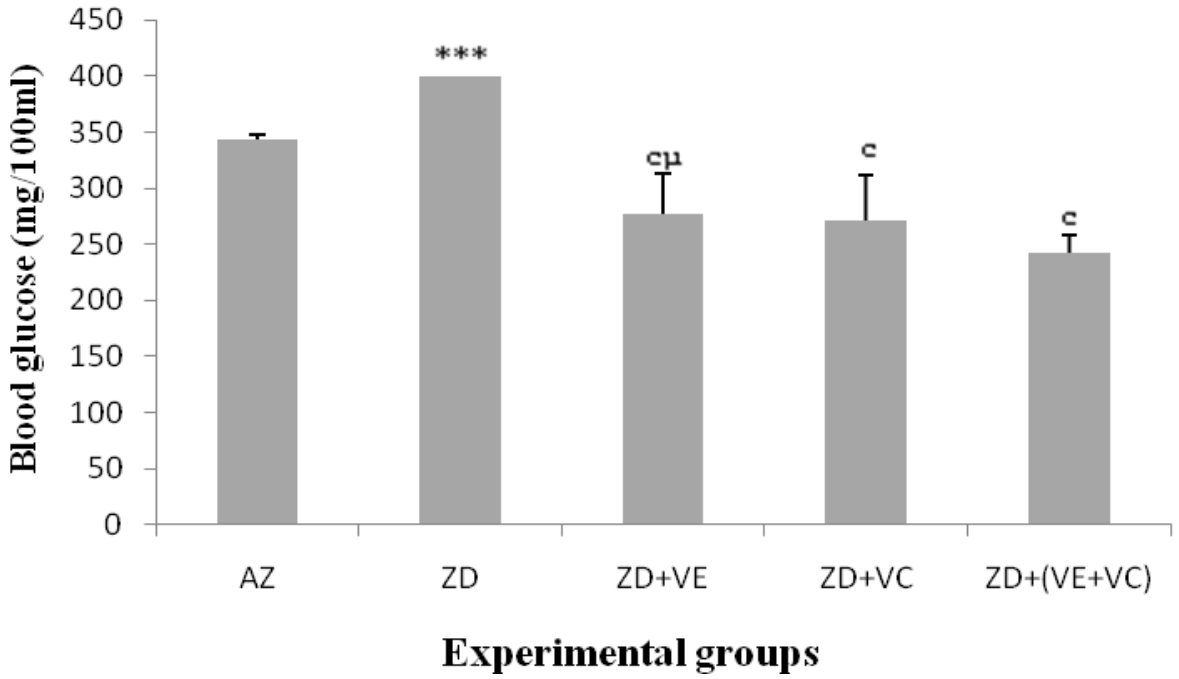

Figure 1. Blood glucose concentration in diabetic rats fed (AZ), (ZD) diets, (ZD+VE), (ZD+VC) and (ZD+VC+VE) after four weeks of treatment. Statistically significant differences from AZ: ***p < 0.001; from ZD: ^{c}p<0.001; from ZD+ (VE+V C): $^{\mu}$p<0.05. Values are given as mean ±SEM for group of 8 animals each.

Table 1. Serum albumin and creatinine levels, Urinary volume, Serum and urinary protein, urea and uric acid concentration of AZ, ZD, ZD+VE, ZD+VC and ZD+ (VE+VC) groups after four weeks of treatment.

Parameter	Experimental groups (n=8)				
	AZ	ZD	ZD+VE	ZD+VC	ZD+ (VE +VC)
Serum					
Total protein (g/l)	83.21± 2.6	76.38±0.59**	83.37±2.0^{c}	83.95 ±1.13^{c}	83.06 ±1.90^{c}
Albumin (g/l)	35.49±0.95	33.83±0.97*	35.46±0.15^{b}	35.82±0.52$^{c\mu}$	35.27 ±0.15^{b}
Urea (g/l)	0.57±0.04	1.02±0.13***	0.44 ±0.0 2^{ck}	0.47 ±0.04^{ck}	0.83±0.06^{a}
Creatinine (mg/l)	7.32±0.50	9.86±0.72***	7.29±0.17$^{c\mu}$	8.14 ±0.14^{bk}	7.58±0.14^{c}
Uric acid (mg/l)	32.00±6.08	57.33±0.57**	43.00±5.00^{b}	53.33 ±1.52^{ak}	36.33 ±2.51^{c}
Urine					
Urinary volume (ml/24 h)	33.25±6.18	50.50±11.35*	28.00 ±3.26^{b}	27.50 ±3.10^{b}	24.00 ±1.63^{b}
Protein (mg/24 h)	648.33±23.97	846.33±82.51*	437.9 ±17.3^{a}	601.33 ±28.92$^{b\mu}$	524.77±33.24^{b}
Urea (mg/24 h)	8.63±0.91	16.11±3.03**	9.23 ±1.82^{b}	10.83 ±0.79^{a}	10.60 ±0.51^{a}
Uric acid (µmol/24 h)	37.00±8.04	57.33±3.68**	38.33 ±11.26^{a}	28.30 ±23.40^{a}	42.15 ±10.77^{a}

Statistically significant differences from AZ: *P < 0.05, **P < 0.01, ***P < 0.001; from ZD: ^{a}P < 0.05, ^{b}P < 0.01, ^{c}P<0.001; from ZD+ (VC+VE): $^{\mu}$P<0.05, ^{K}P < 0.001. Values are given as mean ± SEM, n = 8 number of animals.

urea (p < 0.001) and uric acid (p < 0.01) values were higher than those of AZ rats. Serum albumin (p < 0.01 and p < 0.001) and total protein (p < 0.001) values were significantly higher in ZD + VE and ZD + VC groups in comparison with ZD group. Meanwhile creatinine (p < 0.001 and p < 0. 01), urea (p < 0.001) and uric acid (p < 0.01 and p < 0.05) values were lower in these two groups (ZD + VE and ZD + VC). Moreover combined vitamin E and vitamin C treatements significantly reduced creatinine (p < 0.001), urea (p < 0.05) and uric acid (p < 0.001) and elevated serum albumin (p < 0.01) and total protein (p < 0.001) values compared to ZD group, but administration of vitamin E and vitamin C in association improved creatinine (p < 0.05 and p < 0.001), urea (p <

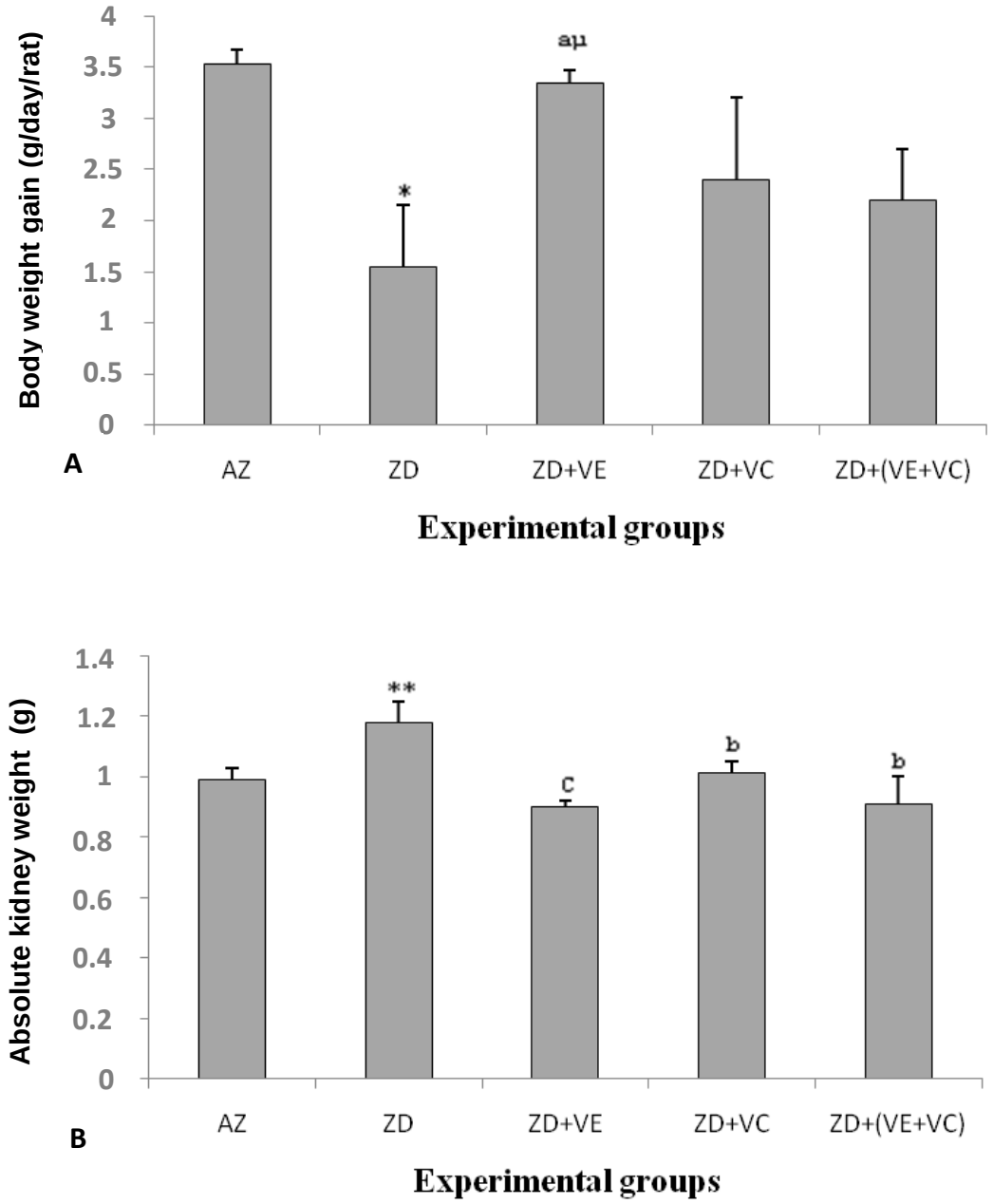

Figure 2. Body (A) and absolute kidney weight (B) of diabetic rats fed (AZ), (ZD) diets, (ZD+VE), (ZD+VC) and (ZD+VC+VE) after four weeks of treatment. Statistically significant differences from AZ: *$p < 0.05$, **$p < 0.01$; from ZD: $^{a}p < 0.05$, $^{b}P < 0.01$, $^{c}p<0.001$; from ZD+ (VE+V C): $^{\mu}P<0.05$. Values are given as mean ± SEM for group of 8 animals each.

0.001 and $p < 0.001$), uric acid ($p < 0.001$) and serum albumin ($p < 0.05$) values in comparison with (ZD+VE) or (ZD+VC).

Urinary biochemical values

Urinary protein, urea and uric acid values are shown in Table 1. Urinary protein ($p < 0.05$), urea ($p < 0.01$) and uric acid ($p < 0.01$) values were higher in ZD group than in AZ group. Urinary protein ($p < 0.05$ and $p < 0.01$), urea ($p < 0.01$ and $p < 0.05$) and uric acid ($p < 0.05$) values were lower in (ZD + VE and ZD + VC) groups respectively in comparison with ZD group. Moreover combined vitamin E and vitamin C treatements reduced, urea ($p < 0.05$) and uric acid ($p < 0.05$) concentrations compared to ZD group and improved urinary protein exerition ($p < 0.05$) in comparison with (ZD+VC).

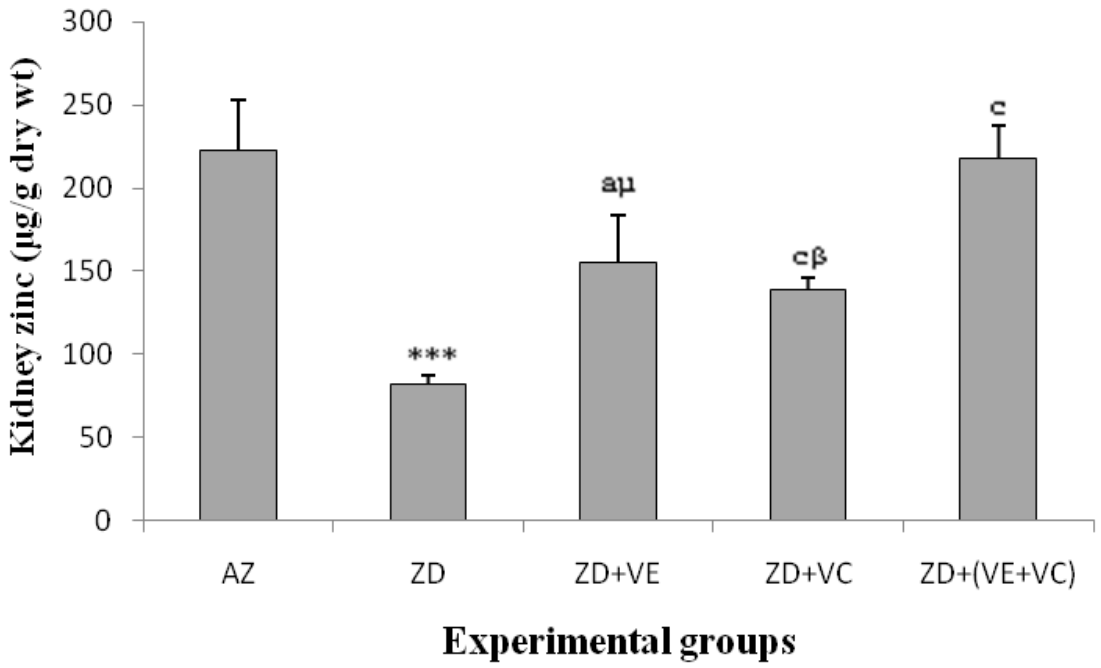

Figure 3. Kidney zinc level in diabetic rats fed (AZ), (ZD) diets, (ZD+VE), (ZD+VC) and ZD+(VC+VE) after four weeks of treatment. Statistically significant differences from AZ: ***$p < 0.001$; from ZD: $^{a}p < 0.05$, $^{c}p<0.001$; from ZD+ (VE+V C): $^{\mu}p<0.05$, $^{\beta}p < 0.01$. Values are given as mean ±SEM for group of 8 animals each.

Oxidative stress parameters

MDA concentration was significantly higher in diabetic animals fed low zinc diet as compared to the adequate zinc group ($P < 0.05$), but markedly declined after vitamin E and vitamin C administration ($P < 0.05$) compared to ZD group (Figure 4). Also, there was a marked decrease in the GSH level ($p < 0.01$), and CAT ($p < 0.01$) activity in ZD rats as compared to AZ group. There was a significant rise of GSH level ($p < 0.01$) and CAT activity ($p < 0.05$ and $p < 0.01$), in kidney of diabetic rats fed zinc deficient diet after vitamin E and vitamin C administration (Figure 4).

In addition administration of vitamin E and vitamin C in association reduced in part MDA and elevated GSH concentrations ($p < 0.01$), and CAT ($p < 0.05$) activity compared with ZD group, on the other part ameliorated CAT activity ($p < 0.05$) when compared with (ZD+VE) or (ZD+VC) groups.

DISCUSSION

The result of this study showed that there were marked reductions in the total body weight as well as elevation in the absolute kidney weight of rats fed the zinc deficient diet (ZD) compared with rats fed the adequate zinc diet (AZ). It was reported that growth retardation was common in zinc defficient bull calves this was due in part to decreased appetite and impaired protéin synthesis. Sun et al. (2006) reported that the relative weight of kidney increased by a zinc deficient diet and found that ZD diet influence organ development. Administration of vitamin C and /or vitamin E minimized body weight loss observed in diabetic rats fed low zinc diet and ameliorated both body and kidney weights.

Blood glucose was affected by low zinc diet. The observed higher blood glucose in the present study of low zinc animals may relate to altered glucose utilisation by tissues or to the increased rate of endogenous glucose production (Hendricks and Mahoney, 1972). The result of this study and those of previous studies show that vitamin E and vitamin C reduce blood glucose levels in diabetic animals. However, vitamin E as an antioxidant may help in the clearance of free radicals responsible for the complications of diabetes mellitus. In addition, it may also promote the absorption or uptake of glucose from the intestine and cells, respectively (Al Shamsi et al., 2004). Vitamin C was reported to help in improving plasma glucose in patients with type 2 diabetes (Afkhami and Shojaoddiny, 2007).

Several studies clearly demonstrated that hyperglycemia is an important causal factor in the development and progression of diabetic kidney disease (Haidara et al., 2009). The diabetic hyperglycemia induces elevation of serum levels of urea, uric acid and creatinine which are considered as significant markers of renal dysfunction (Shind and Goyal, 2003). Our results show significant

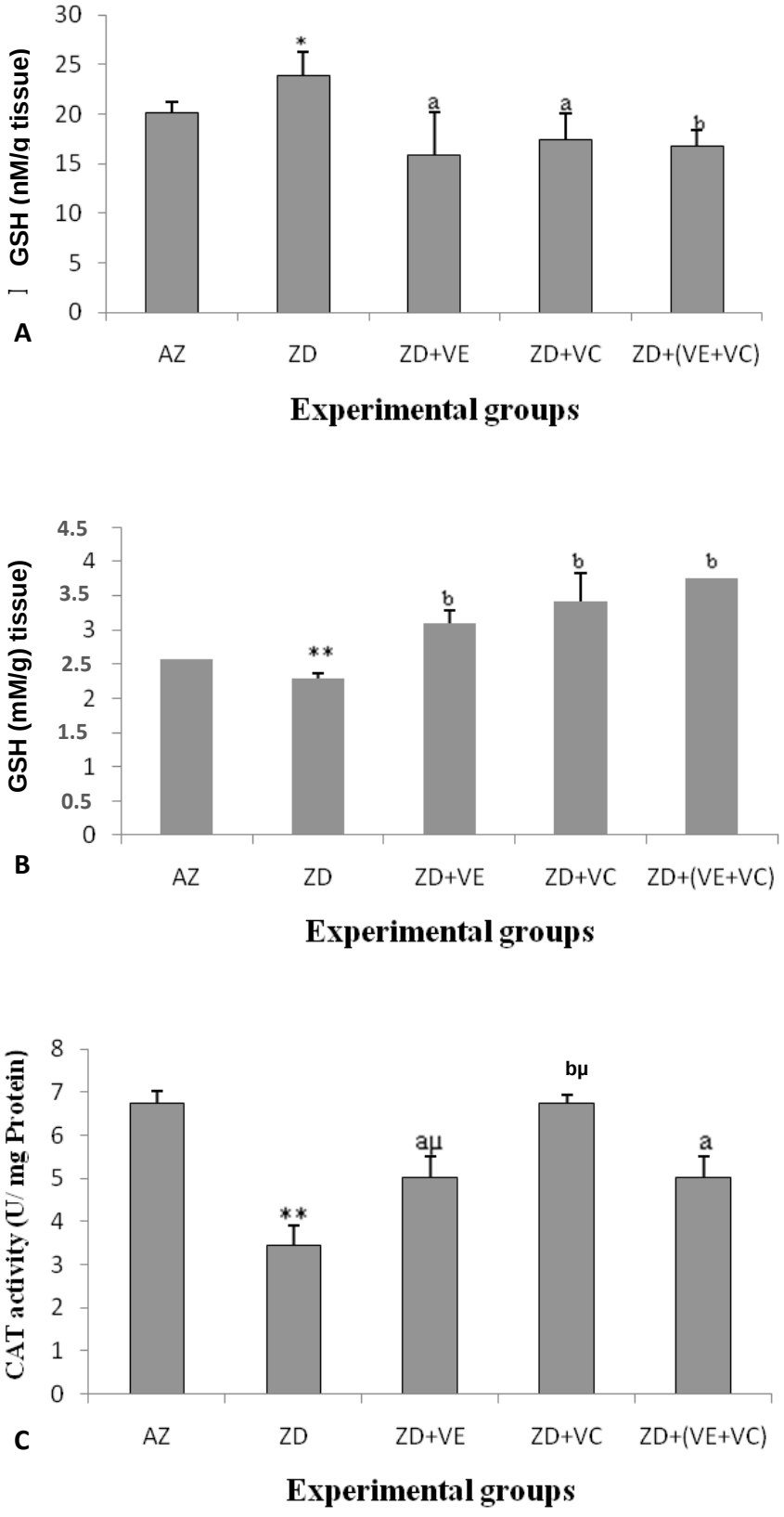

Figure 4. Kidney MDA (A) and GSH levels (B) and CAT activity (C) of AZ, ZD, ZD+VE, ZD+VC and ZD+ (VE+VC) groups after four weeks of treatment. Statistically significant differences from AZ: *p < 0.05, **p < 0.01; from ZD: ^{a}p < 0.05, ^{b}p < 0.01; from ZD+ (VE+V C): $^{\mu}$p<0.05. Values are given as mean ±SEM for group of 8 animals each.

increase in the level of serum urea, uric acid and creatinine in the diabetic rats fed low zinc diet. These results indicated that zinc deficiency elevated renal markers (serum urea nitrogen, uric acid and creatinine), which are found responsible for proper maintenance, functioning of kidney and change in the glomerular filtration rate (Mauer et al., 1981).

Our experimental data indicate that rats receiving a zinc-deficient diet had an increased rate of catabolism. This is based on the findings of elvated concentrations of urinary urea and uric acid. The production of uric acid, an end product of purine metabolism, can be influenced by altering the concentrations of substrates of nucleic acid. The urinary urea is the product of the hydrolytic cleavage of L-arginine by the enzyme arginase. The increased urinary urea in a zinc-deficient rat is due to the increase of hepatic arginase activity, which is a rate-limiting enzyme in urea formation (Jenc and william, 1975). Administration of vitamin E and C improves renal markers. Previous studies (Sutton et al., 1983; Mitch et al., 1981) suggest that vitamin C exerts a uricosuric effect by increasing urinary excretion and reducing serum concentrations of uric acid that at high levels could become crystallized in the joint and kidney and lead to gout and kidney stones.

Several studies have described biological mechanisms by which vitamin C reduces serum uric acid. *In vivo* studies suggest that vitamin C has uricosuric properties, increasing renal fractional clearance of uric acid, thereby reducing serum uric acid (Stein et al., 1976). This is likely due to competitive inhibition of an anion exchange transport system at the proximal tubule in the nephron (Berger et al., 1977). It is also possible that vitamin C increases the glomerular filtration rate by reducing glomerular microvascular ischemia and increasing dilatation of afferent arterioles (Huang et al., 2005).

In this experiment there was a significant reduction of kidney zinc, serum albumin and total protein concentration, but urinary protein excretion was elevated in zinc deficient group. A significant decrease in total protein level might be due to catabolism of protein and/ or malfunction of liver (Harper et al., 1977). In addition, changes in the binding of zinc to plasma proteins may result in an increase in ultrafilterable zinc concentration (Prasad and Oberleas, 1970). Administration of vitamins C and E decreased urinary protein and improves renal damage. These results are in agreement with the results of previous studies (Eun et al., 2007).

In the present study, GSH level and CAT activity were measured in renal tissue to evaluate the changes of antioxidant status in the kidney. Increased renal MDA content and decreased GSH level and CAT activity were found in diabetic rats fed low zinc diet compared to the AZ group. However, administration of vitamin E and C alone or in combination significantly improved these parameters. These vitamins may exert their beneficial effects through antioxidant action (Sadi et al., 2012). Vitamin C also acts as an aldose reductase inhibitor reducing sorbitol conversion and decreasing cellular damage in the kidney (Vincent, 1999). Vitamin E, on the other hand, acts as a non-enzymatic antioxidant and reduces lipid peroxidation and glutathione (Punithavatki et al., 2008; Minamiyama et al., 2008).

Conclusion

Our data shows that vitamin C or vitamin E alone and a combination of them preserved renal antioxidant levels and prevented kidney damage. The decreases in urinary protein excretion and the improvement in renal function in antioxidant vitamins treated groups suggest a major role of oxidative stress in the developing of renal dysfunction in diabetes associated with zinc deficiency.

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Full Length Research Paper

Biological activity of sugarcane pyroligneous acid against *Spodoptera frugiperda* (J.E. Smith, 1797) (Lepidoptera: Noctuidae) larvae

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***Spodoptera frugiperda* (J.E. Smith, 1797) (Lepidoptera: Noctuidae) annually cause enormous loss to the producers and their combat has become a worldwide challenge mainly due to several reports of pesticides resistance. Today, one of the best alternatives used in this combat is the application of natural insecticides such as neem oil and pyroligneous acid. This study demonstrates a method to obtain a hexane fraction from sugarcane pyroligneous acid, which can be easily applied, as well as its effectiveness against *S. frugiperda*. The hexane fraction exhibited LC_{50} of 2206,41 ppm after 24 h of exposure with a linear dose-response, indicating that the fraction can be used as a bio-insecticide against *S. frugiperda*.**

Key words: Wood vinegar, insecticide, *Saccharum officinarum*, fall armyworm, pyroligneous extract.

INTRODUCTION

The fall armyworm, *Spodoptera frugiperda* (Smith, 1797) (Lepidoptera: Noctuidae), is a polyphagous species of tropical and semitropical zones (Sparks, 1979) that causes severe damage to crops (Kamaraj et al., 2008). The control of this pest is usually made with the use of synthetic insecticides (Tavares et al., 2010a; Wititsiri, 2011) which, although effective, can cause various problems, such as the presence of residues in food, poisoning of applicators, and outbreaks of secondary pests (Roel et al., 2000; Berlitz et al., 2012). Furthermore, the continued use of these pesticides may aid in development of a resistant pest population (Munoz et al., 2013).

An alternative for combating crop pests is the use of natural products such as neem (Correia et al., 2009; Tavares et al., 2010b), plant extracts (Roel et al., 2000; Tavares et al., 2009, 2011, 2013), and pyroligneous acid (Azevedo et al., 2007; Tavares et al., 2010a). The latter one is produced from the condensation of smoke from wood carbonization (Mendonça et al., 2006). This product is mainly characterized by low environmental impact and is a good alternative in organic agriculture. The pyroligneous acid, also known as wood vinegar, has been demonstrated to have low mammalian toxicity, lack neurotoxicity, have low persistence in the environment, and have high biodegradability (Céspedes et al., 2000).

The pyroligneous acid is normally composed of sugars, carboxylic acids, phenols (Fengel and Wegener, 1984; Kim et al., 2008), aldehydes, ketones, esters, furans and pyrans derivatives, nitrogen compounds, and other com-

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pounds (Ninomiya et al., 2004), and the major part is constituted by water (85%) and acetic acid (5.1%) (Zanetti et al., 2003). Studies on the pyroligneous acid have reported bactericidal, fungicidal, and termiticidal activity (Mu et al., 2003).

The extract is usually obtained from species used to make charcoal (Cai et al., 2012). In Brazil, the species most used for charcoal production is the *Pinus elliottii* var. *elliottii* Engelm. (Pinaceae) (Porto et al., 2007). One of the viable alternatives for obtaining this extract is from sugarcane bagasse (*Saccharum officinarum* L., Poaceae); this residue has been routinely burned as an energy source (Zandersons et al., 1999), and hence, by a simple condensation of the smoke released, the extract can be obtained, which can be processed and used as a bio-insecticide. This process could avoid the annual emission of millions of tons of gases into the atmosphere (Alves et al., 2010), and produce a product with low environmental impact. The aim of this study was to obtain and process sugarcane pyroligneous acid and evaluate it against *S. frugiperda* larvae.

MATERIALS AND METHODS

Plant material and extract fractionation

The bagasse of *S. officinarum* was collected from Santa Olinda S/A, located in the municipal of Sidrolândia - Mato Grosso do Sul State, Brazil. The pyroligneous acid was obtained from the Laboratory of Pharmacognosy - (Federal University of Mato Grosso do Sul - UFMS) in Campo Grande, Mato Grosso do Sul State, Brazil. For that purpose, the bagasse was placed in an Erlenmeyer flask and completely burned in a muffle at 300°C, and then the smoke was condensed and the extract was collected. The crude extract had its pH neutralized and then was partitioned with hexane; this hexane fraction of the pyroligneous acid (HF-PA) was used in the biological assay.

Bioassay procedures

The eggs of *S. frugiperda* were provided by the Laboratory of Entomology of the Anhanguera University (Uniderp) in Campo Grande, Mato Grosso do Sul State, Brazil. After the eggs hatched, the larvae were placed on artificial diet (Greene et al., 1976) with 12-12-h photoperiod, at 27 ± 3°C, and under 70 ± 5% of relative humidity. The tests were divided into three groups of 10 neonatal larvae for each concentration: Negative group, Artificial diet and acetone; positive group, artificial diet plus neem oil at concentration of 12000 ppm (Neem oil 95%, Serôdia®, Campo Grande, Mato Grosso do Sul State, Brazil) plus acetone; and test group, artificial diet plus the HF-PA (60, 120, 360, 600, 1200, 3600, 6000, 12000, and 36000 ppm) and acetone to dilute the extract.

Statistical analysis

The experiment was monitored at 24 h period, observing mortality in relation to concentrations of HF-PA, and the statistical program used was SPSS Statistics 20.0.0 to apply simple linear regression model ($P < 0.05$). To calculate the LC_{50}, Probit analysis from the logarithms of the concentrations was used.

RESULTS AND DISCUSSION

The yield of pyroligneous extract was approximately 40%. This extract was neutralized once acetic acid constituted approximately 5% of the extract (Zanetti et al., 2003) because this compound could interfere in the bioassay and degrade other compounds present. Another step was the fractionation of the extract; this step was aimed mainly to obtain an extract without water. The HF-PA showed a neutral pH and can be easily concentrated; these characteristics allowed easy development of new products and application of this extract.

The bioassay using HF-PA showed significant change, when compared with the negative control group. All the different concentrations of the extract caused significant mortality in 24 h, which was also observed in the positive control group (neem oil). The highest concentration evaluated (36000 ppm) resulted in 100% death, and the lowest concentration (60 ppm) caused only 10% of deaths (Figure 1). The LC_{50} of HF-PA was 2206,41 ppm. Our results suggested a linear dose-response (Figure 2), which was also confirmed by r^2 (0.83). Neonatal nymphs of *Bemisia tabaci* (Gennadius, 1889) (Hemiptera: Aleyrodidae) showed 66.59% mortality caused by neem oil (Natuneem®) and 67.45% of deaths caused by pyroligneous acid (Pironat®) (Azevedo et al., 2005). Neem oil (Natuneem®) and crude pyroligneous extract (Biopirol7M®) tested on eggs of *S. frugiperda* and *Diatraea saccharalis* (Fabricius, 1794) (Lepidoptera: Crambidae), confirmed mortality of eggs of different ages with different concentrations of the extract, and the extracts' satisfactory effect in controlling these pests (Tavares et al., 2010a, b). However, neem oil (Natuneem®) and commercial crude pyroligneous acid (Biopirol7M® and Pironat®), no significant mortality was observed when the extract was applied to adults of *Anastrepha fraterculus* (Wiedemann, 1830) (Diptera: Tephritidae) (Efrom et al., 2011). These contradictions among different studies are related to the stages of the insects used in the experiments. Another possibility is related to the tolerance of the insect to insecticides in the course of their development (Yu, 1983; Schmutterer, 1990).

One of the apparent causes of the activity against the larvae is the synergistic effect among the compounds (Richards et al., 2010); this synergism occurs between different classes of compounds or structurally similar compounds, and the effects may range from antifeeding effect to toxicity activity (Dyer et al., 2003). The wide range of compound prevents resistance development (Rice, 1993). These secondary metabolites have different sites of action and molecular targets. They interact with important metabolic and enzymatic inhibitors (Céspedes et al., 2000; Torres et al., 2003) against microbial pathogens and invertebrates (Wink and Schimmer, 1999). Thus, for example, some secondary metabolites such as the nitrogenous compounds may act by blocking

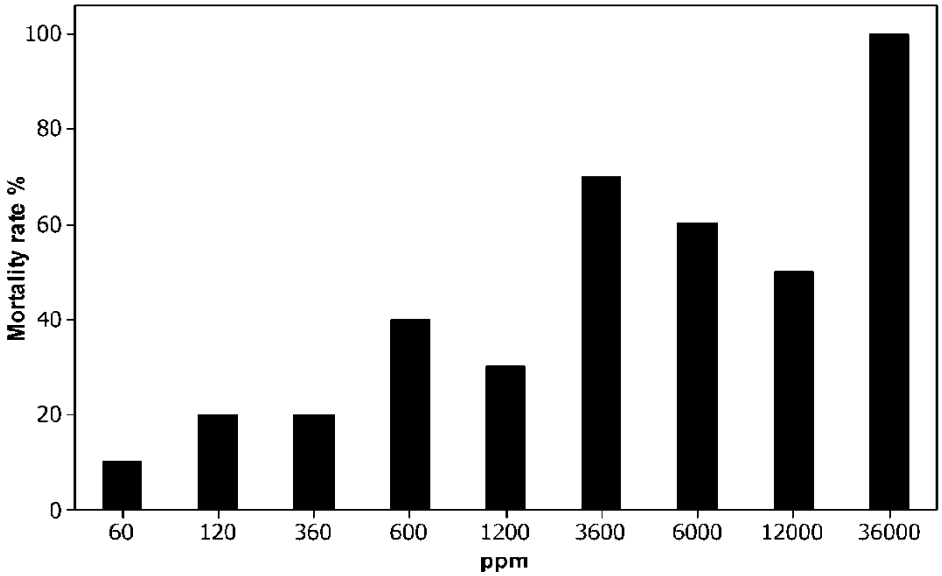

Figure 1. Distribution of percentages of mortality of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) larvae caused by HF-PA.

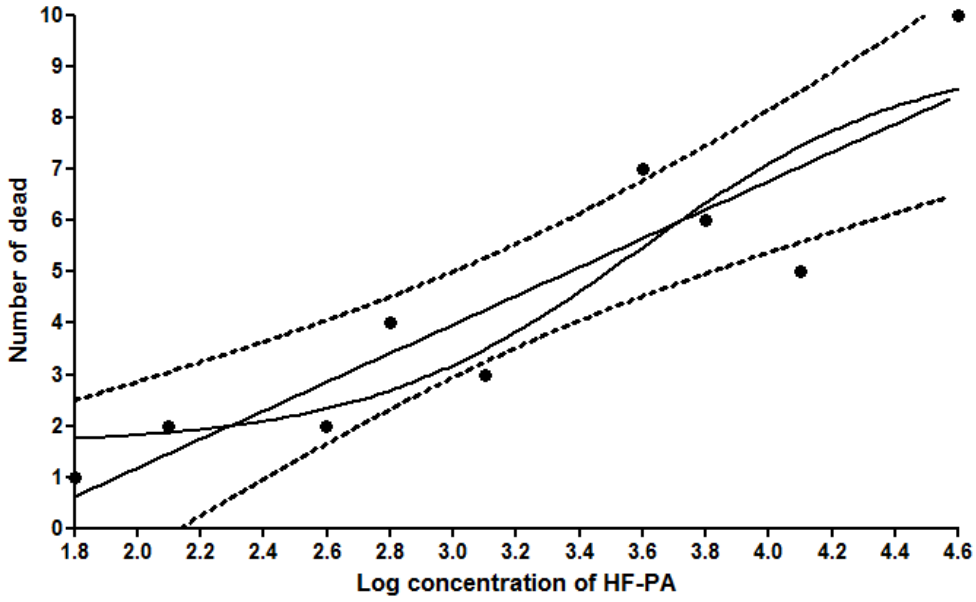

Figure 2. Graph regression analysis (P <0.05) presenting the log of the dose against the number of dead *Spodoptera frugiperda* (Lepidoptera: Noctuidae) larvae during 24 h.

the nervous system activity (Kagabu, 2008), or by inhibiting synthesis of deoxyribonucleic acid (DNA), and modifying the permeability of the membrane by changing the composition with respect to structural proteins (Schmeller et al., 1997). Another mechanism of action of PA-HF could be related to the presence of phenolic compounds, one of the main groups being investigated for pest control (Henn, 1997).

The pyroligneous extract used was toxic to *S. frugiperda* larvae and displayed a linear dose-response with an LC_{50} of 2206,41 ppm. Furthermore, the possibility of preparation of a low-cost fraction of the sugarcane pyroligneous acid was also demonstrated. These results open new possibilities for the fraction to be tested against different pests of crops and agriculture, providing useful application for the smoke produced by the burning of

sugarcane bagasse.

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