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Biological Sciences (Botany & Zoology)

DISCOVERING THOUGHTS AND INVENTING FUTURE

Highlights

Immobilized Degradation

Callosobruchus Chinensis Linn

Behavioral and Physiological

Astragalus verus and Astragalus

Yellow Jellyfish

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Co-Metabolism and Immobilized Degradation of Some Anesthesia Drugs by *A.Fumigatus*

By Amany H. Aboellil & Fawziah M. Albarakaty
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Abstract - In this study, three fungi isolated from some hospitals at Makkah Almoukarramah showed promising degrading capabilities towards some anesthesia drugs (propofol, clonazepam, lidocaine and bucaine) commonly used. Based on polyphasic identification, morphological, biochemical and 18SrRNA molecular identification these isolates are nominated as Aspergillus niger, A. fumigatus and Rhizopus oryzae. In general, propofol and clonazepam, were more liable to the biodegradation process when compared to the other two drugs. A. fumigatus showed the highest degrading capability towards drugs. The highest fungal wet biomass of A.fumigatus was obtained on cultures containing propofol and clonazepam at a final concentration of 2.5 and 1.25mgml-1, respectively and separately. Shaking cultures showed an enhanced degradation when compared to that of static cultures. Moreover, the optimal conditions for drug biodegradation by A.fumigatus were pH4 at 28.5C° and addition of vitamin C to the growth medium. Calcium alginate- immobilized fungal cells of A. fumigatus grown on propofol and clonazepam containing media showed improved higher degradation of the two drugs, compared to those of free fungal cells growing on the same media. On the other hand, a chromatogram of Infrared (IR) for the end products derived from the biodegradation of drugs confirmed that these two drugs are efficiently degraded to certain end products by A. fumigatus that could be categorized into some identified groups.

Keywords : *A. fumigatus, Propofol, Clonazepam, immobilization, DNA, IR.*

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Strictly as per the compliance and regulations of :

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Amany H. Aboellil [α] & Fawziah M. Albarakaty [σ]

Abstract - In this study, three fungi isolated from some hospitals at Makkah Almoukarramah showed promising degrading capabilities towards some anesthesia drugs (propofol, clonazepam, lidocaine and bucaine) commonly used. Based on polyphasic identification, morphological, biochemical and 18SrRNA molecular identification these isolates are nominated as *Aspergillus niger*, *A. fumigatus* and *Rhizopus oryzae*. In general, propofol and clonazepam, were more liable to the biodegradation process when compared to the other two drugs. *A. fumigatus* showed the highest degrading capability towards drugs. The highest fungal wet biomass of *A.fumigatus* was obtained on cultures containing propofol and clonazepam at a final concentration of 2.5 and 1.25mgml-1, respectively and separately. Shaking cultures showed an enhanced degradation when compared to that of static cultures. Moreover, the optimal conditions for drug biodegradation by *A.fumigatus* were pH4 at 28.5C° and addition of vitamin C to the growth medium. Calcium alginate-immobilized fungal cells of *A. fumigatus* grown on propofol and clonazepam containing media showed improved higher degradation of the two drugs, compared to those of free fungal cells growing on the same media. On the other hand, a chromatogram of Infrared (IR) for the end products derived from the biodegradation of drugs confirmed that these two drugs are efficiently degraded to certain end products by *A. fumigatus* that could be categorized into some identified groups.

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I. Introduction

The most important anesthesia drugs having clinical and therapeutic implications are dipriivan (propofol) , clonazepam, lidocaine and bucaine as tablets or injection. Fukada *et al.,* (2008) and Loftus, *etal.,*(2008) reported that microbial infections could occur in drug addicted persons with immune complications .

Some microbes such as *Candida albicans*, *Staphlococcus aureus*, *S.epidermidis*, *Acinetobacter sp*, *A.fumigatus*, *A.oryzae*, *A.niger*, *A.clavatus* and *A.ustus* could grow well in these contaminated drugs. The ability of the aforementioned microbes to sustain life in these drugs was reported by Robertson and Drummer (1995). On the other hand, it was reported that some of microbes such as *Bacillus megaterium*, *B.subtilis, B. cereus, Streptococcus faecalis, Salmonella typhimuriums, Escherichia coli and Pseudomonas aeruginosa* have the ability to develop som physicochemical modifications for the structure of some drugs (Ohsuka *etal.,* 1994).

Several papers have described the antimicrobial activety of local anesthetics on different organisms. Lidocaine is most active against pathogenic bacteria *in vitro* (Erlich, 1961 and Evron, 1980), an effect also confirmed in an *in vivo* model of surgical wound infections (Frelin *etal.,* 1982). Lidocaine & bupivacaine also showed fungicidal activity against *Candida spp*. by damaging the cytoplasm membrane (Galper and Catterwall, 1979). Additionally, both drugs are potent inhibitors of germ tube formation by *C. albicans* as a result of Ca+2 channel blockade. Local anesthetics affect the membrane of *Candida*, impairing its permeability to ions e.g Ca+2, at low concentration while causing lethal damage at high concentrations (Hill,1991).

The objective of this study is to isolate and identify some fungi that are accomplished with four narcotic drugs namely, propofol, clonazepam, lidiocaine and baucaine, routinely used as anesthetic agents in a wide range of hospitals, in Makkah Moukarrama. The aim is extended to determine the biodegradability of these drugs by tested fungi ,in an attempt to present a biotechnological approach in the treatment of drug-addicted person, to help get rid of any residual drugs circulating in their bodies . Moreover, it is to avoid contamination during anesthesia processor in hospital and to bioremediate of these drugs in disposable syringes.

II. Materials and Methods

1. **Drugs used:** anesthesia drugs were propofol, clonazepam, lidocaine, bucaine were purchased with in a pure form (99%) from Sigma-Aldrich, USA company.
2. **Isolation of some fungi associated with the process of anesthesia:**
   i. **Sources of micro-organisms :** Some fungal isolates were isolated from hospitals on Czapek's medium at 28C° for 5-7 days. from old contaminated syringes or ampoule containing the residue of drugs .
   ii. **Preliminary tests to confirm the ability of fungi to grow on some drugs :** by using the method of wells, and measuring the inhibition area, if any.

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iii. **Identifying fungal isolates:** fungal isolates obtained were traditionally identified after their purification and conservation in a suitable media, at the Public Health Laboratory in the capital of the Holy Capital. Some of them were selected to further study, confirming the identification by a molecular characterization of the gene 18SrRNA, at Alexandria Research Center (UNARC) Department of Biotechnology. Alexandria university.

3. **Effect of some different concentrations of some drugs on the growth of the selected fungi by estimating the wet weight :** Suitable Czapeks broth media fungi with different concentrations, of species of each drug separately (1.25 - 2.50 - 3.75 - 5.00 - 8.3 – 10 $mgml^{-1}$). The media inoculated with fungal isolate separately and on all the previous concentrations .Flasks are incubated at a temperature of 28ºC for a period of 5-7 days on shaking incubator at 180 rpm . After the end of the incubation period, the fungal growth could be estimated as biomass .
4. **Preparation of biomass for use in the following further studied experiments :** Fungal cultures were filtered after the incubation period then centrifuged at 2000 rpm , for 10 minutes, and then the precipitate biomass are washed twice by distilled water, try to get rid of water out by using filter papers followed by taking the wet weight (fresh weight g/12ml media). The filtrate is preserved in the refrigerator at a temperature of 4ºC until analyzed by infrared spectrum IR.
5. **Optimization the environmental conditions to improve the process of growth and biodegradation :** Tested fungi had been developed in different environmental conditions in order to improve growth at different concentrations of the drugs between (1.25 - 2.50 - 3.75 - 5.00 - 8.3 – 10 $mgml^{-1}$) were studied . Fungi were incubated at different degrees of temperature ranging from (5,15,28,37 and 40ºC) as well as different the hydrogen ion concentrations pH 4-7-8-10, the aeration by increasing the number cycles of shaking incubator (100-180-220 rpm) . Also the impact of adding different concentrations from ascorbic acid (vitamin C) in the environment 12-36-72-108 mg /12 ml .
6. **The preparation of Biocatalysts** : Cells have been restricted (immobilized) in gels in agar and calcium alginate by dissolving 2g agar and 0.75 g sodium alginate in 60 ml of distilled water at room temperature, sterilized at a temperature of 110ºC for 10 minutes and leave to cool at a temperature of 50º C, then 40 ml fungal suspension is added. 20 ml of this mixture is transferred under aseptic conditions to sterile syringe and injected in the form of drops in a cold sterile solution of calcium chloride of 2% concentration with continuous stirring using a magnetic stirrer for two hours. The gel beads were put in 200 ml of potassium phosphate buffer solution, 1.46% base potassium phosphate, 0.226% hydrogen potassium phosphate, for two hours to remove calcium alginate from beads gel. Then the gel beads is washed with sterile distilled water or sterile saline solution and then filtrate and transferred to Czapecks broth.
7. **Preparation of the selected fungal isolate for examination with scanning electron microscope** : In order to examine the surface view and the interior porous of the restricted cells where it is frozen by cooling and then broken down into two parts and then dried, followed by loading on carbon or gold holder(gold coated prior) to cover the sample and preparing for microscopic examination and photography by the scanning electron microscope at King Saud University in Riyadh in the Central Laboratory of the Company [(JEOL) JSM-6060 LV] (Hayakawa *et al.,* 1991).
8. **Infrared spectrum (IR) analysis of the product of biodegradation of drugs in filtrate of *A.fumigatus* :** Filtrate is prepared resulting from the vital degradation process at the end of incubation, centrifuged at 2000 rpm / min for 15 minutes, to try to precipitate any impurities from the fungal growth or contents of media. To ensure the clearance of the filtrate, it is filtered by Minisart single use filter non-pyrogenic 0.20µm, then getting rid of water by lyophilizing apparatus (to avoid the affect the disturbance during spectrum infrared (IR) measurement through the emergence the peaks of water molecules, where the analysis was performed using Perkin elemrspectrum 100 Japan Model FT-IR spectrometer (King Saud University in Riyadh in the central laboratory of the company).

III. Results

1. **Isolation and Identification of microorganisms associated with anesthesia at hospitals of Makka Almokarrama** : The fungi were isolated, purified and conserved as method mentioned before. It was prepared by subculturing the isolate on Czapek's media, incubated for 5 days at 28Cº. Preliminary tests, using well method to estimate the effect of different drugs on the isolates was done by measuring inhibition zones, if occurred, after incubation for 24-72 hrs at 28Cº. The surface area of inhibition zones were adopted according to its ability to resist the drug. In this study,three fungal isolates were highly satisfactory to grow against anesthetic drug, were selected for identifying morphologically and biochemically. These isolates were *A.fumigatus, A.niger* and *R.oryzea*.
2. **The effect of different concentrations of some aneasthesia drugs on the growth of the tested fungi on shaking culture :** From Table (1) and figure (2) the tested fungi reported different growth rate (represented as fresh weight g/12ml media) under different concentrations for each of propofol, clonazepam, lidocaine and bucaine. The highest growth rates were reported under clonazepam followed by propofol then bucaine and lidocaine

The growth rates of *A.fumigatus* and *A.niger* were the best in all concentration of clonazepam, if compared with the other drugs. *A.fumigatus* gives the highest growth rates of 27.17 and 32.99% at 2.5 $mgml^{-1}$ concentrations of propofol and 1.25 $mgml^{-}$ clonazepam respectively, if compared with the growth of other fungi at the same concentrations of propofol and clonazepam.

3. **Classification of fungal isolates according to its abilities to grow against different concentrations of drug :** Table (2) summarized the *in vitro* sensetivities of the three fungal isolates to the four anesthetic drugs as determined by inhibition zone and growth on different drug–doses . Isolates were classified as drug-phile, resistant, tolerant, sensitive and lethal. *A.fumigatus* and *A.niger* were classed as propofol – resistants & clonazepam – phile, while *R.oryzea* was classed as propofol – sensitive, clonazepam – resistant. Bucaine and lidocaine have lethal effect for *R.oryzea*. Bucaine and lidocaine have negatively effect on fungal growth. *A.niger* and *A.fumigatus* showed reduced but persistent growth under all drug doses of bucaine , therefore they were classed as doses tolerant . *A.fumigatus* was selected to further study.
4. **Confirmation the identification of *A.fumigatus* by molecular method :** Fungal isolate wase polyphasic identified through microscopical examination, biochemical and molecular identification via sequencing of 18SrRNA gene fig (1). DNA sequences of *A.fumigatus* were subjected to blast programme at National Center for Biotechnology Information (NCBI). Initial alignment with blast programme results in selection of some 18SrRNA fungal sequences, already deposited in the Genbank, that showing high similarity with the query sequences derived from the present study. These selected 18SrRNA fungal sequences along with our query sequences were subjected to another alignment (multiple alignment) in Bioedit software to precisely determine which 18SrRNA fungal sequence is highly similar to our query sequence. Moreover, phylogenetic tree was drawn to determine the genetic affiliation of these fungal isolates. This confirm that the isolate is perfect *A.fumigatus*.
5. **Growth rates of *A.fumigatus* as free and immobilized cell cultures at different drug-doses of propofol and clonazepam :** From fig (2) , the highest growth rates of *A.fumigatus* (2.710 and 2.834 g/12 ml) at 2.5 $mgml^{-1}$ and 1.25 $mgml^{-1}$ for propofol and clonazepam , respectively were demonstrated as free cell culture . On immobilized cell culture the growth rate reported increased values 92.45 and 74.84% at the same concentrations, if compared with free cell culture. The immobilized cell culture support the growth rate of *A.fumigatus* in the highest concentrations of propofol and clonazepam by consider values 2.125 and 2.653 g/12ml at 10.0 $mgml^{-1}$ for each drug.
6. **Optimizing cultural conditions involved in growth and biodegradation of anesthesia drug by *A.fumigatus* :** The factors as pHs , temperatures , drug concentrations , antioxidants and aeration rates were optimized to improve the growth of *A.fumigatus* . Table (3) showed that optimum cultural conditions were pH 4 and temperature 28 C° . The growth of *A.fumigatus* was improved by addition of 108 and 12 $mgml^{-1}$ of ascorbic acid as antioxidant on the media containing 2.5 and 1.25 $mgml^{-1}$ of propofol and clonazepam respectively, on orbit shaker for 220 rpm . These previous cultural conditions have been applied on immobilized cell culture for inducing (improving) of biodegradation of anesthetic drugs .
7. **Electron microscopic scannin :** Fig (4a) illustrated the biocatalyst (immobilized cells as a bead) before incubation showing its smooth surface , but fig (b, c) showed the heavy growth of *A.fumigatus* hyphae on the biocatalyst .
8. **IR analysis of the products of the biodegradation by *A.fumigatus* for each of propofol and clonazepam :** Fig (5a) illustrated the IR analysis of the principle drug compound of clonazepam . From fig (5b) the IR peak of absorption was shown at $3292cm^{-1}$ illustrated the NH group which already occurred in drug (clonazepam) also another peak was at $1417cm^{-1}$ confirmed the above group (NH). The group C-Cl was demonstrated by the peak of absorption at $768cm^{-}$. The IR analyses of the filtrate of *A.fumigatus* grown on clonazepam as free cell was shown in fig (5b). A new absorption peak was observed at $1725cm^{-1}$.This is evidence the presence of carbonyl group (C=O) which did not recorded in principle drug. C-Cl group was lacked. The two peaks of 2940, 2890 cm^{-1} were reduced, due to decrease the intensity of aromatic CH. On the other hand, IR of the filtrate of immobilized *A.fumigatus* grown on medium containing clonazepam was shown in fig (5c). The increase in absorption peak at 3363 cm^{-1} illustrate increasing in intensities of NH and C=O groups in compared with the principle compound a peak at 1406 cm^{-1} confirmed rising of intensities of these groups .At first time a new peak at $1350cm^{-1}$ involved in the filtrate of immobilized *A.fumigatus* grown on clonazepam, this peak characterized the group of C-O-C . Two peaks at 1727 and 2940 – 2890 cm^{-1} increased due to high intensities for acidic carbonyl (C=O) and aromatic CH corresponding to the principle compound and the first filtrate.

From fig (5d) the IR analysis absorption illustrated the principle propofol . A peak at $3353cm^{-1}$ was evidence for presence of OH group with H-bonds . A peak for $2850\text{-}2920cm^{-1}$ is due to aromatic OH. On free cell culture, a peak of at 1743 cm^{-1} due to acidic C=O

was indicated fig (5e) , but on immobilized culture fig (5f), a high intensity of carbonyl group C=O was demonstrated.

IV. Discussion

A survey of fungi associated with anesthesia were isolated on specific media, under appropriate conditions, purified and conserved as the above mentioned method. Three isolates are identified as *Aspergillus niger, A. fumigatus, Rhizopus oryzae. A. fumigatus* is the most prevalent airborne fungal pathogen in developed countries and in immunocompromised patients causes a usually fatal invasive aspergillosis (Latge, 2001).The definition of *A. fumigatus* was confirmed by molecular characterization of the gene 18S rRNA. This method is the best definition, through multiplication of gene by PCR. Sequences of genetic for 18S rRNA was found out and compared with their counterparts in the field of Bioedit software Showing 85% similarity. Phylogenetic tree determined the affiliation gene for this isolate.

In this study, the isolated fungi were classified, on its ability to grow in different concentrations of each drug used, as drug-phile, resistant, tolerant, sensitive or lethal, depending on the doses used. lidocane or bucaine have inhibiting effects on *A.niger* and *A.fumigatus* and influential fatal to *R. oryzae*. Lidocaine and bucaine are known to inhibit germ tube formation of *C.albicans*, hypothesizing that the effect is due to blockading ionic channels, particularly calcium channel. Therefore, lidocaine can affect morphology and probably also the pathogenesis of *C. albicans*. (Rodrigues *et al,* 2005).

Pina-Vaz *et al.,* (2000) stated that at lower concentrations, these drugs have a fungistatic activity, due to metabolic impairment, while at higher concentrations they are fungicidal, due to direct damage to the cytoplasmic membrane. Ohsuka *et al.,* (1994) suggested that depolarization of the cytoplasmic membrane, preceded by the permeabilization of the outer membrane for gram-negative bacteria, is associated with antibacterial activity of lidocaine. The drugs have a negative impact on the membranes of organism as a result of an imbalance in the permeability and the demise of polar cell membrane and the leakage of ions of potassium K +. Also, it led to the inhibition of activity of some enzymes such as succinic dehydrogenase .

However, numerous studies over the past several decades have elucidated the supplemental role of local anesthetics as antimicrobial agents. In addition to their anesthetic properties, medications such as bupivacaine (bucaine) and lidocaine have been shown to exhibit bacteriostatic, bactericidal, fungistatic, and fungicidal properties against a wide spectrum of microorganisms (Johnson *et al.,* 2008).

In our study, *A .fumigatus* and *A .niger* were propofol- resistant . *R.oryzea* showed sensitivity to propofol. Güzelant *et al.,* (2008) have found that some types of organisms such as *Candida albicans*, *Staphylococcus aureus*, *Acinetobacter calcoaceticus*, *Escherichia coli*, *Enterobacter cloacae*, *Moraxella osloensis*, *Cunninghamella elegans* and *Pseudomonas aeruginosa*, *Streptomyses* were grown well on propofol , they are resistant to the drug. Propofol infusion promoted budding of *Candida* and the germination of *Aspergillus*, latter forming a lipid layer around the hyphae . Propofol infusion , due to its lipidic vehicle, increased the fungal germination and promoted resistance to antifungals. This effect seems to be related to the reduced access and / or permeabilization to fungal cells by antifungals. (Costa - de - oliveira *et al.,* 2008).

A. fumigatus and *A. niger* have an affinity to clonazepam but *R.oryzea* showed a resistance to drug. It may be due to their ability to degrade the drug and use it as essential material for their growth and their activities therefore, they are drug-phile. Robertson and Drummer (1995) have studied on the degradation of some drug types of clonazepam in the blood by bacterial species of intestine, *Streptococcus faecalis* and *Clostridium perfringens*. He noted the complete degradation of this drug in the blood .The incubation under anaerobic condition led to a reduction of clonazepam and its transformation into 7-aminoclonazepam and the incubation under aerobic conditions gave 3-hydroxyclonazepam, as a key component of the transformation processes. He also found that the process of degradation occurs the same way in animals by the microflora.

Environmental conditions have been improved for enhancing the growth of *A. fumigatus* to best degradation of selected drugs at high concentrations. It has been found that the best concentration of hydrogen ion (pH) is 4, and the best temperature is 28˚C. Adding antioxidant ascorbic acid (vitamin C) to media accelerated the degradation of drugs, when using $2.5 mgml^{-1}$ concentration of propofol and $1.25 mgml^{-1}$ clonazepam . Using shaking culture (220 rpm) helped to improve the degradation of drugs. Güzelant *et al.,* (2008) stated that the temperature is of the important factors to control the activity of microbial, and that the proper temperature promotes the growth of microbes on the drug and lead to an increase in rates of destruction of drugs.

Robertson and Drummer (1995) stated that the rates of metabolism, at 37 C, ranged from 0.1 ng/mL/min of nitrobenzodiazepines for *Streptococcus faecalis* to 8.8 ng/mL/min. The pH had variable effects on the rate of metabolic bioconversion of nitrobenzodiazepines, while increasing temperatures were found to generally increase the rate of nitrobenzodiazepine bioconversion.

Baumgart *et al.*,(2007) noted that the most important chemicals affecting also are antioxidants, which found that intravenous drug abuse led to an increase of antioxidants in blood plasma and this led to increased resistance to large molecules to oxidation. The some fungal species and bacteria can produce some antioxidants, such as carotene and some antioxidant enzymes, such as those produced by bacteria *E.coil*.

Some new technologies have been used to improve the degradation of drugs by microorganism through immobilizing the fungal cells, this immobilized cell is called Biocatalyst (Usha *et al.,* 2010). Calcium alginate used in this study. Modak *et al.,* (2001) has been able to use of the immobilizing process in the detention cells *Pseudommnas putida* for the degradation of phenol. Mattiasson (1983) has proved to that stability enzymatic system of immobilized cells was better than in the free cells, and offers the opportunity to step enzyme multiple accompanied by co-enzyme for the process of degradation in general. On this basis, Wiesel *et al.,* 1993 worked on restricting the entire microbial living cells. This is due to its features, many is that the cells bound to be more resistant to pollution , can be separated metabolites recorded easily, can re-use of biocatalyst recorded more than once, Also, it give the large biomass and protect cells from high concentrations of toxic substances. In our study immobilized cells of *A. fumigatus* has shown resistance, even was able to grow in higher concentrations, it has already grown at a concentration of 10 $mgml^{-1}$ of the medical drug.

The immobilized *A.fumigatus* mycelia (biocatalyst) may be allowed the concentrations of nutrients around the fungus away from the toxicity, of high concentrations of narcotic substances. This accelerates the growth process and leads to flourishing growth surface on gel beads which may speed up the process of degradation of drugs.

According to the IR analyses of propofol and clonazepam, the changes of the original compounds after treatment with exposure to fungal growth were studied. In general, functional groups presented showed fluctuation by decreasing or increasing of its the related peacks. Also, some original funcutional groups disappeared and some new ones appeared, e.g acidic carbonyl (C=O) group was appeared the first time and (C-Cl) group was laked, corresponding to the principle compound of clonazepam.

These changes led to confirm the bioconversion of both drugs by fungal growth. IR has proved the capability of *A.fumigatus* to change the nature of drugs. These indicate that *A.fumigatus* may be biodegraded the drugs to another compounds to avoid its toxicity or to use its derivatives as nutrient.

Also, IR analyses showed that the immobilized cells of *A.fumigatus* recorded the highest degree of degradation for the two drugs than that of the free cells . of *A.fumigatus*.

V. Conclusion

Some drugs may support the growth of some microbes, such as enhancing of propofol for the growth of *A.fumigatus*. The fungus may be able to use the drug clonazepam where it has the ability to convert drug into suitable compound easy to use. Llidocaine and biocaine have to do mutual action, they had the ability to inhibit microbes, where they were working as antimicrobial. Immobilization of the fungus, as biocatalyst under certain circumstances may lead to increased growth of *A.fumigatus* and its ability to degrade some drugs. The vision for the future is to take these advantages of the microorganisms, in a positive step, to treat the addict or the patient, focusing on an attempt to avoid the contamination with microbes during the process of anesthesia, in medical field, to avoid microbial infection. Further studied are necessary to define the clinical significance, prevalence and mechanisms of resistance of these isolates of *A.fumigatus* to drugs .

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Fig 1 : Effect of different concentrations of some anesthesia drugs on the growth of the tested fungi (fresh weight g/12ml) on shaking culture .

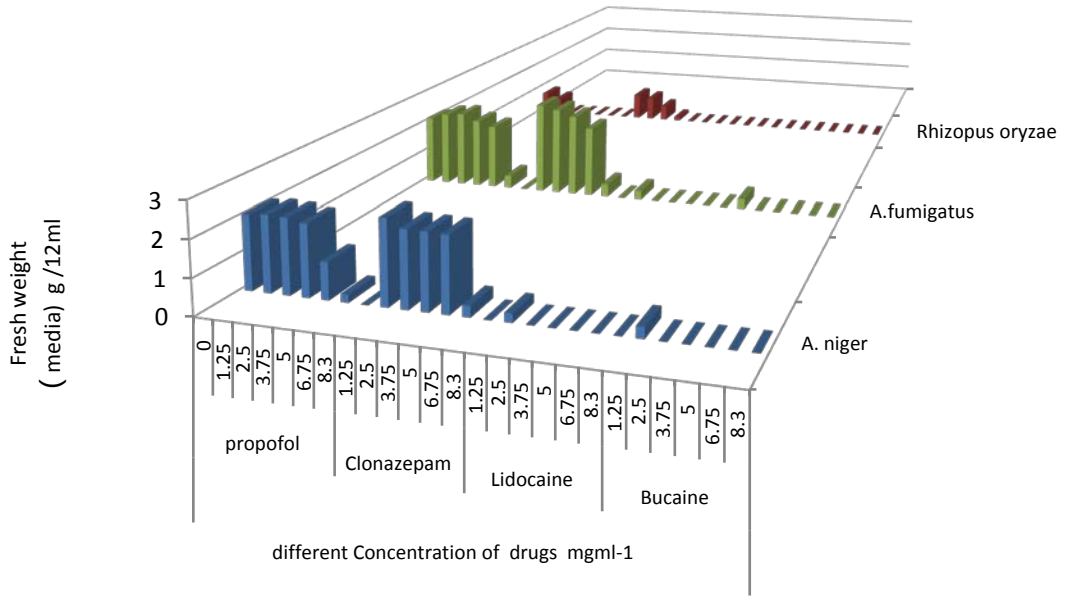


Table 1 : The growth rate (%) of the tested fungi grown on different concentrations of some anesthesia drugs on shaking culture.

The tested fungi / Different concentrations of drugs mgml^{-1}		Growth rate %		
		A. niger	*A. fumigatus*	*R. oryzae*
propofol	control	100	100	100
	1.25	+ 2.12	+7.84	- 24.65
	2.50	+ 0.86	+ 10.18	≠
	3.75	- 3.66	- 1.64	≠
	5.00	- 50.12	- 6.52	≠
	6.75	- 89.25	- 81.84	≠
	8.30	≠	≠	≠
Clonazepam	1.25	+ 13.49	+ 32.99	+ 25.35
	2.50	+ 2.27	+ 27.69	+12.47
	3.75	+ 1.74	+ 18.82	- 28.83
	5.00	+ 0.63	+ 2.72	- 82.53
	6.75	- 84.82	- 81.42	≠
	8.30	≠	≠	≠
Lidocaine	1.25	- 88.05	- 87.10	≠
	2.50	≠	≠	≠
Bucaine	3.75	- 84.82	- 85.45	≠
	5.00	≠	≠	≠

(+) positive growth

(-) negative growth

(≠) no growth

Table 2 : Classification of isolates according to its ability to grow against some anesthetic drugs .

Fungi	Typs of drugs			
	Propofol	Clonazepam	Lidocaine	Bucaine
A.niger	drug – resistant	drug – phile	drug – senstive	drug – tolerant
A.fumigatus	drug – resistant	drug – phile	drug – resistant	drug – tolerant
R. oryzae	drug – sensitive	drug – resistant	drug – lethal	drug – lethal

Fig 2 : showing PCR product of 18SrRNA gene amplified via PCR from one fungal isolates. M: DNA base pair marker. Lane1: PCR product of the fungal isolate nominated biochemically as *A. fumigatus* .

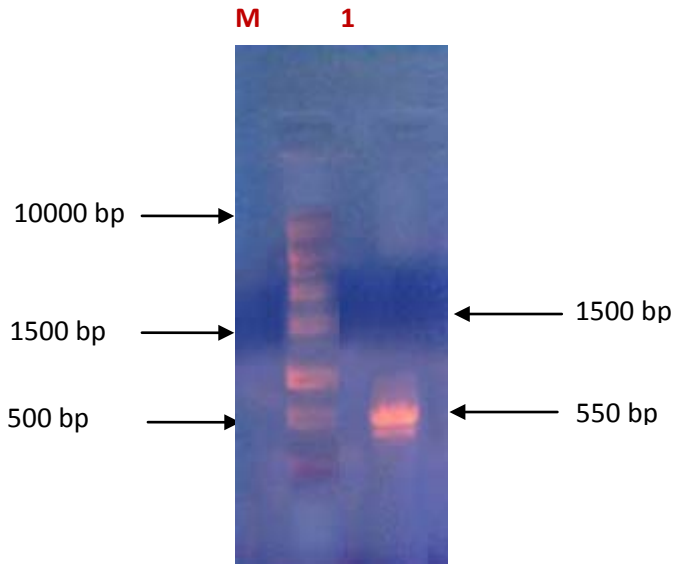


Fig 3 : Comparison between the growth of *A.fumigatus* as free cells and immobilized cells on different concentrations of propofol and clonazepam on shaking culture .

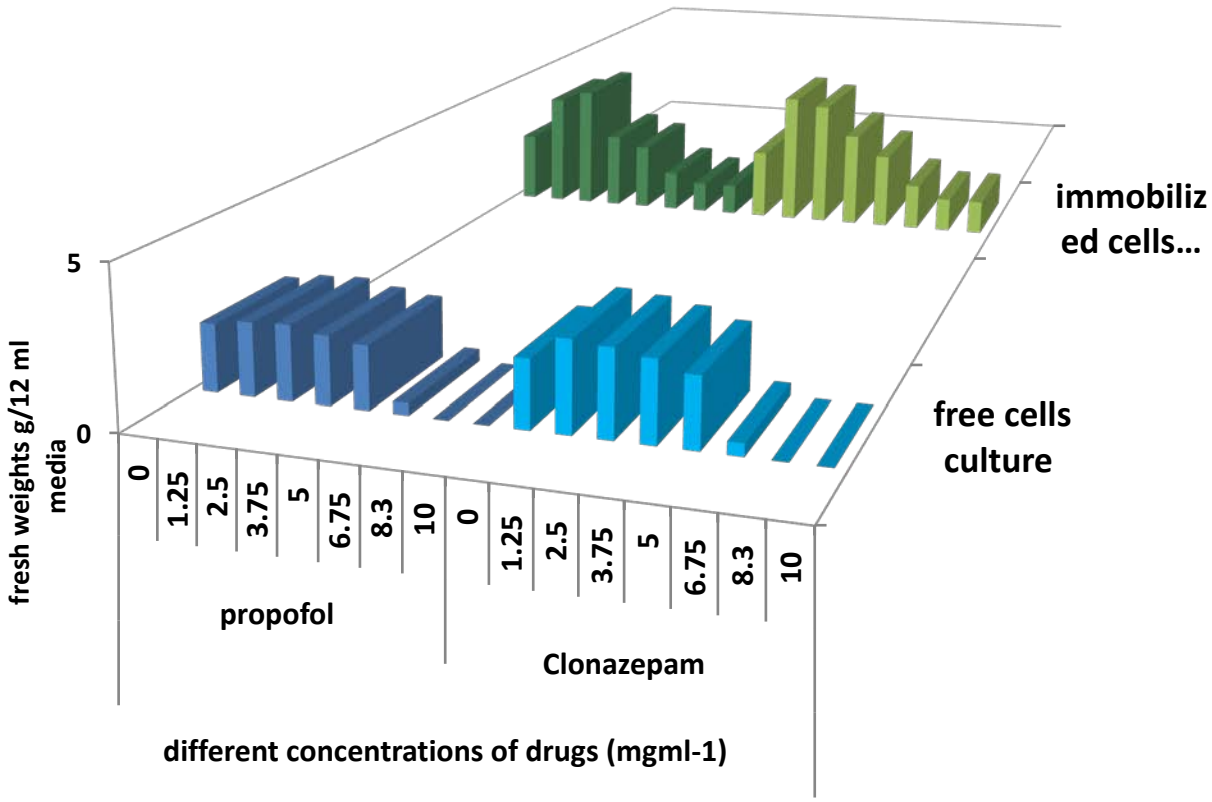


Table 3 : The optimization of environmental conditions for the growth of *A.fumigatus* grown on media containing some anesthesia drugs .

Drugs mgml-1	Concentration of optimal growth	Final concentration in free cell culture	Final concentration in immobilized cell culture	Incubation temperature (C°)	pH	Number of cycles of shaked culture rpm/min	Vitamin C mgml-1
Propofol	2.50	6.75	10.00	28	4	220	108
Clonazepam	1.25	6.75	10.00	28	4	220	12

Fig (4) : Scanning electron micrographs of (A) the surface of calcium alginate bead containing subsurface mycelium of *Aspergillus fumigatus.* (B) the surface of an inoculated calcium alginate bead which was overgrown with mycelia of *A. fumigatus* mycelia during incubation. (C) amplified of (B).

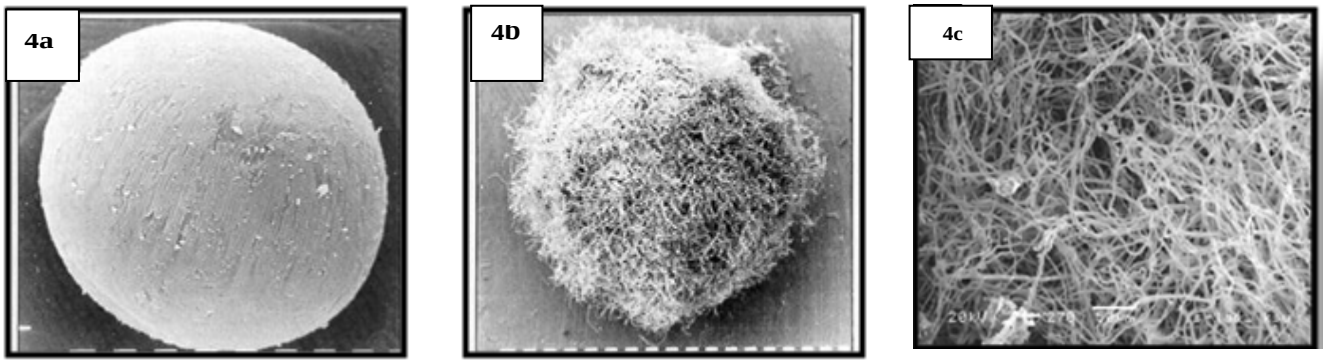
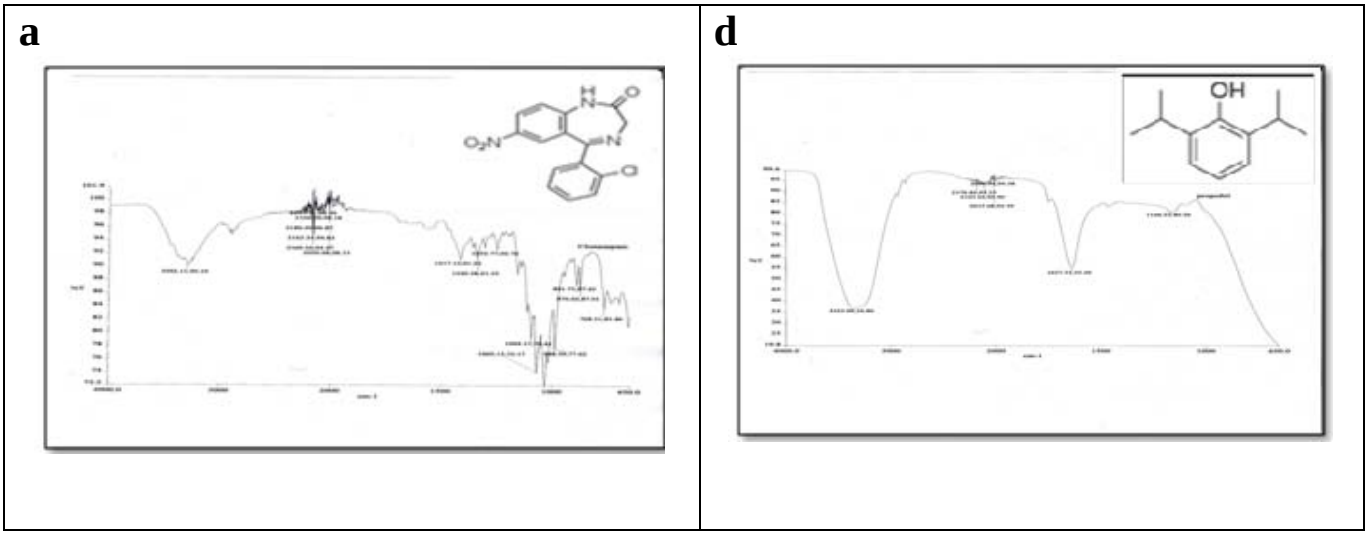


Fig 5 : IR Spectra (a): IR spectrum of clonazepam (b): IR spectrum of degradating products of clonazepam on free cell culture (c): IR spectrum degradating of products of clonazepam on immobilized cell culture (d): IR spectrum of propofol (e): IR spectrum of degradating products of propofol on free cell culture (f): IR spectrum of degradating products of propofol on immobilized cell culture.



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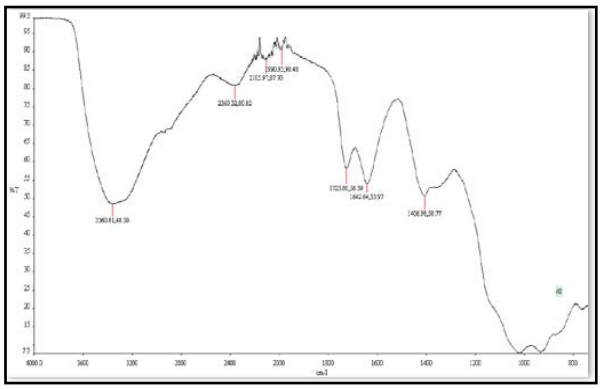

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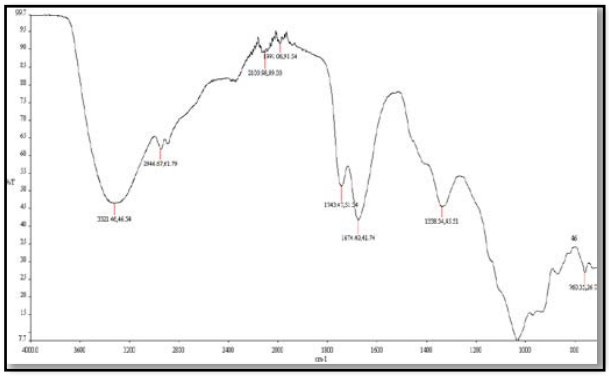

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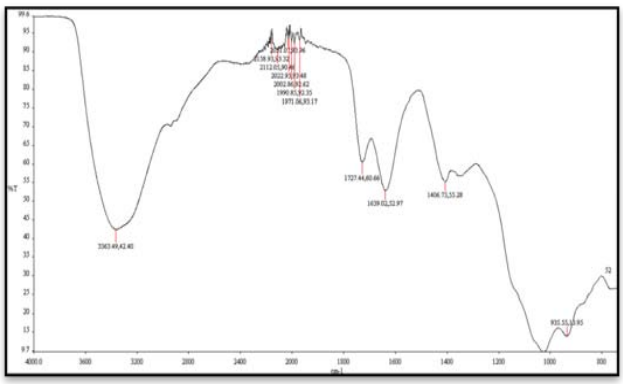

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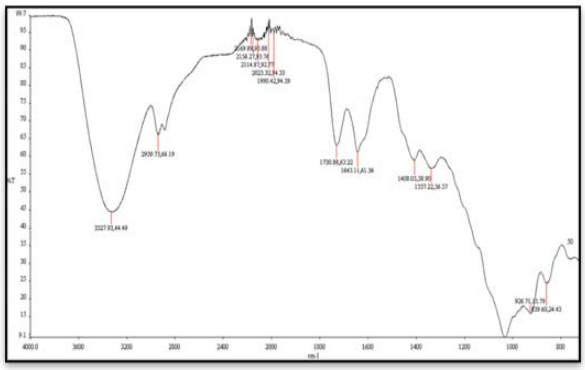

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Effect of Environmental Color on the Behavioral and Physiological Response of Nile Tilapia, *Oreochromis Niloticuss*

By Dalia M. Sabri , Nagwa Elnwishy & Francis Nwonwu
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Abstract - Behavioral response, Acetylecholinesterase activity, total protein level and protein fractions of the Nile tilapia, Oreochromis niloticus were investigated after 37 days exposure period to different environmental colors (Yellow, blue, green, red and darkness). Groups of 10 individuals each with initial body weight of 10.01±0.15 g were reared in 60x30x50 cm) aquarium. Two replicate groups for each color were covered with blue, green, red or black cellophane (no cellophane was used for yellow light). Fish Behavior was observed daily in the containers. Enzyme activity, mortality, total protein, albumin/globulin ratio and protein fractions were measured in 7, 37 and 45 days (7 days recovery). Results showed different behavioral and biological changes in responce to the change of color in all tested parameters. Mortality ratios were also affected by changes in surrounding color. Fish showed preference for blue light followed by green light, while red light was the most unfavorable to fish. Therefore, authors recommend applying blue color lighting in aquaculture system in order to obtain the best conditions for fish production.

Keywords : *AChE, Environmental color, Oreochromis niloticus, Protein fractions, Stress factors, Total protein.*

GJSFR-C Classification : *FOR Code: 060807*

Strictly as per the compliance and regulations of :

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I. Introduction

Tilapia fish is one of the most important fish species in the fisheries world. It is the second most essential group of food fishes in the world after carps. There are about 100 species and subspecies of tilapia with global annual production of 2532407 tons (FAO, 2008) of which aquaculture contributes 92% of the production (FAO, 2008). Although tilapia species are receiving a great attention as they occupy two different market types, being a main fish food in most Asian and African countries and being a high value fish food in Southern United States (Maclean *et al.,* 2002).

Nile Tilapia *Oreochromis niloticus* is also an excellent laboratory animal. In order to optimize the cultivation of this species, it is important to understand and estimate its behavior and performance in culture conditions (Strand *et al*., 2007). This is because the artificial environments could vary from the natural habitats of fish and may cause negative effect on fish feeding activity, health, welfare and growth.

One of the environmental factors that may influence fish performance in culture is environmental color (Brännäs *et al*., 2001). In nature, light intensity and background color can affect feed detection, food conversion rate and feeding success of cultured fish, thus influencing fish growth and mortality (Henne & Watanabe, 2003; Jentoft *et al*., 2006). Furthermore, tank color and light intensity can contribute to fish stress (Rotllant *et al.,* 2003; Papoutsoglou *et al.,* 2005), which may affect their behavior by altering swimming performance, activity levels and habitat utilization (Mesa & Schreck, 1989; Schreck *et al*., 1997).

The common colors of the surrounding environment of fishes are blue, green or near infrared (Levine & MacNichol, 1982) and fish can discriminate against them (Nicol, 1963). Although very few studies have been conducted to understand the effects of background or light color on fish biology, in some fish families, effects of environmental color have been described, such as changes in fright reaction, color attractiveness, survival and growth rate (Tamazouzt *et al*., 2000).

The effect of environmental color on animal physiology and behavior is a developing field. As in earlier studies, environmental color showed both improvement and disruption of fish welfare. These findings are supporting the rising interest to investigate and get a better understanding of the effects of such related rearing conditions on fish performance. Thus, in fisheries, the environmental conditions should definitely be monitored to guarantee improved fish welfare.

While fish environment is composed of a wide range of colors, which many fishes can discriminate against, the present study investigates the effect of different environmental colors on the behaviors, biochemical indicators, and nervous enzyme activity (AChE) of Nile tilapia *Oreochromis niloticus*. The aim is to reach the optimum conditions for application in fish culture techniques. The study was conducted according to the ethical principles in animal research.

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II. Materials and Methods

a) Fish and Experimental design

One hundred Nile tilapia fish, *Oreochromis niloticus* with average body weight of 10.01±0.15 g, were procured from Fish Research Center and brought to Biotechnology Research Center of the Suez Canal University, Egypt) and kept for seven days before experimentation.

The tilapia fish were randomly distributed in 10 (60x30x50 cm) glass aquaria. Pairs of glass aquaria were covered with cellophane of the perspective environmental color; blue (B), green (G), red (R), dark (D) and no cellophane for yellow(Y) color, which was used as the control. Fish aquaria were supplied with illumination source covered with the same color as the aquaria, the light intensity reaching each aquarium was 120 for blue, 120 for green, 150 for red, and 320 lux for yellow. As part of the experimentation, water was aerated and heated to approximately 28°C while the pH ranged from 6.8 to 6.95. The photoperiod was setup from 06:00 to 18:00 hrs Fish were fed on diet pellets (30% crude protein) twice a day at 3% of body weight (Eurell *et al.,* 1978).

Samples were collected after 7 days of exposure to the perspective color, which are respectively designated as (B1), (G1), (R1), (D1) and (Y1)Samples were again collected after 37 days from each aquarium and were designated as (B2), (G2), (R2), (D2) and (Y2). Finally samples were collected after 45 days and labeled (B3), (G3), (R3), (D3) and (Y3) respectively after which the color effect was removed for 7 days of recovery.

b) Blood and serum samples

At the end of each stage of the experiment (after 7, 37 and 45 days), two fish per aquarium were anesthetized using 150 mg l–1 tricane methan sulphonat (MS 222) (Wagner *et al.,* 1997) and immobilized for 3 minutes. All fish samples were handled in the same way. Blood samples were taken individually from the caudal artery by heparinized syringes for further analysis.

Samples were left to clot in a refrigerator at 4ºC for one to two hours and then centrifuged at 4000 rpm for 20 minutes in a cooling centrifuge Supernatants were transferred into dry, clean tubes as serum samples and stored at 20ºC for further analysis.

c) Behavioral response and Clinical examination

Fish behavioral response was performed daily at 08:00 hrs and 14:00 hrs according to Conroy and Hermann (1981).

d) Acetylecholinesterase (AChE) Activity

AChE activity was determined in blood serum immediately after collecting blood samples using the method of Kendel and Bottger (1967) and Den *et al.* (1983).

e) Total Protein and Albumin Level

Serum samples were analyzed to determine total protein content (Henry, 1964) and albumin level (Doumas & Biggs, 1972).

f) Albumin/Globulin Ratio

Globulin levels were calculated mathematically from the difference between serum total protein and albumin level. The albumin:globulin (A:G ratio) was also calculated mathematically.

g) Serum protein fractions

Separation and identification of serum protein fractions were done using SDS/PAGE and BioRAD molecular markers ranging between 214 and 6.8kDa. Blood samples were prepared and injected into a PAGE gel (Laemmli, 1970). Gel photo was captured and analyzed on a Gel documentation analyzer, Ver. 2, 2006, Elmanar Company.

h) Statistical analysis

The variations in Total Protein content (TP), Protein fractions, Albumin/Globulin ratio (A/G ratio), Acetylecholinesterase Activity (AChE), and mortality were tested statistically using the Satistical Package for Social Scientists (SPSS) 18.0 analytical tool.

III. Results

The statistical analysis shows an overall highly significant (P = 0.0001) Wilks' Lambda multivariate ANOVA effect of the treatments on the experimental materials on the investigated parameters.

a) Behavioral Response and Clinical Signs

Results show that fish demonstrated a preference for blue and green color background to red adapted groups. Fish in the Y, B1 and G1 groups were seen moving actively and normally in the aquarium under the short time exposure regime. In contrast, R1 groups showed very aggressive behaviors against each other, and swam with high speed across the aquarium. It was further notable that B1 and B2 groups were the least aggressive among all groups. D1and D2 groups showed very slow movement, less activity, and no aggressive behaviors.

Removing the colors caused a severe abnormal behavior among the fish in group D3. They avoided swimming across the aquarium and did not show keen interest in feeding. Fish in group G3 and B3 showed little interest in feeding for the first three days after removing the color, but fed normally later. Fish in group R3 were swimming normally in the aquarium, but they did not respond to feeding in the first week after removing the color. And showed no aggressive actions when the color was removed.

No changes in skin color were observed in all groups except in group D, fish skin color changed from

grey to dark, and finally to black color when the darkness effect was removed.

b) Acetylecholinesterase (AChE) Activity

Generally, there was a significant ($P < 0.05$) increase in AchE activity after 7 days of exposure to green, red and dark colors, but no significant change was found in fish exposed to blue light for the same period. The highest increase was obtained from R1and D1 treatments for the same short exposure (Figure 1 and Table I).

AChE activity was significantly ($P < 0.05$) increased by red and green color light under the R2 and G2 treatments compared to the control, while it was significantly ($P < 0.05$) decreased by dark color in the D2 exposure. No significant difference was found between blue light treatment in B2 and the control. The highest increase in AChE activity was obtained from the R2 treatment (Figure1 and Table I).

There was a significant ($P < 0.05$) increase in the enzyme activity in groups exposed to green and red colors G3 and R3 when the color effect was removed, and a significant ($P < 0.05$) decrease in the group exposed to blue and to darkness . But, no significant ($P < 0.05$) change was found when the blue light B3 effect was removed (Figure 1 and Table I).

Table I : Variations in AChE concentrations in responce to changes of color(U/L)

7 days treatment		30 days treatment		7 days recovery	
Sample	Concentration	Sample	Concentration	Sample	Concentration
Y	4953,525 ±431,02				
B1	4204,018 ±569,58	B2	4171,588 ±76,91	B3	5004,145 ±10,10
G1	7296,060 ±0,12	G2	5687,228±2394,42	G3	8446,508 ±1341,04
R1	9512,263 ±312,58	R2	7992,605 ±1023,3	R3	7526,205 ±2299,35
D1	8544,483 ±339,33	D2	1691,658 ±36,24	D3	2116,053±55,30

* Significance *(P < 0.05)*
Y= (Y1+Y2+Y3)/3

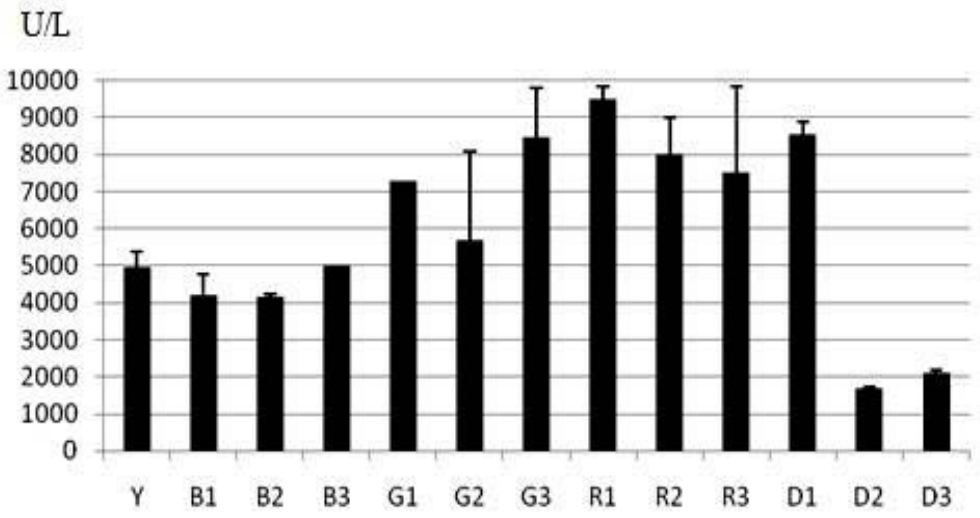

Figure 1 : Variations in AChE concentrations in responce to changes of color(U/L)

Total protein content (TP) was significantly ($P < 0.05$) increased by the surrounding colors in 7 days exposure in groups G1, R1, and D1. The increase in the group B1 was not significant. (Figure 2 and Table II). Delete the paragraph. Meanwhile, long term exposure to green, dark, and red colors showed significant decrease in (TP) in groups exposed to G2, R2, and D2 colors compared to control Y. On the other hand TP showed insignificant decrease in the group exposed to long term blue light B2 (Figure 2, and Table II).

Removing the blue color in group B3 showed significant change in TP compared to the control. Furthermore, a significant increase in group R3 and significant decrease in G3 and D3 were observed after removing the color effects.

Albumin/Globulin ratio was significantly decreased by all colors exposure in the short term exposure period (Table III). The long exposure caused a significant change in G2, R2 and D2 compared to control. The same significance was found in G3, R3 and D3 when the colors were removed, while insignificant decrease was detected in B3. There was slight but statistically insignificant decrease in the group exposed to blue color.

Table II : Variations in proteins content in responce to changes of color (gm /l)

7 days treatment		30 days treatment		7 days recovery	
Sample	Concentration	Sample	Concentration	Sample	Concentration
Y	3,830 ±0,387				
B1	4,040 ±0,031	B2	3,190 ±0,193*	B3	3,463±0,371
G1	4,334±0,122*	G2	3,772 ±0,166	G3	3,427±0,216*
R1	4,246±0,071*	R2	3,213±0,206	R3	4,202±0,089*
D1	4,115±0,067*	D2	3,707±0,07	D3	3,336±0,060*
* Significance *(P < 0.05)* Y= (Y1+Y2+Y3)/3					

Table III : Variations in Albumin/Globulin ratio in responce to changes of color

7 days treatment		30 days treatment		7 days recovery	
Sample	Ratio	Sample	Ratio	Sample	Ratio
Y	1,26				
B1	0,50*	B2	1,29	B3	1.14
G1	1,06*	G2	0.99*	G3	0,35*
R1	0,91*	R2	0,80*	R3	1,56*
D1	0,84*	D2	1,01*	D3	1,65*
* Significance *(P < 0.05)* Y= (Y1+Y2+Y3)/3					

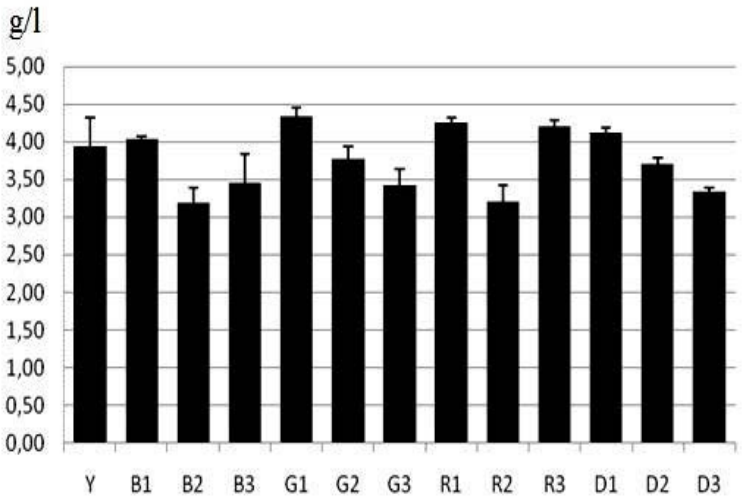

Figure 2 : Variations in proteins content in responce to changes of color (gm /l)

c) Mortality ratio

Mortality was highest in R2, D2 and D3 wile no mortality was observed in R1 or in D1. Removing the darkness resulted in higher rate of mortality in fish even though the removal of all treatments color was made gradually over 3 days. The least effect of color change was observed in the aquaria with blue treatment where no change occurred after removing the color, Also, less mortality was observed when the red color was removed (Figure 3 and Table IV).

Table IV : Variations in mortality ratio in responce to change of color

7 days treatment		30 days treatment		7 days recovery	
Sample	Ratio	Sample	Ratio	Sample	Ratio
Y	3.75				
B1	1.25	B2	2.5	B3	2.5
G1	1.25	G2	3.75	G3	1.25
R1	0	R2	16.3	R3	3.75
D1	0	D2	20	D3	23.8
* Significance (*P* < 0.05) Y= (Y1+Y2+Y3)/3					

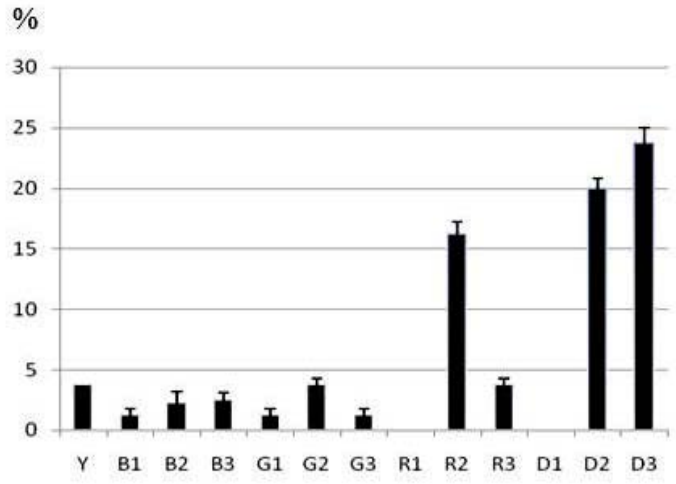

Figure 3 : Variations in mortality ratio in responce to changes of color

d) Serum protein fractions

Examining the protein separation gel revealed the induced expression of HSP90 proteins (95.4 kDa) and HSP70 protein (71.45 kDa) as shown in Figures 4 and 5.

HSP90 and 70 proteins were induced in fish in groups G1 and R1 during the first 7 days of exposure. The highest intensity HSP90 and HSP70 protein were found in R1 unlike in B1 and G1 where these proteins were lowest in detected intensity. The 37 days exposure showed induction of HSP90 and 70 proteins in R2 and D2, while they were not shown B2, G2, or the control Y. However, the intensity of the stress proteins shown in R2 was less than that found in the R1. On the other hand, the stress proteins shown in D2 were higher than the ones found in D1.

The removing of the colorful light resulted in highly increasing HSP90 in D3, and no other proteins were found. Unlike the HSP90, the HSP70 were induced when the colors were slightly removed in B3 and D3. This induction may be explained as a reflection of the stress caused by exposure to a different color after adaptation to blue color for 30 days, the same applies to inducing stress by exposure to light after 30 days of darkness in fish in group D3.

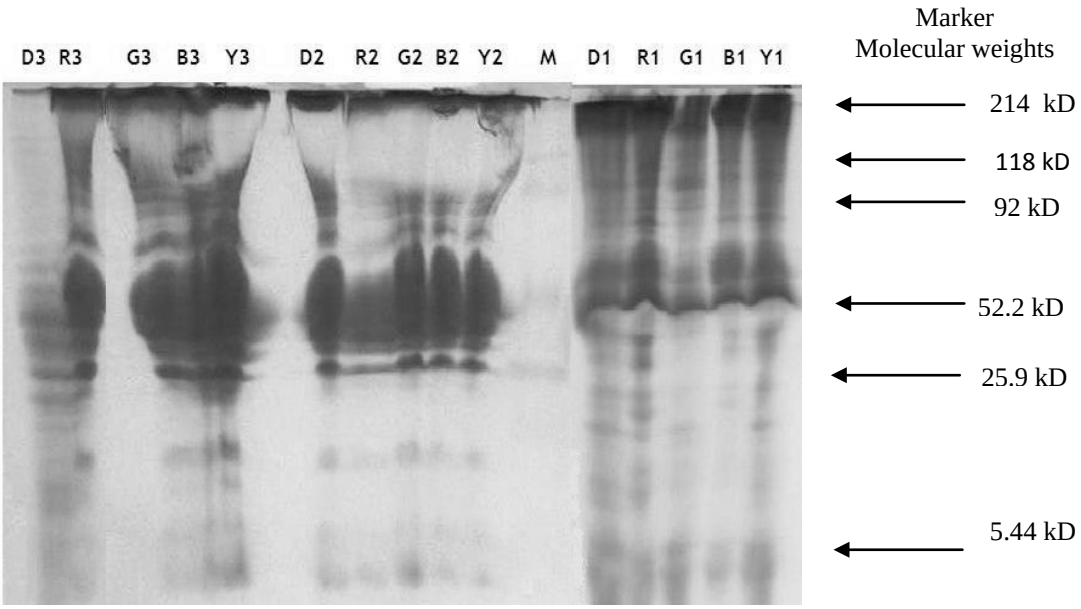

Figure 4 : Different protein fractions in SDS/PAGE responding to changes of color

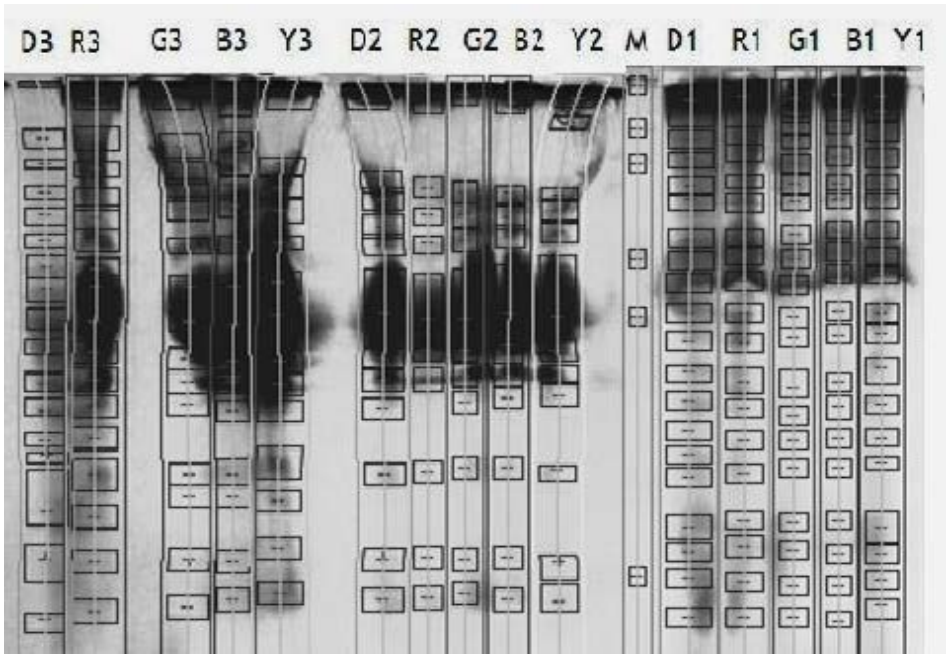

Figure 5 : Analyzed SDS/PAGE gel

M.W	Y	B1	G1	R1	D1	B2	G2	R2	D2	B3	G3	R3	D3
95,4		38466	46620	56832	52472			30800	69384				55562
71,45		28062	22120	48864	44800			23040	52052	48204			43524

IV. Discussion

a) Behavioral Response and Clinical Signs

The results show that color has significant effect on the tested behavioral and biological parameters. This is similar to results reported by Volpato *et al.* (2004) who indicated that color may affect different biological systems. Fish demonstrated a preference for blue and green color as they represent most closely their natural habitat (Levine & MacNichol, 1982) this explains why they were notably less aggressive in the blue color groups. The aggressiveness observed in red adapted fish was not observed when the color was removed, indicating that the aggressiveness behavior against each other was mostly a reflection of the stress induced by red color. In a similar experiment by Staffan (2004), the fish moved freely in tanks with green and blue background, but they did not seem to show similar preference to other colors.

On the other hand, the darkness adapted fish D2 showed less activity this is mostly due to the absence of clear visibility. They were also pale in color compared to the fish from the other groups. The same observations were made by Mairesse *et al.* (2005). In addition, in an experiment on seahorses, Lin *et al.* (2009) demonstrated that different colors and intensity can impact on the survival and skin color change rates of the juvenile seahorses. There by affecting their market. Also, the removal of the dark effect induced stress, and resulted in darker skin formation mostly due to the sympathetic nervous system disturbance, which is probably affected by the light. Color was most likely affected and regulated by a melanophore stimulating hormone (MSH) when the fish was stressed (Van der *et al.*, 2005). Similar results were obtained by Strand *et al.* (2007), who noticed a clear difference in tilapia fish body color kept in black and yellow tanks.

AChE activity was found to be significantly changed in the long term exposure in all treatments. This is mostly caused by the stress effect of light on the activity of AChE, which affects acytelcholine mediated, or cholinergic, neurotransmission (Beauvais *et al.* (2000). AChE is also reported to be affected by environmental changes like water temperature, salinity, water pollution (Elnwishy *et al.,* 2007), as well as being a good and early indicator to pesticide contamination (Chitman *et al.*, 2008). However, blue color exposure resulted in the least significant change in the enzyme, whose activity was reduced in the treatment. This is mostly explained by Volpato and Barreto (2001), who reported that blue light was an effective inhibitor of the stress-induced cortisol response in the Nile tilapia. However the results suggest that blue light may prevent the increase of stress-induced AChE in the Nile tilapia, while darkness and red color may increase stress-induced AChE. The highest AChE activity observed in R1 and D1 may indicate a high state of stress caused by red color and darkness.

Total protein content (TP) provides some information regarding a general status of organs

systems (Kenneth *et al*., 1990). In this research, TP was affected by short term exposure to all colors but not in the control. There was significant decrease in TP in fish exposed to green, dark and red colors for long treatment. This reduction of TP can probably be explained by an intensive use of muscle protein caused by the stress to which fish was exposed in attempt to obtain higher energy than that obtained from carbohydrates, which led to the reduction of total protein content. Also, the significant decrease in total serum proteins and albumin level could be as a result of the increase in protein catabolism (Lebelo *et al*., 2001). It could also be as a result of the fish losing feeding appetite, causing a decrease in energy sources needed for maintaining vital functions, and for combating the stressful conditions, this leads to the metabolism of blood protein as a compensating source of energy (Sabri *et al.,* 2009).

Albumin/Globulin ratio was significantly affected by red and dark color. In fact, the A : G ratio is an index used to track relative changes in the composition of serum or plasma (Mazeoud *et al*.. 1977) where Albumin carries substances through the blood and is important for tissue growth, while Globulins, including alpha, beta, and gamma types, helps in transporting metals in the blood and help protecting the body infection (Svobodová, 1991). Normally, there is a little more albumin than globulin and the ratio is greater than 1. A ratio less than 1 or much greater than 1 can give clues about problems (Kenneth et *al.,* 1990). These values may vary according to the individual laboratory. Thus, the changes in ratio in red adapted fish G2, and R2 and D2 may indicate disorders in fish internal physiology in response to the color effect. Removing the colors still caused disorder indicated by changes in the ratio in G3, R3, D3 and. The low ratio in B3 and G3 could be attributed to the increase of γ-globulin as a specific humoral immune response of fish (Woo, 1996) against color stress effect. Furthermore, due to the highly significant increase in globulin level and the significant decrease in albumin level (Ismail, 2003).

Serum protein fractions: Heat shock protein (Hsp) families have been shown to play critical roles in the stress response of aquatic organisms. These proteins may play a great role in protecting cells when exposed to different kinds of stress. They have broad cytoprotective properties, which are constitutively expressed in cells to maintain a number of critical cellular processes relating to protein folding, and translocation (Iwama *et al*., 1998).

The major classes of HSPs induced in cells in response to stress are the HSP90 and HSP70 families (Zhao & Houry, 2005). HSP90 contributes to various cellular processes including signal transduction, morphological evolution, folding newly synthesized proteins, and the stabilization and refolding of proteins denatured due to stress (Wegele *et al.,* 2004). HSP90 works as a chaperone protein, which helps with the proper folding and assembly of other structural proteins (Carrello *et al.,* 1999; Halliwell & Gutteridge, 1999). As a chaperone HSP 90 also helps move proteins from one place in the cell to another place where they can be more useful. HSP90 is also found to be present in unstressed cells and accounts for 1-2% of the measurable cytosolic proteins (Pratt, 1997). However increases in their production are noticed when stress is induced.

Under various stress conditions, the synthesis of stress-inducible HSP70 enhances the ability of stressed cells to cope with increased concentrations of unfolded and/or denatured proteins. Moreover, HSP70 can effectively inhibit cellular death processes, apoptosis or necrosis, and thereby increase the survival of cells exposed to a wide range of lethal stimuli (Jaattela, 1999). The multiple reactions and chaperone activities help with stressed cells as well as normal cells.

The induction of stress proteins in all fish groups within the first 7 days could be attributed to a different surrounding color. Later, when fish was adapted to the colors in the long term exposure, the stress proteins were reduced in two groups R2 and D2 while it was totally removed in B2 and G2. The intensity of the stress proteins shown in R2 was found less than found in R1. This indicates that even though the red color was still stressful, its effect was slightly reduced by adaptation with time factor. On the other hand, the stress proteins shown in D2 were higher than in D1 reflecting that the effect of darkness may be less adaptable with time factor. The removing of the Blue light resulted in induction of HSP70 in B3 which may be a result of a stress effect on fish caused by removing the color after long adaptation to blue light. Similar adaptation may have happened in the group exposed to darkness, and removing the darkness effect may have resulted in the high induction of stress proteins in D3 even though the color was removed gradually in 3 days.

The overall results of the protein fraction analysis indicate that blue exposure may have the least stressful impact on fish in both the short and long exposure period.

The induction in HSP90s and 70s mostly resulted from exposure to the stress factor of light color for 30 days. However, unstable temperature, hypersomatic pressure, and changing salinity may all lead to the induction of HSP stress proteins synthesis in fish (Takeuchi *et al*., 2000). It is reported also that surrounding water pollution (Elnwishy & Sabri, 2009) and acid or alkali treatment can have the same effect (Kim *et al.,* 2003).

The induction of HSP70 proteins was probably caused by the bound FATP (fatty acid transport proteins) in the HSP70, which freely associates with nascent or misfolded peptides (open lid), causing a conformational change that activates inherent HSP70 ATPase activity. However, these results may indicate potential dysfunctions in protein folding, translocation

of proteins across organellar membranes, and/or disassembly of protein complexes (Bukau, 2006; Craig *et al.,* 2006). They may also point out to a potential possibility of promoting mitosis of dividing cells and suppressing the reactivity of the immune system cells (Browne *et al.,* 2007). However, HSP response may vary in accordance with a variety of factors related to tissue (Rabergh *et al*., 2000), stressor (Airaksinen *et al*., 2003), species (Basu *et al*., 2001; Nakano & Iwama, 2002) and the developmental stage (Martin *et al.,* 2001).

In general, blue light color was found to be the most comfortable color for fish behaviors and growth followed by green light. Results obtained by Volpato *et al.* (2004) suggested that color may affect different biological systems. However, other colors have been shown to improve fish welfare in other species. These include green and blue in *Sardinops caerulea* (Head & Malison, 2000), *Oplegnathusfasciatus*, *Monocanthus cirrhifer*, *Cybium niphonium*, *Spheroides niphobles* and *Sphyraena japonica* in (Tamazouzt *et al*., 2000), and green in *Brycon cephalus* (Papoutsoglou *et al.,* 2000).

Light intensity and light color or colored background has been reported to affect several processes in fish (Stefansson *et al*., 1990 and 1993). However, Volpato and Barreto (2001) suggested that blue and green colors with the similar intensity had strongly different effect on biological processes in tilapia.

These results indicate that culture management should aim to optimize the farming environment to maximize the growth and welfare of fish. Like the light intensity (Volpato & Barreto, 2001), rearing density (Haukenes & Barton, 2004), and feeding schedule (Davis, 2006), which are factors that could potentially alleviate stress levels for cultured fish proper management, and proper practices to improve feed intake in order to maximize growth rates. These experimental units were indeed smaller than normal fish ponds used for aquaculture. It is expected that the application of the results of this laboratory study could be extrapolated to large scale aquaculture fish production. These results further show that with proper management, a blue color light at proper intensity could result in higher feeding and growth rates for aquarium-grown fish.

V. Conclusion

The results show that blue color lightening or background in aquaculture techniques will maximize the growth rates of juvenile tilapia, reduce their stressful behaviors, and minimize their mortality to the lowest rate. This application will improve the culture performance of tilapia, and increase the productivity of fish in the aquaculture industry. On the contrary, exposing fish to red color and darkness will worsen the growth conditions and fish productivity and should be minimized or completely avoided in order to increase aquaculture fish output.

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Colonization Abilities of Microflora to Attach Aquatic Plants

By A.H. Aboellil & Amany H. Aboellil
Universe College, Makkah

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Keywords : *Hydrophytes, epiphytic algae, fungal flora, enzymatic activities.*

GJSFR-C Classification: *FOR Code: 060703*

Strictly as per the compliance and regulations of :

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A.H. Aboellil [α] & Amany H. Aboellil [σ]

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Keywards : Hydrophytes, epiphytic algae, fungal flora, enzymatic activities.

I. Introduction

The aquatic system is a complex and dynamic ecosystem composed of water, plants, soil bed and microflora, etc, in general this microflora colonizes the phylloplane, phyllosphere and rhizosphere of macrohydrophytes. It is may provides dynamic micro environment where bacteria and fungi in association to algal flora with plants detoxify hazardous organic compounds (Walton *et al.*, 1994). Microorganisms possess specific biochemical traits that account for their existence and prevalence in certain environment (Alexander, 1971).

Submerged and floating aquatic plants have different architecture offering different opportunities for epiphytes (Cattaneo, 1998 and Aboellil, 2006 and 2011). Fenchel and Jorgensen (1977) stated that, microorganisms have long been recognized important link between primary and secondary production in detritus-based food webs in aquatic environment. Manyinvestigators studies terrestrial fungal flora inhabitant macrohydrophytes like Gauer*et al.* (1992) and Galabraith (1986) which studied the relative contributions of fungi to water hyacinth decomposition while others like El-Morsy*et al.* (2000) study the occurrence of microfungi of some selected macrophytes from Nile delta of Egypt, Aboellil (2003) surveyed fungal and algal microflorainhabitant different types of hydrophytes in fresh water systems at middle Egypt belt. As far as, we know no one had been classified the fungal flora inhabitant macrohydrophytes according to their degree of attachment to the plant. On the other hand, Burkholder *et al.* (1990) and Cattaneo and Kalff (1978) classify the algal flora inhabitant hydrophytes to loosely attached and tightly attached ones, according to their degrees of attachment.

Kuehn and koehn (1988) stated terrestrial fungi in particular may be enzymatically better adapted than aquatic fungi in the initial colonization and utilization of submerged organic materials. Moreover, fungi may be enhancing or weakening the activity of allelopathic components (Gunnison and Barko, 1989). They possess a powerful system for penetrating the root as endophytes (El-Morsy, 1999b). Moreover cutin, pectin, cellulose is the short difficult structural obstacles to fungal invasion El-Morsy (1999).

Plant/microbial community feed back can have important consequences for species composition of both the plant and microbial communities (Gustafson and Casper, 2004).

The main objectives of the paper was to evaluate and classify the fungal and algal flora which inhabitants different categories of hydrophytes at middle Egypt belt according to their degree of attachment to plants and examine the enzymatic activities of some isolates, that's may help to colonize the hydrophytes surfaces.

II. Materials of Methods

The studied area lies at Mid Egypt (Lat. 30-25 N) selected macrohydrophytes and water samples were collected from different water courses at great Cairo and Beni-Swif districts and representing available different types of eater course (Nile branch, channels, drains and pools). After preliminary survey 4 different hydrophytes were chosen to represent as far as the most abundant hydrophytes, and also monitoring the different categories of hydrophytes, for example *Eichhonniacressipes* (Floating), *Ceratophyllumde - mersum* (Submersed), *Echirochloa Stagninum* (emmersed) and *Azolla* (Ferns).

Isolation of fungus: the studied plants were collected aseptically and transported to lab. Three different technique were used to isolated the fungal flora according to their degree of attachment, 1st one washing

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the sample and dilution technique using 1 g as disks of plants (Loosely attached flora), 2nd disks were punched from the washing sample using sterilized cork borer then planted on nutrient agar (Czapek's-Dox) (moderately attached flora) and finally maceration used to recognized the firmly attached flora (Dickinson, 1967; Salama *et al*., 1986).

Isolation, fixation and identification of algal flora from different chosen hydrophytes per formal according to Prescott (1978).

Physicochemical characteristics of water at studied site were evaluated according to standard methods and recent instruments.

Enzyme activities of fungal flora isolated from different hydrophytes (pectinase, cellulose, lipase and amylase) detected by method described by Sall (1967), Hankin *et al*. (1971), Elwan *et al*. (1977) and whistler *et al*. (1984).

III. Results and Discussion

Regarding to physical and chemical parameters average at studied sites reveals, the transparency of water was ranged between 35-68 cm by using secchi disk. DO_2 of Nile water was reworded the highest values with (5-6mg) comparing to drains which recorded the lowest values (2.1-3.8 mg) pH varied between 7.8-8.6 , while T.S.S. showed the lowest amount at River Nile (280-315 mg) and the highest amount at drain site (725-812 mg) (Table 1).

Fungal flora inhabiting floating hydrophytes *Eichhorniacrassipes* as shown from Table (1) were mainly belonging to 8 genera and about 13 species with total count (70colonies/g). Only *Asperigillus* spp. contributed about 47% of total count of isolated fungi, with high occurrence. Then *Alternariaalternate* and *Paecilomyces* sp. are represented about 14.29% of totalcounts for each with low occurrence. While *Cladosporiumherbarum* and *Fusarium* spp. showed about 10 and 7.14% of total count, respectively. *Trichoderma*, *Phomahemicola* and *Rhizopusnigricans* contributed 2.86, 2.86 and 1.43% of total counts, respectively.

Regarding to attachment degree of isolated flora, *A.alternata*, *C. herbarum*, *Fusarium* spp. and *P. humicola* were isolated after maceration process only so were com concluded that its belonging to firmly attached ones. While *Paecilomyces* sp. and *T. viride* restricted as moderately attached ones, isolated only at immersed stage. On the other hand, *Aspergillus* spp. generally may consider as biphase fungi because it was isolated as loosely and moderately attached fungi to *Eichhorniacrassipes.*

From table 2, fungi isolated from submersed hydrophyte *Ceratophyllumdemersum* were comprised about 16 species with total counts (60 colonies/g) varied between *Asperigillus* species contributed higher than 50% of total count with high occurrence, considered as loosely to moderately attached fungi. *Alternariaalternata*, with low occurrence, represented 18.42% of total count monitoring again as firmly attached fungi as well as in case of *Eichhorniacrassipes*. Also *Fusarium* spp. contributed about 11.67% of total count, showed the same trend. On the other hand, *Curvularia*, *Cladosporium* and *Ulocladim* are of the moderate rank showed 10, 5 and 5% of total count, respectively and partially appeared firmly attached isolated.

Helminthosporium and *P. humicola* were isolated once with low occurrence and considered moderately attached fungus.

Table (3) showed the range of occurance, total count and types of fungi isolated from *Echinochloastagninum*. Instead of the low occurrence of *Alternaria*, it appeared to be most abundant species isolated from *Echinochloa*, it is represent 30% of total count and also recorded as firmly attached fungi while *Fusarium* spp. Contributed 16% of total count and considered as firmly attached fungi. *Asperigullus* here showed loosely attached ones and represented about 28% of total count with moderate occurrence. *Sepedonumchrysosporum* also recorded as moderate attached fungi isolated from the plant.

Helminthosorium and *P. humicola* were isolated once with low occurrence and considered moderately attached fungi.

Regarding to fungal flora isolated from *Azolla* fern, Table (4) confirmed that, again *Alternariaalternata* representing 4% of isolated fungal total count appears to be firmly attached fungi. *Trichoderma viride, Mucor* spp. contributed about 2.7% of total count and considered as moderately attached. The total count of fungi isolated from *Azolla* (84 colonies/g) comprise 12 species. *Aspergillus* spp. contributed 88.1% of total count and appears at 3 types (loosely, moderately and finely attached ones) by different ratio sloped to moderate one.

Difference of fungal flora may be due to the nature of leaching substances of different plants which considered as the media for growing the specific flora. Leaching substances are sugar, amino acids growth substances…etc have important consequences in microbial diversity (Gustafson and Casper, 2004).

From tables (2-5) we can concluded that *A.alternata* recorded only as firmly attached fungi for different investigated hydrophytes, while *Aspergillus* spp., instead of its recorded the most ascendant species its seems to be loosely to moderate attached fungi-*Fusarium* which isolated only from *Ceratophylum* and *Echinochloa* also showed to be finely attached fungi which isolated only by maceration. Newshan *et al*. (1995), were found *Fusarium* spp. implicated in the suppression of *Fusarium* wilt diseases and effecting the plant performance.Generally the above mentioned data came in harmony with El-Morsy *et al*. (2000) which stated that, fungi isolated from selected aquatic macrophysics at Nile Delta of Egypt were typical of terrestrial origin. The majority of isolated species were recorded from the rhizosphere of aquatic macrophytes isolated by Motta (1978) and Gunnison and Barko (1989).

On the other hand,Table (6) showed the enzymatic activities of isolated fungi, may explain to what extent the efficiency of these fungi to attached to hydrophytes depending upon their highly efficiency for decompose pectin. Cellulase, lipid and starch.

As shown from table (6) most isolates showed high to moderate efficiency to secrets pectinase, cellulose, lipase and amylase except some rare cases such *Mucor* which had lacking in secretion of cellulase and lipase, while, *F. moniliforme*, *A. carbanrous* and *A. alternata* showed no activities of lipase.

The best performance of enzymatic activities observed from *P.notatum*, *A. terreus*, *R. nigricans* and *Cladosporumherbarum*, on contrary the less performance showed in *Mucor*, *A. fumigatus* and *A. alternata*

While the moderate performance appeared in cases of *A. flavus*, *A. niger* and *F. moniliforme.*

In general the results obtained from detected the remarkable enzymatic activities of isolated fungi come in harmony with Kuehn and Koehn (1988) stated terrestrial fungi may be enzymaticaly better adapted than aquatic fungi in the initial colonization and utilization of submerged organic materials. Also with El-Morsy*et al*. (2000) which emphasized that the presence active fungal flora in the rhizosphere of aquatic macrophytes indicates their significant role in degradation of organic compounds they may be produced enzymes.

From the previous results, there is no clear relation between the degree of attachment of organisms to plants and their enzymatic potentiality. According to data, it is suggestion that the attachment degrees are due to the capability of the fungus to cause diseases to plants like *Alternaria*, *Fusarium*, *Curvularia*and *Ulocladium*. Therefore, it may be more firmly attached to plant surfaces, but the saprophytic fungi like *Aspergillus*, *Mucor*, *Trichoderma*, *Rhizopus* are more or less loosely or moderately attached to plants.

The fungus perhaps has a high enzymatic system but can not caused diseases. Correlation of hydrophytes genera with microbial inhabitants influenced the enzymatic profiles of microbial communities from these plants.

On the other hand, the common constituents of aquatic systems, epiphyticalgae, which isolated from different chosen hydrophytes included cyanobacteria, chlorophyta and Bacillariophyta. Obtained data revealed that, the loosely attached algae included majority of cyanobactera such as *Oscellatoria* spp., *Lungbya*spp., *Microcystis*spp. and *Anabaena*spp.,in addition to chlorophyta membranes *Pediastrum*spp.,*Scenedesmus* spp.,*Quadrigula* spp., *Botryococcus* and *Cladophora* spp. (Table 7).

While*Coleochaete* and *Melosira*, *Fragilaria*, *Nitzschia*, *Synedra* and *Rhizosolenia* from Bacillariophyta showed as moderately attached ones.

Firmly attached alga includes *Navicula*, *Cymbella*, *Cocconies* represented nonmotilepennates diatoms. Theabove mentioned data come in harmony with Zimba and Hopson (1997) stated, it is not surprising that significantly different removal efficiencies of epiphytes were obtained for the sampling types analyzed (mechanical agitation). Also with Cattaneo and Kalff (1978) and Burkholder *et al*. (1990) identified two distinct components of the epiphytic flora loosely and eighthly attached or adnate component. Goldsborough and Hickman (1991) revealed the importance of efficient removal of epiphyton from host tissues to assess the importance of macrophytes and epiphytes in ecological studies.

In general the data obtained from isolated epiphytic algae from different studied hydrophytes showed that, the main mean of loosely attached alga representing about 56% of algal taxa, while moderately attached ones harvested about 28% of total algal taxa most of them belonging to Bacillariorphyta, while firmly attached ones representing about 16% of total harvest and completely belongs to Bacillariophyta especially nonmotilepennatesdiatoams.

Algal communities collected from under four co-occurring hydrophytes depended on the medium (nutrient), temperature, transparency of water and pH. It can be hypothesizyed that some isolates were present as dormant structures while some others could be in growing forms.

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Table 1 : Physical andchemical parameters average at studied sites

Parameters	Average
Transparency of water	34-68 cm
Demand O_2 (DO_2)	5-6 mgl^{-1}
Low Drain values High	2.1-3.8 mgl^{-1} 725-812 mgl^{-1}
pH	7.8-8.6
Total soluble salts (TSS)	280-315 mg l^{-1}

Table 2 : Fungal flora inhabitant *Eichhornia crassipes* and their occurrence range regarding to its attachment degree to hydrophytes

Organimm	Phylloplane (colonies/g)			Total count (colonies/g)	Total count (%)	No of cases of isolation	Rank of Occurrence
	Washing	Immersion	Maceration				
Alternaria alternata	-	-	10	10	14.29	1	L
Aspergillus sp.	16	12	5	33	47.15	3	H
Aspergillus nodulans	-	1	-	1	1.43	1	L
A. flavus	4	2	-	6	8.57	2	M
A. carbonarius	10	-	-	10	14.29	1	L
A. sydowi	-	1	-	1	1.43	1	L
A. terreus	-	1	-	1	1.43	1	L
A. niger	2	7	5	14	20	3	H
Cladosdorum herbarum	-	-	7	7	10	1	L
Furarium sp.			5	5	7.14	1	L
F. moniliforme			3	3	4.29	1	L
F. oxysporum			2	2	2.86	1	L
Phoma humicola			2	2	2.86	1	L
Rhizopus nigricans		1		1	1.43	1	L
Paecilomyces spp.		10		10	14.29	1	L
Trichoderma viride	2			2	2.86	1	L
Total count	18	23	29	70	100	17	

Rank of occurrence H, High = 3 cases, M, Moderate = 2 cases, L, Low = 1 cases

Table 3 : Fungal flora inhabitant *Ceratophyllum demeritam* and their occurrence range regarding to its attachement degree to hydrophytes

Organimm	Phylloplane			Total count (colonies/g)	Total count (%)	No of cases of isolation	Rank of Occurrence
	Washing	Immersion	Maceration				
Alternaria alternata			7	7	11.67	1	L
Aspergillus spp.	20	9	2	3	51.67	1	H
Aspergillus nodulans	1			1	1.67	1	L
A. flavus	5	3		8	13.33	2	M
A. carbonarius	2			2	33.3	1	L
A. sydowi		1		1	1.67	1	L
A. wentii		1		1	1.67	1	L
A. terreus		1		1	1.67	1	L
A. niger	12	3	2	17	38.3	2	H
Cladosporum herbarum		1	2	3	5.0	2	M
Furarium			7	7	11.67	1	
F. moniliforme			3	3	5.0	1	L
F. oxysporum			4	4	6.67	1	L
Phoma humicola			1	1	1.67	1	L
Rhizopus nigricans		1		1	1.67		L
Helminthosporium sp.			1	1	1.67		L
Ulocoldium sp.		1	2	3	5.0	2	M
Curvularia sp.		1	5	6	10.0	1	M
Total count	20	13	27	60	100	18	

Table 4 : Fungal flora inhabitant *Echinochloa stagninum* and their occurrence range regarding to its attachment degree to hydrophytes

Organimm	Phylloplane (colonies/g)			Total count (colonies/g)	Total count (%)	No of cases of isolation	Rank of Occurrence
	Washing	Immersion	Maceration				
Alternaria alternata			15	15	30	1	L
Aspergillus sp.	12	2		14	28	2	M
Aspergillus nodulans	2			2	4	1	L
A. flavus	3	2		5	10	2	M
A. carbonarius	1			1	2	1	L
A. terreus	1			1	2	1	M
A. niger	1			1	2	1	L
A. wentii	1			1	2	1	L
Cladosdorum herbarum	2		5	7	14	2	M
Furarium sp.			8	8	16	1	L
F. moniliforme			3	3	6	1	L
F. oxysporum			5	5	10	1	L
Rhizopus nigricans		2		2	4	1	L
Sepedonium chrysosporum		2		2	4	1	L
Total count	16	6	28	50	100	16	

Table 5 : Fungal flora inhabitant *Azolla* fern and their occurrence range regarding to its attachment degree to hydrophytes

Organimm	Phylloplane (colonies/g)			Total count (colonies/g)	Total count (%)	No of cases of isolation	Rank of Occurrence
	Washing	Immersion	Maceration				
Alternaria alternata			3	3	3.6	1	L
Aspergillus sp.	28	31	15	74	88.1		H
Aspergillus nodulans		1		1	1.2	1	L
A. flavus	7	20	3	30	35.7	3	H
A. carbonarius	2			2	2.4	1	L
A. sydowi			10	10	12.0	1	L
A. terreus	12	2		14	16.7	2	M
A. niger	7	2	2	11	18.0	2	H
A. ustus		5		5	6.0	1	L
Cladosdorum herbarum		2		2	2.4	2	M
Furarium sp.							L
F. moniliforme							L
F. oxysporum							L
Rhizopus nigricans	1			1	1.2	1	L
Trichoderma viride		3		3	3.6	1	L
Total count	29	37	18	84	100		

Table 6 : Enzymatic activities of fungal isolates from different hydrophytes

Isolate	Pectinase	Cellulase	Lipase	Amylase
Alternria alternata	1+	3+	-	2+
Aspergillus carbonareus	3+	2+	-	3+
A. flavus	4+	2+	2+	4+
A. fumigatus	2+	2+	2+	4+
A. niger	2+	3+	-	2+
Cladospolum herbarum	4+	2+	2+	4+
Fusarium moniliforme	4+	2+	-	3+
Fusaruim oxysporium	-	3+	1+	2+
Helminthosporium sp.	4+	3+	2+	4+
Rhizopus sp.	2+	-	-	3+
Rhizopus nigricans	3+	4+	2+	3+
Sepedonum chrysosporum	1+	1+	4+	2+
Trichoderma viride	3+	1+	±	3+
Ulocladium sp.	4+	2+	3+	4+

Key of table used:

- *= No activity, ± = Slightly activity, 1+ = 1.1 – 1.4 cm in diameter, 2+ = 1.5-2.0 cm, 3+ = 2.1 – 2.5 cm and 4+ = 2.6 – 3 (≥ 2.6 cm)*

Note: Cups were made (4 cups optimal) in each solidified plate using sterile cork-borer (1 cm in diameter).

Table 7 : Isolated epiphytic algae from different studied hydrophytes as their attachment degree

Firmly attached algae	Moderately attached algae	Loosely attached algae
Nivicula spp.	*Coleochaete* spp.	*Oscellatoria* spp.
Cymbella spp.	*Melosira* spp.	*Lungbya* spp.
Cocconies spp.	*Fragilaria* spp.	*Microcystis* spp.
	Nitzchia spp.	*Anabaena* spp.
	Synedra spp.	*Pediastrum* spp.
	Rhizosolenia spp.	*Scenedesmus* spp.
		Guadrigula spp.
		Botryococcus spp.
		Cladophora spp.

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Evaluation of Efficacy of Formulations of Plant Prosopis Juliflora against Callosobruchus Chinensis Linn

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Abstract - An investigation was conducted to evaluate the efficacy of extracts obtained from different parts (bark, leaf and fruit) of P.julifora against C.chinensis. A significant mortality of the insect, up to 66.67%, was obtained in some cases. The adult pests were also subjected to smoke of the different plant parts for assessing results in terms of adult mortality. Here also fruit and leaf formulations were able to cause 70% mortality. Similar encouraging results were found when different plant parts were used as egg laying deterrents (reduced to 6 no. /pair as against a normal of 42.33 no. / pair). Some treatments were capable of lengthening the period of insect development by as much as 10 days. Significant results were obtained for managing adult emergence from host grains were in best case scenario it dropped from 93% to 56%.

Keywords : *Biopesticides, C.chinensis, P.julifora, adult mortality, egg laying, rate of development, adult emergence, plant extracts.*

GJSFR-C Classification: *FOR Code: 060703, 060704*

Strictly as per the compliance and regulations of :

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Shailja Rawat[α] & Meera Srivastava[σ]

Abstract - An investigation was conducted to evaluate the efficacy of extracts obtained from different parts (bark, leaf and fruit) of *P.julifora* against *C.chinensis.* A significant mortality of the insect, up to 66.67%, was obtained in some cases. The adult pests were also subjected to smoke of the different plant parts for assessing results in terms of adult mortality. Here also fruit and leaf formulations were able to cause 70% mortality. Similar encouraging results were found when different plant parts were used as egg laying deterrents (reduced to 6 no. /pair as against a normal of 42.33 no. / pair). Some treatments were capable of lengthening the period of insect development by as much as 10 days. Significant results were obtained for managing adult emergence from host grains were in best case scenario it dropped from 93% to 56%.

Keywords : Biopesticides, C.chinensis, P.julifora, adult mortality, egg laying, rate of development, adult emergence, plant extracts.

I. Introduction

In recent years there has been a growing concern towards the environmental hazards such as toxicity, erosion of beneficial natural enemies and pest resurgence caused by synthetic pesticides. Use of plant bioproducts became an alternative, protecting nature from pesticidal pollution (Prakash et al., 1989, Tiwari et al., 1990). The efforts have been applauded by all, and the efficacy of botanicals has been found against stored grain pests (Rao et al., 1990, Prakash et al., 1990). They are broad spectrum in pest control, safe to apply, unique in action, and can be easily processed and used. A number of plants have been identified for their pesticidal activities. Plants contain a large number of secondary metabolites and those categorized under terpenoids, alkaloids, glycosides, phenols, tannins etc. play a major role in plant defense and cause behavioural and physiological effects on insects. Over the past 50 years, more than 2000 plant species belonging to different families and genera have been reported to contain toxic principles (Solanki & Shanker, 2001). Therefore a study was planned for assessing the efficacy of plant *Prosopis juliflora* on the various life-cycle aspects of *Callosobruchus chinensis*.

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II. Material and Methods

A pure line culture of *C. chinensis* reared on grains of *Phaseolus mungo* was maintained in a BOD incubator maintained at 28 ± 2^{0C} temperature and 70% relative humidity. The test plant was collected from in and around Bikaner city for the study carried out during the period of 2005-2007. The formulations of bark, leaf and fruit of *P.juliflora* were used against the pest. The powdered plant parts were used in three forms viz. liquid extracts, powder suspension, and in the form of smoke by incineration.

a) Liquid formulations

The liquid extract of the plant parts were made in two media, inorganic (water) and organic (petroleum ether).

i. *Aqueous extract*

1g of powdered plant material was kept in a thimble. The thimble was placed in a flask containing 50 ml of distilled water and boiled till the volume reduced to 10 ml. Thus 10 percent concentration was obtained. Further dilutions were made by adding required amount of distilled water for getting lower concentrations viz. 5, 2.5 and 1 percent.

ii. *Ether extract*

1g of dried and powdered plant material was taken in a thimble. It was placed in a soxhlet extraction unit with petroleum ether. It was distilled in the unit. The extract so obtained was made to a fixed volume of 10 ml having concentration of 10 percent. This was used as stock solution. Further dilutions were made to have 5, 2.5 and 1 percent concentration from the stock solution.

b) Powder suspension

The powdered plant parts were weighed to get required concentration of 10, 5, 2.5 and 1 percent and suspension was prepared by adding distilled water.

c) Smoke treatment

The powdered plant material weighed as 10 g was placed in an incineration flask from which a tube led to fumigation chamber measuring 10 liters by volume. The contents of flask were heated causing incineration of the plant material producing smoke, which was allowed to fill the chamber for 10 minutes. 50 adult

insects were placed in a beaker with host grains. The beaker was covered with muslin cloth and placed in the lower chamber of fumigation chamber.

Thus four sets of experiments were laid out:

a. For powder suspension treatment
b. For aqueous extract treatment
c. For ether extract treatment
d. For smoke treatment.

For the first three treatments, 5 g of host grains were taken and treated with 1 ml of the specific extract. Five pairs of the test insects were released into each experimental set of different doses viz. 10, 5, 2.5 and 1 percent. For the study each experimental set was taken in three replicas .For the smoke treatment only one dose was applied.

III. Recording of Observations

The following aspects were studied:

a) Adult mortality (Percent)

The total number of adult insects surviving after the treatment was recorded for three days after infesting with insects. The percent mortality was then calculated.

Adult mortality by smoke treatment (Percent)

The smoke produced by different plant powders was used to fumigate the insects and mortality was noted after 24 and 48 hours of treatment.

b) Egg laying (No. /pair)

The egg laying or fecundity was calculated by counting the total number of eggs laid per pair of adult insects after three days of introduction of the adult into the treated sets.

c) Rate of development (days)

The time taken for the development of adult from the day of egg laying till emergence was recorded as rate of development. This was further averaged to get a single value.

d) Adult emergence (Percent)

The total number of adults which emerged was recorded and percent adult emergence was calculated as:

$$\text{Percent adult emergence} = \frac{E}{T} \times 100$$

Where,

E : Total no. of adults emerged
T : Total no. of eggs laid

The experiments were set in three replicas and were compared with control and normal, where control included grain treated with the particular solvent.

III. Results and Discussions

a) Adult mortality

The percent adult mortality in *C. chinensis* in the sets treated with formulations of *P. juliflora* ranged from 16.67 to 66.67%. The highest adult mortality of 66.67% of the test insect was observed in sets treated with 10% ether extract of leaf. The present findings are supported by earlier works of Mala & Solayappan (2001) who conducted experiments on certain plant extracts including *P. juliflora* against the early shoot borer *Chilo infuscatelus* and found that it caused significantly high mortality of 2nd and 3rd instar larvae after 24h. Significant antibacterial activity of aqueous extract of *P. juliflora* on phytopathogenic *Xanthomonas compestris* has earlier been reported by Satish et al. (1999). Rajappan et al. (2000) also studied the effect of leaf extract of *P. juliflora* and found it to be effective in reducing the population of green leaf hopper *Nephotettix virescens* in both the nursery and the main field.

b) Effect of smoke treatment

A significantly high mortality of more than 50% was also recorded in sets treated with 5 and 10% formulation of leaf. Highest bruchid mortality of 70% resulted after 48 h with treatment of smoke of fruit and leaf of *P. juliflora*, while the smoke of bark of the same plant resulted in lowest mortality of only 14%. The results are in conformation with earlier work of Ghei (2001) who observed that smoke of pods of *Tephrosia, Trigonella* and *Crotolaria* was effective in causing significantly high mortality (66.66 – 100%) of *C. chinensis* and Gupta (2004) who studied the efficacy of *S. nigrum*, *S. surattense* and *W. sominifera* and found that the fruit of *S. nigrum* resulted in maximum adult mortality (85%) of *C. chinensis* after 24 hours. More than 60% adult mortality was observed when the insects were treated with the smoke of leaves of *S. surattense,* root, stem and leaves of *S. nigrum* and root of *W. somnifera* after 48 hours.

c) Egg laying

The oviposition by the test insect in the normal sets was 42.33 eggs/pair. In control sets formulated with distilled water it was 41.22 while it was 40.33 with those formulated with ether. Minimum egg laying by *C. chinensis* (6 eggs/pair) was found in sets treated with 10% aqueous extract of bark, while 10% formulations of bark, fruit, leaf, 5% aqueous extract of bark and leaf and 5% aqueous suspension of fruit were found to moderately reduce egg laying to about 6 to 20 eggs/pair and these were significantly different from normal (42.33 eggs/pair). The results obtained during the present study are in conformation with the works of Kamakshi et al. (2000) who reported significant reduction in the number of eggs laid by *C. maculatus* when treated with *Mentha arvensis, Sesbania glandiflora* and *Ocimum sanctum* as compared to control. Tinzaara et al. (2006) tested the potential of certain botanicals on *Cosmopolites sordidus* and found that oviposition was significantly low on corms treated with *M. azedarach, Tagetes* spp. and *R. communis.*

d) Rate of development

The perusal of the results show that the mean rate of development of *C.chinensis* in normal sets was

26 days while in control sets treated with distilled water it was 27 days and in those treated with ether it was 28 days.

The maximum time taken for development (38.67 days) of *C.chinensis* was recorded from the sets treated with 10% formulation of bark. 5% formulations of bark and 5 and 10% ether extracts of leaf also delayed the development by 10 days. Similar results were obtained when the efficacy of different concentration of commercial neem based insecticide 'Nimbicidine' was evaluated against *T. castaneum* by Das et al. (2006) who observed significant reduction in growth with lengthened developmental period.

e) Adult emergence

During the present study, adult emergence in normal sets was recorded as 96%. Control sets formulated with distilled water showed 95% while those formulated with ether showed 93% emergence of the bruchid The percent adult emergence of the test insect in sets formulated with extracts of *P. juliflora* ranged from 56.81% (in 10% ether extract of bark) to 94.33% (in 1% aqueous suspension of fruit).

Sets treated with 5 and 10% aqueous extract and 10% aqueous suspension of bark was also found to have significant effect in controlling the bruchid emergence. Present findings are supported by earlier work by Mbaiguinam et al. (2006) where total emergence of adults of *C. maculatus* was found to be reduced significantly when treated with extracts of *A. indica*, *R. communis*, *T. nerifolia*, *Balanites eagyptiaca, Moringa oleifera* and *Kaya sanegalensis*. A significant reduction in progeny emergence of *S. zeamais* and *C. maculatus* was observed by Udo et al. (2004) when treated with formulations of *Z. xanthoxyloides.*

On the basis of results obtained and its comparison with normal and control it could be deduced that concentrated extracts of parts of *P.juliflora* can significantly control the infestation by *C.chinensis.* Smoking the warehouses with leaves and fruit of the plant can effectively reduce infestation by causing adult mortality. Concentrated aqueous extract of bark can be mixed with pulses as deterrents of egg laying.

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Study of Physico-Chemical Properties of Kasura Dam from Jalna District (M.S) India

By Pramod.P.Gaike & K.B. Shejule
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Abstract - The present study deals with the physical and chemical properties of the water of Kasura dam is located in Marathwada region (M.S) India. Determination of physico-chemical parameter (Atmospheric Temp, Water. Temp, Rain fal , **Ca ,Cl, CO_2, DO**) were carried out to indentify the nature and quality of the water of Kasura dam year (July 2008-June 2009). Kasura dam is situated 19 km away from South side of Partur city. It lies between **19^0.30'0''** North latitude, **76^0.15'50''** East longitude and altitude. The details of results are discussed in the text.

Keywords : *Physico chemical, Kasura, Marathwada, At.Temp, Wt.Temp.*

GJSFR-C Classification: *FOR Code: 060306*

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Study of Physico-Chemical Properties of Kasura Dam from Jalna District (M.S) India

Pramod.P.Gaike [α] & K.B. Shejule [σ]

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Keywords : Physico chemical, Kasura, Marathwada, At.Temp,Wt.Temp.

I. Introduction

Fresh water has become a scarce commodity due to over exploitation and pollution of water. Increasing population and its necessities have lead to the deterioration of surface and sub surface water .The limnology plays an important role in decision-making processes for problems like dam construction, pollution control, fish and aquaculture practices (Muley and Gaikwad, 1999).

Climatic factors such as rainfall, temperature, pressure and humidity etc play an important role in the geology as well as terrestrial environment. A sound knowledge of these factors help in understanding the complex processes of interaction between the climate and biological processes in water bodies. Temperature is basically important for its affects on certain chemical and biological activities in the organism attributing in aquatic media. The water temperature and air temperature were found to go more or less hand in hand. In Indian subcontinent the temperature in most of the water bodies ranges between 7.8 - 38.5^0C (Singhal et al., 1986). The total life of the world depends on water and hence the hydrological study is very much essential to understand the relationship between its different tropic levels and food webs. Lakes, dams and ponds are important fresh water habitats throughout many regions of the world, although the amount of water in them constitutes only a minute fraction of the total freshwater resource on earth (Christer and Lars-Anders, 2002). In the present study the Kasura dam from Jalna Disrict were chosen since the effluents from the Kasura and Dudhana rivers respectively receives large amount of fertilizers and domestic wastes are directly released into the dam.

II. Materials and Method

In one year study of Kasura dam (July 2008-June 2009) atmospheric temperature was recorded with the help of mercury thermometer, Rainfall data provided by govt. office Di. Jalna, water temperature, dissolved oxygen (DO), (CO_2) were recorded by using Meter Toledo (MX- 300) sensor. These above mentioned parameters were analyzed on the spot, soon after collecting the samples. The estimation of calcium (Ca+), by titriometric method (APHA, 1998.). Cl by titriometric method (APHA, 1998.) with silver nitrate.

III. Result and Disscusion

Month & Station	Rainfall		Atm. Temp		Water Temp		DO		CA		CL		CO_2	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B
July	155	155	26	26	25	25	11.88	11.88	27	27	41.33	41.33	4.7	4.7
Aug	71	71	25	25	24	24	12.34	12.34	22.6	22.6	42.6	42.6	5.2	5.2
Sept	36	36	24	24	23	23	10.76	10.76	18.9	18.9	42.5	42.5	4.8	4.8
Oct	33	33	29	29	25	25	10.81	10.81	16.7	16.7	45.32	45.32	4.2	4.2
Nov	00	00	25	25	22	22	10.72	10.72	17.8	17.8	48.76	48.76	6.2	6.2
Dec	00	00	16	16	16	16	11.56	11.56	18	18	56.32	56.32	8.2	8.2
Jan	00	00	22	22	20	20	11.42	11.42	19.8	19.8	69.68	69.68	9.8	9.8
Feb	00	00	23	23	21	21	10.54	10.54	17.2	17.2	53.73	53.73	9.6	9.6
Mar	00	00	26	26	23	23	10.12	10.12	18.5	18.5	49.76	49.76	10.0	10.0
April	15	15	30	30	28	28	9.82	9.82	29	29	33.5	33.5	10.9	10.9
May	62	62	32	32	29	29	9.37	9.37	26	26	38.7	38.7	11.00	11.00
June	77	77	28	28	25	25	10.67	10.67	30	30	40.2	40.2	4.6	4.6

Table 1 : Monthly value of physico-chemical parameters of Kasura dam.(July 2008-June 2009)

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a) Atmospheric Temperature

The atmospheric temperature recorded at the time of sample collection was ranging from 16ºC to 32ºC. In Kasura dam, maximum temperature recorded in the month of May 32ºC and minimum of 16ºC was recorded in the month of December during (2008-09) at station A & B. Temperature is a physical factor indicated the quality of water. It has effect on growth and distribution of aquatic life, concentration of dissolved gases and chemical solutes; probably it is the only factor having profound influence, either directly or indirectly, on the quality of water and biota. According to Welch (1952) in the tropics usually atmospheric temperature is more than water temperature. In present investigation, in all the two water bodies studied, air temperature exceeded water temperature during the study period.

b) Water temperature

The water temperature recorded at the time of sample collection was ranging from 16ºC to 29ºC at A and B station in Kasura dam, maximum of 29ºC was recorded in May, the minimum of 16ºC was recorded in December during (2008-09) at station A & B.

According to Welch (1952) the response of water temperature to air temperature depends on the size of the water body. The smaller masses of water temperature more quickly than bigger sheets having more surface area and mean depth (Munavar, 1970). Same results were found in relation to Phytoplankton diversity in relation to physic-chemical parameters of Gnanaprakasam temple pond of Chidambaram in (T.N), INDIA K. Thirugnanamoorthy, et.al,. (2009)

c) Rain fall

At Kasura dam catchments area, the annual rain fall recorded (2008-09) was from 0 to 550 mm. The maximum of 155 mm was recorded in the month of July and there was no rain in November, December, January, February and March in year (2008-09) at station A & B.

d) Calcium

In Kasura dam, calcium varied from 15.9 mg/l to 30 mg/l at A and B station in the study period. The maximum of 30 mg/l was recorded in the month of June. The minimum of 16.7 mg/l was recorded in the month of October of (2008-09) at station A & B.

Calcium is considered to be more important because it is an integral part of plant tissue as well as it increase the availability of other ions. High calcium contents in the sediment of the lake support the growth of mollusca. It is also required as a nutrient for various metabolic processes and assists in proper translocation of carbohydrates and facilitates other ions (Wetzel, 1975).

e) Free Carbon Dioxide (CO_2)

The free carbon dioxide of Kasura dam varied from 4.2 mg/l to 11.5 mg/l at A and B station in the study period. The maximum of 11 mg/l was recorded in May and the minimum of 4.2 mg/l was recorded in October during (2008-09) at station A & B.

Free carbon dioxide in water forms carbonic acid (H_2CO_3) which is dissociates in to H^+ and HCO_3 ions. This brings a change in the pH of water as H^+ ions are set free and HCO_3 reacts with calcium to form calcium carbonate which is insoluble in soluble in water. If at this stage, free carbon dioxide is not available, then calcium carbonate will be converted into insoluble calcium carbonate and will be lost to water.

f) Chlorides

In Kasura dam Chlorides varied from 33.5 mg/l to 69.68 mg/l at A and B station in study period. The maximum of 69.68 mg/l was recorded in the month of January and the minimum of 33.5 mg/l was recorded in the month of April of (2008-09) at station A & B. A quality parameter of significance is the chlorides concentration. Chlorine in Free State which is used as disinfectant will be converted in to chlorides or combines with organic matter to form toxic compounds (Adoni, 1985). Rajnarayan et.al, (2007) found same result in investigation of Texi Temple pond in district Etawahva (U.P).

g) Dissolved Oxygen (DO)

The dissolved oxygen in Kasura dam varied from 9.37 mg/l to 13.95 mg/l at A and B station. The maximum of 11.88 mg/l was recorded in the month of July and the minimum of 9.37 mg/l was recorded in the month of May during 2008-09 at station A & B.

Dissolved Oxygen is of great importance to all living aquatic organisms. It is considered as the factor which can reveal the nature of entire ecosystem. Much characteristic of water can be learnt by series of determinations of oxygen than other chemical data. The source of oxygen to any water body is mainly due to physical and biological process.

Generally Dissolved Oxygen is directly to photosynthesis, which is generally high when the sky is clear and the days are long (Sreenivasan ,1976) and an inverse relation between Dissolved Oxygen and rainfall was reported by Manawar (1970). Low Dissolved Oxygen values indicate the biodegradation of organic matter and decay of vegetation (Jameel, 1998).

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Faecal Examinations of Pashmina Goats (Capra siberica) of Ladakh for Nematode Infections

By J. A. Kuchai , M. Z. Chishti H.Tak & B. A. Lone
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Abstract - In the present study an attempt was made to find out the various nematodes and their prevalence infesting the pashmina goats of Ladakh through faecal examinations. The eggs collected from these were identified as Haemonchus contortus, Trichuris ovis, Dictyocaulus filaria and Chabertia ovina. Identification was done on the basis of various morphological and morphometric characters (Yamaguti, 1975; Soulsby, 1982). Of the 70 animals examined 22 (31.42%) were found infected with single or multiple parasite species. It was also observed that among these H. contortus 42.15% was most dominant followed by T. ovis (37.46%) D. filaria (32.24%) and C. ovina (18.78%) respectively. The study also revealed a significant difference with respect to season, wherein higher prevalence (40.00%) was observed during the rainy season as compared to the dry season 22.85%. Similarly an association was observed between sex and age of the host with prevalence of nematode infections. It was also observed that females were more infected (37.14%) as compared to males (25.71%). Likewise young animals were more infected (34.28%) than the adult ones (28.57%). Similarly an association was observed between prevalence and agro-ecology of the study area where in higher values (32.05%) were recorded for comparatively lowland (Kargil) areas as compared to highland (Leh) areas (30.00%).

Keywords : *Pashmina goats, Nematode parasites, Prevalence, Ladakh.*

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J. A. Kuchai [α], M. Z. Chishti H.Tak [σ] & B. A. Lone [ρ]

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Keywords : Pashmina goats, Nematode parasites, Prevalence, Ladakh.

I. Introduction

Faecal examination is one of the important diagnostic tools for detection of helminth infections in all the animals especially in wild animals and in animals who are not being slaughtered, and also in areas where slaughtering is banned. Pashmina goats play an important role in economy throughout the world and satisfy a number of needs of mankind in different ways, a large section of people is directly or indirectly dependent on them so is the case with the people of Ladakh where rearing of domestic animals including Pashmina goats is one of the most important activity for ensuring livelihood for these resource poor people. The wool of these animals is very costly and very suitable to the environmental conditions of this region therefore these animals are reared by the people of this reagion in large numbers. However, unfortunately the production of these animals is being reduced by a number of factors and one among them has been recognized as helminth parasitism. These are responsible for a number of economic losses in a variety of ways as: losses through lower fertility, reduced work capacity, involuntary culling, a reduction in food intake, lower weight gains, milk and meat production, treatment costs and mortality in heavily parasitized animals (Carmichael, 1972; Akerejola *et al.*, 1979). After a century of research into their biology and control, nematode parasites continue to be an important constraint on goat production. Modern anthelmintics, together with an understanding of the epidemiology of parasitism, the immune response and nutritional requirements of goats, currently enable satisfactory management of the problem. However, the increasing incidence of resistance by the parasites to available anthelmintics is challenging task for producers to maintain high levels of productivity in the goat industry. Novel developments for the management of nematode parasites such as vaccines, biological anthelmintics, genetic markers and selective breeding of goats may, in the future, provide additional or alternative means of parasite control. However, such alternative control methods are likely to be more dependent on a sound understanding of the species, lifecycle and population dynamics of the parasites involved and the epidemiology of disease they cause than current methods that rely heavily on broad-spectrum anthelmintics.

II. Materials and Methods

A systematic survey of various farm houses, pastures and local houses was carried by visiting them at regular intervals during the study. The faecal samples for detection of infection were mostly collected directly from the rectum of the host or fresh samples were collected from the pasture in the collection tubes containing 10% formalin. The samples were examined by direct smear and concentration (floatation and sedimentation) methods for the presence of nematode parasite eggs. The counting of eggs was performed by

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McMaster egg counting technique (Urquhart *et al.*, 1966). Identification was done on the basis of various morphological and morphometric characters (Yamaguti, 1975; Soulsby, 1982). To record the prevalence age, sex, body condition of the animals was noted so was the case with locality and season of the study area. The number of total and infected animals was also recorded.

III. Results

The results of the present study show that the Pashmina goats of Ladakh shares the same nematode parasitic infections as those of the common goats and sheep of this region but comparatively with low intensity and prevalence. During the study a total of four species viz; *Haemonchus contortus, Trichuris ovis, Dictyocaulus filaria and Chabertia ovina* have been recovered from the host species of which *H. contortus (*42.15%) was the most prevalent followed by *T. ovis* 37.46% *D. filaria* 32.24% *and C. ovina* 18.78% respectively Table 1.1 while as in case of common goats and sheep of this region. Of the 70 hosts investigated, 22 (31.42%) were found to be infected with one or more parasite species. Most of the cases were reported with a multiple type infection it was also observed that the prevalence of *Dictyocaulus filaria* was increasing with a decrease in temperature, while as the case was reverse with that of *H. contortus*. There was a significant difference in prevalence of parasites with respect to season, where in the prevalence was higher in wet season (40.00%) than in the dry season (22.85%) Table 1.2. Similarly the prevalence was higher in females (37.14%) and young animals (34.28%), as compared to males (25.71%) and adult ones (28.57%) Table 1.3 and 1.4. Also the study show an association between the prevalence and agro-ecology of the study area wherein the infection rate was higher in comparatively lowland areas (Kargil), (32.05%) as compared to high-altitude (Leh), (30.00%) Table 1.5. Furthermore an association was observed in prevalence of parasite and body condition of the host, weak animals were found slightly more infected (32.00%) as compared to the healthy ones (31.11%) Table 1.6. It was also observed that the animals infected with *H. contortus* and *D. filaria* or with one of the either species especially with the later one were much more affected as compared to the other two species so far as the health status is concerned. The animals infected with *D. filaria* were easily diagnosed showing the symptoms of cough, nasal discharge, weight loss, drowsiness, etc.

Table 1.1 : Prevalence on the basis of parasite species

Host	No. Examined	No. Positive	*H. contortus*	*T. ovis*	*D. filaria*	*C. ovina*
P. Goats	70	22 (31.42%)	42.15%	37.46%	32.24%	18.78%

Table1.2 : Prevalence on the basis of Season

Host	No. Examined	Total No. Positive	Wet Season	% age	Dry Season	%age
P. Goats	70	22	14/35	40.00	08/35	22.85

Table1.3 : Prevalence on the basis of Sex of the host

Host	Total No. Examined	Total No. Positive	Males	%age	Females	%age
P. Goats	70	22	09/35	25.71	13/35	37.14

Table1.4 : Prevalence on the basis of Age of the host

Host	Total No. Examined	Total No. Positive	Young	%age	Adult	%age
P. Goats	70	22	12/35	34.28	10/35	28.57

Table1.5 : Prevalence on the basis of Agro-ecology

Host	Total No. Examined	Total No. Positive	Kargil (Lowland)	%age	Leh (high-altitude)	%age
P. Goats	70	22	13/40	32.05	09/30	30.00

Table1.6 : Prevalence on the basis of body conditions of the host

Host	Total No. Examined	Total No. Positive	Healthy	%age	Weak	%age
P. Goats	70	22	14/45	31.11	08/25	32.00

(P= Pashmina; No= Number)

IV. Discussion

This study revealed that pashmina goats of Ladakh are infected with the same parasite species as have been reported from those of the sheep and common goats of this region (Kuchai *et al.*, 2011) as well as from the other two regions of the same state (J&K), (Bali, 1976; Chishti, 1986). The possible reason for the presence of the same species of nenatode parasites in all these animals could be because they share some common grazing, drinking and sheltering areas and therefore the possibility of picking the same eggs from ground along with grass, food, water, etc are also same. However the occurrence of comparatively low prevalence rate in pashmina goats from those of the other small ruminants could be that most of the samples

were collected from farm houses where these animals are dewormed regularly and properly, also these animals are reared more carefully even by the farmers because of their wool production which is very costly. Another possible reason could be the sampling error. The higher prevalence of *H. contortus* as compared to the other three species could be that it is more resistant to antihelmintics as well as to the environmental hazards. The significantly higher prevalence in wet season than that of the dry season is in consent with many reports around the world, (Tembely *et al.*, 1997; Moyo *et al.*, 1996; Fritche *et al.*, 1993; Githigia *et al.*, 2005). This could be due to the existence of a direct relationship between prevalence with rainfall, humidity and temperature. The presence of sufficient rainfall and moisture during wet season favored the survival of infective larvae in pasture and higher probability of uptake of the infective larvae leading to higher prevalence rate Sissay *et al.,* 2007. Similarly the higher prevalence recorded in younger animals as compared to the adult ones is in agreement with most literatures Dunn, 1978, Shah-Fischer and Say, 1989, Kiyyu, 2003, Nwosu *et al.*, 1996, Nganga *et al.,* 2004, from different corners of the world. This could be due to the fact that younger animals are more susceptible to infections than adults. Adult animals may acquire immunity to parasites through frequent challenge and expel the ingested parasites before they establish infectuion; (Dunn, 1978, Shah-Fischer and Say, 1989).The study further revealed that sex of animals show an association with the prevalence of the parasites, the higher prevalence in females than their counter partners could be due to some physiological peculiarities of the female animals, which usually constitute stress factors thus, reducing their immunity to infections, also females happen to be lactating which leads to weakness/ malnutrition as a result of which females are not able to fight against infections to the same extent as those of males (Blood and Radostists, 2000; Kuchai, et al., 2011). The possible reason for slightly higher prevalence in weak animals of the host species could be that these animals usually have a comparatively weak immune system which does not fight with the infections to the same extent as that of a healthy animal's immune system Kuchai *et al.*, 2011.The reason for higher prevalence in lowland areas (Kargil), as compared to high altitude (Leh) could be that these agro-ecological zones are characterized by a hot humid environmental situation that is favorable for the survival of intermediate and infective stages of most of the parasites (Teklye, 1991, Fikru *et al.*, 2006).

V. Acknowledgement

The authors would like to thank the farmers, bouchers and the veterinary hospital staff of the Ladakh for providing material and lab facilities during the study. Thanks are also due to accommodators for providing accommodation.

VI. Conclusion

The present study show that it is beyond the doubt that the pashmina goats of Ladakh make no exception from those of other ruminants so far as helminth infections is concerned which could be responsible for economic losses in a variety of ways, therefore efforts should be made to control helminthiasis which requires a detailed knowledge of these parasites and it is believed that the present study will provide some help for the same. The study also show that season, sex, age and geographical location appear to be the major limiting factors for the prevalence of nematode parasite infections.

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Eco-Phytosociology Method and it New Application in Determination and Discrimination of Intraspecific Diversity, Case Study: *Astragalus Verus and Astragalus Glaucops*

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Abstract - This study carried out for determination and discrimination of intraspecific diversity of Astragalus verus and Astragalus glaucops by Eco-phytosociological method from west of Iran. In this order, application of Endogenous milieu (special station) for data collecting and then their analyzing permit us only determine existence of inter and intraspecific diversity. Then for determinating kind and level of intraspecific diversity (Ecophene, Chemotype, Cytotype, Ecotype …), can use other studies such as: morphological, anatomical, phytochemical, cytological and etc. In this survey, 31 special stations were studied. Then floristic-ecologic data collected from each 31 special stations and analyzed by Anaphyto software (F.C.A, A.H.C, Marquag methods). Comparison of obtained results on multiple coordinate axes from F.C.A method with results from Marquag and A.H.C methods led to determination of 7 main groups of Endogenous milieus (special station). Flavonoid analyses were used for determination kind and level of intraspecific diversity in 7 discriminated groups. Leaves flavonoid components of all collected individuals of Astragalus verus and Astragalus glaucops were investigated by TLC method. Obtained data from flavonoid survey analyzed by MVSP package with WARD and UPGMA methods. Finally, the results of flavonoid studies confirmed the same groups that identified by floristical composition study and showed intraspecific diversity in chemotype level.

Keywords : *Astragalus verus, Astragalus glaucops, Eco-phytosociology method, intraspecific diversity.*

GJSFR-C Classification: *FOR Code: 060203*

Strictly as per the compliance and regulations of :

Eco-Phytosociology Method and it New Application in Determination and Discrimination of Intraspecific Diversity, Case Study*: Astragalus Verus* and *Astragalus Glaucops*

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Abstract - This study carried out for determination and discrimination of intraspecific diversity of *Astragalus verus and Astragalus glaucops* by Eco-phytosociological method from west of Iran. In this order, application of Endogenous milieu (special station) for data collecting and then their analyzing permit us only determine existence of inter and intraspecific diversity. Then for determinating kind and level of intraspecific diversity (Ecophene, Chemotype, Cytotype, Ecotype …), can use other studies such as: morphological, anatomical, phytochemical, cytological and etc. In this survey, 31 special stations were studied. Then floristic-ecologic data collected from each 31 special stations and analyzed by Anaphyto software (F.C.A, A.H.C, Marquag methods). Comparison of obtained results on multiple coordinate axes from F.C.A method with results from Marquag and A.H.C methods led to determination of 7 main groups of Endogenous milieus (special station). Flavonoid analyses were used for determination kind and level of intraspecific diversity in 7 discriminated groups. Leaves flavonoid components of all collected individuals of *Astragalus verus and Astragalus glaucops* were investigated by TLC method. Obtained data from flavonoid survey analyzed by MVSP package with WARD and UPGMA methods. Finally, the results of flavonoid studies confirmed the same groups that identified by floristical composition study and showed intraspecific diversity in chemotype level.

Keywords : Astragalus verus, Astragalus glaucops, Eco-phytosociology method, intraspecific diversity.

I. Introduction

During the past forty years or more much of the attention of continental plant ecologists has been devoted to devising and systematizing methods for the description and classification of plant communities. So impressive is the body of material collected that schemes have been proposed to establish rules for the correct description and nomenclature of vegetation units comparable for those in effect for taxonomic species, genera, families etc. (Barkman, 1950; Du Rietz, 1930, 1936). The view expressed by Tuxen (1942) that the plant can measure habitat factors better than any instrument is symptoma - tic of the skepticism with which the sociologist regards intensive ecological investigation, in spite of the fact that the only exact knowledge which he possesses of the tolerance of species has been obtained by extrapolation (often unjustified) from original instrumental measure - ments.

The knowledge of the floristic composition of an area is a perquisite for any ecological and phytogeographical studies and conservation management activities. In studying any particular piece of vegetation, from an ecological point of view, our first step must be to determine the facts as they exist on the ground: facts regarding the vegetation, on the one hand; facts regarding the habitat, on the other (Nicholes, 1930). If there is any one set of facts which is more susceptible to direct study and exact characterization than any other, it is the floristic composition of the vegetation.

In mentioned studies did not use a special method in plant specimens collecting process, while for collecting correct and precise floristic-ecologic data, we must apply an appropriate method that be according to factors governing nature and can be used for determination and discrimination existence of inter and intraspecific diversity. In this order, we used the unit of study (Endogenous milieu) in Eco-phytosociological method (Atri, 1996, 1999).

The aim of this project, was study on exist of intraspecific diversity (biodiversity point of view) in species, *Astragalus glaucops* and *Astragalus glaucops* in wes Iran. It was carried out from two different aspects, the studies of floristic- ecologic diversity in this species belong to their stations and investigation on the diversity of flavonoid patterns in their populations. The next aim was if floristic- ecologic data and obtained groups for each species can be distinguished intraspecific diversity of the species separately.

II. Eco-Phytosociology Methodology

By 1996, after extensive plant sociological studies in the Iran, Atri had formed definite ideas about the fundamental concepts of his system. Which he published under the title, a presentation of some aspects of the application of neosigmatiste method in pedology, systematics and chorology. In new method

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namely Eco-phytosociology for determine plant association after diagnosis individuals association, in each individual's association; Endogenous milieus determine. Endogenous milieu in Eco-phytosociological method is an area of vegetation that is homogenous view point of floristic- ecologic. Establishment of releves (stands) carry out randomly in each Endogenous milieu for floristic-ecologic data collecting.

In this method, by employing physiognomic criterion, the exiting formations (principle and secondary formations) are specific. By employing the floristic criterion in each formation, the homogenic areas are determines in terms floristic composition and their delimitations are specific on the map as association individuals respect. Then, by using ecological criterion in each association individuals, based on observation of any changes in one or more ecological factors, the exiting endogenic milieu(s) can specific in each association individuals. Then, any endogenic milieus, which show homogeneity in floristic-ecologic term, the releve are place at random. To determine the minimal area of each releve, by using the area-species method on basis of area-species curve and Cain method are apply (Cain, 1959). The necessary floristic-ecologic information and data (including plant species, texture class, OM %, OC%, pH, EC, moisture Altitude, exposition and slope degree) are collect for each releves and are duly enter in the relative forms. In the next stage, the species and samples of soil identify and duly study so that they can prepare to analysis by computer software after labeling and coding of the releves. Must, Pay attention that, each own Endogenous milieu can be one releve or in each Endogenous milieu establish several releve. Finally, data analyses lead to know plant associations of vegetation study.

III. Eco-Phytosociology Applications

Employing ecologic and phytosociologic criteria as eco-phytosociology (Atri, 1996) are not only suitable and exact in the data collection stage to determine the placement of releves, but also it is able to provide results which conform and agree to the rules that govern the nature in the analysis and result interpretation stage. Some Investigations by use this method (Atri, 1996, 1999., Atri et al., 2006, 2007, Fakhre-Tababaei et al, 2000; Safidkon et al, 2003 and 2005; Kalvandi et al, 2004) show that this method can suitable for ecological studies such as chorology, auteocology, pedology, biosystematics, plant diversity (intra and interspecific variations such as, ecotype, cytotype, ecophene, chemotype,…).

IV. Eco-Phytosocilogy and Plant Diversity

The environmental factors and its influence in plant variation (plant diversity) have been extensively studied. Some of these studies include: (Turesson, 1922; Mooney and Billings, 1959; Koch and Bernhardt, 2004; Semmar *et al.*, 2005; Telascrea *et al.*, 2006).

In mentioned studies did not use a special method in plant specimens collecting process, while for collecting correct and precise floristic-ecologic data, we must apply an appropriate method that be according to factors governing nature and can be used for determination and discrimination existence of inter and intraspecific diversity. In this order, we can use the unit of study (Endogenous milieu) in Eco-phytosociological method (Atri, 1996, 1999).

In vegetations study, endogenous milieu determine by physiognomic-floristic-ecologic criteria. Establishment of relieves (stands) carry out randomly in each Endogenous milieus for floristic-ecologic data collecting (figure 1). Finally, data analyses lead to know plant associations of vegetation study. While for studying inter and intraspecific diversity, an Endogenous milieu (special station) determine base on the presence of individual of studied species in its stations (Atri and Asgari Nematian, 2006). Data collecting in inter and intraspecific diversity study is based on floristical composition in each special station (figure 2). floristical composition as floristical marker is good marker, because any kind of changing floristical composition in different special stations show existence of different ecological factors in them, that lead to inter and intraspecific diversity. We have done some research by this method (Atri, 1996, 1999; Atri et al, 2006, Atri et al, 2007).

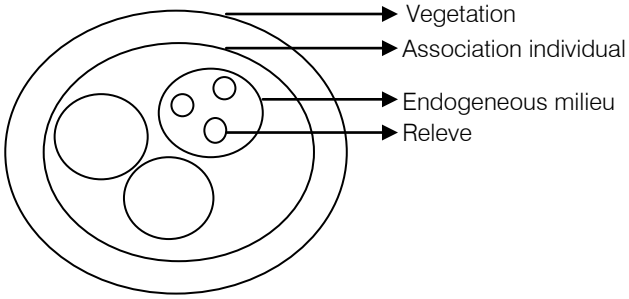

Figure 1 : The use Eco-phytosociology method for determine association

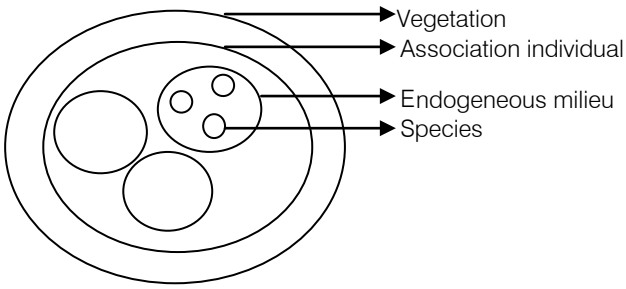

Figure 2 : The use Eco-phytosociology method for determination and discrimination intra and interspecific variations

V. Materials and Methods

Plant materials: At the first phase, different stations of *Astragalus verus and Astragalus glaucops* were determined in the west of Iran by using the accessible references, Herbaria and existence information. Then we referred to the different stations in study area, along 2004-2006 years, in growth season for collecting floristic-ecologic data. Totally between studied stations, 31 stations selected for investigation in Hamadan, Kermanshah, Kordestan and Markazi provinces from west of Iran (Table 1 and 2). Data collecting from 31 selected station carried out by using the unit of study in Eco-phytosociological method (Atri, 1996, 1999; Atri and Asgari Nematian, 2006) that is named Endogenous milieu (special station). In each station, location of establishment for each relive (stand) determined on base of presence of individual study species.

Then for determination of special station of individual study species, minimal area determined by using the area-species method with area-species curve and Cain method (Cain *et al.*, 1959). All ecologic-floristic data (the studied species and its companion species as floristical markers) were collected of each special station. Plant specimens deposited in the Herbarium, of Bu-Ali Sina University in Hamadan, Iran. Studied ecological factors included (elevation, pH, EC, texture of soil, slop direction and slop percent) in each special station.

Flavonoid aglycone study: The plant leaflet of different individuals of *Astragalus verus* and *Astragalus glaucops*, that collected from different special stations in west of Iran, were separated and then ground in a grinder. Flavonoid aglycone analysis was taken on all individuals of *Astragalus glaucops* and *Astragalus glaucops* listed in (Table 1 and 2). Briefly, 2 g dried powder of leaves boiled in 50 mL 2 M HCL for 45 min. Hydrolyzed leaf extracts were allowed to cool to room temperature. The extracts were then washed 3 times with equal volumes of ethyl acetate. The pooled ethyl acetate fractions were evaporated to dryness in a fume hood. The residue of each plant sample was taken up in an equal volume of 95% ethanol (Joseph *et al.*, 2003). The analysis was performed on Silica gel plates 25 Fuelled aluminum CCM (20x20), Gel de silica 60 F_{254} (Merck). Replicate plates were developed in BAW (n-butanol: acetic acid: water, 4:1:5, top layer used). Quercetin, flavone and rutin used as standards. The plates were developed at room temperature in a vertical separating chamber to the height of approximately 14 cm from the start. After drying, visualization was performed by:

- Spraying with 1% methanolic diphenylboryloxyethyl-amine
- and 5% ethanolic polyethyleneglycole 4000

Chromatograms were interpreted in long wave UV light (366 nm), then were measured R_f of each bands (Medica-Saric *et al.*, 2004).

Data analysis: For determination and discrimination of intraspecific diversity of *Astragalus verus* and *Astragalus glaucops*, were applied correspondence, cluster, classification and discriminate analysis. Floristical composition data (as floristical marker) analyzed by using Anaphyto software version 95 (Briane, 1995) by means FCA (Factorielle Correspondence Analysis), AHC (Ascendant Hierarchical Classification) and Marquage methods. In studying phytochemical data, once presence and absence of different bands were determined on chromatogram for different individuals of *Astragalus glaucops* and *Astragalus glaucops*. Then phytochemical data analysis was taken by MVSP softwares by means UPGMA method. Ecological data analyzed by MVSP software with CCA method and Anaphyto software with FCA method.

VI. Results

Floristical results: Obtained results base on floristical composition analyses of 31 special stations showed seven main groups by using FCA method (Fig. 1). Group (A) include special station number 0031, 0022, 0020, 0015, 0014 group (B) number 0002, group (C) numbers 0042, 0043, 0044, 0045, 0046, 0047, 0048, 0049, 0050, 0018, 0007, group (D) number 00392, 0030, 0024, 0023, 0011 group (E) numbers 0032, group (F) number 0041, 0037, 0038, group (G) numbers 0006, 0005, 0004, 0003, 0001. It must indicate that the mentioned 7 groups obtained base on similarity and dissimilarity of their floristic composition (as floristical marker). The obtained results from FCA method completed by AHC and Marquage methods (Fig. 2, 3). These 7 main groups evidence existence of intraspecific diversity for *Astragalus verus* and *Astragalus glaucops* in study area

Flavonoid results: Determination of level and kind of intraspecific diversity were used by flavonoid studies. Prepared chromatograms by TLC method showed different flavonoid bands and also different quantity of bands in different individuals of *Astragalus verus* and *Astragalus glaucops* in study area. Different bands and their R_f measured. Analyzing of flavonoid data separate 5 groups (Fig. 4). The obtained groups of flavonoid results had a good correlation with floristical composition groups that confirm them and showed intraspecific diversity in chemotype level. These 7 groups are different regarding quality, quantity and R_f of flavonoid bands.

Ecological results: Ecological factors data that were collected by applied method analyzed by MVSP software with CCA method. The obtained results showed between studied ecological factors (elevation, pH, EC, texture of soil, slop direction and slop percent) elevation factor has the most important role in separating different determined groups (Fig. 5, 6).

VII. Discussion

Creation of inter and intraspecific diversity are the main origin and storage of speciation. In this order, creation, inter and intraspecific diversity in different levels cause to richness of taxa in an area. For determination and discrimination of inter and intraspecific diversity, should applicate and suitable method that the obtained results of applied method be correct, precision and spends lower expenses (Atri et al, 2006, 2007).

Many studies carried out for determination intraspecific diversity (Turesson, 1922; Mooney and Billings, 1959; Perez-Alonso *et al*., 2003; Koch and Bernhardt, 2004; Semmar *et al*., 2005; Telascrea *et al*., 2006), but most of them do not apply a special method in plant data collecting process and carry out by time consumer and expensive experiments. While, application the floristical marker and the unite of study in Eco-phytosociological method (special station) in this kind of studies led to correct and precision results because that is according to factors governing nature. In the other hand, it prevents of more expenses and time consumer experiments (Fakhre-Tabatabaei *et al*., 2000; Sefidkon *et al*., 2003; Kalvandi *et al*., 2004; Sefidkon *et al*., 2005; Ebrahimzadeh *et al*., 2006; Atri et al, 2006, 2007).

In regard to applied principles in this method for data collecting, we can certainly declare that floristic markers without application other markers can determine intraspecific diversity existence. Then for determinating kind and level of intraspecific diversity (Ecophene, Chemotype, Cytotype, Ecotype …), between obtained floristical groups, we can use other studies such as: morphology, anatomy, phytochemistry, cytology and etc.

Present results show that *Astragalus verus* and *Astragalus glaucops*, has high diversity in the west of Iran. According to our results of floristical analyses, there are 7 distinctive different groups of *Astragalus verus* and *Astragalus glaucops* individuals in study region. At second phase, phytochemical studies create seven kinds of chemotypes which conform and affirm the obtained results of floristical studies.

In picture 1_ 3, Special stations of *Astragalus verus* and *Astragalus glaucops* separated from each others. Group (C) include numbers 0042, 0043, 0044, 0045, 0046, 0047, 0048, 0049, 0050, 0018, 0007 separated by floristic composition was belonged to *Astragalus glaucops* that conformed with flavonoid analysis (Fig 4). On the other hand, flavonoid profile of *Astragalus verus* and *Astragalus glaucops* separated from each others that conformed to floristical composition.

The phenomena such as interactions, substitution, stenoece and euryece nature of species and existence of intra-specific and inter-specific relations, consideration of the ecological factors as the base and pillar by focusing on one or a number of predetermined ecological factors to study vegetation, could not express the existing reality in all times. On the other hand, with respect to homogeneity of the environment for dominant species and its non – homogeneity for other species, the possibility of careful determination of association individuals or the homogenic surface in ecologic- floristic term is low. With respect to the aforesaid instances in studying effective ecological factors on the vegetation, eco-phytosociology method was employed. In this approach, it became possible to determine principal ecological factors. Between studied ecological factors, elevation is the most important ecological factor in creation intraspecific diversity. For *Astragalus glaucops*, elevation starts of 2450m to 2750m. The present study shows that in studying the vegetation and determining ecological factors, employing ecological and phytosociologic criteria as eco-phytosociology (Atri, 1996) are not only suitable and exact in the data collection stage to determine the placement of releves, but also it is able to provide results, which conform and agree to the rules that govern the nature in the analysis and result interpretation stage.

So this study and other studies that done base on this method until to now, show the high efficiency of it in determination and discrimination of inter and intraspecific diversity existence. By using this method after determinating floristic groups, we should characterize kind and level of intraspecific diversity only between obtained floristic-ecologic groups and this in require us of testing all of the individuals of studied species and expending long time and much money in this way.

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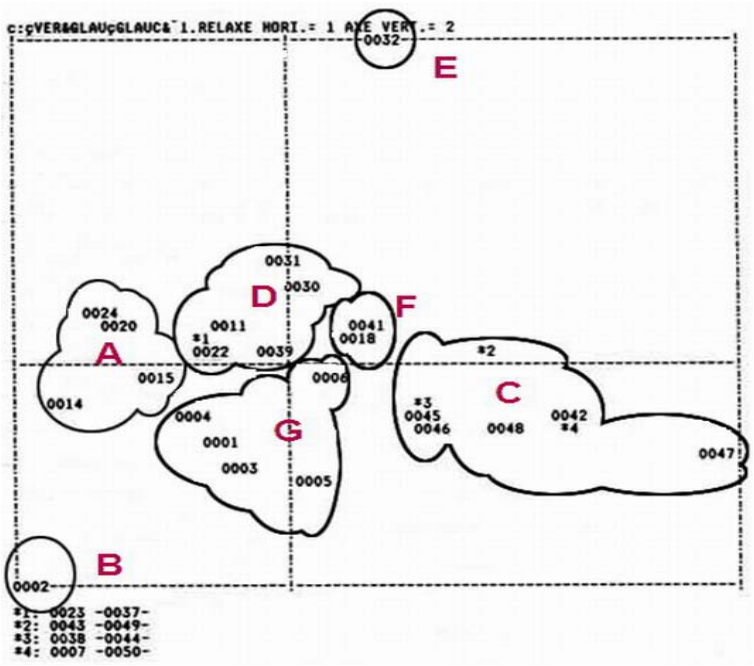


Figure 1: Results of floristical composition data analysis by FCA method on1, axes

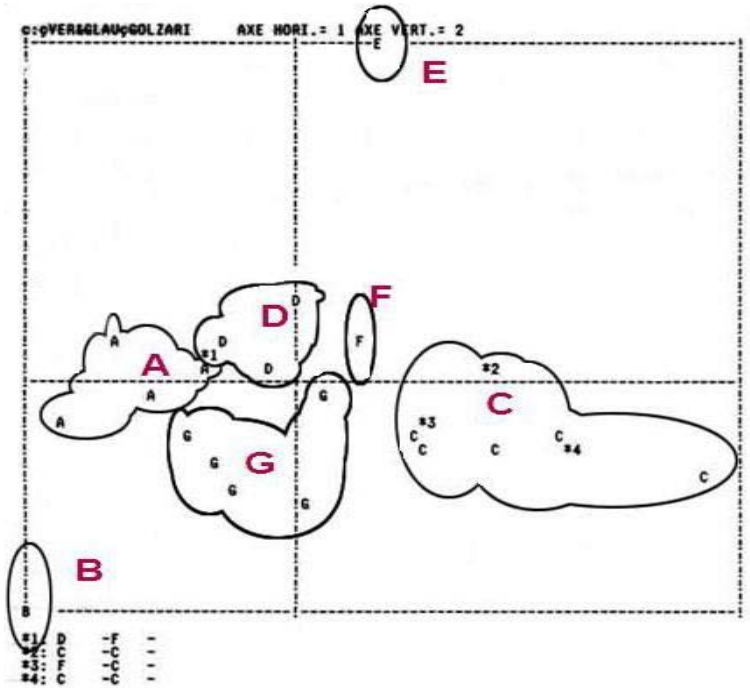
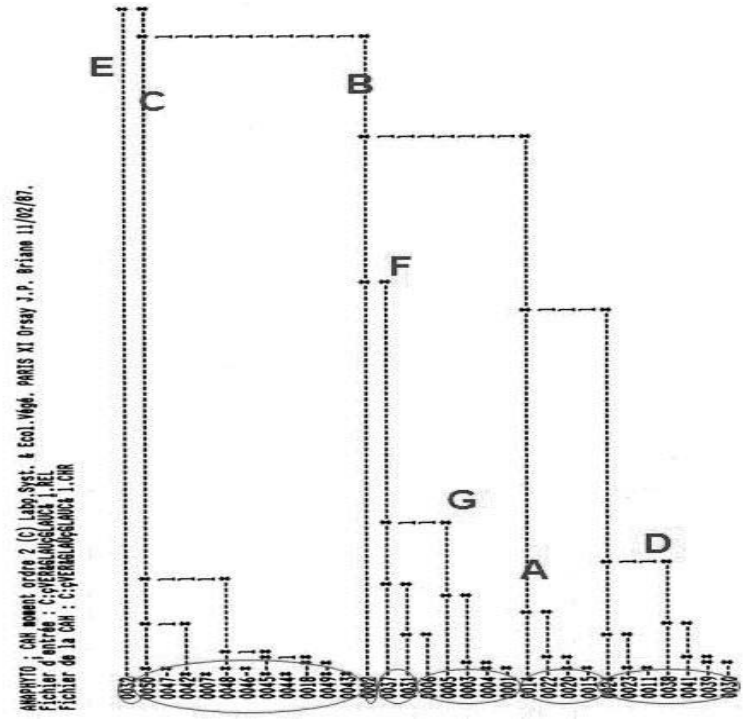


Figure 2 : Results of floristical composition data analysis by Marquage method on1, axes



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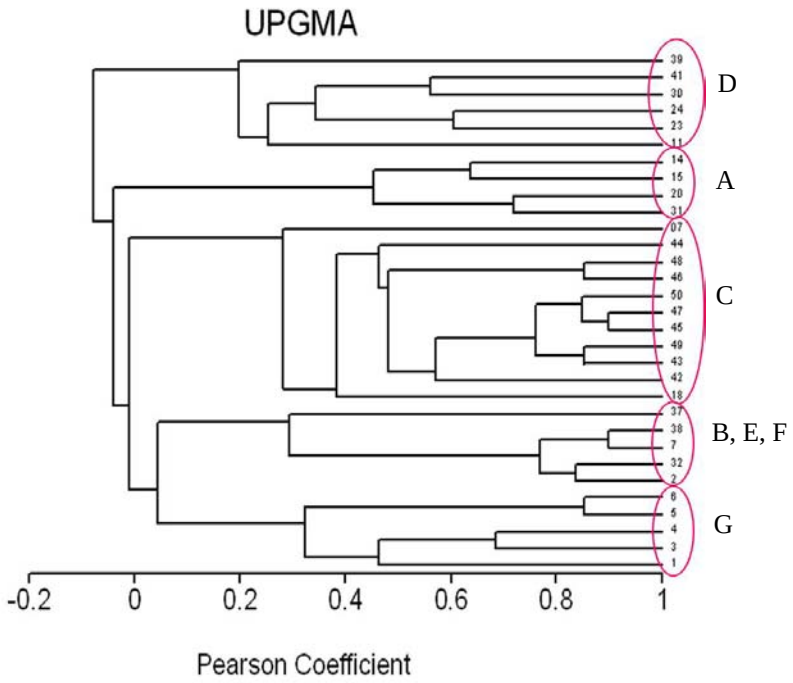

Figure 4. 2 : Resulted cluster of aglycoside flavonoids studies of *Astragalus glaucop and Astragalus verus* individuals by UPGMA method

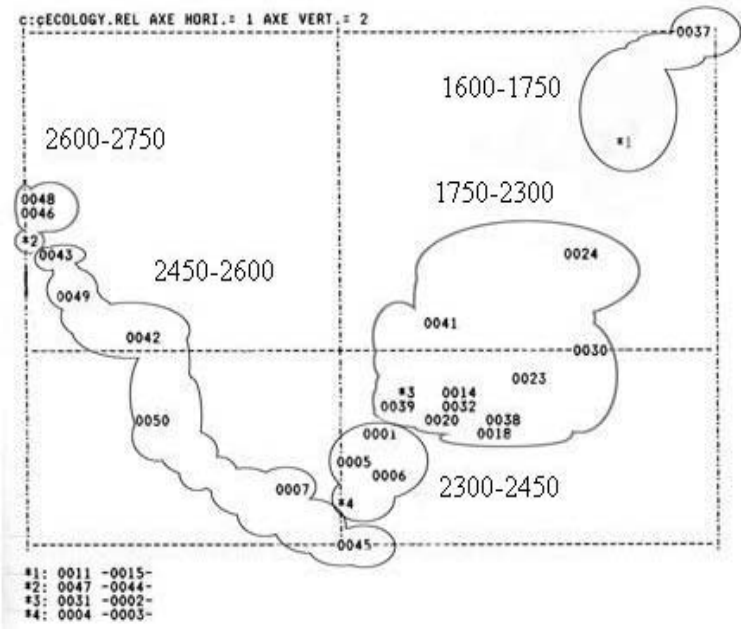

Figure 5 : Results of ecological factors studies by FCA method

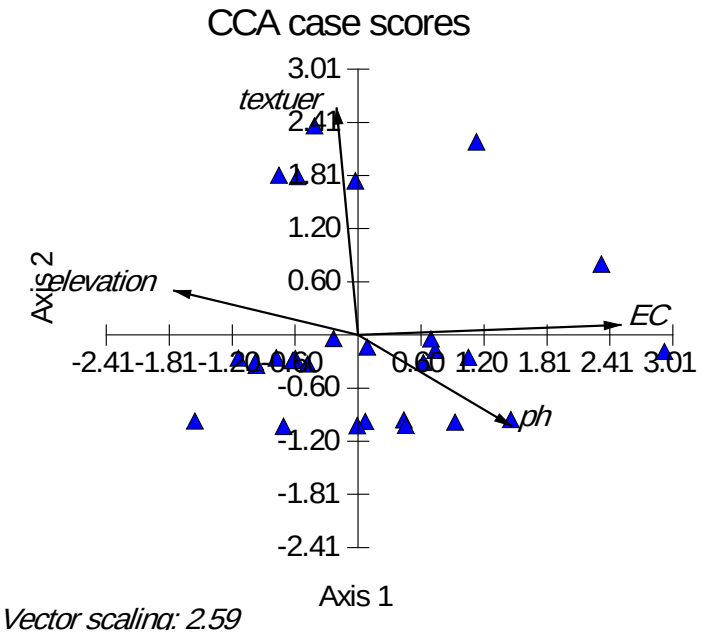

Figure 6 : Results of ecological factors studies by CCA method

Table 1: The different studies special stations for *Astragalus verus*

Releve NO.	Voucher No.	Altitude	Place
1	7286	2425	Hamedan, Asad- Abad
2	7287	2417	Hamedan, Asad- Abad
3	7288	2408	Hamedan, Asad- Abad
4	7289	2424	Hamedan, Asad- Abad
5	7290	2416	Hamedan, Asad- Abad
6	7291	2344	Hamedan, Asad- Abad
7	7292	2563	Hamedan, Alvand
11	7293	1723	Hamedan, Nahavand
14	7294	1898	Hamedan, Razan
15	7295	1723	Hamedan, Razan
20	7296	1898	Hamedan, Touyserkan
23	7298	2213	Hamedan, Divijin
24	7299	2220	Hamedan, Divijin
30	7300	1840	Kermanshah
31	7301	1850	Kermanshah
32	7302	1800	Kermanshah
37	7303	1610	Kordistan, Sanandaj
38	7304	1995	Arak
39	7305	1940	Arak
41	7306	2008	Arak

Table 2 : The different studies special stations for *Astragalus*

Releve NO.	Voucher No.	Altitude	Place
7	7307	2563	Hamedan, Alvand
18	7308	2300	Hamedan, Touyserkan
42	7312	2540	Hamedan, Alvand
43	7310	2536	Hamedan, Alvand
44	7313	2547	Hamedan, Alvand
45	7309	2600	Hamedan, Alvand
46	7311	2650	Hamedan, Alvand
47	7314	2660	Hamedan, Alvand
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Search engines for most searches, use Boolean searching, which is somewhat different from Internet searches. The Boolean search uses "operators," words (and, or, not, and near) that enable you to expand or narrow your affords. Tips for research paper while preparing research paper are very helpful guideline of research paper.

Choice of key words is first tool of tips to write research paper. Research paper writing is an art.A few tips for deciding as strategically as possible about keyword search:

- One should start brainstorming lists of possible keywords before even begin searching. Think about the most important concepts related to research work. Ask, "What words would a source have to include to be truly valuable in research paper?" Then consider synonyms for the important words.
- It may take the discovery of only one relevant paper to let steer in the right keyword direction because in most databases, the keywords under which a research paper is abstracted are listed with the paper.
- One should avoid outdated words.

Keywords are the key that opens a door to research work sources. Keyword searching is an art in which researcher's skills are bound to improve with experience and time.

Numerical Methods: Numerical methods used should be clear and, where appropriate, supported by references.

Acknowledgements: Please make these as concise as possible.

References

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References	Complete and correct format, well organized	Beside the point, Incomplete	Wrong format and structuring

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