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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.

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Review

Status and prospects for improving yam seed systems using temporary immersion bioreactors

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Yam production is constrained by scarcity of clean seed, pests, diseases and low soil fertility in the informal seed system, which is still operational, causing up to 90% yield losses. Although meristem culture can be effective for producing healthy seed yam, its use is limited by slow rate of regeneration and propagation in conventional tissue cultures. In most crops tested, temporary immersion bioreactor systems (TIBs) increased propagation rates. To determine the potential of TIBs in improving the yam seed system, 23 databases were consulted and three returned a total of eight publications with only 2 for *Dioscorea rotundata-cayenensis*. Both plantlets and microtubers can be produced in TIBs, which will facilitate production of quality breeder, foundation and certified seeds and fast-track genetic improvement and the evolution of a formal from informal seed production system. Control of contamination, direct use of field explants, culture of micro-explants like immature embryos and anthers, increasing the size of microtubers produced and standardization for various economically important yam genotypes are knowledge gaps that require immediate research attention. No report has put a cost on yam TIBs, but it will be necessary to use cost-effective TIBs to encourage integration public-private partnerships into emerging formal seed system.

Key words: Tissue culture, healthy seed yam, temporary immersion bioreactors, *dioscorea* spp.

CLONAL PROPAGATION AND BIOREACTORS

Traditionally, the multiplication of genetically identical individuals by asexual methods, otherwise known as clonal propagation, is achieved by cuttings, grafting, layering and tuber portions, among others. The use of tuber portions has been applied to yam for ages, preserving traits of selected genotypes. Micropropagation and clonal propagation which utilize plant tissue culture techniques in a closed, sterile container however occur

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Abbreviations: **TIBs:** Temporary immersion bioreactor systems; **NACGRAB:** National Centre for Genetic Resources and Biotechnology; **YIIFSWA:** Yam Improvement for Income and Food Security in West Africa; **USAID:** United States Agency for International Development; **NAS:** National Academy of Science; **NSF-PEER:** National Science Foundation-Partnership for Enhanced Engagement in Research.

within the laboratory environment. These methods are now being applied industrially for numerous crops (Yam and Arditti, 2009). In the case of yam, reports on micropropagation include organogenesis from pre-formed meristems, immature leaves and nodal culture, and microtuber formation (Balogun and Gueye, 2013). However, yam meristem cultures took more than 1 year to regenerate in some cultures. In conventional micro-propagation, frequent sub-culturing also increases labour costs while small size of culture container (hence nutrients) and insufficient aeration (Ziv, 1991) results in fragile plantlets (Ziv et al., 1998) and sub-optimal propagation rates. The need to improve on these systems caused the emergence of the bioreactor technology.

Bioreactor technology is an advanced tissue culture - an enclosed sterile environment provided with inlets and outlets for air flow under pressure. In most crops tested, better growth performance due to better aeration was reported compared to continuous immersion in medium. Several bioreactor systems have been successfully applied for cultivation of differentiated plant *in vitro* systems (Steingroewer et al., 2013), including liquid-phase (stirred tank, airlift and connective flow bioreactors), gas-phase, hybrid bioreactors, and Tem-porary Immersion Bioreactor systems (TIBs). The TIBs' are uniquely able to provide lower level of shear stress and significantly reduce shoot hyperhydricity culminating in increased productivity. In TIBs, there is timed immersion of plant tissues in liquid medium to allow for culture aeration, which circumvents limitations associated with conventional tissue culture.

THE MAJOR CHALLENGE OF SEED YAM PRODUCTION

In spite of its status as a staple and source of livelihood in West Africa, (FAO, 2013), yam production is still sub-optimal. The major pre-requisites for enhanced yam productivity and storability are high quality seeds of improved genotypes. However, quality seed yam is scarce. This is a consequence of the slow rate of propa-gation (less than 1:10 compared to 1:200 in some cereals) (Mbanaso, 2011) which is also vegetative, encouraging a build-up of an array of fungal, nematode, bacterial and viral diseases and pests.

The situation is further complicated by lack of a formal seed system which has a functional regulatory structure, such that there is scarcity of certified and quality declared seeds. Yam production therefore revolves quite repeatedly, around the use of mixed, undelineated genotypes, pre-infected seed yam and farmlands, causing a build-up of an array of diseases (Winch et al., 1984). This leads to 50 to 90% yield reduction. Consequently, there is a significant demand for clean seed in a market driven seed system and 50 to 70% of production costs (Nweke et al., 1991; Agbaje et al., 2005; Ironkwe, 2005; Coyne, 2010) is spent on purchase of seed.

Other constraints include shrinking land area due to flooding and desertification, which impacts soil fertility, high cost of labour in the absence of mechanization, high post harvest losses, tuber dormancy which prevents off-season production and uncontrolled sprouting after dormancy break which further causes storage losses. However, the challenge of seed scarcity is central to these constraints due to inadequacy of improved genotypes specifically adapted to prevailing challenges, frequently renewed without losing its quality. Global annual production was therefore projected to reach a plateau, having decreased by 11.5% in 2007 (Manyong et al., 1996).

EXISTING SEED YAM PRODUCTION SYSTEM

In the current informal seed system, farmers reserve up to half of the year's harvest for future planting, obtain seeds from fellow farmers or purchase from the market, in decreasing order of preference. Traditional yam propagation is by planting 200 to 500 gram setts, which takes a large portion of the harvest. In the "milking" technique (Okigbo and Ibe, 1973; Okoli et al., 1982), tubers are harvested two-thirds into the growing season without destroying the root system, providing early ware yam for consumption. The parent plant regenerates new small tubers used as seed yam for the following season. This system therefore doubles the propagation ratio relative to other traditional methods although, multipli-cation ratio is still very low.

In the modified minisett technique (Kalu and Erhabor, 1992; Okoli and Akoroda, 1995; Ikeorgu and Igbokwe, 2003; Ikeorgu et al., 2007) use of 25-80 g minisett has reduced the production cost of seed yam (Okoli et al. 1982; Otoo et al., 1987; Oguntade et al., 2010) but rate of adoption is low (Kalu and Erhabor, 1992). More recently, rooting of 20 cm long 3-node vines (Acha et al., 2004; Kikuno et al., 2007; Agele et al., 2010) produced minitubers of 50 to 600 g after 8 months giving a 1:22 propagation ratio.

Multiplication rates are doubled in the partial sectioning technique (Nwosu, 1975), where planted tubers are dug out and sprouted sections excised for field planting but labour requirement is enormous. The layering of vines into soil for tuber production while on the mother plant is unpractical for farm use although up to 1:80 propagation ratio is possible (Acha et al., 2004). All of these macropropagation techniques are genotype-dependent, have no provision for cleaning infected seed yam and tuber dormancy remains a challenge. Sexual seeds are only useful in breeding but not multiplication as the product is different from the parents due to outcrossing and are therefore not true-to-type (Okonkwo, 1985). However, the informal seed system preserves abundant

Table 1. Formal versus informal seed systems.

Item	Informal	Formal
New technology generation	No	Yes
Technology transfer/dissemination	No	Yes
Wide diversity of adapted genetic resources	Yes	Obtainable
Infrastructure for assessing quality seed	No	Yes
Quality control at all levels of seed production	No	Yes
Public sector involvement	No	Yes
Private sector involvement	Yes	Yes
Official recognition of impacts	No	Yes

Source: Extracts from Larinde and Ilboudo (2006); Wekundah (2012)

diversity of landraces, including wild types.

THE CONCEPT OF FORMAL SEED SYSTEM IN YAM

In contrast to the informal system which is based on family heritage in terms of experience, the bases of the formal seed systems are scientific research, new variety selections, field/laboratory seed control and testing which constitute the technology-based aspect while the economic and legal aspects involve production/marketing and rules/regulations, respectively. The vision is that the first 2 aspects can be handled by the public or private sector or a partnership between the two, while the legal aspect is governmental and public sector-managed. Consequently, the formal seed system is all about following the rules: quality control and certification at all stages, the increase in quantity of breeder to foundation seeds and then certified seeds that can be distributed to farmers to ensure the genetic and physiological quality (includes disease and sprouting status) of the seeds. In Nigeria for example, the National Centre for Genetic Resources and Biotechnology (NACGRAB) is responsible for registration of new varieties, involving aspects of confirmation of genetic purity. To date, 19 researchers' varieties (improved) of yam have been officially released and registered by NACGRAB with no landrace varieties attributable to farmers' selections. The National Agricultural Seed Council (NASC) has the mandate for quality control and certification at all levels of breeder, foundation, certified seed production. In partnership with stakeholders, YIIFSWA is facilitating the development and dissemination of quality management protocols in addition to robust and cost-effective virus diagnostic tools which will fast-track establishment of the formal seed system. For yam, national standards are adopted but yet to be applied by NASC inspectors as the formal seed yam system is not yet in place.

FORMAL VERSUS INFORMAL SYSTEM

In as much as the formal seed system has many advantages, many of the quality control aspects are still not being applied for yam. Checking the sprouting ability status of seed yam is a challenge as even different tuber portions may have different sprouting abilities. Use of small whole tubers as seed will facilitate seed quality testing in addition to knowing what the standards are for field delineation of real genetic differences versus nutrient deficiency and disease symptoms (O'Sullivan, 2006, 2010). These overlaps have implications for high variations within yam somatic cells.

A formal seed system, involving adequate monitoring, is adequate for cross pollinated crops which produce seeds different from the parent at each generation. The vegetative propagation of yam is normally expected to produce uniform varieties as with self-pollinated crops for which genetic uniformity is high and informal seed system can thrive, which has helped yam over the years. However, variations are encountered within a yam variety. The genetic basis and rate of somaclonal variation in yam need to be determined. If yam is a transition between genetic uniformity and variation, it will be necessary to combine elements of the formal with informal seed systems. It should be noted that one of the consequences of the formal seed system which is market-driven, is a narrower genetic base resulting from need for genetic purity and uniformity while that of the informal is a wider genetic base due to community drive (Table 1). A good quality, locally adapted farmer-preferred variety is certainly better than an improved, non-adapted and over-selected variety (Otoo, 2003)'.

TISSUE CULTURE AND ITS ROLE IN EVOLUTION OF THE FORMAL SEED SYSTEM

The change from informal to formal seed system will require novel technologies, especially in terms of rapidity of production, certification of seeds as disease-free, propagation and distribution of 'CLEAN' breeder/-foundation/ certified seed yam in large quantities. Such technique should be applicable at most, if not all stages of development and propagation of disease tolerant or

resistant varieties that are adapted to targeted agro-ecologies.

Tissue culture has, and is still being explored for yam propagation. The basis of this is the potential of every plant cell to regenerate into the complete plant. In improving yam micropropagation rates, there are reports on many aspects of conventional tissue culture: organogenesis from pre-formed meristems (Malaurie et al., 1995a, 1995b) in *D. zingiberensis* (Chen et al., 2003), shoot organogenesis from immature leaves (Kohmura et al., 1995) and roots (Twyford and Mantell, 1996) of *D. opposita*, shoot (nodes) culture and microtuber formation in *D. composita D. rotundata and D. alata* (Alizadeh et al., 1998; Balogun et al; 2006; John et al., 1993; Salazar and Hoyos, 2007, Ovono et al., 2007). Tuber pieces were reported not to produce *in vitro* plantlets in *D. rotundata, D. trifida* and *D. cayenensis* (Mitchell et al., 1995) while it did in *D. alata* (Fotso et al., 2013). In addition, aseptic conditions are observed and this cleans the plant of fungi, nematodes and bacteria. Meristem culture combined with heat/cold/chemo therapy is the only technique that is able to clean infected plants from viruses to date, followed by rapid multiplication of superior clones (Mantell et al., 1980; Ng, 1984, 1992; Sengupta et al., 1984, Mitchell et al., 1995). This technique ensures that the viral inoculum is not passed on to subsequent generations.

Conventional tissue culture employs manual introduction into culture vials. However, the slowness of yam propagation *in vivo* is also witnessed in *vitro* cultures and a 1:4 multiplication rate (Chu and Ribeiro, 2002; Borges et al., 2004; Ondo et al., 2007) is reported averagely for yam tissue cultures. Meristem cultures took more than 1 year to regenerate in some cultures. This low multiplication rates limit the use of *in vitro*-produced, virus-tested plantlets in conventional tissue cultures in addition to losses during acclimatization and trans-planting. A high level of contamination is encountered in the culture of tuber pieces, probably due to high load of endophytes in the explants.

There is also limitation of frequent sub-culturing which increases labour costs, limitation to size of culture container (hence nutrients), insufficient aeration, hyper-hydricity and vitrification, a stress condition in tissue-cultured plants, manifested mainly as abnormal leaf functioning (Ziv, 1991; Ziv et al., 1998). Protein- and photo-syntheses, gas exchange, cellulose and lignin synthesis and ethylene production are affected, resulting in fragile plantlets (Ziv et al., 1998) and sub-optimal propagation rates.

TEMPORARY IMMERSION BIOREACTOR SYSTEMS FOR YAM PROPAGATION

Among other factors, culture aeration combined with automation has been proposed to increase productivity and reduce cost in conventional tissue cultures due to significant reduction of contamination and cost of labour respectively; in addition to faster multiplication rate. This suggested a role for temporary immersion bioreactor systems (TIBs) (Cabrera et al., 2011; Watt, 2012).

A bioreactor is an enclosed, sterile environment which is provided with inlets and outlets for airflow under pressure and utilizes liquid medium (Watt, 2012). TIBs are types of bioreactor used for differentiated plant tissues. Different designs of TIBs exist, but most common are the twin flask types, having 2 containers, one for the medium and the other for the cultures (Adelberg and Simpson, 2002). Another type of TIBs is the recipient for automated temporary immersion (RITA) (Alvard et al., 1993) in which the upper container containing the plant is linked to the lower compartment containing the medium and internal pressure regulates the movement of medium up or down such that immersion of cultures can be timed. There is also the Bioreactor of Immersion by Bubbles (BIB) (Soccol et al., 2008) where nutrient and air is provided to cultures by bubbling. In all these cases, the cultures are immersed in the medium in a timed manner, in terms of frequency and duration of immersion to allow for aeration. TIBs require interplay of plant physiology, chemical and physical sciences since components like air compressor for air flow, silicon tubing connections, membrane filters and remote monitoring may be involved. In TIBs, growth is enhanced (Escalona, 2006) since there is lack of continuous immersion in liquid medium. Use of TIBs has been reported for potatoes, pineapple, apple, coffea, lemon grass, oil palm, eucalyptus, strawberry among others (Watt, 2012).

Among databases consulted (Table 2), Scirus, Springer and Google scholar returned a total of 8 reports on yam TIBs from Cuba, Japan, China and France. Four out of 8 reports were on *D. alata*, 1 on *D. fordii,* 1 on *D. opposita* and the last 2 on *D. cayanensis-rotundata*. Yam shoots grown in TIBs had enhanced growth and the leaves had higher photosynthetic pigment content than other techniques (Table 3, Jova et al., 2005, 2011, 2012; Cabrera et al., 2011), The exciting aspect of use of TIBs for yam propagation is that both plantlet and microtuber production are possible (Balogun, 2009), although sprouting was higher in bigger microtubers. This implies that large enough microtubers can be planted directly on the field as reported for *D. alata*. Since *D. rotundata* is the most economically important species in the West African yam belt, it is critical that attention be directed towards its propagation in TIBs and this system be incorporated into the seed system.

PRODUCTION OF CERTIFIED SEEDS

The most important thing in production of certified (breeder, foundation and commercial) seeds yam is that it is true-to-type as described by the breeder. The greatest challenge is that the existing yam diversity is a

Table 2. Databases with articles on yam propagation in temporary immersion bioreactors*

Database	Article
Scirus	2
Springer	1
Social Science research network	0
Worldcat	0
Proquest Dissertation and Thesis database	0
JSTOR	0
AJOL	0
SciDevNet	0
Ideas	0
PubScience	0
Royal Tropical Institute	0
ISI Highlycited.com	0
Gogglescholar	7
Current Agricultural Research Information	0
Agris:International information system for the Agricultural sciences and technology	0
USDA National Aricultural Library	0
CGIAR library	0
Agricola	0
Eidis	0
Findarticles.com	0
Highwire press	0
Directory of Open Access Journals	0
Ingentaconnect.com	0

Although the table shows a total of 10 publications, the 2 articles in Scirus were among the 7 found in Googlescholar'

Table 3. Multiplication rate of yam in conventional tissue culture and temporary immersion bioreactor system.

Product	Genotype	Conventional gelled medium	TIBs	Author
Plantlet	*D. alata*	5.7	10.1	Salazar and Hoyos, 2007
Plantlet	*D. alata*	2.2	4.1	Jova et al., 2005
	D. alata	4.4	8.0	Yan et al., 2011
	D. rotundata-cayenensis	Not compared	8.5	Jova et al., 2008
	D. cayenensis-rotundata	Compared for stem length, no significant difference (6.6)	6.3	Polzin et al., 2013
	D. opposita	Not compared	Not reported	Akita and Ohta, 2002
	D. fordii	2.4	5.0	Yan et al., 2011
Microtuber production (%)	*D. alata*	Not reported	2.8	Jova et al., 2012
	D. alata	6	47	Jova et al., 2005
	D. fordii	8.3	73.8	Yan et al., 2011

component of the existing informal seed yam system and they are not certified. Breeders therefore need to incurporate characterization of their varieties for certification at some stage of variety development for the end product to be confirmed as being same as the initial stock.

The role of tissue culture in this study will be cleaning of breeders' seed before production of foundation seed using meristem culture combined with one or all of the

Plate 1. Left: Seed yam tubers from field-grown plants at Ilushi market in Nigeria (Courtesy: Aighewi, Personal communication); Right: Yam microtubers from conventional tissue culture (Balogun, 2005).

therapies. It will be worthwhile to investigate the possibility of meristem culture in TIBs to overcome the slowness encountered in conventional tissue cultures and this is yet to be reported. The cleaned breeder seeds can in turn be multiplied in TIBs to increase initial stock ofbreeder seed yams, whether as plantlets or small whole microtubers. The challenge here with *D. rotundata* will be to experimentally determine optimum immersion frequency and duration, nutrient/hormone requirement, age of mother plant and number of medium renewals that will be optimum for the two (plantlet or microtubers) products.

If higher quality plantlets are the products of TIBs, losses due to transplanting will be considerably reduced and they can be directly transplanted into macropropagation systems like aeroponics but there might be a waiting time during the dormancy of the minitubers produced from transplanted plantlets. In cases where microtuber production (Plate 1, right) is the target of TIBs, it is important to increase the size and weight of the microtubers to enhance sprouting when directly planted on the field.

It was reported that *in vitro* microtubers do not go into dormancy if immediately put into fresh *in vitro* medium (Ovono et al., 2009) with the advantage that it can be ploughed back into the *in vitro* system for multiple sprout production. However, sprouting ability varied with stage of physiological maturity and size (Balogun, 2009). The NSF-PEER is on-going to standardize the stage of physiological maturity and size of microtubers in photoautotrophic TIBs among farmer-preferred varieties to control their dormancy. The smaller size of the two products and smaller space used is not only cheaper, but also an advantage for easier handling in disease diagnostics and germplasm distribution rather than plants or tubers in a large field or screenhouse.

ENHANCING BREEDING FOR TARGET TRAITS THROUGH TIBS

Ample diversity and efficient selection sieves constitute the core of yam improvement. In spite of wide diversity existing among yam genotypes (Ng and Ng, 1997) 19 yam varieties have been released in Nigeria (Lopez et al., 2012) but those resistant to specific diseases are yet to be identified. This slow rate of variety release (compared to in maize) is due to conventional yam hybridization breeding process, which is from intra-specific crosses, takes six to nine years with only one generation produced per year. In addition, flowering varies with season and location (Hamadina et al., 2009) and is irregular in genotypes, making successful hybridization unpredictable.

There is still a great challenge in inter-specific hybridization due to lack of synchronization of flowering and cross-compatibility, especially in crossing either of *D. rotundata* or *D. cayenensis* to *D. alata.* Other options for creating new variations include genetic transformation, somaclonal variation and mutagenesis while embryo rescue is useful in inter-specific hybridization. This is however constrained by lack of protocols for yam regeneration via somatic embryogenesis. It will be worthwhile to test for ability to regenerate in TIBs since growth is enhanced.

In exploiting mutation breeding for yams, *in vitro* regeneration of plantlets from adventitious buds will facili-

tate production of solid mutants and avoidance of unstable chimeras. Also, yam grown in automated systems can be used to investigate cellular pathways and processes (Jova et al., 2011; Ivanov et al; 2012) as in control of yam tuber dormancy.

Although most landraces are farmer-preferred varieties, a continuously changing agroclimate and new strains of pathogens is a threat to optimum performance of landraces in a specific environment. The challenge will be to introduce disease resistance or environmental adaptation into specific landraces so as to reduce the menace of yield losses due to pests and diseases in yams (Asala et al., 2012). Efficient selection for resistance to toxins from pathogens is possible *in vitro* within minimal space in a controlled environment, as opposed to larger field space with risk of environmental spills.

It is advisable that TIBs be set up in plant protection/-germplasm health units as this will facilitate an understanding of the mechanisms of resistance. This includes *in vitro* screening for resistance to obligate parasites like nematodes which require continuous supply of inoculum, efficient inoculation and diagnostic protocols. There is no nematode-resistant variety of white or water yam to date (Claudius-Cole, Personal communication). Due to enhancement of growth in TIBs, it can be explored for the production of medicinal secondary metabolites like yam steroidal diosgenins (Raju and Rao, 2012) as done for Fenugreek (Rezaeian, 2011).

PRODUCTION OF QUALITY DECLARED SEEDS

Existing farmers' seeds in the current informal seed system stand a chance of harbouring large amounts of disease inoculums. Migration from an informal to a formal seed system will require gradual replacement with quality-declared seeds. Since the starter materials remain the current farmers' planting materials, there must be assemblage of the landraces, identification of different types, disease diagnostics followed by rapid multiplication of disease-free ones and therapy of disease-infected materials. Such rapidity of multiplication can be provided in TIBs, followed by macropropagation in aeroponic systems, further propagation, certification and distribution which will involve a public-private partnership. The beauty of TIBs is that it can also be private-sector managed, facilitating evolution of the formal seed system.

CHALLENGES OF TIBS

The time required to produce the starter stock to feed into yam TIBs is a major challenge. In previous reports on yam culture in TIBs, plantlets already established *in vitro* were used as starter stocks. Although initial *in vitro* establishment takes time, this method ensures reduced or no contamination, and increased survival of plantlets. It will be worthwhile to develop protocols that will permit the use of direct field explants especially for shoot organogenesis. Control of contamination will be critical as done for Eucalyptus and other crops (Thomas, 2004; Watt, 2002). To achieve this, a Hazard Analysis and Critical Control Point Protocol (HACCPP) should be developed for yam culture in TIBs.

Culture of small plant parts in TIBs is also a challenge. This includes use for somatic embryogenesis, meristem, anther and immature embryo culture as it will require supports that are inert, non absorbent or fibrous. In resistance breeding, use of TIBs may be limited to toxin-producing pathogens or obligate parasites like viruses. In the case of viruses, protocols for inoculating *in vitro* calli or other materials with virus for screening will have to be developed followed by an efficient regeneration protocol. In terms of cost, cheaper sources of culture vessels and power need to be explored. The "glass jar temporary immersion bioreactor" and the "horizontal disposable temporary immersion bioreactor" (''Box-in-Bag'') were developed for purposes of cost reduction for coffea cultivation (Ducos et al., 2008). With TIBs at the heart of yam seed systems, it will be necessary to standardize the protocols for different economically important genotypes.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Coupled biochemical genetic and karyomorphological analyses for taxonomic classification - A case study of *Schizothorax* species complex (Teleostei: Cyprinidae)

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Biochemical genetic and karyomorphological studies were evaluated for taxonomic importance. Five *Schizothorax* species namely: *Schizothorax niger*, *Schizothorax curvifrons*, *Schizothorax esocinus*, *Schizothorax labiatus* and *Schizothorax plagiostomus* were studied in this experiment as a model for serum proteins and chromosomal analysis. Serum protein band patterns of *Schizothorax* species complex were studied by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Their electrophoretograms revealed similarities as well as differences in the number and molecular weight of protein bands. *S. esocinus* showed five bands, *S. curvifrons* five, *S. niger* seven, *S. labiatus* and *S. plagiostomus* each showed six bands; they also showed species characteristic bands. Karyotypic study of these was carried out. The diploid chromosome numbers recorded were 98 in *S. niger* (24 m + 32 sm + 22 st + 20 t), 98 in *S. esocinus* (30 m + 22 sm + 10 st + 36 t), 98 in *S. labiatus* (24 m + 20 sm + 2 st + 52 t), 96 in *S. plagiostomus* (24 m + 18 sm + 5 4t) and 94 in *S. curvifrons* (26 m + 20 sm + 20 st + 28 t). Coupled biochemical genetic and karyomorphological analysis proved a good taxonomic tool as the results were decisive in establishing the species status of these species despite their overlapping morphological characters.

Key words: *Schizothorax*, serum proteins, karyotype, taxonomy, biochemical.

INTRODUCTION

Taxonomy provides a vocabulary to discuss the world (Knapp et al., 2002). Most morphological features are plastic and have the potential of being modified by the environmental conditions (Svardson, 1965; Fowler, 1970).

Due to the existence of morphological plasticity among individuals, conventional morphological characters are often found to be deceiving in exact detection of a species (Menon, 1989). Hence, a detailed knowledge of cytogenetic make up is necessary to establish evolutionary relationships between various species, genera, families or orders of animals and plants (Farooq et al.,

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2011).

The comparison about morphological characters is often not adequate for taxonomists to make decisions. By using electrophoretic methods, taxonomic activities have increased rapidly in animal systematics. Proteins are the products of gene action (Crick, 1963; Nirenberg et al., 1963; Ochoa, 1963) and are used as genetic markers which play a significant role in assigning taxonomic status. Genetic markers are superior to artificial markers and tags as they are natural and can be found in all the stages of animal (Kapila and Kapila, 1996). Blood serum proteins have been found to be reasonably conservative among the characteristics of taxonomic importance. Serum proteins can present taxonomic values when serum proteins of different fish species are examined electrophoretically. Therefore, the discrimination between related taxa can be easily made by their electrophoretic results of serum proteins (Theophilus and Rao, 1998).

A morphotaxonomic classification should be replaced by karyotaxonomic classification, for latter being least affected by environmental distortions (Campos, 1972). Genetic information assists in solving problems of identity and defining conservation units for species. Cytogenetic analysis in fish have allowed to determine sex chromosomes (Moreira-Filho et al., 1993; Devlin and Nagahama 2002; Molina and Galetti, 2007) and to perform inferences on cytotaxonomic (Bertollo et al., 2000; Bertollo et al., 2004) and evolutionary issues (Demirok and Unlu, 2001). Karyological studies have also provided basic information on the number, size and morphology of chromosomes (Tan et al., 2004) which is important to undertake chromosome manipulation in fish (Khan et al., 2000).

Schizothorax heckel (Cypriniformes: Cyprinidae) comprises many species that inhabit the reservoirs of Central Asia from Turkmenistan and Eastern Persia in the West to the far reaches of Mekong and Yangtzein the East. Taxonomic status of fishes of the subfamily Schizothoracinae has remained highly controversial, though creditable work has been done by the earlier workers on Kashmir Valley fishes, prominent among them has been that of Hora (1934), Mukerji (1936), Heckel (1838), Silas (1960), Das and Subla (1663, 1964), Qadri et al. (1983), Yousuf et al. (1990), Yousuf (1995), Kullander et al. (1999) etc.

However, most of the above work was based on morphometric and meristic cha-racters. The occurrences of overlapping external charac-ters and the possibility of hybrids have caused further complications. These inspired us to work on SDS-PAGE of blood serum proteins and karyomorphology for com-parative taxonomic study of Kashmir valley *Schizothorax* and also evaluate the role of the present study as a taxonomic tool. The present study has unravelled their real taxonomic status and has paved the way for their evaluation for other studies viz. nutritional biochemistry, aquaculture, etc. The study has also shown that the biochemical, genetic and cytogenetic methods are corroborating and a good taxonomic tool to solve taxonomic problems.

MATERIALS AND METHODS

Collection of fish

Sample fishes were caught alive with an electric fisher and were also bought from the local fish markets. They were transported to the limnology and fisheries laboratory of CORD, University of Kashmir, where they were kept in fully aerated aquaria.

Serum protein analysis

Blood was taken by cardiac puncture of fish with syringe (40 fishes in total; eight of each species, Wt. 200 g ± 10/fish). In order to prevent haemolysis, blood was poured into centrifugation tubes after removing the needle. The blood sample was allowed to clot and thereafter centrifuged at 5000 rpm for 10 min. Separated serum was used for protein estimation and further for electrophoresis (SDS-PAGE). Protein concentration was determined by using the method of Lowery et al. (1952) with BSA as a standard protein.

SDS-PAGE was performed according to the Laemmli (1970) and O'Farrell (1975) methods. Proteins were separated on 12 - 8 cm and 1 mm thick slab gel. Slab gel consists of stacking gel in which proteins are stocked and running gel part in which proteins are resolved. Running gel contained 10% and stacking gel contained 5% polyacrylamide. Each sample was mixed with a sample buffer which consisted of 0.5 M Tris-Cl (PH 6.8), 10% SDS, BPB, 10% glycerol, beta-mercaptoethanol. The sera were mixed with sample buffer as described by Laemmli (1970). Equal amount of sample was loaded in each lane. Electrode buffer solution was made from 0.025 M Tris, 0.192 M glycine and 0.1% SDS at pH 8.3. A current of 70 V was applied to stacking gel. After tracking dye reached separating gel, voltage was adjusted at 110 V. After electrophoresis, gels were stained with 0.25 g Coomassie Brilliant Blue R250 in a solution of methanol (45 ml), ddH2O (45 ml) and glacial acetic acid (10 ml) and destained with a solution of glacial acetic acid (10 ml), methanol (10 ml), ddH2O (80 ml). Gel was scanned in a densitometer.

Cytogenetic analysis:

Air dried chromosome preparation method with some modifications was used as described by Thorgaard and Disney (1990). Fish (30 specimens, six of each species) received two doses of phytohemagglutinin (PHA) injections (4 $\mu g g^{-1}$ BW), in a 20 h interval at 20°C. 8 h after the second PHA injection, colchicine was injected intraperitoneally, 0.05% at 0.1 ml/100 g BW to depress the mitotic division at the metaphase stage and left for 2 to 3 h before sacrificing.

The fish were anesthetized by 300 ppm clove oil for 40 s, their anterior kidney was removed, homogenized and hypotonised simultaneously by potassium chloride, 0.56% for 35 min at room temperature. Suspensions were centrifuged at 1000 rpm for 10 min. Supernatant was removed and the cells were fixed by cold fresh Carnoy fixative (3:1 methanol and glacial acetic acid). This fixation process was repeated three times and the cold fresh Carnoy was replaced at 30 min intervals. Smears were prepared on cold lamellae using splash method from 1 m height and air dried for 24 h, then stained with 2% Giemsa.

Chromosomal analysis

Leica DM LS2 trinoccular photomicroscope with 1000 X magnification

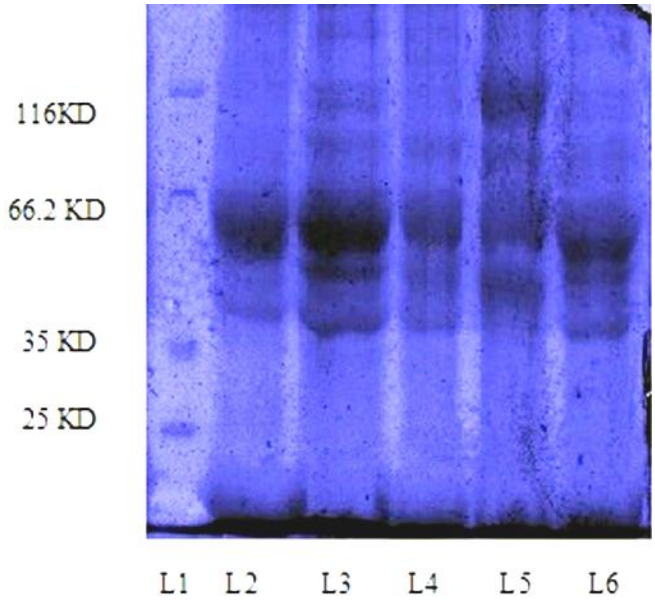

Figure **1.** SDS-PAGE (10% gel) showing electrophoretic pattern of serum proteins observed in *S. curvifrons* (L2), *S. labiatus* (L3), *S. niger* (L4), *S. esocinus* (L5) and *S. plagiostomus* (L6). L1 shows the separation of marker protein.

lens oil immersion was used for taking the photographs and analyzing the chromosomes. Eighty metaphase plates were counted per species and a proper plate was selected to obtain karyotype formulae. Microsoft Excel 2010 software was used to calculate the centromeric indices and to draw ideogram. For each chromosome, centromeric index, arm ratio and total length were calculated as described by Levan et al. (1964) and the fundamental arm number was also calculated. Chromosomes were classified into metacentric, sub-metacentric, sub-telocentric and telocentric following the method of Levan et al. (1964).

RESULTS

Biochemical genetic analysis

SDS-PAGE patterns of serum proteins of five species of *Schizothorax* studied are shown in Figure 1. In the SDS-PAGE electrophoretograms, *S. niger*, *S. curvifrons*, *S. esocinus*, *S. labiatus* and *S. plagiostomus* showed 7, 5, 5, 6 and 6 bands, respectively. Molecular weights of protein bands estimated (Table 1) were as 121, 117.5, 97.5, 83, 66.2, 52.8 and 41.5 KDa in *S. niger*, 121, 97.5, 66.2, 52.8 and 45.8 KDa in *S. curvifrons*, 121, 115, 83, 66.2 and 52.8 KDa in *S. esocinus*, 121, 97.5, 83, 66.2, 52.8 and 41.5 KDa in *S. labiatus*, 120, 96, 83, 64, 52.8 and 45.8 KDa in *S. plagiostomus*.

Karyomorphological study

Schizothorax niger revealed a diploid number of 98 comprising 12 metacentric pairs, 16 sub-metacentric pairs, 11 sub-telocentric pairs and 10 telocentric pairs of chromosomes (Figure 2). *Schizothorax esocinus* showed a diploid complement of 98 comprising 15 metacentric chromosome pairs, 11 sub-metacentric pairs, 5 sub-telocentric pairs and 18 telocentric pairs (Figure 3). *Schizothorax labiatus* also revealed a diploid complement of 98 that comprised 12 metacentric pairs, 10 sub-metacentric pairs, 1 sub-telocentric pair and 26 telocentric pairs (Figure 4). *Schizothorax plagiostomus* showed asomatic complement of 96 chromosomes comprised of 12 metacentric pairs, 9 sub-metacentric pairs and 27 telocentric pairs (Figure 5). The diploid chromosomal complement of *Schizothorax curvifrons* comprised 94 chromosomes; 13 metacentric pairs, 10 sub-metacentric pairs, 10 sub-telocentric pairs and 14 telocentric pairs (Figure 6).

DISCUSSION

Our study on comparative analysis of serum proteins of five species of *Schizothrax* found in Kashmir valley (*S. niger*, *S. curvifrons*, *S. esocinus*, *S. labiatus* and *S. plagiostomus*) revealed that the amount of serum protein differed considerably among them. Diet might be the contributor factor to the variable amount of protein in sera (Badawi, 1971).

The present SDS-PAGE serum protein, study of five species of *Schizothorax* revealed similarities as well as differences in the number and molecular weight of protein bands among the five species studied. Protein differences

Table 1. Molecular weight (in KDa) of protein bands in electrophoretograms of five fishes.

Fraction number/band number	*S. niger*	*S. curvifrons*	*S. esocinus*	*S. labiatus*	*S. plagiostomus*
1	121	121	121	121	-
2	-	-	-	-	120
3	117.5	-	-	-	-
4	-	-	115	-	-
5	97.5	97.5	-	97.5	-
6	-	-	-	-	96
7	83	-	83	83	83
8	66.2	66.2	66.2	66.2	-
9	-	-	-	-	64
10	52.8	52.8	52.8	52.8	52.8
11	-	45.8	-	-	45.8
12	41.5	-	-	41.5	-

Figure 2. Karyotype of *S. niger* (m = metacentric; sm = sub-metacentric; st = sub-telocentric; t = telocentric).

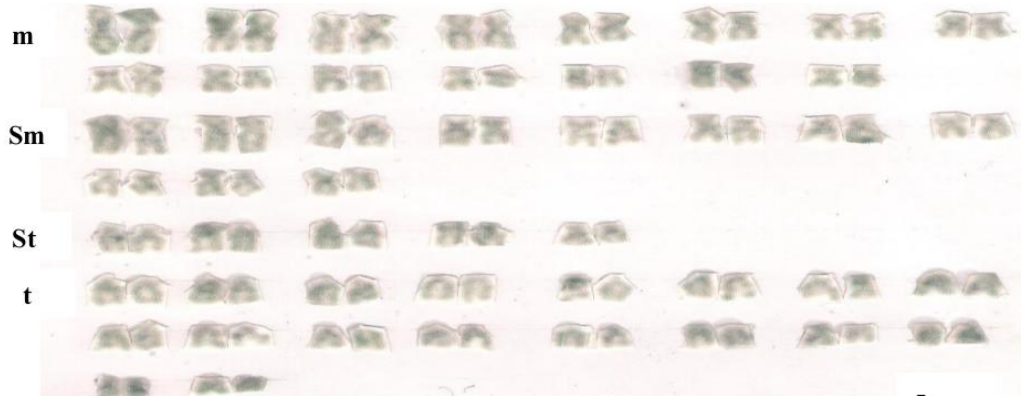

Figure 3. Karyotype of *S. esocinus.* (m = metacentric; sm = sub-metacentric; st = sub-telocentric; t = telocentric).

between species are specific for individuals representing a group. This could elucidate taxonomic controversies in the cases of disputed species (Smith et al., 1979). *S. esocinus* and *S. curvifrons* showed same number of bands and similarity in molecular weight of certain bands (121, 66.2 and 52.8 KDa) but differed in bands 115 and

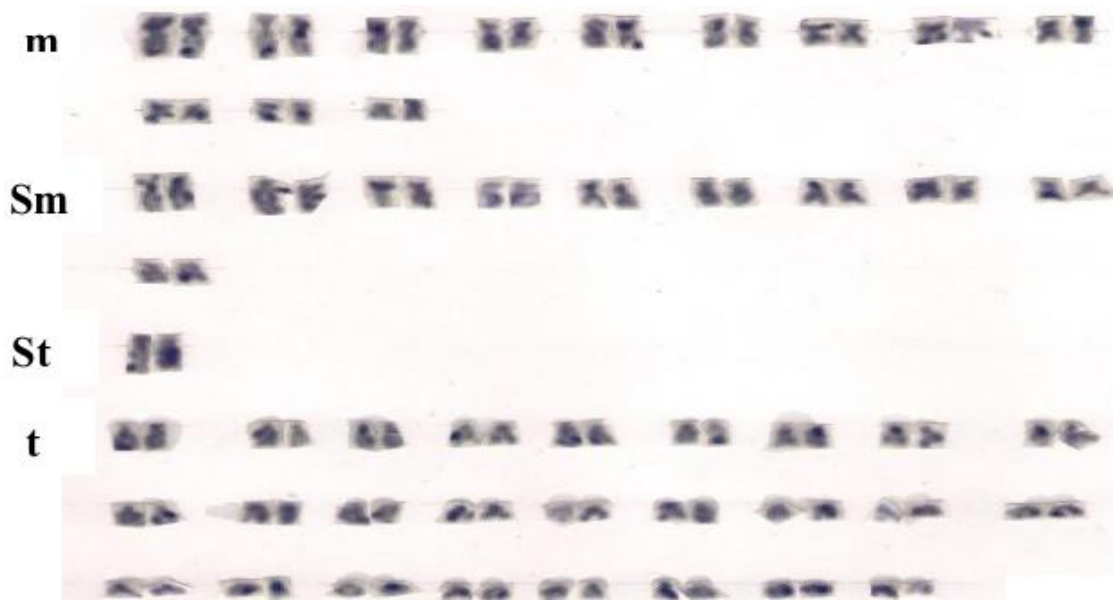

Figure 4. Karyotype of *S. labiatus* (m = metacentric; sm = sub-metacentric; st = sub-telocentric; t = telocentric).

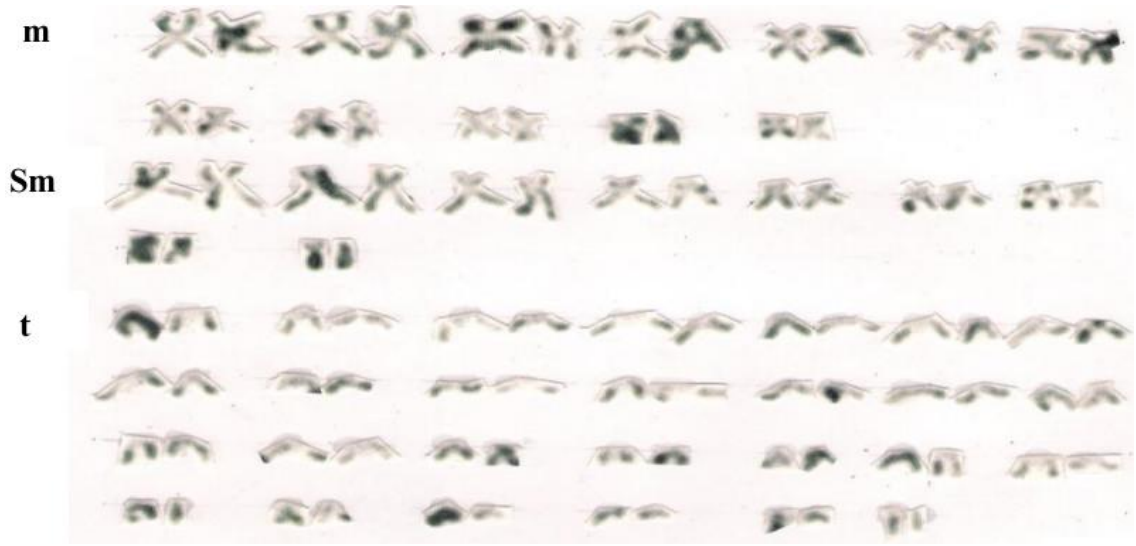

Figure 5. Karyotype of *S. plagiostomus* (m = metacentric; sm = sub-metacentric; st = sub-telocentric; t = telocentric).

83 KDa (found in *S. esocinus* but absent in *S. curvifrons*) and bands of molecular weight 97.5 and 45.8 KDa (found in *S. curvifrons* but absent in *S. esocinus*). Closely related species not only share many electrophoretic alleles, but also differ at some gene loci at which they are fixed for different alleles (Smith et al., 1990).

S. labiatus and *S. plagiostomus* are similar in having same number of bands that is, 6 and sharing bands of molecular weight 83, and 52.8 KDa, but differed in 121, 97.5, 66.2 and 41.5 KDa (found in *S. labiatus* but absent in *S. plagiostomus*) and bands of molecular weight 120, 96, 64 and 45.8 KDa (found in *S. plagiostomus* but absent in *S. labiatus*). If two different species have same number of electrophoretic fractions, further close comparison of percentage age relative mobility (Rf) values and corresponding molecular weight of one or more fractions could reveal species-specific differences. Species specificity of a protein should mean primarily the electrophoretic mobility differences of one or more bands (Verghese and Jayasankar, 1999). In our study, although

Figure 6. Karyotype of *S. curvifrons* (m = metacentric; sm = sub-metacentric; st = sub-telocentric; t = telocentric).

S. curvifrons and *S. esocinus* showed same number of bands that is 5; they differed in the Rf values of individual fractions. Similarly *S. labiatus* and *S. plagiostomus* each depicted 6 bands, but they also differed in the Rf values of individual fractions or bands. Differing in their relative mobilities, they possessed different banding patterns, hence their patterns were species diagnostic. *S. plagiostomus* showed characteristic bands of molecular weight 120, 96 and 64 KDa. Band 115 KDa was found characteristic to *S. esocinus*, while *S. niger* depicted a characteristic band of molecular weight 117.5 KDa. The electrophoretic patterns, therefore, confirm their status as valid species which have undergone speciation long back that is all the five species revealed bands in the range 19.5 to 39 KDa and originated from the same genetic stock. The fractional differences have taxonomic significance at the level of population, sub-species or species.

Three out of the five species analysed in the present study namely: *S. niger, S. esocinus* and *S. labiatus* revealed a diploid number of 98 and a fundamental arm number (FN) of 154, 150 and 142, respectively. The variation in the fundamental arm number without change in the 2 n may be attributed to the intra chromosomal changes involving pericentric and paraentric inversion and centromeric shifts (Rishi et al., 1998). Variation in the karyotypic configuration of *S. niger* and *S. esocinus* can be easily explained by centric fusion and fission events. It is evident from the karyotype of these two species that there has been simultaneous fusion of telocentric and fission of metacentric chromosomes in *S. esocinus* which resulted in the karyotype of *S. niger*. This is because *S. niger* is having more biarmed chromosomes than *S. esocinus* and karyotype with biarmed chromosomes are generally regarded to represent a derived condition (Ohno et al., 1968; Ohno 1970; Denton 1973; Gold 1979). Same types of chromosomal rearrangements seem to have framed the karyotype of *S. labiatus*.

Other two species namely: *S. plagiostomus* and *S. curvifrons* revealed a diploid number of 96 and 94 and FN of 138 and 140 respectively. Decrease in the 2 n in these species may be attributed to the Robertsonian arrangements and change in FN to pericentric inversion (Choudhury et al., 1982).

Highest number of biarmed chromosomes was found in *S. niger* followed by *S. esocinus*, *S. curvifrons, S. plagiostomus* and *S. labiatus* whereas highest number of sub-telocentrics were found in the order of *S. plagiostomus* and *S. labiatus* (equal), *S. curvifrons, S. esocinus* and *S. niger*. The primitive teleost karyotype is thought to have consisted of 46-48 acrocentrics (Nayyar, 1966; Ohno et al., 1968; Ohno 1970; Fitzsimons, 1972; Legrande, 1975) and the karyotypes with biarmed chromosomes are regarded to represent a derived condition (Ohno et al., 1968; Ohno, 1970; Denton, 1973; Gold, 1979). Keeping this into consideration, *S. plagiostomus* and *S. labiatus* seem to be primitive fishes when compared to *S. niger*, *S. esocinus* and *S. curvifrons* which possess a more derived karyotype. The trend of gradual increase in the FN from 138 in *S. plagiostomus* to 154 in *S. niger* supports the above assertion.

The overall dissimilarity in the 2 n, karyotypic configuration and FN points to the role of almost all types of chromosomal rearrangements in the karyological evolution of Schizothoracinae, however deviation in the chromosome number can be possibly dislocation hypothesis of evolution of chromosomes according to Navashin's (1932).

This hypothesis explains that each chromosome is monocentric and an evolutionary change in the chromosome number must involve duplication of centromere together with a region within it while a decrease in the number must mean a permanent loss from the karyotype of the region containing centromere. Family Cyprinidae points to the role of almost all types of chromo-

somal rearrangements for their karyological evolution (Rishi et al., 1998).

The present study conclusively confirms the specific status of the five species of *Schizothorax* on the basis of their serum proteins and genetic material. The study negates the proposition of Silas (1960) regarding the taxonomic status of *Schizothorax niger* and *Schizothorax curvifrons*, who had combined these two species into a single species *Schizothorax niger* treating *curvifrons* and *niger* as two varieties or sub-species. The present study has clearly shown that these two species of fish be treated as distinct species and not varieties or sub-species of the same species. The present study has also cleared the taxonomic position of *Schizothorax plagiostomus* side by side *S. richardsonii* for a long time; the two species were regarded as synonyms. A comparison of the two species clearly indicates that the two species are distinct from each other as proposed by Kullander et al. (1999).

Coupled biochemical genetic and karyomorphological analyses constitute a good taxonomical tool that the classifications should be based on. In fact genetic changes proceed morphological ones (Howell and Villa 1976), which also render support to this notion and morphological based classifications, should be re-evaluated using these methods.

Conflict of interests

The author(s) have not declared any conflict of interests.

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Utilization of chitinolytic bacterial isolates to control anthracnose of cocoa leaf caused by *Colletotrichum gloeosporioides*

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***Colletotrichum* spp. are causal agents of anthracnose in many plant species. Biological control of *Colletotrichum* spp. utilizing bacterial isolates and fungi has been reported. However, chitinolytic bacterial isolate utilization to control anthracnose of cocoa leaf has not seemingly been studied yet. In this study, we used chitinolytic bacterial isolates to reduce anthracnose severity and incidence caused by *Colletotrichum gloeosporioides*. Identification of the chitinolytic isolates was conducted for their morphological and biochemical traits, and the sequencing of 16S rRNA was to know their related species. Assay of antagonistic bacterial chitinolytic to *C. gloeosporioides* was conducted in minimum salt medium agar with 2% colloidal chitin as sole carbon source. To examine ability of the chitinolytic isolates in reducing anthracnose severity and incidence, cocoa leaves were treated with the isolates prior infestation of the conidia. Identification of 16S rRNA showed that KR05, LK08, BK13, BK15 and BK17 isolates were *Enterobacter* sp., *Enterobacter cloacae*, *Bacillus* sp., *Enterobacter* sp., and *Bacillus* sp., respectively. All chitinolytic isolates inhibited growth of *C. gloeosporioides in vitro* to some extent. Microscopic studies showed morphological abnormalities of *C. gloeosporioides* hyphae that is, broken, lytic, rolled, twisted, curled and abnormal branching of hyphae as a result of antagonistic mechanism caused by the chitinolytic isolates. All chitinolytic isolates were able to reduce anthracnose severity and incidence on cocoa leaves from 0.8 to 3.2% and 4 to 12%, respectively.**

Key words: Anthracnose, biological control, chitinolytic bacteria, *C. gloeosporioides*, cocoa.

INTRODUCTION

The genus *Colletotrichum* represents an economically important group of fungal plant pathogens and is recorded from approximately 2.200 plant host species (Farr and Rossman, 2009). As plant pathogens *Colletotrichum* spp. are the principal cause of anthracnose as well as pre- and postharvest fruit rots, damping-off and blossom and seedling blight diseases (Bailey and Jeger, 1992). One species, *C. gloeosporioides*, causes

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leaf spots in plantation plant such as cacao (Rojas et al., 2010; Yee and Sariah, 1993) and para rubber tree (Adekunle and Ogbebor, 2009), and also attacks other economic plants/fruits such as yellow passion fruit (Anaruma et al., 2010), mango (Nelson, 2008), avocado, and almond (Freeman et al., 1996).

Control of this disease has been largely dependent on chemical fungicides inhibiting vegetative pathogen growth. However, the chemical practices to overcome plant disease problem have adverse environmental effects affecting non-target organisms and causing health hazards to humans, besides demanding high costs (Ningthoujam et al., 2009). Biological control of plant pathogens has been shown to have potential to control over many diseases in plant. Biological control may operate through antibiosis, competition, predation or parasitism (Alabouvette et al., 2006; Ozbay and Newman, 2004). As a general method, biological control using antagonistic microorganisms has been successfully demonstrated in a number of plant species. Antagonistic microorganisms, by their interactions with various plant pathogens, play a major role in microbial equilibrium and serve as powerful agents for the biological control of diseases (Ozbay and Newman, 2004; Alabouvette et al., 2006). The lytic activity of bacteria is one of a number of mechanisms that has been implicated in biocontrol (Alabouvette et al., 2006). Recently, the use of naturally occurring bacteria (Sangeetha et al., 2010; Akinbode and Ikotun, 2008; Mahadtanapuk et al., 2007) and antagonistic fungi (Adekunle and Ogbebor, 2009; Akinbode and Ikotun, 2008; Shovan et al., 2008) for biocontrol of *Colletotrichum* spp. has been proposed.

A number of fungi are particularly susceptible to be degraded by microorganisms (Kim et al., 2008). Mycolytic enzymes producing by microorganisms such as chitinase have great potential in solving such problems (Anitha and Rabeeth, 2010; Patel et al., 2007; Gohel et al., 2006). For this purpose of employing such chitinolytic bacterial isolates for biological control of anthracnose in cocoa leaves caused by *C. gloeosporioides*, assay on their ability to inhibit the fungal growth on agar and on cocoa leaves were conducted. Abnormal hyphae of *C. gloeosporioides* as a result of antagonistic assay were also observed. To our knowledge, possible utilization of chitinolytic bacteria in controlling *C. gloeosporioides* causal agent of anthracnose in cocoa leaves has not been reported.

MATERIALS AND METHODS

Chitinolytic bacterial isolates

Bacterial isolates KR05, LK08, BK13, BK15 and BK17 used in this study were collection from Microbiology Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara. These isolates were not morphologically and biochemically characterized except that of KR05 and LK08 (Suryanto et al., 2011). The isolates were kept at 30°C in a modified salt medium (0.7 g K_2HPO_4, 0.3 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.01 g $FeSO_4.7H_2O$, 0.001 g $ZnSO_4$, and 0.001 g $MnCl_2$ in 1.000 ml) containing 2% (w/v) chitin colloidal (MSMC) agar, with a pH of 6.8.

Examination of cell morphology and biochemical properties

Cell shape and Gram staining were evaluated using a microscope, while colony shape was observed directly. Motility was observed using semi-solid medium sulfide indole motility. Biochemical properties were examined including gelatin test using gelatin nutrient medium, citrate test using Simons citrate agar, catalase test using 3% H_2O_2 solution, and starch metabolism using starch agar.

16S rRNA gene of chitinolytic bacterial isolate identification

Bacterial DNA was prepared following the manufacturer's protocol of Wizard® Genomic DNA Purification Kit (Promega). The 16S-rRNA genes were polymerase chain reaction (PCR)-amplified using specific primers of 63f and 1387r (Marchesi et al., 1998) from the bacterial DNA. PCR reactions were performed in thermal cycler using the following conditions: initial denaturation for 1 min at 95°C; annealing (1 min at 50°C), and extension (1.5 min at 72°C); prolong the extension (5 min at 72°C). PCR product was purified using QIAquick PCR Purification Kit Protocol (Qiagen). Part of the genes of 16S-rRNA was pre-sequenced using thermo cycler. Pre-sequencing reaction products was precipitated using manufacturer's protocol of Pellet Paint® NF Co-Precipitant (Novagen). The sequencing reaction was run on ABI PRISM® 310 Genetic Analyzer (Applied Biosystem). The sequence was analyzed using Blast (http://blast.ncbi.nlm.nih.gov.).

Isolation and reisolation of *C. gloeosporioides* of cocoa leaves

Cocoa leaves showing symptoms of leaf spot disease were collected from mature cocoa trees. For re-isolation after conidia infestation, leaves with symptom were taken. Pieces of the infected leaf, 3 x 3 mm were surface disinfected for few seconds in 70% alcohol and in 1% aqueous sodium hypochlorite for 5 min, and then rinsed thoroughly with sterile distilled water (SDW). Surface-disinfected leaves were grown on potato dextrose agar (PDA) for 72 h at ±25 to 30°C. Growing fungal colonies were then purified. Culture of the isolate was sub-cultured onto and maintained on PDA throughout this study. Identification morphological characteristics such as colony, mycelia as well as shape and size of conidia under light microscopy observation were carried out.

Assay of bacterial-fungal antagonism

The ability of the chitinolytic bacterial isolates to inhibit *C. gloeosporioides* growth was conducted *in vitro*. Fungal culture was grown at the center of MSMC agar. Two pieces of paper discs immersed with ≈ 10^8 cells/ml of bacterial suspension were placed in the opposite direction about 3.5 cm from the fungal culture. Culture was incubated at ±25 to 30°C. Inhibitory activity was determined based on the inhibition zone formed around bacterial colonies on medium MSMC. Inhibition zone was measured from three to seven days of incubation as the radius of the normal fungal growth subtracted the radius of the inhibited fungal growth. The assay was replicated four times.

Observation of hyphal abnormality

Inhibited hyphae of *C. gloeosporioides* of antagonistic assay were cut by 1 cm^2. The hyphae were examined under light microscope and compared with normal ones.

Table 1. Identification and characterization of the chitinolytic bacterial isolates.

Character	Bacterial isolate				
	BK13	BK15	BK17	KR05*	LK08*
Colony shape	Entire, flat	Irregular, flat	Entire, flat		
Colony color	White	White	Yellowish		
Cell shape	Rod	Rod	Rod		
Biochemical traits					
Gram	+	-	+		
Motility	+	+	+		
Gelatine	-	-	-		
Citrate	+	+	+		
Catalase	+	+	+		
Starch	+	-	+		
16S rRNA gene partial	*Bacillus* sp.	*Enterobacter* sp.	*Bacillus* sp.	*Enterobacter* sp.	*Enterobacter cloacae*

*Morphological and biochemical characterization was previously described (Suryanto et al., 2011).

Assay of chitinolytic bacterial isolates to *C. gloeosporioides* on cocoa leaves

Chitinolytic bacterial isolates were sub-cultured in nutrient agar for two days. Bacterial suspension was prepared in 0.09% NaCl with cell density of ≈ 10^8 cells/ml. The isolated *C. gloeosporioides* was sub-cultured on PDA. Fungal colony was removed and put into 10 ml of 0.09% NaCl. Conidia were harvested by filtering them through muslin cloth to remove mycelia. Conidia concentration was then determined using a hemocytometer and adjusted to ≈ $2x10^5$ conidia/ml. All microbial cultures were incubated at ±25 to 30°C.

10 ml of chitinolytic bacterial suspension was thoroughly spray-inoculated into cocoa leaf surface of three-month old trees. Conidia suspension was applied after bacterial overnight- application. The leaf was then wrapped with transparent wrapping plastic. (+) Control was treated similarly but without chitinolytic bacterial inoculation, while (-) control had no conidia and chitinolytic bacterial inoculation. The work was replicated five times.

Observation on disease incidence and disease severity

Observation was conducted after 1 week of application every week for 1 month on 5 upper leaves below 2 tip-leaves. The Disease Incidence (DI) was measured using this following equation (Cooke, 2006):

$$DI = \frac{\text{Number of infected plant units}}{\text{Total number of plant units assessed}} \times 100\%$$

Meanwhile, the disease severity (DS) was determined according to alternative rating scale proposed by Bowen (2007) in which scale 0 = no symptom, scale 1 = 0 to 20% disease severity range, scale 2 = 20 to 40%, scale 3 = 40 to 60%, scale 4 = 60 to 80% and scale 5 = 80 to 100%, respectively. DS was measured using the equation proposed by Kranz (1988) as follows:

$$DS = \frac{\Sigma (axb)}{NZ} \times 100\%$$

Σ (axb) = Sum of the symptomatic plant and their corresponding score scale, N = total number of sampled plant and Z = highest score scale.

RESULTS

Characterization and identification of chitinolytic bacterial isolates

Identification of chitinolytic bacteria showed that KR05, LK08 and BK15 and except that of colony color BK13 and BK17 possessed similar characteristics. This indicated different species of bacteria (Table 1). Two isolates were Gram-positive, while three was Gram-negative. Chitinolytic bacteria spread among Gram-negative and Gram -positive (Anitha and Rabeeth, 2010; Singh et al., 2008; Metcalfe et al., 2002; Folders et al., 2001).

Identification of part of 16S rRNA gene revealed that KR05, LK08, BK13, BK15 and BK17 were closed to *Enterobacter* sp., *Enterobacter cloacae*, *Bacillus* sp., *Enterobacter* sp., and *Bacillus* sp. with similarity (forward-reverse) of 95 to 99, 94 to 98, 95 to 86, 96 to 99 and 97 to 98%, respectively.

Isolation and reisolation of *C. gloeosporioides* of cocoa leaf

Manifestation of anthracnose caused by *C. gloeosporioides* on cocoa leaf was shown as brownish lesions and chlorotic haloes symptoms (Figure 1a). These lesions had brown centers and then coalesced to rot. Identification of the isolate showed that the fungus had whitish colony (Figure 1b), with septate hyphae, and capsule-like conidia containing one cell (Figure 1c.). Reisolation of infected cocoa leaf showing anthracnose symptom revealed the same fungus.

Assay of bacterial-fungal antagonism

To know chitinolytic isolate ability to inhibit growth of *C.*

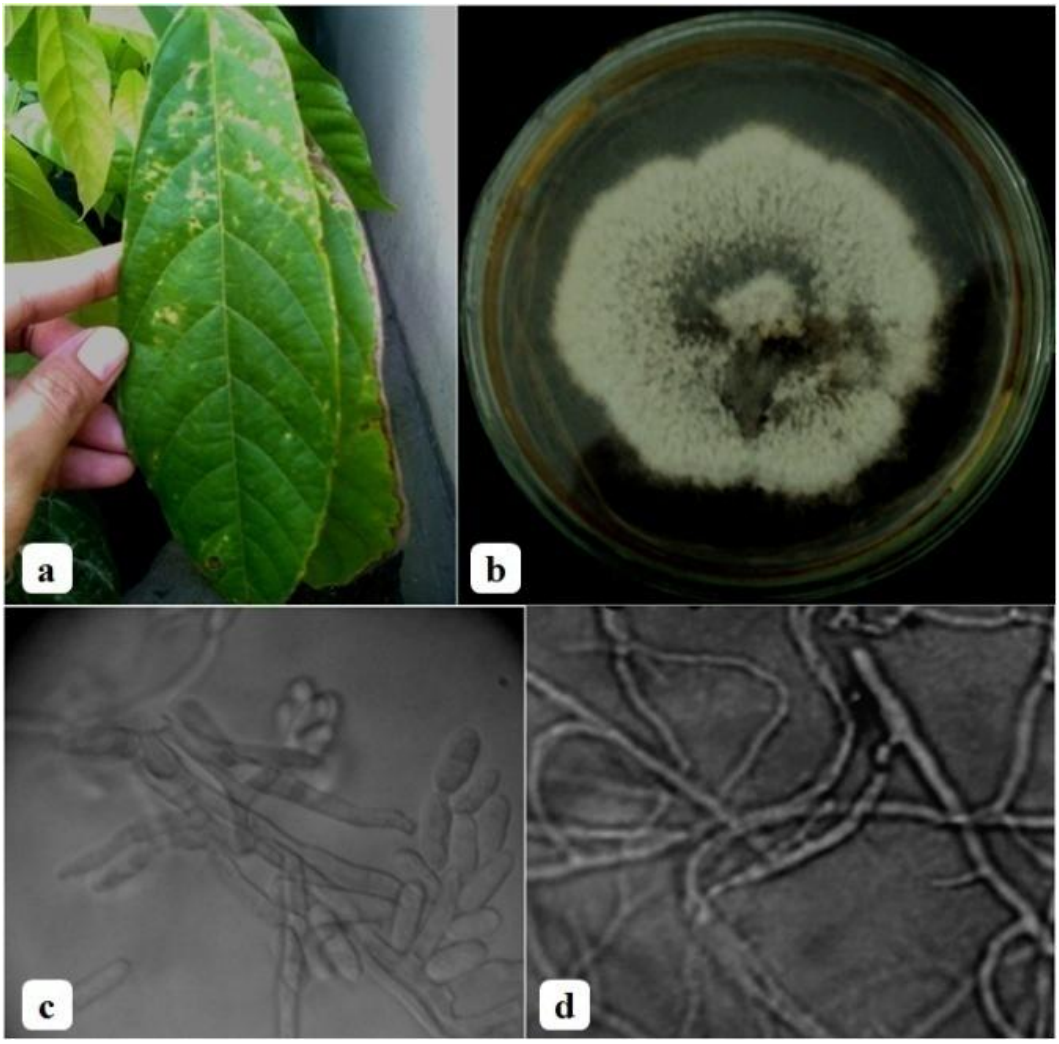

Figure 1. (a) Disease symptom on treated leaf. **(b)** Colony on PDA. **(c)** Conidia and hyphae. **(d)** Abnormal hyphae.

gloeosporioides hyphae, examination was conducted by growing chitinolytic isolates next to the fungi in chitin containing media. All isolates showed ability to inhibit growth of *C. gloeosporioides* hyphae to some extent (Table 2). Inhibition zone increased during incubation time, and was observed on 3-days of incubation and continued to 7-days of incubation, or more (Kim et al., 2008). This indicated that chitinase was still produced and diffused to the media to degrade fungal hyphae. The lytic activity of bacteria is one of a number of mechanisms that has been implicated in biocontrol (Anitha and Rabeeth et al., 2010; Gohel et al., 2006).

Observation of hyphal abnormality

Microscopic observation of fungal hyphae after the antagonistic assay showed the occurrence of abnormality hyphae. Abnormal hyphae were marked with broken, lytic, rolled, twisted, curled and abnormal branching of hyphae (Figure 2). Getha and Vikineswary (2002) reported that hyphal distortion like lytic of hyphal ends, swollen hyphae, abnormal branching of hyphae and the formation of hyphal protuberances is observed after contacted *Fusarium oxysporum* f.sp. *cubense* hyphae with *Streptomyces violaceusniger* strain G10.

Control of *C. gloeosporioides* on cocoa leaves

Efficacy of chitinolytic isolates to reduce infestation of *C. gloeosporioides* was conducted on cocoa leaves. All chitinolytic isolates showed reduce disease severity (Table 3) and disease incidence (Table 4) compared to that of (+) control. Unsterilized leaves of (-) control of cocoa leaves might result in contamination o f the leaves

Table 2. Inhibition zone of antagonistic assay of chitinolytic bacteria to *C. gloeosporioides.*

Isolate	Inhibition zone (mm) of days				
	3	4	5	6	7
KR05	6.0	6.2	8.7	17.1	28.9
LK08	10.0	9.9	12.4	18.3	30.5
BK13	6.1	6.5	9.6	18.8	31.8
BK15	6.1	6.4	8.4	17.7	28.7
BK17	6.1	6.1	9.5	18.5	29.9

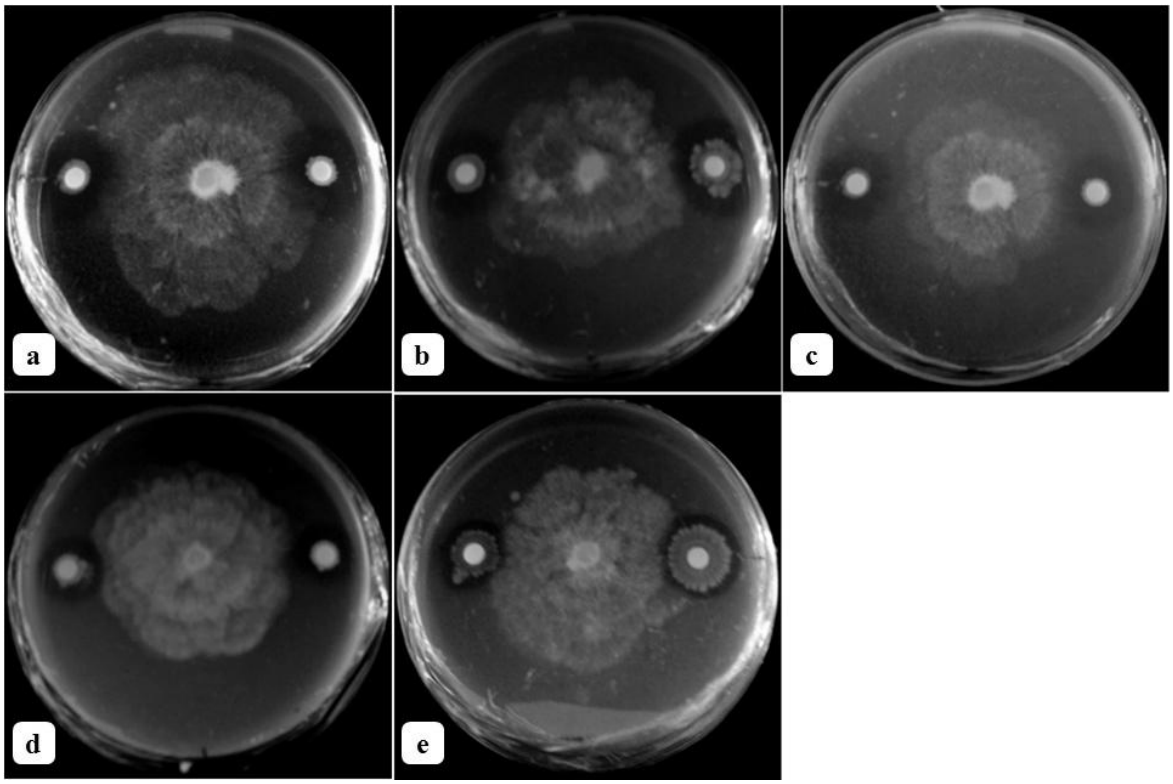

Figure 2. Antagonistic assay of **(a)** KR05, **(b)** LK08, **(c)** BK13, **(d)** BK15 and **(e)** BK17 on *C. gloeosporioides in vitro.*

to *C. gloeosporioides*. The fungus may spread by strong wind currents that dislodge spores from agitated leaves, by nursery workers handling diseased plants, and by movement of slugs or other pests (Uchida and Kadooka, 1997). Guyot et al. (2005) reported that the conidia of *C. gloeosporioides* disperse for several tens of meters in rubber tree. However, disease severity and disease incidence were still weak rather than of (+) control, in which fungal infestation was purposely inoculated. The maximum reduction of disease severity and disease incidence was shown by LK08 and BK17 by 0.8%, followed by BK13 and BK15 by 0.8 to 1.6 and 2.4 to 3.2%, respectively after eight weeks of the conidia infestation. The highest disease severity and disease incidence increased to 16 and 20%, respectively in (+) control, in which the conidia were infested without chitinolytic bacterial inoculation.

DISCUSSION

Biological control using microorganism has been studied intensively since not many alternatives for control are available. Various microbial antagonists have been investigated as potential antifungal biocontrol agents of plant diseases. Certain strain of microorganism has been reported to succesfully suppress the growth of plant pathogen (Adekunle and Ogbebor, 2009; Kim et al., 2008; Mahadtanapuk et al., 2007; Soytong et al., 2005).

Our study on utilization of chitinolytic bacterial isolates

Table 3. Disease severity on cocoa leaf caused by *C. gloeosporioides.*

Treatment	Disease severity (%)/week			
	1	2	3	4
KR05	1.6	1.6	1.6	2.4
LK08	0.8	0.8	0.8	0.8
BK13	0.8	0.8	0.8	1.6
BK15	2.4	2.4	3.2	3.2
BK17	0.8	0.8	0.8	0.8
(+) Control	15.2	15.2	15.2	16
(-) Control	3.2	3.2	3.2	3.2

Table 4. Disease incidence on cocoa leaf caused by *C. gloeosporioides.*

Treatment	Disease incidence (%)/week			
	1	2	3	4
KR05	8	8	8	12
LK08	4	4	4	4
BK13	4	4	4	8
BK15	8	8	8	8
BK17	4	4	4	8
(+) control	20	20	20	20
(-) control	8	8	8	8

as potential biological control agent to reduce disease severity and disease incidence caused by *C. gloeosporioides* on cocoa leaves showed that the isolates were capable of declining infestation of the fungi. Species in the genus *Colletotrichum* are common in the environment and frequently parasitize higher plants, causing anthracnose disease (Rojas et al., 2010; Nelson, 2008; Freeman et al., 1996; Yee and Sariah, 1993; Bailey and Jeger, 1992). The disease can occur on leaves, stems, and both preharvest and postharvest fruit (Ivey et al., 2004).

In vitro assay of antagonism showed that chitinolytic isolates inhibited fungal growth to some extent. Chitinase, antifungal protein, might play an important role to lytic fungal cell wall. Clear zone around the bacterial isolate colonies demonstrated ability of their chitinases to degrade chitin as C-source. Different ability of the chitinolytic bacterial isolates to inhibit fungal growth might be due to different chitinase produced by the isolates. Molecular and biochemical characterizations have revealed that chitinases, similar to other glycosyl hydro-lases, are molecular in nature and can differ according to their structural organization. Enzymes can vary both within and between microbes (Kobayashi et al., 2002; Metcalfe et al., 2002). Bacterial genera *Achromobacter, Bacillus, Chromobacterium, Pseudomonas* and *Vibrio,* along with bacteria from the *Flavobacterium-Cytophaga* group and the *Enterobacterioceae* family (Donderski and Brzezińska (2001), and *Streptomyces* (Anitha and Rabeeth, 2010) are the common chitinase producing bacteria. As shown by 16S rRNA gene identification, our isolates were *Enterobacter* spp., *Enterobacter cloacae*, and *Bacillus* spp.

Recently, the use of naturally occurring bacteria (Sangeetha et al., 2010; Akinbode and Ikotun, 2008; Mahadtanapuk et al., 2007) and antagonistic fungi (Adekunle and Ogbebor, 2009; Akinbode and Ikotun, 2008; Shovan et al., 2008) for biocontrol of *Colletotrichum* spp. has been recognized. The efficacy of chitinolytic bacterial isolates against different pathogens has been proven earlier (Maisuria et al., 2008; Gohel et al., 2006).

Our previous study have shown that several local chitinolytic bacterial isolates inhibited the growth of pathogenic fungi *Ganoderma boninense*, *Penicillium citrinum* and *F. oxysporum in vitro* (Suryanto et al., 2011). In this study, we observed that all tested chitinolytic bacterial isolates effectively suppressed the growth, and decreased anthracnose severity and intensity caused by *C. gloeosporioides*.

Application of chitinolytic bacteria on cocoa leaves before *C. gloeosporioides* conidia infestation reduced disease severity and disease incidence. This could be due in part to chitinase produced by the isolates that inhibited the fungal growth. Chitinolytic bacteria often show antago-nistic association with fungi (Gohel et al., 2006). Microbial antagonism implies direct interaction between two microorganisms sharing the same ecological niche (Alabouvette, 2006). Antagonistic effects responsible for disease suppression in biological control

results either from microbial interactions directed against the pathogen, mainly during its saprophytic phase, or from an indirect action through induced resistance of the host plant (Alabouvette, 2006).

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Full Length Research Paper

Addition of grape extracts can enhance quality of natural seasonings by changing their physicochemical properties

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The present study was aimed to evaluate the application of different concentrations of grape extract on the physicochemical properties of natural seasonings. The results show that increase in concentration of grape extract (0 to 20%) significantly reduced the lightness (34.75 to 28.3) and yellowness (15.33 to 10.64), however increased the redness (0.98 to 1.5) values of natural seasonings. The mineral contents of zinc and iron were raised by the addition of grape extracts but potassium, magnesium, calcium and sodium were reduced. The antioxidant activity, as 1,1-diphenyl-2-picrylhydrazyl (DPPH), of seasonings increased by up to 6.21% with the addition of 20% grape concentration extract. Total phenolic content was also significantly increased with the addition of concentrated grape extract. Reactive oxygen species (ROS) as superoxide anion and hydroxyl radical were significantly reduced with the addition of grape extract. The amino acids analysis showed that concentration of few amino acids like aspartic acid, proline, alanine, cysteine, valine, methionine, isoleucine, leucine, and lysine were elevated, conversely while threonine, serine, glutamic acid, glycine, tyrosine, phenylalanine, histidine, and arginine were found declined with higher proportion of grape extract in the seasoning. Results suggest that addition of grape extract may enhance the physicochemical properties as well as health potential of natural seasonings.

Key words: Grape extracts, natural seasonings, physicochemical properties, quality characteristics.

INTRODUCTION

The terms spices and herbs, also known as seasonings, are usually used together to describe bark, buds, flowers, leaves, fruits, bulbs, roots or seeds derived from a group of aromatic plants. In general, spices are highly aromatic due to their high contents of essential oils, whereas herbs are low in essential oils and usually used to produce

delicate or subtle flavors in food preparations (Chi and Wu, 2007). Spices have been recognized to possess medicinal properties and their uses in traditional system of medicines have been known since long. The use of spices might be whole spices, ground spices, or isolates from their extract (Srinivasan et al., 2004). Cooking with herbs, spices and seasonings can add flavor and variety to food. Seasonings are used as food additive for the purpose of flavor, medicine, color or as a preservative, that kills harmful bacteria or prevent their growth (Ernst and Pittler, 2000). Spices, like vegetables, fruit, and medicinal herbs, are known to possess a variety of antioxidant effects and properties (Zheng and Wang, 2001).

Most of the spices and herbs analyzed have very high antioxidant content (Paur et al., 2011). Phenolic compounds in the tested spices contribute significantly to their antioxidant capacity (Shan et al., 2005). Various spice-derived ingredients possess potential inhibitors of lipid peroxidation in cell and low density lipoprotein cholesterol in human (Naidu and Thippeswamy, 2002). It has been demonstrated that the antioxidant activity of bioactive compounds found in herbs and spices could play an important role in suppressing viral replication, inhibiting allergy and arthritis, preventing cancer and heart diseases (Aggarwal et al., 2002). Consumers' interest in food formulations containing "natural" ingredients has motivated the food industry to evaluate the effectiveness of naturally occurring components of food for functional purposes as compared to synthetic ones.

The nutritional and medicinal properties of spices and herbal plants may be interlinked through phytochemicals, both nutrient and non-nutrient (Ranhotra et al., 1998). Although spices are used primarily for their desirable flavor and odor, they may play other important roles in the food systems. From antiquity, in addition to spices and their derivatives being used for flavoring foods and beverages and for medication, they have also been highly valued for their use as antimicrobials (Koedam, 1986; Özcan, 2004), and antidiabetics (Tundis et al., 2010). Spices and herbs can be practical for antimicrobial effect as protecting seafood from the risk of contamination by *Vibrio parahaemolyticus*, a foodborne pathogen (Yano et al., 2006). Antioxidant food database (Carlsen et al., 2010) developed from the analysis of 3,100 foods, beverages, spices, and herbs, shows that the spices and herbs are the most antioxidant-rich products in the human diet, some of them exceptionally high. Various fruits, herbs, and spices have demonstrated the anti-inflammatory activity (Mueller et al., 2010). As grape is one of the major fruits in the world conferring health benefits due to their antioxidant activity (Kedage et al., 2007), phytosterols (Ruggieroa et al., 2013), also contains a characteristic color value for seasonings, the present study was aimed to evaluate the physicochemical changes in the natural seasonings by addition of various concentrations of grape extracts.

MATERIALS AND METHODS

Grape (*Vitis vinifera* L.) berries, *Saccharina japonica* powder, anchovy powder, shiitake (*Lentinus edodes*) powder, tomato (*Lycopersicum esculentum* Mill) puree, parched soybean (*Glycine max* L.) powder, hot pepper (*Capsicum annuum* L.) seed, Chinese radish (*Raphanus sativus* L.), root of Chinese bellflower (*Platycodon granidiflorum* A, DC.), lotus root (*Nelumbinis rhzoma*), sesame powder, roasted salt, grain syrup, black sugar, soy sauce and water were purchased from local markets in Korea.

Chemicals and reagents

Falin-Ciocalteu reagent, gallic acid, 1,1-Diphenyl-2-pricrylhydrazyl (DPPH), pyrogallol, 2-deoxyribose, thiobarbituric acid (TBA), trichloroacetic acid (TCA), ethylenediaminetetraacetic acid (EDTA) and phosphate buffer were purchased from Sigma Chemical co. (St. Louis, Mo, USA). Iron (II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) was purchased from Acro Orgamics (NJ, USA). Anhydrous sodium carbonate was purchased from J. T. Baker (NJ, USA). Ethanol and hydrogen peroxide were purchased from Merk (Darmstadt, Germany). All reagents used in the study were of analytical grade.

Preparation of grape natural seasoning samples

The ingredients of natural seasoning prepared for the experiment are given in Table 1. Grape berries cv. Muscat Bailey A, grown at Yeongcheon, Gyeongsangbuk-do, Korea, was harvested at the commercial maturity stage and transported to the laboratory. The fruits were washed several times with tap water. Fruit pulps and skins were separated from seed and heated in a pan over hot plate (Prestige Euro ER-822W, Sunny Tech Ltd, Korea) for 30 min and concentrated under vacuum to a final weight of 85%. The grape concentrates and the other ingredients mentioned in Table 1 were mixed thoroughly and simultaneously heated in a pan over hot plate (Prestige Euro ER-822W, Sunny Tech Ltd, Korea) for 45 min. The control seasoning sample was prepared by heating the ingredients (mentioned in Table 1 without grape extract) in a pan over hot plate (Prestige Euro ER-822W, Sunny Tech Ltd, Korea) for 45 min. The mixtures heated with different concentrations of ingredients were cooled to room temperature and freeze-dried (Virtis, 10-146·MRBA model). The freeze-dried mixture was milled with Speed Rotor Mill (Model: KT-02A) into powder and passed through a 100-mesh sieve. The strained samples were packed into airtight sample bottles and stored in refrigerator until analysis.

Extraction

Dried natural seasonings (1 g) were extracted in water (50 ml) for 4 h by using a Soxhlet extractor (Soxtec System HT6, Tecator,

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Table 1. Recipe of natural seasoning prepared by adding different grape concentrated extracts (%).

Ingredient	Sample[1)]				
	NSG-0	NSG-5	NSG-10	NSG-15	NSG-20
Grape concentrated juice	-	5	10	15	20
Saccharina japonica	3.1	3.0	2.9	2.7	2.6
Anchovy powder	2.3	2.3	2.2	2.0	1.9
Shiitake (*Lentinus edodes*) powder	3.2	3.0	2.8	2.7	2.5
Tomato puree	1.5	1.5	1.4	1.3	1.3
Parched soybean powder	1.5	1.5	1.4	1.3	1.3
Sesame powder	1.3	1.0	1.0	0.9	0.9
Hot pepper seed	0.8	0.8	0.7	0.7	0.6
Chinese radish	1.5	1.5	1.4	1.3	1.3
Doraji (root of Chinese bellflower)	0.2	0.1	0.1	0.1	0.1
Roasted salt	3.1	3.0	2.8	2.7	2.5
Grain syrup	7.7	7.3	6.9	6.5	6.2
Black sugar	1.5	1.5	1.4	1.3	1.3
Lotus root	0.2	0.2	0.2	0.1	0.1
Soy sauce	46.2	43.9	41.6	39.3	36.9
Water	25.7	24.4	23.1	21.8	20.5

[1)]NSG-0, natural seasoning prepared by adding no grape concentrated extracts; NSG-5, natural seasoning prepared by adding 5% grape concentrated extract; NSG-10, natural seasoning prepared by adding 10% grape concentrated extract; NSG-15, natural seasoning prepared by adding 15% grape concentrated extract; NSG-20, natural seasoning prepared by adding 20% grape concentrated extract.

Hoganas, Sweden) under reflux conditions. The residues were then extracted in boiling water (50 ml). The extracts were filtered through Whatman No. 4 filter paper and then concentrated using a rotary evaporator (Büchi Rotavapor R-144, Switzerland) to get the extracts. The water extracts yielded 15.3% (w/w) were analyzed for their antioxidant activities.

Color measurement

L^* (lightness), a^* (redness, + or greenness, -), and b^* (yellowness, + or blueness, -) values of grape natural seasonings were measured using a Chroma Meter (CR-300, Minolta Corp., Japan). A Minolta calibration plate (Y_{CIE} = 94.5, X_{CIE} = 0.3160, Y_{CIE} = 0.330) and a Hunter laboratory standard plate (L^* = 82.13, a^* = - 5.24, b^* = - 0.55) were used to standardize the instrument with D65 illuminant. Color was measured directly on three zones of grape natural seasonings and the average was calculated (Son et al., 2013).

Determination of mineral content

Five hundred (500) mg of sample was taken into a cup and 15 ml of nitric acid (HNO_3) was added. The solution was diluted with distilled water. Mineral concentrations were determined by using Inductively Coupled Plasma Atomic Emission Spectrometer (ICP AES: Varian Vista) (Skujins, 1998). The instrument was calibrated using known standards for each mineral. Average values of two replicate samples were reported (Son et al., 2013).

Radical scavenging activity using DPPH

The scavenging activity of the extract from natural seasonings was measured with DPPH radicals according to the method of Blois (1958) with some modifications. DPPH solution was prepared at the concentration of 4 x 10^{-4}M in ethanol. A 0.1 ml aliquot of extract was mixed with 2.9 ml of DPPH solution and the mixture was incubated in the room temperature for 30 min. After standing for 30 min, absorbance was recorded at 516 nm by UV-VIS spectrophotometer (Opron 3000 Hanson Tech. Co. Ltd., Seoul, Korea). The inhibitory percentage of the DPPH radical by the samples was calculated according to Shyu and Hwang (2002) as:

$$\text{Scavenging effect (\%)} = [(A_0 - (A - A_b))/A_0] \times 100$$

Where, A_0 is the absorbance of DPPH without sample (control), A is the absorbance of sample and DPPH, and A_b is the absorbance of sample without DPPH (blank).

Superoxide anion scavenging activity

Superoxide anion (O_2^-) scavenging activity was determined by measuring the inhibition of the auto-oxidation of pyrogallol following the method of Marklund and Marklund (1974) with a slight modification. A 0.3 g aliquot of sample and 2.61 ml of 50 mM phosphate buffer (pH 8.24) were added into freshly prepared 90 µL of 3 mM pyrogallol (dissolved in 10 mM HCl). The absorbance value was measured at 325 nm to determine the inhibition rate of pyrogallol auto-oxidation. The superoxide anion scavenging activity was calculated with inhibition rate of pyrogallol auto-oxidation, which was determined by the difference (the absorbance at 10 min - the absorbance at the starting time) in absorbance (325 nm) of each extract recorded at every 1 min interval for 10 min.

Hydroxyl radical scavenging activity

Hydroxyl radical (HO^-) scavenging activity was determined according to the 2-deoxyribose oxidation method (Chung et al., 1997) with

Table 2. Hunters color values of natural seasoning prepared by adding different grape concentrated extracts.

Color value[2)]	Sample[1)]				
	NSG-0	NSG-5	NSG-10	NSG-15	NSG-20
L (Lightness)	34.75 ± 1.01[a3)]	33.00 ± 1.11[ab]	31.36 ± 0.80[bc]	30.82 ± 1.01[c]	28.30 ± 1.32[d]
a (Redness)	+0.98 ± 0.12[b]	+0.79 ± 0.11[bc]	+0.68 ± 0.11[c]	+1.03 ± 0.21[b]	+1.50 ± 0.11[a]
b (Yellowness)	+15.33 ± 1.33[a]	+14.27 ± 1.00[a]	+13.81 ± 1.32[ab]	+12.05 ± 0.98[b]	+10.64 ± 0.63[b]

[1)]Abbreviations are defined in Table 1. [2)]L: lightness (100, white ; 0, black), a: redness (-, green; +, red), b: yellowness (-, blue; +, yellow). [3)]Values are mean±standard deviation of triplicate experiments. [a-d)]The values followed by the different superscripts in the same row are significantly different, according to Tukey test ($p<0.05$).

some modifications. Fenton reaction in the presence of $FeSO_4.7H_2O$ was used to generate hydroxyl radical. A reaction mixture containing each 0.2 ml of 10 mM $FeSO_4.7H_2O$, 10 mM EDTA and 10 mM 2-deoxyribose was mixed with 0.2 ml of the extract solution and 0.1 M phosphate buffer (pH 7.4) was added into the reaction mixture to make the final volume of 1.8 ml. Then 0.2 ml of 10 mM H_2O_2 was added to the reaction mixture and incubated at 37°C for 4 h.

Then after that each 1 ml of 2.8% TCA and 1.0% TBA were added to the incubated mixture. Finally, the mixture was placed into a boiling water bath for 10 min and absorbance reading was taken at 532 nm.

Determination of total phenolic content

The amount of total phenolics (TPH) was determined using the Folin-Ciocalteu method (Zheng and Wang, 2001). A calibration curve of gallic acid was prepared, and the results were expressed as mg GAE (gallic acid equivalents)/g sample. Briefly, 5 ml of distilled water was put into a 10 ml volumetric flask. A suitable volume of the natural seasoning extract was transferred into the volumetric flask.

A 0.2 ml aliquot of Folin-Ciocalteu reagent was added into the flask and carefully mixed. After 3 min, 0.4 ml of saturated Na_2CO_3 solution was added, carefully mixed and made up to volume with distilled water. After 1 h of reaction in the dark, the absorbance was measured at 725 nm using a spectrophotometer (Hewlett-Packard 8452A diode-array).

Determination of amino acid content

Amino acid contents were analyzed following the procedure of Je et al. (2005) with some modification. Briefly, 1 g of freeze-dried sample powder was hydrolyzed with 6 N HCl (10 ml) in a sealed-vacuum ampoule at 110°C for 24 h. The HCl was removed from the hydrolyzed sample on a rotary evaporator, brought to a known volume (5 ml) with 0.2 M sodium citrate buffer (pH 2.2).

The sample was passed through a C-18 Sep Pak (Waters Co. Milford, USA) cartridge and filtered through a 0.22 µM membrane filter (Millipore, USA). Amino acids were determined on an auto-matic amino acid analyzer (Biochrom-20, Pharacia, Biotech Co., Sweden).

Statistical analysis

Data were subjected to one-way or two-way analysis of variance (ANOVA) when required using Statistix version 4.0 package (Analytical Software, AZ, USA). Differences between means at $p<0.05$ were analyzed using the Tukey test.

RESULTS AND DISCUSSION

Color of natural seasoning

Lightness (L), redness (a), and yellowness (b) color expressions of different natural seasonings prepared by adding concentrated grape extracts varied significantly depending on the concentration of the grape extract (Table 2). The seasonings showed a significant reduction in lightness and yellowness, whereas redness value was increased significantly with the increased amount of grape concentrated extract. Increase in concentration of the grape extract from 0 to 20% substantially reduced lightness (from 34.75 to 28.30) and yellowness (from 15.33 to 10.64). However, redness value was increased from 0.98 to 1.50 with the addition of 0 and 20% grape extract, respectively. This trend of color expression demonstrated that addition of higher concentration of grape extract would promote development of darker color of the natural seasonings. The darker color of seasonings at higher concentration of grape concentrated extract might be because of color of grapes itself and chemical reactions with other ingredients. Natural colorant areas like anthocyanins, betalains, chlorophylls, carotenoids, flavonoids, monascus, hemes, quinones, biliproteins, safflower, turmeric may be found as such and a variety of hues can be obtained ranging from green through yellow, orange, red, blue, and violet, depending on the source of colorant (Francis and Markakis, 1989). Color is a key factor in natural seasonings as different consumers prefer different hues of colors. Addition of grape extract improved the darkness of natural seasonings.

Mineral content of grape natural seasoning

The mineral contents of the natural seasonings were significantly varied with the concentration of the grape extract added in them (Table 3). Contents of minerals like K, Mg, Ca, and Na were significantly decreased while those of Fe, and Zn were increased after addition of grape extract to the natural seasonings. The seasonings were rich in K, the most abundant mineral element (ranging 26042.2 mg/100 g at 0% grape extract to 13759.6 mg/100 g at 20% grape extract) followed by Na (19454.4

Table 3. Mineral content of natural seasoning prepared by adding different grape concentrated extracts (mg/100 g sample).

Element	Sample[1)]				
	NSG-0	NSG-5	NSG-10	NSG-15	NSG-20
K	26042.2 ± 8.9[a2)]	25730.3 ± 10.1[b]	23688.3 ± 9.2[c]	18951.5 ± 6.7[d]	13759.6 ± 9.3[e]
Mg	3192.2 ± 3.7[a]	2882.3 ± 5.9[b]	2859.2 ± 9.1[c]	2800.3 ± 8.2[e]	2821.8 ± 7.1[d]
Ca	4295.5 ± 5.1[a]	3737.3 ± 3.1[e]	3756.6 ± 2.1[d]	3866.6 ± 6.2[c]	4147,7 ± 3.0[b]
Na	19454.4 ± 10.2[a]	17246.9 ± 9.9[b]	16304.5 ± 9.1[c]	12882.5 ± 13.1[e]	12988.3 ± 8.1[d]
Fe	43.3 ± 1.2[c]	58.1 ± 0.9[b]	68.5 ± 1.7[a]	69.7 ± 0.8[a]	69.8 ± 1.2[a]
Zn	25.2 ± 0.5[c]	24.2 ± 0.7[c]	28.6 ± 0.6[ab]	29.3 ± 0.9[a]	27.6 ± 0.4[b]
Mn	ND[3)]	ND	ND	ND	ND

[1)]Abbreviations are defined in Table 1. [2)]Quoted values are mean±standard deviation of duplicate experiments. [3)]ND: Not detectable. [a-e)]The values followed by the different superscripts in the same row are significantly different, according to Tukey test ($p<0.05$).

Table 4. Scavenging activity of reactive oxygen species and total phenolic contents of natural seasoning prepared by adding different grape concentrated extracts.

Sample[1)]	% Inhibition[2)]			Total phenolics (mg GAE[3)]/g sample)
	DPPH	O_2^-	HO^-	
NSG-0	67.56 ± 0.31[c4)]	19.17 ± 0.34[a]	50.56 ± 1.83[a]	153.3 ± 2.2[d]
NSG-5	67.87 ± 0.39[c]	17.21 ± 0.46[b]	50.41 ± 2.21[a]	148.8 ± 2.8[d]
NSG-10	69.63 ± 0.23[b]	8.82 ± 1.02[c]	38.25 ± 1.82[b]	164.0 ± 2.3[c]
NSG-15	69.91 ± 0.16[b]	5.91 ± 0.12[d]	39.05 ± 1.66[b]	201.2 ± 1.1[b]
NSG-20	73.77 ± 1.01[a]	5.41 ± 0.22[e]	37.51 ± 0.61[b]	229.2 ± 1.5[a]

[1)]Abbreviations are defined in Table 1. [2)]DPPH: DPPH free radical scavenging activity; O_2^-: superoxide anion scavenging activity; HO^-: hydroxyl radical scavenging activity. [3)]GAE: gallic acid equivalent. [4)]Quoted values are mean±standard deviation of duplicate experiments. [-e)]The values followed by the different superscripts in the same column are significantly different, according to Tukey test ($p<0.05$).

mg/100 g at 0% grape extract and 12988.3 mg/100 g at 20% grape extract). Zn and Fe were the least detected mineral elements in the seasonings; increased a little with the addition of grape extract from 0 to 20%. The increase in Zn and Fe content in grape extract added seasonings might be due to relatively higher content of these minerals in grape extract. It has been indicated that food seasonings contain plant sterols and/or stanols or their derivatives together with minerals, such as magnesium, calcium and potassium. Ingestion of food supplied with phytosterol leads to a significant decrease in both cholesterol level (Weingartner et al., 2008). The decrease is synergic, that is, larger than that expected from the sum of the effects of plant sterols and minerals (Karppanen et al., 2000) since grape berries contain phytosterols (Ruggieroa et al., 2013). Thus addition of grape extract to the natural seasonings may enhance its nutritional and medicinal values even some of the minerals were significantly decreased.

Scavenging activities and total phenolic contents of natural seasoning

Scavenging activities of reactive oxygen species in the natural seasonings were determined by analyzing DPPH, O_2^-, HO^- and total phenolic content (Table 4). DPPH radical scavenging activities (73.77%) and total phenolic content (229.2 mg/g sample) were recorded the highest in the seasonings containing 20% grape extract whereas value of O_2^-, and HO^- decreased with the increased concentration of grape extract. Addition of 5% concentrated grape extract did not show significant differences from the samples with no grape extracts in terms of DPPH, HO^-, and total phenolic content. The increase in DPPH radical scavenging activity of the natural seasonings was accompanied by a significant reduction in O_2^- and OH^- activities in all the samples. The O_2^- content was the highest (19.17%) in the seasonings with no concentrated grape extract added and the least (5.41%) when the natural seasonings were prepared by addition of 20% concentrated grape extract. The HO^- levels were also reduced when the amount of concentrated grape extract added in preparation of the natural seasoning was increased from 5 to 20%. In effect, significant scavenging of reactive oxygen species (O_2^- and HO^-) could be obtained in natural seasonings when 5 to 20% concentration of grape extract is added during preparation of the seasonings.

Table 5. Amino acids content (mg of amino acid per 100 g of sample) in natural seasonings prepared by adding different grape concentrated extracts.

Amino acid	Sample[1)]				
	NSG-0	NSG-5	NSG-10	NSG-15	NSG-20
Aspartic acid	4762.65[2)]	3138.49	4237.23	4241.89	4890.42
Threonine	1959.49	1149.31	1825.50	1584.32	1910.36
Serine	1953.80	1165.03	1801.41	1606.97	1909.92
Glutamic acid	6532.34	4675.94	6294.40	5587.96	6333.75
Proline	3185.39	451.76	3276.03	1855.69	3347.68
Glycine	2098.01	1319.91	1975.84	1787.60	2096.51
Alanine	2329.25	1495.15	2500.53	2221.01	2701.87
Cysteine	391.68	203.05	367.05	612.76	538.27
Valine	2260.78	1385.30	2382.38	2009.98	2316.45
Methionine	828.80	444.24	863.49	1017.91	1053.35
Isoleucine	2278.61	1150.29	2094.28	1954.22	2304.52
Leucine	3041.21	1851.56	3047.12	2757.16	3080.90
Tyrosine	1228.53	718.45	1113.92	1006.11	1166.47
Phenylalanine	2278.93	1396.35	2087.71	1823.80	2196.33
Histidine	1387.87	815.18	1237.72	1036.73	1233.45
Lysine	2535.10	1573.84	2350.16	2261.34	2619.90
Arginine	2442.28	1505.52	2255.45	2031.60	2347.34

[1)]Abbreviations are defined in Table 1. [2)]Values are the means of duplicate experiments.

Hydroxyl radical, superoxide anion radical, hydrogen peroxide, oxygen singlet, hypochlorite, nitric oxide radical, and peroxynitrite radical are highly reactive species, and capable of damaging biologically relevant molecules such as DNA, proteins, carbohydrates, and lipids (Young and Woodside, 2001). Alteration of lipids, proteins, and DNA by free radicals triggers a number of human diseases. Negative effects of free radicals can be corrected by the application of external source of antioxidants. Synthetic antioxidants like butylated hydroxytoluene and butylated hydroxyanisole have recently been reported to be dangerous for human health (Lobo et al., 2010). Grape berries are rich in antioxidants (Kedage et al., 2007) including melatonin (Vitalini et al., 2013), novel anti-oxidant. Thus addition of grape concentrated extract could increase the antioxidant potential of natural seasonings.

Total amino acid content of natural seasoning

Among the amino acids analyzed, glutamic acid (range 4675.94 - 6532.34 mg/100 g) was the most abundant followed by aspartic acid (range 3138.9 - 4890.42 mg/100 g) in the natural seasonings depending on the amount of concentrated grape extract added during preparation of the seasoning. Cysteine was the lowest constituent amino acid (range 203.05 - 612.76 mg/100 g) in the seasonings. Some of the amino acids contents were increased while others decreased with the addition of different concentrations of grape extract in the natural seasoning (Table 5). Amino acids are the building blocks of protein (Ekeanyanwu, 2013) that plays an important role in biochemical, biophysical and physiological functions. The deficiency of proteins leads to weakness, anaemia, protein energy malnutrition (kwashiorkor and marasmus), delayed wound and fracture healing and also decreased resistance to infections.

Glutamic acid, glycine, alanine, proline and aspartic acid are recognized as being important in the taste and their presence in the natural seasoning with grape extract will enhance the properties of the seasoning (Choi et al., 1996). The results of this study showed that contents of amino acids aspartic acid, proline, alanine, cysteine, valine, methionine, isoleucine, leucine, and lysine could be increased in the natural seasonings with the addition of grape concentration extract.

Conclusion

Natural seasonings prepared by adding different concentrations of grape extracts could be used as good sources of antioxidants, proteins and minerals supplement in the human diet. This study established the physicochemical properties of natural seasonings when different concentra-

tions of grape extracts were added during preparation. Although some of the desirable physicochemical parameters were continuously improved with the gradual increase in the amount of grape extract, concentration beyond 20% resulted in poor quality seasonings by reducing their powdery structure making them difficult to use properly. Results of this study suggest that concentration of grape extract up to 20% could be added in natural seasonings to impart better physicochemical properties as well as to enhance their nutritional and medicinal value.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Effect of various growth regulators on growth of yam (*Dioscorea trifida* L.) *in vitro* shoot tips

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In this work, we observed the effect of hormonal content of four culture media on the growth of *Dioscorea trifida* shoot tips. Medium S included 0.6 µM 6-benzylaminopurine, 1.07 µM naphthalene acetic acid, and 0.23 µM gibberellic acid (GA_3), medium EBR 0.1 µM 24-Epibrassinolide and 0.23 µM GA_3, medium T 25 µM meta-topolin and 0.23 µM GA_3 and medium ZR 25 µM zeatin riboside and 0.23 µM GA_3. After 2 months in culture, shoot length was highest on medium EBR and ZR, with values of 11.63 and 11.30 mm, respectively, intermediate on medium S (9.70 mm) and lowest on medium T (3.07 mm). Oxidation reached a similar level on medium S, T and ZR (2.17 - 2.40) while it was only 1.63 on medium EBR. Well-developed shoots were obtained on medium EBR with an average of 1.93 leaves and 0.70 roots per shoot. Shoots were less developed on medium S and ZR, with an average of 1.30 leaves and 0.20 roots per shoot on medium S and 1.60 leaves and 0.00 roots per shoot on medium ZR. On medium T, buds showed a tuberized aspect and no leaves or roots were produced. In conclusion, medium EBR proved superior to the three other media tested based on the parameters tested.

Key words: Yam, *Dioscorea trifida*, shoot tip growth, 6-benzylaminopurine, 24-Epibrassinolide, meta-topolin, zeatin riboside.

INTRODUCTION

Yam, a multi-species, polyploid and vegetatively propagated crop, is an economically important staple food for more than 300 million people in low income, food-deficit countries of the tropics (Gedil and Sartie, 2010). Out of the more than 600 yam species identified, 10 are generally cultivated as food crop including *Dioscorea alata, D. rotundata, D. cayenensis, D. bulbifera, D. esculenta, D. opposita-japonica, D. nummularia, D. pentaphylla, D. transversa* and *D. trifida*. *D. trifida* L. (cush-cush yam) is believed to originate from the Guyana region of South America; it is by far the most important of the indigenous American yams.

Because of its vegetative mode of propagation, the field gene bank is the traditional *ex situ* method for conserving yam germplasm (Ng and Ng, 1999). However, such collections remain exposed to attacks by pests and pathogens, both in the field and during tuber storage, and viruses can accumulate progressively in plants, leading to significant yield decreases and ultimately to loss of accessions. Moreover, field gene banks are very costly to maintain properly, requiring large land areas, high input and labor resources (Ng and Ng, 1999).

In the case of yam, *in vitro* culture techniques have been developed for virus eradication by means of

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meristem culture (Mantell et al., 1980), multiplication (Mantell et al., 1978), distribution, and conservation of germplasm using *in vitro* plantlets and microtubers (Ng and Ng, 1999). For yam medium-term storage, *in vitro* slow growth protocols have been developed, which have been applied to several thousand accessions (Dumet et al., 2013). For long-term conservation of vegetatively propagated plants such as yam, cryopreservation (liquid nitrogen, -196°C) is currently is the only safe and cost-effective option (Engelmann, 2004). Indeed, at this temperature, all cell divisions stop and metabolism comes to a halt. Explants can thus be conserved for extended periods (several thousand years) without modification or alteration, sheltered from contamination, in a limited volume and with reduced maintenance.

Cryopreservation protocols have been developed for *D. alata*, *D. cayenensis* and *D. rotundata* shoot tips using different techniques including vitrification (Mandal et al., 2008; Mukherjee et al., 2009; Leunufna and Keller, 2005), encapsulation-dehydration (Mandal et al., 1996; Malaurie et al., 1998; Mandal et al., 2008) and droplet-vitrification (Leunufna and Keller, 2005). A droplet-vitrification protocol jointly established by the International Institute of Tropical Agriculture (IITA, Ibadan, Nigeria) and our laboratory has been applied to a total of 42 *D. cayenensis*, *D. rotundata*, *D. alata*, *D. bulbifera* and *D. mangotiana* accessions, with an average recovery of 29% (Gueye et al., unpublished results). Cryopreservation experiments performed in IRD Montpellier with *D. trifida* shoot tips showed that, even though positive results were obtained with droplet-vitrification, no reproducible protocol was yet available for this species (Engelmann-Sylvestre et al. unpublished results). In all these reports, the authors mentioned that if high survival could be consistently achieved, regeneration of whole plantlets from cryopreserved shoot tips was highly variable, depending on the species and the technique used, and was generally much lower than survival. An additional problem identified during regeneration of cryopreserved yam shoot tips was the occurrence of severe oxidation phenomena, which had a strong negative impact on re-growth.

Modifying the hormonal content of culture media can have a dramatic impact on re-growth of plant material (George, 1993). Among the numerous plant growth regulators (PGRs) identified, cytokinins, including natural ones such as zeatin (Z) or zeatin riboside (ZR), and synthetic cytokinin analogues such as 6-benzylaminopurine (BAP) have been broadly used for decades for their stimulatory effect on cell division, adventitious shoot formation and axillary shoot proli-feration (George, 1993). Topolins, another category of naturally occurring aromatic cytokinins, and especially the meta-topolin and its derivatives, have been employed for culture initiation, protocol optimization and for counteracting various *in vitro* induced physiological disorders in many species (Amoo et al., 2011; Aremu et al., 2012). Topolins have been reported to increase shoot multiplication, maintain histogenic stability, improve rooting efficiency and subsequently reduce production costs (Bogaert et al., 2006). Of more recent use in *in vitro* culture are brassinosteroids (BRs), a class of plant steroid hormones, which possess significant growth-controlling activity, and are involved in the promotion of cell elongation, cell division, differentiation, disease resistance, stress tolerance, and senescence throughout the plant life cycle (Bajguz and Hayat, 2009). BRs have been reported to help modulating the plant antioxidant defense system and thus scavenging the free radicals and help the plant protecting itself from oxidative stress (Verma et al., 2012). BRs have also been found to have an activity *in vitro.* They were reported to increase the rate of cell division and colony formation of Chinese cabbage mesophyll protoplasts (Nakajima et al., 1996) and *Petunia hybrida* protoplasts (Oh and Clouse, 1998). BRs proved also to be essential for the differentiation of isolated *Zinnia* mesophyll cells into tracheary elements (Iwasaki and Shibaoka, 1991) and in the morphogenesis of *Arabidopsis* (Li et al., 1996).

In this study, we compared the effect of four culture media on re-growth of *D. trifida* shoot tips: 1) the recovery medium used in the droplet-vitrification protocol developed in IRD, which contained BAP, naphtalene acetic acid (NAA) and gibberellic acid (GA_3); 2) a medium containing topolin and GA_3; 3) a medium containing Z and GA_3; and 4) a medium containing 24-epibrassinolide and GA_3. Our objective was to study if these changes in PGR content in the recovery medium were effective in stimulating shoot tip re-growth and in reducing oxidation.

MATERIALS AND METHODS

This study was performed using *in vitro* shoot cultures of *D. trifida* accession N° 278, provided by the Institut National de la Recherche Agronomique (INRA) Guadeloupe, French West Indies. Mother-plants were sub-cultured every 3 to 5 months on medium containing Murashige and Skoog (MS, 1962) basal salts and vitamins, 3% sucrose, 0.2% activated charcoal and 0.7% agar. The pH was adjusted to 5.8 ± 0.1 and the medium was autoclaved for 20 min at 120°C. Cultures were maintained at 27 ± 1°C under a 12 h light/12 h dark photoperiod and a light intensity of 50 µmol m^{-2} s^{-1}.

Single node cultures were transferred to yam multiplication medium (YMM) consisting of MS basal salts and vitamins (Murashige and Skoog, 1962), 0.476 µM KIN, 0.164 µM L-cysteine, 3% sucrose and 0.7% agar. After 3 weeks, shoot tips (1-2 mm in length) were excised under the binocular microscope and transferred on four different culture media for re-growth.

The first medium was the standard yam meristem medium (medium S) used for shoot tip regeneration after cryopreservation, which consisted of MS mineral salts and vitamins (Murashige and Skoog, 1962), 0.164 µM L-cysteine, 0.22 mM adenine hemisulfate, 0.6 µM BAP, 1.07 µM NAA, 0.23 µM GA_3 (filter-sterilized), 3% sucrose and 0.7% agar. In the other three media, BAP and NAA were replaced by 0.1 µM 24-Epibrassinolide (Sigma-Aldrich ref. E1641, filter-sterilized, medium EBR); 25 µM meta-topolin (Duchefa Biochemie ref. T0941, filter-sterilized, medium T); or 25 µM zeatin riboside (trans-isomer, Duchefa Biochemie ref. Z0937, filter-sterilized, medium ZR).

Table 1. Effect of culture medium on shoot length and oxidation level after 1 month and on shoot length, oxidation level, number of leaves and roots produced per shoot at 2 months.

Medium	1 month		2 months			
	Shoot (mm)	Oxidation	Shoot (mm)	Oxidation	Leaves/ shoot	Roots/ shoot
S	2.93^{b}	1.00^{a}	9.70^{b}	2.40^{a}	1.30^{c}	0.20^{b}
EBR	5.50^{a}	0.17^{c}	11.63^{a}	1.63^{b}	1.93^{a}	0.70^{a}
T	1.30^{c}	0.90^{ab}	3.07^{c}	2.17^{a}	0.00^{d}	0.00^{b}
ZR	4.77^{a}	0.67^{b}	11.30^{ab}	2.30^{a}	1.60^{b}	0.00^{b}

S: 0.6 µM BAP + 1.07 µM NAA + 0.23 µM GA_3; EBR: 0.1 µM24-epibrassinolide + 0.23 µM GA_3; T: 25 µM meta-topolin + 0.23 µM GA_3; ZR 25 µM zeatin riboside + 0.23 µM GA_3. In columns, figures followed by different letters are significantly different ($p < 0.05$).

Shoot tips were kept in the dark for 1 week, and then transferred to the culture conditions employed for mother-plants.

Observations performed and statistical analysis of results

The experiment was performed once, with three replicates of 10 shoot tips per experimental condition. After 1 and 2 months of culture on medium S, EBR, T or ZR, the size of shoot tips (mm) was measured and the oxidation level was evaluated using a scale from 0 (no oxidation) to 3 (very high oxidation). One-way ANOVA was performed to compare the growth of shoot tips and oxidation levels on the four media tested. Means were statistically differentiated using Duncan test at a significance level of $p \leq 0.05$.

RESULTS

After one month in culture, growth of shoot tips was highest on medium EBR and ZR, with shoot tips lengths of 5.50 and 4.77 mm, respectively, intermediate on medium S (2.93 mm) and lowest on medium T (1.30 mm) (Table 1). Oxidation was highest on medium S and T, with values of 1.00 and 0.90, respectively, intermediate on medium ZR (0.67) and lowest on medium EBR (0.17).

After 2 months in culture, shoot length was highest on medium EBR and ZR, with values of 11.63 and 11.30 mm, respectively, intermediate on medium S (9.70 mm) and lowest on medium T (3.07 mm). Oxidation reached a similar level on medium S, T and ZR (2.17 - 2.40) while it was only 1.63 on medium EBR. Well-developed shoots were obtained on medium EBR with an average of 1.93 leaves and 0.70 roots per shoot (Figure 1A, Table 1). Shoots were less developed on medium S and ZR, with an average of 1.30 leaves and 0.20 roots per shoot on medium S (Figure 1B, Table 1) and 1.60 leaves and 0.00 roots per shoot on medium ZR (Figure 1C, Table 1). On medium T, buds showed a tuberized aspect and no leaves or roots were produced (Figure 1D, Table 1).

DISCUSSION

These experiments showed that the hormonal content of the shoot tip culture medium had a strong impact on their growth pattern. Medium EBR, which included 24-epibrassinolide, led to the production of well-developed shoots with leaves and roots and strongly limited oxidation. This result illustrated the role of BRs in stimulating the plant antioxidant system (Verma et al., 2012) and showed for the first time the positive effect of 24-epibrassinolide on shoot tip morphogenesis. Media S and ZR produced slightly shorter shoots with a lower number of leaves per shoot and very few (medium S) or no roots (medium ZR) and a higher oxidation level. The lower results obtained with two media may be due either to the non-optimal concentrations of the PGRs they contained and/or to their lower effect on shoot tip morphogenesis and reduction of oxidation, compared to medium EBR. Finally, medium T had a detrimental effect on the development of shoot tips, as it induced the production of tuberized shoots and led to an oxidation level comparable to that noted with media S and ZR. Despite their positive effect on shoot regeneration which has been observed with many species (Aremu et al., 2012), Bogaert et al. (2006) noted the unfavorable effect of topolin on shoot regeneration and multiplication of *Rosa* hybrid cultures. The induction of tuberization in shoot tips by their exposure to meta-topolin may be of interest for medium-term storage and international exchange of yam germplasm in the form of microtubers.

In conclusion, experiments should now be performed to observe if medium EBR, which provided the best results with control shoot tips, has also a beneficial effect on growth recovery of cryopreserved shoot tips of *D. trifida* accession N° 278. The effect of this medium should also be tested on the regeneration of other *D. trifida* accessions and of other *Dioscorea* species.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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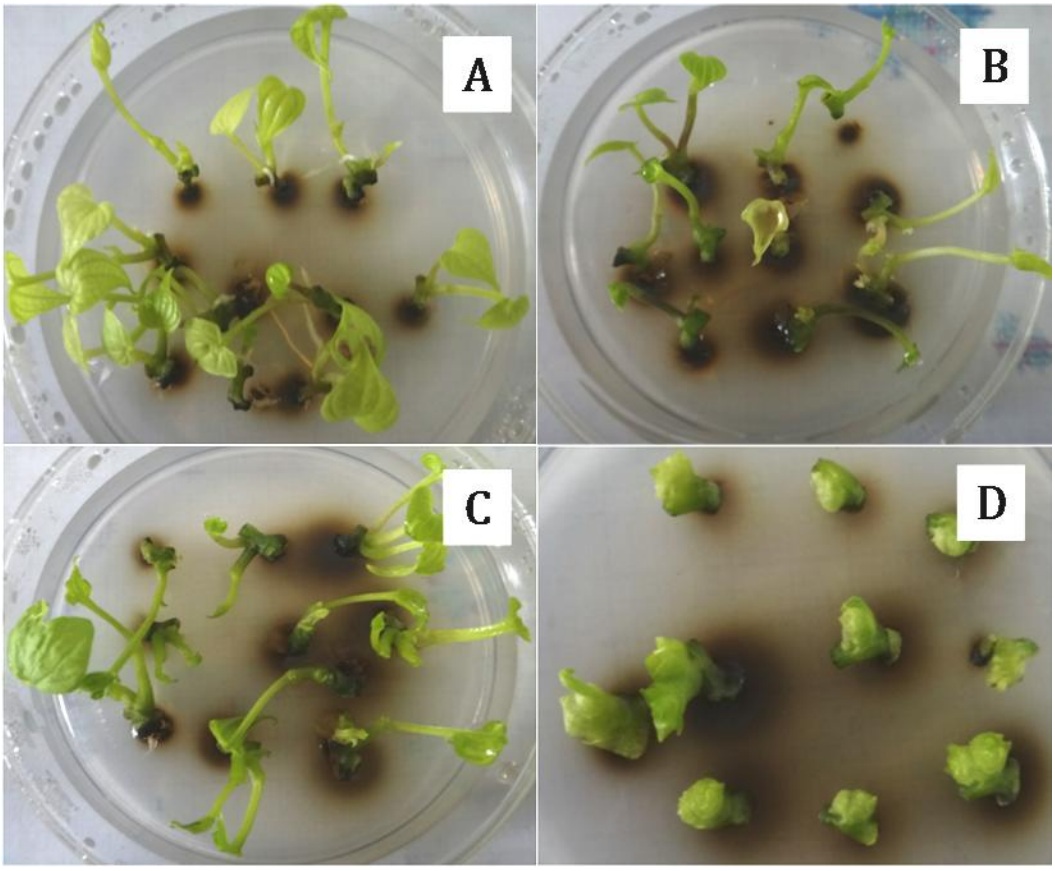

Figure 1. Effect of culture medium on shoot development after 2 months. A: medium EBR (0.1 µM24-epibrassinolide + 0.23 µM GA_3); B: medium S (0.6 µM BAP + 1.07 µM NAA + 0.23 µM GA_3); C: medium ZR (25 µM zeatin riboside + 0.23 µM GA_3); D: medium T (25 µM meta-topolin + 0.23 µM GA_3).

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Full Length Research Paper

Shooting responses of potato (*Solanum tuberosum* L.) varieties in liquid and solid media

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Potato seeds degenerate over time due to systemic diseases (viruses and bacteria wilt), whose detections are mostly evident during later stages of epidemiology. Potato varieties released by Institute of Agricultural Research for Development (IRAD) Bambui recorded a drop in yields of approximately 50% within 20 years indicating that the varieties had started degenerating and thus required cleaning. A study was conducted in 2012 and 2013 at IRAD Bambui (Cameroon) to evaluate the rate of shooting in four improved IRAD potato varieties: Cipira, Tubira, Mafo and Bambui wonder. Liquid and solid media stocks prepared according to the International Potato Center (CIP)'s protocol were used. A randomized complete block design with four replications was used. Number of shot out nodes and average shoot length were collected after 28 days. The average number of shot out nodes in liquid and solid media in all the varieties ranged from 87.5 to 100, with the solid medium yielding better results, 97.5 to 100% in all the potato varieties as compared to liquid medium, 8.75 to 97.5%. The average shoots length in the liquid and solid media ranged from 4.95 to 10.56 cm. The analysis of variance revealed that there were significant differences at p = 0.05 between potato varieties studied. The liquid medium showed better results (8.49 to 10.5 cm) in all the varieties compared to the solid medium (4.59 to 6.08 cm) but the plantlets suffered from vitrification and asphyxiation forming callus. From the results obtained, the following conclusions may be drawn: shoot proliferation of potato tissues are reduced in stationary liquid medium as a result of vitrification and asphyxiation; potato nodal cuttings have faster growth in liquid medium than in solid medium. It is therefore recommended that solid medium should be used over stationary liquid medium in potato micropropagation because the loss of plant tissues in the liquid medium as a result of vitrification and asphyxiation can be very high.

Key words: Potato, media, growth, shoots, Cameroon.

INTRODUCTION

Potato (*Solanum tuberosum* L.) is the fourth most important food crop in the world after wheat, rice and maize. Its production represents almost half of the world's annual output of all roots and tubers with a production figure of

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321.69 million tons (FAOSTAT, 2013). Potato is eaten by 6475 million people worldwide and is part of the diet of half a billion consumers in developing countries (CIP, 1995). In Cameroon, potato is grown in the highland zones (5°26.51 to 6°01.6)N and (10°03.8 to 10°18.5)E between 1000 to 3000 m above sea level in six of the country's 10 regions (Njualem, 2010). The crop occupies the fifth range among the starchy staples and the third among the root and tuber crops. It occupies a relatively small land area (72.103 ha) compared to other staples. The North West and West regions are the highest potato producers in Cameroon accounting for more than 80% of the national production of 435.354 tons. The crop is one of the main sources of revenues for farmers of these regions. Although, the bulk of the harvest is eaten locally or sold in local markets, significant quantities are exported to neighboring countries such as Gabon, Equatorial Guinea, Central African Republic and Chad (Demo et al., 2000). Despite increases of potato production in the tropics, yields are generally low (Burton, 1989). Potato is attacked by 210 different diseases from the field to storage (Beukema et al., 1990). Many potato programs have failed due to degeneration generally caused by systemic diseases (viruses and bacteria wilt), whose detection are mostly evident only during later stage of the disease.

The Institute of Agricultural Research for Development (IRAD) has an ambitious potato seeds program at its Bambui Regional Center in Cameroon, which is a center of excellent within West and Central Africa, owing to its climatic conditions which is suitable for potato seed production. In 1992, the potato program of IRAD Bambui released potato varieties, six of which have been vulgarized (Cipira, Tubira, Mafo, Bambui wonder, Jacob and IRAD). These varieties were selected with major genes as the main sources of resistance to major diseases (Njualem et al., 2001). At the time of release, average yield ranged from 25 to 35 t/ha depending on the variety. In 2001, a yield drop of 15 t/ha was reported in some of these varieties (IRAD, 2006). The varieties had started degenerating and required cleaning.

Many approaches have been developed to clean potato seeds as soon as degeneration is apparent. Among these techniques is the meristem culture. A successful meristem culture usually starts with potato varieties that show promise of shooting (Kuria et al., 2008) in liquid and solid media. An investigation was conducted to ascertain the shooting performance of four IRAD improved potato varieties in liquid and solid media, so that the varieties are included in the IRAD potato seed production program.

MATERIALS AND METHODS

The experiments were conducted at IRAD Bambui plant tissue culture laboratory in 2012 and 2013. IRAD Bambui is one of the five Regional Centers of the Institute for Agricultural Research for Development in Cameroon. Bambui is a village in the Northwest region of Cameroon located at 1600 m above sea level with an average annual rainfall of 1500 to 2000 mm and an average temperature of 21 to 24°C.

Plant material

Four improved Cameroon potato varieties: Cipira and Tubira released in 1992 through a collaborative research project between IRAD Cameroon and the CIP Lima, Peru (Martin et al., 1995); Mafo and Bambui wonder released in 2005 (IRAD, 2006) were used for the study. These varieties are resistant to late blight, tolerant to viruses and bacteria wilt, and adapted and high yielding in the Cameroon highland ecological conditions. The criteria of selection were based on yield, dry matter contents, disease resistance among others. The explants were derived from 32 days old mother plantlets of the four potato varieties selected from the growth chamber. The mother plantlets were selected according to the number of nodes (four or more nodes) and plant vigor (size and health).

Culture media

Two nutrient media were prepared under sterile conditions: 500 ml of liquid medium with no gelling agent (agar) and 500 ml of solid medium with agar. Both media were prepared using stock solutions according to International Potato Centre (CIP)'s laboratory manual (Toledo et al., 1998). Each culture medium was dispersed into 50 ml test tubes (Pyrex co.) (2 $ml.tube^{-1}$) and sealed with aluminum foil and autoclaved at 121°C for 15 min.

Experimental design

A complete randomized block design (CRBD) with four replications was used for the study. Each replication contained 10 culture node segments and forty nodes were cultured for each potato variety per medium type.

Cultural techniques

The tissues culture operations were carried out under aseptic conditions inside a laminar air flow chamber, previously cleaned with 70% alcohol. The mouths of the test tubes containing the mother plantlets were quickly flamed on a spirit lamp and the plantlets carefully removed from the test tubes using a pair of sterilized forceps. The plantlets were then placed on sterilized tissue paper and the stems cut into single node segments. All roots were removed. The average single node cutting was 3 to 5 mm long. Using the tip of a scaple, the isolated nodes were carefully transferred onto the surface of the freshly prepared propagation media. The nodes were planted upright in the solid medium, taking care not to push them below the surface. In the liquid medium, the nodes were allowed to float taking advantage of the surface tension. The test tubes were re-sealed with aluminum foil. All test tubes were labeled according to potato variety, with date of culturing and replication numbers. The cultured nodes were then transferred to the growth chamber, protected from direct sun light and illuminated by fluorescent bulbs. The temperature within the growth chamber was maintained at 27°C with 16 h of light per day (80 $\mu Mol.m^{-2}.s^{-1}$). Humidity of 68% within the chamber was regulated with the aid of an air conditioner. The cultured nodes were allowed to grow for 28 days during which the following variables were collected on weekly basis: number of shot out nodes

Table 1. Average number of shot out of 4 potato varieties cultured in solid medium for 4 weeks.

Variety	Week 1	Week 2	Week 3	Week 4
Cipira	10.00 ± 0.00[a]	10.00 ± 0.00[a]	10.00 ± 0.00[a]	10.00 ± 0.00[a]
Tubira	9.50 ± 0.29[a]	9.75 ± 0.25[a]	9.75 ± 0.25[a]	10.00 ± 0.00[a]
Mafo	10.00 ± 0.00[a]	10.00 ± 0.00[a]	10.00 ± 0.00[a]	10.00 ± 0.00[a]
Bambui wonder	9.50 ± 0.50[a]	9.75 ± 0.25[a]	9.75 ± 0.25[a]	9.75 ± 0.25[a]

Means in a column followed by the same letter are not significantly different at p = 0.05.

Table 2. Average number of shot out of 4 potato varieties cultured in liquid medium for 4 weeks.

Variety	Week 1	Week 2	Week 3	Week 4
Cipira	8.00 ± 0.00[a]	8.00 ± 0.48[a]	8.75 ± 0.48[a]	8.75 ± 0.48[a]
Tubira	9.25 ± 0.25[b]	9.75 ± 0.25[b]	9.75 ± 0.25[a]	9.75 ± 0.25[a]
Mafo	9.75 ± 0.25[b]	9.75 ± 0.25[b]	9.75 ± 0.25[a]	9.75 ± 0.25[a]
Bambui wonder	7.75 ± 0.48[a]	8.75 ± 0.48[a]	8.75 ± 0.48[a]	8.75 ± 0.48[a]

Means in a column followed by the same letter are not significantly different at p = 0.05.

by counts and average shoot length by measuring the length of the longest shoot in centimeter with a meter rule.

Statistical analysis

Data obtained were analyzed using the SPSS-17 Statistical package and mean differences were separated using Duncan alpha multiple test (p = 0.05).

RESULTS

Number of shot out nodes

In solid medium

Table 1 represents the average shot out nodes of four potato varieties cultured in solid medium for four weeks. The average ranged from 9.50 out of 10.00 in Tubira and Bambui wonder within the first week, to 10.00 out of 10.00 in Cipira and Mafo in the fourth week (Table 1). One week after culture all the nodes of Cipira and Mafo had shot out (100%). Tubira and Bambui wonder each had an average number of shot out nodes of 9.50 out of 100.00 (90%). In the fourth week, all the nodes of Tubira had shot out while the average shot out nodes of Bambui wonder remained at 9.75 out of 10.00 from week 2 to 4. However, there were no significant differences at p = 0.05 between varieties (Table 1).

In liquid medium

Table 2 represents the average number of shot out nodes of four potato varieties cultured in liquid medium for four weeks. It ranged from 7.75 out of 10.00 in Bambui wonder, within the first week, to 9.75 out of 10.00 in Tubira and Mafo in the fourth week (Table 2). One week after culturing, the average number of shot out nodes ranged from 7.75 out of 10.00 (77.5%) in Bambui wonder, to 9.75 out of 10.00 (97.5%) in Mafo. The average of Tubira and Mafo were significantly different at p = 0.05 from those of Cipira and Bambui wonder (Table 2). In the second week after culture, all varieties had reached their maximum number of shot out nodes which ranged from 8.57 out of 10.00 (85.7%) in Cipira and Bambui wonder, to 9.75 out of 10.00 (97.5%) in Tubira and Mafo. The averages of Tubira and Mafo were significantly different at p = 0.05 from those of Cipira and Bambui wonder (Table 2).

Shoot length

In solid medium

The average shoot length of nodes of the four potato varieties cultured in solid medium increased continuously from week 1 to 4 (Table 3). It ranged from 0.72 cm in Bambui wonder, in the first week, to 6.08 cm in Mafo in the fourth week. In the first week, the average shoot length varied from 0.72 cm in Bambui wonder to 3.28 cm in Mafo. The average shoot length of Mafo was significantly different at p = 0.05 from the averages of the other three varieties (Table 3). In the fourth week, the average shoot length of the nodes varied from 4.95 cm in Tubira, to 6.08 cm in Mafo, with no significant difference at p = 0.05 between varieties (Table 3, Figure 1A and B).

Table 3. Average shoots length (cm) of 4 potato varieties cultured in solid medium for 4 week.

Variety	Week 1	Week 2	Week 3	Week 4
Cipira	1.72 ± 1.57[a]	3.05 ± 0.25[a]	4.11 ± 0.28[ab]	4.94 ± 0.30[a]
Tubira	1.37 ± 0.09[a]	3.58 ± 0.32[ab]	4.59 ± 0.28[ab]	4.59 ± 0.28[a]
Mafo	3.28 ± 0.91[a]	4.22 ± 0.49[b]	5.37 ± 0.62[b]	6.08 ± 0.72[a]
Bambui wonder	0.72 ± 0.11[a]	2.49 ± 0.36[a]	3.97 ± 0.40[a]	5.11 ± 0.41[a]

Means in a column followed by the same letter are not significantly different at p = 0.05.

Figure 1. Mafo variety, four weeks after culture in solid (A) and liquid (B) media.

Table 4. Average shoots length (cm) of 4 potato varieties cultured in liquid medium for 4 weeks.

Variety	Week 1	Week 2	Week 3	Week 4
Cipira	1.48 ± 0.20[a]	4.69 ± 0.64[a]	7.52 ± 0.77[ab]	9.13 ± 0.72[ab]
Tubira	1.68 ± 0.54[a]	5.53 ± 0.58[ab]	5.88 ± 0.82[a]	8.78 ± 0.73[ab]
Mafo	3.05 ± 0.48[b]	7.00 ± 0.42[b]	9.35 ± 0.45[b]	10.56 ± 0.62[a]
Bambui wonder	0.84 ± 0.28[a]	4.20 ± 0.46[a]	6.72 ± 0.72[a]	8.49 ± 0.38[a]

Means in a column followed by the same letter are not significantly different at p = 0.05.

In liquid medium

Table 4 represents the average shoot length of 4 potato varieties cultured in liquid medium. All the 4 potato varieties showed a continuous increase in the average shoot length from week 1 to 4. It ranged from 0.84 cm in Bambui wonder in the first week, to 10.56 cm in Mafo in the fourth week (Table 4). In the first week, the average shoot length ranged from 0.84 cm in Bambui wonder, to 3.05 cm in Mafo. The average shoot length of Mafo was significantly different at p = 0.05 from the averages of the other three varieties (Table 4). In the fourth week, the average shoot length of the notes ranged from 8.49 cm in Bambui wonder to 10.56 cm in Mafo. Cipira and Tubira were significantly different at p = 0.05 from Mafo and Bambui wonder, both of which were also significantly different from each other (Table 4).

DISCUSSION

A single node of an *in-vitro* plantlet placed in an appropriate medium will induce the development of an axillary bud (shoot) (Espinoza et al., 1992). These shoots could be produced in five to seven days (Kyazev, 1983); this was in agreement with the results obtained in the study after one week culturing where by all nodes (100%) of Cipira and Mafo had shot out while Tubira and Bambui wonder had a 95% average shot out nodes in the solid medium. These results were better than those obtained in the liquid medium in the range of 7.75 to 9.75 shot out nodes out of 10.00. The differences might have been due to varietal genetic make-up in the varieties. In the 4th week after culturing, all the nodes of three (Cipira, Tubira and Mafo) varieties cultured in the solid medium had shot out while all nodes in the liquid medium were in the range of 8.75 to 9.75 out of 10.00. These results differ from those of Sandal et al. (2001) who realized better shooting in many plant species in liquid than in solid media. The lower average number of shot out nodes in liquid medium could have been due to vitrification (or hyperhydricity) and asphyxiation of tissues (Pierik, 1997) which caused some of the cultured nodes to form callus (Figures 2, 3 and 4). Callus formation tends to stop shooting of the nodes in liquid medium.

The results of the study show that the average shoot length of nodes for all potato varieties cultured in liquid medium were better than those in the solid medium. The averages after four weeks of cultured ranged from 8.49 to 10.56 cm in liquid medium and 4.59 to 6.08 cm in solid medium. These results agree with those of Kuria et al. (2008) who reported higher biomass accumulation in liquid media than in solid media. The availability and ease of uptake of water and nutrients as well as the close contact between the explants and the medium could have led to faster growth of plantlet (Mbiyu et al., 2012). However, not all plant species will grow normally on stationary liquid media as soaking or hyperhydricity may result. Generally, Mafo had the longest average shoot lengths from week 1 to 4 in both liquid and solid media while Tubira and bambui wonder had the shortest lengths after four weeks of culture in solid and liquid media, respectively. The significant difference in shoot length between the different potato varieties could be explained by the fact that varieties respond differently to shooting due to genetic variation among them.

Conclusion

Based on the foregoing study, the following conclusions may be drawn:

1. The four Cameroonian improved potato varieties (Cipira, Tubira, Mafo and Bambui wonder) responded positively to *in vitro* culture. Mafo variety had the best performance in culture in both liquid and solid media in terms of number of shot out nodes and shoot lengths.
2. Shoots proliferation of potato tissues is reduced in stationary liquid medium probably as a result of vitrification (hyperhydricity) and asphyxiation.
3. Potato nodal cuttings have faster growths in liquid medium than in solid medium hence subculturing can be done more frequently with liquid media.

Conflict of interests

The author(s) have not declared any conflict of interests.

Recommendations

From the research results, the following are recommended:

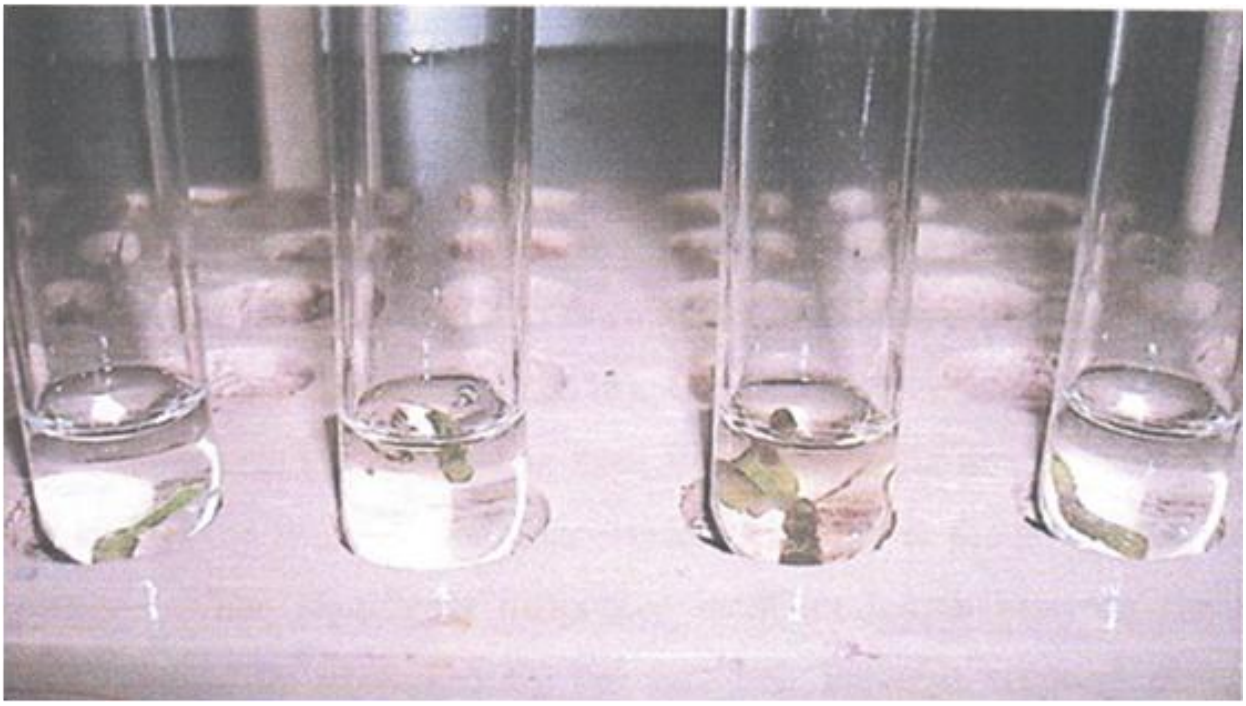

Figure 2. Asphyxiation of culture nodes of Cipira in liquid medium.

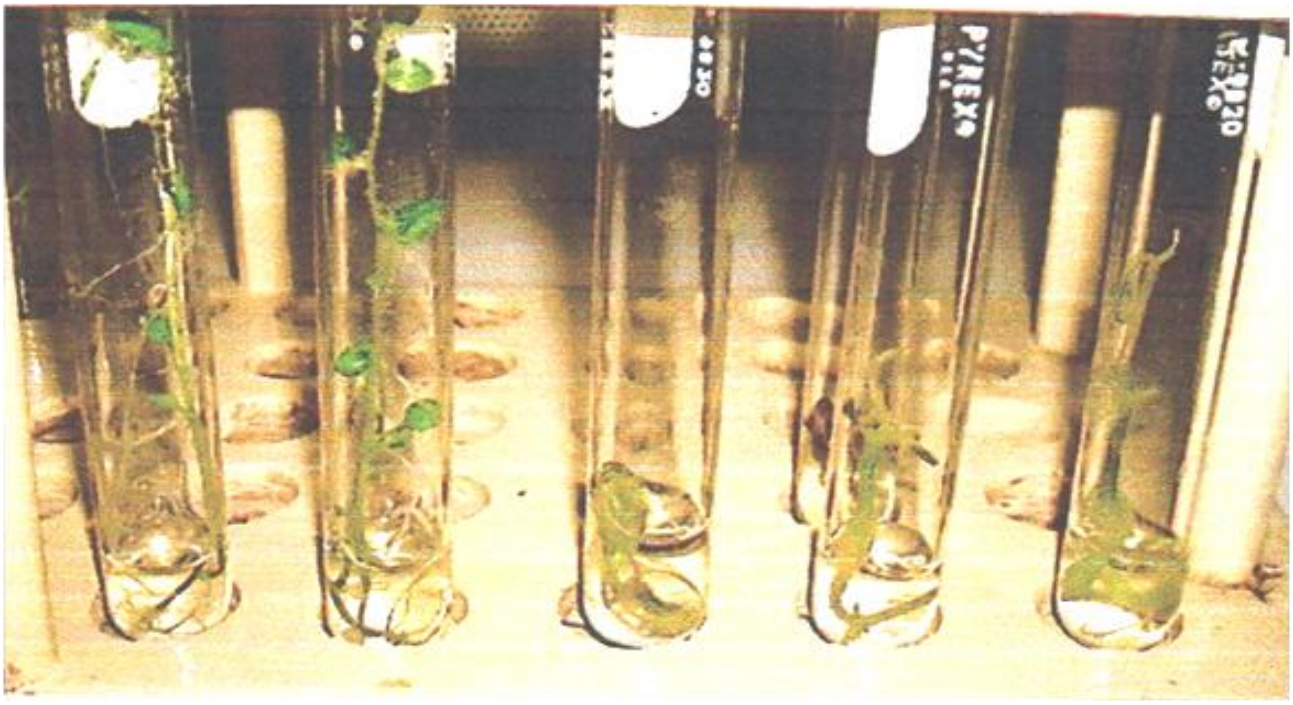

Figure 3. Vitrification of Tubira in liquid medium (left tubes).

1. The four improved varieties (Cipira, Tubira, Mafo and Bambui wonder) should be used for meristem culture to clean the plant varieties for Cameroon.
2. Solid medium should be preferentially used than in

Figure 4. Callus formation on plantlets of Bambui wonder variety in liquid medium.

stationary liquid medium in potato micropropagation

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Assessment of *Phaseolus vulgaris* L and *Vigna unguiculata* (L.) Walp leaves for antifungal metabolites against two bean fungal pathogens *Colletotricum lindemuthianum* and *Phaeoisariopsis griseola*

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The antifungal potential of hydro-ethanolic leaf extracts of bean varieties was analyzed by bioautography, assessing the contribution of defense molecules of proteic nature against two bean fungal pathogens *Colletotricum lindemuthianum* and *Phaeoisariopsis griseola*, also, the Rf values and relative activities of separated compounds were determined; these compounds were in a range of medium polarity to high polarity. The bioactivity expressed by the studied bean varieties could be correlated with the presence of proteic nature compounds, in joint action with secondary plant metabolites. There was some similarity in the chemical composition of the components of the extracts. The varieties G18350, G14241, G1320 CIAT and *Vigna ungiculata* were the most promising for isolating antifungal compounds. The results demonstrate the value of bioautography as a simple and cheap method to examine plant extracts with antifungal activity.

Key words: *Phaseolus vulgaris L*, *Vigna unguiculata* (L.) Walp, bioautography, peptides, secondary metabolites, *Colletotricum lindemuthianum, Phaeoisariopsis griseola,* antifungal defense.

INTRODUCTION

Beans are the most important grain legumes for direct human consumption in the world. Bean represents one third of the total world production of pulses (19.3 Mt/year; FAOSTAT, 2007) and the total production exceeds 23 million metric tonnes (MT) of which 7 million MT are produced in Latin America and Central Africa where it is a staple food for many people due to its energy, protein, dietary fiber and minerals content (Haytowitz et al., 1981; Norton et al., 1985; Broughton et al., 2003; Bitocchi et al., 2012).

One of the relevant factors associated with significant poor yield in most areas of bean production is phytopathogen attack (Santoyo et al., 2010). More than 200 species of phytopathogens that cause plant disease

have been reported, nonetheless only some of them cause considerable economic losses (Venegas, 2002). Despite the environmental implications associated with the excessive use, chemical fungicides remain the first line of defense against fungal pathogens, this problem involves many researchers to seek viable, safe and effective alternatives in controlling pests and diseases affecting crop plants of commercial interest, which has led to the introduction of improved varieties. To produce these varieties, the investigation has been focused in searching natural compounds with biological activity.

From this perspective, legumes have been extensively studied phytochemically finding out several types of compounds such as alkaloids, non-protein amino acids, amines, flavonoids, isoflavonoids, coumarins, phenylpropanoids, anthraquinones, terpenes, cyanogenic glycolsides, protease inhibitors, chitinases and lectins (Carlini and Grossi-de-Sa', 2002; Wink and Mohamed, 2003). Other researchers have detected proteins that protect legumes from pathogens and predators, such as arcelin, α-amylase inhibitor, vicilins, trypsin inhibitor, canatoxin, soybean cystatins, chymotrypsin inhibitors, lysozymes, ribosome inactivating proteins, antifungal proteins, and small cysteine-rich proteins, including plant defensins (Chen et al., 2002; Ye and Ng, 2003; Wang et al., 2005, 2007, 2012; Wong et al., 2012).

Among the species of the genus *Phaseolus*, there are representative phytoalexins like isoflavones, isoflavans, pterocarpans (phaseollin), coumestans and isoflavanones (kievitone), substances that are produced as a consequence of microorganism attacks (Mazid et al., 2011), therefore are considered as one of the most important defensive mechanisms in plants, considering that, Durango et al. (2002) demonstrated a link between phytoalexin accumulation and resistance/susceptibility to pathogenic microorganisms. The identification of high levels of arcelin in the seeds of the bean line called RAZ-2 shows *Phaseolus* as a potential source of defensins that could be explored for different applications (Pusztai et al., 1993; Montoya et al., 2010).

Although there have been a number of investigations related to the bean, little is known about the preventive management of pathogens. Considering that there have been scarce reports about biological activity in the leaves of beans, and that the studies have been done mainly in seeds, this study sought to evaluate the antifungal potential of hidro-ethanolic extracts of bean leaves; assessing the contribution of defense molecules of proteic nature and understanding the immune system of plants would allow farmers to create better yielding crop plants.

MATERIALS AND METHODS

Plant material

Phaseolus vulgaris seeds varieties G5734; G5747; G18350; G5694; G5732; G51094; G14241; G1320; G2233; G5038; G6030; G22164, G2333 and G10474 were provided by the Germplasm Bank of the International Center for Tropical Agriculture (CIAT). These varieties have been previously described as resistant to Angular leaf spot, Anthracnose and Rust diseases (Wahome et al., 2011). Cowpea bean was supply by local farmers.

Microorganisms

Colletotricum lindemuthianum (Cl 600 andean, Cl 242 Mesoamerican) and *Phaeoisariopsis griseola* (Pg 286 andean, Pg 305 Mesoamerican) fungi, were provided by the CIAT, these fungi were recovered by transferring them into a Petri dish (10 cm of diameter) with 20 ml of culture medium potato dextrose agar (PDA) for *C. lindemuthianum* and V8 agar for *P. griseola*, (de Oliveira et al., 2011) leading to incubation at 20 and 24°C, respectively.

Propagation of the seeds

There was a planting of all the varieties keeping a permanent control of growth. The spread of seeds was conducted on a greenhouse separating the material to avoid unwanted crossing between them (Tinivella et al., 2009). Eight days after planting, the number of germinated seeds was counted and fifteen days after, it was established which of them had continued to mature, thereby obtaining the percentages of germination and seed maturation. Analyses were performed in duplicate.

Considering the germination, maturation and plant resistance to the pathogenicity test, the best seeds (Cowpea, *Phaseolus vulgaris* varieties G18350, G5732, G5747, G51094, G14241, G10474, G22164 and G2333) were propagated for obtain sufficient material, this propagation was developed under field conditions, separating each cultivar. Only varieties that developed successfully were used in subsequent trials.

Pathogenicity test

When the plants were around one month old the pathogenicity test with *C. lindemuthianum* and *Phaeoisariopsis griseola* was performed (Bugeme et al., 2009). For this assay, leaves of healthy plants were collected; each one of them was disinfected with sodium hypochlorite (2%) for 2 min, and washed with sterile distilled water. At the same time circles of absorbent paper were cut, they were moistened with sterile distilled water and placed in the bottom of each Petri dish, together with the leaves who were then inoculated with the sprinkling of a spore suspension (2.4×10^5CFU/mL). The Petri dishes were sealed and incubated at 28 ±

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Abbreviations: MTT, Methyl thiazolyl tetrazolium; **C. l,** *Colletotricum lindemuthianum;* ***P. g,*** *Phaeoisariopsis griseola;* **PDA,** potato dextrose agar; **CIAT,** International Center for Tropical Agriculture.

Table 1. Germination and maturing rates of bean varieties with resistance to pathogens.

Varieties	Resistance to	Average germination rate (%)	Average maturing rate (%)
G5734	*U. phaseoli*	90	100
G5747	*U. phaseoli*	80	75
G18350	*U. phaseoli*	100	100
G5732	*U. phaseoli*	60	100
G5694	*P. griseola*	30	100
G51094	*P. griseola*	90	100
G14241	*P. griseola*	90	88.8
G10474	*P. griseola*	100	100
G1320	*C. lindemuthianum*	60	100
G2233	*C. lindemuthianum*	80	100
G5038	*C. lindemuthianum*	40	25
G6030	*C. lindemuthianum*	70	57.14
G22164	*C. lindemuthianum*	70	100
G2333	*C. lindemuthianum*	100	100
Cowpea	*nd*	100	100

nd: not determinate. The coefficient of variation were minor or equal to 10%

2°C in the dark, throughout the incubation period. Each test was performed in duplicate.

Metabolite extraction of beans leafs

Leaves of the *Phaseolus vulgaris* varieties G18350, G14241, G1320 and cowpea were exposed to cleaning treatment (sodium hypochlorite 2%), and each g of left in contact with the mixture extractor ethanol/water 2:5 used 10 mL of the extraction mixture submitting to mechanical agitation

Bioautography

Chromatograms were performed on Thin Layer Cromatography (TLC) plate silica gel 60 F_{254} (Merck 2 × 6 cm), each plate was loaded with 5 µl of each extracts. The solvent systems dichloromethane-methanol (8:2) and water-propanol (5:5) gave good results for the fraction of secondary metabolites and peptides, therefore, were used to further study. All chromatograms were developed from the extracts under the same working conditions; a group of them was used to perform the bioautography, while the others served as controls for the identification of active compounds after application of the revealing agents: vanillin, Liebermann-Burchard and Shinoda test for secondary metabolites and ninhydrin/butanol/acetic acid for peptides.

Once obtained, the chromatography plates were dried to completely remove the solvent. Each chromatogram was placed on a Petri dish and inoculated culture prepared with tetracycline (0.1%) and the fungi object of study, at a concentration of 2.2×10^5 UFC/ml, was sprayed over all the plate; then *Colletotricum lindemuthianum* were incubated at 20°C for and 24°C and *Phaeoisariopsis griseola*, both for 24 h time after which the aqueous solution of methylthiazolyltetrazolium (MTT) was sprayed to detect the dehydrogenase activity. The inhibition was evaluated for 24 or 48 h after spraying with MTT (Schmourlo et al., 2005). This test was run in triplicate.

Proteins and secondary metabolites determination

The presence of peptide bonds was verified by the biuret test (Gornall et al., 1949). The detection of primary and secondary amines was performed by Ninhydrin test (Kaiser et al., 1980). Other tests were the Shinoda test (Mallikharjuna et al., 2007), and the Liebermann-Burchard reaction (Coelho and Alves, 1946) obtaining positive results for flavonoids and steroids, respectively. These assays were realized for observation if there were secondary metabolites that could be involved in the antifungal defense of the plant.

The protein content was determined from the extracts by the Lowry method, with bovine serum albumin as the standard, following the methodology proposed by Rutten et al. (1987). This method is consists of the complex made by the phenolic group of tyrosine and trytophan residues (amino acid) in a protein with Folin-Ciocalteau; this method is sensitive down to about 10 µg/ml (Sreekumar, 2010).

Data analysis

For those data related for the germination rate, including the maturity of different varieties, the coefficient data of variation and standard deviation for each of the measurements were made with the zones of inhibition measured in the autobiogram report.

RESULTS AND DISCUSSION

Performance and endurance of the seeds

Table 1 showed the germination and maturation rates of resistant bean varieties. The values evidence that the varieties resistant to *P. griseola* showed better germination reaching maturity as well. However, the Cowpea bean was the most remarkable, not only for its high germination and maturity (Gómez, 2011) but also because of its sturdiness comparing to other varieties that showed chlorosis and their stems were thin.

These results could be explained due to the wide adaptation of cowpea, which is an important grain legume

Figure 1. Pathogenicity test performed with *C. lindemuthianum.* A: blank; B: *P. vulgaris* variety G1320; C: *P. vulgaris* variety G18350; D: *P. vulgaris* variety G14241; E: cowpea.

throughout the tropics and subtropics, covering Asia, Africa, and Central and South America, as well as parts of Southern Europe and the United States (Maredia and Raitzer, 2006). The crop has considerable adaptation to high temperatures and drought, it is also tolerant of low fertility, due to its high rates of nitrogen fixation, effective symbiosis with mycorrhizae and its ability to withstand acid and alkaline soil conditions, at the same time it is shade-tolerant (Ehlers and Hall, 1997). In all cases the error rate associated with germination and mature rats did not exceed 10%.

Compared to cowpea, the varieties provided by the CIAT are not known to have any resistance to biotic or abiotic stresses; even some of them came from areas that present quite different climatological characteristics from Colombia, which explains the reason why these seeds were not adapted to neither greenhouse nor field conditions in Ibagué city.

The varieties G18350, G14241, G1320 and cowpea, showed the highest production, best morphological characteristics, great resistance to pests and developed more foliage, reasons why they were chosen for continuing the search for antimicrobial activity of the peptides. It is also worth mentioning that each of them has resistance to a particular fungus.

Pathogenicity test

In this assay, the varieties resistant to *U. phaseoli* were the ones who showed further deterioration, being strongly affected when evaluated against *C. lindemuthianum*. Still, as it was expected, the varieties described by the CIAT as those resistant to *C. lindemuthianum*, although were affected by the fungi, showed less damage compared to other varieties. In spite of this, cowpea showed the highest resistant to the microorganism attack even if it is compared to the resistant varieties provided by the CIAT. This could be explained by the presence of defensins, such as ϒ-thioninas present in cowpea seed involved in antibacterial and antifungal activities (Carvalho et al., 2001; Franco et al., 2006). Figure 1 exhibits the results of pathogenicity test performed with C*. lindemuthianum* only for the chosen varieties.

Figure 1 shows pathogenicity test performed with C*. lindemuthianum.* A: blank; B: G1320; C: G18350; D: G14241; E: Cowpea.

The Figure 2 shows the pathogenicity test performed with *P. griseola* for the elected varieties. The variety G18350 showed the highest resistance of all, although it was not expected to have that property, opposed to this variety G14241 which was considered having resistance to this fungus, was very affected. Once again, cowpea exhibited high resistance to the fungi.

Figure 2 shoes pathogenicity test performed with *P. griseola.* A: blank; B: G1320; C: G18350; D: G14241; E: Cowpea.

Protein determination

The protein content in leaves was evaluated in the varieties that showed increased resistance to field and laboratory conditions, even if the bioactivity was evident on single fungal specie. The results obtained allow to sort the varieties according to their protein content as follows: G18350 (4.16 ± 0.25 mg/g) > G1320 (2.41 ± 0.11 mg/g) > G14241 (2.29 ± 0.05 mg/g) and cowpea (1.75 ± 0.09 mg/g).

It is notorious that although the G18350 variety expressed the highest protein content its bioactivity was only against *P. griseola*, whereas Cowpea revealed the lower content of this metabolite, having resistance to both of the fungal species tested, this means that the bioactivity could be not only the result of the protein activity but also of secondary metabolites contained in these bean species, such as flavonoids and anthocyanins that are related to protection from attack by

Figure 2. Pathogenicity test performed with *P. griseola.* A: blank; B: *P. vulgaris* variety G1320; C: *P. vulgaris* variety G18350; D: *P. vulgaris* variety G14241; E: cowpea.

pathogens and insects that have been reported in previous studies (Lattanzio et al., 2000; Makoi et al., 2010).

The antimicrobial activity of plant peptides from bean species and others plants has been widely demonstrated (Lipkin et al., 2005; Loeza et al., 2008; Wu et al., 2011; Chan et al., 2012), in proteins the bioactivity has been detected mostly in seeds, as evidenced by the work of Chen et al., (2002), Louis et al. (2007) and Wua et al. (2011). However, thus far, few studies have been interested in finding this functionality in leaves, making this work one of the first developed for this purpose; the interest increases if it is considered that this research was focused on the search of proteins in leaves of several bean varieties, one of the most widely consumed legume in developing countries.

Bioautography results

Bioautography was used to separate the antifungal compounds to obtain more information on the diversity of antifungal compounds present in hidro-ethanolic extracts of different bean varieties. Inhibition zones were observed as white spots on a purple-blue background, indicating where reduction of MTT to the coloured formazan did not take place due to the presence of compounds inhibiting the growth of tested fungi.

In some cases organisms did not grow too well, being difficult to detect inhibition zones. The most likely available explanation is that the growth of *P. griseola* and *C. lidemuthianum* at laboratory conditions in Ibagué city is very slow. In other cases there were growth, but apparently there was no inhibition, these could be explained by the disruption of synergism between active constituents caused by TLC. In spite of the differences observed in the zones of inhibition and the high values of diversions standard obtained, it is of highlight that the results were consisting of the different replies and in all the cases they presented zones of inhibition that were associated with a value of certain Rf.

As shown in the Table 2, in all cases, the four extracts developed in dichloromethane-methanol presented metabolites with Rf values of 0.1; 0.24 ± 0.005; 0.67 ± 0.01 and 0.82 ± 0.016, showing antifungal compounds that were very active against *P. griseola* on a range from 0.67 ± 0.01 to the solvent front.

On the other hand, the metabolites present on the bioautography test against *C. lindemuthianum* did not exhibit a similar pattern among varieties, presenting inhibition zones with Rf values of 0.56 (G1320); 0.92 (G14241) and 0.84 (cowpea). In this case G18350 did not evidence any antifungal activity, which is consistent with the results revealed by this variety on the pathogenicity test against *C. lindemuthianum,* where it was the most affected variety. Figure 3 shows the bioautograms of the secondary metabolites against *P. griseola* and *C. lindemuthianum*.

Table 2 shows antifungal activity of hidro-ethanolic leaf extracts of bean varieties by direct bioautography.
C. l = *C. lindemuthianum,* P. g = *P.griseola*

Figure 3 shows bioautography of bean varieties extracted with water-ethanol separated by dichloromethane-methanol and sprayed with vanillin (top), *P. griseola* (center) and *C. lindemuthianum* (bottom). A: G1320; B: G18350; C: G14241; D: cowpea. White areas indicate where reduction of MTT to the coloured formazan did not take place due to the presence of compounds that inhibited the growth of the fungi.

In what is related to the bioautography test developed in water: propanol for the separation of peptide compounds, it was observed that all the extracts presented Rf values of 0.55 ± 0.01; 0.65 ± 0.02; 0.81 ± 0.05 and 0.91 ± 0.01, the last one was not observed in G18350. All the varieties were active against *P. griseola* on a range from 0.66 ± 0,005 to the solvent front, except for G14241; this is in line with the pathogenicity test where this variety was highly affected.

It is interesting to note that all the varieties presented different patterns against *C. lindemuthianum*, displaying

Table 2. Antifungal activity of hidro-ethanolic leaf extracts of bean varieties by direct bioautography.

Parameter	Rf value	G1320		Rf value	G18350		Rf value	G14241		Rf value	COWPEA	
		$\bar{X}$ +/- ð Diameter of inhibition zone (mm)			$\bar{X}$ +/- ð Diameter of inhibition zone (mm)			$\bar{X}$ +/- ð Diameter of inhibition zone (mm)			$\bar{X}$ +/- ð Diameter of inhibition zone (mm)	
		C. l	P. g		C. l	P. g		C. l	P. g		C. l	P. g
	0.08			0.04			0.04			0.1		
	0.1			0.1			0.1			0.24		
	0.24			0.14			0.18			0.34		
	0.28			0.26			0.26			0.68		20 +/- 5.3
	0.4			0.4			0.4			0.74		
Dichloromethane-methanol	0.56	25 +/- 7.1		0.5			0.42			0.84	10 +/- 3.4	
	0.68		16 +/- 4.6	0.6			0.52					
	0.76			0.66		16 +/-3.6	0.6					
	0.82			0.72			0.64		18 +/- 6.1			
	0.9			0.76			0.68					
				0.82			0.8					
				0.88			0.92	5 +/- 1.3				
	0.54	0.5		0.56			0.52			0.36		
	0.6			0.6			0.56			0.56	20 +/- 5.8	21 +/- 4.9
Water-propanol	0.66		17 +/- 5.1	0.66	15+/- 4.3	17+/- 5.4	0.63			0.68		
	0.74			0.88			0.8	13 +/- 4.2		0.76		
	0.8	10 +/- 2.8					0.92			0.92		
	0.9											

C.l = *C. lindemuthianum,* P.g = *P.griseola*

inhibition zones with Rf values of 0.66 (G18350) 0.8 (G14241) and 0.56 (cowpea). However, unlike the others, G1320 showed three inhibition zones (Rf values: 0.1; 0.54 and 0.8); of them, the lowest (0.1) did not show the characteristic coloration of peptides revealed with ninhydrin, although it showed inhibition against *C. lindemuthianum* (Figure 4), indicating that it is a non-protein character compound. The high inhibition on this variety could be related to the results revealed on the pathogenicity test against *C. lindemuthianum* (Figure 1), where it did not show much damage. Figure 4 shows the bioautograms of the peptides against *P. griseola* and *C. lindemuthianum*.

Another important observation is that Rf values of 0.66 in G18350 and 0.56 in cowpea presented antifungal compounds with bioactivity in both fungal species.

Figure 4 shows bioautography of bean varieties extracted with water-ethanol separated by water:propanol and sprayed with vanillin (top), *P. griseola* (center) and *C. lindemuthianum* (bottom). A: G1320; B: G18350; C: G14241; D: cowpea. White areas indicate where reduction of MTT to the coloured formazan did not take place due to the presence of compounds that inhibited the growth of the fungi.

Some researchers (Hancock and Chapple, 1999; Reuter et al., 2009) argue that the fractions of a positively charged peptide, bind to the negative portion of the bacterial cell membrane (phosphatidylglycerol and cardiolipin,

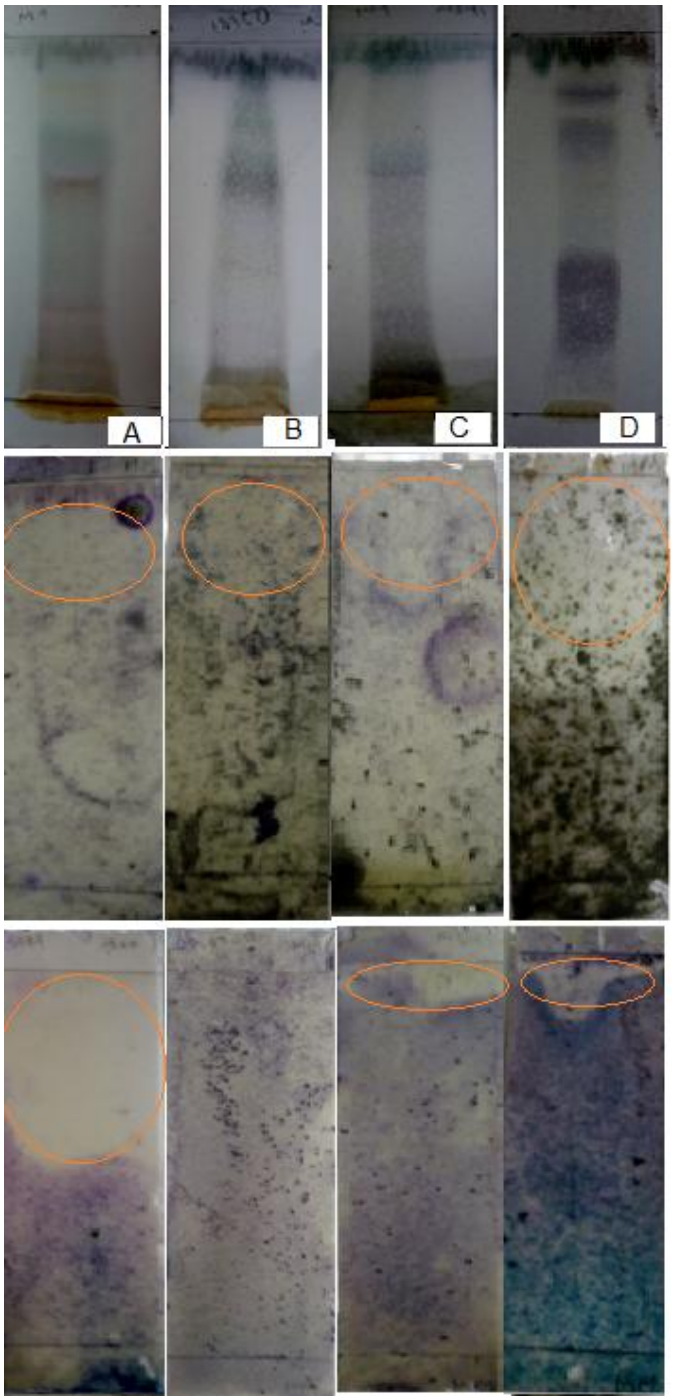

Figure 3. Bioautography of bean varieties extracted with water-ethanol separated by dichloromethane-methanol and sprayed with vanillin (top), *P. griseola* (center) and *C. lindemuthianum* (bottom). A: G1320; B: G18350; C: G14241; D: Cowpea. White areas indicate where reduction of MTT to the coloured formazan did not take place due to the presence of compounds that inhibited the growth of the fungi.

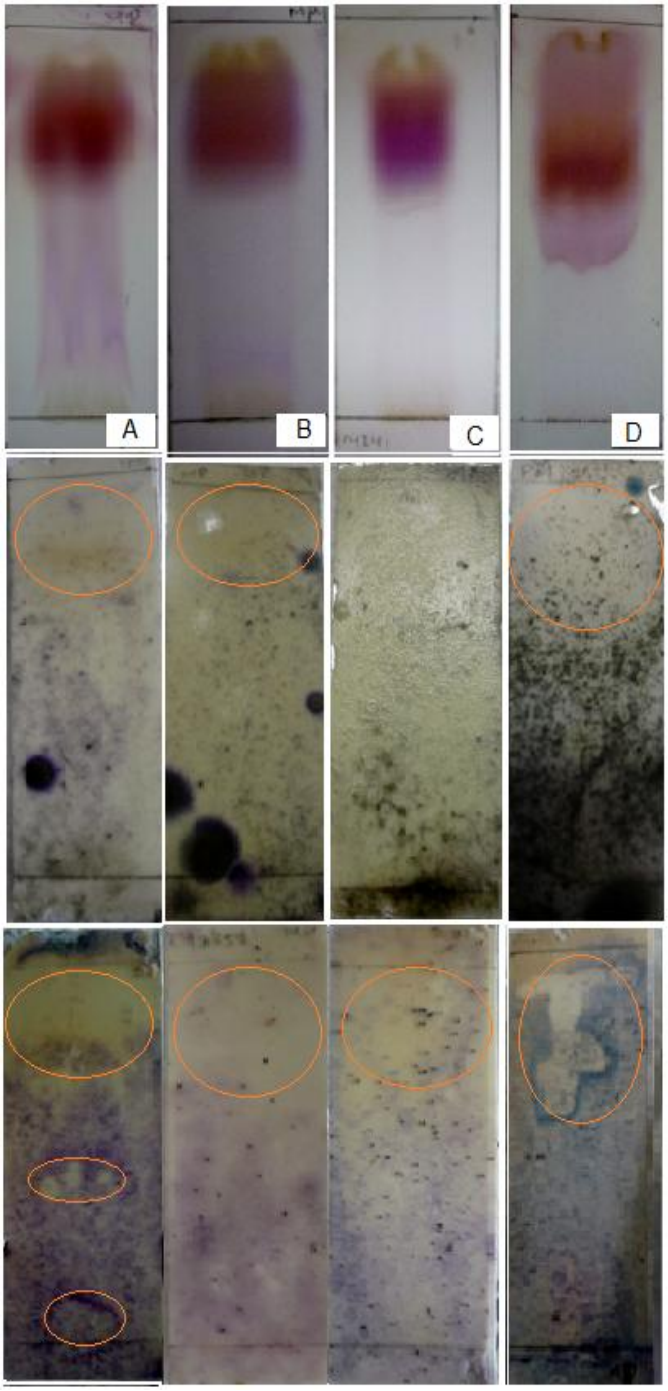

Figure 4. Bioautography of bean varieties extracted with water-ethanol separated by water:propanol and sprayed with vanillin (top), *P. griseola* (center) and *C. lindemuthianum* (bottom). A: G1320; B: G18350; C: G14241; D: Cowpea. White areas indicate where reduction of MTT to the coloured formazan did not take place due to the presence of compounds that inhibited the growth of the fungi.

among others) causing pores resulting in the death of the microorganism. A similar mechanism may also occur in case of fungi inclining to induced hyperbranching of fungal hyphae, plasma membrane permeabilization

and induction of apoptosis (Thevissen et al., 2003; Sagaram et al., 2011; Katrijin et al., 2011; Wilmes et al., 2011).

In general, the low activity of the samples using bioautography assay could be explained by a weak selectivity of the extract components against the microorganisms chosen for this study, or by the very low concentration of the active compounds in the crude extract under the tested conditions. The results obtained in the bioautography test suggested that the bioactive compounds of the varieties studied are in a range of medium polarity (dichloromethane 8: methanol 2) to high polarity (water:propanol).

Although the bioautography assay has not been of wide application in our country, this methodology has certain advantages when compared with other trials to test the antimicrobial activity of extracts or compounds of plant origin, such as, direct isolation of active constituents in complex mixtures using small amounts of material, greater sensitivity, and it can provide useful information about the nature of the active compounds.

Conclusions

The bioactivity expressed by the studied bean varieties could be correlated with the presence of protein nature compounds, perhaps in joint action with secondary plant metabolites, resulting in the varieties: G1320, G14241, G18350 and cowpea, as the most promising interest in the search for bioactive compounds, all of which would constitute a scientific basis for a better understanding of the bean immune system, allowing inroads to improving varieties of study.

This study is constituted as one of the few developed in the country, using the bioautography as a method for the determination of bioactive compounds in plants, this being a simple and economic technique that saves time and requires no sophisticated equipment. This work, besides being pioneer in the search for bioactive protein compounds in bean leaves the results obtained through it may contribute to decrease the difficulties present in the screening of antifungal compounds from plants.

Conflict of interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Increasing the extraction efficiency of algal lipid for biodiesel production: Novel application of algal viruses

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Various studies have been conducted recently using microalgal system for the production of algal lipid for biodiesel production. This study aimed at increasing the extraction efficiency of algal lipid from *Chlorella* sp. by the application of *Chlorella* viruses. The calorific value of lipid from *Chlorella* sp. has been reported to be higher than that of fresh water microalgae, making it a potential candidate to be used as biofuel. This is the first report on novel application of microalgal viruses for improving the extraction efficiency of algal lipid for biodiesel production through viral lysis of *Chlorella vulgaris*. The algal lipid extraction efficiency, increased by 11.68% in the case of *Chlorella* virus treated *C. vulgaris* when compared with *Chlorella* virus untreated *C. vulgaris*. The application of *Chlorella* virus and the viral encoded lytic enzymes for increasing the extraction efficiency of algal lipid will be helpful in optimizing algal biofuel industry in the future.

Key words: Micro-algal system, biodiesel, algal lipid, *Chlorella* viruses, viral lysis.

INTRODUCTION

Microalgae are a promising candidate for biodiesel production. Microalgae have comparatively high oil content, biomass productivity and are more sustainable and environmentally friendly than petroleum-derived fuels (Hu et al., 2008). Some microalgae are known to produce high amounts of lipids and can be applied in bioprocess to produce alternative oils for biodiesel manufacture (Ratledge, 2005). Nevertheless, the microalgal biodiesel has not been widely commercialized mainly due to its high costs. The microalgal biodiesel production can be optimized for cost effectiveness and increasing the algal lipid extraction efficiency (Li et al., 2008; Pittman et al., 2011).

Chlorella viruses have large icosahedral capsids with an internal membrane enclosing their ds DNA genome that infect certain strains of the unicellular green alga *Chlorella* (chlorovirus or *Chlorella* virus; Phycodnaviridae) which are ubiquitous in natural environments (Yamada et al., 1991; Zhang et al., 1988; Wulfmeyer et al., 2012). In a normal lytic cycle, virus particles attach to the surface of host *Chlorella* cells and degrade the cell wall at the point of attachment; the viral core is then released into

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the host cytoplasm, leaving an empty capsid on the cell wall. Within 6 to 8 h, post infection, mature viral progenies exit the cells after cell lysis. Both the initial and final stages of the viral replication cycle obviously require cell wall-degrading activities, but little is known about the nature and origin of the enzymes that degrade the cell wall (Van Etten et al., 1991).

A common characteristic of virus-sensitive *Chlorella* strains are a rigid cell wall containing uronic acids and glucosamine in addition to other polysaccharides such as glucose, rhamnose, galactose, xylose, arabinose, mannose and glycoprotein matrix providing the cells with a formidable defense against its environment (Kapaun et al., 1992; Kapaun and Reisser, 1995; Gerken et al., 2013). Based on different microalgae and culture conditions such as temperature, nutrient and light intensity; microalgal lipid content and composition are varied (Converti et al., 2009; Solovchenco et al., 2008). The oil content of some microalgae such as *Scenedesmus* sp., *Chlorella* sp., *Neochloris oleoabundans* can be from 20 to 50% of total cell dry weight (Gouveia and Oliveira, 2009), revealing the significant potential of biodiesel production. Fatty acid methyl esters (FAME) originating from vegetable oils and animal fats are known as biodiesel, which have also been characterized in microalgae and are non-toxic. The exploitation of microalgae for commercial biodiesel production is not yet a commercial reality and still warrants further research and development (Benemann, 2008).

Algae for biodiesel have been studied for many years for production of hydrogen, methane, vegetable oils (triglycerides, for biodiesel), hydrocarbons and ethanol. The high calorific value lipids generated by the microalgae and cyanobacteria are used for biodiesel (Illman et al., 2000). A mechanism that could concept-tually scale-up the yield of biodiesel precursors has not yet been demonstrated effectively (Benemann, 2008). Beside the selection of candidate microalgal strains for high production of biodiesel precursors, the present research challenge is also on increasing the extraction efficiency of biodiesel precursors (Sierra et al., 2008) from algae *Chlorella vulgaris* by using a novel approach like *Chlorella* virus mediated lysis.

MATERIALS AND METHODS

Isolation and identification of microalgae

Recently, a report demonstrated that the calorific value of *C. vulgaris* was found to be 28 kJ/g, which was highest among the freshwater microalgae making it a promising candidate to be exploited for alternate fuel (Scragg et al., 2002). Fifteen (15) algal strains were isolated from different aquatic ecosystems in and around Nagpur; out of which one was identified as *C. vulgaris*. The microalgae was identified by light microscopy analysis (Leica DM2500 Wien, Austria) and was used for further experimental studies.

Chlorella vulgaris genome sequencing and sequence analysis

The microalgal species was confirmed by partial sequence of 18S rRNA of microalgae using outsourcing the sequencing services to Macrogen Inc. Seoul, South Korea. The sequencing was performed using the primers and conditions as prescribed by Hoshina and Fujiwara (2012). The partial nucleotide sequence was subjected to Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990).

Propogation of *C. vulgaris* microalgae

The isolated *C. vulgaris* microalgae was propagated in nitrate depleted Bold Basal Media (Rippka, 1988) at 28 ± 2°C and light intensity of 350 µmol m^{-2} s^{-1} for a week using a light meter and subsequently sub-cultured (Scragg et al., 2002). The sub-cultured microalgae strain of isolated *C. vulgaris* in exponential phase was subjected to further study as depicted in the workflow (Figure 1).

Isolation of *C. vulgaris* specific virus by plaque assay method

The surface water sample collected from Ambazari Lake, Nagpur, Maharashtra, India was used for isolation of *C. vulgaris* specific virus. The water sample was filtered through 0.2 µm polycarbonate membranes (Millipore, India.); 100 µl of the filtrate was inoculated into 900-µl exponentially growing culture of isolated *C. vulgaris*. The cultures were incubated for a week. The virus was isolated from the lysed culture using the plaque assay procedure using Bold Basal Media with 1% agar (Figure 2). In brief, a 100 µl lysed culture was mixed with 200 µl of the host cells at a concentration of 1×10^8 to 2×10^8 cells per ml. The mixture was poured with 5.5 ml of 1% Bold Basal Media and incubated in the light at 28 ± 2°C (Van Etten, 1982).

Purification and enrichment of *C. vulgaris* specific virus

The single plaque was selected for enrichment in exponentially growing *C. vulgaris* culture in Bold Basal Media and was incubated at 28 ± 2°C and light intensity of 350 µmol m^{-2} s^{-1} for one week (Rippka, 1988).

Extraction of lipid from micro-algal biomass

Extraction and estimation of lipid content from micro-algal biomass with and without algal virus treatment was carried out by following the standard protocol of Bligh and Dyer (1959). Each experiment was done in triplicate. The mean and standard deviation of the experimental results was calculated using MS-Excel.

RESULTS AND DISCUSSION

Chlorella vulgaris genome sequencing and sequence analysis

The microalgae partial sequence accession was subjected to NCBI-BLAST that showed 100 and 99% identity with other *C. vulgaris* nucleotide sequences submitted to genbank with accession numbers AB699112.1 and JX185298.1 respectively.

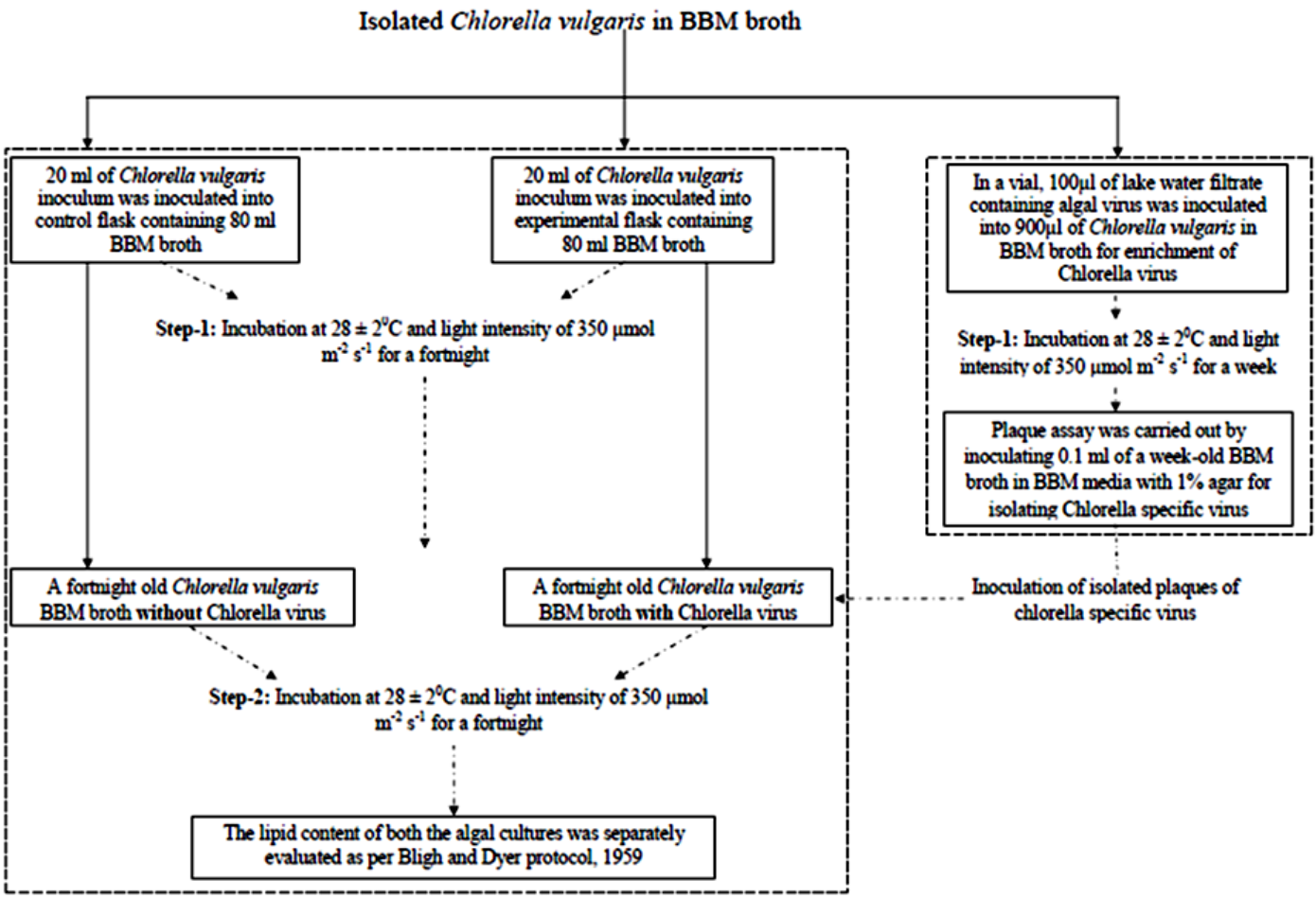

Figure 1. Workflow for increasing the extraction efficiency of bio-diesel precursor from algae through application of algal viruses.

Figure 2. *Chlorella vulgaris* specific algal virus isolated on bold basal media with 1% agar.

Table 1. Lipid content of *C. vulgaris*.

Experimental set	Lipid content (g g^{-1})	Percentage lipid content (%)
C. vulgaris treated with Chlorella virus (Experimental)	0.291 ± 0.017	29.1
C. vulgaris not treated with Chlorella virus (Control)	0.257 ± 0.02	25.7

*Data indicates the mean ± SD which was measured from five replicates.

Extraction of lipid from algal virus treated and untreated microalgal culture

C. vulgaris showed promising lipid extraction efficiency when treated with *Chlorella* virus. The lipid yield of *C. vulgaris* treated with *Chlorella* virus was found to be 0.291 ± 0.017 g/g and the lipid yield of *C. vulgaris* not treated with *Chlorella* virus was found to be 0.257 ± 0.02 g/g of biomass. Hence, algal lysis due to virus yielded an increased percentage of lipid content that is 29.1%, which was significantly higher than the untreated *C. vulgaris* that is 25.7%. The result implied that algal lipid extraction efficiency, increased by 11.68% in the case of *Chlorella* virus treated *C. vulgaris* when compared with *Chlorella* virus untreated *C. vulgaris* as shown in Table 1.

Productions of precursors for biodiesel have already been well established (Chiu et al., 2008). This study reports for the first time the application of *Chlorella* virus for increasing algal lipid extraction efficiency from *C. vulgaris*. We found from our results that *Chlorella* virus could be helpful in increasing the extraction efficiency of the algal lipid through viral mediated lysis of *C. vulgaris* under optimum conditions. We observed that the application of *Chlorella* viruses could effectively increase the extraction efficiency of algal lipid from *C. vulgaris*; similarly, viruses can be isolated against other microalgae for enhanced algal lipid recovery.

However, this method has some limitations as far as efficiency is concerned, such as development of resistance after frequent use of viruses (*Chlorella* viruses, in this case); lysogenic conversion of the viruses within their specific hosts; and the unpredictability of viral lysis event in the culture. The limitations can be minimized; as resistant algal strain can be screened for lytic viruses from environmental samples, which is simple and affordable, lysogen formation may be minimized by maintaining optimum conditions for algal growth. The lysis events can be monitored by microscopic observation of culture after viral addition and incubation for desired period depending on culture volume. Nevertheless, we considered that there is need to explore fluid dynamics between two fluids for improving the extraction efficiency of algal lipid for ultimately potentiating the biodiesel production. Further algal virus encoded products in the form of formulations can also be employed for efficient lysis of biodiesel producing algae commercially.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Protective effect of *Allium sativa* extract against carbon tetrachloride- induced hepatic oxidative stress and hyperlipidemia in rats

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Pathogenesis of several chronic liver diseases has been attributed to overwhelmed antioxidant protective system against reactive oxygen species (ROS). The present study ascertained the capacity of short-term administration of ethanolic extract of *Allium sativa* to neutralize ROS and ameliorate hyperlipidemia. Hyperlipidemia was induced in rats by single intra-peritoneal injection of CCl_4 (dosage = 2.0 mL/kg), followed by treatment with ethanolic extract of *A. sativa* (dosage: 200 and 400 mg/kg) at a regular interval of 16 for 64 h. Blood samples were drawn from the rats at *t* = 0 h and *t* = 76 h, that is, 12 h after the end of 64 h treatment with CCl_4/*A. sativa* extract, to ascertain for hepatic function and serum lipid profile (SLP). In addition, liver post mitochondrial supernatant (PMS) fraction was measured for oxidative stress indicators: lipid peroxidation (LPOx), superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) and reduced glutathione (GSH). On the average, short-term administration of ethanolic extract of *A. sativa* caused reduction of SLP in the following magnitude: total cholesterol (TC) = 19.48%, triacylglycerol (TAG) = 48.59%, VLDL-C = 48.57%, LDL-C = 19.49% and increase in HDL-C = 32.43%. Also, improvement in oxidative stress indicators gave SOD = 10.20%, GPx = 30.92%, CAT = 18.18%, LPOx = 35.92% and GSH = 51.09%. Although the administration of *A. sativa* extract to the rats did not restore full therapeutic benefits within the experimental time (*t* = 76 h), the capacity of the plant extract to ameliorate oxidative stress and hyperlipidemia in the animals was fairly at par with the standard hepatic drug-hepaticum.

Key words: *Allium sativa,* hepatocyte, hyperlipidemia, lipid profile, oxidative stress.

INTRODUCTION

The liver is often referred to as an organ of homeostasis by virtue of the fact that the metabolic concern of the hepatocyte is to ensure constancy in the internal environment of vertebrates. The capability of the liver to achieve this physiologic feat is hinged on high vascularization of the organ, capacity to serve as storage site for macromolecules and micronutrients as well as abode for enzymes involved in carbohydrate, protein and lipid metabolism. In addition, the central roles of the liver in xenobiotic and endogenous detoxification reactions have been well reported (Sugatani et al., 2006; Shaker et al., 2010; Singh et al., 2011). The biosynthesis of most

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plasma lipoproteins and apolipoproteins occur in the hepatocytes (Mensenkamp et al., 2000; Jiang et al., 2006). Therefore, agents/factors that compromise hepatocellular functionality and integrity alter plasma lipid profile patterns (Wolf, 1999; Ramcharran et al., 2011). Hyperlipidemia describes the elevation in plasma lipid components; triacylglycerol (TAG), low-density lipoprotein cholesterol (VLDL-C), low-density lipoprotein cholesterol (LDL-C) and total cholesterol (TC), but reduced levels of high-density lipoprotein cholesterol (HDL-C) (Ochani and D'Mello, 2009; Kaur and Meena, 2013). According to Shaker et al. (2010), hepatic dysfunction is associated with acute hepatitis, hepatocellular carcinoma, apoptosis, necrosis, inflammation, immune response, fibrosis, ischemia, altered gene expression and regeneration.

The hepatocyte is well furnished with antioxidant defense systems. Notwithstanding, pathogenesis of several chronic liver diseases has been attributed to overwhelmed antioxidant protective system by accumulation and elevated levels of ROS (Czuczejko et al., 2003; Novo et al., 2006; Chikezie, 2011). Notably, the antioxidant scavenging enzymes include superoxide dismutase (SOD), catalase (CAT), glutathione *S*-transferase (GST) and glutathione peroxidase (GPx), which offer primary protection to the hepatocyte and by extension, to other peripheral tissues, against oxidative injury (Halliwell, 1994; Bonnefont-Rousselot et al., 2000; Avti et al., 2006; Pasupathi et al., 2009). Some non-enzymatic antioxidants are reduced glutathione (GSH), α-tocopherol, β-carotene and ascorbate (Avti et al., 2006; Surapanen, 2007; Singh et al., 2011; Necib et al., 2013).

Despite disparities in the distribution and metabolism plasma lipoprotein between humans and rats (Uetrecht, 2006), the use of animal model as tool for lipid and biomedical research is reliable and still popular. Also, applications of plant extracts for the treatment/-management of lipidemia have been severally reported with promising prospects (Kaur and Meena, 2013; Reach and Ernst, 1995).

Accordingly, among several medicinal benefits, *A. sativa* (garlic) have been demonstrated to be an agent of glycemic control (Banerjee and Maulik, 2002; El-Demerdash et al., 2005; Ibegbulem and Chikezie, 2013). The phytochemical and nutritive contents, coupled with previously reported medicinal usefulness of *A. sativa* extract (Auer et al., 1990; Reach and Ernst, 1995; Qidwai et al., 2000; Ibegbulem and Chikezie, 2013) informed the trial of *A. sativa* extract in the present investigation. The present study ascertained the capacity of short-term administration of ethanolic extract of *Allium sativa* to neutralize ROS and ameliorate hyperlipidemia in CCl_4 induced hyperlipidemic rats.

MATERIALS AND METHODS

Collection of plant samples and preparation of extract

Fresh samples of *A. sativa* were obtained in July, 2012 from a local market at Umoziri-Inyishi, Imo State, Nigeria. The plant specimen was identified and authenticated by Dr. F.N. Mbagwu at the Herbarium of the Department of Plant Science and Biotechnology, Imo State University, Owerri, Nigeria. A voucher specimen was deposited at the Herbarium for reference purposes. Ethanol/water extract (1:2 *v/v*) of *A. sativa* was prepared by methods of Ibegbulem and Chikezie (2013) with modifications according to Lam et al. (2003). Fresh bulbs of *A. sativa* were washed under a continuous stream of distilled water for 15 min and air-dried at room temperature (25 ± 5°C) for 5 h. The bulbs were chopped and further dried for 5 h in an oven at 60°C and subsequently ground with ceramic mortar and pestle. Twenty-five grams (25 g) of the pulverized specimen was suspended in 250 mL of ethanol/water mixture (1:2 *v/v*) in stoppered flask and allowed to stand in a thermostatically controlled water bath at 40°C for 24 h. The suspension was filtered with Whatman No. 24 filter paper, concentrated in a rotary evaporator at 50°C and dried in vacuum desiccator. The yield was calculated to be 3.6% (*w/w*). The extract was re-dissolved in 20 mL of PBS (pH = 7.4) and incubated at 37°C for 30 min with thorough shaking. The dissolved content was quickly frozen at -80°C before lyophilization. The required amount of lyophilized extract was reconstituted in 400 µL distilled water (DW) and administered by intra peritoneal injection to the rats at doses of 200 and 400 mg/kg (Giri et al., 2012**)** at regular time intervals of 16 to 64 h.

Experimental animals

Male rats *Rattus norvegicus* (8-10 weeks old) weighing 150 to 200 g were generous gift from Professor A.A. Uwakwe (Department of Biochemistry, University of Port Harcourt, Nigeria). The rats were maintained at 25 ± 5°C, 30-55% of relative humidity on a 12-h light/12-h dark cycle, with access to water and food *ad libitum* for two weeks acclimatization period. The handling of the animals was in accordance with the standard principles of laboratory animal care of the United States National Institutes of Health (NIH, 1978).

Study design

The animals were deprived of food and water for 16 h before commencement of treatments (control and test experiments) as previously described (Ibegbulem and Chikezie, 2013). Also, hyperlipidemia was induced in the rats by single intra-peritoneal injection of CCl_4 (dosage = 2.0 mL/kg) 16 h before commencement of study. A total of 20 rats were categorized into five groups of four (n = 4) each as follows:

(i) Group C1: Control/Normal rats received only DW (vehicle; 2.0 mL/kg/16 h, i. p.) for 64 h.
(ii) Group C2: Control/Hyperlipidemic rats received 2.0 mL/kg CCl_4 + DW (vehicle; 2.0 mL/kg/16 h, i. p.) for 64 h.
(iii) Group T1: Hyperlipidemic rats received 2.0 mL/kg CCl_4 + *A. sativa* (200 mg/kg/16 h, i. p.) for 64 h.
(iv) Group T2: Hyperlipidemic rats received 2.0 mL/kg CCl_4 + *A. sativa* (400 mg/kg/16 h, i. p.) for 64 h.
(v) Group T3: Hyperlipidemic rats received 2.0 mL/kg CCl_4 + Hepaticum (100 mg/kg/16 h, i. p.) for 64 h.

Collection of blood

Blood samples were drawn from the tail vein of each rat prior to anaesthetization under light ether that is, at experimental t = 0 h for measurement of serum lipid profile (SLP) and levels of γ-glutamyl transferase (γ-GT), alanine transaminase (ALT) and aspartate transaminase (AST) activities. Finally, blood samples were obtained

by carotid artery puncture for measurement of SLP and enzyme activities 12 h after the end of 64 h treatment with *A. sativa* extract that is (*t* = 76 h).

Serum lipid profile

Total cholesterol (TC), triacylglycerol (TAG) and high-density lipoprotein cholesterol (HDL-C) were determined using commercial kits (Randox Laboratory Ltd., UK). Low-density lipoprotein cholesterol (LDL-C) concentration was determined by difference according to the formula described by Friedewald et al. (1972): LDL-C = TC - (HDL-C) - (TAG/5), as reported by Shaker et al. (2010). Very low-density lipoprotein cholesterol (VLDL-C) concentration was estimated using the methods of Burnstein and Sammaille (1960), where the value in mg/dL is based on the assumption that in fasting animals, the VLDL-C to TAG ratio is relatively fixed at 1:5 (Ibegbulem and Chikezie, 2013). Atherogenic index (AI), which was a measure of atherogenesis in normal and treated rats was calculated thus: [TC-(HDL-C)]/(HDL-C) (Suanarunsawat et al., 2011).

Serum enzyme assay

AST and ALT activities were measured using the automated enzymatic methods (EliTech Diagnostic, Sees, France); whereas, γ-GT activity was according to the methods as described by Fiala et al. (1972).

Preparation of liver homogenates

Organ homogenate was prepared according the procedures of Adekunle et al. (2013). Quickly, the liver was excised and placed between blotting papers to remove accompanying blood. Next, the organ was rinsed in 1.15% KCl solution to obliterate residual hemoglobin molecules. The sample was homogenized using a Teflon homogenizer in aqueous $K_2PO_4/KHPO_4$ buffer (0.1 M; pH = 7.4); in 4:1 volume of buffer to organ weight. Subsequently, the homogenate was centrifuged at 10,000 × *g* for 20 min at 4°C to obtain the post mitochondrial supernatant (PMS) fraction and collected into sample bottles. The PMS fraction was finally stored at -80°C before used for analyses. The homogenate was used to assay the following oxidative stress indicators: Lipid peroxidation (LPOx), superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) and GSH. Protein concentration was measured at λ_{max} = 595 nm by methods of Bradford (1976) using bovine serum albumin as standard.

Lipid peroxidation

Measurement of LPOx was according to the methods of Ohkawa et al. (1979) with minor modifications according to Chikezie (2011). Briefly, the reaction mixture consist of PMS fraction in Tris-HCl buffer (50 mM, pH = 7.4), ter-butyl hydroperoxide (BHP) (500 µM in ethanol) and 1.0 mM $FeSO_4$. The reaction mixture was incubated for 90 min at 37°C, after which the reaction was stopped by introducing 0.2 mL of 8% sodium dodecyl sulfate (SDS) followed by 1.5 mL of 20% acetic acid (pH = 3.5). The quantity of malondialdehyde (MDA) produced during the incubation period was determined by adding 1.5 mL of 0.8% thiobarbituric acid (TBA) and further heating the mixture at 95°C for 45 min. After cooling to 24°C, the mixture was centrifuged at 3,000 × *g* for 10 min. The TBA reactive substances (TBARS) were measured in supernatant at λ_{max} = 532 nm; molar extinction coefficient (Σ) = 1.53×10^5 M^{-1} cm^{-1}. The level of LPOx was expressed in terms of nM of TBARS per 90 min/mg protein.

Superoxide dismutase

SOD was estimated according to the methods of Kono (1978). Briefly, the reaction mixture containing solution A (50 mM Na_2CO_3, 0.1 mM EDTA, pH = 10.0), solution B (96 µM nitrobluetetrazolium [NBT] in solution A), and solution C (0.6% Triton X-100 in solution A) were incubated at 37°C for 10 min. Reaction was started by introducing 100 µL of solution D (20 mM hydroxylamine hydrochloride, pH = 6.0). The rate of NBT dye reduction by $O_2^{\cdot-}$ anion generated due to photo-activation of hydroxylamine hydrochloride was measured at λ_{max} = 560 nm in the absence of PMS fraction. Next, 10 µL aliquot of PMS were added to the reaction mixture and 50% inhibition in the rate of NBT reduction by SOD present in the enzyme source was measured. A unit (U) of SOD activity was defined by the 50% inhibition of NBT. SOD activity was expressed in U/mg protein.

Glutathione peroxidase

GPx activity was measured by the method of Paglia and Valentine (1967). Briefly, the reaction mixture contained aliquot of PMS in 50 mM $K_2PO_4/KHPO_4$ buffer (pH = 7.0), 1.0 mM EDTA, 1.0 mM NaN_3, 0.2 mM NADPH, 1.0 U glutathione reductase, and 1.0 mM GSH. The reaction mixture was allowed to equilibrate at 25°C for 5 min. The reaction was started by introducing 0.1 mL of 2.5 mM H_2O_2. Increase in absorbance at λ_{max} = 340 nm was recorded for 5 min. The change in absorbance was defined as nanomoles of NADPH oxidized to NADP; Σ = 6.2×10^3 M^{-1} cm^{-1} at λ_{max} = 340 nm. The levels of GPx were expressed in terms of nmole NADPH consumed/min/mg protein (U/mg protein).

Catalase

Measurement of PMS fraction CAT activity was according to the method of Luck (1963). The final reaction volume of 3.0 mL contained 0.05 M Tris-buffer, 5 mM EDTA (pH = 7.0), and 10 mM H_2O_2 (in 0.1 M $K_2PO_4/KHPO_4$ buffer; pH = 7.0). A hundred microliters (100 µL) aliquot of the PMS fraction was added to the above mixture. The rate of change of absorbance per min at λ_{max} = 240 nm was recorded for 5 min. CAT activity was calculated using Σ = 43.6 $M^{-1}cm^{-1}$ and expressed in terms of mole H_2O_2 consumed/min/mg protein (U/mg protein).

Reduced glutathione

Level of GSH in organ homogenate was determined by the procedure according to Moron et al. (1979) with minor modification. The 100 µL aliquot of the PMS fraction was mixed with 25% of $CHCl_3$ and centrifuged at 2000 ×*g* for 15 min to precipitate proteins. The supernatant was aspirated and diluted to 1.0 mL with 0.2 M $Na_2PO_4/NaHPO_4$ buffer (pH = 8.0). Later, 2.0 mL of 0.6 mM 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB) was added. The absorbance of the developed yellow-colour complex maintained at 25 ± 5°C was measured at λ_{max} = 405 nm after 10 min. A standard curve was obtained with standard GSH. The level of GSH was expressed as µgGSH/mg protein.

Statistical analysis

The data collected were analyzed by the analysis of variance procedure while treatment means were separated by the least

Table 1. Serum γ-GT, ALT and AST activities of normal and hyperlipidemic rats treated with *A. sativa* extract.

Group	Enzyme activity (U/L)					
	t = 0 h			*t* = 76 h		
	γ-GT	ALT	AST	γ-GT	ALT	AST
C1	17.89±0.95[a]	44.09±1.04[a]	94.98±1.35[a]	17.78±0.75[a]	46.14±1.64[a]	98.18±1.81[a]
C2	38.08±1.05[b]	75.68±0.95[b]	123.68±1.99[b]	41.09±1.01[b]	68.07±1.04[b]	124.94±2.64[b]
T1	39.99±1.00[b,,c]	71.89±1.57[b,c]	130.80±0.94[b,c]	27.06±1.96[c]	55.67±1.25[c]	115.89±1.95[b,c]
T2	40.49±0.68[b,c,d]	73.91±1.05[b,c,d]	125.23±1.22[b,c,d]	25.41±0.77[c,d]	50.03±1.75[c,d]	109.96±1.62[b,c,d]
T3	41.43±0.99[b,c,d,e]	75.11±0.98[b,c,d,e]	123.70±1.09[b,c,d,e]	20.98±0.92[a,e]	47.09±0.99[a,d,e]	102.08±1.91[d,e]

The mean ± S.D of three (*n* = 3) determinations. Means in the columns with the same letter are not significantly different at $p > 0.05$ according to LSD. γ-GT, Levels of γ-glutamyl transferase; ALT, alanine transaminase; AST, aspartate transaminase.

significance difference (LSD) incorporated in the statistical analysis system (SAS) package of 9.1 version (2006).

RESULTS

At the end of the experimental time, *t* = 76 h, serum γ-GT, ALT and AST activities of group C1 ($C1_{\gamma\text{-GT}}$, $C1_{ALT}$ and $C1_{AST}$) did not show significant difference ($p > 0.05$) compared to corresponding enzyme activities at *t* = 0 h. Table 1 shows that the ratio of $C1_{ALT}$ activity to $C1_{AST}$ activity at *t* = 0 h and *t* = 76 h was 1:2 approx. In addition, relative marginal variation in $C1_{\gamma\text{-GT}}$ activity within the experimental time was 0.73%; $p > 0.05$. Although γ-GT, ALT and AST activities of group C2 were significantly ($p < 0.05$) elevated compared to group C1, group C2 exhibited marginal variations in the three serum enzyme activities at *t* = 76 h compared to the values at *t* = 0 h; increase in $C2_{\gamma\text{-GT}}$ activity = 7.90%, decrease in $C2_{ALT}$ activity = 10.06% and increase in $C2_{ALT}$ activity = 1.02%. However, group C2 serum γ-GT, ALT and AST activities were relatively elevated at *t* = 76 h compared to groups C1, T1, T2 and T3. Specifically, at *t* = 76 h, $C2_{\gamma\text{-GT}}$, $C2_{ALT}$ and $C2_{AST}$ activities represented 2.31, 1.48 and 1.27 folds increase in corresponding enzyme activity compared to group C1; $p < 0.05$.

At the beginning of the experiment; that is, at *t* = 0 h, serum γ-GT, ALT and AST activities of rats in groups C2, T1, T2 and T3 were comparatively not significantly ($p > 0.05$) different. The three serum enzyme activities were within the range: γ-GT = 38.08 ± 1.05 - 41.43 ± 0.99 U/L; ALT = 71.89 ± 1.57 - 75.68 ± 0.95 U/L and AST = 123.68 ± 1.99 - 130.80 ± 0.94 U/L (Table 1). Furthermore, within the experimental time, serum γ-GT, ALT and AST activities of groups C2, T1, T2, and T3 were significantly different ($p < 0.05$) compared to group C1. Specifically, $C2_{\gamma\text{-GT}}$ activity represented 2.4 folds increase compared to $C1_{\gamma\text{-GT}}$ activity at *t* = 76 h; $p < 0.05$. Again, at *t* = 0 h, serum γ-GT, ALT and AST activities of groups T1, T2, and T3 were significantly different ($p < 0.05$) compared to group C2; whereas, at *t* = 76 h, the three serum enzymes activities were not significantly different ($p > 0.05$).

Although at *t* = 76 h, $T1_{\gamma\text{-GT}}$ activity was significantly elevated compared to $C1_{\gamma\text{-GT}}$ activity, serum $T1_{\gamma\text{-GT}}$ represented 32.33% decrease in enzyme activity relative to $T1_{\gamma\text{-GT}}$ activity at *t* = 0 h. Likewise, decreases in serum enzyme activities at *t* = 76 h relative to *t* = 0 h were: $T1_{ALT}$ activity = 22.56% and $T1_{AST}$ activity =11.40%. The reduction in serum enzyme activities in group T2 was in the order: $T2_{\gamma\text{-GT}}$ activity = 37.24% > $T2_{ALT}$ activity = 32.31% > $T2_{AST}$ activity = 12.19%. $T3_{\gamma\text{-GT}}$ activity at *t* = 76 h represented 2 folds decrease compared to $T3_{\gamma\text{-GT}}$ activity at *t* = 76 h. $T3_{ALT}$ and $T3_{AST}$ activities at *t* = 76 h decreased by 1.60 and 1.20 folds respectively, compared to corresponding enzyme activity at *t* = 0 h.

Although, $T1_{ALT}$ activity and was not significantly different ($p > 0.05$) from $T2_{ALT}$ activity; these values represented corresponding 18.29 and 26.57% reduction in enzyme activities relative to $C1_{ALT}$ activity; $p < 0.05$. Conversely, $T3_{ALT}$ activity = 47.09 ± 0.99 U/L < $C1_{ALT}$ activity = 46.14 ± 1.64 U/L; $p > 0.05$ (Table 1). Likewise, $T3_{ALT}$ activity was not significantly different ($p > 0.05$) from $T2_{ALT}$ activity. Peak value of serum AST activity was registered in group C2; $C2_{AST}$ activity = 124.94 ± 2.64 U/L (Table 1). Serum AST activity was in the order: $T1_{AST}$ activity = 115.89 ± 1.95 U/L > $T2_{AST}$ activity = 109.96 ± 1.62 U/L > $T3_{AST}$ activity = 102.08 ± 1.91 U/L (Table 1). These values corresponded to 7.34, 11.99 and 18.30% reduction in $T1_{AST}$, $T2_{AST}$ and $T3_{AST}$ activities respectively, compared to $C2_{AST}$ activity.

Furthermore, compared to $C_{2\gamma\text{-GT}}$ activity, $T1\gamma_{\text{-GT}}$ activity was lower ($p < 0.05$), which was 59.20% reduction in enzyme activity. However, $T1_{\gamma\text{-GT}}$ activity was raised compared to $C1_{\gamma\text{-GT}}$ activity; *t* = 76 h, $T1_{\gamma\text{-GT}}$ activity = 27.06 ± 1.96 U/L > $C1_{\gamma\text{-GT}}$ activity =17.78 ± 0.75 U/L; $p < 0.05$ (Table 1). $T2\gamma_{\text{-GT}}$ activity was lower than $T1_{\gamma\text{-GT}}$ activity by 6.10%; $p > 0.05$. Nevertheless, $T2_{\gamma\text{-GT}}$ activity was significantly ($p < 0.05$) lower than $C2_{\gamma\text{-GT}}$ activity. $T3\gamma_{\text{-GT}}$ activity was not significantly ($p > 0.05$) different from $C1_{\gamma\text{-GT}}$ activity; specifically, $T3_{\gamma\text{-GT}}$ activity = 20.98 ± 0.92 U/L >$C1_{\gamma\text{-GT}}$ activity =17.78 ± 0.75 U/L; *t* = 76 h (Table 1). $C2_{ALT}$ activity was highest, representing 1.48 folds increase in enzyme activity compared to $C1_{ALT}$ activity ($p < 0.05$).

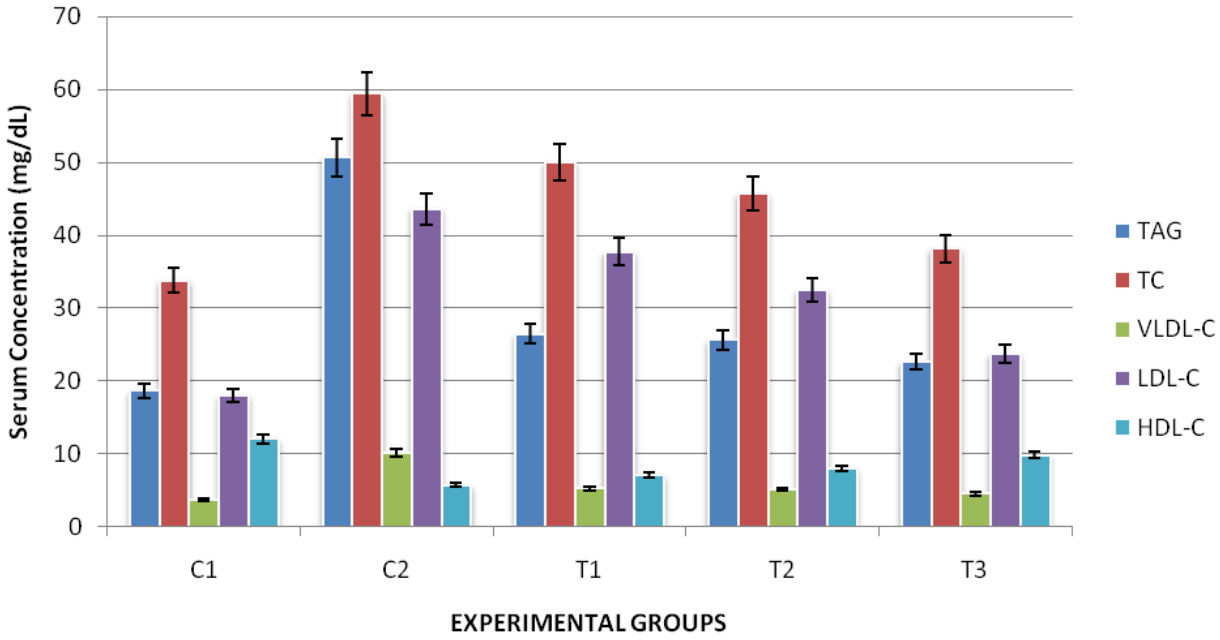

Figure 1. SLP at *t* = 76 h of normal and hyperlipidemic rats treated with *A. sativa* extract. TC, total cholesterol; TAG, triacylglycerol; VLDL-C, very low density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol.

Table 2. Atherogenic index at *t* = 76 h of normal and hyperlipidemic rats treated with *A. sativa* extract.

Group	C1	C2	T1	T2	T3
AI	0.54	7.90	2.74	2.20	1.30

SLP indicated $C1_{TC}$ = 33.75±1.02 mg/dL (Figure 1), of which serum concentrations of VLDL-C, LDL-C and HDL-C accounted for 11.02, 53.21 and 35.76% of TC concentration respectively; AI = 0.54 (Table 2). $C2_{SLP}$ showed that serum lipids concentrations were profoundly altered. For instance, serum TAG, TC, VLDL-C and LDL-C concentrations were significantly ($p< 0.05$) elevated in group C2 by factors of 2.72, 1.76, 2.72 and 2.42 respectively, compared to group C1. The reduced levels of serum HDL-C in group C2 caused corresponding increase in AI (Table 2). Generally, $T1_{SLP}$ was not significantly different ($p> 0.05$) from $T2_{SLP}$. However, these values represented significant ($p< 0.05$) alteration in $T1_{SLP}$ compared to $C1_{SLP}$. The use of group C2 as reference point indicated decreased $T1_{TAG}$ and $T1_{VLDL-C}$ ($p< 0.05$); whereas, $T1_{TC}$ and $T1_{LDL-C}$ ($p> 0.05$). Furthermore, $T1_{HDL-C}$ was elevated ($p> 0.05$). Accordingly, $T2_{SLP}$ was significantly different ($p < 0.05$) from $C2_{SLP}$. Conversely, $T3_{SLP}$ showed no significant difference ($p> 0.05$) compared to $T2_{SLP}$, except in LDL-C concentration. An overview of Table 2 shows that the AI was in the order: C2 > T1 > T2 > T3 > C1.

Hepatocyte $C2_{SOD}$ gave the highest level of enzyme activity, representing 3.77 folds increase in activity compared to $C1_{SOD}$ activity ($p< 0.05$). Furthermore, hepatocyte $T1_{SOD}$, $T2_{SOD}$ and $T3_{SOD}$ exhibited elevated activities, which was significantly different ($p< 0.05$) from $C1_{SOD}$ activity. However, hepatocyte $T1_{SOD}$ and $T2_{SOD}$ activities were reduced compared to $C2_{SOD}$ activity ($p> 0.05$). Specifically, $T3_{SOD}$ activity gave 0.77±0.07 U/mg protein (Table 3), corresponding to 21.48% reduction in SOD activity compared to $C2_{SOD}$ activity. $C2_{GPx}$, $T1_{GPx}$, $T2_{GPx}$ and $T3_{GPx}$ activities were reduced relative to $C1_{GPx}$ activity. GPx showed progressive increase in enzyme activity in the order: $T3_{GPx}$ = 7.09±0.08 U/mg protein> $T2_{GPx}$ = 6.440.09U/mg protein> $T1_{GPx}$ = 6.39±0.14U/mg protein> $C2_{GPx}$= 4.90±0.10 U/mg protein (Table 3). A cursory look at Table 3 shows that hepatocyte CAT activity of the various experimental groups followed the same pattern as hepatocyte GPx activity. $T1_{CAT}$, $T2_{CAT}$ and $T3_{CAT}$ activities were reduced compared to $C1_{CAT}$ activity ($p> 0.05$). However, levels of activity of $T1_{CAT}$, $T2_{CAT}$ and $T3_{CAT}$ were not significantly different ($p> 0.05$).

Table 4 shows that hepatocyte level of $C2_{LPOx}$ doubled that of $C1_{LPOx}$. However, levels of $T1_{LPOx}$, $T2_{LPOx}$ and $T3_{LPOx}$ were not significantly different ($p> 0.05$), but with values significantly lower than those of $C2_{LPOx}$; $p< 0.05$ and $C1_{LPOx}$; $p > 0.05$. Level of $C2_{GSH}$ was relatively

Table 3. Effects of *A. sativa* extract on hepatocyte SOD, GPx and CAT activities at *t* = 76 h.

Group	SOD	Enzyme activity (U/mg protein)	
		GPx	CAT
C1	0.26±0.05[a]	14.89±0.04[a]	8.17±0.51[a]
C2	0.98±0.04[b]	4.90±0.10[b]	4.51±0.31[b]
T1	0.87±0.02[b,c]	6.39±0.14[b,c]	5.17±0.34[b,c]
T2	0.89±0.05[b,c,d]	6.44±0.09[b,c,d]	5.49±0.39[b,c,d]
T3	0.77±0.07[c,d,e]	7.09±0.08[b,c,d,e]	5.97±0.31[b,c,d.e]

The mean ± S.D of three (*n* = 3) determinations. Means in the columns with the same letter are not significantly different at $p > 0.05$ according to LSD.

Table 4. Effects of *A. sativa* extract on hepatocyte LPOx and GSH levels at *t* = 76 h.

Parameter	Group				
	C1	C2	T1	T2	T3
[LPOx]	9.31±0.84[a]	18.82±0.65[b]	12.16±0.57[b,c]	11.96±0.63[b,c,d]	10.52±0.77[b,c,d,e]
[GSH]	19.56±0.95[a]	6.41±0.11[b]	9.05±0.35[b,c]	10.32±0.85[c,d]	12.78±0.55[c,d,e]

The mean ± S.D of three (*n* = 3) determinations. Means in the rows with the same letter are not significantly different at $p > 0.05$ according to LSD. [GSH] = µgGSH/mg protein; [LPOx] = nM of TBARS per 90 min/mg protein.

lowest, whereas $C1_{GSH}$ registered the highest concentration. Table 4 shows progressive increase in levels of hepatocyte GSH in the order: $T3_{GSH}$ =12.78±0.55 µgGSH/mg protein> $T2_{GSH}$ = 10.32±0.85µgGSH/mg protein> $T1_{GSH}$ =9.05±0.35 µgGSH/mg protein, $p > 0.05$.

DISCUSSION

Short-term administration of CCl_4 to the experimental rats induced hepatocellular damage typified by raised levels of diagnostic liver functional enzymes in serum; γ-GT, ALT and AST (Table 1). The measurement of serum γ-GT, ALT and AST activities as a basis for ascertaining and confirmation of hepatocellular damage and dysfunction have been widely reported (Sugatani et al., 2006; Abdel-Moneim and Ghafeer, 2007; Shaker et al., 2010; Singh et al., 2011; Al-Dosari, 2011). The serum γ-GT, ALT and AST activities in groups T1, T2 and T3 relative to the group C2 was obvious indication of improvement of functional status of rats in groups T1, T2 and T3. The results of the present study confirm ROS as promoters of hepatic damage, which was indicated by disturbances in antioxidant defense systems and alterations of biopsy oxidative stress indicators. The mechanism by which CCl_4 compromised hepatic functionality and integrity was previously suggested by Shaker et al. (2010). They reported that the biotransformation of CCl_4 caused the production of highly unstable free radicals (CCl_3 or CCl_3O_2) that engendered endoplasmic reticulum lipid peroxidation and cellular damage. Mayes (1983) in another report stated that the short-term hepatotoxic effect of CCl_4 was because of the capability of CCl_4 to inhibit secretory mechanisms and conjugation of lipids with apolipoproteins within the hepatocytes and thereby causing fatty liver. In this regard, preceding studies have revealed distortions in plasma lipoproteins and lipid profile in animals with induced hepatocellular damage or impairments (Ooi et al., 2005; Jiang et al., 2006; Ramcharran et al., 2011). The reports presented here show perturbation in SLP patterns in the experimental rats, which was in concordance with previous observations. The alterations in SLP were reflections of compromised structural and functional integrity of the hepatocytes. Ooi et al. (2005) had previously suggested the clinical representation of serum low levels of HDL-C as a reflection of pathologic conditions and evaluation of severity of hepatic dysfunction.

The hyperlipidemic ameliorative property of *A. sativa* extract is exemplified by its serum TC, TAG, VLDL-C and LDL-C lowering effect in a dose dependent manner (Figure 1) in the experimental rat groups (T1 and T2). In similar manner, Lau et al. (1983) had demonstrated by animal and human studies that component of garlic extract lowered plasma TC and TAG levels with changes in blood lipoproteins and coagulation parameters. They further posited that available data suggested that garlic may be of value in either the prevention or treatment of atherosclerotic diseases.

In another study, El-Demerdash et al. (2005) reported the presence of cysteine derivatives, notably, S-alkyl cysteine sulfoxides in *A. sativa*. They noted that during extraction process, these compounds are converted by allinase to thiosulfinates and polysulfides compounds,

which possess hypocholesterolaemic, antidiabetic, antibiotic and fibrinolytic properties.

The pattern of AI of the various experimental groups (Table 2) showed the propensity of hyperlipidemia, occasioned by hepatic injury and dysfunction, to promote atherogenic conditions. Studies have confirmed that hyperlipidemia elicits oxidative stress in organs such as the heart, kidney and liver (Suanarunsawat et al., 2011; Shaker et al., 2010), which is a major contributing factor in the etiology of atherosclerosis, hypertension, diabetes and several degenerative diseases (Vijayakumar et al., 2004; Du et al., 2010). In addition, ROS cause the oxidation of LDL-C, engendering cytotoxic events in endothelial cells and selective accumulation of modified LDL-C (Torres et al., 1999). This pathologic event is one of the various major contributing and causative factors of atherosclerosis. The present study shows the capacity of *A. sativa* extract to reverse oxidative stress and hyperlipidemia in the experimental rats (T1 and T2), which was comparable to those treated with the standard hypolipidemic drug-hepaticum (T3). However, the short-term treatments did not provide for the experimental animals the requisite and anticipated full therapeutic benefits. Nevertheless, previous authors have reported the therapeutic usefulness of *A. sativa* in the treatment and management of cardiovascular diseases (Mahmoodi et al., 2006), hypertension (Benavides et al., 2007), Alzheimer's disease (Peng et al., 2002), inflammation, thrombosis (Fukao et al., 2007) malignancy (Hsing et al., 2002) fatty liver (Sahebkar, 2011) and as antimicrobial (Gull et al., 2012).

Liver biopsy showed perturbations of enzymatic (SOD, GPx and CAT) and non-enzymatic (LPOx and GSH) oxidative stress indicators of experimental rats (Tables 3 and 4). In agreement with the present findings, Đurendić-Brenesel et al. (2013) reported increased SOD activity in the liver homogenates of the hyperlipidemic rats (Table 3). The reduced levels of $C2_{GPx}$ and $C2_{CAT}$ activities were the effect of raised and overwhelming levels of ROS (El-Demerdash et al., 2005; Avti et al., 2006); ROS has inhibitory effect on ROS scavenging enzymes such as CAT and GPx activities (Hassan and Fridovich, 1978; Avti et al., 2006). Consequently, raised levels of cytotoxic ROS engendered membrane lipid peroxidation with the production of associated by-products such as malondialdehyde (MDA) and 4-hydroxyalkenals (4HNE) (Shaker et al., 2010; Al-Dosari, 2011; Đurendić-Brenesel et al., 2013).

Depleting $C2_{GSH}$ concentration confirmed increased oxidative stress (Surapaneni, 2007; Abdel-Moneim and Ghafeer, 2007) mediated by ROS oxidation of sulfhydryl groups that are essential in cellular enzymatic cofactor and non-enzymatic reduction pathways. The present investigations show that administration of *A. sativa* extract caused relief in oxidative stress to the experimental rats, indicated by decreased SOD but increased GPx and CAT activities, coupled with decreased LPOx but increased GSH content in groups T1 and T2 compared to group C2 (Tables 3). Equally, oxidative stress indicators showed that short-term administration of *A. sativa* extract did not restore full therapeutic benefits to the experimental rats. However, the capacities of the two experimental doses (200 and 400 mg/kg) of *A. sativa* extractto ameliorate oxidative stress were comparable to the standard hepatic drug-hepaticum.

Previous studies have shown that Buckwheat (*Fagopyrum esculentum*) (Đurendić-Brenesel et al., 2013), *Ocimum sanctum* L. (Suanarunsawat et al., 2011) and Roselle (*Hibiscus sabdariffa* Linn) (Ochani and D'Mello, 2009) share similar antioxidant phytochemical profile with *A. sativa* extract (Ibegbulem and Chikezie, 2013). Accordingly, the presence of phytochemicals such phenolics, tannins and flavonoids in *A. sativa* extract, coupled with high content of antioxidant element-selenium (http.//www.complete-herbal.com/details/garlic.htm; Banerjee and Maulik, 2002) contributed to the antioxidant property of*A.sativa* extract.

Although the administration of *A. sativa* extract to the rats did not restore full therapeutic benefits within the experimental time (t = 76 h), the capacity of the plant extract to ameliorate oxidative stress and hyperlipidemia in the animals was fairly at par with the standard hepatic drug-hepaticum.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Antiproliferative activity of cytotoxic tuber lectins from *Solanum tuberosum* against experimentally induced Ehrlich ascites carcinoma in mice

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Cytotoxicity of tuber lectins from two potato cultivars was assessed and their anti-tumor potential against experimentally induced Ehrlich ascites carcinoma in Swiss albino mice was evaluated. Twenty (20) kDa chitin-binding lectins from *Solanum tuberosum* tubers, STL-S and STL-D were purified through ion-exchange and affinity chromatographic methods, hemagglutinating activity and blood group specificity of the lectins were checked whereas the cytotoxicity was determined using brine shrimp (*Artemia salina* L.) nauplii lethality assay. The lectins showed no specificity to animal and human erythrocytes. LC_{50} values for STL-S and STL-D were found to be 75 and 90 µg/ml, respectively with a dose-dependent intermediary toxic effect. After inducing ascites by intraperitoneal propagation, the Swiss albino mice were treated by administering the lectins at a dose of 1.38 mg/kg/day for five consecutive days. STL-S and STL-D showed 79.84 and 83.04% of growth inhibition of EAC cells, respectively. Additionally, hemoglobin and RBC levels became considerably increased with a drop off in the WBC levels in the treated mice group indicating moderate anticancer activities exhibited by the potato lectins.

Key words: Chitin-binding lectins, antitumor activity, LC_{50}, cell growth inhibition.

INTRODUCTION

Plant lectins have been widely studied for pharmacological applications as well as their immuno-potentiating and anti-tumor activities (Karasaki et al., 2001; Suen et al., 2000; Chang et al., 2007; Li et al., 2011; Dhuna et al., 2005; Liu et al., 2009; Zhang et al., 2010; Faheina-Martins et al., 2011) due to their effects on animal cells.

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Abbreviations: STL-S and STL-D, Lectins from potato cultivars Sheelbilatee and Deshi, respectively; **GlcNAc,** *N*-acetyl D-glucosamine; **DEAE,** diethylaminoethyl; **SDS-PAGE,** sodium dodecyl sulfate polyacrylamide gel electrophoresis; **EAC,** Ehrlich's ascites carcinoma; **RBC,** red blood cells; **WBC,** white blood cells; **LC_{50},** lethal concentration 50 (concentration in water having 50% chance of causing death to aquatic life).

Lectins from different sources were found to have an effect on cell functions. There are well-established relationships in their biological effects on a number of tumor cell lines.

The degree of glycosylation of malignant tumors is related with metastasis and differs from benign cells. This difference can be identified by lectins through specific recognition to glycoconjugates.

Particularly, plant lectins have antitumor activities as those can bind to cancer cell membrane proteins and receptors. They can inhibit cell growth or kill the cells by creating cytotoxic environment and through the activation of apoptotic pathways (Kiss et al., 1997; De Mejia and Prisecaru, 2005; Ferriz-Martinez et al., 2010). Lectins from different sources inhibit cancer cells growth depending on their concentration. At lower concentrations, they exert an immunostimulatory effect whereas at high concentrations, a cytotoxic effect is shown to take place (Lyu et al., 2001, 2002; Pryme and Bardocz, 2001).

Plant lectins can be subdivided into four major families; the legume lectins, the chitin-binding lectins composed of hevein domains, the type 2 ribosome-inactivating proteins and the monocot mannose-binding lectins. Each family has its own characteristic carbohydrate recognition domain. Chitin-binding lectins can bind to chitin oligomers or *N*-acetyl-chito-oligosaccharides (GlcNAc)n, where, n = 2 to 5 (Van Damme et al., 1998) and have distinct biological activities including antineoplastic activities (Abdullaev and de Mejia, 1997; Yao et al., 2010; Wang et al., 2000).

A number of chitin-binding lectins are found in Gramineae and Solanaceae species (Van Damme et al., 1998). There are a variety of lectins recognizing oligomeric *N*-acetyl D-glucosamine (GlcNAc) as chitobiose (GlcNAc disaccharide), chitotriose (GlcNAc trisaccharide) and chitin (GlcNAc polymer) in fruit bodies and tubers of the plant family Solanaceae. In case of potatoes (*Solanum tuberosum* L.), there are multiple varieties of chitin-binding lectins with the molecular mass of 45 to 65 kDa in different cultivars (Allen et al., 1996). They were specifically reported to be glycoproteins with an unusual ratio of glycan portions of 50% by weight.

In Bangladesh, potato is regarded as one of the main foods and about 27 cultivars with familiar local names are cultivated in different parts of the country according to cooking habits with different physiological and morphological properties (Salahuddin, 2000). The cultivar named 'Deshi' is a representative variety in this country with relatively small size (2 to 4 cm in diameter) and bright red in color whereas the Sheelbilatee cultivar is more elongated and irregular in shape. It is pale red and stickier than the Deshi cultivar.

As the detailed characterization of pharmacological activities of these varieties have not been reported in literature, the present work describes isolation of chitin-binding lectins from two local potato prototypes cultivated in Bangladesh and their significant *in vivo* antiproliferative activities against Ehrlich ascites carcinoma cells in mice.

MATERIALS AND METHODS

Materials

The tubers of indigenous potato cultivars 'Sheelbilatee' and 'Deshi' were collected from Rangpur and bought from the local market in Rajshahi, respectively. The specimens were identified by Prof. M. Manzoor Hussain, Department of Botany, University of Rajshahi, Bangladesh. Potato tubers were stored in a refrigerator at 4°C.

Chemicals and reagents

DEAE-cellulose and chitin were procured from Wako Pure Chemical Industries Ltd. (Osaka, Japan). All other chemicals and reagents used throughout this study were of analytical grade from BDH, UK; Merck, Germany and Sigma Aldrich, US.

Test animals and ethical clearance

Adult male Swiss albino mice, 6 to 8 weeks old with 25 (± 5) g body weight were bought from animal resource branch of the International Centre for Diarrheal Disease Research, Bangladesh (ICDDR'B). The protocol was approved by the Institutional Animal, Medical Ethics, Bio-safety and Bio-security Committee (IAMEBBC) for experimentations on animals, humans, microbes and living natural sources (286/320 -IAMEBBC/IBSc), Institute of Biological Sciences, University of Rajshahi, Bangladesh. Animals were housed in polypropylene cages containing sterile paddy husk as bedding material under hygienic conditions with a maximum of 10 animals in a cage. They were maintained under standard laboratory conditions (temperature 25±2°C, relative humidity 48% with dark and light cycle 12/12 h). The mice were allowed free access to standard dry mice food-pellets and water *ad libitum*. The mice were acclimatized to laboratory conditions for 10 days before commencement of the experiment.

Purification of potato lectins

300 g of potato tubers were peeled off, sliced and homogenized in 10 mM Tris-HCl (pH 8.2) containing 50 mM NaCl. The homogenates were centrifuged at 12,000 × *g* for 20 min; the supernatants were collected and dialyzed overnight against distilled water and 10 mM Tris-HCl buffer at 4°C. Crude supernatants were applied to diethylaminoethyl (DEAE)-cellulose column (2 × 25 cm) and eluted by a linear gradient of 0 to 400 mM NaCl containing 10 mM Tris-HCl buffer. The fractions (2.5 ml each) having hemagglutination activity were subjected on to a chitin column (2 × 25 cm) previously equilibrated with the same buffer and were eluted by 0.5 M acetic acid (Allen et al., 1996; Allen and Neuberger, 1973). The pH of the eluted fractions was neutralized by the addition of the aliquot of 1 M Tris-HCl buffer (pH 8.2) in each tube and then dialyzed overnight. The molecular mass and hemagglutination activity of the purified lectins from both potato cultivars were determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using 15% separating gel under reducing condition (Laemmli, 1970).

Hemagglutination assay and blood group specificity

The hemagglutination assay was performed in 96-well microtiter U-bottomed plates in a final volume of 100 µl containing 50 µl of protein solution serially diluted with equal amount of hemagglutination buffer (20 mM Tris-HCl buffer, pH 7.8 containing 0.9% NaCl and 10 mM $CaCl_2$) and 50 µl of 2% red blood cells (RBCs)

suspension previously washed with 0.15 M NaCl. After a gentle shaking, the plate was kept at room temperature for 30 min. The visual agglutination titer of the maximum dilution giving the positive agglutination was recorded.

RBCs of human (A, B, AB and O groups), rat, chicken and cow were collected, washed in Tris-buffered saline (10 mM Tris-HCl, 150 mM NaCl. pH 8.2) and centrifuged at 3,000 rpm for 10 min. The erythrocyte pellet was washed thrice and re-suspended in the same saline to make a 2% RBC suspension.

Brine shrimp nauplii lethality assay

Lethality assay was studied using brine shrimp (*Artemia salina* L.) nauplii. The *Artemia* cysts were hatched in artificial seawater at 28°C under constant light and aeration. The artificial sea water was prepared by dissolving 38 g of NaCl in 1 L of DW and pH was adjusted to 7.0 by sodium tetraborate. The cysts were incubated in a glass tube with 1 g cysts per liter of artificial seawater. After a period of 48 h, the aeration was halted, and the light was directed to the bottom of the tube. The phototropic nature of nauplii caused them to migrate in the direction of light toward the bottom of the tube, facilitating their separation from unhatched cysts. 60, 125, 250, 500, and 1000 µl of STL-S and STL-D vials were taken containing artificial sea water and 10 *Artemia* nauplii were added to each vial. Finally the volume of each vial was adjusted to 4 ml by the addition of artificial sea water. The experiments were performed in triplicate, and negative control was the artificial water and the *Artemia* nauplii in the absence of the lectin. After 24 h, the number of dead nauplii was counted for each concentration and the LC_{50} values were determined by using Probit analysis as described by Finney (Finney, 1971).

Ehrlich's ascites carcinoma (EAC) cell growth inhibition assay

Ehrlich ascites carcinoma cells were maintained in Swiss albino mice by a bi-weekly intraperitoneal propagation. The cells were diluted with normal saline and adjusted to a number of 3×10^6 cells/ml by the help of a hemocytometer. Then, 0.1 ml of tumor cells having viability above 90% was injected intraperitoneally to each mouse. After 24 h of tumor inoculation, the mice were randomly distributed into three groups consisting of 6 mice per group. Two groups of the mice were treated with STL-S and STL-D at a concentration of 1.38 mg/kg/day for five consecutive days while the remaining group was used as a negative control (injected with 10 mM Tris-HCl buffer). Mice in each group were sacrificed on the 6^{th} day and the total intraperitoneal tumor cells were harvested by normal saline (0.98%). Viable cells were first identified with Trypan blue and then counted by a hemocytometer under an inverted microscope (XDS-1R, Optica, Italy). The percentage of inhibition was calculated by using the following formula:

Percentage of inhibition = 100 - {(cells from lectin treated mice/cells from control mice) × 100}

Hematological assay

To elucidate the cytotoxic effect of potato lectins on EAC-bearing and control mice, the study of hematological parameters were carried out. Treatment started after 24 h of tumor transplantation and continued for 10 consecutive days as the previous experiment. On the 12^{th} day of tumor transplantation, blood was collected from each mouse by tail puncture to determine the hematological parameters namely: WBC, RBC and hemoglobin content following standard methods using appropriate cell dilution fluids and hemocytometer (Rusia and Sook, 1988).

Statistical analysis

The experimental results are presented as mean ± SEM for three replicates for studied parameters. One way analysis of variance (ANOVA) was used to calculate the data followed by Dunnett 't' test using statistical package for social sciences (SPSS) software (Chicago, IL) version 10.

RESULTS AND DISCUSSION

Purification of potato lectins

The crude supernatants extracted from both potato cultivars had strong hemagglutinating activity against mice erythrocytes. In anion-exchange chromatography, the supernatants were applied to the DEAE-cellulose column and four peaks were eluted from the column by the linear gradient of NaCl concentration (0-400 mM). Peaks with significant hemagglutinating activity were collected together and applied separately on to the chitin column. After washing the chitin columns with 10 mM Tris-HCl buffer extensively, one single peak was eluted with the help of 500 mM acetic acid. Eluted fractions were collected, neutralized using 1 M Tris-HCl buffer and dialyzed against 10 mM Tris-HCl buffer. SDS-PAGE showed that, both STL-S and STL-D consisted of a major 20 kDa band with two other faint 22 and 17 kDa bands in reducing condition (Figure 1).

Twenty (20) kDa potato lectins isolated from both the cultivars were found to be non-specific to human and animal erythrocytes. Similar result was found in case of ADL which is a 15 kDa *N*-acetyl- D-glucosamine specific lectin with antiproliferative activity (Kaur et al., 2005). It also agglutinated all the tested erythrocyte samples from human and animals. Another GlcNAc-specific mitogenic lectin, BBL was reported to have this property (Banerjee et al., 2004). Besides different sugar-binding lectins like NNTL, PCSL, TRA and *Ficus cunia* lectin also possess these two common properties, non-specificity and antiproliferative activity (Kabir et al., 2011; Chen et al., 2009; Bhowal et al., 2004; Ray et al., 1992).

Hemagglutination and blood group specificity assay

STL-S and STL-D showed no specificity to any type of blood and agglutinated RBC cells from every blood type significantly. This result supports the previous findings that this lectin agglutinates erythrocytes of different animals including mice, chickens, cows, sheep, horses, rabbits as well as the human (A, B, AB and O groups) erythrocytes indicating its non-specificity to any blood group (Sharon et al., 2000). The minimum protein concentration to agglutinate Swiss albino mice erythrocytes was 4 µg/ml.

Brine shrimp nauplii lethality assay

Higher mortality rates were found for STL-S comparing to

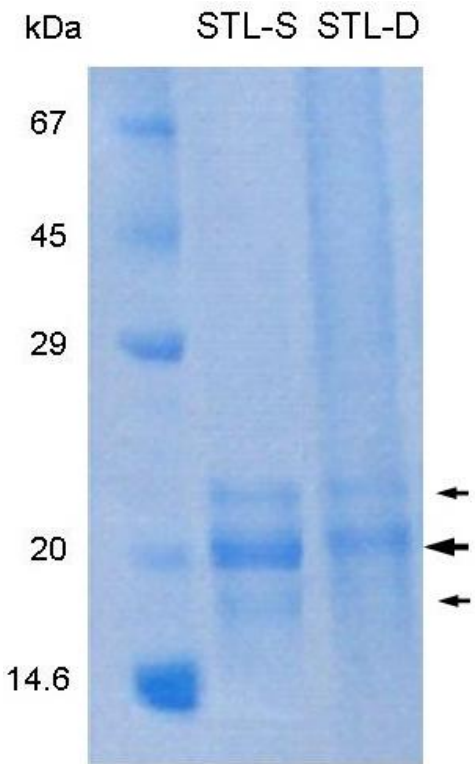

Figure 1. The molecular mass of potato lectins by SDS-PAGE. Standard protein markers: Bovine serum albumin (67 kDa), Ovalbumin (45 kDa), Carbonic anhydrase (29 kDa), Trypsin inhibitor (20 kDa) and Lysozyme (14 kDa). Lectins from Sheelbilatee potato cultivar (STL-S) and Deshi potato cultivar (STL-D): Arrows indicate major (20 kDa) and minor (22 kDa and 17 kDa) proteins.

STL-D. The mortality rate was found to be increased with the increase of concentration of the lectin as shown in Figure 2. The LC_{50} values were determined as 75 µg/ml for STL-S and 90 µg/ml for STL-D. Both these lectins have a molecular weight of around 20 kDa and showed similar inhibitory effects on EAC cell growth; but STL-S was found to be slightly more toxic than STL-D. May be this difference took place because of their genetic diversity. Plant lectins are reported with a wide range of cytotoxicity values. A lectin from *Curcuma amarissima* rhizomes showed *in vitro* antiproliferative activity against a breast cancer cell line (BT 474) with an LC_{50} value of approximately 21.1 µg (Kheeree et al., 2010). Highly toxic lectins were isolated from *Abrus pulchellus* and Mulberry seeds having LC_{50} values of 3.5 mg/ml and 21.87 µg/ml whereas other lectins from the seeds of *Dioclea lasiophylla* and tubers of *Nymphaea nouchali* had much lower values like 45.85 and 120 µg/ml, respectively (Ramos et al., 1998; Absar et al., 2005; Pinto-Junior et al., 2013; Kabir et al., 2012). Presence of a non-toxic lectin from *Sebastiania jacobinensis* bark to *Artemia nauplii* is also reported (Vaz et al., 2010). Even two lectins (Halilectin 1 and Halilectin 2) from the same source, a marine sponge, had been purified with quite different toxicities (6.4 and 142.1 µg/ml) (Carneiro et al., 2013). Comparing different LC_{50} values of these lectins, it became evident that STL-S and STL-D exhibited an intermediary toxic effect against *Artemia* nauplii.

Ehrlich's ascites carcinoma (EAC) cell growth inhibition assay

At the dose of 1.38 mg/kg/day, significant growth inhibition of tumor cells was found by STL-S and STL-D with a reduction percentage of 79.84 and 83.04% respectively. Figure 3 shows the reduced numbers of viable EAC cells in the lectin-treated mice comparing the control mice group.

Plant lectins are recently being used to evaluate novel cancer therapeutics as well as to identify predictive biomarkers in early stages of drug development (Chang and Lei, 2008). A lectin from *Ficus cunia* agglutinated EAC cells and agglutination was inhibited by chitin oligosaccharides [(l → 4) linked β-GlcNAc] and glycopeptides containing GlcNAc residues indicate the presence of these oligosaccharides as receptors (Ray et al., 1992).

Several typical GlcNAc-binding lectins such as mistletoe lectin and wheat germ agglutinin were found to possess significant antitumor activities that can stimulate apoptosis to kill the cancer cells (Liu et al., 2010). Each lectin displays variation in their antitumor activities due to the differences in sugar specificity (Yan et al., 2010). Like mistletoe lectin, STL-S and STL-D are also capable of binding with GlcNAc polymers and exerting antitumor effects.

The aggregated cancer cells may become trapped in microvascular vessels, which facilitate their extravasation. These lectins showed a resemblance with another lectin, TRA that agglutinated EAC cells with a minimum concentration of 3.12 µg/ml. The glycan part of TRA contained mannose (16.8%), GlcNAc (1.07%) and arabinose (0.67%). This may be a consequence to the change in surface structure of the tumor cells, occurring with the progress in tumor growth or because of the clustering of the lectin receptors present on cell surfaces (Alderson and Green, 1978; Mastromarino et al., 1980).

It became evident that these two potato lectins (STL-S and STL-D) had inhibitory effects on EAC cell growth (79.84 and 83.04%) at a dose of 1.38 mg/kg/day (equivalent to 50 µg/day). Some other lectins like Jackfruit lectin inhibited EAC cell growth by 21.8, 40.2 and 57.5% at 50, 100 and 150 µg/day respectively (Ahmed et al., 1988). Pea lectin showed 44 and 63% decrease in EAC cell growth at 1.4 mg/kg/day and 2.8 mg/kg/day (Kabir et al., 2013). *Nymphaea nouchali* tuber lectin (NNTL) was found to inhibit EAC cells by 56 and

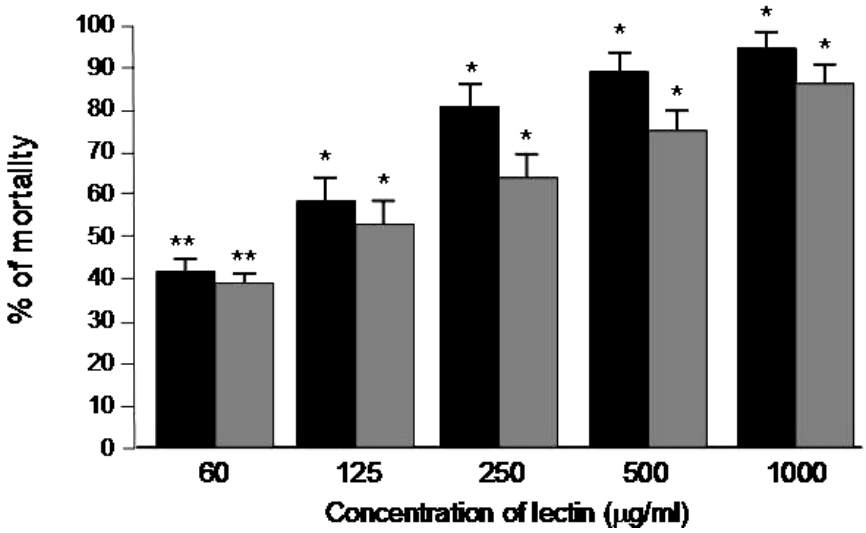

Figure 2. Toxic effect of STL-S and STL-D at different concentrations (60, 125, 250, 500 and 1000 µg/ml) on *Artemia* nauplii. Black and gray bars indicate the mortality rate for STL-S and STL-D, respectively. Error bars: SE calculated from three independent experiments (n = 6). *, P <0.05 and **, P <0.01; P <0.05 were considered statistically significant when compared with control.

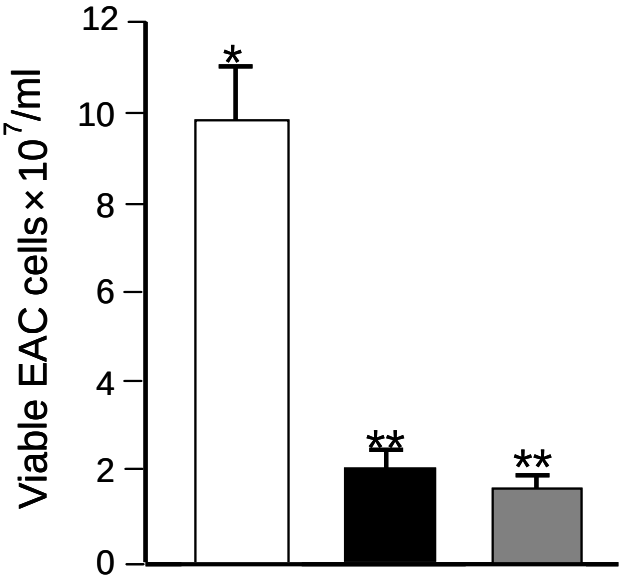

Figure 3. Number of EAC cells in non-treated (control) mice and lectin-treated mice counted by a light microscope on day 6 of tumor inoculation. White, black and gray bars indicate the values for non-treated (control), STL-S treated and STL-D treated mice, respectively. Error bars: SE calculated from three independent experiments (n = 6). *, P <0.05 and **, P <0.01; P <0.05 were considered statistically significant when compared with control.

76% at a dose of 1.5 mg/kg/day and 3.0 mg/kg/day while *Trichosanthes cucumerina* seed lectin (TCSL) showed 28 and 72% growth inhibition against the same cell line (Kabir et al., 2011; Kabir et al., 2012). All these lectins were found weaker than STL-S and STL-D in terms of activity.

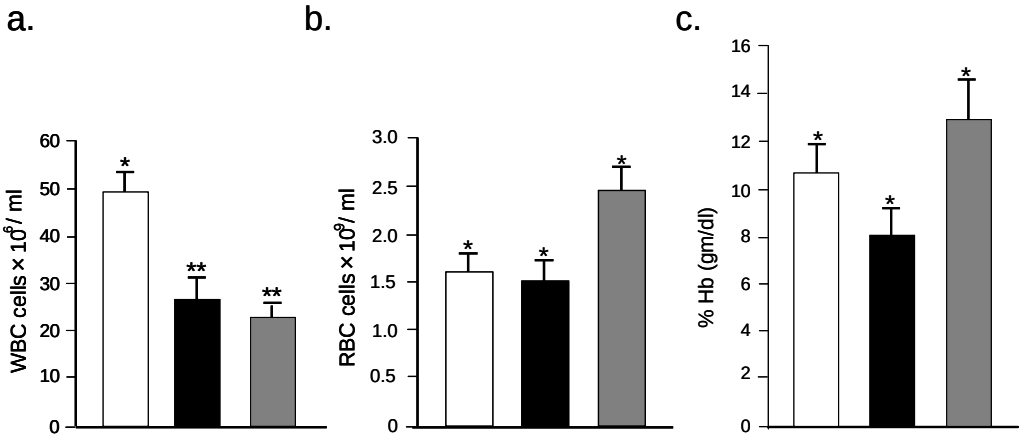

Figure 4. Hematological parameters of ECA-bearing non-treated (control) mice and lectin-treated mice on day 12 of tumor inoculation. White, black and gray bars show the values for non-treated (control), STL-S treated and STL-D treated mice, respectively. Error bars: SE calculated from three independent experiments (n = 6). *, P <0.05 and **, P <0.01; P <0.05 were considered statistically significant when compared with control.

Hematological assay

WBCs count was increased in the control mice as compared to the treated mice (Figure 4a). The RBCs count for the mice treated with STL-D was higher than the control mice but unexpectedly the mice treated with STL-S were found to have even lesser number of RBC cells than the control mice (Figure 4b). This result was corresponded by the amount of hemoglobin found in the treated mice group comparing the control group (Figure 4c).

In tumor-bearing mice, WBC count generally increases while anemia takes place due to the reduction in RBC or hemoglobin percentage and this may occur either due to iron deficiency or due to hemolytic or myelopathic conditions (Fenninger and Mider, 1954). In the present study, both STL-S and STL-D decreased WBC count in the treated mice. STL-D increased the RBC count and hemoglobin level though this phenomenon was particularly absent in case of STL-S. The additional cytotoxicity of STL-S compared to STL-D can be responsible for this. It might also have happened due to the specificity of lectin-induced cellular cytotoxicity. A lectin triggers different clones of effector cells selectively, each with its own unique cytotoxic capability. Therefore, the specificity involves more than the presence of receptors on a RBC target for any particular lectin (MacDermott et al., 1976).

Conclusion

The present study indicates that potato lectins exhibited moderate anticancer activities. To our knowledge, there is no report available discussing growth inhibition of EAC cells mediated by potato lectins. However, further investigations are required to understand these phenomena considering all the relevant hypotheses and to declare potato lectins as potent anticancer agents despite their cytotoxicity.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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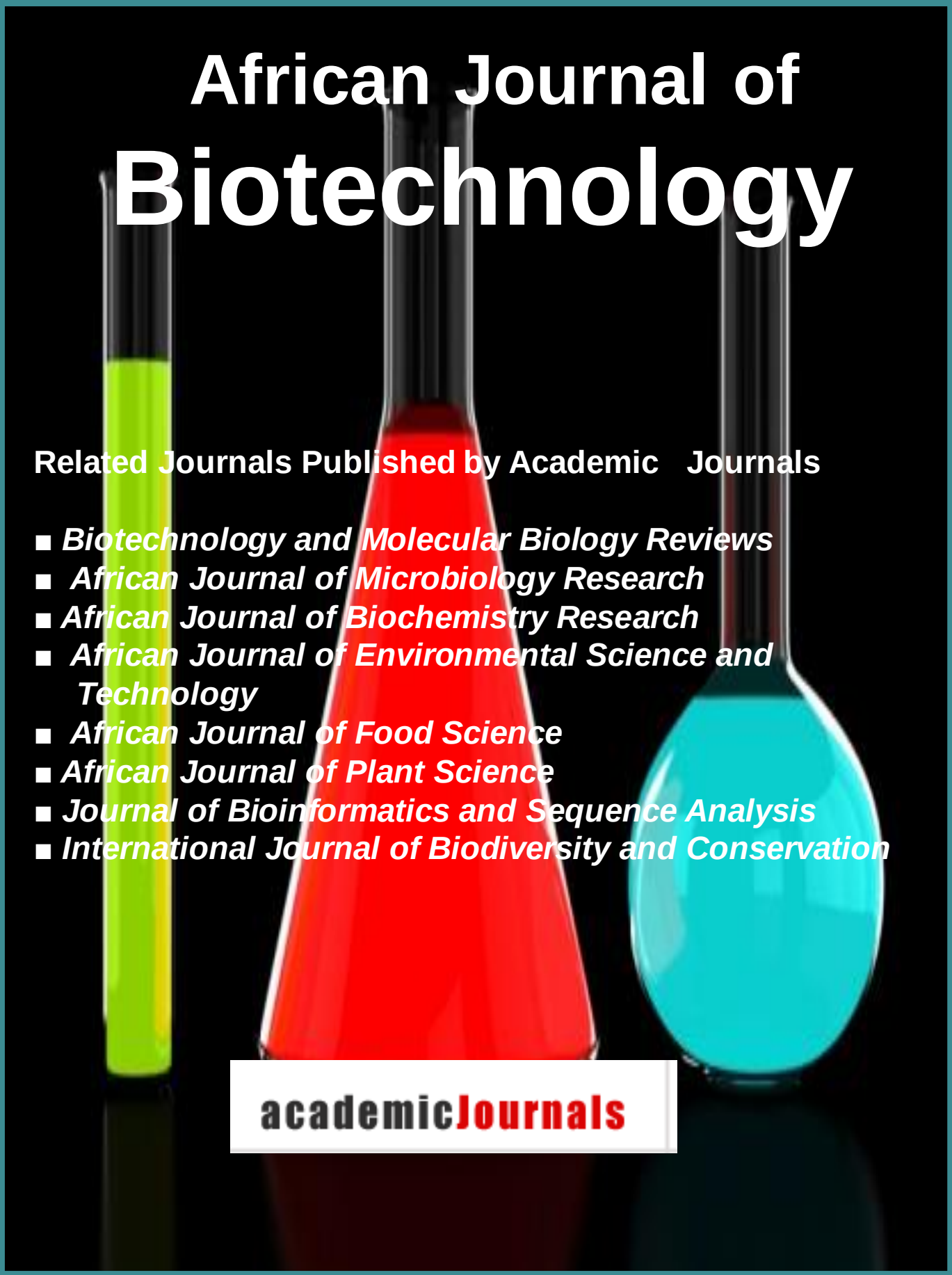

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