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Redundant and Non-Redundant Functions of Actin Depolymerizing Factor (ADF) and Cofilin in Metastasis- Review

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Abstract

Tumor cell motility is the hallmark of invasion and an essential step in metastasis. Cellular changes that occur during the progression of cancer affect proteins that drive actin dynamics; these changes modulate cell cycle progression and lead to more invasive cancers. Actin depolymerizing factor (ADF)/cofilins (actin dynamizing proteins) and their regulatory proteins are involved in the initiation of early steps in cell motility. ADF/cofilins play important roles in various stages of cancer progression including cell polarization and polarized migration, escape from apoptosis, and secretion of metalloproteases, all of which are important in metastasis. Vertebrates express ADF, cofilin-1 and cofilin-2, and even though ADF and cofilin have many qualitatively similar biochemical properties, they differ quantitatively in actin interaction and in some types of regulation and, thus, are not functionally identical. This review compares the activities of these two proteins with respect to how they may function during tumor cell invasion. Understanding the molecular pathways of tumor invasion will provide new diagnostic approaches and targets for the treatment of metastatic cancer.

الملخص

تعتبر حركة الخلايا السرطانية السمة المميزة و الخطوة الأساسية في تكوين الأورام الخبيثة. تؤثر التغيرات الخلوية التي تحدث خلال تطور السرطان في البروتينات التي تتحكم بألياف الاكتين، و هذه التغيرات تعدل من دورة حياة الخلية و تؤدي الى تحول الورم من أولي إلى ثانوي أو خبيث. تلعب البروتينات (ADF/cofilin) و البروتينات المتحكمة بهم دورا مهما في استهلال حركة الخلايا و كذلك تلعب دورا أساسيا في تطور مرض السرطان الخبيث من خلال التحكم بشكل الخلايا و هجرتها و تجنب موت الخلايا المبرمج و إفراز الإنزيمات المحللة للنسيج ما بين الخلايا. تتكون عائلة (ADF/cofilin) في الفقاريات من ثلاث بروتينات هي: ADF و cofilin-1 و cofilin-2 ، يتشابه ADF و cofilin-1في كثير من الصفات البيوكيميائية النوعية و لكن يختلفوا في الصفات البيوكيميائية الكمية وأيضا من حيث تنظيمهما مما يجعلهما غير متطابقين من حيث الوظيفة. تهدف هذه النشرة إلى مقارنة دور كل من ADF و cofilin-1 في عملية تكوين الأورام الخبيثة مما يساعد في فهم تكون الأورام الخبيثة و بالتالي على تطوير علاجات جديدة لمرض السرطان.

Keywords: Actin Dynamics, Cell Motility, Polarization, Cancer, Invadopodia, LIM Kinase, Slingshot.

1. Introduction

Cell polarization and movement are fundamental features of embryonic development in multicellular organisms. They are required for establishing tissue patterns and in other processes such as the response of macrophages to pathogens, tissue repair (Nubler-Jung *et al.*, 1987) and *in vitro* wound healing (Grande-Garcia *et al.,* 2007). They are also important for disease processes such as the metastasis of tumor cells. Cells change their shape and migrate in response to guidance cues (Nobes and Hall, 1999). It is generally accepted that cell movement is mostly dependent on the dynamic reorganization of the actin cytoskeleton, which entails polymerization of actin at the leading edge, and actin bundling and myosin-based contractility at the rear (Machesky and Cooper, 1999; Ishizaki *et al.,* 2001).

Actin polymerization and depolymerization must be regulated spatially and temporally to produce motility. Polymerization requires the formation of free barbed ends (the fast-growing ends), the production of which is tightly regulated *in vivo* (DesMarais *et al.,* 2005). New free barbed ends can arise from uncapping or severing of existing actin filaments (F-actin) and *de novo* nucleation (Zwolak *et al.,* 2010). These processes are dependent upon actin-binding proteins (ABP), of which the members of the ADF/cofilin (AC) family of proteins are essential (reviewed in Maciver and Hussey, 2002; Ono, 2003; Bernstein and Bamburg, 2010).

Vertebrates express three isoforms of the AC family: actin depolymerizing factor (ADF) (Bamburg *et al*., 1980), cofilin-1 (Nishida *et al.*, 1984) and cofilin-2 (Ono *et al.*, 1994). Most researchers working with AC proteins focus

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on the role of cofilin-1 during cell motility, mainly because cofilin is the major form present (Wang *et al.*, 2008; Oser and Condeelis, 2009; Quintela-Fandino *et al.*, 2010). However, even though ADF and cofilin have many qualitatively similar biochemical properties, they differ quantitatively in their interactions with actin as well as in some aspects of their regulation and, thus, are not functionally identical. This current review aims at comparing the activities of these two proteins during tumor cell invasion and will try to answer one important question: do ADF and cofilin have redundant and/or nonredundant activities during metastasis?

2. Cancer invasion and metastasis

Cancer is a group of diseases characterized by unregulated cell growth and invasion and a spread of cells from primary sites to other body sites. It involves dynamic changes in the genome; certain mutations produce oncogenes with dominant gain of function and tumor suppressor genes with recessive loss of function (Ruddon, 2007).

There are six hallmarks of cancer: growth signal autonomy, evasion of growth inhibitory signals, evasion of apoptosis, unlimited replicative potential, angiogenesis and invasion and metastasis (Ruddon, 2007). Metastasis remains the cause of 90% of deaths from solid tumors (reviewed in Gupta and Massagué, 2006).

Metastasis is believed to emerge from a genetically diversified cancer-cell population under the selective pressures of the surrounding environment. If proved to be true, this may explain why millions of cells might be released by a tumor into the circulation every day, but few of these cells will colonize a distant organ (reviewed in Gupta and Massagué, 2006). In order to achieve a successful metastasis, cancer cells have to invade basement membrane, intravasate into the blood stream, disseminate through the circulation, and extravasate into distant sites (Ruddon, 2007).

Invasive tumor cells acquire a migratory phenotype and exhibit a distinct gene expression profile, called the invasion signature, in which genes associated with proliferation and apoptosis are downregulated, while a set of motility-related genes are coordinately upregulated (Philippar *et al.,* 2008). In invasive mammary carcinoma cells, for example, the invasion signature includes a network of actin-regulatory proteins, including the Arp2/3 complex and cofilin that drive formation of membrane protrusions important for invasion, motility, and chemotaxis (Wang *et al.*, 2007).

3. Actin organization during metastasis

Tumor cell motility is the hallmark of invasion and an essential step in metastasis (reviewed in Gupta and Massagué, 2006). Cellular changes that occur during the progression of cancer affect proteins that drive actin dynamics; these changes modulate cell cycle progression and lead to more invasive cancers. Spatially controlled assembly of actin filaments, in response to stimuli, generates cell protrusions called lamellipodia, filopodia, and invadopodia. Motile cells use these extensions to explore the extracellular space and find their way toward their targets (reviewed in Carlier *et al.,* 2003).

3.1. Actin Cytoskeleton

3.1.1. Actin structure and dynamics

Globular (G-) monomeric actin, a 43 KDa protein, has a bound adenine nucleotide that lies in a deep cleft in the center of G-actin. It is occupied by ATP, ADP-Pi, or ADP depending on nucleotide exchange factors and the assembly state of the subunit (reviewed in dos Remedois *et al.,* 2003). Under physiological salt concentrations G-actin assembles to form F-actin (Pfaendtner *et al.,* 2010).

The conventional view of the actin filament is a two-start, right-handed long-pitch helix. In this model, there are 12-14 monomers per half turn and a half pitch of 360-390 Å. Actin filaments have a distinct structural polarity that was first noticed when the filaments were decorated with the heavy meromyosin or S1 fragments of myosin, both of which appear as arrowheads along the filament (Huxley, 1963; Nachmias and Huxley, 1970; Svitkina and Borisy, 1998), giving rise to the "barbed" and "pointed" end nomenclature used to identify opposite filament ends. Assembly studies on decorated actin filaments showed the barbed end to be the faster growing end (Fujiwara *et al.,* 2002).

Actin polymerization *in vitro* shows an initial delay (lag phase) that is due to the instability of actin dimers and the slow formation of stable actin trimers (nucleation). This phase is followed by an elongation phase during which actin monomers are assembled into filaments that grow from both ends, but faster at the barbed end. A lag phase in assembly does not occur *in vivo* because spontaneous nucleation is suppressed by actin monomer sequestering proteins. G-actin is incorporated into a growing filament in its ATP-bound form. ATP is then hydrolyzed to ADP-P_i. A conformational change accompanies the release of the phosphate, which is a much slower step *in vitro*. At a monomer concentration between the critical concentration of the barbed end (0.1 μM) and the pointed end (0.7 μM), the barbed end grows while the pointed end shrinks, resulting in a steady-state process called treadmilling (Fujiwara *et al.,* 2002). Although there is no change in polymer mass, befitting the term "steady state," individual filaments may grow faster or slower than average, resulting in a dynamic filament population.

3.2. Actin organization at the leading edge of migratory cells

3.2.1. Structure of actin cytoskeleton in lamellipodia and filopodia

Cells reach out with various membrane protrusions as they crawl and interact with their environment. Lamellipodia (Figure 1) contain an actin network within 1-3 μm of the leading edge of a migratory cell characterized by adjacent zones of actin filament polymerization and depolymerization. Lamellipodia protrude when the actin filaments, at the leading edge, are short and highly branched or cross-linked; lamellipodia contain transmembrane proteins that can adhere to substrata allowing cells to gain traction and move (Schafer, 2004). Behind the lamellipodium is a lamella (Figure 1) which

extends 10-15 µm from near the leading edge towards the cell interior (Delorme *et al.,* 2007).

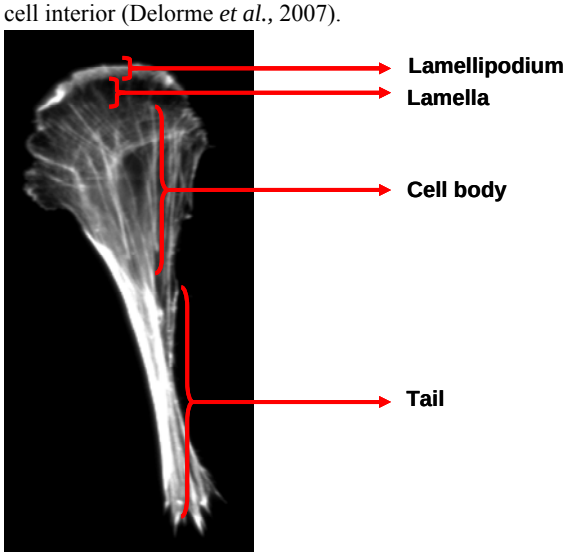

Figure 1. Cell regions of a migratory cell. Polarized chick embryo cardiac fibroblast was fixed and stained for F-actin with fluorescently-labeled phalloidin. The cell has a lamellipodium which is the actin-rich region at the front, followed by a lamella (faint region), a cell body and the tail at the rear. Scale bar, 10 µM.

Filopodia are long thin membranous protrusions (0.5 to several µm in length) containing bundles of actin filaments of near uniform polarity (Mallavarapu and Mitchison, 1999). F-actin in filopodia is cross-linked with fascin (Machesky and Li, 2010). The barbed ends of the actin filaments in filopodia are distal to the cell body, i.e., toward the tip of the filopodium (Cramer *et al.,* 1997, Svitkina *et al.,* 1997). Filopodia seem to be used by many cell types as a sensing organelle to explore the extracellular environment and the surface of other cells, identify appropriate targets for adhesion, and then transform guidance cue signals into intracellular signals for traction force generation (Xue *et al.,* 2010).

3.2.2. Regulation of actin cytoskeleton in lamellipodia and filopodia

Elongation of the barbed ends (pointing towards the membrane) of actin filaments drives membrane protrusion. The assembly and disassembly of the actin filament/network at the lamellipodia has been formulated into a "dendritic-nucleation model" (Mullins *et al.,* 1998). In this model, actin assembles predominantly at the leading edge because the concentration of uncapped barbed ends is both high and rate-limiting for polymerization. There are three proposed mechanisms for the formation of the dendritic filament array: 1) Arp2/3 complex, activated by WASp or another activator, binds laterally along pre-existing filaments on ATP- or ADP-Pi-actin subunits, promoting growth of a new filament at a ~70° angle to the original filament; 2) barbed end branching through the interaction between the Arp2/3 complex and the barbed end of a filament; 3) filamin-mediated cross-linking of the actin meshwork which is a non-Arp2/3 process (reviewed in Small *et al.,* 2008; Bugyi and Carlier, 2010). These may all work to some extent within the same lamellipodium or be used to dramatically different extents by different cell types, or by the same cell under different conditions.

Replenishment of the pool of G-actin for polymerization occurs by depolymerization of the network in regions more distant to the cell edge. Two likely mechanisms contribute to the rapid depolymerization of F-actin. The first is the removal of the Arp2/3 complex from pointed ends, facilitating depolymerization (Gupton *et al.,* 2005). The other mechanism is actin filament severing by ADF/cofilin (Delorme *et al.,* 2007) and/or gelsolin (Larson *et al.,* 2005).

In contrast to the lamellipodium, a lamella contains non-homogeneous regions of actin polymerization and depolymerization. In cells without a lamellipodium, the lamella reaches all the way to the leading edge (Ponti *et al.,* 2005). Tropomyosin (TM) and myosin II are signature molecules of the lamella and are absent from lamellipodia (Ponti *et al.,* 2004). Long TM-decorated actin filaments comprise the contractile actomyosin network, which promotes contraction in central cell regions, and which, in turn, restricts lamellipodium formation to specific persistent sites at the cell edge. The junction between the lamellipodium and lamella is characterized by substrate adhesions that translate actomyosin contraction in the lamella into cell pulling forces on the extracellular matrix (ECM) (Ponti *et al.,* 2004; Gupton *et al.,* 2005).

According to the convergent-elongation model of filopodia formation (Svitkina *et al.,* 2003), filopodia arise from the lamellipodial network by filament elongation from specific precursor sites, which are generated by lateral translocation and conversion of a dendritic array of actin filaments. This model suggests a dependence of filopodia formation on pre-existing lamellipodia. However, other studies have found that cells silenced for expression of Arp2/3 and WASp exhibited normal filopodial protrusion; these filopodia might be a function of the formin proteins, known nucleators of actin filaments (Peng *et al.,* 2003; Steffen *et al.,* 2006).

3.3. Actin organization in invadopodia

3.3.1. Structure of actin cytoskeleton in invadopodia

Cell migration through the ECM involves proteolytic degradation of ECM components. This proteolysis is mediated by specialized actin-rich membrane structures adherant to ECM called invadopodia and podosomes. Cell types that form podosomes include monocytic, endothelial, and smooth muscle cells, whereas invadopodia are formed by transformed and invasive cancer cells (Furmaniak-Kazmierczak *et al.,* 2007; Linder, 2007). Recent studies show that invading tumor cells at sites of ECM degradation form cell membrane protrusions 8 µm wide and 2 µm deep into the ECM containing multiple slender invadopodia, each about 0.8 µm diameter and 2 µm in length (Artym *et al.,* 2006). The formation of invadopodia has been correlated to the degree of tumor cell invasiveness (Artym *et al.,* 2009).

Podosomes usually consist of a core column of actin filaments that extends upwards from the ventral cell surface into the cytoplasm forming a characteristic ring-like structure, whereas invadopodia are long finger-like membrane extensions containing a meshwork of microfilaments that penetrate into the ECM from the

ventral side of the plasma membrane (Ayala *et al.,* 2006; Weaver, 2006). Invadopodia are enriched in actin and actin-associated proteins such as cortactin, Arp2/3, WASp, cofilin, and capping proteins; other proteins enriched in invadopodia include dynamin, cell adhesion molecules, such as integrins, tyrosine kinases, small GTPases, and soluble and membrane-bound proteases (Artym *et al.,* 2006). Invadopodia enhance the invasive potential of cells by concentrating metalloproteinases (ECMMPs) and serine proteases that degrade the ECM (Furmaniak-Kazmierczak *et al.,* 2007).

The broad spectrum of proteins that localize to invadopodia and the function of invadopodia in ECM degradation defines the invadopodium as a unique cellular structure characterized by coordinated interaction and interplay of molecules for cell adhesion, actin nucleation and polymerization, directed protease trafficking, endocytosis, and exocytosis. The extracellular matrix degradation coupled with internalization suggests that invadopodia actively remodel their surrounding microenvironment (Weaver, 2006; Artym *et al.,* 2010).

3.3.2. Regulation of actin cytoskeleton in invadopodia

The current model for invadopodia formation has several steps. The initial stages of assembly involve the formation of a primary invadopodial membrane process that extends from the ventral cell membrane toward the ECM. The tip of the invadopodia flattens as it interacts with the ECM, and it undergoes constant rapid ruffling. Invadopodia precursors are assembled by actin polymerization machinery containing Cdc42, WASp, the Arp2/3 complex, and cortactin in response to extracellular stimuli (Yamaguchi *et al.,* 2005; Weaver, 2006, Artym *et al.,* 2010). Cofilin severing increases the number of free barbed ends to initiate actin polymerization, which enhances further invadopodial growth (reviewed in Oser and Condeelis, 2009). Finally these structures gather matrix metalloproteinases to mature into functional invadopodia (Yamaguchi *et al.,* 2010). *In vitro*, invadopodia are induced to form by plating cells on 3D matrix cushions in the presence of growth factors, and are typically recognized by colocalization of invadopodia markers with degradation of the surrounding fluorescently-labeled ECM (Figure 2) (Weaver, 2006, Artym *et al.,* 2009).

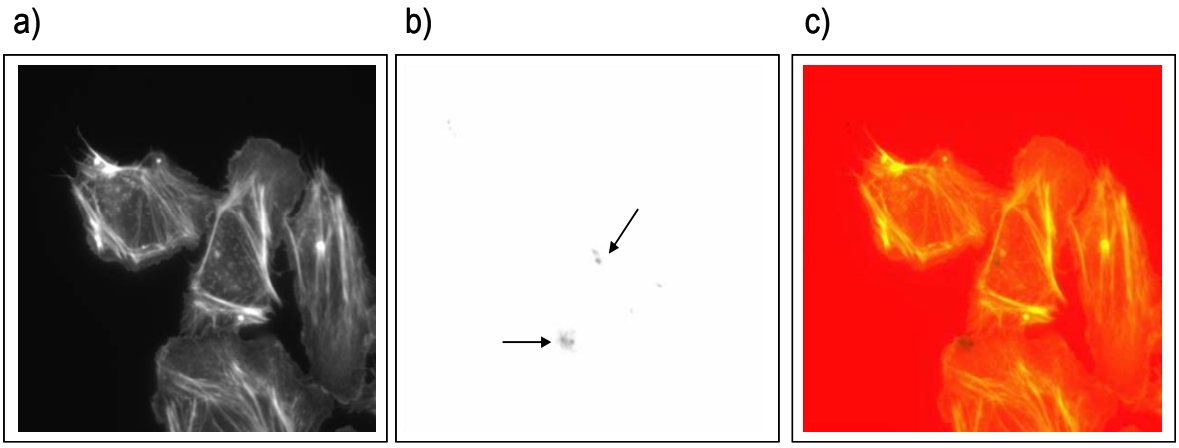

Figure 2. Invadopodia formation in mammary adenocarcinoma MTLn3 cell. (a) MTLn3 breast cancer cells assemble actin filaments to invade into a fluorescently-labeled gelatin film. (b) Matrix digestion is evident as the dark sites where the gelatin has been digested by invadopodia. (c) Merged image of a and b, actin filaments (green) and fluorescently-labeled gelatin (red). Scale bar, 10 μM.

4. ADF/cofilin proteins during metastasis

The first step of migration is actin polymerization, which drives the formation of cell protrusions, which adhere to the extracellular matrix, define the direction of migration, and initiate cell crawling (Nobes and Hall, 1999). ADF/cofilin proteins and their regulatory proteins modulate actin assembly in most mammalian cells and are involved in the initiation of the early steps in the motility cycle (reviewed in Van Troys *et al.*, 2008). ADF/cofilin proteins have molecular masses between 13-19 kDa (reviewed in Bamburg, 1999). ADF was named because of its ability to depolymerize F-actin and form a complex with G-actin in a 1:1 ratio, while cofilin was named because it co-sediments with F-actin. However, these are pH-dependent activities, and both proteins can bind and co-sediment with F-actin as well as sever filaments and increase dynamics of turnover, but to different extents.

ADF/cofilin proteins bind preferentially to ADP-F-actin (Kudryashov *et al*., 2010). They enhance the turnover of actin subunits in actin filaments by binding to a slightly twisted form of F-actin, thus stabilizing the twisted state, enhancing severing and providing more filaments ends to nucleate growth or enhance disassembly (Andrianantoandro and Pollard, 2006). After disassembly, Srv2/CAP and profilin induce a rapid exchange of ATP for ADP on the G-actin, releasing ADF/cofilin proteins for recycling to F-actin to repeat their dynamizing effects (Bertling *et al.,* 2007). Profilin, a major ATP-actin binding protein, promotes the addition of actin onto filament barbed ends (Didry *et al.*, 1998).

Of the three ADF/cofilin isoforms, ADF is the most efficient in turning over actin filaments (Vartiainen *et al.,* 2002). Cofilin-1 is more efficient than ADF in nucleation and severing of F-actin (reviewed in Bernstein and Bamburg, 2010). However, the severing activity of cofilin-

1 shows a biphasic concentration dependence, first increasing, and then decreasing with cofilin concentration, i.e., at high molar ratio with actin, cofilin-1 promotes filament assembly (Pavlov *et al.,* 2007). Cofilin-2 has weaker F-actin depolymerization activity than ADF and cofilin-1 and promotes filament assembly, rather than disassembly (Chen *et al.,* 2004; Nakashima *et al.,* 2005).

In cultured cells, the housekeeping functions of cofilin that are blocked by cofilin knockdown can be rescued by expressing ADF; the opposite is also true, cofilin can rescue ADF down regulation (Hotulainen *et al.,* 2005). In contrast, in more complex conditions such as during specific developmental or physiological processes, different AC isoforms display distinct effects which demonstrate that these isoforms have qualitatively similar but quantitatively different effects on actin dynamics (Lehman *et al.,* 2000; Ono *et al.,* 2003; Gurniak *et al.,* 2005).

4.1. Structure of ADF/cofilin

ADF and cofilin from a single organism share about 70% sequence identity (reviewed in Bamburg, 1999). Vertebrate AC proteins have a nuclear localization sequence (NLS) which allows them to chaperone actin into the nucleus (Matsuzaki *et al.,* 1988; Iida *et al.,* 1992). ADF/cofilin proteins have a characteristic single domain called the ADF-homology domain (ADF-H) (reviewed in Van Troys *et al.,* 2008). The ADF-H consists of a four stranded mixed β-sheet surrounded by four α-helices (Lappalainen *et al.*, 1997; reviewed in Maloney *et al.,* 2008).

Cofilin-1 has four cysteine (Cys) residues (C39, C80, C139 and C147) that are targets of oxidation and can form specific intramolecular disulfide bonds (C39-C80 and C139-C147). The formation of both intramolecular disuflide bonds is required to eliminate actin binding (Klamt *et al.,* 2009). Oxidized cofilin-1 gets translocated to mitochnodria where it causes the release of cytochrome c, an early step in apoptosis (reviewed in Bernstein and Bamburg, 2010). ADF has seven Cys residues but only three (C39, C80 and C147) are conserved with those in cofilin-1 (reviewed in Bernstein and Bamburg, 2010). Thus, ADF might not be targeted to mitochondria during oxidative stress, even though this has not been directly tested.

Recently, cofilin-1 was found to be a substrate for v-Src (activated Src tyrosine kinase) phosphorylation on tyrosine (Y) 68 (Y68) (Yoo *et al.,* 2010). This post-translational modification of cofilin makes it a target for the ubiquitin-proteosome pathway. Cofilin degradation affects its cellular functions on actin dynamics such as enhanced cell spreading and the G-actin/F-actin ratio (Yoo *et al.,* 2010). ADF has a phenylalanine (F) residue at position 68 (F68) and thus is not a substrate for Src-mediated phosphorylation and accompanying ubiquitin degradation (reviewed in Bernstein and Bamburg, 2010).

4.2. Tissue/Cellular Distribution of ADF/cofilin

The relative expression levels of the three ADF/cofilin proteins (ADF, cofilin-1 and cofilin-2) vary in a spatial and temporal-specific manner (Gurniak *et al.,* 2005). During the development of mice, cofilin-1 is the predominant one and it remains ubiquitously expressed in most adult tissues (Gurniak *et al.,* 2005). ADF is expressed at low levels during mouse embryonic development with the highest levels found in embryonic heart and the adaxial region of somites of E10.5 embryos (Gurniak *et al.,* 2005). ADF is upregulated after birth in epithelial and endothelial tissues (Gurniak *et al.,* 2005; Vartiainen *et al.,* 2002). In the chick system, which has only ADF and cofilin-2, ADF becomes post-natally upregulated in the nervous system and in epithelial and endothelial tissues such as intestine, kidney and testis (Bamburg and Bray, 1987).

In late embryogenesis and after birth, cofilin-2 replaces cofilin-1 in striated muscle, becoming the major isoform expressed in differentiated skeletal muscle and cardiac muscle (reviewed in Van Troys *et al.,* 2008). The human cofilin-2 gene yields two mRNAs with identical coding sequence. The first (cof-2b) is strongly expressed in skeletal muscle and heart and the second (cof-2a) is expressed at lower levels in other tissues (Thirion *et al.* 2001; reviewed in Maloney *et al.,* 2008).

ADF and cofilin often show diffuse immunostaining in resting cells; they get translocated upon stimulation to the leading edge of motile cells such as *Dictyostelium*, neutrophils, HL-60 cells and chick cardiac fibroblasts (Bamburg and Bray, 1987; Aizawa et al., 1995; Suzuki et al., 1995; Djafarzadeh and Niggli 1997; Dawe et al., 2003). Inactive cofilin is found to be highest in the perinuclear region in motile colon adenocarcinoma NRK39 and LS180 cell lines, whereas active cofilin is localized with actin filaments at the periphery (Nowak *et al.,* 2010). In resting breast adenocarcinoma MTLn3 cells, cofilin is distributed diffusely in the cytoplasm. However, upon stimulation with epidermal growth factor (EGF), cofilin is recruited to the leading edge (Chan *et al.,* 2000). A similar situation is observed in morphologically polarized neurons, which have a higher ratio of total cofilin to the inactive phospho-cofilin in the growth cones of their more rapidly extending axons, compared with growth cones of slower growing minor processes (Garvalov *et al.,* 2007). In spontaneously polarizing chick cardiac fibroblasts, cofilin is required for the formation of oriented actin-filament bundles in the cell body, a process needed to coordinate the spatial location of the cell rear and front during fibroblast polarization (Mseka *et al.,* 2007).

4.3. Regulation of ADF/cofilin

ADF and cofilin from metazoans are complexly regulated. One principal mechanism is their inhibition by phosphorylation on a serine residue near the N-terminus (Ser3 of encoded sequence). The phosphorylated form does not bind to either G- or F-actin (Ressad *et al.,* 1998; Blanchoin *et al.,* 2000; Bamburg and Wiggan, 2002). There are two major kinase families that phosphorylate ADF/cofilin proteins in animal cells, the LIM kinases (LIMK) and testicular kinases (TESK). LIMKs are downstream effectors of the Rho-family GTPases, they can be activated by phosphorylation by the Rac- and Cdc42-activated kinase PAK, or by the Rho kinase ROCK (reviewed in Van Troys *et al.,* 2008). The dephosphorylation of ADF and cofilin is regulated by several phosphatases, particularly the slingshot phosphatase (SSH) family and chronophin (CIN), also

known as pyridoxal-5-phosphate phosphatase (Figure 3) (Huang *et al.,* 2006).

G- and F-actin binding of ADF/cofilin is inhibited by phosphoinositides, because the phosphoinositide-binding site, a large positively charged surface, overlaps with the G- and F-actin binding sites. ADF/cofilin proteins do not display phosphoinositide specificity; i.e., they bind phosphatidylinositol 4,5-bis-phosphate ($PI4,5P_2$), phosphatidylinositol-3,4-bis-phosphate ($PI3,4P_2$) and phosphatidyinositol 3,4,5-tris-phosphate ($PI3,4,5P_3$) and have been proposed to act as a sensor for PIP_2-density on the plasma membrane (Zhao *et al.,* 2010). The $PI4,5P_2$-hydrolysing enzyme phospholipase C (PLC) releases active cofilin from the membrane in various stimulated cells (Matsui *et al.,* 2001; Zhou *et al.,* 2007).

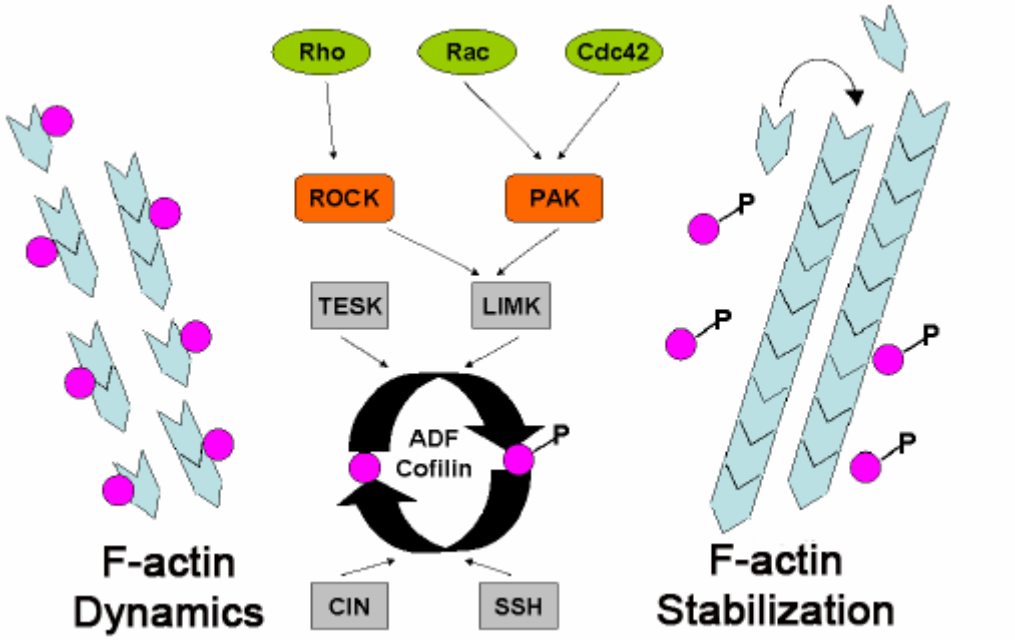

Figure 3. Regulation of ADF/cofilin. AC proteins enhance the turnover of actin filaments by inducing filament severing. LIMK and TESK phosphorylate AC at Ser3 inhibiting their actin-binding activity, while slingshot (SSH) and chronophin dephosphorylate AC proteins activating them.

AC proteins are pH-dependent in their interactions with F-actin; at pH< 7.0, both ADF and cofilin, when present in excess over actin, slightly increase the G-actin pool while binding and co-sedimenting with F-actin at a stoichiometry of 1:1 with actin subunits. Increasing intracellular pH results in more ADF colocalizing with G-actin, but the pH shift has little effect on cofilin, which remains mostly F-actin associated (Bernstein *et al.,* 2000).

The activities of ADF and cofilin are regulated by other mechanisms including interaction with actin-interacting protein 1 (Aip1) which binds the ADF/cofilin/actin complex. Aip1 enhances the ability of ADF/cofilin proteins to sever actin filaments, and may also accelerate depolymerization by capping their barbed ends (Okada *et al.,* 2002; Mohri *et al.,* 2006; Kile *et al.,* 2007). Other ADF/cofilin regulatory mechanisms include competition with tropomyosin for actin binding (DesMarais *et al.,* 2002; Bryce *et al.,* 2003; Kuhn and Bamburg, 2008), compartmentalization (Nebl *et al.,* 1996) and differential stabilization of ADF and cofilin mRNAs by the actin monomer pool (Minamide *et al.,* 1997).

Recently, it has been found that cofilin-1 is regulated by microRNAs (miRNAs) which are short (21-22 nucleotides) noncoding RNAs that bind the 3' untranslated region (UTR) of mRNAs and mainly repress translation. Two miRNAs (miR-103 or miR-107) repress cofilin-1 translation and when their levels are reduced in transgenic mouse model, cofilin proteins levels are increased accompanied with the formation of rod-like structures (Yao *et al.,* 2010). There is no sequence homology between miR-103 or miR-107 and ADF cDNA, suggesting a specific targeting of cofilin and not ADF by these miRNAs.

4.4. Role of ADF and cofilin during metastasis

Trying to decipher from the literature the specific role(s) of ADF and cofilin-1 during metastasis is challenging because most researchers, studying the role of these proteins during invasion, focus on cofilin-1. Although ADF and cofilin can substitute for one another for most housekeeping activities in cultured cells (Hotulainen *et al.,* 2005), this is not always the case during development. Cofilin-1 null mice are not viable despite the fact that ADF is upregulated (Gurniak *et al.,* 2005). In contrast, ADF null mice are viable but show abnormal corneal thickening, suggesting that cofilin-1 can rescue the lack of ADF except in corneal epithelial cells (Ikeda *et al.,* 2003). This finding demonstrates that AC isoforms are not completely redundant. However, in ureteric bud (UB) epithelium ADF and cofilin show considerable functional overlap; deletion of cofilin-1 in UB epithelium or an inactivating mutation in ADF had no effect on renal

morphogenesis, but simultaneous lack of both genes arrested branching morphogenesis at an early stage (Kuure *et al.,* 2010).

Silencing cofilin-1 in a highly invasive colorectal cancer cell line (Isreco1) did not interfere with its ability to undergo transwell migration across collagen in response to a chemotactic attractant, whereas silencing of ADF, which represented only 17% of the total ADF/cofilin, significantly inhibited transwell migration, strongly suggesting different cellular functions of each protein (Estornes *et al.,* 2007). ADF generates a larger actin monomer pool than cofilin (Yeoh *et al.,* 2002); thus activation of ADF in cell regions away from the leading edge could be necessary to provide a pool of subunits that maintains leading edge protrusion during migration.

Too much or too little cofilin activity inhibits membrane protrusions required for motility and chemotaxis. However, the literature in this area is sometimes confusing. For example, a moderate (two-to four-fold) overexpression of cofilin at the protein level increases the velocity of cell migration in *Dictyostelium* (Aizawa *et al.,* 1995) and human glioblastoma cells (Yap *et al.,* 2005), but higher levels of expression inhibit cell motility (Lee *et al.,* 2000). Expression of wild type or a non-phosphorylatable cofilin mutant in which serine3 has been mutated to alanine (S3A) increases melanoma cell invasion through a reconstituted basement membrane (Dang *et al.,* 2006). There are, however, several studies showing that cofilin is down regulated in other cancers and that its overexpression is antagonistic to invasion. Furthermore, expression of LIMK1 in human breast cancer cells, which should lower active AC, enhanced cell proliferation, invasiveness and *in vitro* angiogenesis (Bagheri-Yarmand *et al.,* 2006).

Further confusing information on the role of AC proteins comes from other cancer studies. In support to the more active cofilin in invasive cancers is the report that the amount of phosphorylated cofilin is decreased in cell lines derived from T-cell lymphoma (Jurkat) and carcinomas from the cervix (HeLa), colon (KM12) liver (HepG2) and kidney (COS1) (Nebl *et al.,* 1996). Increased levels of cofilin mRNA and protein are detected, using proteomic and cDNA microarray approaches, in clinical tumor samples of oral squamous-cell carcinoma (Turhani *et al.,* 2006), renal cell carcinoma (Unwin *et al.,* 2003), and ovarian cancer (Martoglio *et al.,* 2000); cofilin-1 was found to be significantly increased in the saliva of patients of head and neck squamous cell carcinoma (Dowling *et al.,* 2008). On the other hand, suppression of LIMK2 in human fibrosarcoma cells, which should increase active cofilin, limits their migration and efficiency to form dense colonies without affecting cell proliferation rate or viability (Suyama *et al.,* 2004; Vlecken and Bagowski, 2009).

These discrepancies are most readily explained if every cell has an optimum amount and activity of cofilin that is required to counter balance the effects of other proteins that antagonize cofilin's ability to sever and dynamize F-actin. Among these other proteins are tropomyosins, most of which bind and stabilize the untwisted form of actin and compete with ADF/cofilin for binding (DesMarais *et al.,* 2002), and cortactin, which binds to ATP and ADP-Pi forms of actin subunits in F-actin (Bryce *et al.,* 2005) and slows down the Pi release, which is necessary to generate the ADP-actin form recognized by ADF and cofilin (Oser *et al.,* 2009). Furthermore, cortactin directly binds cofilin, and releases it in a cortactin phosphorylation–dependent manner (Oser *et al.,* 2009). Thus different cell types could have cofilin activities that lie on opposite sides of the peak of a bell shaped curve that describes optimal cofilin activity for polarized migration. In some tumor cells, cofilin would need to be upregulated or activated to shift toward optimal activity whereas in other cell types this shift would require its inactivation or down regulation.

In addition or alternatively, in different cells the optimal activity of ADF and cofilin may need to be adjusted and will depend on the ratio of ADF/cofilin in each cell type or perhaps even within each cell. Also, it would be interesting to know how Aip1 changes along with ADF and cofilin in tumor cells. Mutational studies of Aip1 showed that the severing and capping activities are differently affected by point mutation, indicating that these activities might be uncoupled (Mohri *et al.,* 2006). Thus, further investigations are needed to understand which activity of Aip1 is required for ADF/cofilin-mediated metastasis. Further studies are also required to better understand the collaboration between tropomyosin isoforms, profilin, ADF/cofilin and Aip1 in organizing the actin cytoskeleton in tumor cells, as was done for studying the role of these proteins in sarcomeric actin organization (Yamashiro *et al.*, 2008).

Invasive cancer cells form invadopodia which aid in the cell's ability to escape from a surrounding basal lamina and pass across the endothelial cell barrier of capillaries (Artym *et al.,* 2009). The formation of invadopodia requires cofilin for initiation, stabilization and maturation (Yamaguchi *et al.,* 2005). Silencing cofilin expression interferes with long-lived invadopodia and decreases matrix degradation activity in metastatic carcinoma cells. When unpolarized highly metastatic breast tumor cells (MTLn3) become polarized by stimulation, unphosphorylated (active) ADF/cofilin is released from PI-4, 5-P_2 at the membrane. Growth factors, such as EGF, locally activate phospholipase C, cleaving membrane PI-4, 5-P_2 and freeing cofilin to sever filaments, giving rise to the increase in F-actin barbed ends that occurs about 1 min. after treatment; the formation of the barbed ends is necessary to initiate assembly of an invadopodium, the structure that is needed for tumor cells to escape into the vasculature (Mouneimne *et al.,* 2004; Sidani *et al.,* 2007, van Rheenen *et al.,* 2007).

In conclusion, determining the expression status of cofilin alone is insufficient to describe the characteristics of tumour cells, such as proliferation, migration and invasion. Rather, the balanced contribution of cofilin and ADF and other molecules in the ADF/cofilin regulatory pathways has to be taken into consideration. In addition, there is a great need of investigating not only single components of the ADF/cofilin pathways, but rather multiple key regulators and the final output of any single component.

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Survey and Determination of Aflatoxin Levels in Stored Peanut in Sudan

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Abstract

Sixty samples of stored peanut kernels were collected from four different locations in Sudan namely Mayo city, Umbaddah city in Khartoum state, Al-Helalia city, and Al-Managel city in Al-Jazeera state. The kernels were examined for contamination with aflatoxin. All samples were subjected to microbiological analysis by culturing them on suitable growth medium "Sabouraud's Dextrose Agar" and chemical analysis by Thin-Layer Chromatography (TLC) technique. Thirty five samples (58.33%) gave positive readings with TLC technique, and in culture, *Aspergillus flavus* was isolated from twenty six samples (43.33%). The concentration of aflatoxin B_1 in these samples were ranged from low to very high, in range of (17.57-404.00 µg/Kg kernel).

الملخص

أجريت هذه الدراسة بهدف تحديد مدى تلوث الفول السوداني المأخوذ من مناطق عديدة بالسودان بالسم الفطري المعروف بالأفلاتوكسين ب$_1$.
جُمعت ستون عينة من الفول السوداني المخزون من مناطق مختلفة بالسودان شملت ولايتي الخرطوم (مدينة مايو ومدينة أم بدة) وولاية الجزيرة (مدينة الهلالية ومدينة المناقل) بمعدل خمسة عشر عينة من كل مدينة.
كل العينات أختبرت عن طريق التحليل الميكروبي للتعرف على الفطريات المصاحبة وذلك بواسطة الزراعة على وسط أجار السابرود ديكستروز وكذلك عن طريق التحليل الكيميائي بواسطة تقنية التصوير الملون ذات الطبقة الرقيقة لمعرفة ما إذا كانت العينات ملوثة بسم الافلاتوكسين ب$_1$.
إحتوت خمس وثلاثون عينة بنسبة (58.33%) من مجموع عينات الفول السوداني المخزون على سم الافلاتوكسين ب$_1$ عن طريق تقنية التصوير الملون ذات الطبقة الرقيقة. أما في التحليل الميكروبي فقد عُزل فطر (*Aspergillus flavus*) من ست وعشرون عينة بنسبة (43.33%).
تراوح تركيز سم الافلاتوكسين ب$_1$ في العينات المختبرة مابين منخفض، عالي، وعالي جداً في مدى يتراوح ما بين (17.57-404.00 مايكروجرام/كجم) في عينات حبيبات الفول السوداني المخزون، بما يؤكد إرتفاع تلك النسب عن المستوى المسموح وما يمثله من خطورة محتملة للإنسان والحيوان.

Keywords: *Key words*: Aflatoxin, Mycotoxins, Peanuts, *Aspergillus flavus*, TLC.

1. Introduction

Aflatoxin is the name for a group of toxins known as B_1, B_2, G_1, G_2, M_1 and M_2 (carcinogenic compounds) that are produced mainly by two fungi called *Aspergillus flavus* and *Aspergillus parasiticus*. These toxins occur naturally and have been found in a wide range of commodities (including peanuts) used for animal and human consumption. Depending on their levels, toxins can severely affect the liver and induce a human carcinogen, i.e., causes cancer. In many developing countries, aflatoxin is a major health risk to both humans and animals due to the high levels of the contaminated products consumed (Wright *et al*., 2002).

The causative agents grow on food and feed grains at a moisture level of 15% or greater in the presence of warm temperatures (21˚C- 37˚C or 70˚-100˚F). The toxin can be found in a variety of grains but most often occurs in peanut and corn. Contamination can occur while the grain is standing in the field, at and soon after harvesting and during storage before or after the grain is processed into food or feed (Allen, 2003).

A few months after the death of more than 100.000 young Turkeys in poultry farms in England, an apparently new disease that was termed "Turkey X disease" appeared. Speculations made during 1960 regarding the nature of the toxin suggested that it might be of fungal origin. In fact, the toxin-producing fungus was identified as *Aspergillus flavus* in 1961 and the toxin was given the name Aflatoxins by virtue of its origin (*A. flavus* → Afla) (Liu *et al*., 2005).

The chemical structure of aflatoxin is coumarin nucleus linked to a bifuran and either a pentanone, as in AFB_1 and the dihydro derivative AFB_2, or a six – member lactone, as

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in AFG_1 and its corresponding derivative AFG_2 (Sanz *et al*., 1989)

The economic impact of aflatoxins was derived directly from crop, livestock losses, and, , indirectly, from the cost of regulatory programs designed to reduce risks to animal and human health. The Food and Agriculture Organization (FAO) estimates that 25 % of the world's food crops are affected by mycotoxins, of which the most notorious are aflatoxins. Aflatoxins losses to livestock and poultry producers from aflatoxin-contaminated feeds include death and the more subtle effects of immune system suppression, reduced growth rates, and losses in feed efficiency. Other adverse economic effects of aflatoxins include lower yield for food and fiber crops (Anon, 1989)

In an attempt to harmonize the current tolerances to aflatoxin which exist in different countries, the working group on mycotoxins of the World Health Organization (WHO) and Food Agricultural Organization (FAO) proposed maximum limits of 15µg/Kg for total aflatoxins in raw groundnuts based on a sample size of 20 Kg (Bhat *et al*., 1996).

The potential economic problems associated with a level of 10µg/Kg and the public health implications of a level of 15µg/Kg, as compared to 10µg/Kg for aflatoxins in foods, are two main issues in the setting of maximum levels for aflatoxins in groundnuts intended for further processing. Many countries considered the level of 15µg/Kg to be a reasonable limit that could be achieved by producing countries, thus facilitating international trade, considering that a lower level would constitute a trade barrier due to the finding of the the evaluation of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) that this may not offer a significant improvement in public health. However, genotoxic properties of aflatoxin, uncertainties in risk assessment, the As Low As is Reasonable Achievable (ALARA) principle, and inadequate data on the effect of a level of 10µg/Kg on the availability of groundnuts in the world market support the continued consideration of the lower level. A situation can be envisaged from a recent Multi-Centric National Study in India of aflatoxin contamination in maize and groundnut. The study indicated that 21% of the contaminated groundnut samples available in the Indian market were not suitable for human consumption as they contained aflatoxin B_1 above the Indian permissible limit of 30µg/Kg (Bhat *et al*., 1997).

1.1. Objectives

The objectives of this study are to:

1. Isolate the fungi which produce aflatoxin B_1 from stored peanut kernels.
2. Measure the quantity of aflatoxin B_1 in stored peanut kernels.

2. Material and Methods

2.1. Microbiological analysis

Peanut samples were collected from four different locations in Sudan by taking 100 gm from each sample and mixing them to obtain the representative sample. One gram of each sample was tested for fungal isolation by inoculating the Sabouraud dextrose agar plates with the suspension of peanut kernels using dilution method by taking 0.5 ml from each dilution; they were placed into Petri dishes and incubated at 28˚C for 3-5 days (Hayate and Idris, 2000). After the incubation period, the growing fungal cultures were examined microscopically using Lactophenol Cotton Blue (LPCB) stain and classified by reporting the culture characteristics at the face and reverse side of the inoculated Petri dishes (Cheesbrough, 1984).

2.2. Chemical analysis

The chemical analysis techniques were performed according to those described by the Association of Official Analytical Chemist (AOAC, 1999).

2.2.1. Extraction of aflatoxin from peanut kernels

Twenty grams Peanut samples were ground and placed in 250 ml conical flask, 100 ml of methanol (55%), and 40 ml of petroleum ether were added and blended for 2 minutes at a high speed. The mixture was left standing undisturbed in the blender for 30 minutes. Twenty five ml were pipetted from the aqueous methanol phase into 250 ml flask, and 25 ml of chloroform were added, covered with a stopper and shaken for 1 minute. The layers were left to separate and the bottom chloroform layer was drained into 100 ml glass beaker, placed into a water bath to evaporate the solvent. The extract was dissolved in 200µl benzene-acetonitril (98:2) for spotting on TLC plate (AOAC, 1999).

2.2.2. Detection of aflatoxin using Thin-Layer Chromatography(TLC)

20µl from the previously prepared sample was spotted on imaginary line 1cm from the bottom edge of TLC plate and 20µls of the aflatoxin reference standard solution. The plate was placed in a tank containing a mixture of acetone-chloroform (5:95) for 10 minutes at 23-25˚C, removed, allowed to dry at room temperature, and illuminated from above by placing its flat, coated side up, on a long wave ultraviolet lamp Chromato-Vue cabinet. The fluorescent spots were observed and the retention factor (R_f) was calculated as in formula (i), recorded to determine the concentration of aflatoxin B_1 after spraying the plate with 50% sulphuric acid solution. The results were calculated applying formula (ii).

Formula (i)

$$R_f = \frac{\text{distance moved by compound}}{\text{distance moved by solvent}}$$

Formula (ii)

Concentration of aflatoxin B_1 in µ/Kg = (S× Y× V)/ (X× W)

Where:

S ≡ µl aflatoxin B_1 standard equal to unknown.

Y ≡ concentration of aflatoxin B_1standard µg/ml.

V ≡ µl of final dilution of sample extract.

X ≡ µl of sample extract spotted to giving fluorescent intensity equal to S (B_1 standard).

W ≡ weight of sample in gram of original sample contain in final extract.

The R_f of aflatoxin B_1 is ranged between 0.4-0.7(AOAC, 1999).

3. Results

Fifteen stored peanut kernel samples, which were collected from Mayo city, Khartoum state, contained aflatoxin B_1 by using chromatographical technique (Fig.1) and they had a range of aflatoxin B_1 concentration from (17.57 µg/ Kg – 67.33 µg/ Kg Table 1), but not all samples containing the aflatoxin B_1 producing-agents (*Aspergillus flavus*). This fungus was isolated from six samples (40%) only and the others contained *Aspergillus niger*, (Table 2). While eight samples (53.33%) of stored peanut kernels collected from Umbaddah city, Khartoum state, appeared positive with chromatographical technique with toxin concentration ranged from (44.89 µg/ Kg – 404.00 µg/ Kg (Table 3), and nine samples (60%) yielded *Aspergillus flavus*, and the rest samples were contaminated with *Aspergillus niger* (Table 4).

The samples which were collected from Al-Jazeera state, notably from Al-Helalia city, showed positive results to the chemical analysis in seven samples (46.67%) with toxin concentration ranged from (36.70 µg/ Kg – 101.00 µg/ Kg (Table 5). The fungal isolated from these samples showed that only six samples (40%) contained *Aspergillus flavus* and the rest were contaminated with *Aspergillus niger* (Table 6). The last location in Al-Jazeera state is Al-Managel city; from this location five samples (33.33%) showed positive in chemical analysis with toxin concentration, ranging from (25.25 µg/ Kg - 80.80 µg/ Kg (Table 7). These samples were also positive in microbiological analysis and the others contained *Aspergillus niger* (Table 8).

The thirty five samples (58.33%) from all the samples (sixty samples) of stored peanut kernels were contaminated with aflatoxin B_1, and all samples (ten samples) after storage time of twenty four months were positively in the chromatographical technique, but not in microbiological analysis except for one sample. While the 73.33% (11 samples from 18) after twelve months storage time showed positive result for chemical analysis, and 66.67% (10 samples) showed positive to the microbiological analysis. In contrast, 35.71% (10 samples from 28) of six months storage time were positive to the chemical analysis and 39.29% (11 samples) were positive in microbiological analysis. Seventy five percent (3 samples from 4) of two months storage time showed positive in both chemical and microbiological analysis. All samples which contained aflatoxin B_1 were visible after spraying with 50% sulphuric acid as brown spots while the others were detected as yellowish-brown spots.

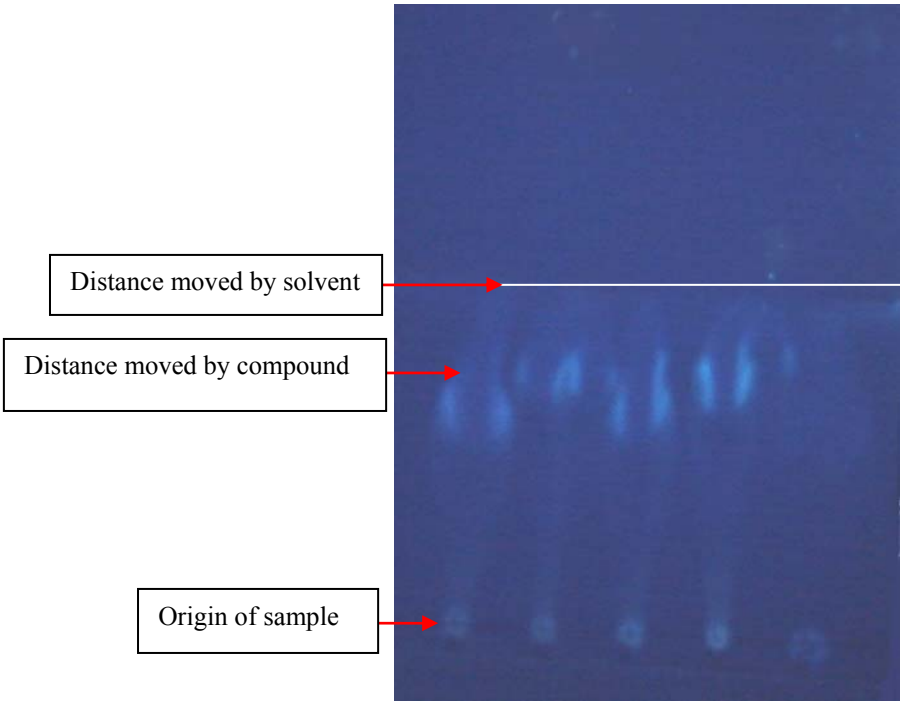

Figure 1. The positive result of aflatoxin B_1 under U.V. light of stored peanut kernels with R_f equal 0.66.

Table **1**. Screening of aflatoxin B_1 (AFB_1) in stored peanut kernels from Mayo city, Khartoum state.

No of sample	Storage time/Months	Weight of extract/g	R_f	Conc. of AFB_1 (µg/Kg)	Appearance under U.V (650 nm wave length)	Visual color
1.	6	0.60	0.61	67.33	Fluorescent	Brown
2.	6	0.80	0.61	50.50	Fluorescent	Brown
3.	6	0.90	o.70	44.89	Fluorescent	Brown
4.	6	0.30	0.67	134.66	Fluorescent	Brown
5.	6	0.60	0.69	67.33	Fluorescent	Brown
6.	24	2.20	o.61	18.36	Fluorescent	Brown
7.	24	1.20	0.66	33.67	Fluorescent	Brown
8.	24	1.90	0.66	21.26	Fluorescent	Brown
9.	24	1.70	0.58	23.76	Fluorescent	Brown
10.	24	1.90	0.45	21.26	Fluorescent	Brown
11.	24	0.80	0.55	50.50	Fluorescent	Brown
12.	24	1.20	0.55	33.67	Fluorescent	Brown
13.	24	1.80	0.55	22.44	Fluorescent	Brown
14.	24	1.90	0.50	21.26	Fluorescent	Brown
15.	24	2.30	0.53	17.57	Fluorescent	Brown

$R_f \equiv$ Retention Fa

Table **2**. Fungi isolated from stored peanut kernels collected from Mayo city, Khartoum state.

No. of sample	Fungi isolated
1.	*Aspergillus flavus*
2.	*Aspergillus flavus*
3.	*Aspergillus flavus*
4.	*Aspergillus flavus*
5.	*Aspergillus flavus*
6.	*Aspergillus niger*
7.	*Aspergillus niger*
8.	*Aspergillus niger*
9.	*Aspergillus niger*
10.	*Aspergillus niger*
11.	*Aspergillus niger*
12.	*Aspergillus niger*
13.	*Aspergillus niger*
14.	*Aspergillus niger*
15.	*Aspergillus flavus*

Table **3**. Screening of aflatoxin B_1 in stored peanut kernels from Umbaddah city, Khartoum state.

No of sample	Storage time/Months	Weight of extract/g	R_f	Conc. of AFB_1 (µg/Kg)	Appearance under U.V (650 nm wave length)	Visual color
1.	12	0.50	0.66	80.80	Fluorescent	Brown
2.	12	0.30	0.70	134.66	Fluorescent	Brown
3.	12	0.40	0.70	101.00	Fluorescent	Brown
4.	12	0.30	0.00	0.00	Fluorescent	Brown
5.	12	0.70	0.66	57.71	Fluorescent	Brown
6.	12	0.60	0.48	67.33	Fluorescent	Brown
7.	12	0.70	0.89	0.00	Fluorescent	Yellow
8.	2	0.90	0.82	0.00	Fluorescent	Yellow
9.	2	0.60	o.78	67.33	Fluorescent	Brown
10.	2	0.90	0.71	44.89	Fluorescent	Brown
11.	2	0.10	0.67	404.00	Fluorescent	Brown
12.	6	1.00	0.00	0.00	Inflorescent	Yellow
13.	6	0.50	0.00	0.00	Inflorescent	Yellow
14.	6	1.00	0.00	0.00	Inflorescent	Yellow
15.	6	0.10	0.00	0.00	Inflorescent	Yellow

$R_f \equiv$ Retention Factor.

Table **4**. Fungi isolated from stored peanut kernels collected from Umbaddah city, Khartoum state.

No. of sample	Fungi isolated
1.	*Aspergillus flavus*
2.	*Aspergillus flavus*
3.	*Aspergillus flavus*
4.	*Aspergillus niger*
5.	*Aspergillus flavus*
6.	*Aspergillus flavus*
7.	*Aspergillus niger*
8.	*Aspergillus niger*
9.	*Aspergillus flavus*
10.	*Aspergillus flavus*
11.	*Aspergillus flavus*
12.	*Aspergillus niger*
13.	*Aspergillus flavus*
14.	*Aspergillus niger*
15.	*Aspergillus niger*

Table **5**. Screening of aflatoxin B_1 in stored peanut kernels from Al-Helalia city, Al-Jazeera state.

No of sample	Storage time/Months	Weight of extract/g	R_f	Conc. of AFB_1 (µg/Kg)	Appearance under U.V (650 nm wave length)	Visual color
1.	6	0.30	0.00	0.00	Inflorescent	Yellow
2.	12	1.00	0.71	40.40	Fluorescent	Brown
3.	12	1.00	o.71	40.40	Fluorescent	Brown
4.	12	1.10	0.71	36.70	Fluorescent	Brown
5.	12	1.10	0.71	36.70	Fluorescent	Brown
6.	12	1.00	0.71	40.40	Fluorescent	Brown
7.	12	0.80	0.66	50.50	Fluorescent	Brown
8.	12	1.00	0.87	0.00	Fluorescent	Yellow
9.	12	1.10	0.87	0.00	Fluorescent	Yellow
10.	12	0.90	0.9	0.00	Fluorescent	Yellow
11.	12	1.30	0.97	0.00	Fluorescent	Yellow
12.	12	1.30	0.97	0.00	Fluorescent	Yellow
13.	6	0.40	0.78	101.00	Fluorescent	Brown
14.	6	0.20	0.00	0.00	Inflorescent	Yellow
15.	6	0.30	0.00	0.00	Inflorescent	Yellow

$R_f \equiv$ Retention Factor.

Table **6.** Fungi isolated from stored peanut kernels collected from Al-Helalia city, Al-Jazeera state.

No. of sample	Fungi isolated
1.	*Aspergillus flavus*
2.	*Aspergillus flavus*
3.	*Aspergillus flavus*
4.	*Aspergillus flavus*
5.	*Aspergillus flavus*
6.	*Aspergillus flavus*
7.	*Aspergillus niger*
8.	*Aspergillus niger*
9.	*Aspergillus niger*
10.	*Aspergillus niger*
11.	*Aspergillus niger*
12.	*Aspergillus niger*
13.	*Aspergillus niger*
14.	*Aspergillus niger*
15.	*Aspergillus niger*

Table **7**. Screening of aflatoxin B_1 in stored peanut kernels from Al-Managel city, Al-Jazeera state.

No of sample	Storage time/Months	Weight of extract/g	R_f	Conc. of AFB_1 (µg/Kg)	Appearance under U.V (650 nm wave length)	Visual color
1.	6	1.00	1.03	0.00	Fluorescent	Yellow
2.	6	1.00	0.71	40.40	Fluorescent	Brown
3.	6	1.60	0.77	25.25	Fluorescent	Brown
4.	6	1.00	0.75	40.40	Fluorescent	Brown
5.	6	1.20	0.81	0.00	Fluorescent	Yellow
6.	6	1.00	0.78	40.40	Fluorescent	Brown
7.	6	0.50	0.69	80.80	Fluorescent	Brown
8.	6	1.30	0.82	0.00	Fluorescent	Yellow
9.	6	1.40	0.88	0.00	Fluorescent	Yellow
10.	6	1.60	0.88	0.00	Fluorescent	Yellow
11.	6	1.70	0.91	0.00	Fluorescent	Yellow
12.	6	0.60	0.88	0.00	Fluorescent	Yellow
13.	6	0.40	0.00	0.00	Inflorescent	Yellow
14.	6	0.20	0.00	0.00	Inflorescent	Yellow
15.	6	0.30	0.00	0.00	Inflorescent	Yellow

$R_f \equiv$ Retention Factor.

Table **8**. Fungi isolated from stored peanut kernels collected from Al-Managel city, Al-Jazeera state.

No. of sample	Fungi isolated
1.	*Aspergillus niger*
2.	*Aspergillus flavus*
3.	*Aspergillus flavus*
4.	*Aspergillus flavus*
5.	*Aspergillus niger*
6.	*Aspergillus flavus*
7.	*Aspergillus flavus*
8.	*Aspergillus niger*
9.	*Aspergillus niger*
10.	*Aspergillus niger*
11.	*Aspergillus niger*
12.	*Aspergillus niger*
13.	*Aspergillus niger*
14.	*Aspergillus niger*
15.	*Aspergillus niger*

4. Discussion

This study demonstrated the wide contamination frequency with aflatoxin in peanut samples from different locations in Sudan. Levels of aflatoxin were high in most locations. These results are similar to those obtained by Lund *et al*. (2000) who reported that the 27 samples (23.5%) of peanut and peanut products of one hundred and fifteen (27/115) showed positive to aflatoxin B_1 with a range of (1.6 – 26.0 µg/ Kg). This range is similar to the twenty two samples (18.33%) of the one hundred and twenty samples of peanut and peanut butter studied in this study. Also Suliman *et al*. (2007) reported that the 73/145 (50%) stored peanut kernels showed positive to aflatoxin B_1 with ranges of (0.8 – 547.5 µg/ Kg); this resembles the range shown in our study (13.47 – 404.00 µg/ Kg).

A survey done in Philippines on peanut-based products revealed that 60% of the samples were positive for aflatoxin B_1 in range of 1.00 - 244 µg/ Kg (Ali *et al*., 1999). In addition, there are several surveys that show a relatively lower level of contamination of aflatoxin B_1 in peanuts and their products. Siame *et al*., (1998) who did a study in Botswana, Africa, reported that the levels of aflatoxin B_1 in a range of (0.8 – 16.00 µg/ Kg) for the raw shelled peanut samples and for peanut butter were (3.2 – 16.00 µg/ Kg). In Tokyo, Japan, Tabata *et al*., (1993) found that several peanut products were contaminated by aflatoxin B_1 in a range of (0.4 – 21.7 µg/ Kg).

According to Ali *et al*. (1999), when the initial content of aflatoxin was high in the raw shelled peanut, a high level of aflatoxin contamination can be expected in its final products such as peanut candy and peanut butter. On the other hand, the low level of aflatoxin contamination in the peanut products has always been associated with the use of high quality raw materials (raw shelled peanut) that contain an acceptable low initial level of aflatoxin. Besides, various peanut processing techniques, such as shelling, drying under sunlight, boiling with salty water, and roasting, were also found to be useful in reducing the aflatoxin content in the products (Yazdanpanah *et al*., 2005).

The percentage of samples (53.33%) for the occurrence of aflatoxin B_1 in this survey was almost similarr to that in several previous studies such as Ali, (2000) who reported the contamination of aflatoxin B_1 in 56% of raw peanut samples, 50% of peanut butter samples, and 50% of other peanut products samples.

5. Conclusion

The levels of aflatoxin B_1 contamination in stored peanut kernel samples, collected from Mayo city, Umbaddah city, Al-Helalia city, and Al-Managel city in Sudan were very high and exceeded the maximum permitted levels according to the WHO/FAO food regulations of 1996, but not all the samples that showed positive results to chemical analysis contained *Aspergillus flavus*.

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Genetic Diversity Analysis of *Achillea fragrantissima* (Forskal) Schultz Bip Populations Collected From Different Regions of Jordan Using RAPD Markers

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Abstract

Medicinal plants are still used traditionally to cure some illnesses. *Achillea fragrantissima* is among medicinal species used by rural communities in Jordan. The genetic diversity of this species was studied using Random Amplified Polymorphic DNA (RAPD) markers of five populations sampled from five locations in Jordan. Fifteen polymorphic primers were used for the construction of the dendrogram and the similarity matrix. A total of 2599 bands were obtained, 420 of them were polymorphic. Similarity values between the populations samples ranged from 0.58 between the samples of Madaba (1 population) to 0.02 between Shoubak (3 populations) and Mwaqqar (2 populations). High similarity values were also recorded between Madaba (1population) and Mafraq (1 population), two samples of Ma'an (3), two samples of Madaba (1), two samples of Mwaqqar, Mafraq (1), and Madaba (1) populations with values from 0.54 to 0.49, respectively. Four main clusters were obtained based on UPGMA dendrogram. Both Mwaqqar2 and Ma'an 3 populations formed separate sub-clusters. This emphasizes the presence of genetic diversity among the studied populations. RAPD analysis has the ability to discriminate between *Achillea fragrantissima* populations.

Keywords: *Achillea fragrantissima*, genetic diversity and RAPD markers, polymorphism.

1. Introduction

Flora of Jordan is rich and diverse with medicinal and aromatic plants as well as herbs and spices. A total of 485 species of these plants belonging to 330 genera and 99 families were reported by Oran, 1994. These plants have been used by local people in folk medicine for curing human and animal illnesses. These species also contribute to increasing the income of poor people, especially women, who are involved in most of the fieldwork, processing and marketing of medicinal plants (Rawashdeh, 2007). *Achillea fragrantissima* is a medicinal shrub plant that belongs to the Asteraceae family (Sahin *et al*., 2006). *A. fragrantissima* species is diploid and contains this number of chromosomes: of 2n =18 (Rawashdeh *et al*., 2009 c; Rawashdeh, 2007). It is known as a sweet-smelling Lavender Cotton (Al-Esawi, 1998) and as one of the important medicinal and aromatic plant species in Jordan. Al-Esawi (1998) described *A. fragrantissima* as a perennial herb, 30-60 cm long, forming a hemispherical bushy growth, covered by minute, soft to woolly hairs, strongly aromatic. Leaves are simple, 0.5-1.5 cm long, with serrate margins, densely, woolly at lower surface. Flower heads are yellow, less than 1 cm in diameter, arranged in a flattened top, medium, compact inflorescence. It flowers from June to October. This species is mainly distributed in the desert and the dry areas of Jordan especially along runoff water places and is found in Eastern desert and Wadi Rum. *A. fragrantissima* is known by local communities as Qaisoum or Qisum (Mustafa, 1991) and is the only *Achillea* species used for medicinal purposes in Jordan. It is mainly used for treating diabetes, intestinal colic, lowering blood cholesterol level and as a carminative, dysmenorrheal and various infections (Atyat, 1993; Hammed, 1993; Mustafa, 1991; Kelly *et al*., 1988).

Several species of *Achillea* are grown for their flowers; their inflorescences are a rich source of active substances such as essential oils, sesquiterpene lactones, flavonoids and tannins (Špinarová and Petříková, 2003). *A. fragrantissima* is used for anti-inflammation, anti-nosebleeds, anti-excessive menstruation and hemohorrids (Atyat, 1993). Abu-Rabia (2005) reported that the boiled leaves of *A. fragrantissima* were used to treat smallpox (Jadra, Jadari) through using them in washing the whole body. Also, it was showed that the leaves had the most

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prominent cytotoxic activity (above 96%) against both types of melanoma cell lines (Sathiyamoorthy *et al*., 1999).

Many different molecular markers techniques were used for assessing genetic diversity (Karp and Edwards, 1997). Among these, the Amplified Fragment Length Polymorphism (AFLP) method has been successfully employed for fingerprinting varieties and cultivars (Vos *et al.,* 1995) and for studying the genetic diversity among and within different populations of *A. fragrantissima* (Rawashdeh *et al*., 2009 b**;** Rawashdeh, 2007).

Morphologic diversity in the field, genetic relatedness using AFLP markers, morphological diversity, chromosome counting, and essential oil composition were studied among and between *A. fragrantissima* populations collected from 15 monitoring areas in Jordan (Rawashdeh *et al*., 2009 a; Rawashdeh *et al*., 2009 b; Rawashdeh, *et al*., 2009 c; Rawashdeh, 2007). The results showed the existence of an association between morphology and molecular analysis, especially in the populations of Shoubak and Ma'an, which form separate groups in the analysis.

RAPD was used to detect the genetic variation among *A. fragrantissima, A. biebersteinii* and *A. santolina,* which have antimicrobial activity using four primers OPB01, OPB10, OPB15 and OPB18. The results showed 35% similarity between *A. fragrantissima,* and *A. santolina* (Kharma, 2004). RAPD was used for fingerprinting of five populations of *A. fragrantissima* in Egypt (Morsy, 2007), revealing that differences in locations were particularly reflected on DNA fingerprints.

This study aims at elucidating the genetic relationships among *A. fragrantissima* populations using RAPD technique.

2. Materials and methods

2.1. Plant material

The study evaluated a total of 50 samples of *A. fragrantissima* composed of 10 samples from each of the populations collected from different habitats in Ma'an (3), Shoubak (1), Madaba (1), Mwaqqar and Mafraq.

2.2. DNA isolation

Following the procedure described by Doyle and Doyle (1987) with minor modifications, total cellular DNA was extracted from the fresh leaves of *A. fragrantissima* populations when they were at the seedling stage. Approximately 20 mg of fresh leaves of "30-days-old" *A. fragrantissima* plants were grounded in liquid nitrogen and mixed with 600 µl of freshly and preheated 2x CTAB solution with 0.8g PVPP in 2ml tubes then placed at 65ºC for 30 min. The mixture was added to 600 µl of chlorophorm/isoamyl alcohol (24:1), vortexed for few seconds, and then centrifuged at 13,000g for 10 min. The supernatant was placed in 2ml tubes with 600ml isopropanol, and then shaken until the thread of DNA appeared, then centrifuged for 20 min at 13,000g. The solution is poured in tubes and left to dry, then 600 µl of cooled 70% ethanol was added to the solution and placed in the refrigerator (–20ºC) overnight. Next day, ethanol was poured in the dried tubes and 150µl of TE was added; the whole mixture was placed at 65ºC for 60 min. Four micolitters of RNAase (10mg/ml) were added per tube and left for 60 min at 37ºC. The DNA quantity was measured using S2100 UV/VIS DIODE-Array-Spectrophotometer, machine Version 1.7.

2.3. PCR amplification

The stored DNA at -20ºC was selected for PCR reaction. PCR reaction was performed as described by Williams *et al*. (1990) with 10-mer ologonucleotides synthesized by Operon technologies (Almeda, Calif.). The final volume of 25µl contained 10 x buffer with $MgCl_2$, 20ng of total genomic DNA, 0.25 mM dNTPs (Promega), 100 µM of primers, 1.5mM $MgCl_2$ and 1U of *Taq* polymerase. Amplification was carried out in thermocycler (MJ Research, USA, Model PCT-200), one cycle of 1 min at 94°C followed by 44 cycles, each consisting of a denaturation step for 1min at 94°C, followed by an annealing step for 1min at 36°C and an extension step for 2 min at 72°C, followed by a further extension step for 5 min at 72°C. After the final cycle, the samples were cooled at 4°C. Samples of 10 µl RAPD-PCR product were analyzed by electrophoresis on 1.4% a garose gel and the

amplified products were detected after staining by ethidium bromide. Forty-four RAPD primers, corresponding to kits A, B, C, D, N, T and Z (Table 1), were used to study the genetic variation and diversity among the *A. fragrantissima* populations. Fifteen RAPD primers which showed polymorphism were used to construct the dendrogram (Table 2).

2.4. Data analysis

RAPD bands were manually scored as present (1) or absent (0) for estimating the similarity among all the tested samples. The matrix of similarity, based on Jaccard's method, and similarity coefficients, based on Nei and Li (1979), were calculated and the dendrogram gained clustering according to the Unweighted Pair-Group Method with Arithmetic Averages (UPGMA) using SPSS, V. (11.0), software. The polymorphism percentage was estimated by dividing the number of polymorphic bands over the total number of bands.

3. Results

Forty-four RAPD primers (Table 1) were used for initial assessment of genetic variation in five populations of *Achillea fragrantissima*. A total of 50 samples (10 samples per population) collected from five regions in Jordan were amplified using 15 polymorphic RAPD oligonucleotides indicated in Table 2. The total number of bands, the number of polymorphic bands and the percent of polymorphism per primer are shown in Table 2. A total of 2599 RAPD fragments were consistently recognized and 420 (16%) of them were polymorphic for all population samples (Table 2). The number of polymorphic bands ranged from 34 for OPC03 to 21 for OPN14. The percent of polymorphism ranged from 13% for OPT16 to 21 for OPT15 with an average of 16.4 % polymorphism (Table 2). The total number of the bands amplified ranged from 233 bands (OPT16) to 120 bands (OPD18).

Table 1. Primers names and their sequences as used in this study

Primer name	Sequence 5'-3'	Primer name	Sequence 5'-3'
OPA09	GGGTAACGCC	23. OPC18	TGAGTGGGTG
OPA10	GTGATCGCAG	24. OPC20	ACTTCGCCAC
OPA13	CAGCACCCAC	25. OPD04	TCTGGTGAGG
OPA15	TTCCGAACCC	26. PD06	ACCTGAACGG
OPA16	AGCCAGCGAA	27. OPD10	GGTTCACACC
OPA18	AGGTGACCGT	28. OPD11	AGCGCCATTG
OPA20	GTTGCGATCC	29. OPD14	CTTCCCCAAG
OPB01	GTTTCGCTCC	30. OPD16	AGGGCGTAAG
OPB04	GGACTGGAGT	31. OPD18	GAGAGCCAAC
OPB05	TGCGCCCTTC	32. OPD20	ACCCGGTCAC
OPB06	TGCTCTGCCC	33. OPN14	TCGTGCGGGT
OPB08	GTCCACACGG	34. OPN16	AAGCGACCTG
OPB09	TGGGGGACTC	35. OPT03	TCCACTCCTG
OPB10	CTGCTGGGAC	36. OPT05	GGGTTTGGCA
OPB12	CCTTGACGCA	37. OPT10	CCTTCGGAAG
OPB13	TTCCCCCGCT	38. OPT12	GGGTGTGTAG
OPB14	TCCGCTCTGG	39. OPT15	GGATGCCACT
OPB17	AGGGAACGAG	40. OPT16	GGTGAACGCT
OPB19	ACCCCCGAAG	41. OPT19	GTCCGTATGG
OPC03	GGGGGTCTTT	42. OPZ07	CCAGGAGGAC
OPC09	CTCACCGTCC	43. OPZ10	CCGACAAACC
OPC10	TGTCTGGGTG	44. OPZ18	AGGGTCTGTG

Table 2. Total bands, number of polymorphic bands, and percent polymorphism per primer of 15 polymorphic RAPD primers as used in this study

Primer name	Total bands/primer	Number of polymorphic bands	% of polymorphism
OPA09	200	33	16
OPA10	173	25	14
OPB01	165	30	18
OPB06	175	26	15
OPB19	166	24	14
OPC03	206	34	17
OPD18	120	21	18
OPD20	184	24	13
OPN14	186	34	18
OPN16	164	25	15
OPT03	187	27	14
OPT05	129	22	17
OPT10	161	32	20
OPT16	233	31	13
OPT15	150	32	21
Total	**2599**	**420**	**243** **Mean: 16.4**

Similarity matrix values ranged from (0.58) between the samples of Madaba (1 population) to (0.02) between Shoubak (3 populations) and Mwaqqar (2 populations). High similarity values were also recorded between Madaba (1 population) and Mafraq (1 population), two samples of Ma´an (3 populations), two samples of Madaba (1 population), two samples of Mwaqqar population and Mafraq (1) and Madaba (1), which were 0.54, 0.51, 0.50, 0.49 and 0.49, respectively (Table 3). The dendrogram based on UPGMA analysis grouped the five populations into three main clusters (Figure 1) with Jaccard's similarity coefficients. The first main cluster included 10 samples of Madaba (1 population), 8 samples of Mafraq (1 population) and one sample of Ma´an (3 populations). The second cluster contained 10 samples of Shoubak (3 populations), 9 samples of Ma´an (3 populations) while the third cluster included 10 Mwaqqar (3 populations) and two samples of Mafraq (1 population) (Figure 1).

4. Discussion

High polymorphism that is obtained in this study indicates the presence of genetic variation among the *Achillea fragrantissima* populations collected from different districts in Jordan. This variation is attributed to the differences in the number of alleles per locus/or loci and their distribution within the population. The presence of a large range of similarity values (0.58-0.02) between the tested samples revealed that the differences in the ecosystems of *Achillea fragrantissima* might be reflected on RAPD-PCR. Similar results were obtained by Morsy (2007) when fingerprinting five populations of *Achillea fragrantissima* using RAPD-PCR. In this study, eight samples out of 10 of Shoubak populations formed a separate sub-cluster, which is in agreement with the findings by Rawashdeh *et al.*, (2009 b) using AFLP markers. This result indicates that a genetic diversity is found among the studied populations. RAPD analysis has a potential for studying the genetic diversity among and within *Achillea fragrantissima* populations. However, the application of biochemical and genetic systems play a great role in studying the status of wild plant species and in investigating their evolution and migration from their centers of distribution. Combining several of molecular patterns such as proteins, isoenzymes and AFLP, SSRs and ISSR profiling will allow discriminating between all populations and species of *Achillea* genus in the future.

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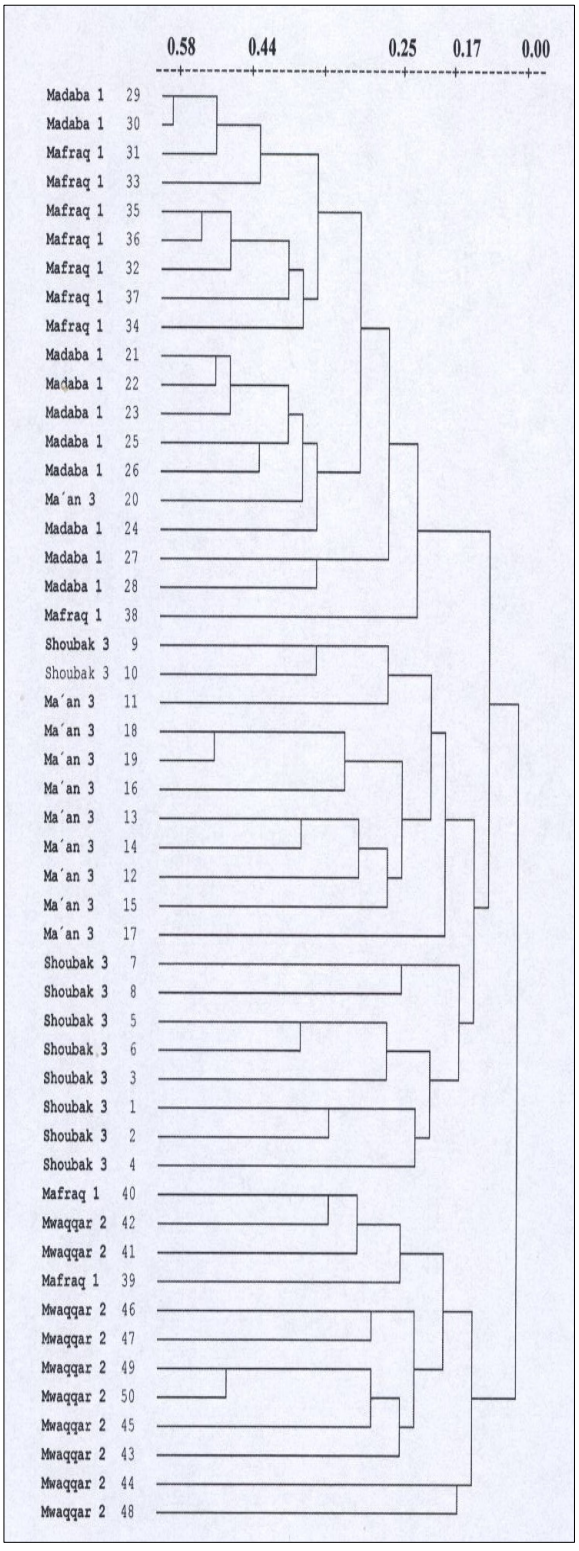

Figure 1. A dendrogram among five locations of medicinal plant species "*Achillea fragrantissima*" using fifteen polymorphic RAPD primers, based on Jaccards´ coefficient of similarity. The disconnected line is the point where the dendrogram is divided.

Table 3 continues next page……

Table 3: RAPD similarity matrix based on similarity coefficient of the amplified bands for *Achillea fragrantissima* species.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1	1																													
2	0.35	1																												
3	0.22	0.28	1																											
4	0.22	0.24	0.21	1																										
5	0.16	0.19	0.30	0.25	1																									
6	0.18	0.21	0.26	0.22	039	1																								
7	0.16	0.15	0.16	0.13	0.16	0.16	1																							
8	0.20	0.15	0.20	0.19	0.17	0.22	0.25	1																						
9	0.16	0.14	0.21	0.18	0.24	0.23	0.19	0.30	1																					
10	0.11	0.12	0.15	0.15	0.20	0.14	0.14	0.20	0.36	1																				
11	0.18	0.17	0.22	0.21	0.21	0.21	0.18	0.20	0.26	0.27	1																			
12	0.15	0.13	0.11	0.20	0.15	0.14	0.09	0.11	0.21	0.23	0.24	1																		
13	0.16	0.19	0.15	0.16	0.18	0.17	0.16	0.21	0.28	0.31	0.25	0.33	1																	
14	0.19	0.19	0.12	0.16	0.13	0.15	0.14	0.16	0.20	0.21	0.27	0.31	0.40	1																
15	0.14	0.14	0.10	0.22	0.14	0.12	0.15	0.13	0.17	0.22	0.20	0.22	0.28	0.30	1															
16	0.11	0.10	0.13	0.14	0.13	0.14	0.14	0.11	0.19	0.16	0.22	0.24	0.28	0.23	030	1														
17	0.15	0.12	0.11	0.13	0.11	0.13	0.14	0.12	0.20	0.17	0.16	0.19	0.21	0.21	0.18	0.20	1													
18	0.14	0.15	0.11	0.14	0.11	0.08	0.13	0.11	0.22	0.18	0.17	0.25	0.32	0.27	0.19	0.34	0.22	1												
19	0.17	0.16	0.14	0.19	0.11	0.11	0.15	0.12	0.17	0.13	0.17	0.24	0.24	0.26	0.22	0.31	0.18	0.51	1											
20	0.14	0.10	0.09	0.10	0.09	0.12	0.04	0.07	0.11	0.12	0.18	0.20	0.11	0.17	0.17	0.18	0.16	0.17	0.19	1										
21	0.14	0.12	0.09	0.12	0.12	0.13	0.05	0.07	0.11	0.10	0.17	0.25	0.13	0.17	0.16	0.14	0.11	0.17	0.21	0.39	1									
22	0.18	0.13	0.10	0.13	0.10	0.13	0.05	0.07	0.12	0.07	0.14	0.18	0.10	0.17	0.14	0.15	0.11	0.19	0.20	0.42	0.50	1								
23	0.15	0.12	0.12	0.18	0.11	0.10	0.03	0.08	0.15	0.08	0.19	0.17	0.12	0.18	0.15	0.14	0.16	0.16	0.14	0.38	0.48	0.49	1							
24	0.15	0.13	0.16	0.13	0.11	0.14	0.09	0.11	0.15	0.09	0.20	0.18	0.14	0.16	0.12	0.15	0.15	0.16	0.16	0.32	0.32	0.41	0.41	1						
25	0.12	0.09	0.07	0.11	0.06	0.08	0.05	0.09	0.11	0.08	0.11	0.15	0.11	0.17	0.14	0.13	0.11	0.15	0.17	0.35	0.38	0.48	0.40	0.33	1					
26	0.11	0.10	0.09	0.11	0.08	0.11	0.08	0.10	0.10	0.07	0.16	0.16	0.13	0.17	0.13	0.15	0.10	0.17	0.21	0.37	0.45	0.42	0.39	0.42	0.44	1				
27	0.12	0.11	0.12	0.13	0.12	0.18	0.07	0.06	0.08	0.08	0.18	0.16	0.15	0.17	0.16	0.14	0.16	0.12	0.17	0.26	0.31	0.28	0.29	0.28	0.25	0.34	1			
28	0.18	0.10	0.10	0.12	0.08	0.12	0.11	0.06	0.12	0.11	0.16	0.18	0.12	0.14	0.16	0.13	0.20	0.15	0.18	0.29	0.28	0.26	0.25	0.31	0.24	0.33	0.36	1		
29	0.15	0.08	0.10	0.15	0.08	0.10	0.06	0.08	0.11	0.14	0.17	0.21	0.10	0.18	0.14	0.17	0.13	0.16	0.18	0.31	0.36	0.34	0.29	0.32	0.40	0.38	0.30	0.32	1	
30	0.17	0.12	0.14	0.15	0.09	0.15	0.05	0.06	0.10	0.11	0.20	0.17	0.15	0.16	0.17	0.18	0.11	0.13	0.19	0.36	0.38	0.35	0.30	0.32	0.32	0.40	0.38	0.35	0.58	1
31	0.13	0.15	0.14	0.16	0.10	0.11	0.07	0.07	0.10	0.12	0.14	0.20	0.14	0.16	0.16	0.18	0.14	0.20	0.22	0.32	0.44	0.37	0.35	0.29	0.33	0.38	0.29	0.33	0.49	0.54
32	0.22	0.15	0.17	0.16	0.16	0.14	0.06	0.09	0.14	0.18	0.17	0.22	0.17	0.21	0.19	0.22	0.14	0.19	0.21	0.34	0.34	0.37	0.36	0.27	0.32	0.28	0.24	0.29	0.41	0.49
33	0.12	0.10	0.13	0.17	0.11	0.16	0.06	0.09	0.12	0.12	0.18	0.17	0.14	0.14	0.13	0.15	0.17	0.14	0.17	0.29	0.36	0.39	0.35	0.26	0.41	0.35	0.38	0.30	0.42	0.46
34	0.16	0.13	0.09	0.21	0.15	0.12	0.06	0.09	0.12	0.14	0.17	0.16	0.14	009	0.08	0.10	0.08	0.15	0.13	0.23	0.32	0.29	0.33	0.20	0.26	0.27	0.18	0.21	0.28	0.34
35	0.15	0.13	0.10	0.19	0.15	0.15	0.6	0.09	0.12	0.16	0.12	0.19	0.15	0.15	0.12	0.15	0.12	0.19	0.15	0.25	0.34	0.33	0.34	0.21	0.28	0.26	0.19	0.18	0.37	0.41
36	018	0.13	0.15	0.15	0.15	0.14	0.06	0.11	0.13	0.15	0.16	0.23	0.14	0.16	0.13	015	0.17	0.17	0.19	0.32	0.34	0.41	0.32	0.25	0.32	0.31	0.23	0.22	0.32	0.36
37	0.14	0.14	0.15	0.18	0.14	0.14	0.09	0.11	0.14	0.10	0.15	0.14	0.11	0.14	0.13	0.12	0.10	0.13	0.12	0.28	0.24	0.29	0.27	0.18	0.25	0.24	0.16	0.18	0.35	0.32
38	0.17	0.16	0.14	0.21	0.11	0.13	0.06	0.08	0.09	0.13	0.12	0.17	0.10	0.14	0.11	0.12	0.14	0.11	0.15	0.21	0.25	0.25	0.23	0.14	0.21	0.19	0.16	0.16	0.19	0.23
39	0.07	0.047	0.07	0.07	0.07	0.10	0.05	0.07	0.12	0.13	0.12	0.09	0.05	0.06	0.07	0.07	0.08	0.13	0.08	0.10	0.09	0.08	0.07	0.12	0.04	0.11	0.10	0.14	0.09	0.09
40	0.09	0.09	0.07	0.10	0.08	0.14	0.07	0.07	0.08	0.17	0.10	0.08	0.06	0.13	0.09	0.09	0.09	0.12	0.11	0.15	0.13	0.14	0.14	0.10	0.10	0.14	0.14	0.13	0.14	0.14
41	0.08	0.06	0.07	0.11	0.06	0.10	0.09	0.08	0.12	0.14	0.12	0.19	0.12	0.16	0.16	0.13	0.16	0.16	0.15	0.13	0.11	0.12	0.09	0.13	0.10	0.11	0.11	0.14	0.16	0.12
42	0.08	0.06	0.04	0.08	0.05	0.10	0.06	0.06	0.11	0.12	0.12	0.11	0.09	0.12	0.12	0.10	0.12	0.11	0.10	0.12	0.10	0.11	0.10	0.09	0.08	0.10	0.11	0.16	0.11	0.10
43	0.06	0.06	0.07	0.09	0.07	0.06	0.08	0.05	0.09	0.13	0.10	0.15	0.07	0.08	0.10	0.11	0.06	0.09	0.08	0.12	0.11	0.09	0.09	0.07	0.06	0.09	0.10	0.11	0.12	0.13
44	0.08	0.07	0.09	0.10	0.07	0.09	0.07	0.09	0.11	0.08	0.10	0.16	0.09	0.18	0.12	0.08	0.12	0.10	0.14	0.11	0.08	0.06	0.07	0.06	0.07	0.07	0.11	0.14	0.09	0.06
45	0.13	0.08	0.09	0.09	0.11	0.14	0.08	0.09	0.14	0.12	0.11	0.11	0.04	0.11	0.09	0.07	0.11	0.07	0.09	0.10	0.12	0.13	0.13	0.08	0.09	0.09	0.13	0.11	0.12	0.08
46	0.06	0.05	0.03	0.09	0.07	0.11	0.09	0.08	0.07	0.09	0.06	0.09	0.07	0.07	0.07	0.04	0.08	0.06	0.06	00.6	0.07	0.05	0.07	0.06	0.05	0.10	0.07	0.06	0.07	0.05
47	0.04	0.02	0.20	0.06	0.04	0.05	0.06	0.05	0.05	0.09	0.04	0.05	0.03	0.02	0.07	0.02	0.03	0.03	0.03	0.05	0.04	0.07	0.04	0.07	0.08	0.05	0.07	0.07	0.06	0.07
48	0.06	0.07	0.08	00.8	0.10	0.08	0.07	0.10	0.06	0.09	0.10	0.10	0.08	0.08	0.11	0.05	0.09	0.11	0.10	0.20	0.06	0.04	0.04	0.04	0.02	0.07	0.10	0.09	0.06	0.07
49	0.07	0.04	0.05	0.08	0.09	0.10	0.07	0.05	0.06	0.07	0.08	0.08	0.03	0.06	0.07	0.04	0.09	0.06	0.03	0.04	0.07	0.06	0.06	0.06	0.05	0.06	0.08	0.11	0.11	0.09
50	0.07	0.4	0.05	0.09	0.08	0.11	0.05	0.05	0.07	0.11	0.11	0.10	0.05	0.07	0.07	0.03	0.07	0.05	0.05	0.06	0.09	0.09	0.07	0.08	0.06	0.07	0.08	0.09	0.12	0.11

Table 3: RAPD similarity matrix based on similarity coefficient of the amplified bands for *Achillea fragrantissima* species continues……..

	31	32	33	34	35	36	12	38	39	40	41	42	43	44	45	46	47	48	49	50
31	1																			
32	0.45	1																		
33	0.46	0.41	1																	
34	0.32	0.34	0.38	1																
35	0.41	0.48	0.41	0.49	1															
36	0.38	0.49	0.42	0.36	0.53	1														
37	0.27	0.38	0.31	0.35	0.44	0.41	1													
38	0.30	0.31	0.24	0.26	0.29	0.32	0.30	1												
39	0.09	0.12	0.07	0.12	0.07	0.08	0.07	0.13	1											
40	0.14	0.12	0.13	0.14	0.13	0.12	0.15	0.13	0.29	1										
41	0.15	0.13	0.13	0.09	0.09	0.11	0.10	0.14	0.25	0.33	1									
42	0.10	0.07	0.09	0.10	0.10	0.07	0.12	0.08	0.26	0.34	0.29	1								
43	0.11	0.13	0.11	0.09	0.10	0.09	0.14	0.09	0.16	0.22	0.23	0.25	1							
44	0.07	0.06	0.06	0.04	0.05	0.06	0.08	0.09	0.16	0.18	0.17	0.15	0.12	1						
45	0.08	0.13	0.14	0.10	0.14	0.15	0.16	0.12	0.15	0.16	0.23	0.19	0.24	0.23	1					
46	0.06	0.07	0.08	0.08	0.08	0.12	0.11	0.09	0.15	0.23	0.19	0.19	0.22	0.18	0.25	1				
47	0.03	0.05	0.08	0.07	0.09	0.06	0.15	0.05	0.18	0.15	0.14	0.24	0.27	0.16	0.16	0.29	1			
48	0.07	0.05	0.06	0.04	0.06	0.07	0.09	0.07	0.10	0.08	0.15	0.13	0.16	0.17	0.15	0.12	0.21	1		
49	0.08	0.07	0.08	0.10	0.12	0.12	0.13	0.12	0.12	0.13	0.11	0.21	0.17	0.11	0.25	0.23	0.18	0.10	1	
50	0.08	0.09	0.08	0.11	0.11	0.11	0.16	0.14	0.20	0.19	0.21	0.28	0.34	0.17	0.31	0.30	0.27	014	0.49	1

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The Effect of Certain Environmental Factors on Growth and β-Carotene Production by *Dunaliella sp.* Isolated from the Dead Sea

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Abstract

Microalga *Dunaliella sp.* was isolated and identified from ponds of the south-east shores of the Dead Sea. The most suitable media for the isolated *Dunaliella sp.* was found to be M1 (modified BG-11) which gave the highest growth and β-carotene production. The effects of different physical (temperature and light intensity) and chemical (different nitrogenous and sulfate compounds) factors were tested. The best salinity for *Dunaliella* growth was 2.5 % NaCl, while the maximum β-carotene to chlorophyll *a* (Chl. *a*) ratio was found in high salinities: Dead Sea water-M1 (DSw-M1) dilution (1:1), 10% NaCl and DSw-M1 (3:1). By using $NaNO_3$ at 40 mg N l^{-1} concentration as a nitrogen source and $MgSO_4$ at 25 mg l^{-1} concentration as a sulfate source, the maximum growth and β-carotene production was obtained. In response to different light intensities, the maximum growth was obtained at 61 µmol s^{-1} m^{-2}, and the maximum β-carotene production was at 200 µmol s^{-1} m^{-2}, while the maximum β-carotene to chlorophyll *a* ratio was recorded in cells grown at 1000 µmol s^{-1} m^{-2}.

الملخص

تم في هذه الدراسة عزل الطحالب الدقيقة المحبة للملوحة "دونياليلا" من السبخات المائية المالحة والمتناثرة في الجزء الجنوبي من البحر الميت. حيث كان أفضل وسط غذائي من المواد اللاعضوية لهذه الطحالب هو M1 (BG-11). وكان أفضل نمو للطحالب عند درجة حرارة 20 C م ، بينما ماتت معظم الخلايا عند درجة حرارة 40 و 50 C م . وقد تم دراسة تأثير عوامل وظروف فيزيائية وكيميائية مختلفة على نمو هذه الطحالب وانتاجها لمادتي البيتاكروتين والكلوروفيل . وقد سجل أعلى نمو لهذه الطحالب عند استخدام كلوريد الصوديوم بنسبة 2.5 % ، بينما كانت أعلى نسبة بيتاكاروتين/كلوروفيل أ عند تراكيز الملوحة العالية DSw- , 10 % M1 (1:1), DSw-M1 (3:1) . تم الحصول على أعلى نمو ونسبة انتاج البيتاكاروتين عند استعمال نترات الصوديوم (كمصدر للنيتروجين) بتركيز 40 ملغ نيتروجين/لتر ، وكبريتات المغنيسيوم (كمصدر للكبريت) بتركيز 25 ملغ/ل. وبعد دراسة تأثير شدة الضوء وجد أن أعلى نمو تم تسجيله عند شدة اضاءة 61 (مايكرومول/ث.م2) وأعلى انتاج للبيتاكاروتين كان عند شدة اضاءة 200 (مايكرومول/ث.م2) بينما كانت أعلى نسبة بيتاكاروتين/كلوروفيل أ عند شدة اضاءة 1000 (مايكرومول/ث.م2).

Keywords: Dunaliella, β - Carotene, Dead Sea, Halophiles

1. Introduction

The Dead Sea is the lowest exposed surface on earth (416 m below sea level) and is one of the world's saltiest lakes, with a total dissolved salt concentration of 340 gl^{-1}. It is considered sterile and, therefore, unsuitable for fishery (Gavrieli *et al*., 1999).

The eukaryotic algae *Dunaliella* was first described by Teodoresco in 1905, and first reported to be present in the Dead Sea by Elazari-Volcani in 1940 (Oren, 1999). *Dunaliella* is a unicellular, motile, green microalgae which lacks a rigid cell wall and has a single large cup-shaped chloroplast that fills the posterior part of the cell (Butcher, 1959; Javor, 1989).

Great interest in *Dunaliella* has arisen because of its ability to withstand various environment stresses, especially those associated with hypersaline conditions. Its halotolerance is predominantly mediated by an accumulation of glycerol as an osmoregulator (AL-Hasan *et al*., 1987; Ginzburg, 1987).

A number of studies have revealed that growth (Ben-Amotz and Avron, 1983; Ginzburg, 1987) and pigment compositions (Ben-Amotz *et al*., 1989) of this algae are affected by halostress conditions. It was found that the β-carotene to chlorophyll *a* ratio gradually increased with an increase in NaCl concentration, and, as a result, the algae changed its appearance from green to deep orange (Ben-Amotz and Avron, 1983; AL-Hasan *et al*., 1987).

Overproduction of β-carotene is induced by high light intensity (Kleinegris *et al.*, 2010) and by other environmental factors such as nutrient deprivation, or high salt concentration (Ben-Amotz and Avron, 1983; Raja *et al.*, 2007; Macias-Sa'nchez *et al*., 2009). β-Carotene was found to be accumulated in oil globules in the interthylakoid space of the chloroplast and it is composed of two major stereoisomers: all-*trans* and 9-*cis*

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β-carotene (Ben-Amotz *et al*., 1989; Macias-Sa'nchez *et al*., 2009; Kleinegris *et al*., 2010).

From the other side of the Dead Sea facing Jordan, *Dunaliella sp.* was isolated. So, the effect of certain environmental conditions on growth and β-carotene is reported.

2. Material and methods

2.1. Sampling and growth conditions

Sampling was carried out during spring season (April-May, 2003) from ponds present at the east-south basin of the Dead Sea. All collected samples were examined microscopically for the presence of a green algae *Dunaliella* and a preliminary cultivation was carried out using inorganic medium (AL-Hasan *et al*., 1987). Identification of the isolated and cultivated *Dunaliella* was carried out morphologically according to Butcher (1959).

Dunaliella cells were harvested by centrifugation at 3500 rpm for 15 min and washed several times with sterile 10% (w/v) NaCl solution to minimize bacterial contamination. Cells were then cultivated in an inorganic medium containing 5 mM $MgSO_4$, 0.3 mM $CaCl_2$, 5 mM KNO_3, 0.2 KH_2PO_4, 1.5 µM $FeCl_3$, 50 mM $NaHCO_3$, 30 µM EDTA, 5% NaCl, 300 U polymyxin B. ml^{-1}, 150 U penicillin G. ml^{-1}, 1000 U streptomycin. ml^{-1}, pH 8 (AL-Hasan *et al*., 1987). The antibiotics were sterilized separately and added to eliminate residual bacteria. Cultures were incubated at room temperature 25±2 C˚ with continuous slow stirring using a magnetic stirrer. Cultures were sparkled with sterile air. A constant illumination of 61 µmol m^{-2} s^{-1} was provided at the surface of the vessels using cool white fluorescent lamps.

Five different types of media were tested for their effects on the growth of *Dunaliella sp.*; these are: **M1** (BG-11 medium, Stanier *et al*. (1971), **M2** (f2 medium, Jeffrey and LeRoi (1997), **M3** (f/2 medium, Guillard (1962), **M4** medium of Ben-Amotz *et al*. (1989), and **M5** of Sallal *et al*. (1987).

2.2. Growth parameters and pigments

Cell number was determined using a haemocytometer (Jeffrey and LeRoi, 1997). Chlorophyll *a* and β-carotene were extracted from algal pellet with 80% (v/v) acetone according to (Ben-Amotz and Avron, 1983). $\boldsymbol{E}^{1\%}$ of 87.67 at 664 nm and $\boldsymbol{E}^{1\%}$ of 2273 at 480 nm have been used to calculate chlorophyll *a* and β-carotene concentrations, respectively (Ben-Amotz and Avron, 1983 and Jeffrey *et al*., 1997).

2.3. Cultivation of Dunailella cells in different nutrient media

M1 medium with different NaCl concentrations, 1.25%, 2.5%, 5%, 10%, 20%, and 30%, was used to grow *Dunailella sp.* Different dilutions of Dead Sea water and M1 medium were also used (DSw : M1) (1:1) and (3:1). Cultures were incubated at room temperature 25±2 C˚ under constant light illumination of 61 µmol m^{-2} s^{-1}.

Different nitrogenous sources such as $NaNO_3$, NH_4Cl, $Ca(NO_3)_2$, and NH_4NO_3 were prepared with the following concentrations: 10, 20, 30, 40, 50 mg Nl^{-1} in 250 ml conical flasks containing M1 nitrogen-free medium. Various concentrations of $MgSO_4$: 0, 25, 50, 75 mg/l were also prepared in M1 sulfate-free media. All cultures had 2.5% NaCl concentration, and were incubated at room temperature 25±2 C˚ and constant light illumination of 61 µmol m^{-2} s^{-1}.

Dunaliella culture grown in M1 medium were also illuminated at different light intensities: 61, 200, and 1000 µmol m^{-2} s^{-1} using cool white fluorescent lamps and Halogen lamp (Phoenix electric, China). Light intensity was measured using a photometer (LI-COR model LI-189, USA).

2.4. Pigment analysis

TLC was carried out for the extracted pigments as described by Stahl (1965). Two developing solvents were applied separately on TLC aluminum sheets of silica gel: (1) *n*-hexane : acetone : *iso*-propanol (69:30:1 v/v/v) to resolve individual pigments, (2) petroleum ether : acetone (97: 3 v/v) to resolve β,β-carotene (Repeta and Bjornland, 1997).

2.5. β-Carotene crystallization

β-Carotene crystallization was performed as described by Repeta and Bjornland (1997). 2 ml extract was dissolved in 10 ml benzene and cooled to a solid phase at - 20 C˚. 30 ml of pre-cooled methanol was added on the top of the solid benzene layer and the biphasic system was left at – 20 ˚C, for 1 to 3 days. The epiphasic methanol layer will slowly dissolve the solid benzene and the β-carotene will get crystallized at the interface between the layers.

3. Results

Collected samples from brine ponds at the east-south basin of the Dead Sea were examined microscopically for the presence of a green algae *Dunaliella* which was isolated and cultivated in M1 medium. *Dunaliella* cells were identified as *Dunaliella sp.* according to the description given by Butcher (1959).

In TLC, two structurally related β,β-carotene (β-carotene) and β,ε-carotene (α-carotene) were resolved using the developing solvent 1 (Fig. 1a) in addition to four other spots with different colors (blue-green, green, faint green, and faint yellow) and different $\boldsymbol{R_f}$ values (Fig. 1a). Using developing solvent 2, a yellow-orange β,β-carotene spot with a $\boldsymbol{R_f}$ value= 0.82, was also resolved (Fig. 1b).

Dunaliella cells were cultivated in 5 different types of media (M1, M2, M3, M4 and M5) under constant illumination and at room temperature 25±2 ˚C. Through 14 days, the maximum growth and β-carotene production were obtained with M1 media as shown in Fig. 2. Chlorophyll *a* was 7.5 mgl^{-1} while β-carotene was 5.2 mgl^{-1} (Fig.2). M5 media gave the maximum β-carotene to chlorophyll *a* ratio 0.8 while M4 gave the lowest ratio 0.6 as shown in Table1 and Fig2.

Table 1 shows the average number of *Dunaliella* / ml grown in five different media. The maximum number of *Dunaliella* cells was found in M1 medium, which agrees with the result obtained in Fig. 2. M5 medium gave $4.8x10^6$ cell/ml in comparison with $6.5x10^6$ cells/ml in

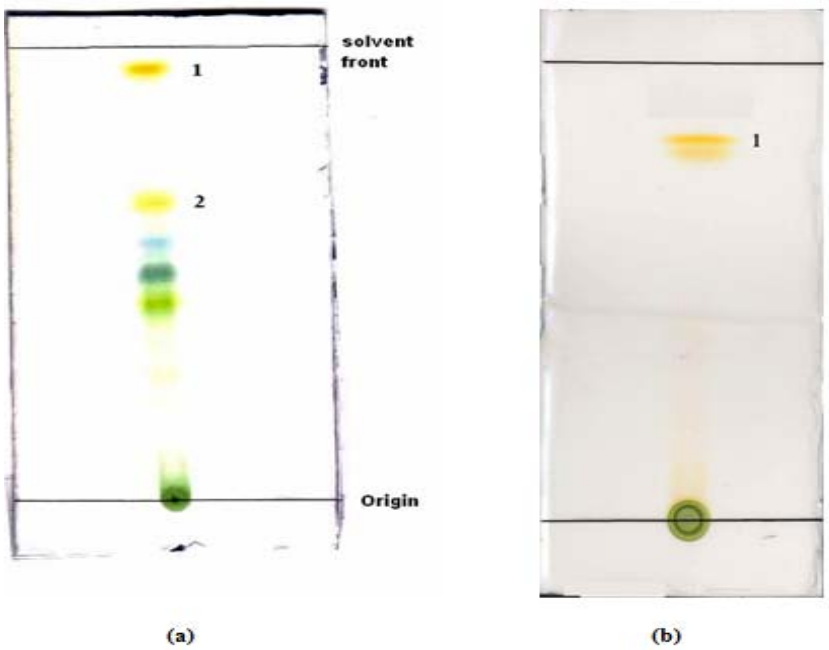

Figure 1. Thin layer chromatography (TLC) plates showing:

(a) The structurally related β,β- and β,ε-carotenes (1,2 respectively). Stationary phase: silica G, Mobile phase: *n*-hexane : acetone: *i*-propanol. (b)β,β-carotene (1) with R_f=0.82. Stationary phase: as in (a). Mobile phase: petroleum ether : acetone.

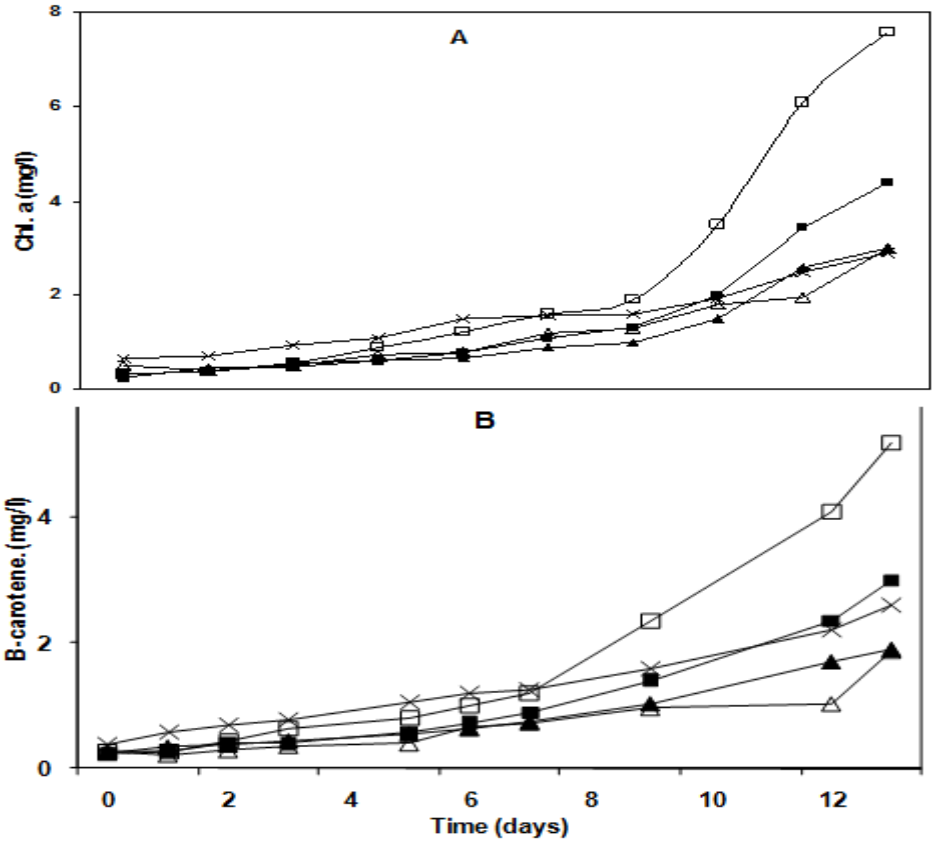

Figure 2. Growth (A) and β-carotene (B) of *Dunaliella sp.* in five different types of media: M1 □, M2 ■, M3 ▲ , M4 △, and M5 ✕ .

medium M1, while other types of media gave less numbers than these two types of media after 14 days of growth (Table 1).

Table 1. The average number of *Dunaliella sp*. cells grown in different types of media. ($x10^6$/ml) .

Days / Media	0	2	5	7	9	12	14
M1	0.5	1.7	1.5	3.9	4.1	6.4	6.5
M2	0.5	1.2	1.6	1.7	2.3	2.6	2.9
M3	0.45	1.6	2.0	2.1	2.2	2.4	2.4
M4	0.6	1.6	1.7	2.2	3.0	3.8	4.4
M5	0.5	1.2	1.4	1.9	2.7	3.1	4.8

Maximum growth was obtained at 20 ˚C where chlorophyll *a* and β-carotene concentrations were 3.4 and 2.1 mgl^{-1}, respectively, after 10 days. A slight growth was observed at 30 C˚, while no growth observed at 40 C˚ and 50 C˚.

The highest growth of *Dunaliella* cells was found toat 40 $mgNl^{-1}$. However $NaNO_3$ enhanced the highest growth and β-carotene production compared to other nitrogenous compounds used (Fig. 3). The different concentrations presented in this figure were the highest for each nitrogenous compound.

Sodium nitrate ($NaNO_3$) at a concentration of 40 $mgNl^{-1}$ gave 5.17 mgl^{-1} and 4 mgl^{-1} for chlorophyll *a* and β-carotene, respectively . However, the maximum β-carotene/chlorophyll *a* ratio was found to be 0.82 at 20 $mgNl^{-1}$ (Fig. 3).

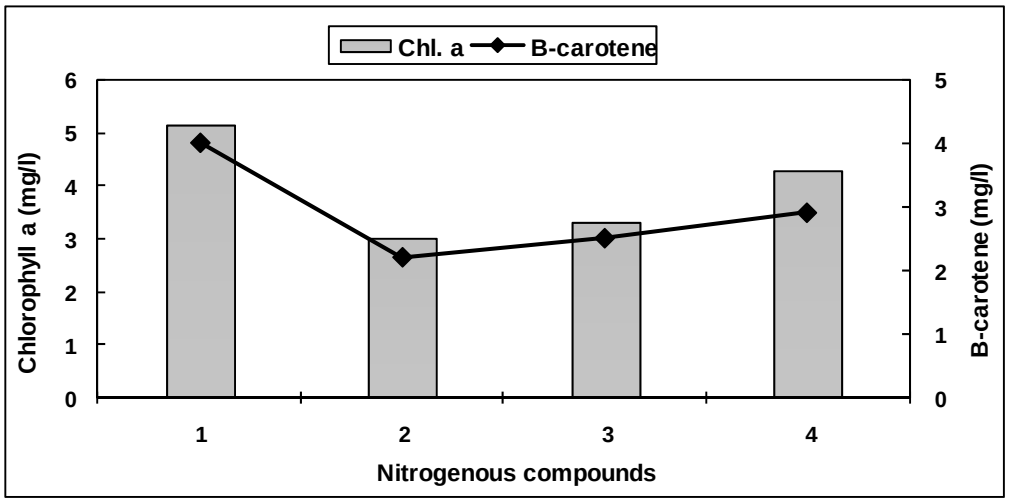

Figure 3. Growth and β-carotene production after 15 days of growth using M1 medium supplemented with different nitrogenous compounds: 1, 40 mg NL^{-1} $NaNO_3$; 2, 40 mg NL^{-1} $Ca(NO_3)_2$; 3, 40 mg NL^{-1} NH_4NO_3; and 4, 50 mg NL^{-1} NH_4Cl.

The effect of different concentrations of magnesium sulphate on *Dunaliella sp.* was studied. *Dunaliella* growth and β-carotene production were found to be the highest at 25 mgl^{-1} $MgSO_4$. So, chlorophyll *a* and β-carotene concentration were 3.4 mgl^{-1} and 2.4 mgl^{-1}, respectively (data not shown).

The maximum chlorophyll *a* and β-carotene production were obtained at 2.5% NaCl with 5 mg/l and 4.2 mg/l chlorophyll *a* and β-carotene respectively (Fig. 4). However, the decrease in chlorophyll *a* under laboratory conditions was noticed in 30% and DSw-M1 (3:1)(0.2mg/l) (Fig. 4), an increase in β-carotene production was 2.5mg/l noticed in *Dunaliella* grown in DSw-M1 (1:1) as compared to DSw-M1 (3:1). The best ratio of β-carotene/chlorophyll *a* was recorded in culture grown in DSw-M1 (1:1) which was 1.1.

Effects of different light intensities on *Dunaliella* growth are shown in Fig. (5). Chlorophyll *a* contents increase in *Dunaliella* with 4 and 2.6 mgl^{-1}, respectively, at both 61 and 200 µmol $s^{-1}m^{-2}$ after 12 days culture old, but *Dunaliella* chlorophyll *a* content decreased to 0.14 mgl^{-1} under 1000 µmol $s^{-1}m^{-2}$ light intensity (Fig. 5). *Dunaliella* grown under 1000 µmol $s^{-1}m^{-2}$ gave the maximum β-carotene/chlorophyll *a* ratio (1.25) at day 8, while cells grown under 61 µmol $s^{-1}m^{-2}$ gave 0.66 ratio.

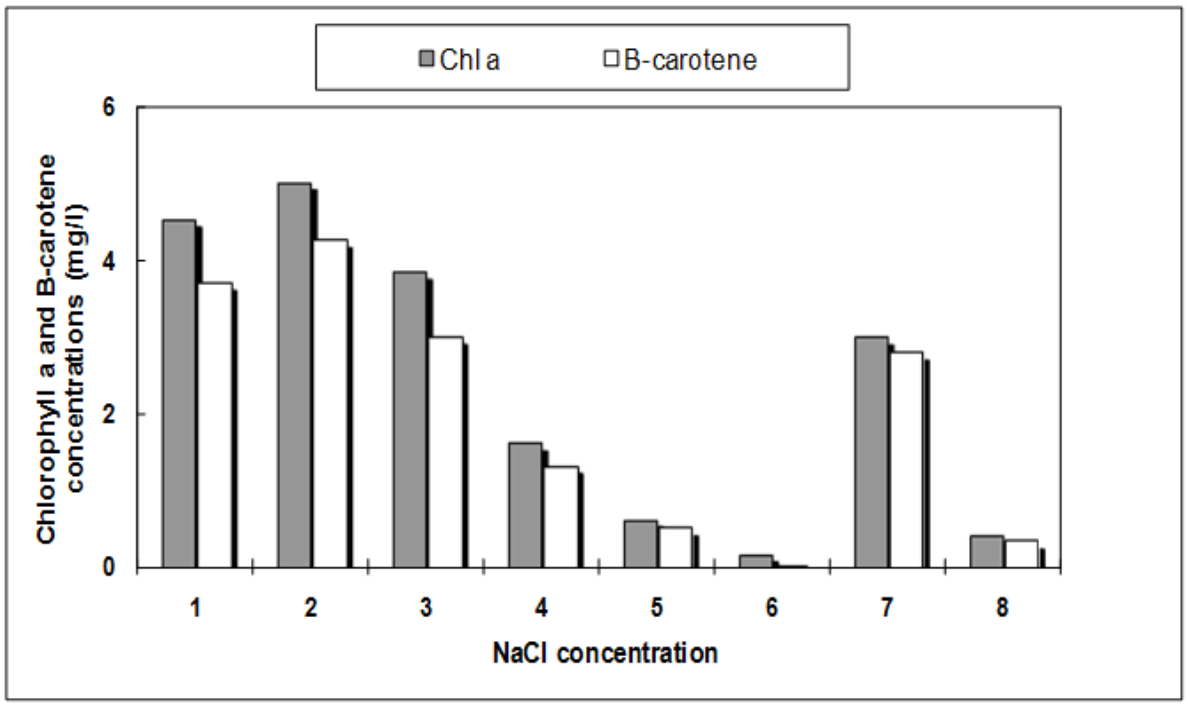

Figure 4. Effect of different NaCl concentrations: 1, 1.25%; 2, 2.5%; 3, 5%; 4, 10%; 5, 20%; 6, 30%; 7, 1:1 (DSw:M1); 8, 3:1 (DSw:M1) on the growth and β-carotene production of *Dunaliella sp.*

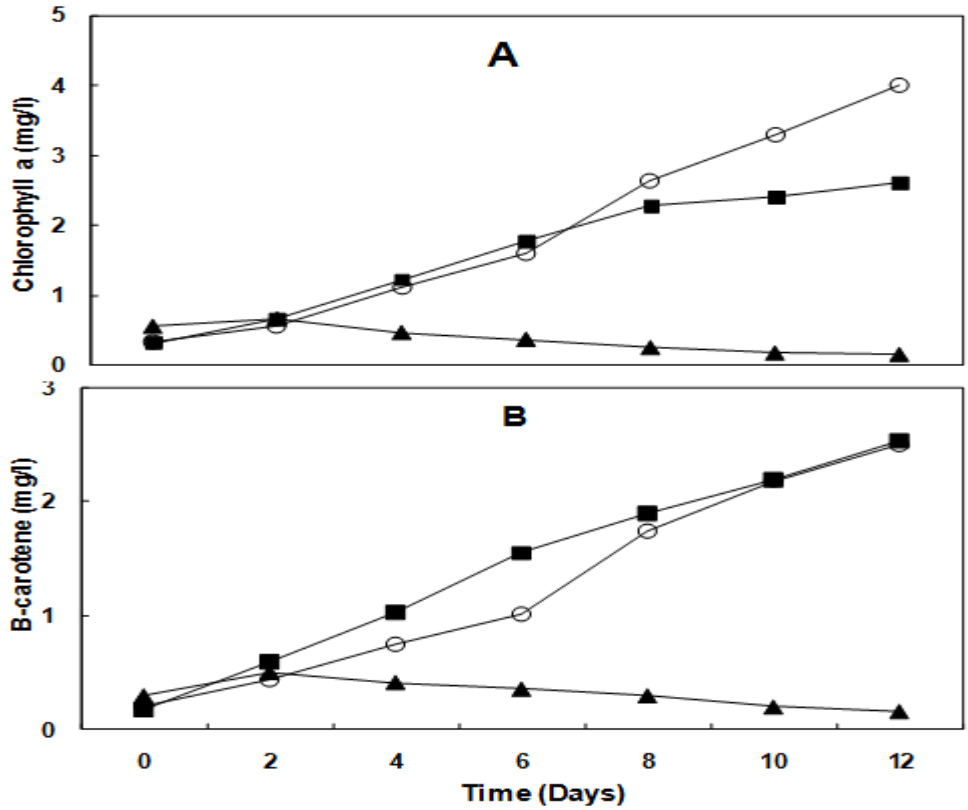

Figure 5. Effect of different light intensities on *Dunaliella sp.* growth (A) and β-carotene production (B). ¤ 61 µmol s^{-1} m^{-2}, ■ 200 µmol s^{-1} m^{-2}, ▲ 1000 µmol s^{-1} m^{-2}.

4. Discussion

Dunaliella sp. culture in this study remained green in color at all tested conditions. According to Sammy (1993) *D. viridis* remained green at all salinities, but he reported "a red flowering" growth of *Dunaliella salina*. Al-Hasan *et al.* (1987) recorded a red *Dunaliella* forms in Kuwait salt marshes during summer, while in winter *Dunaliella* cells turned green and became smaller in size.

The different species of *Dunaliella* have different optimal growth temperatures. *Dunaliella salina* was found to grow optimally at 30˚C, *D. viridis* at 37˚C, and *D. tertiolecta* at 20˚C (Brown and Browitzka, 1979). Other *Dunaliella* strain (AL-Hasan *et al*., 1987) remained viable at 55˚C during summer and at 12˚C during winter. In this study, *Dunaliella* cells were found to grow optimally at 20˚C.

Dunaliella sp. were found to grow better in $NaNO_3$ and NH_4Cl than in NH_4NO_3 (AL-Hasan and Sallal, 1985), which agrees with the results in this study as presented in Fig. 4. Gibor (1956) found that both *D. salina* and *D. viridis* grow much better with NO_3^--N than with NH_4^+-N; however, *D. salina* from the Great Salt Lake preferred NH_4^+ - N over NO_3 - N (Post, 1977).

The depletion of sulfate in the medium causes *Dunaliella* cells to stop dividing and start accumulating β-carotene inside the cells (Lers *et al*., 1990; Phadwal and Singh, 2003), and, as a consequence, an increase in β-carotene/chlorophyll a ratio (Ben-Amotz and Avron, 1983). These results are in agreement with this study where β-carotene production and β-carotene/chlorophyll *a* ratio increased at low sulfate concentration 25 mgl^{-1} $MgSO_4$.

In this study, *Dunaliella* cells grow optimally at 2.5 % salinity and tolerated up to 20% salinity (Fig. 4). In many studies, the major species of *Dunaliella* were found to have different salt concentrations. *D. viridis* grow optimally in 5.8 – 8.9% salinity and tolerate up to 23.2% salinity (Browitzka *et al*., 1977), while *Dunaliella salina* isolated from Kuwait marshes had an optimum growth at 2.5 – 5% (w/v) NaCl and the growth continued up to 30% NaCl (AL-Hasan *et al*., 1987). The strain of *Dunaliella tertiolecta* tolerated 0.5 – 34% NaCl (Wegmann, 1981).

The β-carotene/chlorophyll *a* ratio in this study was the highest at high salinities DSw-M1 (1:1), 10 % and DSw-M1 (3:1); this agrees with many studies that reported that β-carotene to chlorophyll *a* ratio increased in high NaCl concentration (Ben-Amotz and Avron, 1983). Javor (1989) reported that carotenoid content continued to increase in cells with respect to salinity in medium with >10% NaCl. Other study found that the highest β-carotene production per cell was obtained at 2 M NaCl in *D. salina* and *D. bardawil* in comparison with 1 and 3 M NaCl concentrations (Gomez *et al*., 2003).

Optimal light conditions for both growth and β-carotene production have been reported for several strains of *Dunaliella* (Van Auken and McNulty, 1973; AL-Hasan and Sallal, 1985; Javor, 1989). Light intensity is the major induced factor for β-carotene production, which is highly effective in protecting *Dunaliella* cells against photoinhibition due to the ability of β-carotene to quench damaging singlet oxygen and hydroxyl radicals (Ben-Amotz *et al*., 1989; Prescott *et al*., 2005). This is in accordance with the results of this study: β-carotene production increased at high light intensity (200 $\mu mol\ m^{-2}\ s^{-1}$) (Fig. 5), and the highest β-carotene/chlorophyll *a* ratio was obtained at 1000 $\mu mol\ m^{-2}\ s^{-1}$. Hejazi and Wijffels (2003) also reported that β-carotene content of the *Dunaliella salina* cells increased by increasing the light intensity.

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Growth Rate, Proximate Composition and Fatty Acid Profile of Juvenile kutum *Rutilus frisii kutum* Under Light/Dark Cycles

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Abstract

This study investigated the growth rate, proximate composition, and fatty acid profile in juvenile of kutum *Rutilus frisii kutum* in different dark-light cycles. Fish were stocked randomly in eighteen 2000-L fiberglass tanks and received six photoperiod regimes: natural photoperiod, 24L (light):0D (day), 16L:8D, 12L:12D, 8L:16D and 0L:24D. Application of continuous dark (0L:24D) and an intermediate photoperiod (12L:12D) resulted in the highest specific growth rate (SGR) as well as lowest feed conversion ratio (FCR) ($P < 0.05$) in kutum. The quantity of crude protein and ash of kutum subjected in continuous dark (0L:24D), exhibited higher rate than other photoperiod regimes. The proportion of DHA (docosahexaenoic acid) +EPA (eicosapentaenoic acid) content ranged from 3.20% in natural photoperiod to 3.52% in continuous dark, n-3/n-6 and PUFA/SFA fatty acids ratio slightly ranged from 0.15-0.18, and 0.99-1.08 in all photoperiod regimes, respectively.

Keywords: Dark-light cycle · Kutum, *Rutilus frisii kutum* · Proximate composition · Fatty acid · Growth performance.

1. Introduction

Environmental and nutritional factors notably influence fish growth. In addition to temperature, light/dark cycle is an important factor that affects organisms including fish. Alteration of artificial photoperiod can induce physiological and immunological changes in fishes (Valenzuela *et al.*, 2008). These changes can be recognized through hormonal variation and alteration of blood parameters, such as cell number and volume (Zarejabad *et al.*, 2009). Taylor *et al.* (2006) believed that artificial light regimes have the ability to increase growth rates up to 25% in farmed rainbow trout *Oncorhynchus mykiss*.

The effects of photoperiod on growth rate and other variables have been studied in various species (Krakenes *et al.*, 1991; Imsland *et al.*, 1995; Davis *et al.*, 1999; Jonassen et al., 2000; Kissil et al., 2001; Petit et al., 2003; Norberg et al., 2004; Bayarri *et al.*, 2004; Taylor *et al.*, 2006; Valenzuela *et al.*, 2006; Ruchin, 2007; Bani *et al.*, 2009; Ghomi *et al.*, 2010a; Ghomi *et al.*, 2010b).

kutum *Rutilus frisii kutum* is a commercially important fish available in the southern waters of the Caspian Sea and some lakes of Turkey with high market acceptance. A decrease in water levels in the Caspian Sea from the 1950s has led to a drastic decline in the stocks (Afraei Bandpei, 2010). Artificial propagations of kutum are carried out every year to produce fingerlings to be released into the rivers in the Caspian Sea and the amount of restocking and catching were 187.1 million individual fish and 14835 ton in 2008, respectively (Abdolhay *et al.*, 2010).

There are many studies on the effect of photoperiod on teleosts; however, physiological responses of kutum juvenile in captivity condition to different photoperiod regimes are not well known. Thus, this study aims at measuring the growth rate, proximate composition, and fatty acid profile in juvenile of kutum in different dark-light cycle.

2. Materials and Methods

2.1. Experimental materials and fish

Kutum juveniles (1.39 ± 0.3 g) were provided from a local hatchery (Rajaee Fish Farm Center, Sari, Iran) and fed with a grinded basal diet of carp (32% protein, 10% fat, 15% ash, 5% fiber, and 11% moisture) (Isfahan Mokamel, Iran) 10% of body weight. The juveniles acclimatized in the two 2000-L fiberglass storage tanks and fed basal diet (4 times daily) for 1 week prior to the commencement of the experiment. Acclimated fish were distributed randomly in eighteen 2000-L fiberglass tanks (with three replicates for each treatment) each of which was filled each with 1000 L of well water. Fish received six photoperiod regimes: natural photoperiod, 24L (L = light):0D (D = dark), 16L:8D, 12L:12D, 8L:16D and 0L:24D. All tanks were covered by black nylon sheets. Light cycles were provided by incandescent lamps (20 W, Nama Noor, Tehran, Iran), which were installed 1 m above water surface, equipped with digital timers (Everflourish Electrical Co, China).

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During the experimental period, aeration was continuously supplied and different physico-chemical parameters of the rearing water such as temperature, dissolved oxygen, and pH were routinely monitored. The pH and temperature varied in the range of 7.9-8.3 and 17-24 ºC, respectively. Water flow was 5 l/min and the dissolved oxygen content was more than 7.0 mg/l. The fish were stocked at 200/tank and cultured for 60 days.

Sampling was carried out at the beginning of study and on days 30 and 60 after rearing by removing the 50 juveniles of each treatment at each time. The specific growth rate and survival were measured by counting the number of juveniles at each sampling time, measuring the weight to nearest 0.01 g. The SGR and survival rate were calculated as follows: SGR (%g/day)= 100× ($\ln W_t$ - $\ln W_o$)/t; and survival (%)= (N_t-N_o) ×100, where W_t and W_o are the weight of the juveniles at day t and at the beginning of the experiment, respectively. N_t and N_o are the number of juveniles at the end (t) and beginning (o) of the study. Feed conversion ratio (FCR) was calculated as FCR= Fc/ΔW, where Fc is the total dry food consumed by the fish and ΔW is the total weight gained (g).

2.2. Proximate composition

Moisture content was determined by drying the 5 g minced fish at 105 °C until a constant weight was obtained (AOAC, 2005). Lipid was extracted according to Kinsella *et al.* (1977). Fifty g of whole body were homogenized in a warring blender (32BL79, New Hartford, Connecticut, USA) for 2 min with a mixture of 50 ml chloroform and 100 ml methanol. Then 50 ml of chloroform were added and further homogenized. Finally, 50 ml of distilled water were added to the mixture and blended for 30 sec. The homogenate was filtered through a whatman No. 4 filter paper into a decantor (Witeg, Germany). The lower fraction was then collected and filtered. It was then transferred to a rotary evaporator (Rotavapor R-114, BÜCHI, Switzerland) for solvent evaporation. Lipid content was expressed as gram per 100 g wet whole body. Crude protein content was calculated by converting the nitrogen content determined by Kjeldahl method (6.2×N) (AOAC, 2005). Ash content was determined gravimetrically in a furnace by heating at 550°C to constant weight (AOAC, 2005).

2.3. Fatty acid profile

Fatty acid methyl ester (FAME) was prepared following the method of Timms (1978). Lipid samples (0.2 g) were weighed and diluted with 4 ml of hexane followed by the addition of 0.2 ml of sodium methoxide in a sealed tube. The mixture was then shaken using a vortex for 1 s and left for about 30 min until it separated into two phases. The top layer, FAME was then taken for analysis by using Trace GC (Thermo Finnigan, Italy). The GC conditions were as follows: capillary column (Bpx-70 60 m x 0.25 mm i. d. × 0.25µm), the split ratio was 80:1. Injection port temperature was 250 °C; flame ionization detector temperature was 270 °C. Oven temperature was set at 194 ºC for 90 minutes. Flow rate of carrier gas (helium) was 1 mL/min and the make up gas was N_2 (30 ml/min). The sample size injected for each analysis was 1 µL. Samples were manually injected into the GC port. Compounds were identified in comparison to retention times of known standard (Vingering and Ledoux, 2009).

2.4. Data analysis

The ANOVA assumptions of normality, homogeneity of variance (Little and Hills 1978) were examined using the Shapiro-Wilk and the Levene tests, respectively. The differences between groups were analyzed by using one-way ANOVA and Duncan's multiple range tests at $P < 0.05$.

3. Results and Discussion

Mean weight of fish during experimental duration under different regimes of photoperiod has been presented in Figure 1. Continuous dark (0L:24D) gained the greatest final weight of fish among all photoperiod regimes ($P < 0.05$). According to Figure 2, application of continuous dark (0L:24D) and an intermediate photoperiod (12L:12D) resulted in the highest specific growth rate (SGR) as well as the lowest feed conversion ratio (FCR) ($P < 0.05$) in kutum. The treatment 5 (8L:16D) insignificantly exhibited the lowest mortality among all photoperiod treatments. A significant decrease in SGR can be seen in the natural photoperiod (Fig. 2). These results in the better growth rate of an intermediate photoperiod (12L:12D) were in accordance with some species. Pavlidis *et al.* (1999) pointed out that the highest growth rate and food utilization efficiency in *Pagrus pagrus* were recorded under 12 h photoperiod, while the negative growth was observed in the dark. Also, the highest weight gain for European eel *Anguilla anguilla* observed under 12 h light/dark regime and decreased food coefficient in the dark (Meske, 1982). Similarly, the maximum SGR and minimum FCR have been observed by an intermediate light/dark cycle (12L:12D) ($P < 0.05$) in beluga sturgeon *Huso huso* (Ghomi *et al.*, 2010a). The highest survival rate (89%) in juvenile bluga sturgeon *Huso huso* was observed in the 12L:12D period, but differences in growth were insignificant for the photoperiod regimes (Bani *et al.*, 2009). The maximum growth rate of juvenile Siberian sturgeon, *Acipenser baerii*, was in 12, 16, and 24 h photoperiod (Ruchin, 2007). Thus, exerting either continuous dark (0L:24D) or an intermediate photoperiod (12L:12D) may cause a better condition for growth of kutum juvenile.

In contrast, Ghomi *et al.* (2010a) pointed out that the continuous dark (0L:24D) and the continuous light (24L:0D) significantly ($P < 0.05$) reduced the final weight of fish in beluga sturgeon *Huso huso* and are not compatible with the physiological condition of this species. A physiological deterioration can be recognized in carp yearlings in the dark as well as their better physiological state in the 12D:12L, 16D:8L, and 0D:24L variants (Ruchin, 2006). Similarly, Semenkova and Trenkler (1993) reported that mean weight of 4-month old beluga sturgeon *Huso huso* exposed to a 24 h photoperiod was 15% lower compared to a 16 h photoperiod. The highest growth rate of *Acipenser nudiventris* was observed under a 24 h photoperiod (Ponomarenko *et al.*, 1992). Juvenile rainbow trout *Oncorhynchus mykiss* exposed to 18L:6D grew to a significantly heavier mean weight than the other treatments (Taylor *et al.*, 2005).

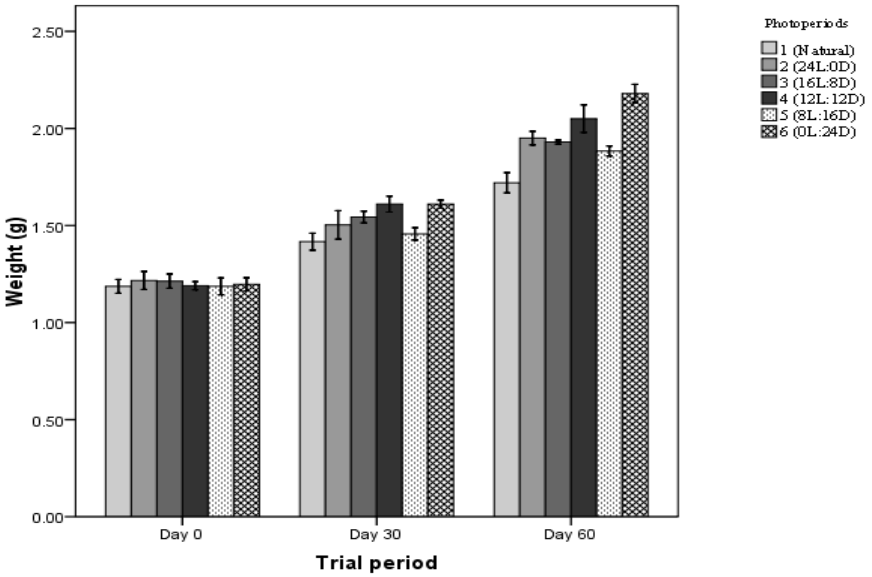

Figure. 1. Mean weight of kutum during 60 days trial under different regimes of photoperiod

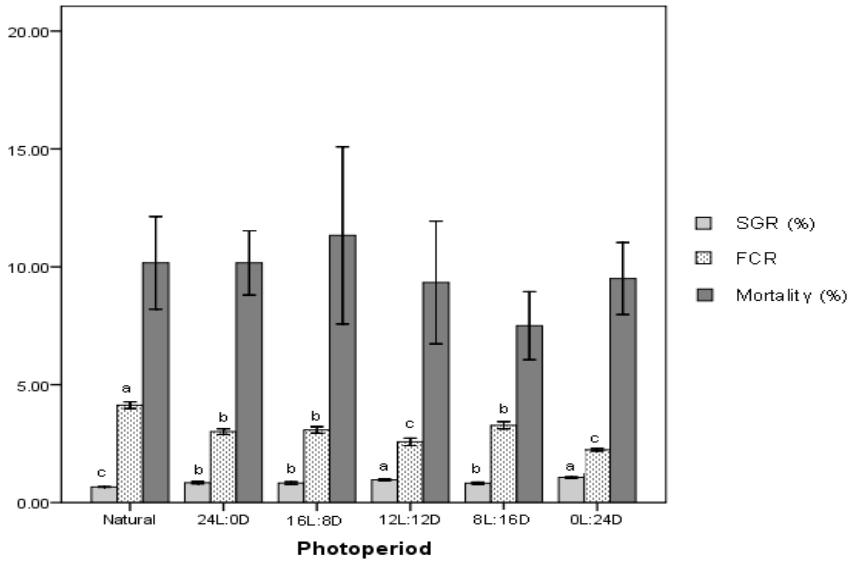

Figure. 2. SGR, FCR and mortality of fish under different regimes of photoperiod. Mean values with the same letter for same labeled columns are not significantly different ($P > 0.05$).

Proximate composition of fish is shown in Table 1. The crude fat, crude protein, ash and moisture content of kutum under various photoperiods were significantly different from each other ($P < 0.05$). This is the first report for the investigation of the photoperiod effect on proximate composition and fatty acid profile in kutum. The highest crude fat content in this study for kutum was 5.01% for treatment 3 (16L:8D). Mean protein content of kutum was in the range of 14.44% (for treatment 3 (16L:8D)) and 15.38% (for treatment 6 (0L:24D)) (Table 1). The maximum amount of moisture (75.26%) and ash (2.19%) was gained for treatment 4 (12L:12D) and 6 (0L:24D), respectively. Consequently, the quantity of crude protein and ash of kutum subjected in continuous dark (0L:24D) exhibited a higher rate than other photoperiod regimes. The proximate content values for the kutum in this study were mostly in the range of previous studies. Pirestani *et al.* (2009) and Keyvan et al. (2008) reported the amount of fat, protein, ash and moisture content in kutum (6.70%, 21.40%, 1.30%, 72.40%) and (3.21%, 21.80%, 1.29%, 75.90%), respectively. Kutum belongs to low-fat fish species, and by increasing the fat content, the rate of moisture was reduced ($r = -0.656$, $P < 0.01$) (Table 1). This fact was in agreement with Osman *et al.* (2001) which pointed out that low-fat fish species have higher water content. The increase in the moisture content was found to be associated with increased protein and ash contents in fish in this experiment (Table 1).

The fatty acid composition of fish under different photoperiod is summarized in Table 2. The fatty acid composition of kutum ranged from 0.37% for C22:4n-6 to 17.30% for C18:1n-9. A good indicator for comparing nutritional values of oils is the ratio of n-3/n-6. In the present study, n-3/n-6 ratio ranged from 0.15 to 0.18 (Table 2) which was not close to the range (0.5-3.80) given by Henderson and Tocher (1987) for freshwater species. Freshwater fish are generally characterized by high levels of n-6 PUFA, especially linoleic acid (18:2n-6) and arachidonic acid (20:4n-6) (Ozogul *et al.*, 2007). The same result was observed in the present study for linoleic acid (18:2n-6) and arachidonic acid (20:4n-6) up to the rate of 16.48% and 4.35%, respectively. Since freshwater fish contain lower proportions of long-chain n-3 PUFA than marine fish (Rahman *et al.* 1995), the ratio of total n-3 to n-6 fatty acids is much higher for marine fish than freshwater fish, varying from 5 to 10 or more (Ozogul *et al.*, 2007).

Table 1. Proximate composition (g/100g wet weight) of kutum subjected to different photoperiod regimes, and intercorrelation among proximate parameters

Photoperiod regimes	Moisture (%)	Crude protein (%)	Crude fat (%)	Ash (%)
1 (Natural)	73.51±0.49^{c}[1]	15.16±0.10^{ab}	4.36±0.06^{c}	1.77±0.02^{c}
2 (24L:0D)	72.75±0.05^{d}	14.75±0.26^{c}	4.77±0.02^{b}	1.66±0.00^{c}
3 (16L:8D)	73.05±0.03^{d}	14.44±0.05^{d}	5.01±0.09^{a}	1.65±0.00^{c}
4 (12L:12D)	75.26±0.35^{a}	15.22±0.10^{a}	4.33±0.04^{c}	1.96±0.06^{b}
5 (8L:16D)	74.19±0.02^{b}	14.94±0.15^{bc}	4.62±0.11^{b}	1.71±0.20^{c}
6 (0L:24D)	74.29±0.10^{b}	15.38±0.02^{a}	4.37±0.12^{c}	2.19±0.03^{a}
Intercorrelation				
Moisture	1	0.645**[2]	-0.656**	0.631**
Crude protein		1	-0.848**	0.677**
Crude fat			1	-0.688**
Ash				1

[1] Mean values with the same letter for each column are not significantly different ($P > 0.05$).
[2] Significant at $P < 0.01$.

Table 2. Fatty acid composition (% total fatty acids by peak area) of kutum subjected to different photoperiod regimes

	Photoperiod regimes					
Fatty acid	Natural	24L:0D	16L:8D	12L:12D	8L:16D	0L:24D
C14:0	3.23	4.44	3.80	3.65	4.12	3.98
C16:0	15.75	16.10	15.08	16.30	15.60	16.13
C18:0	8.35	8.46	7.17	7.90	7.68	7.83
Σ SFA[*]	27.73	29.00	26.05	27.85	27.40	27.94
C16:1n-7	1.77	1.85	1.63	1.68	1.60	1.56
C18:1n-7	7.04	8.35	9.52	9.03	7.30	8.10
C18:1n-9	15.10	16.20	14.25	15.10	17.30	16.15
C20:1n-9	4.75	4.63	4.84	5.07	4.81	5.17
Σ MUFA [*]	28.66	31.03	30.24	30.88	31.01	30.98
C18:2n-6	15.70	16.35	15.84	16.48	16.36	16.10
C20:2n-6	3.65	3.72	3.83	3.80	3.89	2.95
C20:4n-6	4.17	4.23	4.04	3.86	4.11	4.35
C20:5n-3 (EPA)	1.05	1.33	1.41	1.38	1.47	1.39
C22:4n-6	0.39	0.38	0.40	0.45	0.36	0.37
C22:5n-3	0.59	0.65	0.80	0.71	0.42	0.82
C22:6n-3 (DHA)	2.15	2.07	1.91	1.95	2.04	2.13
Σ PUFA [*]	27.70	28.73	28.23	28.63	28.65	28.11
n-3/n-6	0.15	0.16	0.17	0.16	0.15	0.18
EPA+DHA	3.20	3.40	3.32	3.33	3.51	3.52
PUFA/SFA	0.99	0.99	1.08	1.02	1.04	1.00

[*] SFA – saturated fatty acids, MUFA – monounsaturated fatty acids, PUFA – polyunsaturated fatty acids.

Fish lipids have useful polyunsaturated fatty acids, particularly n-3 fatty acids, which play an important role in human health promotion. Mozaffarian *et al.* (2005) believed that DHA and EPA had been reported to have preventive effects on human coronary artery disease. The proportion of DHA and EPA content ranged from 3.20% in natural photoperiod to 3.52% in continuous dark (Table 2). The amount of the DHA+EPA content in our study was lower than that of some other species. The low levels of DHA and EPA in the kutum juvenile farmed in freshwater, in present study, were not surprising when compared with their seawater un-growing size (11.69%, reported by Pirestani *et al.*, 2010) because seawater fish obtain higher omega-3 fatty acid from more diversity of seafood. The fatty acid composition of different individual fish of the same species can vary because of diet, location, gender,

and environmental conditions (Gruger, 1967). EPA+DHA content was found to be 7.88% for *Acipenser oxyrinchus desotoi* (Chen *et al.* 1995), 15.71% for cultured sturgeon (*A. baerii*, *A. naccarii,* and *A. transmontanus*; Badiani *et al.*, 1996). EPA+DHA content in *Clarias gariepinus* and *Tilapia zillii* was 4.2% and 4%, respectively (Osibona *et al.*, 2009). Ozogul *et al.* (2007) reported EPA+DHA mean content was various in *Clarias gariepinus* (8.82%), *Cyprinus carpio* (14.07%), *Siluris glanis* (17.56%), *Tinca tinca* (25.51%), *Rutilus frisii Kutum* (23.5%), *Sander lucioperca* (28.39%). Since the minimum value of the PUFA/SFA ratio recommended is 0.45 (HMSO 1994), in present study, PUFA/SFA fatty acids ratio was slightly ranged from 0.99 to 1.08 in all photoperiod regimes (Table 2).

As a conclusion, artificial regulations of photoperiod regimes may cause a better condition for the growth of kutum juvenile, significantly affecting proximate composition, but they did not affect fatty acid, comparing indicators like PUFA/SFA, n-3/n-6, and EPA+DHA content in kutum.

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Evaluation of Body Iron and Oxidative Stress Status in Smoker/Hypertensive/ Diabetic Patients Suffering Acute Myocardial Infarction Episode

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Abstract

The relationship between serum ferritin, body iron indices, and the coronary heart disease (CHD) or stroke remains controversial. The role of diabetes mellitus, smoking, and hypertension on serum ferritin and other iron monitoring molecules in acute myocardial infarction (AMI) has been under active consideration. The present study addresses the alterations in the body iron status and the lipid peroxidation (LPO) activity in AMI, particularly in the diabetics, hypertensives, and smokers. The study also evaluates the correlation between them. This study is comprised of AMI patients with or without diabetes/hypertension/smoking and healthy controls of ages 29 to 79. Blood hemoglobin, hematocrit (HCT) values, serum iron, total iron binding capacity (TIBC), ferritin and erythrocytes LPO were analyzed. An elevation in total iron, ferritin and erythrocyte LPO, and a decline in TIBC were observed in AMI patients irrespective of whether they are with or without diabetes, hypertension, or smoking while Haemoglobin (Hb) decreased in AMI non-smokers, and HCT remained unchanged when compared to controls. A positive correlation existed between total iron, ferritin and erythrocyte LPO, between hemoglobin and hematocrit, but a negative correlation of TIBC with ferritin and LPO in the AMI experimental groups is observed. A statistical significant increase in Hb and HCT values were noted in AMI smokers in comparison to AMI non-smokers, while other parameters remained unchanged between the complimentary AMI groups. AMI females had lower levels of Hb and HCT than AMI males. Diabetes, hypertension, smoking, and AMI are inflammatory processes. Elevated Ferritin, an acute phase reaction protein and associated LPO activity might be attributed to AMI progression.

Keywords: Smoking; Diabetes; Hypertension**;** Acute myocardial infarction (AMI); Lipid peroxidation (LPO); Ferritin; Total iron binding capacity (TIBC).

1. Introduction

High blood pressure is no less than an epidemic and it remains a major cause of cardiovascular morbidity and mortality worldwide (Wang and Wang,2004; Lawes *et al.*, 2006). It is still unknown whether iron itself has a role in the development of hypertension or not. Recently, non-haem iron has been reported to have an inverse relation with blood pressure while heme iron intake, on the other hand, elevates blood pressure (Tzoulaki, *et al.,* 2008). Serum ferritin and dietary iron are reported to be associated with the increased incidence of myocardial infarction (Klipstein-Grobusch, *et al.,* 1999a). Recently, a link has been established between increased dietary iron intake, particularly eating red meat as well as increased body iron stores, and the development of diabetes. A causative link with iron overload is suggested to improve insulin sensitivity and insulin secretion with frequent blood donation and decreased iron stores (Jiang, *et al*., 2004; Fernandez-Real, *et al.,* 2005). The role of tissue iron and elevated body iron stores in causing type 2 diabetes and or the pathogenesis of its important complications, particularly diabetic nephropathy and cardiovascular disease is now being investigated elaborately. Several epidemiological studies have analyzed the involvement of iron in coronary heart disease but with inconclusive results (Vander, *et al.,* 2005; Tavani *et al.,* 2006; Qi, *et al*., 2007). Smoking, current or past, has been associated with higher plasma/serum ferritin concentrations among various populations (Rodger, *et al.,* 1985; Touitou, *et al*, 1985). The mechanism of increased plasma ferritin concentrations among current smokers remains to be investigated. Considering the inconclusive literature reports on the role of ferritin, indices of body iron stores, in relevance to the development of acute myocardial infarction, the present study aims at investigating and evaluating the total iron, ferritin, total iron binding capacity (TIBC), hematocrit (HCT), hemoglobin (Hb) and lipid peroxidationin activity (LPO), and their correlations, if any, in acute myocardial

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infarction (AMI) group of patients with or without hypertension/ smoking/ diabetes.

Despite of the traditional use of *Aloysia triphylla* as an analgesic, no systemic studies concerning the antinociceptive effects are available. In the present study, we are reporting the antinociceptive effects of two flavonoids (artemitin and hesperidin) which were isolated from the *Aloysia triphylla*.

2. Materials and Methods

2.1. Subjects

This study includes patients who were admitted in the Jamahyria hospital, Benghazi, during the period from June, 2007 to December, 2007 with acute myocardial infarction (AMI) and were between 29–59 years old with a mean value ± S.D., 55±13. The patients were subjected to clinical examination. The detailed medical history, blood pressure and ECG tracing were recorded. These patients (AMI patients) were divided into the following subgroups: AMI Smokers (10), AMI hypertensive (10), AMI diabetics (10), AMI non-smokers (20); AMI normotensives (20), and AMI non-diabetics (20); with AMI only males (21) and with AMI only females (9). The control group included 30 (15 males and 15 females), healthy subjects with a mean age 50±10 for males, 48.5±10.5 for females. They were normotensive nonsmoking and non-diabetics with normal ECG tracing and were devoid of heart, liver, kidney and any other endocrine related diseases.

2.2. Criteria for smoking patients

The selection criteria for the smoking group with AMI included those who had a history of smoking of ten or more cigarettes per day during the last ten years or more.

2.3. Criteria for Hypertension and anemia

Hypertension was defined as a resting systolic blood pressure >140 mm Hg and diastolic blood pressure >90 mm Hg, and anaemia was defined, using World Health Organization (WHO) criteria, as a hematocrit value at initial presentation < 39 % for men and < 36 % for women.

2.4. Criteria for diabetes mellitus

The AMI patients having fasting blood glucose level more than 126mg/dl and post absortive blood glucose levels above 200mg/dl were included.

2.5. Exclusion criteria

None of the subjects in the study groups and the control group had a previous history of myocardial infarction, angina, coronary artery surgery, transient ischemic attack and peripheral arterial diseases, liver, kidney and thyroid related diseases. None of the study subjects had undergone blood transfusions/ donations throughout their life time.

The present study was conducted after obtaining the written informed consent of the participating subjects. The study was approved by the ethical committee of the hospital, and procedures were followed in accordance with the ethical standards laid down on human experimentation (institutional and national) and with the Helsinki Declaration of 1975.

2.6. Blood specimen collection

Blood samples were collected from the study subjects and the controls after overnight fasting. One part of the blood was placed into plain tube for the serum separation by centrifugation at 3500 rpm for 10 minutes and stored at -30 C till the analysis was conducted. Another blood aliquot was collected in EDTA containing tubes for the plasma separation by centrifugation. The packed cells were washed with an equal volume of the physiological saline and centrifuged again. The supernatant was removed and the cells were analyzed for the lipid peroxidation.

2.7. Analytical methods

The complete blood count, hemoglobin (Hb), serum iron (Makino, 1988), (TIBC) total iron binding capacity (Ramsey, 1958), ferritin (Zuyderhoudt and Linthorst, 1984) and the (LPO) lipid peroxidation in erythrocytes (Quinlan *et al.*, 1988) were analyzed by employing the authentic methods. The hemoglobin (Hb) and the hematocrit values (HCT) were estimated by the established hematological methods (Dacie and Lewis, 1986).

2.8. Statistical analysis

All the biochemical parameters were statistically analyzed using software SPSS version 11 (statistical package for social sciences) for calculation of student's 't'test for obtaining the *p* values and pearson's correlation coefficient 'r'.

3. Results

The data on serum iron, total iron binding capacity (TIBC), ferritin, blood hemoglobin, hematocrit and erythrocyte lipid peroxidation in the AMI patients, AMI males and females, AMI smokers/hypertensives/diabetics patients, AMI non-smokers/ normotensive/non-diabetics patients and the healthy controls, Correlation coefficient between these experimental groups are presented in table-1-4.

3.1. Body iron status and LPO activity in AMI patients

The AMI patients showed a significant increase in serum iron, ferritin and erythrocyte LPO activity along with a significant decline in serum TIBC when compared to healthy controls. The hemoglobin and hematocrit values remained unaltered in AMI patients as shown in table I.

3.2. Body iron status and LPO activity in AMI males and females patients

No significant difference was noticed in serum iron, TIBC, ferritin and erythrocyte LPO activity in AMI males and females while a significant decrease in hemoglobin and hematocrit values was observed in female AMI patients when compared to male AMI patients as shown in table 2.

3.3. Body iron status and LPO activity in AMI non-smokers, AMI normotensive and AMI non-diabetic patients

The AMI non-smokers, AMI normotensive and AMI non-diabetic patients groups when compared with corresponding healthy nonsmoking, normotensive and non-diabetic controls, there was a significant increase in

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Table 1: Iron status, hematocrit and erythrocyte lipid peroxidation activity (Mean values±SD) in AMI patients

Parameters	Controls N=30	AMI Patients N=30
Serum Iron (mg/dL)	62.07±18.05	79.50±22.34 $p< 0.002$
Serum TIBC (mg/dL)	341.43±47.36	313.60±49.41 $p< 0.03$
Serum Ferritin (ng/dL)	71.60±33.23	185.50±57.92 $p <0.001$
Hemoglobin (g/dL)	13.07±1.67	12.73±1.57
Hematocrit (%)	38.26±5.33	37.57±4.77
Erythrocyte LPO (Absorptive value)	0.36±0.14	0.50±0.32 $p<0,03$

AMI: Acute myocardial infarction

TIBC:Total iron binding capacity ; LPO: erythrocyte lipid peroxidation

$P<0.05$, significant when compared to control group; N, the number of subjects

the level of the serum iron and serum ferritin while the TIBC decreased significantly with the exception of AMI normotensiove patients where the decline was statistically nonsignificant. The erythrocytes lipid peroxidation activity was raised in these groups but it was observed to be significant only in AMI smoker group. There was a significant decrease in serum hemoglobin in AMI nonsmoker group while it remained unchanged in AMI normotensive and AMI nondiabetic groups. Hematocrit levels of AMI nonsmokers, AMI normotensive and AMI nondiabetic patients groups did not differ significantly from that of healthy controls. The data are presented in table3.

3.4. Body iron status and LPO activity in AMI smoking patients

The AMI smoker patients group showed a significant increase in the level of serum ferritin and non-significant changes in the levels of serum iron, serum TIBC, hemoglobin, hematocrit and erythrocyte lipid per-oxidation when compared to nonsmoking healthy controls. AMI smokers group showed a significant increase in hemoglobin and hematocrit values than the AMI nonsmoking group, while iron, TIBC and ferritin and lipid per-oxidation (LPO) activity remained unchanged when compared to AMI nonsmoker group of patients. The data are given in table 3.

3.5. Body iron status and LPO activity in AMI hypertensive group of patients

There was a significant increase in total serum iron, ferritin levels, and LPO activity in AMI hypertensive patients while TIBC though declined, yet remained statistically unchanged when compared to healthy controls. Hemoglobin and hematocrit levels did not alter when compared to healthy controls. None of the parameters altered in AMI hypertensive patients when compared to AMI normotensive patients. The data are shown in table 3.

3.6. Body iron status and LPO activity in AMI diabetic group of patients

AMI diabetic group of patients observed a significant increase in iron, ferritin, and LPO activity while the decline in TBIC was not statistically significant when compared to healthy controls. Hemoglobin and hematocrit values remained unaltered as compared to healthy controls. None of the parameters studied differed in their values in AMI diabetics when compared to AMI nondiabetics. The data are exhibited in table 3.

3.7. Coefficient correlations between parameters within different groups

The data on correlation coefficient and p values in various parameters in AMI smokers, AMI hypertensives, AMI diabetics and AMI non-smokers, AMI normotensives, AMI non-diabetics patient groups are presented in table 4.

There were positive correlations of iron with serum ferritin, LPO activity and a negative correlation between serum TIBC and ferritin in AMI smokers, AMI hypertensives, AMI diabetics patients and in AMI non-smokers, AMI normotensives, AMI nondiabetics patients groups. Hemoglobin showed a positive correlation with hematocrit in all these experimental groups.

Table 2: Iron status, hematocrit and erythrocyte lipid peroxidation activity (Mean values±SD) in AMI patients, both males and females

Parameters	AMI Patients Males (N=21)	AMI Patients Females (N=(9)
Serum Iron (ug/dL)	80.19±23.55	77.89±20.46
Serum TIBC (mg/dL)	315.00±52.51	310.33±44.05
Serum Ferritin (ng/dL)	187.52±61.98	180.78±50.20
Hemoglobin (g/dL)	13.26±1.54	11.5±0.73 p <0.003
Hematocrit (%)	39.04±4.75	34.12±0.31 p <0.05
Erythrocyte LPO (Absorptive value)	0.52±0.33	0.45±0.31

AMI: Acute myocardial infarction; TIBC: Total iron binding capacity

LPO: erythrocyte lipid peroxidation; N: Number of subjects

p<0.05 denotes significant on comparison with AMI males

p>0.05 denotes non-significant on comparison with AMI males

Table 3: Iron status, hematocrit and erythrocyte lipid peroxidation activity (Mean values±SD) in AMI patients and controls.

Parameters	Control N=30	AMI Non Diabetics N=20	AMI Diabetics N=10	AMI Non Smokers N=20	AMI Smokers N=10	AMI Normo-tensive N=20	AMI Hyper-tensive N=10
Serum Iron (μg/dL)	62±18	81±23 p<0.003	77±22 p<0.02	81.20±21.68 p<0.001	76.10±24.42	77±24 P<0.02	85±17 P<0.001
Serum TIBC (mg/dL)	341±47	308±46 p<0.03	320±54	310.50±47.20 p<0.03	319.80±55.68	316±51	309±47 P<0.05
S. Ferritin (ng/dL)	72±33	190±58 p<0.001	180±59 p<0.001	189.45±59.87 p<0.001	177.60±56.03 p<0.001;	188±64 p<0.01	181±46 p<0.001
LPO (absorptive value)	0.36±0.14	0.49±0.03	0.52±0.32 p<0.03	0.54±0.32 p<0.001	0.43±0.33	0.47±0.34	0.56±0.29 p<0.01;
Hemoglobin (g/dL)	13±1.6	13±1.8	12±0.99	12.00±0.91 p<0.001	14.19±1.6 3 p2<0.001	12.7±1.5	13±1.8
Hemato- crit(%)	38±5.3	37.8±2.5	36±3.40	35.60±3.54	41.51±4.57 p2<0.001	37.4±4.5	37.7±5.5

AMI: Acute myocardial infarction; TIBC: Total iron binding capacity;

LPO: erythrocyte lipid peroxidation; N: Number of subjects;

Non significant at p>0. 05

p<0.05, significant when compared with controls

p_1<0.05, significant when compared with AMI non-diabetic patients

p2<0.05, significant when compared with AMI non-smoker patients

p3<0.05, significant when compared with AMI normotensive patients

Table.4. Correlation coefficient (r) between the Analytes in AMI patients

Coefficient Correlation	AMI Groups					
	Diabetics	NonDiabetics	Smokers	Nonsmokers	Hypertensive	Normotensive
Iron Vs TIBC	r, -0.75 p<003	r,-0.54 p<0.03	r,-0.51 p<0.13	r,-0.71 p< 0.001	r,-0.71 p< 0.02	r,-0.62 p< 0.04
Iron Vs Ferritin	r, 0.70 p<0.01	r, 0.80 p<0.001	r,0.81 p0.01	r,0.74 p< 0.001	r,0.67 p< 0.03	r,0.81 p< 0.001
Iron Vs Hb	r,0.39 p<0.18	r,-0.14 p<0.59	r,-0.04 p< 0.92	r,-0.22 p< 0.35	r,0.05 p< 0.89	r,-0.27 p< 0.26
Iron Vs HCT	r,0.29 p<0.33	r,-0.07 p<0.80	r,0.01 p< 0.99	r,-0.09 p< 0.72	r,0.09 p< 0.81	r,-0.20 p< 0.41
Iron Vs LPO	r,0.62 p<0.02	r,0.78 p<0.001	r,0.79 p<0.01	r,0.65 p< 0.002	r,0.40 p< 0.25	r,0.78 p<0.01
TIBC Vs Ferritin	r,-0.71 p<0.01	r,-0.72 p<0.001	r.0.70 p<0.03	r,-0.73 p< 0.001	r,-0.60 p< 0.07	r,-0.77 p< 0.001
TBIC Vs Hb	r,0.29 p<0.36	r,0.15 p< 0.56	r,0.02 p< 0.97	r,0.19 p< 0.41	r,0.14 p< 0.71	r,0.14 p< 0.56
TBIC Vs HCT	r,0.11 p<0.72	r,0.06 p <0.82	r.0.07 p< 0.85	r,0.02 p< 0.94	r,0.08 p< 0.85	r,0.03 p< 0.91
TBIC Vs LPO	r,-0.73 p<0.01	r,-0.56 p <0.02	r,-0.54 p< 0.11	r,-0.68 p< 0.001	r,-0.56 p< 0.09	r,-0.65 p <0.002
Ferritin Vs Hb	r,-0.30 p<0.32	r,-0.19 p< 0.46	r,- 0.28 p< 0.43	r,-0.06 p< 0.80	r,-0.22 p< 0.55	r,-0.17 p< 0.48
Ferritin Vs HCT	r,-0.21 p<0.50	r,-0.09 p< 0.74	r,-0.24 p< 0.50	r,0.07 p< 0.78	r,-0.17 p< 0.64	r,-0.06 p< 0.79
Ferritin Vs LPO	r,0.59 p<0.04	r,0.85 p< 0.001	r,0.91 p<0.001	r,0.64 p< 0.003	r,0.46 p< 0.19	r,0.83 p< 0.001
Hb Vs HCT	r,0.93 p<0.001	r,0.95 p< 0.001	r,0.97 p<0.001	r,0.89 p< 0.001	r,0.98 p< 0.001	r,0.92 p< 0.001
Hb Vs LPO	r,-0.21 p<0.49	r,-0.23 p< 0.38	r,-0.31 p< 0.38	r,-0.01 p< 0.95	r,-0.05 p< 0.89	r,-0.31 p< 0.19
HCT Vs LPO	r,-0.06 p<0.86	r,0.18 p< 0.49	r,-0.24 p< 0.50	r,0.06 p< 0.80	r,0.03 p< 0.93	r,-0.24 p< 0.31

Non significant at p>0. 05

p<0.05, significant when compared with controls

4. Discussion

Epidemiological studies have established risk factors for coronary heart disease which include cigarette smoking, hypertension, diabetes and elevated serum cholesterol levels. Hypertension was strongly associated with both ischemic heart disease and stroke mortality not just in middle age but also among people in their seventies and eighties (Lewington, *et al.,* 2002). The present study observed a significant elevation in the levels of ferritin and total serum iron and a decline in iron binding capacity in AMI patients irrespective of whether the patients were smokers or nonsmokers; hypertensives or normotensive and diabetics or non-diabetics when compared to healthy controls (table 1-3). Excessive iron has been proposed to be a potent risk factor for CHD, especially for acute myocardial infarction in several earlier investigations (Salonen, *et al*., 1992; Salonen, *et al*., 1994) which provide support to the present findings. A couple of reports showed a marked increase in ferritin levels in smoker population (Touitou, *et al*., 1985; Rodger *et al.,*1985). Serum ferritin may additively affect ischemic heart disease risk in the elderly. The risk of acute myocardial infarction was associated with the highest tertile of ferritin which was most evident in current or former smokers along with other risk factors (Klipstein-Grobusch *et al.,* 1999). In diabetics, serum ferritin levels are elevated, which may increase the risk of coronary heart disease (Mert *et al*., 2005**)**

An analysis of NHANES III data showed elevated serum ferritin in persons with newly diagnosed or previously diagnosed diabetes and in all ethnic groups and age groups combined (Ford and Cogswell,1999). The serum ferritin level has been found to be associated with decreased insulin sensitivity and increased fasting serum insulin and blood glucose (Fernandez-Real *et al.*, 1998; Ford and Cogswell,1999). Iron depletion improves vascular dysfunction in type 2 diabetic patients with high ferritin concentrations (Fernandez *et al.*, 2002.). Raised ferritin levels among patients of myocardial infarction suggest a role of increased iron stores in myocardial infarction but iron overload was not an independent risk factor for coronary heart disease patients (Clys *et al*, 2002). In myocardial infarction, a gradual increase in

serum ferritin levels can be detected. Furthermore, a significant increase in ferritin content can be found in peripheral blood monocytes. Peripheral blood monocytes activated by steroids during stress could be the cause of increased serum ferritin levels following AMI (Moroz *et al.*, 1997). Body iron storage in males increases progressively with a proportional rise in serum ferritin, whereas serum ferritin levels are lower and more stable in females during reproductive period. Ferritin levels only increase after the menopause. In the present study, ferritin levels were not statistically different in AMI females compared to males AMI patients group. It might be due to the inclusion of post-menopausal age group subjects in the present study. However, the levels of Hb and HCT were significantly higher in males AMI patients (table II). The difference in hemorheological properties in female blood is caused by the increased concentration of younger red blood cells (RBCs) and the reduced population of older RBCs. Higher viscosity, increased RBC aggregation and decreased RBC deformability are observed in male compared with female blood (Mert *et al.*, 2005).

Iron induced oxidative stress in the form of increased generation of reactive oxygen species, in a series of fenton like reactions, does not make the pancreatic islets only but it also makes the vascular endothelium vulnerable to dysfunctional injury, leading to the development of diabetes and increased susceptibility to AMI. Moreover, total serum iron in acute myocardial infarction with or without hypertension, with or without diabetes, with or without smoking correlated positively with the elevation observed in ferritin concentration and negatively with a decline in total iron binding capacity in the present study (table 4). It suggests that due to an acute reaction response to inflammation and with the more availability of free iron on account of declined total iron binding capacity, the formation of ferritin gets markedly induced in a protective, body defense measure to sequester the free iron along with its known anti-oxidative properties (Crichton *et al.*, 2002). It is the free ionic form of iron that is harmful due to its pro-oxidative properties, which generates reactive free radicals. Superoxide produced during oxidative stress can mobilize free catalytic iron from ferritin (Halliwell, 1994) and facilitate the formation of Hydroxyl ion ($OH^{\cdot}$). Reactive oxygen species, superoxide cause lipid peroxidation and endothelial dysfunction in vessels (Salonen *et al.*, 1994).

In addition, hypertension, which is considered to be a risk factor, may exacerbate the inflammatory response on the arterial wall by increasing oxidative stress, production of oxygen-free radicals and recruitment of mononuclear cells (Chobanian and Alexander, 1996). Endothelial alterations occur early in hypertension with enhanced adherence of leukocytes to the endothelial surface and increased endothelial permeability. The risk of coronary heart disease, doubles with each increment of blood pressure by 20/10 mm Hg, beginning beyond 115/75 mm Hg. Elevated blood pressure is associated with macrophage accumulation, stimulation of smooth muscle cell proliferation and enhanced expression of cytokines and growth factors in the intima (Lewington *et al.*, 2002). Inflammatory processes play a role in the initiation of unstable coronary artery disease because they destabilize the atherosclerotic plaque and enhance the formation of thrombus (Ross,1999).

Increased LPO activity in AMI patients of experimental groups when compared to healthy controls, in the present study, supports the generation of reactive free radicals. The erythrocyte value of LPO in the smoker group significantly increased when compared to normal healthy non-smokers individuals. In smokers, a series of free radical chain reactions were aggravated, the dynamic balance between oxidation and antioxidation was seriously disrupted, and oxidative stress was clearly exacerbated (Zhou *et al.*, 2000). The lipid peroxidation activity correlated positively with elevated total iron and ferritin levels in the present study. It was further observed that the iron homeostasis was mainly affected by altering hemoglobin concentrations, which were increased on smoking (Northrop-Clewes and Thurnham, 2007). Similar elevation in the hemoglobin level was obtained in the AMI smoking patients group when compared to the AMI nonsmoking patients group in the present study. Further hemoglobin and hematocrit values significantly correlated positively in both AMI groups with or without smoking in the present study. The increased hemoglobin could be attributed to the effect of smoking which increases carboxyhemoglobin, thereby decreasing the oxygen carrying capacity of red blood cells thus leading to tissue hypoxia. Hypoxia stimulates erythropoietin production, which induces hyperplasia of the bone marrow and to the development of secondary polycythemia (El-Zyadi, 2006). There is also a higher risk of cardiovascular disease due to decreased oxygen delivery (Kameneva, 2007). Fe^{3+} is released from ferritin and other scavengers in rough proportion to the amounts of iron stores by leukocyte-derived oxygen radicals or by oxygen-free redox systems such as hydroquinones in cigarette smoke (Northrop-Clewes CA, Thurnham *et al.*, 1995; Reif, 1992; Thomas *et al.*, 1985; Abdalla *et al.*, 1992; Biemond *et al.*, 1986). This process is stimulated by disturbances of oxygen supply in atherosclerotic lesions (Crawford and Blankenhorn, 1991). Susceptibility of LDL to oxidative modification increases as a function of serum ferritin and dietary iron supplementation and decreases in response to experimental iron depletion (Craig *et al.*,1995; Vander *et al.*, 2005)). The capacity of prominent iron stores to amplify lipid-induced atherogenesis has been verified in a hypercholesterolemic animal model (Araujo *et al.*, 1995).

Several studies have demonstrated a direct association between increased iron intake, body iron stores, and cardiovascular risk in the general population. Increased intake of heme iron is associated with increased cardiovascular events (Vander *et al.*, 2005; Ascherio *et al.*, 1994; Lee *et al.*, 2005; Ramakrishnan *et al.*, 2002). Increased body iron stores are associated with myocardial infarction in a prospective epidemiological study (Tuomainen *et al.*, 1998). However, no significant difference was observed in these parameters between AMI patients with or without smoking; AMI with or without diabetes; AMI with or without hypertension when compared in the present study. It indicated that the diabetes and AMI, hypertension and AMI, smoking and AMI did not show synergic effect in the alterations of the iron status and LPO activity in the all AMI experimental groups. The present study concludes that the significant

alterations in the iron and lipid peroxidation activity in AMI shown in the subgroups of AMI patients may contribute along with other risk factors to the development and precipitation of AMI.

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In vitro Free Radical Scavenging and Brine Shrimp Lethality Bioassay of Aqueous Extract of *Ficus racemosa* Seed

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Abstract

The present work was accomplished to explore the free radical scavenging and cytotoxic potential of the freeze-dried aqueous extract of *Ficus racemosa* seed using 1, 1-diphenyl-2-picrylhydrazyl (DPPH), and brine shrimp lethality bioassay method. In both methods, *F. racemosa* showed a significant activity. In case of free radical scavenging activity, it showed a potent radical scavenging activity with IC_{50} value of 22.10 µg/ml. The cytotoxic activity of the extract was moderate having LC_{50} value of 4.04 µg/ml.

Keywords: 1, 1-diphenyl-2-picrylhydrazyl (DPPH), Brine shrimp, Cytotoxicity, *Ficus racemosa*, Moraceae, Radical scavenging.

1. Introduction

In developing countries, especially in rural contexts, people usually turn to traditional healers when in diseased conditions, and plants of ethnobotanical origin are often presented for use. Investigations into the chemical and biological activities of plants during the past two centuries have yielded compounds for the development of modern synthetic organic chemistry and the emergence of medicinal chemistry as a major route for the discovery of novel and more effective therapeutic agents (Roja and Rao, 2000). Thus, plants are considered as one of the most important and interesting subjects that should be explored for the discovery and development of newer and safer drug candidates.

Several members of the genus *Ficus* (family: Moraceae) are being used traditionally in a wide variety of ethnomedical remedies. One of them is *Ficus racemosa* syn. *Ficus glomerata* (Gular; Udumbara) (Kulkarni and Shahida, 2004) is widely distributed all over India, northern Australia, and other parts of Asia.

Ficus racemosa Linn. (Moraceae), commonly known as 'cluster fig', is used widely in Indian folk medicine for the treatment of various diseases, including jaundice, dysentery, diabetes, diarrhea and inflammatory conditions (Anonymous, 1952).

Apart from the usage in traditional medicine, scientific studies indicate that *F. racemosa* possesses various biological effects such as hepatoprotective (Mandal *et al.,* 2003), chemopreventive (Khan and Sultana, 2005), antidiabetic (Rao *et al.,* 2002a), antiinflammatory (Mandal *et al.,* 2000), antipyretic (Rao *et al.,* 2002b), antitussive (Rao *et al*, 2003), and antidiuretic (Ratnasooriya *et al.,* 2003). The bark has also been evaluated for cytotoxic effects using 1BR3, Hep G2, HL-60 cell lines and found to be safe and less toxic than aspirin, a commonly consumed anti-inflammatory drug (Li *et al.,* 2004).

Previously, it was reported that *F. racemosa* stem bark possesses excellent antioxidant properties *in vitro, ex vivo* (Ahmed and Urooj, 2009a) and *in vivo* in streptozotocin-induced diabetic rats (Ahmed and Urooj, 2009b). Besides, *in vitro* antioxidant activity of *F. racemosa* stem bark has also been reported by Veerapur *et al.,* 2007. However, to the best of our knowledge, free radical scavenging and brine shrimp lethality bioassay of *F. racemosa* seeds extract have not been reported previously. The objective of this work was to explore the free radical scavenging and brine shrimp lethality bioassay of the freeze-dried aqueous extract of *F. racemosa* seeds.

2. Materials and Methods

2.1. Collection and Identification of the plant

The fresh seed of *Ficus racemosa* was collected during February 2009 from the area of Purana Palton, Dhaka. The plant was identified by the National Herbarium where a voucher specimen was deposited having the accession number of 34479.

2.2. Drying and Pulverization

The fresh seed was first washed with water to remove adhering dirt and then cut into small pieces, sun dried for 4

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days. After complete drying, the entire portion was pulverized into a coarse powder with the help of a grinding machine and was stored in an airtight container for further use.

2.3. Extraction of Plant Material

Ten grams of powdered seeds were mixed with 1000 ml distilled water, boiled for 10 min and then cooled for 15 min. Thereafter, the aqueous extract was filtered using a Millipore filter (Millipore 0.2mm) to remove particulate matter. The filtrate was then freeze-dried from BCSIR (Bangladesh Council of Scientific and Industrial Research), Dhaka Bangladesh.

2.4. Screening for free radical scavenging activity

Free radical scavenging activities of the seeds of aqueous extract were determined based on their scavenging potential of the stable DPPH free radical in both qualitative and quantitative assay.

i) Qualitative assay: A suitably diluted stock solutions of extracts were spotted on pre-coated silica gel TLC plates and the plates were developed in solvent systems of different polarities (polar, medium polar and non-polar) to resolve both polar and non-polar components of the extracts. The plates were dried at room temperature and were sprayed with 0.02% DPPH in ethanol. Bleaching of DPPH by the resolved band was observed for 10 minutes and the color changes (yellow on purple background) were noted (Sadhu *et al.*, 2003).

ii) Quantitative assay: The antioxidant activity of the seeds extract of *F. racemosa* was determined using the 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay (Hasan *et. al,* 2006; Koleva *et. al,* 2002; Lee *et. al,* 2003). DPPH offers a convenient and accurate method for titrating the oxidizable groups of natural or synthetic anti-oxidants (Cao *et al.,* 1997). DPPH solution was prepared in 95% methanol. The crude extracts of *F. racemosa* were mixed with 95% methanol to prepare the stock solution (5 mg/50mL).

The concentration of the sample solutions was 100µg/ml. The test samples were prepared from stock solution by dilution with methanol to attain a concentration of 20 µg/ml, 40 µg/ml, 60 µg/ml, 80 µg/ml and 100 µg/ml. Freshly prepared DPPH solution (0.004% w/v) was added in each of these test tubes containing *F. racemosa* extract and after 20 min, the absorbance was taken at 517 nm. Ascorbic acid was used as a positive control. The DPPH solution without sample solution was used as control. 95% methanol was used as blank. Percent scavenging of the DPPH free radical was measured using the following equation:

% DPPH radical scavenging (%) = [1-(As/Ac)] ×100.

where, Ac=absorbance of control, As =absorbance of sample solution.

Then the percentage of inhibition was plotted against respective concentrations used and IC_{50} value was calculated from the graph using Microsoft Excel 2007.

2.5. Brine shrimp lethality bioassay

Brine shrimp lethality bioassay is widely used in the bioassay for the bioactive compounds (Meyer *et al,* 1982; Zhao *et al,* 1992). The brine shrimp, *Artemia salina*, was used as a convenient monitor for the screening.

The eggs of the brine shrimp, *A. salina*, were collected from an aquarium shop (Dhaka, Bangladesh) and hatched in artificial seawater (3.8% NaCl solution) for 48 hr to mature shrimp called nauplii. The cytotoxicity assay was performed on brine shrimp nauplii using Meyer method. The test samples (extract) were prepared by dissolving in DMSO (not more than 50 µl in 5 ml solution) plus sea water (3.8% NaCl in water) to attain log concentrations of – 0.11 µg/ml, 0.19 µg/ml, 0.49 µg/ml, 0.89 µg/ml, 1.09 µg/ml, 1.40 µg/ml, 1.70 µg/ml, 2.00 µg/ml, 2.30 µg/ml and 2.60 µg/ml. A vial containing 50µl DMSO diluted to 5ml was used as a control. Standard vincristine sulfate was used as positive control (Hossain *et al,* 2004; Khan *et a*l, 2008; Nikkon *et al,* 2003). Then matured shrimps were applied to each of all experimental vials and control vial. After 24 hours, the vials were inspected using a magnifying glass and the number of surviving nauplii in each vial were counted. The lethal concentrations of plant extract resulting in 50% mortality of the brine shrimp (LC_{50}) from the 24 h counts and the dose-response data were transformed into a straight line by means of a trend line fit linear regression analysis (MS Excel version 7); the LC_{50} was derived from the best-fit line obtained.

3. Results

3.1. The result of free radical scavenging activity

i) Qualitative assay: The color changes (yellow on purple background) on the TLC plates were observed due to the bleaching of DPPH by the resolved bands.

ii) Quantitative assay: The aqueous extract of *F. racemosa* seed showed potential free radical scavenging activity having an IC_{50} value of 22.10 µg/ml (Fig 1). On the other hand the free radical scavenging activity of standard (ascorbic acid) was having an IC_{50} value of 15.93 µg/ml.

3.2. The Result of Brine Shrimp Lethality Bioassay

The lethality of the aqueous extracts of *F. racemosa* seed to brine shrimp was determined on *A. salina* after 24 hours of exposure to the test solutions and the positive control, vincristine sulfate by following the procedure of Meyer *et al.,* 1982. The aqueous extract of *F. racemosa* showed potential cytotoxic activity having an LC_{50} value of 4.04 µg/ml in contrast to the LC_{50} value of standard vincristine sulfate of 0.397 µg/ml (Fig 2).

4. Discussion

Antioxidant deficiency and excess free radical production have been implicated in human hypertension in numerous epidemiological, observational and interventional studies. Herbal antioxidants have been gaining prime importance in the antiradical drug discovery due to lesser side effects as reviewed extensively by many authors (Arora *et al.,* 2005, Meenal *et al.,* 2006). Therefore, the antiradical activity of *F. racemosa* seed extract was studied, as the tendencies of electron or hydrogen donation are critical factors in characterizing antioxidant activity that involves free radical scavenging (Hu *et al.,* 2000). The DPPH radical has been widely used to test the potential of compounds as free-radical

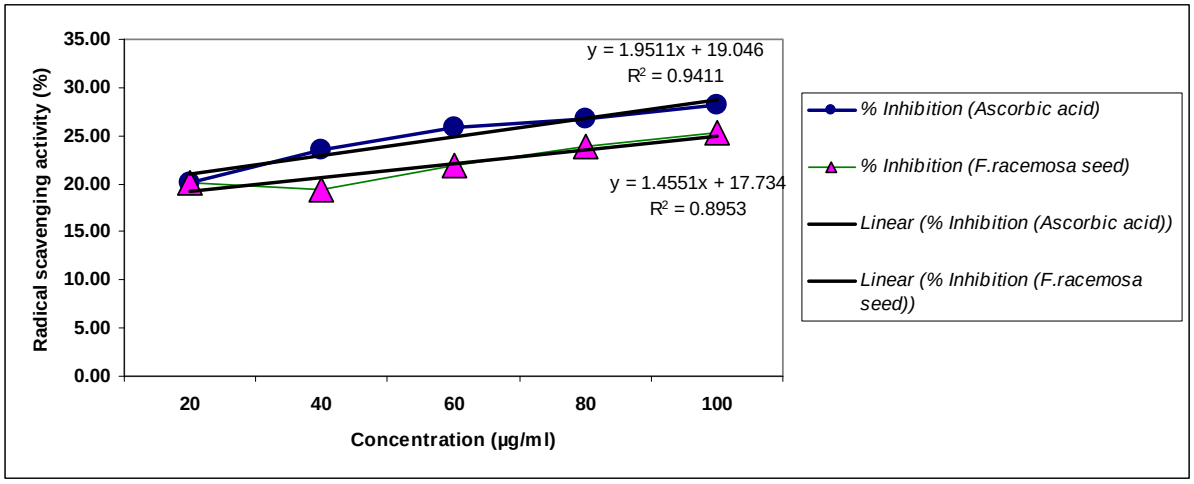

Figure 1: IC $_{50}$ value of aqueous extract of *F. racemosa* seed and ascorbic acid for radical scavenging activity

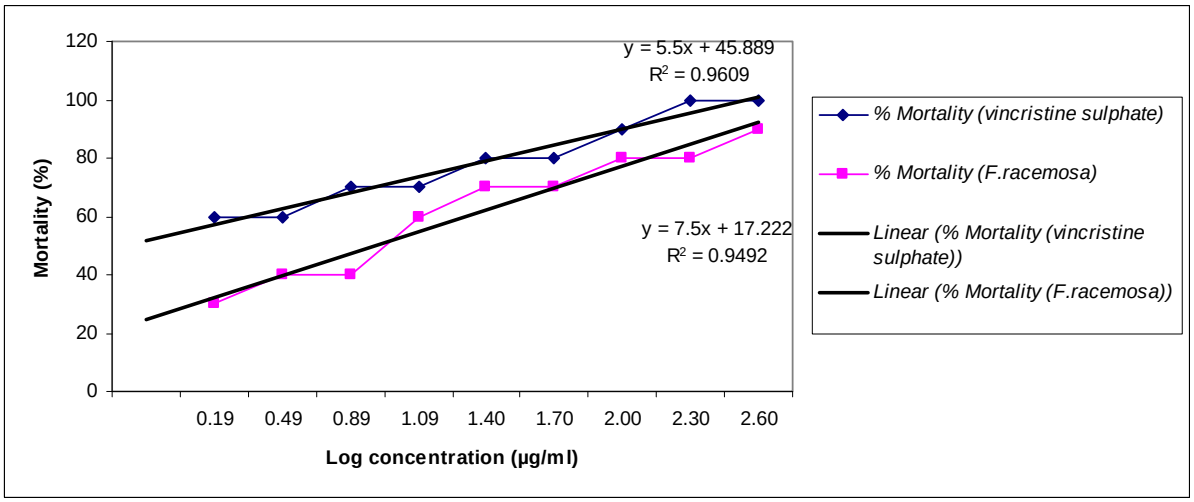

Figure 2: LC $_{50}$ value of aqueous extract of *F. racemosa* seed and vincristine sulfate against the brine shrimp, *Artemia salina*.

scavengers or hydrogen donors and to evaluate the antioxidative activity of plant extracts and foods (Porto *et al.,* 2000 and Soares *et al.,* 1997). It is a free radical donor that accepts an electron or hydrogen to become a stable diamagnetic molecule.

The present observation that *F. racemosa* seeds extract has free radical scavenging activity can be correlated with other studies done by Ahmed and Urooj, 2009a and Veerapur *et al.,* 2007. This potent antioxidant activity of *F. racemosa* seed may be due to the presence of antioxidant and chemopreventive principles such as, racemosic acid, bergenin, tannins, kaempferol, rutin, bergapten, psoralenes, ficusin, coumarin and phenolic glycosides that have been previously isolated from the bark of this plant (Baruah and Gohain, 1992; Li *et al.,* 2004).

A general bioassay that appears capable of detecting a broad spectrum of bioactivity present in crude extracts is the brine shrimp lethality bioassay (BSLT). The technique is easily mastered, of little cost, and utilizes small amount of test material. The aim of this method is to provide a front-line screen that can be backed up by more specific and more expensive bioassays once the active compounds have been isolated. It appears that BSLT is predictive of cytotoxicity and pesticidal activity (Ghisalberti, 1993). The result obtained from the brine shrimp lethality bioassay of *F. racemosa* can be used as a guide for the isolation of cytotoxic compounds from the aqueous extract of the seeds of this plant.

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Effect of Seasonal Monsoons on Calanoid Copepod in Chabahar Bay-Gulf of Oman

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Abstract

Seasonal abundance and diversity of calanoid copepod was studied from zooplankton samples collected between August 2007 and May 2008 in Chabahar Bay (one of the Iranian coasts of Gulf of Oman located on the southeastern Iran (25° 17' 45"N- 60° 37' 45" E). This area is influenced by seasonal monsoons. Five stations were investigated along the Bay. Zooplankton was collected with vertical plankton tows using 100-µm mesh net. The net was towed obliquely from a depth near the bottom to the surface. The main peak of calanoid density was recorded in SW-monsoon with an average of (445.19 ± 126.16 individual. m^{-3}) and formed 62.40% of the total copepod while pre-monsoon was characterized by the lowest calanoid density (153.75 ± 54.20 ind.m^{-3}) and formed 26.73% of the total copepod. The results suggested calanoid abundance regulated by variations in chlorophyll (*a*) concentrations and temperature. Overall, 45 calanoid species representing 11 families and 15 genera were identified during four surveys. SW-monsoon was characterized by highest diversity index (H'=1.95) and species richness (D= 1.66). Pre-monsoon showed lowest numerous of calanoid species (mean 13), diversity index (H'=1.42), and species richness (D=1.39)

Keywords: calanoid, abundance, diversity, monsoon, Chabahar Bay, Gulf of Oman.

1. Introduction

Chabahar Bay is a small semi-enclosed and sub-tropical bay on the south-eastern coasts of Iran (25° 17' 45"N- 60° 37' 45" E). The Bay surface area is 290 km2 with 14 km wide, located between of Chabahar and Konarak (Figure 1). The average depth of the bay is 12 m (ranges from 8-22m). This Bay is connected to the Indian Ocean by the Gulf of Oman. Therefore, the effect of Indian monsoonal winds on this area is remarkable. The year is divided into periods of northeast (NE) monsoon, southwest (SW) monsoon, and its following inter-monsoon periods (post-south west monsoon(post-monsoon), and pre-southwest monsoon (pre-monsoon) (Wyrtki, 1973; Wilson, 2000). This bay is one of the five major ports in the Arabian Sea and Gulf of Oman, located in naturally suitable fish sites. Nonetheless, very scant published information is available on the Bay (Wilson, 2000). Calanoid copepods are the representative taxa of the pelagic mesozooplankton and are both highly diverse and abundant (Angel, 1994). In spite of the abundant evidence about the importance of zooplankton in marine pelagic food webs and the importance of calanoids as food for larval fish (Runge, 1988; Motta *et al.*, 1995), there is limited information about the zooplankton and calanoid density, abundance, diversity and composition in Chabahar Bay. However, there is some information based on copepoda in Mussa creeks by Savari *et al.*, 2003; Savari *et al.*,2004, and on total zooplankton in ROPME Sea (AL-Khabbaz & Fahmi, 1994), in Arabian Sea (Smith, 1995; Baars,1999), in waters of Pakistan (Kazmi, 2004; Saravankumar, 2007), and in Arabian Sea and Indian Ocean (Pillai *et al.*, 1973; Madhupratap, 1987; Tiwari & Nair 1993; Mwaluma *et al.*,2003; madhu *et al.*,2007). The above researchers observed calanoid as the major component of copepod.

The present study is the first in this region to examine the abundance and diversity of calanoid copepod (with temporal-spatial variation) and the impact of environmental factors on their abundance.

2. Materials and methods

Zooplankton sampling conducted during four oceanography cruises: August 2007 (SW-monsoon), November 2007 (post-monsoon), February 2008 (NE-monsoon), and May 2008 (pre-monsoon). Five stations were investigated throughout the Chabahar Bay. Two stations (St 1 and 2) were located far from shore waters with 22 m depth, other two stations were near the shore with 6m depth (St. 3 and 5), and the final station (St. 4) was located in the middle of the Bay with 12m depth. Zooplankton was collected by using 100-µm mesh nets equipped with Hydrobios flow meter. Plankton samples fixed immediately in 4-5% formalin, buffered to a حٌ of 8 with sodium tetraborate (borax). The species diversity was calculated using Shannon–Weaver diversity index

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3. Results

The values of different parameters are shown in Figure 2. The mean of water temperature varied from (20.52 ± 0.20°C) in NE-monsoon to (29.92 ± 0.05° C) in SW-monsoon. The mean of salinity ranged from (36.7 ± 0.06) in SW-monsoon to (36.91) in pre-monsoon. The minimum and maximum values of chlorophyll (*a*) concentrations were noticed (0.77 ± 0.08 mg. m^{-3}) to (1.84 ± 0.92 mg. m^{-3}) in SW-monsoon and NE-monsoon, respectively (Figure 2c). The average of dissolved oxygen (DO) ranged from 5.66 ± 0.05 $ml.l^{-1}$ in SW-monsoon to 8.80 ± 0.03 $ml.l^{-1}$ in NE-monsoon. Pre-monsoon season showed the maximum Silicate (SiO_4) concentration (av. 0.031 ± 0.006 mg. m^{-3}) whereas the minimum was during the NE monsoon (av.0.017 ± 0.05 mg. m^{-3}). The variation of nitrate (NO_3) concentration was (av. 0.026 ± 0.004 mg. m^{-3}) in pre-monsoon to (av. 0.002 ± 0.0002 mg. m^{-3}) in post-monsoon. Minimum and maximum values of phosphate (PO_4) concentration were from (av. 0.015 ± 0.006 mg. m^{-3}) during the NE-monsoon season to (av.0.008 ± 0.002 mg. m^{-3}) during the SW-monsoon season.

Calanoid was the major component of copepod during SW-monsoon comprising 62.40% of total copepod and NE-monsoon (comprising 44.96%). The lowest abundance of calanoid was observed in pre-monsoon (26.73%). In post–monsoon calanoid copepods represented 33.72% of total copepod numbers. Among calanoids, the species belong to following families: Acartidae, Paracalanidae, Centropagidae, Clausocalanidae, Pontellidae, Eucalanidae, Euchaetidae, Lucicutiidae, Pseudodiaptomidae, Temoridae, and Candaciidae. A significant increase was noticed in calanoid abundance in SW-monsoon (445.19 ± 176.13 $ind.m^{-3}$) as compared to NE-monsoon (219.19 ± 37.09 $ind.m^{-3}$), post-monsoon (160.61 ± 43.15 $ind.m^{-3}$), and pre-monsoon seasons (153.56 ± 54.20) ($p<0.05$) (Figure 3a).). Spatial variation based on calanoid abundance during four study periods was significant (Figure 3b).

Overall, 45 calanoid species representing 11 families and 15 genera were identified during four periods in the Chabahar Bay. Some species appeared only in one season and showed lowest abundance. *Lucicutia flavicormis* and *Lucicutia gaussae* were observed during post-monsoon at stations 1 and 3 (comprising 0.01%). *Paracanadica truncate* , *Euchatea marina* was noticed at station 1 during NE-monsoon (comprising 0.01%) as rare calanoid species. *Bestiolina similis*, *Delius nudus* appeared at station 2 in pre-monsoon (comprising 0.04 %). Dominant species in Chabahar Bay are presented in table 1**.** *Temora turbinata* and *Centropages tenuremis* flourished in NE- monsoon as major species (~12%).

SW-monsoon was characterized by highest diversity index (H'=1.95) and species richness (D= 1.66). Pre-monsoon showed the lowest abundance of calanoid species (mean 13), diversity index (H'=1.42) and species richness (D=1.39) (Table 2).

Pearson correlation showed that physico-chemical parameters such as salinity, chlorophyll(*a*) concentration, and pH do not provide any significant correlation with calanoid abundance, whereas calanoid showed a significant positive correlation with temperature ($P < 0.05$, Table 3). Chlorophyll (*a*) concentration showed a significant correlation with phosphate.

Results of cluster analyses based on calanoid density revealed the presence of 2 main groups (*I* and *II*). Station 5 separated from others and group *II* comprised stations 1, 2, 3, and 4 (Figure 4).

4. Discussion

The seasonal variations in abundance of calanoid copepod in Chabahar Bay appear to be regulated by environmental parameters (Fazeli, 2008). In this study, the water temperature was from 20.53 ± 0.20 °C during NE-monsoon to 29.92 ± 0.05 °C during SW-monsoon. The positive association between temperature and total calanoid abundance ($p<0.05$) reveals that temperature is the major factor controlling the abundance of calanoid in Chabahar Bay. This result is in agreement with the findings of Rezai *et al.*, 2004 and Li *et al.*, 2008 about Malacca strait and Taiwan Strait.

Boucher *et al.* (1987) and Williams *et al.* (1994) believe that copepod abundance and distributions are influenced by hydrographic conditions in tropical waters. Nonetheless, salinity, pH and DO appear to play a minor role in influencing abundance and distribution patterns of calanoida in Chabahar Bay. No significant relationship was noticed between those parameters and total calanoid abundance. An opposite trend was observed in some studies in Arabian Sea and Indian Ocean (Pillai *et al.*, 1973; Madhupratap, 1987, 1984; Tiwari & Nair 1993). Those studies showed that salinity plays a key role in copepod abundance.

The results of this investigation support the hypothesis that food importance (e.g. phytoplankton) regulates the abundance of calanoid. The positive association between chlorophyll (*a*) and phosphate concentration implies the increase of chlorophyll(*a*) in high phosphate concentrations. Eduardo (1998) suggests that copepod abundance was, at least in part, regulated by food availability (e.g. phytoplankton); Tranter (1973) said that zooplankton increased after a higher lag time of higher phytoplankton. Therefore, we conclude that calanoid copepod increases after a lag time of higher phytoplankton (chlorophyll (*a*) concentration) and phytoplankton increases after the periods of higher phosphate concentration in the Bay. A similar trend was observed in some studies in some parts of the Indian Ocean by Baars (1999), Madhu *et al.* (2007), and Saravankumar (2007).

Species diversity was generally high in stations far from the shore (St 1 and 2) than those near the shore (St 3 and 5). Also, species richness was increasingly enhanced in stations that are far from the shore and in those in the middle of the Bay. Because of the organisms living in near shore waters seem to be adapted to high food

Table1. Major calanoid copepod species in the Chabahar Bay during monsoonal seasons (Sw.m=SWmonsoon, post.m=postmonsoon, NEmonsoon=NEmonsoon and pre.m=premonsoon) (+++: highly abundant (>1000 m^{-3}), moderately abundant (++:100-1000 m^{-3}), Presence (+:1-100.m^{-3}) and (-: absence)

Species	SW.m	post.m	NE.m	pre.m
Paracalanus elegans	+++	+++	+++	++
*P.*sp	++	+++	-	++
Acrocalanus longicornis	+++	++	++	-
A.gracilis	+++	+	+++	-
*A.*sp.	+++	+++	+++	++
Temora turbinata	++	+++	+++	+++
Pseuodiaptomus sp.	++	-	+++	++
Centropages tenuiremis	++	+++	+++	++
Labidocera. sp.	+++	+	++	-
Pseuodiaptomus marinus	-	-	++	-
Lucicutieae flavicornis	-	+	-	-

Table 2. Diversity indices (H) and species richness (D) of calanoida.

Stations	SW-monsoon	post- monsoon	NE- monsoon	pre-monsoon
1	2.36 (2.46)	2.15 (2.20)	2.62 (2.82)	1.59 (1.06)
2	2.82 (2.50)	2.59 (2.27)	1.60 (0.97)	1.49 (0.57)
3	1.61 (0.65)	1.92 (1.15)	1.56 (0.56)	1.33 (0.48)
4	2.39 (1.84)	1.67 (1.36)	1.72 (1.15)	1.63 (0.96)
5	0.59 (0.86)	1.55 (0.78)	1.71 (1.23)	1.10 (0.41)
Mean	1.95 (1.66)	1.97 (1.55)	1.84 (1.34)	1.42 (1.39)
N.species	30	29	31	13

Table 3: Pearson correlation of major environmental parameters and calanoid copepod density ('*' significant at 0.05 level; '**' significant at 0.01 level)

Variables	Temperature	Salinity	Chl(*a*)	pH	DO	SiO_4	NO_3	PO_4
Calanoid density	0.48*	0.20	-0.23	-0.32	-0.21	-	-	-
Chl(*a*)	-0.38	0.06	1	0.27	0.33	0.05	0.05	0.93**

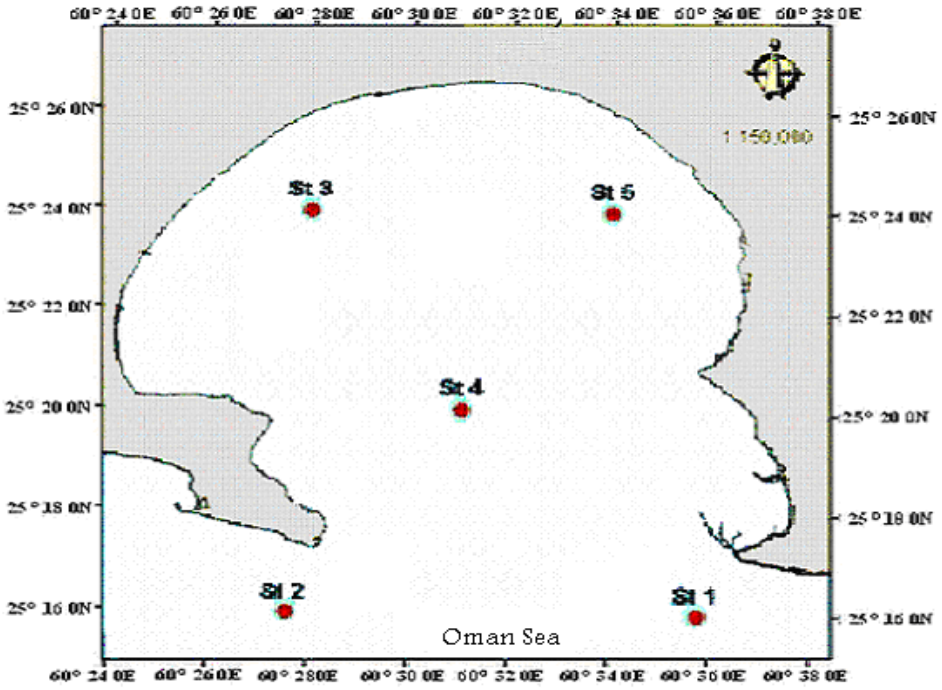

Figure 1.Sampling stations (the numbers show stations)

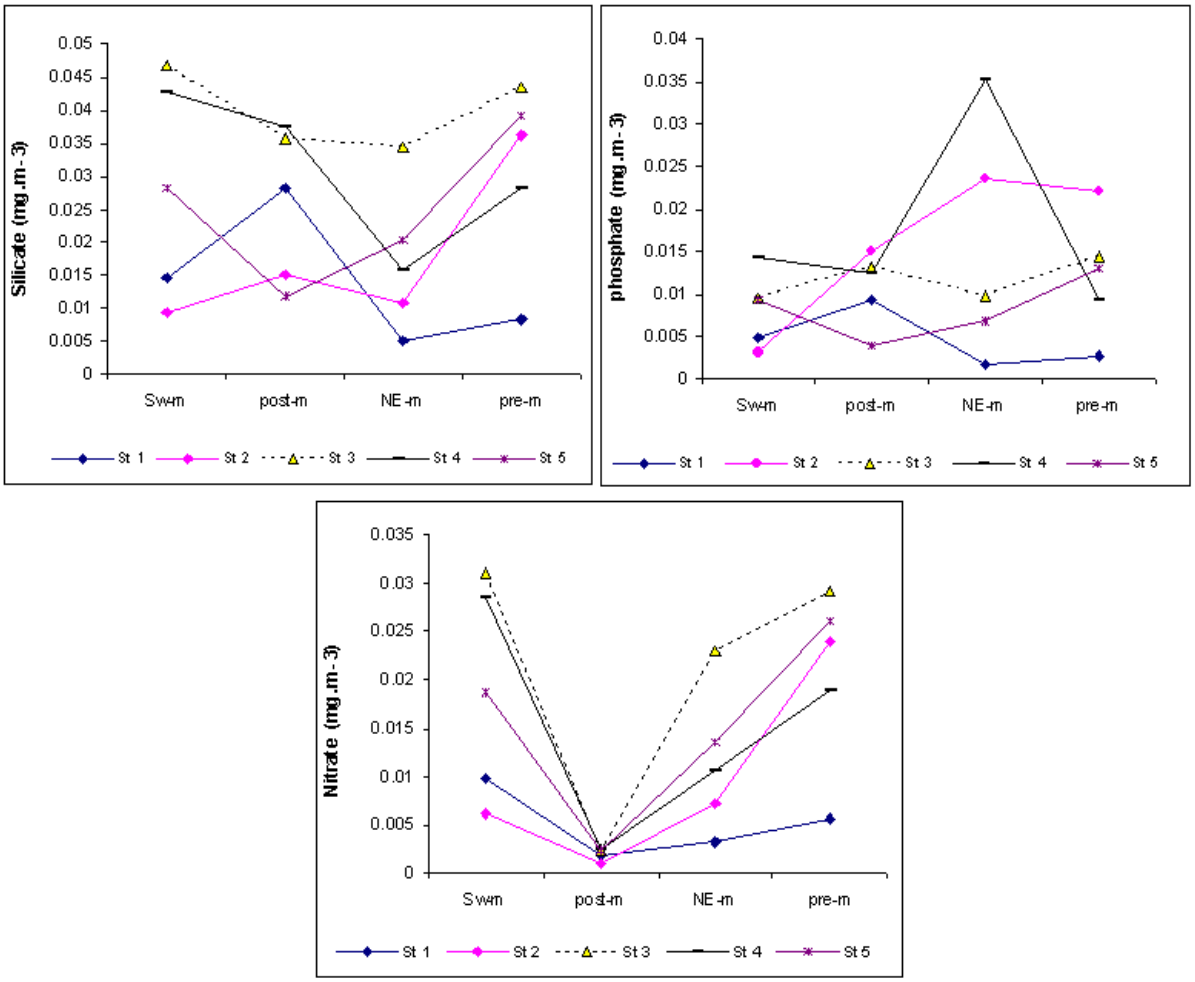

Figure 2. Distribution of major physico-chemical variables during monsoonal seasons (x axis as seasons and Y axis as physico-chemical variables) continues next page……

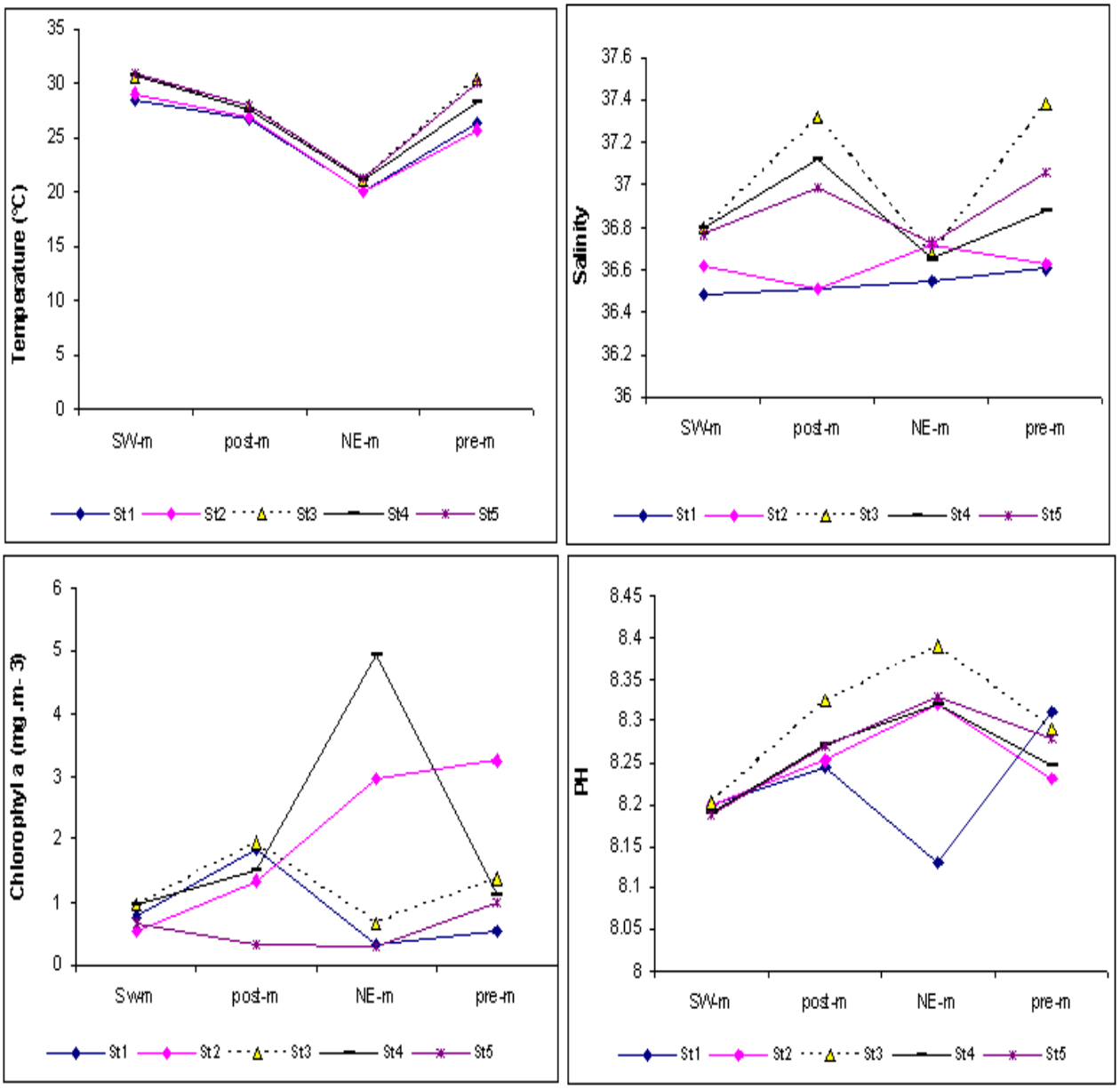

Figure 2. Distribution of major physico-chemical variables during monsoonal seasons (x axis as seasons and Y axis as physico-chemical variables)

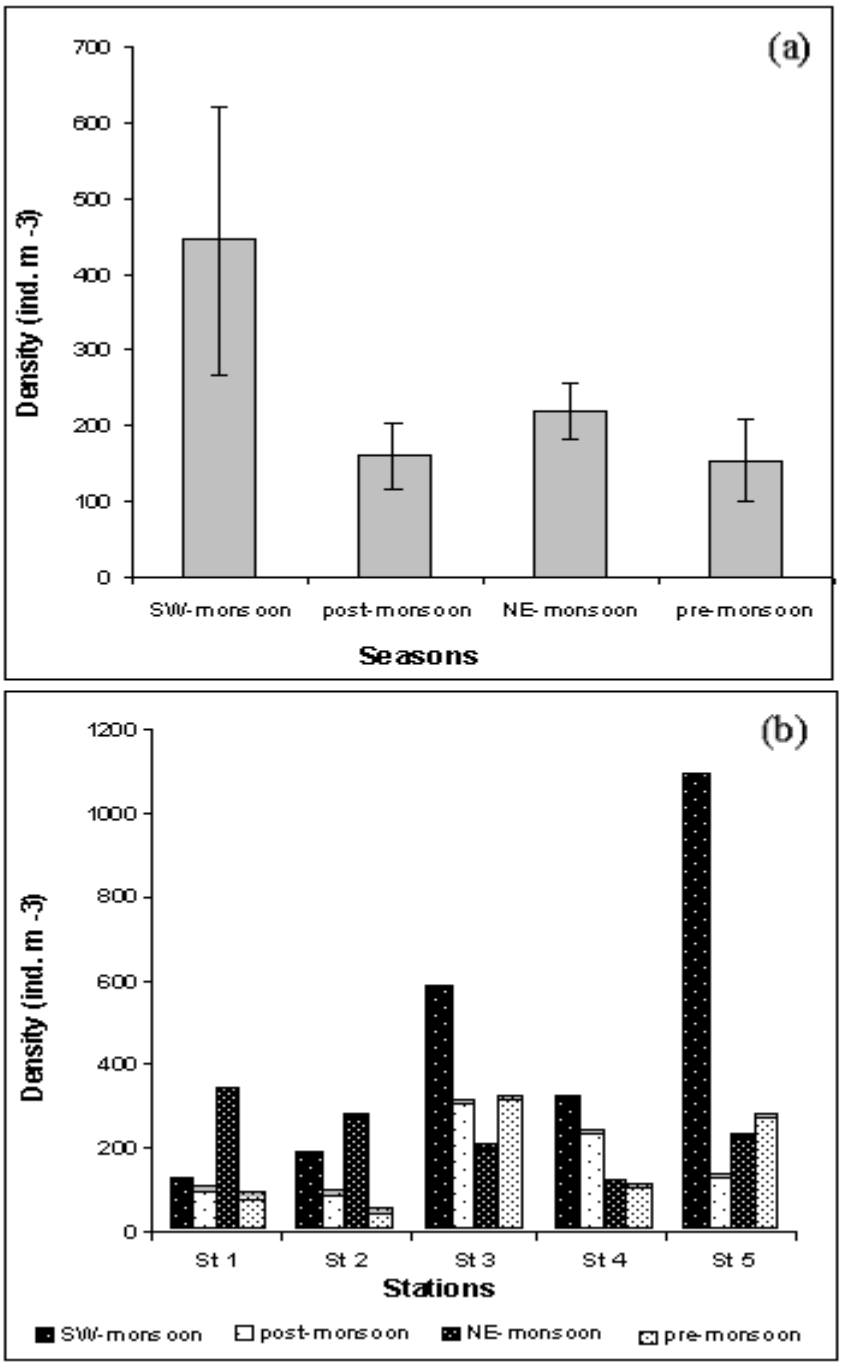

Figure 3. Abundance of calanoid density in Chabahar Bay in stations (a) and seasons (b).

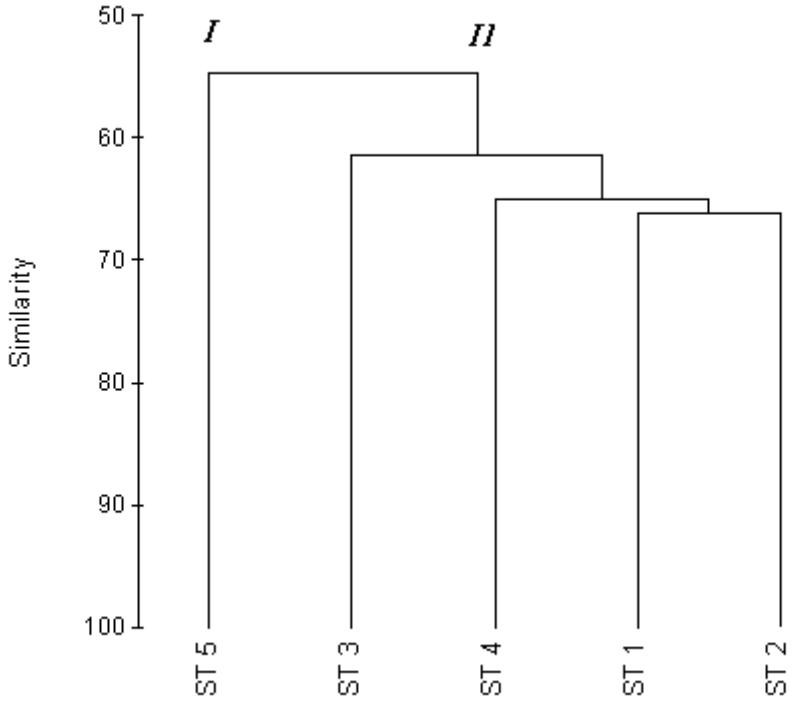

Figure 4. Cluster analyses showing similarity of stations based on calanoid density in Chabahar Bay.

concentration they are unable to thrive in offshore area where food levels are low (Somoue *et al.*, 2005).

Spatial and temporal variations in zooplankton have been associated with changes in phytoplankton standing stocks (Al-Najjar, 2000) and with combined effects of regional climatology and local hydrographic variables (Aoki *et al.*, 1990; Tomosada & Odate, 1995) as observed in other marine habitats including Indian Ocean (Haury, 1988; Piontkovski *et al.*, 1995).

According to the studies, spatial and temporal variations in calanoid copepod throughout the Chabahar Bay can be related to variations in the environmental variability. The temperature and chlorophyll (*a*) concentration play a major role in determining the spatial and temporal patterns of calanoid distribution and abundance.

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