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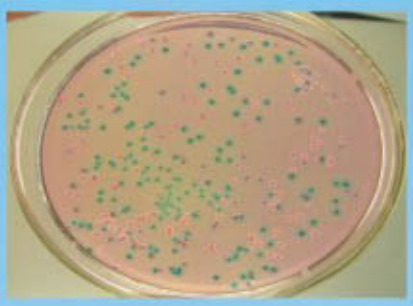



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Interdisciplinary Researches

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Schizophrenia and Other Psychoses in Polish Middletown

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Abstract: The most of studies conducted up till now have shown a frequent occurrence of schizophrenia and other psychoses in cities compared to rural and suburban areas. The following research work was done on the basis of address data in a medium size town of people placed in psychiatric hospitals schizophrenia and other psychoses in the years 1989-2002. ICD9 criteria were initially used for hospital diagnosis, and then ICD10 criteria. To study the differences among particular districts of the town the test for two proportions was conducted. The analyzed group of persons amounted to 380 patients, including 169 men and 211 women. It indicated that patients more frequently lived in more urbanized and postindustrial districts of town as well as men aged 20-29 years old in postindustrial districts. It should be mentioned that more of patients placed the inner city, but also in postindustrial district and in districts with city housing projects.

Key words: Schizophrenia, hospitalized patients, middletown, stratification, epidemiology.

1. Introduction

Studies conducted up till now have shown a frequent occurrence of schizophrenia in cities compared to rural areas, as well as a frequent occurrence of the same in inner cities compared to suburban areas [1-9]. Such is the case in a pioneer study in the social aspects of mental disorders by Faris and Dunham [1]. A brief summary of the results obtained by the authors show a regular decrease from the center to the periphery of the city. For instance, they examined the distribution of the different types of schizophrenia within the large city as Chicago. The higher risk of prevalence of schizophrenia is between lonely and unemployment people [10]. The purpose of the following work was to study the placement of people in psychiatric hospitals as a result of schizophrenia and other psychoses in areas of the medium size Polish town of Tarnowskie Góry, as well as selection of their social demographic data.

The city of Tarnowskie Góry has 62,000 inhabitants. The city of Tanowskie Góry lies in The Silesian Voivodeship in the Upper Silesian industrial region. The Voivodeship has 4.7 million inhabitants and is the most densely populated area in Poland with 381 inhabitants per km^2, 78.8% of the population lives in cities and the region is the most urbanized in Poland. It is a city of historical tradition, inasmuch as King John Sobieski passed through it in 1683. The city has market square around which the small streets of the city spread out. After Poland's economic transformation in 1989 changes into a market economy visible began. There was an increase in service-based companies and a succession of territories formed around the market square in accordance with Burgess's theory [11]. Industries began to have problems and unemployment appeared. The housing in Tarnowskie Góry is public (communist era projects), traditional townhouses,

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and single-family homes. Many of the building require restoration. The city districts did not gain complete independence from the city center as a result of poorly developed service and cultural life, even though after 1989 there was a material improvement.

Town Districts:

I-"Śródmieście" (Inner city). Categories: the main places of employment are the large railway junction, clothing factories, and chemical plants a certain distance from the city centre that are on the verge of being shut down.

II-"Stare Tarnowice" consisting of single-family homes and a large council housing complex. There is a general hospital with a psychiatric ward.

III-"Osada Jana" with older single- and multi-family housing, which is the second largest council housing complex.

IV-"Lasowice", a large industrialized district with town-houses, single-family homes and a council housing complex.

V-"Sowice" which neighbours "Lasowice" (IV), also largely industrialized with predominantly decrepit town-houses. It also has "social-care apartments" for persons evicted from other districts.

VI-"Bobrowniki" possesses a brickyard and sports recreation centre where in the past dolomite was extracted. It has single- and multi-housing, as well as town and council housing.

VII-"Repty" is an area with state-owned farmland. It possesses single-family housing, a beech-tree forest and also a large rehabilitation hospital.

VIII-"Pniowiec" has areas rich in greenery, adjacent to water and forests. Single-family homes predominate here.

IX-"Strzybnica" has few industrial plants and areas with farm land, town-houses, single- and multi-family homes and council housing.

X-"Rybna" is a district of single- and multi-family housing, neighbouring "Strzybnica" (VI), with many tillable fields and woods.

XI-"Opatowice" is the smallest district of the town with slightly isolated housing, mainly single-family.

2. Materials and Methods

Research was based on admission books and on computer databases in psychiatric wards in Tarnowskie Góry, as well is psychiatric hospitals in the not too distant region of Toszek and Lubliniec. Among those placed in psychiatric hospitals in the years 1989-2002, persons diagnosed with schizophrenia or other psychoses were selected in the city of Tarnowskie Góry on the basis of their place of their residence. Hospitalization indexes were counted in particular districts of the city. Then the dependence among particular districts of the city was studied for test for two proportion ($P = 0.025$). ICD9 criteria were initially used for hospital diagnosis, and then ICD10 criteria [12]. Group analysis of the persons amounted to 380 patients with schizophrenia or other psychoses, 169 men and 211 women. The research protocol was approved by the institutional ethical committee.

3. Results

The analyzed group of persons amounted to 380 patients suffered from schizophrenia or other psychoses, 169 men and 211 women. The analysis was based on studying the relation between the number of illnesses which found their finish in hospital care, and their place of residence in the years 1989-2002. The numerical force of the ill was defined by counting the number of persons with diagnosed psychiatric data and then their grouping with regard to place residence in a defined area of the city. The numerical index of those hospitalized for schizophrenia or other psychoses is presented in Table 1. The graphical image of indexes along with the statistical dependencies among studied districts is presented in Table 2.

As data from Table 1 shows, in the majority of the city districts significant statistical differences were

Table 1 The percentage of persons placed under psychiatric care as a consequence of schizophrenia and other psychoses in relation to the number of inhabitants in a given district in the years 1989-2002.

City districts	% hospitalized	Men %	Women %	Number of inhibitants	*P*
Śródmieście (I)	0.78	0.32	0.45	22,725	0.04
Stare Tarnowice (II)	0.56	0.27	0.28	13,428	0.41
Osada Jana (III)	0.64	0.24	0.41	5,891	0.08
Lasowice (IV)	0.49	0.31	0.17	5,123	0.05
Sowice (V)	0.68	0.43	0.26	1,170	0.20
Bobrowniki (VI)	0.20	0.10	0.10	3,947	0.46
Repty (VII)	0.44	0.28	0.17	1,804	0.20
Pniowiec (VIII)	0.36	0.27	0.09	1,121	0.14
Strzybnica (IX)	0.49	0.15	0.34	6,572	0.02*
Rybna (X)	0.15	0.00	0.15	2,014	0.05
Opatowice (XI)	0.29	0.14	0.14	703	0.48
Miasto	0.59	0.26	0.33	64,498	0.08

* $P < 0.025$.

Table 2 Comparison in all the city districts of persons placed in psychiatric hospitals as a consequence of schizophrenia and other psychoses in the years 1989-2002.

City districts	Stare Tarnowice	Osada Jana	Lasowice	Sowice	Bobrowniki	Repty	Pniowiec	Strzybnica	Rybna	Opatowice
Śródmieście (I)	0.01*	0.14	0.01*	0.36	0.00*	0.05	0.05	0.01*	0.001*	0.07
Stare Tarnowice (II)		0.23	0.28	0.29	0.002*	0.26	0.19	0.26	0.01*	0.17
Osada Jana (III)			0.14	0.44	0.001*	0.16	0.12	0.12	0.004*	0.12
Lasowice (IV)				0.20	0.01*	0.41	0.28	0.50	0.02*	0.23
Sowice (V)					0.0047*	0.19	0.14	0.19	0.01*	0.12
Bobrowniki (VI)						0.05	0.17	0.01*	0.32	0.33
Repty (VII)							0.36	0.40	0.04	0.28
Pniowiec (VIII)								0.28	0.12	0.40
Strzybnica (IX)									0.02*	0.23
Rybna (X)										0.23

* $P < 0.025$.

observed with regard to sex between "Śródmieście" (I) and "Strzybnica" (IX). Data contained in Table 2 graphically show the assessment indexes of persons hospitilized between "Śródmieście" (I) and "Stare Tarnowice" (II) and "Lasowice" (IV). The significant statistical differences were observed between more urbanized districts (I-V) to the less, including those with rural housing (VI. IX. X). The significant statistical differences were observed between I and II, III as well as III a V in women but not in men (Tables 3 and 4).

In the 20-29 age group the significant statistical differences with regard to sex apply to "Lasowice" (IV) with the majority of the hospitalized cases being men ($P = 0.0067$) between districts IV and V to districts II and IX (Table 5), but in women between "Pniowiec"(VIII) and "Lasowice" (IV) ($P = 0.0162$) (Table 5).

In men 30-39 age group there are significant statistical differences between "Śródmieście" (I) and "Strzybnica" (IX) ($P = 0.0093$). In men 40-49 age group there are significant statistical differences between "Śródmieście" (I) i "Bobrowniki" (VI) ($P = 0.0207$), but in women between "Śródmieście" (I) i "Osada Jana" (III) ($P = 0.0195$) and between "Lasowice" (IV) and "Osada Jana" (III) ($P = 0.0184$). In the 50-59 age group the significant statistical differences with regard to sex apply to "Śródmieście"

Table 3 Comparison between districts of the city of the number of men placed in psychiatric hospitals as a consequence of schizophrenia and other psychoses in the years 1989-2002.

City districts	Stare Tarnowice	Osada Jana	Lasowice	Sowice	Bobrowniki	Repty	Pniowiec	Strzybnica	Rybna	Opatowice
Śródmieście (I)	0.19	0.13	0.43	0.28	0.01*	0.35	0.36	0.01*	0.005*	0.19
Stare Tarnowice (II)		0.31	0.34	0.18	0.02*	0.50	0.48	0.04	0.01*	0.25
Osada Jana (III)			0.23	0.13	0.06	0.39	0.43	0.14	0.01*	0.31
Lasowice (IV)				0.27	0.02*	0.40	0.40	0.03	0.01*	0.21
Sowice (V)					0.0093*	0.24	0.25	0.02*	0.002*	0.14
Bobrowniki (VI)						0.06	0.09	0.24	0.08	0.38
Repty (VII)							0.48	0.13	0.01*	0.26
Pniowiec (VIII)								0.19	0.01*	0.29
Strzybnica (IX)									0.04	0.47
Rybna (X)										0.04

* $P < 0.025$.

Table 4 Comparison between districts of the city of the number of women placed in psychiatric hospitals as a consequence of schizophrenia and other psychoses in the years 1989-2002.

City districts	Stare Tarnowice	Osada Jana	Lasowice	Sowice	Bobrowniki	Repty	Pniowiec	Strzybnica	Rybna	Opatowice
Śródmieście (I)	0.01*	0.32	0.002*	0.16	0.001*	0.04	0.03	0.09	0.02*	0.11
Stare Tarnowice (II)		0.08	0.10	0.43	0.02*	0.18	0.11	0.26	0.14	0.24
Osada Jana (III)			0.01*	0.22	0.002*	0.06	0.05	0.25	0.04	0.14
Lasowice (IV)				0.28	0.18	0.47	0.25	0.05	0.40	0.42
Sowice (V)					0.10	0.29	0.17	0.33	0.25	0.30
Bobrowniki (VI)						0.26	0.45	0.01*	0.30	0.38
Repty (VII)							0.29	0.12	0.45	0.45
Pniowiec (VIII)								0.08	0.33	0.37
Strzybnica (IX)									0.09	0.19
Rybna (X)										0.48

* $P < 0.025$.

Table 5 Comparison between districts of the city of the number of men age 20-29 placed in psychiatric hospitals as a consequence of schizophrenia and other psychoses in the years 1989-2002.

City districts	Stare Tarnowice	Osada Jana	Lasowice	Sowice	Bobrowniki	Repty	Pniowiec	Strzybnica	Rybna	Opatowice
Śródmieście (I)	0.05	0.36	0.14	0.11	0.33	0.40	0.41	0.05	0.11	0.24
Stare Tarnowice (II)		0.05	0.01*	0.01*	0.27	0.29	0.15	0.27	0.22	0.32
Osada Jana (III)			0.30	0.20	0.26	0.34	0.48	0.04	0.09	0.22
Lasowice (IV)				0.32	0.14	0.24	0.40	0.01*	0.06	0.18
Sowice (V)					0.10	0.17	0.29	0.01*	0.03	0.13
Bobrowniki (VI)						0.47	0.32	0.15	0.15	0.27
Repty (VII)							0.37	0.16	0.14	0.26
Pniowiec (VIII)								0.08	0.09	0.21
Strzybnica (IX)									0.29	0.37
Rybna (X)										

* $P < 0.025$.

(I) (P = 0.0003) and for all town. In the men, there are significant statistical differences between "Rybna"(X) and "Śródmieście" (I) (P = 0.0120) and "Bobrowniki" (VI) (P = 0.0236), but in women between "Śródmieście" (I) and "Strzybnica" (IX) (P = 0.0181). In men and women older than 60 there are significant differences with regard to sex apply to "Strzybnica" (IX) (P = 0.0191) with more number of men. In men, the significant differences were obtained between "Śródmieście" (I) and "Stare Tarnowice" (II) (P = 0.0138), between "Sowice" (V) and "Stare Tarnowice" (II) (P = 0.0148), between "Pniowiec" (VIII) and "Stare Tarnowice" (II) (P = 0.0129), but in women between "Śródmieście" (I) and "Stare Tarnowice" (II) (P = 0.0005), "Bobrowniki" (VI) (P = 0.0119), "Strzybnica" (IX) (P = 0.0018), between "Lasowice" (IV) and "Strzybnica" (0.0248), and between "Sowice" (V) and "Strzybnica" (IX) (P = 0.0089).

4. Discussion

As the results of the study show the majority of schizophrenia-related problems appear as a consequence of not only living in the inner-city areas "Śródmieście" (I), but also as a consequence of the living in public housing that is not as attractive as it once was and often in the vicinity of either closed industrial plants or of plants that have laid workers off. Therefore in districts with townhouses where there is a marked predominance of public housing (projects) such as "Śródmieście" (I) "Sowice" (V), and "Osada Jana" (III), these problems appear more often. Whereas townhouses, even those requiring large amounts of renovation, can give dwellers a greater sense of belonging to a local community, but also of greater economic safety connected with lower rents. Indeed, there are situations where health problems predominate in the inner city district "Śródmieście" (I), but it should be noted, for example, that the highest hospitalization indexes for men as a result of schizophrenia appear in the "Sowice" district, which has a post-industrial character with dilapidated buildings, long rows of townhouses or project blocks with livestock not uncommonly nearby. In studies conducted in Baltimore and the State of Maryland three groups of data were selected: 1) urban, 2) suburban and 3) rural. The frequency of schizophrenia decreases relative to the order listed above [13]. Widerlöw et al. studied urban, suburban, and city districts. The studies dealt with the Stockholm from its outskirts to its city center [14]. The indexes for the occurrence of mental illness in specific areas were as follows: for rural areas 3.4/1000, for suburban 5.6/1000 and for urban 6.6/1000. Among those ill with schizophrenia men clearly dominated in relation to women in rural areas, then in urban and suburban. In studies relating to the occurrence of schizophrenia in the district of Camberwell, in south London, carried out from 1965 to 1984, comparing a group of non-psychotic patients and conducted according to place of birth and occupation. In comparison to the control group those suffering from schizophrenia more frequently were born in socially disadvantaged areas [15]. This also corresponds with the theory of schizophrenia arising in the poorest persons from the lowest social classes, which may have a connection with a lack of supporting factors in the fetal stage as well as in early development. Faris and Dunham in their classic study on mental disturbances in city areas found that there is an increase in the occurrence of schizophrenia in inner city areas and a decline in all directions the further from the center [1]. In 1965 Dunham (1969) no longer found this dependency in Detroit, which sent the author in the direction of the phenomenon of social selection in contrast to his earlier hypotheses of social conditions in the phenomenon of the dominance of mental illness in the inner city areas [16]. In subsequent work dealing with Chicago Levy and Rowitz did not find a marked pattern for the previous results of Dunham's work in the placement of those suffering from schizophrenia

[17]. The areas of more frequent occurrences of schizophrenia were spread through out the city. These authors suggest that Faris and Dunham's observations of the ill were marred by a lack of a centralized psychiatric hospital, which is why there is a dearth of data on ill persons treated privately and in clinics. Levy and Rowitz observe that the construction of highways and the development of industry have altered the social order of persons suffering from schizophrenia more differently than they did when Faris and Dunham first conducted their pioneer studies [17]. In studies conducted in Nottingham, cases of schizophrenia occurred more frequently in the most impoverished parts of the city [18]. Dauncey et al. in their observations fund that the mobility of those afflicted with schizophrenia took place before they began psychiatric treatment for the first time. These observations apply to tests on new cases of schizophrenia in the years 1978-1980 [19]. The majority of the patients were male in the general age group of 15-34. As to the question of sex, a few studies were carried out, showing a higher frequency of schizophrenia among men than among women in urban areas [20, 21]. Our studies in this age group gave similar results, whereas the hospitalization of women occurred in age groups higher than 40. In analytic studies dealing with the factors connected with the risk of the occurrence of schizophrenia, it was found that urbanization is a major factor in the risk for both people born in the cities as well as to those who had already moved there at the time of their hospitalization [9, 22]. Sundquist et al. studied the relation between the level of urbanization of less densely populated areas and the appearance of psychosis and depression [23]. They also took civil status, social-economic status and education level into consideration in their study. They found a significant statistical differences, particularly in the appearance of psychosis in relation to population density in both men and women.

5. Conclusions

It indicated that patients more frequently lived in more urbanized and in postindustrial districts of town as well as men aged 20-29 years in postindustrial districts. It should be mentioned that more of patients placed the inner city, but also in postindustrial district and in districts with city housing projects.

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Space and Temporal Distribution Analysis of Interictal Spike in Epilepsy

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Abstract: Stereo-electroencephalography (SEEG) is the main investigation method for pre-surgical evaluation of patients suffering from drug-resistant partial epilepsy. SEEG signals reflect two types of paroxysmal activity: ictal activity and interictal activity or interictal spikes (IS). The relationship between IS and ictal activity is an essential and recurrent question in epiletology. In this paper, we present a distributed and parallel architecture for space and temporal distribution analysis of IS, based on a distributed and collaborative methodology. The proposed approach exploits the SEEG data using vector analysis of the corresponding signals among multi-agents system. The objective is to present a new method to analyze and classify IS during wakefulness (W), light sleep (LS) and deep sleep (DS) stages. Temporal and spatial relationships between IS and seizure onset zone are compared during wakefulness, light sleep and deep sleep. Results show that space and temporal distribution for real data are not random but correlated.

Key words: Epilepsy, sleep stage, stereo-electroencephalography (SEEG), interictal spike, signal processing, multi-agent system.

1. Introduction

Epilepsy is a chronic disease, a consequence of many and various causes. It is a complex pathology that the symptoms exteriorized by the patient depend directly on the cerebral structures involved in the propagation of the seizure. Around 60% epilepsy are partial and 20-25% of partial epilepsy are drug-resistant, and a surgical treatment can be necessary [1]. Therefore the spikes space and temporal distribution can help in pre-chirurgical evaluation.

Among the investigation methods allowing determining the origin of the paroxysmal discharges, stereo-electroencephalography (SEEG) is essential. This standard method (SEEG) makes it possible to directly collect intracerebral signals of an excellent temporal resolution, which inform about the electrical activity of the concerned structures as shown in Fig. 1. SEEG signals reflect two types of paroxysmal activities: ictal activity and interictal activity (interictal spikes). The interictal spikes (IS) are observed at 1 percent of the non-epileptic subjects and around 60-90% of the epileptic subjects (Bourien, Schaul and Binnie) [2-4]. They are a complementary source of information in the diagnosis and localization of epilepsy. They are characterized by a brief initial phase, sharp and strong amplitude and occur as transitional events (< 1 second). The interictal spikes occurrence is higher than the seizures frequency. They appear in wave but sometimes they appear isolated. The IS are classified in spikes or waves according to their duration, between 20 and 70 ms, 70 and 200 ms respectively. Their amplitude, significantly higher than those background activity's, features them. The clinical characterizations of the IS are based on their density, morphology, and topography. The study of relationship between interictal and ictal activity brings a significant complement in the pre-surgical

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evaluation of patients suffering from drug-resistant partial epilepsy. The relationship between the IS and the seizures are badly known, in spite of the fact that these two types of paroxysmal activity are qualitatively taken into account in the visual analysis of SEEG signals [5].

In this paper, the authors analyzed the dynamics of interactions between different explored cerebral areas during pre-surgical evaluation. The authors present a distributed and parallel architecture for spatio-temporal distribution analysis of IS through a distributed and collaborative approach.

One major step is to design a distributed platform able to follow the dynamics of cerebral structures networks, which are co-activated in order to show the various reorganizations of cerebral structures interactions following various states of patients. We can answer some of following questions: how is it possible to model and simulate situated interactions between cerebral structures? How to simultaneously provide views of locally and globally situated phenomenon? Is it possible to define a detailed spatiotemporal representation in a vector environment?

These issues will be addressed in the next section.

2. Materials and Methods

In this section the authors explain the method to design platform for the analyze space and temporal distribution of IS. This method consists of transposing the vector EEG signals processing in a distributed and collaborative vector platform. Each channel (SEEG signal) is associated with an autonomous process and all entities system cooperate to analyze the whole system. Our approach is based on cooperative and auto-organized mechanisms at the local level (mono-IS) and global phenomenon analysis (multi-IS) as explained in section 2.3.

2.1 SEEG Signal Recording

Stereo-electroencephalography (SEEG) method is shown in Fig. 1. This method of investigation used in epilepsy, make it possible to better understand the mechanisms involved of the initiation of the paroxystic discharges in a given subset of cerebral structures and their propagation to other structures. SEEG signals recorded show sharp variations of ictal activity and of interictal activity, illustrating the various cerebral activities. The method uses a high temporal resolution to provide the accurate localization of distinct interictal activities in each explored structure. SEEG signals inform various peculiarities of a structural entity, an organ or a system. Their interpretation is multi-factor and therefore, it isn't easy; the normality for a given modality varies from subject to subject according the age, the history and protocols of examinations (rest, physical stress, stimulations, etc.).

2.2 Related Works

Many methods have been developed to establish functional connections between brain areas in the frequency domain by using EEG or MEG recordings [6]. Additionally numerous methods have been made to detect a reliable spike, some approaches measure the "sharpness" of the EEG signal [7, 8], while other use nonlinear modelling method [9] or wavelets and

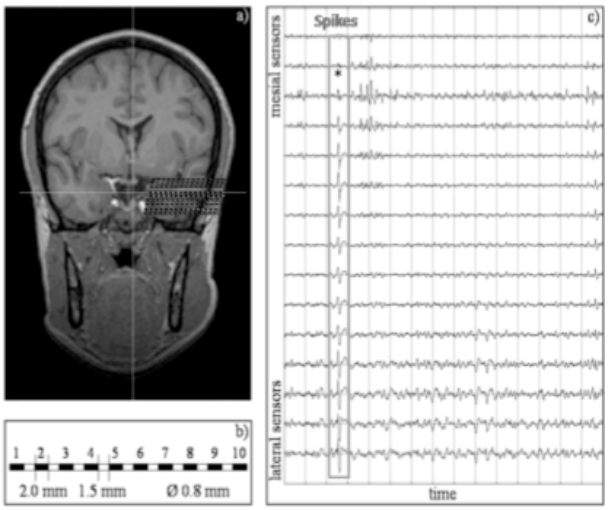

Fig. 1 Intracerebral EEG and interictal spikes distribution. a) MRI showing the implementation of electrodes. b) Plan of an electrode with staged contacts. c) Example of recording SEEG.

time-frequency approaches [10] to characterized the occurrence of IS.

Valenti and Cazamajou [11] propose a data mining classification technique to build an automatic detection of IS. Their method didn't however take into account the precise signal form (morphology). In spatiotemporal analysis field, Badier and Chauvel [12] proposed a spatiotemporal mapping technique to study the IS during the pre-surgical evaluation of their epileptogenic zone prior to surgery. Asano's work [13] shows that overall spike frequency may be increased during the sleep, but the spatial distribution of spike frequency appears similar during wakefulness and sleep in children with focal seizures. In conclusion, the authors have variability of results, heterogeneity of signals and methods (scalp/intracerebral EEG, automatic/manual detection …) for interictal spikes analysis.

2.3 Method Description

2.3.1 Global Description

The method of distributed signal processing is not a well-explored area, however numerous solutions allowing to better analyze problem of space and temporal distribution have been proposed. We characterize the topological properties of the cerebral networks by using a theoretical graph approach following Watts and Strogatz [14]. We consider each depth-EEG channel as an object (process) with a dynamical state, which depends on its cerebral structure activities. The cerebral structures network is considered as a system that can generate, on a temporal sliding window, interictal spikes (IS) in the form of various combinations. Let N be the number of selected SEEG channels. We consider SEEG signals recording at a vector signal $S(t) = [S_1(t) \dots S_N(t)]$ observed on an interval $[0, T]$. The method consists of: (i) characterizing IS on each SEEG channel $S_i(t)$; (ii) determining the temporal relations between the various channels; (iii) studying the organization of subsets of co-actived structures (SCAS); (iv) finally analyzing spatiotemporal distribution of IS. We further analyze statistically the SCAS and we perform a global representation of SCAS dynamics as described below.

We note $i = 1, \dots, N$ the channel index, T is the observation duration, n_i is the number of spike detected on channel i, $j = 1, \dots, n_i$ is the index of detected event and $t_{i,j}$ is detection time of spike j on channel i. Our approach associates to each signal $S_i(t)$ (Fig. 2), an agent (noted C_i) where is implemented the local treatment for channel i.

The implemented approach is situated, reactive, cooperative and decentralized. Calculations, control mechanisms and signal processing algorithms are spatially distributed in the various system entities (agents). Each process (channel) has a local memory, and is able to communicate with each other by sending and receiving messages. Messages can be ordering in multicast or point-to-point communication according to the sender choice. All process runs in parallel and in a concerted way. Process Manager is used to coordinate processing. The method proceeds in four steps: (i) automatic detection of monochannel interictal spikes (mono-IS); (ii) collaborative formation of subsets of co-activated structures (multichannel interictal spikes multi-IS); (iii) automatic extraction of statistical co-activated structures; (iv) global representation of spatiotemporal distribution of IS.

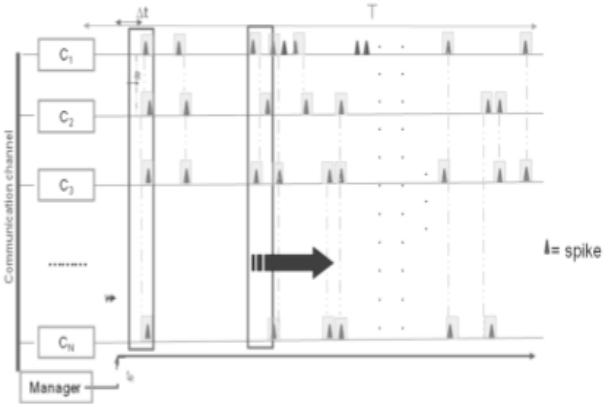

Fig. 2 Multichannel event formation, distributive collaborative approach.

2.3.2 Detection of Monochannel Interictal Spikes (mono-IS)

The automatic detection of mono-IS is made as follows. At each sample time in each channel, the mean value of squared modulus of a wavelet filter bank outputs is calculated. The amplitude of this quantity q(t) is random with high mean value during spike and low mean value during background SEEG. In the second stage, a Page-Hinkley algorithm [2, 16] was used to automatically estimate time instants corresponding to abrupt changes of q(t). Fig. 3 shows the interictal spike detection for each channel.

2.3.3 Formation of Subsets of Co-Actived Structures (SCAS or multi-IS)

The authors consider that two processes (channels) are in interaction if they detect IS at the same time. The collaborative approach is proposed to extract SCAS. The analysis is made by cycle that is explored in parallel and synchronous way on a sliding window *w*, which has a size of Δt. A cycle comprises three successive steps: (i) identification of the reference time of cycle tR, which corresponds to the estimate time to first spike detected after the previous cycle; (ii) sliding the window at this time (tR); (iii) extraction SCAS by grouping all the channels, which detected a spike in this window (w). SCAS can be constituted by one or several structures (Fig. 4).

To group together cerebral structures, which detect a spike at the same time, each channel informs the other channels of its detection on the current sliding window (w). The size of the window determines the time delay below which we consider two detections of two different channels as co-actived.

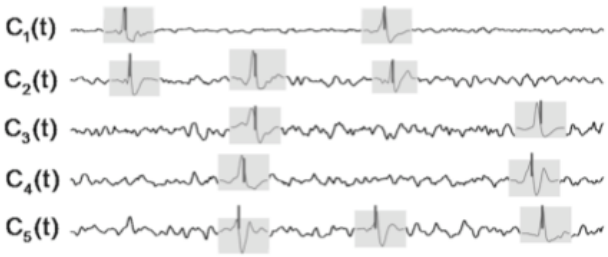

Fig. 3 Monochannel detection of interictal spikes (mono-IS).

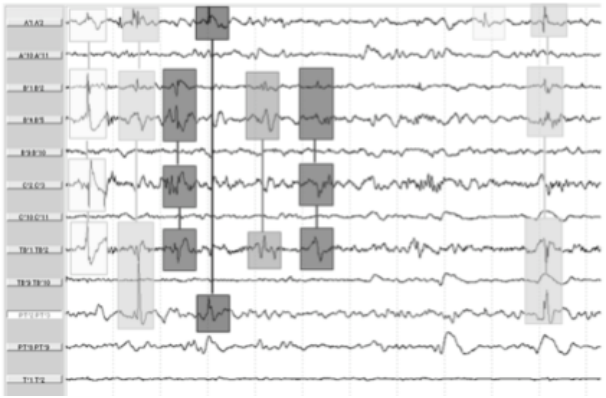

Fig. 4 Multichannel event formation (multi-IS or SCAS), classification and counting in distributive collaborative approach developed in Madkit.

2.3.4 Simulation and Statistical Analysis

The density distribution of spikes is one of the most important aspect considered in IS analysis. To show that this distribution obeys on specific rules depending on the patient, the authors tried to simulate a random distribution following an exponential law, called Poisson distribution. The simulation consists of regenerating the same number of spikes on each channel (each agent signal) randomly. The times ($t^s_{i,j}$) where the simulated spikes occur, are determined by equation (1):

$$t^s_{i,j+1} = t^s_{i,j} + \frac{1}{\lambda_i}\log(Random) \quad (1)$$

where $0 < Random < 1$ and λ_i is the average of interval between spikes. For each channel C_i we generate a simulate channel $C_{i,s}$.

The statistical analysis enumerates number of detections, detection times and probability of apparition for each SCAS. The results of statistical analysis and simulation are presented in section 5.

2.3.5 Graph Proprieties of Subset of Co-Actived Structures (SCAS)

Milgram who studied the structure of social networks [14], has first identified the concept of "*Small World Phenomenon*" in 1967. The study of these networks was revived and extended in many other areas by Watts and Strogatz [15]. Our work tries to explain whether multichannel interictal spikes

distribution can be considered as a "*Small World Phenomenon*" or not. The structural properties of small word network are quantified by two parameters: the characteristic path length L and the clustering coefficient C. Characteristic path length L is the number of vertex in the shortest path between two vertices, the value of L is averaged over all pairs of vertices. Clustering coefficient C takes values in the range [0, 1] and it measures the tendency of the network to form highly interconnected regions.

Watts and Strogatz mathematically formulated "*Small World Phenomenon*" graphs [14].

The clustering index C for vertices introduced by Watts is defined as follows [15]. Given a vertex v, which has K_V neighbours in the network, its vertex clustering coefficient C_V is defined by equation (2):

$$C_V = \frac{2 \times r_V}{k_V(k_V - 1)} \tag{2}$$

with r_V is the amount of the vertices link connected to v (neighbours of v).

where $r_V = \sum e_{ij}, i \neq v; j \neq v$; i and j are directly connected to v and e_{ij} define the link of i to j.

C_V measures the "probability" that two co-activated structures with another structure can be co-activated between them. The clustering index C of a graph is given by the following equation (3):

$$C = \frac{1}{N}\sum_{v=1}^{N} C_V \tag{3}$$

The authors use Floyd's algorithm to evaluate the shortest path between all pairs of vertices.

5. Results

In order to analyze and simulate the IS distribution, the system is implemented in "Madkit" (Multi-agent development kit), a distributed and generic platform, developed by Gutknecht and Ferber [17]. To present preliminary results, the data used in this study were recorded from 4 patients (BRE, MAL, PAS, LAU) suffering from a temporal lobe epilepsy (TLE). Temporal and spatial relationships between IS and seizure onset zone are compared during wakefulness, light sleep and deep sleep.

The Fig. 5 represents the percentage of seizure occurring for the four patients during wakefulness and sleep stages.

The Table 1 presents the mean spike rate per hour during the different stages. As depicted in this table, the spike rate increase with sleep depth. The global spike rate increases from wakefulness towards sleep (W→LS→DS) for all patients.

Fig. 6 shows the maximum number of cerebral structures involved during the three stages (W, LS and DS). Fig. 6 presents also the results on simulated data for the first patient (BRE). Simulation consists of generating the same number of spikes in each channel by random distribution model. These results show that space and temporal distribution for real data are not random but correlated; the distribution follows a well-determined law.

Fig. 7 represents graphs that model the cerebral structures interactions networks at any time and figure 8 is a global representation of analysis.

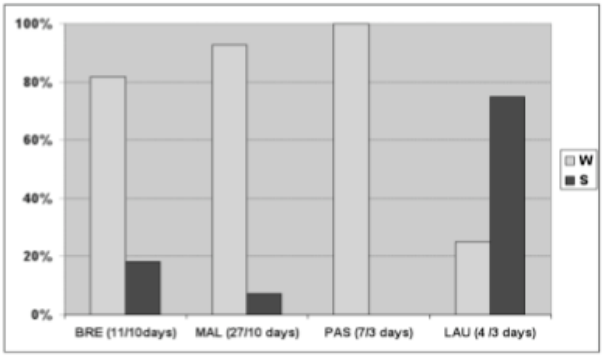

Fig. 5 Percentage of seizures occurring for patients during wakefulness (W) vs. sleep (S). Three patients are seizures mainly during W, one patient is seizures mainly during S.

Table 1 Mean spike rate (/h) during wakefulness (W), light sleep (LS) and deep sleep stages (DS).

Stage type	Patient 1 (BRE)	Patient 2 (MAL)	Patient 3 (PAS)	Patient 4 (LAU)
W	2,542	2,368	2,062	678
LS	5,052	5,062	6,027	1,291
DS	11,490	14,565	9,133	4,582

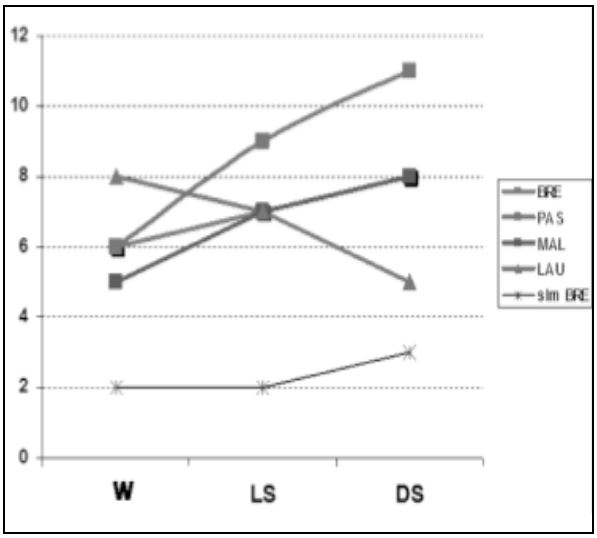

Fig. 6 Maximum number of cerebral structures involved during wakefulness (W), light sleep (LS) and deep sleep (DS) stages.

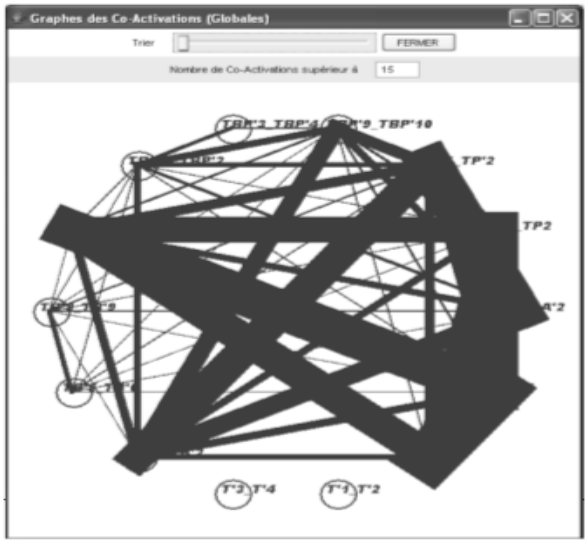

Fig. 8 Global graph shows frequency of intracerebral structures generate EPICs at same time.

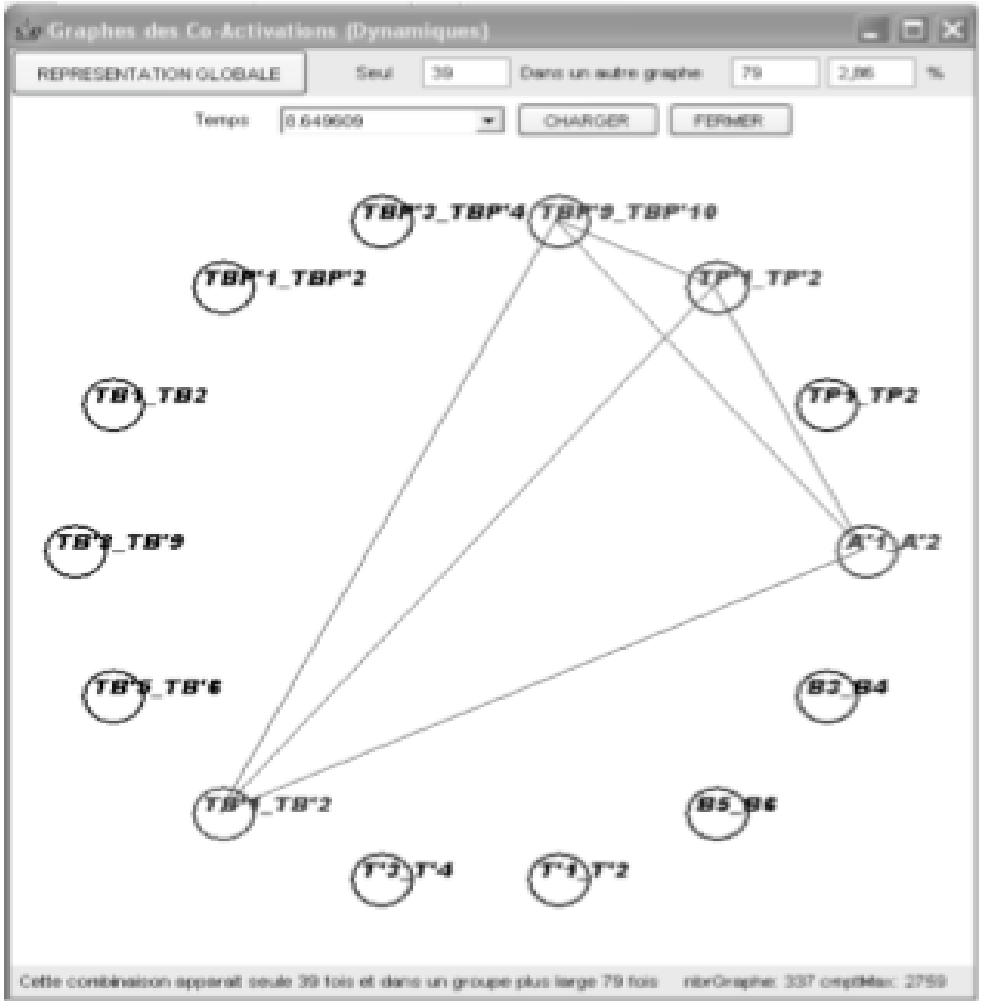

Fig. 7 Representation of one multichannel IS (SCAS) detected at t = 8.649609 s included left structures: internal temporal pole (TP'), internal and external entorhinal cortex (TB') and amygdale (A').

Table 2 Comparison between real and simulated data for first patient 2 (MAL).

Stage	Data type	IS number	SCAS number	N_1	N_2	N_3	N_4	N_5	N_6	N_7	N_8
Wakefulness (W)	Real	2368	107	10	30	30	21	3	7	0	3
	Simulation	2368	39	11	20	0	0	0	0	0	0
Light sleep (LS)	Real	5062	90	10	29	25	15	7	0	0	0
	Simulation	5062	58	9	30	17	1	0	0	0	0
Deep sleep (DS)	Real	14565	119	10	32	38	24	10	1	1	0
	Simulation	14565	81	10	37	33	1	0	0	0	0

N_n is the number of different SCAS included n structures.

The multi-IS (SCAS) event in Fig. 7 appears only 39 times and it is included in another graph 79 times. The platform offers access to every mono-IS event and/or multi-IS event identified during analysis. This representation also offers access to statistical information of each event: the number of times where it appears alone, the number of times where it appears included in another graph (other event). Statistical information concerns the probability of occurrence of each event, the number of events and the number of different events.

The platform can also automatically generate simulated data according to the model presented in section 2.3.4. We applied the detection, formation and counting SCAS events on simulated data and the results show that the probability of having two brain structures (simulation) that discharge at the same time is almost zero (Table 2). This proves that the organization of multi-IS event is not random.

A "*Small World Phenomenon*" has two properties: (1) its L is much smaller than a given value noted L_{random} which is the characteristic path length of a random graph with the same number of vertices; and (2) its C is much larger than a given value noted C_{random}, the clustering coefficient of a random graph ($L << L_{random}$ and $C >> C_{random}$).

The authors' results show that cerebral interictal spikes networks present small word characteristics. The probability of observing several cerebral structures generating EPICs in same time is almost zero for the simulated data. This means that $L_{random} \to \infty$ and $C_{random} \to 0$ ($r_V = 0$).

Table 2 also shows that the probability of observing several cerebral structures (SCAS) generating EPICs in the same time is smaller for the simulated data and important for the real data. The random law for simulated data shows that the underlying phenomenon is not random.

6. Conclusions

In this paper we have presented a new method to analyze and classify interictal spikes during wakefulness, light sleep and deep sleep. The results show that IS distribution is not random and sleep may alter the overall frequency of interictal spike. The authors' approach makes the following contributions. Firstly, it allows the designer for a distributed and parallel application to specify the desired temporal behaviour of system, Secondly, it shows local and global representation of IS distribution for various states of patients. Thirdly it measures the relationship between IS and ictal discharges in human drug-resistant partial epilepsy. These results indicate that an analysis of sleep causes changes in depth spike activity. This can be helpful in improving predictions concerning epileptogenicity.

The perspectives of this work concern a more significant exploitation of the potentialities of the cooperative agents approach by integrating larger cohort of patients (lateral temporal epilepsy, extra-temporal epilepsy and so on). It would be also interesting to analyze the morphological spike.

Acknowledgments

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Extraprofessional and Occupational Risk Factors for Colorectal Cancer

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Abstract: Colorectal cancers (CRC) account for frequent and serious cancers which result from the interaction between individual genetic factors and environmental factors, and in particular widely studied nutritional ones. The role of other occupational factors remains a controversial subject. The objective of this study is to evaluate the possible impact of occupational factors on the risk of developing CRC. **Materials and Methods:** This is a retrospective case-control study. The cases and the control group were enlisted in the general surgical ward of Farhat Hached Teaching Hospital of Sousse (Tunisia) during the period extending from 2004 to 2008, and they were age and gender-matched. The data were analyzed using SPSS 11.0 software with a signification threshold fixed at 5%. A univariate analysis was carried out as well as a multiple binary logistical regression. **Results**: During the period of the study, 40 cases of colorectal cancers have been colligated including 28 men and 12 women with a sex ratio of 0.43. The average age of the cases was 61.55 ± 13.3 years and 60.40 ± 12.84 years for the control group, with a non significant difference (P = 0.69). The univariate analysis has objectivized significant associations between colorectal cancer and the housing conditions, the neoplastic and digestive family history, the occupational activity sector, exposure to pesticides, and lack of periodic medical supervision. After logistical regression, the occurrence risk of CRC was significantly associated with: alcohol and smoking (ORa = 3.43; Pa = 0.05), meat consumption (ORa = 3.34; Pa = 0.03), exposure to pesticides (ORa = 20.44; Pa = 0.012) and lack of periodic medical supervision (OR = 7.45; P = 0.004). **Conclusion:** The occupational risk factors might play a role in the etiopathogenesis of colorectal cancers. With regard to our study, pesticides seem to be most implicated and necessitate suitable preventive measures. Nevertheless, it seems useful to multiply the studies to a much larger scale in order to further explore such relationship and to further reinforce the prevention of such serious disease.

Key words: Colorectal cancer, occupational risk factors, extraprofessional factors, epidemiology.

1. Introduction

Colorectal cancers (CRC) represent the fourth most common cancer in the world, and their incidence has been constantly increasing over the last years in the world except in the United States [1, 2]. It is the second most common incident form of cancer in Europe in 2006 [3] and is in the United States, the third leading cause of cancer death in each sex and second overall in men and women combined [4]. At current rates, approximately 5%-6% of individuals will develop a cancer of the colon or rectum within their lifetime [4]. Despite important progress in the management of this cancer, cure is obtained only in one out of two cases [5]. Many studies evaluated the role of genetic, dietary and behavioral factors in the genesis of CRC; however, those which are interested in the environmental factors and in particular the occupational ones remain rare and often indicate contradictory results. In fact, colorectal cancers are not often attributed to a particular occupational exposure; yet, a higher risk

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was noticed among workers exposed to asbestos, wood dust, metal oils, some pesticides and organic solvents [6, 7]. Thus, the effect of occupational factors remains poorly identified. Yet, their role does not seem to be insignificant [4] and their identification is essential to any presentation approach aiming at reducing the incidence of such terrible cancer.

In order to understand better this phenomenon and reduce its extent, it is necessary to study the factors implicated in the carcinogenesis and the etiopathogenesis of CRC.

In this work, we propose to evaluate the extraprofessional and occupational factors influencing the risk of developing CRC.

2. Materials and Methods

A retrospective case-control inquiry has been conducted. The cases were patients admitted to the General Surgical ward of Farhat Hached Teaching Hospital of Sousse (Tunisia) during the period extending from Jan. 1, 2004 to Jan. 31, 2008 for different complaints. In these patients the diagnosis of colorectal cancer (CRC) was made and histologically confirmed. The eligibility criteria were: the practice of an occupational activity at the time of the inquiry or the previous practice of an occupational activity during at least one year, and the consent to answer the questionnaire.

As far as the exclusion criteria are concerned, they were: age younger than 18 years (subjects supposed to be professionally inactive), and patients who died before the inquiry and whose questionnaire could not be filled by their relatives.

Concerning the control-subjects, they were enlisted among the professionally active patients or those who have been active for at least one year, and they were admitted to the General Surgical ward of Farhat Hached Teaching Hospital of Sousse (Tunisia) for other non-cancerous pathologies during the same period as the cases. They were age and gender-matched.

As for data collection, we have prepared a questionnaire specifying:

- The anthropometric and social characteristics of the patient;
- Lifestyle and habits (smoking, alcohol, diet, physical activity).

Red meat and plant fibers consumption was subdivided into three categories. It was considered: low if the consumption is ≤ 1 time/weak, average if it is between 2 and 3 times/weak and frequent if it is ≥ 4 times/weak.

Regarding water consumption, it was considered: low if the daily consumption is < 1 L, average if it is between 1 and 2 L, and frequent if it is > 2 L. Regarding physical activity, we have taken account mainly sports practice or not.

The socio-economic level was arbitrarily evaluated and considered as follows: high if the questioned person lives in a villa and owns a car, and low in case of lack of villa and car.

- Occupational history: we have looked for the possible occupational exposure to factors incriminated, in the literature, in the occurrence of colorectal cancers namely: mineral oils, metal dust, plastics/resin, pesticides, asbestos, cement dust, textile dust, abrasive products, fiberglass, solvents, paints, wood dust, exhaust gas, soot, ionizing radiation, and electromagnetic waves. A semi-quantitative estimation of exposure to these factors has been done taking into consideration: the frequent daily exposure, duration and level of exposure evaluated and rated as follows:
- Normal base level: 0;
- Slight exposure: 1;
- Moderate exposure: 2 (direct contact with the product);
- Important exposure: 3 (very close handling).

An exposure index (I) for each substance incriminated in the literature was calculated in order to roughly evaluate the intensity of exposure of every patient to the substance. The exposure index (I) is the

result of multiplication of number of hours of exposure per day (H), annual exposure duration (D) and exposure level (N). → I = H × D × N.

Medical data (personal and family medico-surgical history, circumstances of discovery of the disease, the made diagnosis and histological confirmation, received treatment) have been collected from the medico-surgical file of every admitted patient.

For all the missing data, a questionnaire complement was realized by direct contact with the patients or their parents according to the following modalities: history-taking on the occasion of readmission to the ward or on the occasion of a surgery outpatient consultation during the regular follow-up of patients; direct contact with patients or the members of their families by visiting their homes, and telephone contact with the patients or members of their families.

The data collection and the interview were performed by the same person responsible for the inquiry. The data entry and the analysis of results were realized by SPSS 11.0 software.

At first, we carried out a descriptive analysis of the cases and control groups: for this reason, we represented the qualitative variables by the percentage and the quantitative variables by the average with the standard deviation when the distribution was Gaussian, otherwise, by the median and the extremes. Then, we carried out a comparative study. As for the univariate analysis, Chi-squared test, Fischer's exact test, and Student's *t*-test were used. The factors deducted by the univariate analysis were introduced in a multiple binary model of logistic regression in order to identify the independent factors influencing the risk of developing colorectal cancers. The significance level was fixed at 0.05.

3. Results

111 cases of colorectal cancers were brought to the General Surgical ward of the Teaching Hospital of Farhat Hached of Sousse (Tunisia) between the beginning of 2004 and the end of 2008 (i.e. 27 cases/year) divided into 60 men and 51 women, the sex ratio being 1.17.

Eight files tagged colorectal cancers were abandoned because of lack of occupational history and exposures. A 10-year-old patient (mesenchymal tumor of the colon) was excluded from our study (professionally inactive). 16 patients died before our inquiry and there were no contact details in the files permitting to contact their families. 46 files were incomplete after unanswered phone calls, and mails with no reply.

Thus, in all: 40 files could have been used.

3.1 General Characteristics

The forty patients carrying CRC were divided into 12 men and 28 women with a sex ratio of 0.43. The average age of cases was 61.55 ± 13.34 yeas versus 60.40 ± 12.84 years for the controls with a non significant difference ($P = 0.69$). For the majority of cases and controls as well, the primary (elementary) education level was the most represented (37.5) with no significant difference among both groups. Colorectal cancer occurrence risk was higher in those who are uneducated OR = 1.6; IC at 95% = 0.6-4.5 ($P = 0.32$). The majority of cases and controls had low socioeconomic level (92.5% versus 87.5% respectively). Alcohol and tobacco consumption was found in 30% of the cases versus 15% in controls with no statistically significant difference ($P = 0.1$). The risk of occurrence of CRC was higher in case of alcohol and tobacco use with an OR = 2.43; IC at 95% = 0.8-7.29. The weekly consumption of red meat was considered average to frequent in 55% of the cases versus 40% in the controls with a statistically no significant difference ($P = 0.18$). Concerning the risk of occurrence of CRC, it was higher in the subjects consuming much red meat weekly: OR = 1.8; IC at 95% = 0.75-4.45.

Regarding vegetable fibers intake, it was low in 15% of the cases versus 27.5% of the controls with a

statistically no significant difference (P = 0.17). The daily water consumption was low in 12.5% of the cases versus 25% of the controls with a statistically no significant difference (P = 0.15). Regular physical activity was exercised only by 12.5% of the cases versus 7.5% of the controls with a statistically no significant difference (P = 0.7).

3.2 Medico-Surgical Data

12.5% of the cases presented with family history of gastrointestinal cancer (3 cases of colon cancer, one case of gastric cancer and one case of pancreatic cancer) and 5% of cases of extra-digestive cancers (2 cases of endometrial cancer).

No case of polyposis coli or malformation syndrome was noticed in our sample.

No family history of cancer was reported in the controls with a statistically significant difference (P = 0.018).

Personal digestive histories were noticed in 50% of the cases versus 30% of the controls with a statistically no significant difference (P = 0.15).

Colic localization was the most frequent being 43% of the cases (n = 17), followed by rectal localization: 37% of the cases (n = 15) and the recto-sigmoid junction 20% of the cases (n = 8).

Moderately primary differentiated Lieberkuhnien adenocarcinoma was the most frequent histological type accounting for 57.5% of the cases. Locoregional metastases were noticed in 37.5% of the cases and liver metastases in 10% of them.

The majority of our patients have undergone a radical treatment (87.5%). The course of treatment was favorable for most of our patients (57% of the cases) and stabilization was observed in 25%.

Occupational characteristics:

Most of the questioned cases were working in the agriculture sector (32.5%) versus 12.5% of the controls with a statistically significant difference (P = 0.007). Concerning the occurrence risk of CRC, it is higher in those who are working at present or previously in the agriculture sector: OR = 4.84, IC at 95% = 1.4-16.4. The building and public works sector ranked second among work sectors for our cases (10%) followed by the metallurgy and car industries occupying both the third position (2.5% of the cases for each). Most of the cases as well as the controls were represented by workers (30% versus 25% respectively) and semi-skilled workers (42.5% versus 42.5 respectively) with no statistically significant difference (P = 0.44).

Average seniority at the workstation was of 27.46 ± 15.74 years and 27.07 ± 13.12 years respectively for both cases and controls with no statistically significant difference (P = 0.9).

More than a half (57.5%) of the cases was exposed to products implicated in the genesis of CRC versus 37.5% of the controls with no statistically significant difference (Table 1) (P = 0.073). The occurrence risk of CRC was higher in case of product handling implicated in the genesis of this cancer with an OR = 2.25; IC at 95% = 0.92-5.52.

Table 1 Distribution of cases and controls according to exposure to products implicated in the genesis of CRC.

Products	Cases		Controls	
	Number	Percentage	Number	Percentage
Pesticides	10	25	1	2.5
Cement	4	10	4	10
Metal dust	1	2.5	-	-
Asbestos	1	2.5	-	-
Wood dust	1	2.5	-	-
Textile dust	1	2.5	6	15
Solvents and paints	2	5	5	12.5
Electromagnetic waves	1	2.5	-	-
Car exhaust	1	2.5	-	-

Among the products involved in the genesis of colorectal cancers, pesticides were the most represented in the cases: one quarter of the cases was exposed to pesticides versus 2.5% only of the controls with a statistically significant difference ($P = 0.003$).

The average index of exposure to pesticides was of 375.92 ± 482.44 for the cases versus 141.3 ± 230.81 for the controls with a statistically significant difference ($P = 0.007$).

The occurrence risk of CRC was higher in those who were exposed to pesticides with an OR = 13; IC at 95% = 1.57-107.2.

Most of the cases (90%) and controls (82.5%) did not use means of protection during their occupational activities with no statistically significant difference ($P = 0.33$). The occurrence risk of CRC was higher in those who did not use means of protection OR = 1.91; IC at 95% = 0.52-7.1.

There were only 10% of the cases (4 patients) and 40% of controls (16 patients) who received during their occupational activities a periodic medical surveillance with a statistically significant difference among both groups ($P = 0.02$).

The occurrence risk of CRC was higher in those who did not receive a medical surveillance: OR = 6; IC = 1.78-20.14.

In the univariate analysis, comparing the group of patients having CRC with the controls group with regard to the different variables allowed to identify three extra professional factors significantly influencing the occurrence risk of CRC in a statistically significant way, and they are: habitat type, neoplastic family history, and digestive and neoplastic personal history (Table 2).

Five occupational factors significantly influenced the occurrence risk of CRC in the univariate analysis and they were: working in an agriculture sector, the occupational exposure to products implicated in the literature in the genesis of CRC, the occupational exposure to pesticides, the index of exposure to pesticides and the periodic medical surveillance (Table 3).

After multiple binary logistic regression, four variables were significantly associated with the occurrence risk of CRC, among which two were

Table 2 Extraprofessional factors influencing the risk for colorectal cancers.

Variables	Sub variables	Cases (%)	Controls (%)	*P*	OR	IC at 95%	ORa*	ICa	*P*a
Education level	Illiterate	13 (32.5)	9 (22.5)	0.32	1.6	0.6-4.5	-	-	
	Educated	27 (67.5)	31 (77.5)						
Habitat	Villa	10 (25)	21 (52.5)	0.012	0.3	0.11-0.77	0.74	0.21-2.56	0.63
	Others	30 (75)	19 (47.5)						
Means of transport	Motorized	23 (58)	21 (52.5)	0.65	1.22	0.5-2.95	-	-	-
	Not motorized	17 (42)	19 (47.5)						
Tobacco/alcohol	Alcohol and smoking	12 (30)	6 (15)	0.1	2.43	0.8-7.29	3.43	0.96-12.28	0.05*
	No alcohol or smoking	28 (70)	34 (85)						
Socioeconomic level	High	3 (7.5)	5 (12.5)	0.7	0.5	0.12-2.55	-	-	-
	Low	27 (92.5)	35 (87.5)						
Weekly meat consumption	Important	22 (55)	16 (40)	0.18	1.8	0.75-4.45	3.34	1.11-10.04	0.03*
	Not important	18 (45)	24 (60)						
Weekly fibers consumption	Low	6 (15)	11 (27.5)	0.17	0.46	0.5-1.4	0.47	0.12-1.87	0.28
	High	34 (85)	29 (72.5)						
Daily water consumption	Low	5 (12.5)	10 (25)	0.15	0.4	0.05-1.52	0.4	0.13-2.22	0.55
	High	35 (88.5)	30 (75)						
Physical activity	Absent	35 (88.5)	37 (92.5)	0.7	-	-	-	-	-
	Present	5 (12.5)	3 (7.5)						
Neoplastic family history	Yes	7 (17.5)	0 (0)	0.018	2.21	1.71-2.85	0	-	0.99
	No	33 (82.5)	40 (100)						
Personal digestive history	Yes	20 (50)	12 (30)	0.15	2.33	0.93-5.83	2.64	0.82-8.44	0.1
	No	20 (50)	28 (70)						

*: Adjusted OR.

Table 3 Occupational factors influencing the risk for colorectal cancers.

Occupational factors		Cases (%)	Controls (%)	*P*	OR (IC)	*P*a	ORa (ICa)*
Activity sector	Profession at risk:						
	Yes	23 (57.5)	19 (47.5)	0.37	1.49 (0.62-3.61)	-	-
	No	17 (52.5)	21 (52.5)				
	Agriculture:						
	Yes	13 (32.5)	5 (12.5)	0.007	4.84 (1.4-16.4)	0.84	1.22 (0.17-8.63)
	No	27 (67.5)	35 (87.5)				
	Textile:						
	Yes	1 (2.5)	6 (15)	0.048	0.14 (0.01-1.26)	0.98	0
	No	39 (97.5)	34 (85)				
	Building:						
	Yes	4 (10)	6 (15)	0.5	0.63 (0.16-2.43)	-	-
	No	36 (90)	34 (85)				
	Metallurgic industry:						
	Yes	1 (2.5)	0	0.24	0.48 (0.38-0.6)	1	0
	No	39 (97.5)	40 (100)				
Implicated products	Exposed to implicated products:						
	Yes	23 (57.5)	15 (37.5)	0.073	2.25 (0.92-5.52)	0.84	1.17 (0.23-5.94)
	No	17 (42.5)	25 (62.5)				
	Pesticides:						
	Yes	10 (25)	1 (2.5)	0.003	13 (1.57-107.22)	0.012*	20.44 (1.97-212.46)
	No	30 (75)	39 (97.5)				
	Cement:						
	Yes	4 (10)	4 (10)	1	1 (0.23-4.31)	-	-
	No	36 (90)	36 (90)				
	Painting:						
	Yes	1 (2.5)	5 (12.5)	0.2	0.18 (0.02-1.61)	0.99	0
	No	39 (97.5)	35 (87.5)				
	Textile dust:						
	Yes	1 (2.5)	6 (15)	0.11	0.14 (0.017-1.26)	0.98	0
	No	39 (97.5)	34 (85)				
Exposure characteristics	Means of protection:						
	Yes	4 (10)	7 (17.5)	0.33	1.91 (0.52-7.12)	-	-
	No	36 (90)	33 (82.5)				
	Periodic medical supervision:						
	Yes	4 (10)	16 (40)	0.02	6 (1.78-20.14)	0.004*	7.45 (1.92-28.87)
	No	36 (9)	24 (60)				
Pesticides exposure index		375.92 ± 482.44	141.3 ± 230.81	0.007		0.81	0.98 (0.86-1.12)

*: Adjusted OR.

extraprofessional: alcohol and tobacco (ORa = 3.43; ICa at 95% = 0.96-12.28) and red meat consumption (ORa = 3.34; ICa at 95% = 1.11-10.04), and two were occupational: occupational exposure to pesticides (ORa = 20.44; ICa at 95% = 1.97-212.46) and the periodic medical surveillance (ORa = 7.45; ICa at 95% = 1.92-28.87).

4. Discussion

The authors' study admits some limitations: the selection bias since only patients admitted the General Surgical ward of the Teaching Hospital of Farhat Hached of Sousse (Tunisia) were included and in whom the diagnosis of CRC was made and histologically confirmed. CRC patients admitted or followed up in other hospital wards of the same teaching hospital or in other hospitals or in private clinics were not also included. The small size of our studied sample is likewise another limitation which explains the difficulty in recruiting the cases and having a direct interview with them or with the members of their families despite the many attempts and notifications. The lack of a national cancer registry also increases the difficulty in recruiting the cases.

Besides, the retrospective nature of our study makes it difficult to establish the causality links between an implicated factor and the CRC risk of occurrence. The recall biases, as well as the lack of a quantitative evaluation of the exposures are other limitations to the extrapolation of the authors' results.

However, the authors have included in their inquiry that the patients in whom the diagnosis of CRC was histologically confirmed, they have evaluated the exposure in the same way in the cases and controls and have taken into account also both the occupational and extraprofessional factors in the analysis of CRC risk so as to limit the effects of confounding variables.

The study allowed to deduce that the alcohol-tobacco population is more exposed to CRC risk and that red meat frequent consumption increases this risk as well as the occupational exposure to pesticides mainly in the absence of a periodic medical surveillance.

4.1 Alcohol-Tobacco

Several epidemiologic studies associated alcohol consumption with the increasing risk of colorectal cancer [8-10]. Such association is above all in case of heavy drinking [11]. In a meta-analysis carried out in 2004, Cho et al. [12] demonstrated that the consumption of more than 45 gr of alcohol per day induces an increase of 41% of risk for colorectal cancer. Even if several studies indicated an increasing risk for colorectal cancer related to smoking [13, 14], it seemed that this latter was most frequently associated with developing adenomas rather than developing colorectal cancers [15].

4.2 Diet

Diet has been considered for a long time as the most important environmental influence for colorectal cancer [2, 8, 11]. As a general rule, a high consumption of red meat was associated with an increasing risk for CRC [1, 4, 16-18]. A recent meta-analysis of prospective studies carried out by Chan et al. which pointed out that the risk for colorectal cancer increases with a growing consumption of red meat [18]. In a systematic review of 13 prospective studies, Sandhu et al. showed that the increase of 100 gr of red meat daily consumption increases the risk for CRC from 12 to 17% [19]. Red meat is rich in heme and iron. Free iron produces highly instable and mutagenic free radicals [11]. Other mechanisms of action were evoked such as: endogenous insulin secretion which is mutagenic, the release and increase of total or saturated fats as well as the release of heterocyclic amines [4, 19]. This impact of red meat consumption on the risk for CRC was equally found in the results of our study. Moreover, diets rich in fruits and vegetables or fibers were linked to a reduced risk for CRC [2, 4, 8, 11, 20, 21]. The mechanisms of the protective effect of fruits and vegetables are secondary to their richness in fibers which seem to act by: the dilution of the fecal content, the decrease of transit time, the influence on the transformation of bile acids, the increase of stools weight, the reduction of colonic pH and the production of short-chains fatty acids [4, 11].

4.3 Occupational Exposures

Colorectal cancer is not usually considered as a disease of occupational origin [6]. Some authors have yet indicated an association between the occupational exposure to some toxins and notably colon cancer.

4.3.1 Pesticides

In our study, the exposure to pesticides was strongly linked to the risk of developing CRC. It is important to point out that there are no details concerning the exact nature of handled pesticides. Actually, the agriculture sector where there is the highest use of these products constitutes in Tunisia a sector where the workers are in most cases little or not educated and who break away from medical supervision, and hence, the difficulty to identify the exact nature of the handled products. In the literature,

many studies have been carried out so as to evaluate the link between pesticides and CRC, and the results were often contradictory [6]. Many studies are in favor of an increasing risk [6, 22-24]; among them is the study carried out in 2007 in the USA by Lee et al., dealing with 56,813 pesticide applicators [6], which indicates an increase of risk for colorectal cancer due to the intense exposure to chlorpyrifos and a significant increase of risk for colon cancer due to the exposure to aldicarb. Besides, in the pesticide applicator cohort of Iowa and North Carolina, Rusiecki et al. [25] did not objectify the significant association between the exposure to permethrin (pyrethroid insecticide) and the risk for CRC.

4.3.2 Asbestos

In the literature, there is a controversy concerning the relationship between the exposure to asbestos and CRC [26]. Some authors have pointed to a weak association between asbestos exposure and this cancer [27]. In their study, Garabrant et al. doubt such possibility [28], and so do Gamble et al. who notably signal the absence of dose-response gradient as a major argument against such relationship [29].

A meta-analysis of 20 cohorts exposed to asbestos indicates that the exposure to amphibole fibers may be associated with colorectal cancer whereas fibers of the type serpentine are not linked to it [30]. Moreover, in a meta-analysis conducted by Weiss [31] grouping together 30 studies of published cohort aiming at assessing the link between asbestos exposure and the risk for CRC, we note that the overall relative risk was of 0.99. A study conducted by Reid et al. among former workers in a mine of crocidolite (blue asbestos) in Western Australia, and which comprised details about smoking, did not indicate the association between the cumulative exposure to crocidolite asbestos and stomach, colorectal or upper aerodigestive tract cancers [32]. The small size of our population might account for the fact that we could not find significant results with regard to the exposure to asbestos.

4.3.3 Other Toxins

Several other toxins (such as wood dust, metal oils, textile dust, acrylates and solvents) were studied in order to prove their role in the genesis of CRC [6, 7, 33, 34]. Nevertheless, the results remain inconclusive and contradictory.

5. Conclusion

The results of epidemiological, clinical and experimental studies give prominence to the existence of several occupational, nutritional or lifestyle factors influencing the risk for CRC. In terms of our inquiry, the factors that were significantly associated with an increased risk to develop CRC after logistical regression were: alcohol-smoking, meat consumption, exposure to pesticides and the lack of periodic medical supervision. The adequate prevention which can be highlighted necessitates inter alia: incitement to reduce alcohol and smoking, dietary advice to reduce red meat consumption, and an increased awareness of occupational use of pesticides with the necessity to control their use, respect the prescribed doses, and insist on the wearing of protective equipment as well as the periodic medical supervision.

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Cross-Sectional Study of the Prevalence of Obesity Among Adults in Constantine

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Abstract: In Algeria, as in all countries in the developing world, obesity has become more and more common in adults, suggesting a worsening of risk factors for cardiovascular disease and diabetes for these generations. This study aims to assess the prevalence of obesity by anthropometry in adults in the city of Constantine over the year 2010-2011, and to explore its relationship with certain determining factors. A cross-sectional study with cluster sampling and stratification on sex allowed us to estimate the frequency of all categories of BMI. For this purpose, 320 adults living in Constantine were interviewed. In 2011, the prevalence of obesity was 35.84%. Women are more affected by obesity than men (40.83% vs. 22.08%). The prevalence of abdominal obesity, according to IDF, was 81.0% for women vs. 56.2% for men; according to NCEP ATP III, it was 64.3% in women vs. 35.9% in men. Obesity is more common in families of low socioeconomic status. Similarly, level of education and obesity are inversely correlated. People who have a sedentary lifestyle are more likely to be obese than those who are physically active. Obesity is a serious condition that results in significant health care costs. There is a need to undertake epidemiological surveillance in childhood. Urgent preventive actions are required. Promotion of a healthy balanced diet and physical exercise is a priority in the prevention of obesity.

Key words: Obesity, prevalence, adults, socio-economic factors, socio-cultural factors.

1. Introduction

Until the last century, being overweight was an aesthetic criteria associated with an image of wealth and social success, and a state of good health. In parallel with economic development and industrialization, Western countries have been faced with a rapidly increasing prevalence of obesity with its health consequences quickly becoming apparent. The World Health Organization (WHO) defines obesity as "an excess of body fat that causes adverse health effects". This condition is due to a chronic imbalance between energy intake and expenditure which in turn is linked to many factors: genetic, metabolic, behavioral and environmental. If we consider the rapid rise in obesity, we are inclined to believe that it is the behavioral and environmental factors, rather than biological changes, which have led to the epidemic [1, 2].

Currently ubiquitous, obesity is found more in urban areas of developing countries [3, 4]. The city of Constantine was not spared by the increase in the number of obese individuals and the epidemiology of obesity is not sufficiently understood because of the lack of structure and monitoring programs in Algeria. However a number of factors suggest that the situation is not different from the one prevailing in countries of similar development. The first indication was the national health survey conducted in 1990 which highlighted a change in morbidity profile in favor of non-communicable diseases, later confirmed by the National Health Survey of 2005 [5]. These observations were made against a background of

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profound socioeconomic, cultural and behavioral changes. Demographically, the population of Algeria is composed mainly of young adults (41.5% are 20-59 years of age) and the dramatic improvement in life expectancy over the past 40 years announces a gradual aging of the same population and an increased burden of chronic conditions.

Thus, as in many countries, the status of overweight and obesity is a concern in Algeria. It is important to carry out, on a regular basis, cross-sectional studies using nationally representative samples.

This would facilitate international comparisons of obesity rates in adults, help predict the scale that obesity will take in the future, and enable the evaluation of intervention strategies. These studies should document the values of BMI and waist circumference and progressively evaluate the effectiveness of the various intervention strategies that are in progress.

In this context, the present study was designed to assess the epidemiological characteristics of overweight and obesity in adults living in Constantine by analyzing anthropometric data obtained using a questionnaire.

The objective of the study was to determine the prevalence of obesity in an adult population of eastern Algeria and to identify the factors most associated with this condition in the region.

2. Materials and Methods

2.1 Location

The study took place in the town of Constantine (Qacentina in Arabic) a metropolis of the north-east of Algeria, capital of the city of Constantine. It is considered the third largest city in the country in terms of population. Formerly named Cirta, Constantine is also known as the "city of suspended bridges". The city of Constantine is located in northern Algeria at 36°24′ N latitude and 3°48′ E longitude and it is located 439 km from the capital Algiers, 80 km south of Skikda and 212 km north of Biskra. The city lies on a plateau at 649 meters above sea level. At the last census of 2009, the resident population of the town of Constantine was 448,374 inhabitants. The adult population aged 16 and older is 340,428. This population spreads over 10 sectors.

2.2 Type of Investigation and Study Population

This is a cross-sectional descriptive study that took place during the year 2010-2011. The study population consisted of people of both sexes aged 16 to 65 years and residing in Constantine. It consisted of 23.87% men and 76.08% women. The sample size, determined with the help of the National Statistics Office, was 320 adult subjects from ordinary households (families or individuals living in single dwelling). To create this sample, we conducted a cluster sampling in two stages. In the first stage the unit was the district. In the second degree, the survey unit was the private household regardless of size. A list of the districts of Constantine was used as the basis for this survey.

Exclusion criteria were:

- a current illness;
- amenorrhea.

2.3 Data Collection

Data were collected through individual interviews, by weighing and measurement of height, waist and hip sizes. For this purpose, we used scales and a measuring rod graduated from 0 to 200 centimeters. Lastly, a questionnaire was handed to the survey respondents. The first part of this questionnaire was intended to primarily capture demographic, socioeconomic and sociocultural information. The second part captured information on the frequency of food consumption and agrid for recording anthropometric measurements.

2.4 Anthropometric Data

The anthropometric assessment is based on:

BMI: Body Mass Index or Quételet's Index is used to evaluate patient's weight and degree of obesity. The

values obtained were divided into five groups according to the criteria used by WHO (Table 1) [6].

Waist circumference: this simple clinical measure is important because it has been shown to be well correlated with the amount of intra-abdominal fat, which is associated with an increased risk of metabolic and cardiovascular diseases [7]. Waist circumference is taken at the umbilicus, while standing, slightly breathing out and legs slightly apart. According to the IDF waist circumference greater than or equal to 94 cm in European men and 80 cm in European women are signs of abdominal obesity. According to the US NCEP ATP III waist circumference equal or greater than to 102 cm in American men and 88 cm in American women is a sign of abdominal obesity.

The waist/hip circumference ratio (WHR): the hip is measured at the widest section between the waist and upper thighs. A WHR equal or greater than 1 for men and 0.85 for women is a sign of abdominal obesity.

2.4 Data Processing and Statistical Analysis

Data analysis was carried out using the following software: spss v16, excel 2007, stat plus 2007.

3. Results and Discussion

3.1 Sample Description

The age of respondents ranged from 16 to 65 years and older, the average age was 39.07 years for men and 38.82 years for women. Approximately 29.55% of the subjects were 16-24 years of age. This predominance was found in both sexes.

In the surveyed population, 23.87% were males and 76.08% were females. Married subjects represented 57.23% whilst 42.77% were unattached (single, divorced, widowed or separated). The percentage of married women and men were 59.09% and 51.32%, respectively. The number of respondents who were either illiterate or had a primary level of education represented 24.53% of the sample. Respondents with higher education, above the Baccalauréat, represented 37.11% (Table 2).

Of those surveyed 45.91% were active and this category was mostly represented by men (73.68%). The analysis of the population by employment status shows an over representation by the unemployed (48.42% of men and 59.09% of women), 23.68% were students, 17.1% were workers, 13.15% were MSPs

Table 1 Interpretation of body mass index.

Index	(kg/m^2)	Interpretation of body mass
Less than	18.5	Underweight
Between	18.5 and 24.9	Normal weight
Between	25 and 29.9	Overweight
Greater than	30	Obesity
Between	30 and 34.9	Obesity class I
Between	35 and 39.9	Obesity class II
Equal to or greater than	40	Obesity class III

Table 2 Sample distribution by level of education.

	Higher	Secondary	Middle	Primary + Illitrate
Number of respondents	118	76	46	78
%	37.11%	23.90%	14.47%	24.53%
Women %	33.88%	24.79%	14.05%	27.27%
Men %	47.37%	21.05%	15.79%	15.79%

(managers and skilled professionals) and 10.52% of retirees (Table 3).

Recreational physical activity at least 2 hours/week was reported by 16.35% of the studied population sample (34.21% for men vs. 10.74% for women). The time spent watching television was less than 60 minutes for 19.50% of those surveyed and more than 60 minutes for 80.50% of them.

3.2 Weight Status of Respondents

The mean BMI was 27.76, it was higher in women than in men (28.42 vs. 25.65) and it increased steadily with age. As shown on Table 4, 33.33% of the sample had normal weight, 4.09% were underweight, 26.73% were overweight, and 29.87% were obese class I and II, and 5.97% were obese class III. Men were more overweight than women (27.63% vs. 26.45%). The prevalence of obesity was 35.84% in the population aged 16 to 65 years and older. The prevalence reported that our study is much higher than that reported in 2005, it was indeed estimated to be 21- 24% in adults [5]. The overall prevalence of obesity was significantly different between men (22.37%) and women (40.08%).

Table 3 Distribution of population by socio-professional categories.

	Men	Women	All
Security workers	2.63%	0%	0.62%
Craftsman	5.26%	0.82%	2.2%
Retailers	10.52%	0.82%	3.14%
MSPs	13.15%	9.91%	10.69%
Workers	17.1%	5.37%	8.17%
Teachers	1.31%	0.31%	
Students	23.68 %	20.24%	21.06%
Retirees	10.52%	3.71%	5.34%
Unemployed	15.78%	59.09 %	48.42%

Table 4 Sample distribution according to BMI.

Distribution according to BMI	%
Thinness	4.09%
Normal	33.33%
Pre-obesity	26.73%
Obesity classes I and II	29.87%
Obesity class III	5.97%

Our results show that obesity is becoming more prevalent and the same trend is also observed with overweight (26.73%). Progression of overweight may result in more of the population becoming obese later. These results are alarming compared to studies in other countries where the same age group was investigated. In Morocco (2009), 13.3% of the population is obese [8]. In Tunisia obesity affects 15% of the population and 40% live with excess weight. Therefore, the prevalence reported in the Maghreb and North Africa seems lower than that observed in our study. However, these data are difficult to compare because of the heterogeneity of references and their possible evolution. In the United States, between 1993 and 2008 the prevalence of obesity increased from 14.1% to 26.7%. In 2007 and 2009, the prevalence of obesity in Canada was 24.1%, that is to say more than 10 percentage points lower than that reported in the U.S. (34.4%). In Brazil, the rate of overweight increased from about 22% in 1974 to 34% in 1989. In 2009 the prevalence of obesity in France was 14.05% ± 0.4% (31.9% are overweight) [9]. By comparing our results with those of Tahina [10] who studied obesity in adults aged 35 to 70 years, we note that in our sample 67.05% of adults aged 35 and older are overweight (55.90%).

The prevalence of abdominal obesity in this study according to IDF was 81.0% for women vs. 56.2% for men that of abdominal obesity according to NCEP ATP III were 64.3% for women vs. 35.9% for men. It is interesting to note that in this sample of adults in Constantine, based on the thresholds proposed in the literature, we identify more women than men being at risk for metabolic and cardiovascular diseases due to abdominal adipose tissue distribution. Based on the knowledge that cardiovascular mortality and morbidity are higher in men, this finding leads to the conclusion that the proposed thresholds in women are probably inadequate.

3.3 Investigation of Related Factors

3.3.1 Obesity and Demographic Factors

The study showed that the factors most related to BMI are age and gender. Age ranges of 34 to 44 and 44 to 54 years were most affected by obesity with a prevalence of 30.78% and 21.66%, respectively. Those over 40 were especially affected. In Constantine, the prevalence of obesity was significantly higher in women (40.83% vs. 22.08%) (P = 0.0045). The prevalence of obesity was significantly different between married and unmarried subjects (48.35% versus 19.85%) ($P < 0.0001$).

In Morocco 20.9% of women and 7.8% of men are affected. Between 2007 and 2009, the prevalence in Canada was 24.3% and 23.9% for men and women, respectively. In the U.S., rates for men were 32.6% and 36.2% in women. In France the prevalence of obesity has increased regardless of gender. But the relative increase in the prevalence of obesity between 1997 and 2009 is higher in women (81.9%) than men (57.9%). Studies done in China yielded the same results as those of Constantine: women are more obese than men [11]. The prevalence in the Mexican population is comparable to that of women in the United States (25%). The same trend of similarity exists for the male population between the two countries with only 15% and 19% of men being obese in Mexico and the United States, respectively. By comparing our results with Tahina, we note that in our sample the prevalence of overweight for the age of 35 years and over is 71.42% for men and 65.62% for women. However, it is women who are obese with a percentage of 70.10% (30.09% in 2005) against a prevalence of 41.17% (9.07% in 2005) in men.

3.3.2 Obesity and Socio-Cultural Factors

Obesity reflects social inequality and its prevalence is strongly influenced by social status. Thus, there is an inverse relationship between household income and prevalence of obesity. Similarly, educational attainment and obesity are inversely correlated. This survey showed that 24.53% of the study population were uneducated (illiterate or elementary level of education) and that obesity concerns 56% of them, the majority being represented by women. By contrast, obesity affects only 36.16% ($P < 0.0001$) of subjects who have middle, secondary and higher education.

In modern societies, energy expenditure has significantly declined. The evolution of our way of life has led to an ever-growing sedentary lifestyle. Several prospective studies have shown that the time spent in sedentary occupations, regardless of the usual level of physical activity is associated with weight gain over time. In this study, 16.35% of respondents were physically active (34.21% men vs. 10.74% women). Overall, the prevalence is not significantly greater (P = 0.1714) among those who didn't do exercise (26.92%) compared to those who are physically active (37.97%). The prevalence of obesity increases with the length of time spent watching television, 37.97% of the sedentary individuals were obese, compared to 26.92% of non-sedentary individuals.

3.3.3 Obesity and Socioeconomic Factors: The Profession

The prevalence of obesity is higher among the unemployed (75.44%) than among those who are active (24.56%): MSPs (managers and skilled professionals) 21.93%; 14.91% for workers and 13.16% for students. Whilst there is no difference for the occurrence of obesity between different professions, it would be interesting to see after categorization of activities the difference between persons performing activities that require physical effort and those in occupations requiring less physical strength. This is a well known effect of energy expenditure [12, 13].

3.3.4 Perception of Obesity

In relation to general wellbeing:

- It is good to be obese = 6.4%;
- It is bad to be obese = 62.7%;
- No opinion = 30.9%.

In relation to health:

- A health hazard = 91.3%;
- Safe = 7.3%;
- No opinion = 1.4%.

4. Conclusions

The prevalence of obesity is increasing in the city of Constantine (35.84%). Overall, the prevalence of obesity increased significantly ($P = 0.0045$) by gender (40.83% in women vs. 22.08% in men). Individuals aged 25-34 and 45-54 years are most affected by obesity. Prevalence is higher after 40 years. People with overweight and potentially prone to becoming obese, represent 26.73% of the sample. Men are more overweight than women (27.63% vs. 26.45%). Age, sex, marital status, occupation and educational level were factors that played a role in the prevalence of obesity in this study. The public perception of obesity indicates an awareness of the health risk, amongst the obese and non-obese.

After studying the determinant factors of obesity, it is questionable whether it is a pathological phenomenon or a physiological adaptation to changes in our lifestyle. Our results confirm the existence of obesity and overweight among adults in Constantine and highlight its emergence and growing trend among the youngest population. These results must be confirmed by studies at the national level in order to establish the actual frequency of this phenomenon in Algeria. The proportions of overweight in our study are similar to those of other countries, but this remains to be confirmed. It is necessary to perform the analysis of all data generated to date to determine the actual development of obesity and overweight. Although the rise in obesity and overweight in Constantine is less pronounced than that observed in European countries and the United States, it is none the less a very real problem. The consequences of these changes on health are worrying and they will have, in the long-term, an economic impact. To counter this epidemic, prevention and support are to be defined at the national level. This prevention program will be based on information and education that should begin in elementary school.

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Impact of the Liaison Committee of Food and Nutrition on the Quality of Patients' Meals

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Abstract: At hospital, nutrition represents an important value of care, particularly for patients at risk. However, it is observed that for various reasons, the restoration of the hospitalized patients is often neglected in the privileges of the medical care. The establishment of a Liaison Committee of Food and Nutrition (LCFN) within a health establishment has shown according to several works, its positive impact in improving the hygienic quality and nutritional dishes served to patients. In this framework, we conducted a quasi-experimental study into CHU F hached Sousse of Tunisia type (before/after, creation LCFN) in order to assess the role of such structure (LCFN) in the qualification and the improvement of patients' food. Our study was based during the two phases (2007/2010) on the same methodology. Thus we have conducted an audit of observation of hygiene practices along the distribution chain of patient's meals and the samples for microbiological analyzes from food, surfaces, equipment and personnel's hands.The results obtained have shown, in one hand, a degradation of the average rate of contamination for the bacteriological analyzes, and in the other hand, an evolution of the rate of hygiene standards respected. According to this study, the role played by the LCFN is becoming more and clearer in the improvement of the hygienic quality of patients' dishes without forgetting the impact of improving the nutritional quality and Hedonics.

Key words: Hygiene, food, patient, CLFN.

1. Introduction

Nutrition is a public health issue. In many cases, eating well enables to stay healthy in the short, medium and long term. In care chain, the hospital has the particularity to take charge of patients in order to cure them as well as providing them with a number of services during their stay, such as restoration [1]. These hospital meals must realize four objectives: health safety, quality of service, organoleptic and nutritional quality and finally the productivity [2]. However, the problems posed by hospitalized patients' nutritional care are dating back for a long time and are always topical [3]. Indeed, hospital restoration owns specifics constraints, dominated mainly by its capacity to adapt meals of each patient to his pathology. As a result, distribution and preparation need a lot of labor.

Indeed, in France, nearly half of the hospitalized patients are malnourished or at risk to be. However, malnutrition can have serious consequences in terms of mortality, morbidity, increasing duration of hospitalization and additional costs, without forgetting the impact on life's quality. As well, it seems now sure that a bad quality hospital's restoration aggravates the slimming of malnourished patients [4].

Absence of communication and coordination

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among actors in food-nutrition process has been identified and restraint as a predictor of this alarming situation. To deal with that, various actions have been developed in several countries of the world. The creation of a Liaison Committee Food Nutrition (LCFN), in the establishment of health, bringing together all the personnel involved in hospital restoration, has shown its major role in improving the quality of meals served to hospitalized patients. In fact, this structure allows ensuring a better food security and a better nutritional care [5, 6]. We conducted our study in a comprehensive approach to improve the quality of services within our hospital (CHU F hached Sousse) and in order to evaluate the role of LCFN in improvement of hospital meals' health [7].

2. Materials and Methods

Our work is performed in CHU F hached Sousse (City of Tunisian East Center), in which are hospitalized around 30,000 patients per year and it serves, in average, 1,350 meals a day. Nevertheless, for six years, the filling of dishes, their distribution in health-care units and their recovery are insured by agents of the private company sub contractor.

2.1 Type and Study Population

It is a quasi-experimental study type before-after the creation of a LCFN in our hospital. We have adopted the same methodology and we have respected the same inclusion criteria and the same number of samples during the 2 phases of the experimentation carried out respectively in 2007 and in 2010. We have carried out during the 2 phases of the study:

- Observation audit of practices along the dishes distribution chain to hospitalized patients in the services, including food and nutrition, plays a fundamental role in the provision of medical care (Service of cardiology, service of carcinologie, pediatric service and service of endocrinology);
- Microbiological levies of food and environment in which they were prepared and distributed.

2.2 Measured Variables and Measuring Instruments

Measured variables and measuring instruments have been performed during the 2 phases of the study, according to the same methodology:

2.2.1 Audit of Hygiene Along the Chain of Distribution

It is an observation audit of steps that constitute the meals distribution chain to hospitalized patients in the services included in our study. It has lasted 1 month in 2007 and 2010. These steps, counted nine, are:

- Loading trays;
- Loading trolleys;
- Routing to units of care;
- Arrival to units of care;
- Service and consumption by patients;
- Collection of waste and return to the central kitchen;
- Waste management;
- Washing of utensils and carts;
- And arrangement of the equipment.

The audit was conducted by using an observation grid with evaluation criteria of hygiene standards application in restoration, referring to the regulatory texts [8].

Answers to this grid are binary type (yes/no). Then, compliance degree of observed practices with standards of each step, expressed as a percentage, was calculated by relating the number of positive responses (yes) in relation to the whole of the criteria evaluated. The data have been collected during the regular visits of meals distribution chain provided by two external auditors in the service of restoration (researcher in food hygiene and hygiene's technician) [9].

2.2.2 Microbiological Analysis

Microbiological levies have concerned, the meals ready to consumption, and the environment in which food is produced.

(1) Microbiological analysis of food:

The microbiological analysis of foods has concerned 66 dishes for patients in four services and

distributed between the three meals (breakfast, lunch and dinner): 30 dishes were taken from the central kitchen of the hospital at time of manufacturing trays and loading trucks. The other dishes (36 dishes) were taken at patients' beds, just before their consumption.

(2) Microbiological analysis of surfaces and premises

It consists in analyzing hygienic situation at the distribution meals chain's level through the microbiological samples from the surfaces of premises, equipment and hands of personnel. It is more specifically:

• On the premises: 46 levy were carried out at the place of finished dishes establishment (central kitchen), at care units (Offices and rooms of patients) and at health grouping personnel.

• As for the equipment: 32 samples have concerned utensils, work tables and trolleys.

• In the end, 12 samples in the hands of personnel who are carrying trays, and those who are routing the trolleys and serving the dishes to patients.

Researched germs found in levy of foodstuffs and environment are presented in Table 1.

The results are considered non-compliant if the number of germs presented exceeds the threshold tolerated.

2.3 Statistical Analysis

Data seizure and analysis were carried out using Epi-info 5 software. Audit results and bacteriological analyzes are expressed as percentages. The comparison of these results before the creation of the LCFN (2007) and after its establishment (2010) was carried out using the test of Chi2 for a risk of error α of 5%. We have seen a significant difference in the results between 2007 and 2010 if $P \leq 0.05$.

Fig. 1 Levy of foodstuffs.

Fig. 2 Levy of surfaces.

Table 1 Plan of sampling for bacteriological analyzes.

	Number of samples
Foodstuffs	66
Surfaces of premises	46
Equipements	32
Mains du personnel	12
Total	156

Analyzes provided—C FECAL and C Totals —Enterobacteriaceae (*Salmonella*, *E. coli*) —STAPHYLOCOCCI pathogenic germ—anaerobic Sulfito —reducers (Clostroduim)—yeast and mold.

3. Results

3.1 Audit of Hygiene

Our study have shown that compliance with the standards of hygiene along the dishes distribution chain to patients has improved significantly after the creation of the LCFN from 30% in 2007 to 50% in 2010 ($P = 0.001$).

3.2 Bacteriological Analysis

3.2.1 Global rate of Nonconformity

The global rate of nonconformity of foodstuffs, surfaces, equipment and personnel's hands levies has decreased significantly between the two phase of the study from 34% before LCFN to 14% after LCFN, with presence of a whole variety of bacteria and germs ($P = 0.001$).

3.2.2 Foodstuffs

Our study could not show a significant reduction in

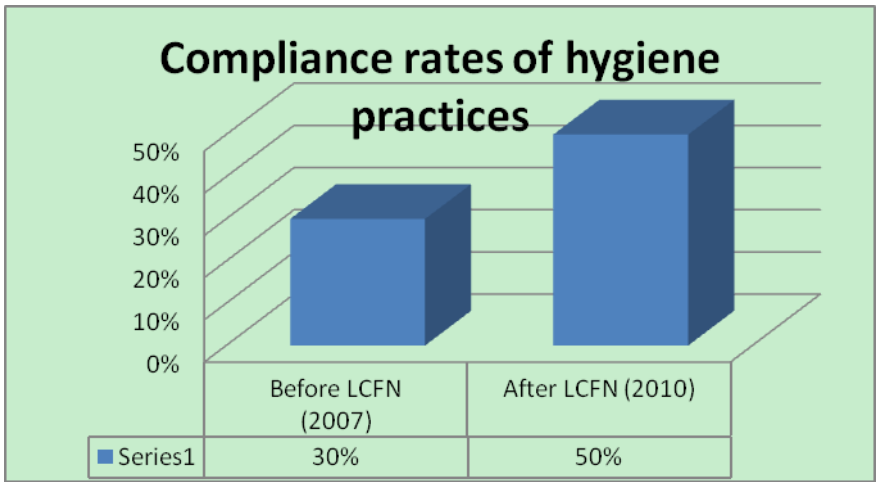

Fig. 3 Comparisons of global rate of compliance with hygiene practices between the two phases.

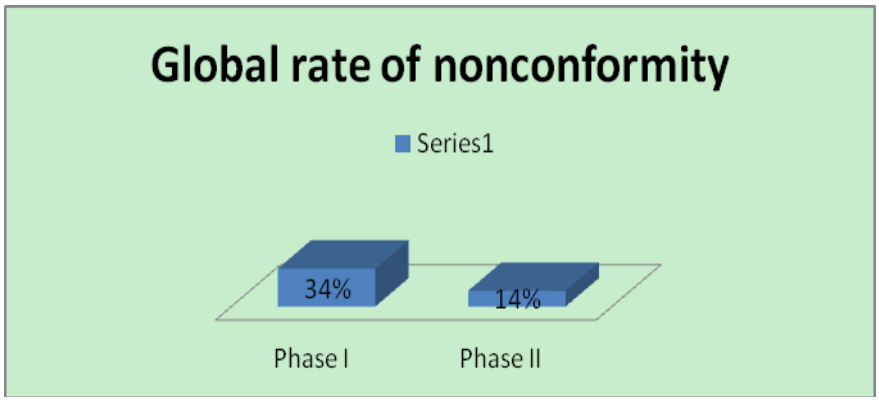

Fig. 4 Global rate of Nonconformity.

noncompliance rates of food, between the two phases of the study. Indeed, the rate of noncompliance has increased from 39% in 2007 to 23% in 2010.

(1) Loading trays

Before the LCFN creation within our hospital, 37% of foodstuffs samples during platters loading were positive and therefore noncompliant. However, after LCFN establishment, this rate has increased to 27%.

(2) Units of care

During the first phase the rate of non-compliance was 39%, while in the second phase this rate was lowered to 19%.

3.2.3 Surfaces of the Premises

The noncompliance rate of surfaces analyzes and premises was 28% for the first phase (before LCFN), and was lowered to 1.5% for the second phase (after LCFN).

3.2.4 Equipment of the Kitche

In the first part and before LCFN, the non-compliance rate was 37.5%, while the rate was 19% in the second part.

3.2.5 Surfaces of Hands

The levies from the surfaces of hands, during the first phase, were given a noncompliance rate of 25%, while the rate becomes zero, during the second phase, and all levies were conform to the standards.

4. Discussion

All above achievements have served to provide a strategy to optimize hygiene quality and meals hedonic and to create a qualified sanitary system and services in order to control the appropriate level of risk.

Table 2 Rate of contamination of foodstuffs by various germs.

Result \ Service	1st measures: Phase I				2nd measures: Phase II			
	NC	C	T-N	Rate of NC	NC	C	T-N	Rate of NC
Breakfast	3	7	10	30%	1	9	10	10%
Lunch	4	6	10	40%	2	6	10	40%
Dinner	4	6	10	40%	3	7	10	30%
Total	11	19	30	37%	8	22	30	27%

NC: No corresponding; C: compliance; T-N: Total number.

Table 3 Rate of food contamination of foodstuffs at the time of the load of trays.

Result \ Service	1st measures: Phase I				2nd measures: Phase II			
	NC	C	T-N	Rate of NC	NC	C	T-N	Rate of NC
Breakfast	4	8	12	33%	2	10	12	17%
Lunch	5	7	12	42%	2	10	12	17%
Dinner	5	7	12	42%	3	9	12	25%
Total	14	22	36	39%	7	29	36	19 %

NC: No corresponding; C: compliance; T-N: Total number.

Table 4 Rate of contamination of premises by various germs.

Result \ Service	1st measures: Phase I				2nd measures: Phase II			
	NC	C	T-N	Rate of NC	NC	C	T-N	Rate of NC
Premises of implementation of the finished dishes	4	6	10	40%	0	10	10	0%
The units of care	4	20	24	17%	1	23	24	4.5%
Cloakroom of the staff and the shower-rooms	5	7	12	42%	0	12	12	0%
Total	13	33	46	28%	1	45	46	1.5%

NC: No corresponding; C: compliance; T-N: Total number.

Table 5 Rate of contamination of equipments by various germs.

Result \ Service	1st measures: Phase I				2nd measures: Phase II			
	NC	C	T- N	Rate of NC	NC	C	T-N	Rate of NC
Utensils containing the warm preparations	2	2	4	50%	1	3	4	25%
Utensils containing the cold preparations	2	2	4	50%	1	3	4	25%
Utensils containing the milk	2	2	4	50%	1	3	4	25%
Trays	2	2	4	50%	1	3	4	25%
Desk	1	3	4	25%	2	2	4	50%
The wagons	3	9	12	25%	0	12	12	0%
Total	12	20	32	37.5%	6	26	32	19%

NC: No corresponding; C: compliance; T-N: Total number.

One of the important tools and the most recommended in this case is the Liaison Committee of Food and Nutrition (LCFN) whose purpose is to coordinate and evaluate the actions of concerned professionals for a better patients' nutritional care.

At the outset, it had to be based on these results of the first phase to convince all stakeholders in the hospital food chain of the need for the creation of a LCFN [4, 10].

The first meeting for establishing the LCFN was in March 2008, then every two months, in average, a meeting have had place, in order to validate and activate the procedures of work and to qualify the patients dishes distribution chain; so that we could reach our aim and achieve our objectives about nutrition and food at hospital [11].

This experience has enabled us to improve the nutritional care of CHU F hached Sousse, by insisting

on the fact that hospital meals must be very good before their consummation.

4.1 The Audit of Hygiene

The rate of hygiene standards respected, at the hospital food chain, experienced a significant development and achieved 50%, which is due to the improvement of hygienic conditions in the kitchen, in accordance with the standards [8].

The main action was to raise awareness, to train and inform the staff of the food chain of the need for the application of the rules of hygiene and the risk it may take place in the case of the negligence of the instruction. A risk which may put the lives of patients already weakened in game.

4.2 The Bacteriological Analysis

The overall rate of noncompliance clearly shows an important and a significant improvement of the bacteriological quality of food served to patients. For the first phase, nearly a quarter of the bacteriological analyzes results have proven not to comply with the related microbiological criteria, which indicates that the risk of foodborne illness is fairly important. But the second phase has given a contamination rate of 15%; that's why, it becomes clear that the contamination rate have been significantly degraded. The result is an improvement of the hygiene standards application in the food chain [12, 13].

The statistical tests have shown the relationship between the creation of CLAN and the improvement of the bacteriological quality of patients' dishes. In fact, the statistical test Chi2 is very significant. That leads us to conclude that the number of samples complied with the bacteriological standards, after improvement of hygienic conditions, is much higher than those toke before. Such a test, therefore has participated in validation of hypothesis and elimination of another (alternative hypothesis) [14].

Thus, several studies have shown the important role that could have germs contamination of hospital food within the hospital infection. As Loiseau-Marolleau and Laforest noticed: "food contamination by pathogenic or potentially pathogenic bacteria brings me the question of their possible implantation in the digestive tract and their role in the hospital infection and in the transfer of resistance to antimicrobial products" [15]. According to authors' work mentioned above, it is mainly the strains encountered during the course of the meat and poultry contamination, which could be the cause. This leads us to conclude that the overall contamination's rate of 24% can participate with others factors, at hospital, in the evolution of infectious risk related to food. But the degradation of this overall rate, down to 15%, will probably influence infectious risk [12]. Therefore, it becomes clear that any positive intervention along the food chain such as the menu by the LCFN is directly and in the right direction on the improvement of the bacteriological quality of food served to patients. This is the same action that will give the application of the HACCP approach in the same context, and this was proven by studies in this direction [16].

5. Conclusions

In a first time, the LCFN of the CHU F hached Sousse has allowed us to begin correcting the inefficiencies through the qualification of the hospital meals. Indeed, our approach (situation diagnosis, followed by LCFN activation), in spite of the invested time, constitutes a starting point for further investigations: at prevention of foodborne illness and optimization of experimental protocols, also for the establishment of food quality and security's system.

We hope that the result of LCFN's activities may be used as soon as possible by those responsible of hospital restoration in CHU F hached Sousse; it is for patients' safety and for this point, all efforts are justified.

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Effect of Different Storage Periods on Egg Quality Traits of Ducks

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Abstract: The present study was conducted in each 20 eggs stored at 65 °F with 75% relative humidity, for a period of 5 weeks (T-1), 4 weeks (T-2), 3 weeks (T-3), 2 weeks (T-4) and 1 week (T-5) with broad end up. The external qualities of eggs like egg weight, length, width and volume were measured. After recording the external qualities the eggs were broken on a glass plate for studying the internal qualities. Storage period had no statistically significant effect on egg weight (g), egg volume (%), shell thickness (mm), shell weight (g) and shell percentage. Egg weight values determined in this present study was between 61.10 and 64.91 g. The egg volume (%), egg shell thickness (mm), shell weight (g) and shell percentage for different storage periods were 87.46 and 98.52, 0.28 and 0.34, 5.29 and 5.98; and 8.16 and 9.44 respectively. Yolk weight (g), yolk percentage and albumen percentage was significantly affected ($P \leq 0.01$) by storage period and the values ranging between 32.20 and 36.40, 48.59 and 58.53 and 32.01 and 43.24 respectively. Storage period had no statistically significant effects on egg shape index and specific gravity. The storage periods on yolk index, yolk colour, albumen index and Haugh unit were statistically significant ($P \leq 0.01$). Yolk index value decreased significantly when storage period was lengthened. This study concluded that yolk index, yolk colour, albumen index, albumen percentage and Haugh unit were decreased with increase in storage time.

Key words: Duck egg, storage period, quality.

1. Introduction

Eggs quality traits are influenced by many factors, including genetic and environmental ones [1]. Both internal and external egg traits can further be significantly influenced by the length of storage period. Lengthing of the storage periods leads to unfourable physiochemical changes of egg content [2]. In hens, it was demonstrated that eggs stored for more than 10 days were characterized by worse white and yolk indices and lower number of Haugh units, in comparison with those examined on the day of laying [3]. Only limited information is available on egg quality traits changes of ducks during storage. Hence this study was carried out to analyse the quality changes of duck eggs storage for several days.

2. Materials and Methods

The present study was conducted at Institute of Poultry Production and Management, TANUVAS, Chennai-51 in the year 2011. The ducks were reared under intensive system of management. The each 20 collected eggs were stored at 65 °F with 75% relative humidity, for a period of 5 weeks (T-1), 4 weeks (T-2), 3 weeks (T-3), 2 weeks (T-4) and 1 week (T-5) with broad end up. The external qualities of eggs like egg weight, length, width and volume were measured. After recording the external qualities the eggs were broken on a glass plate for studying the internal

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qualities. The height of thick albumen and yolk were measured using Ames Tripod stand micrometer. The length and width of thick albumen and yolk were measured using a vernier caliper. The egg yolk colour was identified by comparing with Roche yolk colour fan. Haugh unit was calculated as per Haugh [4]. The yolk was separated from the albumen and weighed. The shell weight was recorded. The shell thickness was measured after removing the sub membranes using a paper gauge meter. The data were analyzed as per the standard statistical procedures [5].

3. Results and Discussion

3.1 Quantitative Traits

Effect of different storage periods on egg quality of ducks are presented in Table 1. Storage period had no statistically significant effect on egg weight (g), egg volume (%), shell thickness (mm), shell weight (g) and shell percentage. Egg weight values determined in this present study was between 61.10 and 64.91 g. The mean egg weight recorded was slightly lesser than the value (66.1 g) [6] and higher than the value (60.55 g) [7]. The egg volume (%), egg shell thickness (mm), shell weight (g) and shell percentage for different storage periods were 87.46 and 98.52, 0.28 and 0.34, 5.29 and 5.98; and 8.16 and 9.44 respectively. The lowest egg weight was observed numerically for eggs stored for 5 weeks and the highest egg weight was for eggs stored between 1 and 3 weeks. This may be due to storage of eggs may loose weight due to water loss. The mean shell thickness and shell percentage of this study was similar [6, 7]. Yolk weight (g), yolk percentage and albumen percentage was significantly affected ($P \leq 0.01$) by storage period and the values ranging between 32.20 and 36.40, 48.59 and 58.53 and 32.01 and 43.24 respectively. When a storage period was lengthened, the yolk percentage increased because albumen loses more moisture than yolk. Albumen percentage of this study was closely in agreement with the value reported [7, 8] in indigenous ducks. The yolk percentage of this present study was higher than the value (28.67) reported [7].

Table 1 Effect of different storage periods on egg quality traits of ducks (Mean ± SE).

Quality traits	T-1 (n = 20)	T-2 (n = 20)	T-3 (n = 20)	T-4 (n = 20)	T-5 (n = 20)	Overall mean (n = 100)
Quantitative traits						
Egg weight (g)^{NS}	61.10 ± 1.44	62.76 ± 1.34	64.45 ± 0.92	64.91 ± 0.93	64.00 ± 0.68	63.44 ± 0.71
Egg volume (%)^{NS}	87.46 ± 4.40	94.00 ± 1.46	97.43 ± 2.25	98.52 ± 1.70	96.80 ± 2.36	94.84 ± 1.74
Egg length(mm)^{NS}	58.55 ± 1.28	58.30 ± 0.66	65.50 ± 0.95	58.95 ± 0.44	57.70 ± 0.50	59.80 ± 0.54
Egg width(mm)^{NS}	44.48 ± 0.55	42.30 ± 0.55	49.50 ± 0.95	45.10 ± 0.42	44.24 ± 0.33	45.12 ± 0.42
Shell thickness (mm)^{NS}	0.29 ± 0.01	0.31 ± 0.02	0.28 ± 0.01	0.34 ± 0.01	0.32 ± 0.02	0.31 ± 0.00
Shell weight (g)^{NS}	5.39 ± 0.11	5.98 ± 0.35	5.29 ± 0.28	5.93 ± 0.25	5.96 ± 0.23	5.71 ± 0.16
Shell percentage ^{NS}	8.90 ± 0.38	9.44 ± 0.38	8.16 ± 0.37	9.10 ± 0.33	9.29 ± 0.31	8.98 ± 0.21
Yolk weight (g)**	32.20^{b} ± 0.61	36.40^{a} ± 0.59	36.40^{a} ± 0.59	32.50^{b} ± 0.65	33.60^{b} ± 0.60	34.22 ± 0.40
Yolk percentage**	52.98^{b} ± 0.96	58.53^{a} ± 1.57	48.59^{c} ± 1.04	50.18^{b} ± 1.04	52.57^{b} ± 1.02	54.18 ± 0.84
Albumen percentage**	38.11^{b} ± 1.09	32.01^{c} ± 1.36	43.24^{a} ± 1.00	42.03^{b} ± 1.25	40.46^{b} ± 1.07	36.82 ± 0.84
Qualitative traits						
Shape index ^{NS}	76.18 ± 1.33	72.59 ± 0.93	75.52 ± 0.35	76.51 ± 0.57	76.68 ± 0.28	75.50 ± 0.40
Specific gravity^{NS}	1.27 ± 0.01	1.06 ± 0.01	1.03 ± 0.02	1.02 ± 0.01	1.03 ± 0.02	1.08 ± 0.01
Yolk index**	0.33^{b} ± 0.04	0.34^{b} ± 0.01	0.51^{a} ± 0.01	0.45^{b} ± 0.01	0.36^{b} ± 0.02	0.40 ± 0.01
Yolk colour**	6.50^{c} ± 0.25	6.40^{c} ± 0.27	7.30^{b} ± 0.20	6.80^{c} ± 0.20	8.10^{a} ± 0.16	6.96 ± 0.15
Albumen index**	0.09^{ab} ± 0.01	0.05^{c} ± 0.01	0.06^{b} ± 0.02	0.06^{b} ± 0.02	0.10^{a} ± 0.01	0.07 ± 0.00
Haugh unit**	99.20^{c} ± 1.30	101.05^{b} ± 1.00	103.50^{b} ± 1.03	104.57^{b} ± 0.91	108.90^{a} ± 0.43	103.45 ± 0.77

Means bearing different superscripts in the same rows different significantly.

NS: Not significant ($P > 0.05$); ** Highly significant ($P \leq 0.01$).

3.2 Qualitative Traits

Storage period had no statistically significant effects on egg shape index and specific gravity and the value varied between 72.59 and 76.68; and 1.02 and 1.27 respectively. Shape index and specific gravity recorded in our study was similar with the values reported [7, 9]. The storage periods on yolk index, yolk colour, albumen index and Haugh unit were statistically significant ($P \leq 0.01$).Yolk index value decreased significantly when storage period was lengthened. For period of one week, 4 weeks and 5 weeks storage the yolk indexes and albumen indexes were 0.36, 0.34 and 0.33; and 0.10, 0.05 and 0.09 respectively. The general decline in yolk index and albumen index is in agreement with the finding [10, 11] who observed a decline in albumen and yolk indices with increase in storage time. Haugh unit values decreased as did yolk and albumen index and varied between 99.20 and 108.90. Storage of eggs results in lower the albumen height and hence lowers the Haugh unit [12]. Haugh unit value recorded in our study was higher than the value (78.99) reported [9].

4. Conclusions

From the study, it was observed that yolk index, yolk colour, albumen index, albumen percentage and Haugh unit decreased with increase in storage time while yolk weight and yolk percentage increased. Egg weight, egg volume, shell thickness, shell weight, shell percentage, shape index and specific gravity not affected significantly ($P > 0.05$) by storage time.

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Alkaloids Production from Callus of *Hyoscyamus niger* L. *in Vitro*

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Abstract: Callus cultures of *Hyoscyamus niger* L. were initiated from leaf segments cultured on Murashige and Skoog (MS) medium supplemented with 0.5 mg/L Benzyl Adenine (BA) and 0, 1, 2 and 3 mg/L Naphthalene acetic acid (NAA). Half of cultures were incubated under light of 16 hr/day, while the other half was incubated under complete darkness. The incubation temperature was 25 ± 1 °C in both incubation conditions. The fresh and dry weight of the produced callus was obtained after six weeks of incubation. Callus produced were recultured on medium that gave the highest production of callus. Constant weight (300 mg) of callus was cultured in each of these medium supplemented with abiotic elicitor of 50 g/L sucrose, 200 mg/L NaCl, 50 or 100 mg/L proline and 2 mg/L BA each one added separately and incubated under complete darkness. The fresh and dry weights of callus were measured after six weeks. HPLC was used to determine the tropane alkaloids (Hyoscyamine and Scopolamine). The results showed that the significant highest average of fresh and dry weight of callus (112 and 89.6 mg) achieved using the medium contained 0.5 mg/L BA and 2 mg/L NAA under dark condition. The amount of fresh and dry weight of callus produced under dark condition was significantly higher than that produced under light condition, with increase in percentage of 51.3 and 37.62% respectively. The addition of abiotic elicitors caused reduction in both fresh and dry weight of callus, therefore the highest fresh weight average was 1,727 mg using 100 mg/L proline. The results indicated that addition of 50 or 100 mg/L proline led to increase in Hyoscyamine concentration of 58.03 and 21.37% respectively compared with the control. While other abiotic elicitors were found to cause reduction in Hyoscyamine concentration. Percentage of Scopolamine concentration were increased to 129.03, 166.94, 205.51 and 149.20% after addition of sucrose (50 g/L), NaCl (200 mg/L) and proline (50 or 100 mg/L) respectively compared with the control.

Key words : *Hyoscyamus niger* L., alkaloids, hyoscyamine, scopolamine, abiotic elicitors.

1. Introduction

Plant considers one of the most important sources of medicines for thousands of years. Even today, the World Health Organization estimates that up to 80% of people still rely mainly on traditional remedies such as herbs which contain different active ingredients. Biotechnological tools are important for multiplication and genetic manipulation of the medicinal plants through callus inductions, cell suspension in bioreactors, *in vitro* regeneration of plantlets and genetic transformations [1, 2]. The major advantages to produce a valuable secondary product in plant cell culture, rather than *in vivo* in the whole plants are: Production can be more reliable, simpler and more predictable; Isolation of the phytochemicals can be rapid and efficient as compared to extraction from complex whole plants; Compounds produced *in vitro* can directly parallel compounds in the whole plants; Interfering compounds that occur in the field-grown plant can be avoided in cell cultures and cell cultures can yield a source of defined standard phytochemicals in large volumes; Cell cultures are a super model to test elicitation [3, 4].

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Black henbane, *Hyoscyamus niger* L. which has long been known as a medicinal herb belongs to the Solanaceae family [5]. The chemical constituents of *H. niger* include alkaloids, saponins, lignans, coumarinolignans, flavonoids and some other non alkaloids compounds [6]. Tropane alkaloids, (Hyoscyamine and Scopolamine) are widely used in medicine for their mydriatic, anticholinergic, antispasmodic, analgesic and sedative properties [7, 8]. Scopolamine has a higher demand being the more valuable alkaloids due to its fewer side effects and higher physiological activity [9]. In most cases, the natural yields of the tropane alkaloids are too low for commercialization. There remains a need to increase alkaloids production rates for commercial exploitation [10]. Plant cells and callus cultures have been extensively used to explore the possibility of production useful secondary metabolites through biotechnological methods [11].

Elicitations are considered to be an important strategy towards improved *in vitro* production of secondary metabolites. Various biotic and abiotic factors added to the medium of callus production influence their production by activating genes for de novo synthesis or by stimulation the physiological processes leading to enhanced accumulation of such products [12-15].

The aims of this investigation are to study the influence of abiotic elicitors to enhance the amount of essential tropane alkaloids (Hyoscyamine and Scopolamine) in callus tissue of *H. niger*, and to determine the presence of those alkaloids compounds in callus extracts using HPLC technique.

2. Materials and Methods

This work was carried out at Biotechnology Research Center, Al-Nahrain University, Baghdad, Iraq during 2009-2011. Plants of *Hyoscyamus niger* were collected from North of Iraq and identified by Prof. Dr Ali Al-Musawi, plant taxonomist at College of Science, Baghdad University.

2.1 Tropane Alkaloids Standards

Hyoscyamine and scopolamine (hyoscine) standards were obtained from Sigma-Aldrich Company, USA.

2.2 Seed Dormancy and Sterilization

Black henbane, *H. niger* seeds have low germination rate under normal laboratory conditions, so they were soaked in 5 mg/L Gibberellic acid (GA_3) for 6 h in order to break dormancy in the seed stage and enhance germination [16]. After treatment with GA_3, seeds were washed with tap water and surface sterilized in 70% ethanol for 2 min, and followed by 50% commercial bleach Clorox (6% sodium hypochlorite) with a drops of tween-20 for 10 min. After the bleach solution was discarded, seeds were washed once with 70% ethanol and 4 times with sterile distilled water. The sterilized seeds were placed on basal MS medium (Murashige and Skoog) [17], supplemented with 7 g/L agar and 30 g/L sucrose for germination.

2.3 Explants Culture and Callus Induction

Four weeks old seedlings were used after wards for preparation explants. Leaf segments of 0.5 cm^2 were placed on MS medium supplemented with BA 0.5 mg/L and different concentrations of NAA (0, 1, 2 and 3 mg/L). Cultures were divided to two groups. The first group was incubated at 25 ± 1 °C under light condition (2,000 lux, 16 h/day), the second group was incubated under dark condition. After 6 weeks the best callus production and fast growing were selected for further work. These callus were sub cultured each 4 weeks on fresh media (supplemented with 0.5 mg/L BA and 2 mg/L NAA) and for four months continuously to maintain callus stock.

2.4 Abiotic Elicitors Used

Equal amount of callus (300 mg) was cultured on same medium which used in callus maintenance. Four abiotic elicitors have been added to the medium

separately, sucrose 5%, sodium chloride (NaCl) at concentration of 200 mg/L, proline as amino acid was used with two concentrations 50 and 100 mg/L, and 2 mg/L of BA as plant regulator hormone. Fresh and dry weights callus were measured after 6 weeks of incubation and alkaloids contents were determined.

2.5 Extraction of Tropane Alkaloids

Leaves of *Hyoscyamus niger* plant age 3 months, and callus of elicitors treatments were collected and dried in oven for 2 days at 60 °C and powdered in mortar. Alkaloids extraction was carried out as described in [5, 10]. Leaves and callus (200 mg each) were soaked overnight in ethanol 28% and NH_4OH (9:1) mixture, then centrifuged for 3 min at 1,500 rpm. Extraction with the basic alcohol was repeated twice and evaporated to dryness at 45 °C. The residue was dissolved in 1.5 mL of 0.1 N HCl and the acidic aqueous solution was filtered and made alkaline with diluted KOH (final pH 8-9). Chloroform (6 mL) was added and the tube was shaking vigorously for 30 sec, then centrifuged for 2 min at 1,500 rpm. The lower layer which contained the alkaloids was pipette twice with 6 mL of chloroform, then evaporated to dryness at 40 °C. The dry residue was dissolved in 99% methanol to make it ready to be used for alkaloids hyoscyamine and scopolamine content determination using High Performance Liquid Chromatography (HPLC).

2.6 HPLC Analysis of Tropane Alkaloids

Tropane alkaloids were analyzed by HPLC (Cecil Company, England), by using an ODS (C18) column (25 cm × 4.6 mm, partial size 5 μm).

The mobile phase contained (Methanol: 0.1 M K_2HPO_4), pH: 7.2; flow rate: 1.0 mL/min; UV-detector at 210 nm; analyzed by external standard.

2.7 Statistical Analysis

All experiments were done with minimum of 20 replicates per treatment. Significance of treatment effects was determined using analysis of variance (ANOVA) followed by LSD test ($P \leq 0.05$) to determine significant differences among treatment means.

3. Results and Discussion

3.1 Callus Initiation

The results indicated the presence of significant effects of growth regulators used on the fresh callus weight initiated from leaf segments of *Hyoscyamus niger* L. incubated under light or dark condition. Both medium contained 2 or 3 mg/L NAA and 0.5 mg/L BA were found to be significantly superior compared with other medium used, the average callus weight were 798 and 710 mg respectively. The lowest callus fresh weight obtained was 275 mg using medium contained 1 mg/L NAA, while no callus initiation was found at the control treatment which did not contain NAA and BA. The result depicted in Fig. 1 also showed that there was significant difference in the average callus weight initiated under dark condition (615 mg) compared with that initiated under light condition (299 mg), with an increase percentage of 51.39%.

The results in Fig. 2 illustrated the interaction between incubation conditions and growth regulators, which found to be highly significant. The highest average of callus weight was 1,112 mg at medium contained 2 mg/L NAA and 0.5 mg/L BA and incubated under dark condition. This treatment was significantly differ with all other interactions except that contained 3 mg/L NAA and 0.5 mg/L BA incubated at complete darkness, in which the average callus weight reached 911 mg. At the control treatment (without NAA and BA) all cultures did not respond to initiate callus, either incubated under light or dark conditions. The lowest amount of callus (237 mg) was found to be initiated at treatment of 1 mg/L NAA and incubated under light condition, with significant differences with most interactions.

The dry callus weight, which almost behaves like the fresh weight, results indicated superiority of

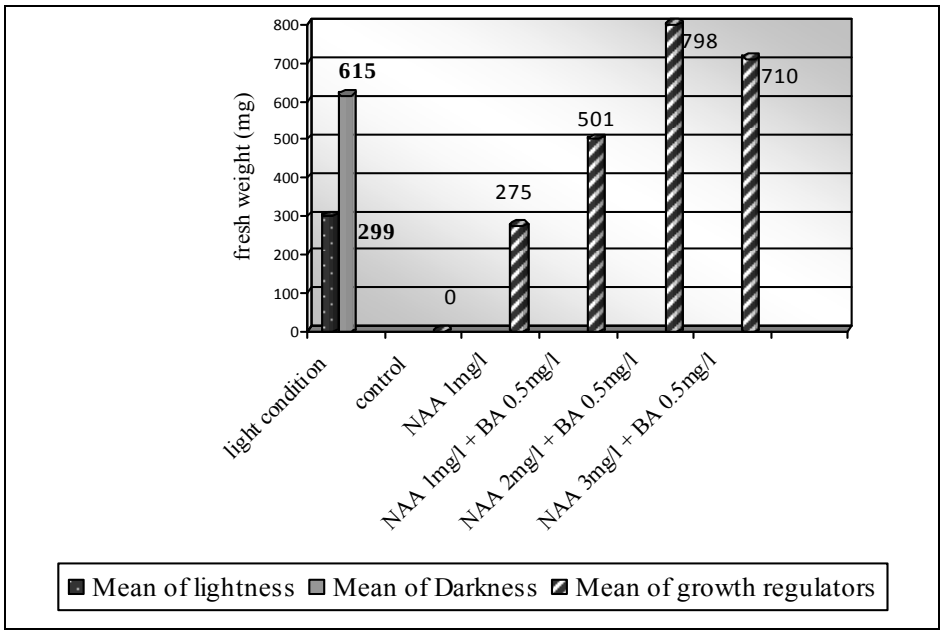

Fig. 1 Effect of growth regulators and lightness condition on the mean of fresh weight of *Hyoscyamus niger* L. grown on MS solid medium for 6 weeks.

LSD at (0.05) level Lightness condition: 57; Regulators: 90.2.

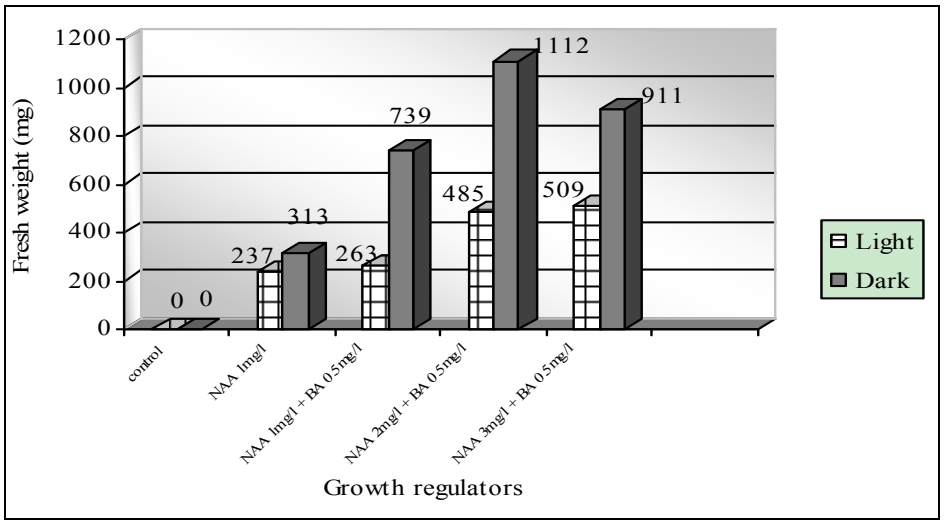

Fig. 2 Interaction between concentration of growth regulators and lightness on callus fresh weight of *Hyoscyamus niger* L. grown on MS solid medium for 4 weeks.

LSD at (0.05) level Interaction between regulators and lightness: 127.5.

medium contained 2 mg/L NAA and 0.5 mg/L BA to produce 67.6 mg of callus with significant differences with all other treatments (Fig. 3). Result of control treatment showed no sign of callus initiation, what so ever the incubation condition, in addition the lowest amount (28.9 mg) of dry callus was found at treatment contained 1 mg/L NAA and 0.5 mg/L BA. The results also showed the presence of significant differences in amount of dry callus weight produced depending on incubation conditions. The amount of dry callus produced under dark condition was 47.52 mg, differ significantly with that of 29.64 mg under light condition, with increase percentage of 37.625%.

The results of Fig. 4 indicated the presence of significant interactions between incubation conditions and growth regulators on callus dry weight production,

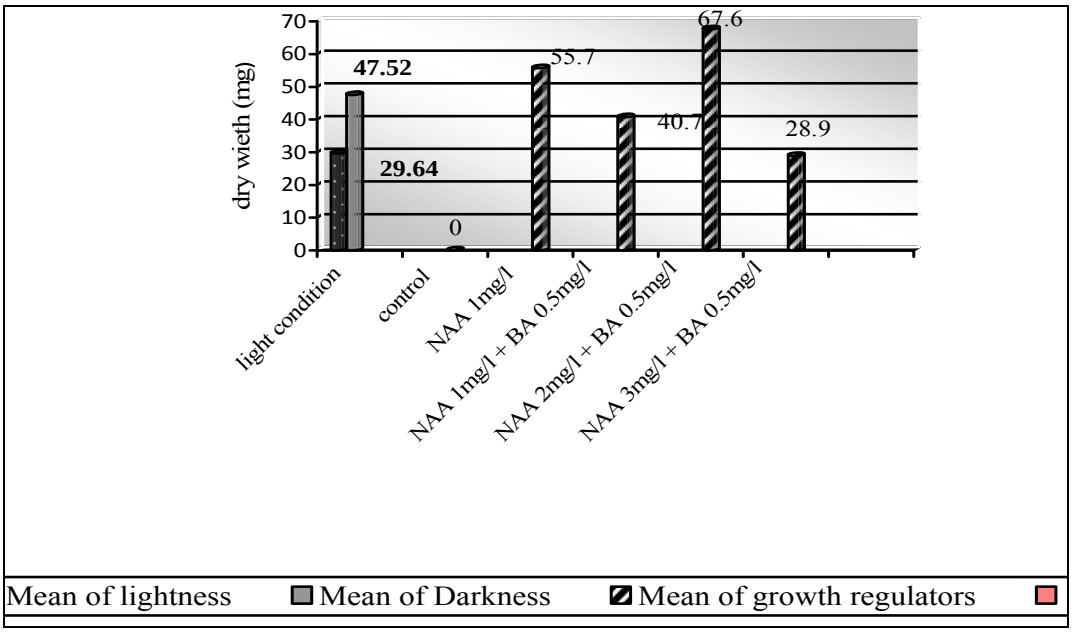

Fig. 3 Effect of growth regulators and lightness condition on the mean of dry weight of *Hyoscyamus niger* L. grown on MS solid medium for 6 weeks.

LSD at (0.05) level Lightness condition: 3.443; Regulators: 5.443.

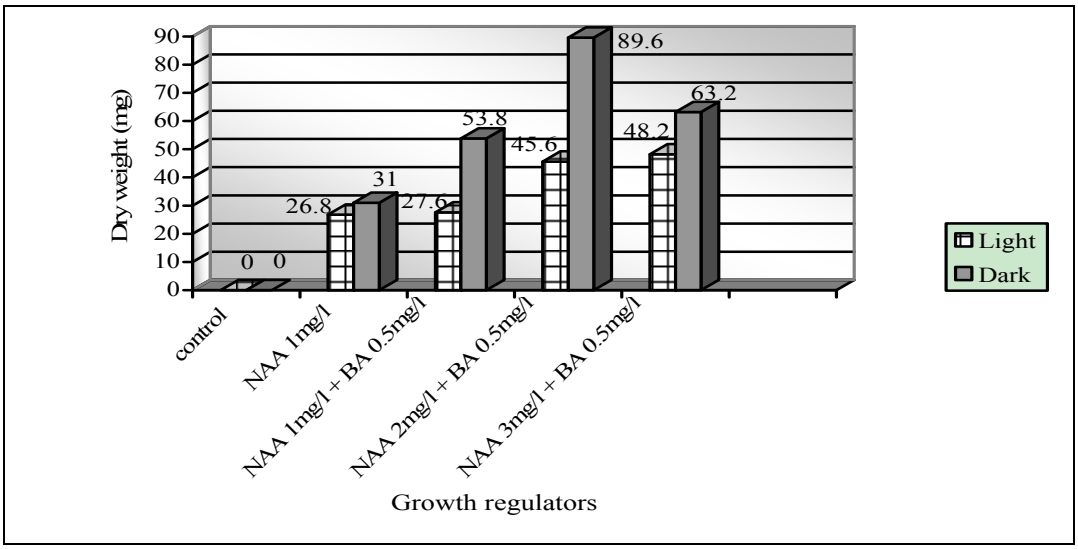

Fig. 4 Interaction between concentration of growth regulators and lightness on callus dry weight of *Hyoscyamus niger* L. grown on MS solid medium for 4 weeks.

LSD at (0.05) level Interaction between regulators and lightness: 7.698.

treatment contained 2 mg/L NAA and 0.5 mg/L BA incubated under dark condition which produce significant amount of callus (89.6 mg) compared with all other treatments. There was no callus produced by the control treatments, either incubated under dark or light conditions. The lowest amount of dry callus (26.8 mg) was achieved at treatment contained 1mg/L NAA and incubated under light.

Generally, the results were in harmony with that depicted before concerning that Auxins and Cytokines have significant impact on callus production [18, 19] mentioned that addition of 2 mg/L NAA and 0.5 mg/L Kinetin (Kin.) led to produce the highest amount of fresh and dry weight of callus of *H. muticus*. While,

the best concentrations of NAA and BA added to MS medium to produce the highest amount of *Datura metel* callus, were 1 mg/L in cell suspension cultures [20]. The results depicted in this investigation concerning the effect of incubation conditions in callus production, indicated the presence of differences with that obtained by Ibrahim et al. [18] which showed that callus production of *H. muticus* under light condition was higher than that under dark condition. While Yaimada and Hghimoto[10] found that the rates of callus growth of *H. niger*, under dark or light were almost identical. However, Stimart [21] stated that callus from H*osta scape* did not formed except under dark condition. Therefore, callus production under light or dark conditions depend on plant species, explants and internal growth regulators [22].

The effects of supplemented abiotic elicitors to the medium on fresh and dry weight of callus produced, Figs. 5, 6 illustrated the results obtained which indicated

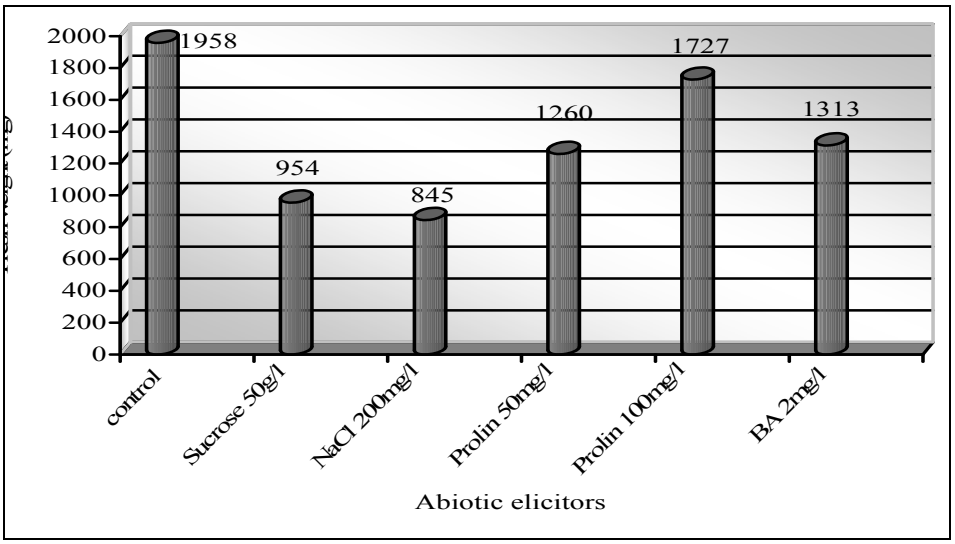

Fig. 5 Influence of abiotic elicitors on fresh weight of callus growth of *Hyoscyamus niger* L. grown on MS solid medium for 6 weeks.

LSD at (0.05) level for fresh weight: 295.3.

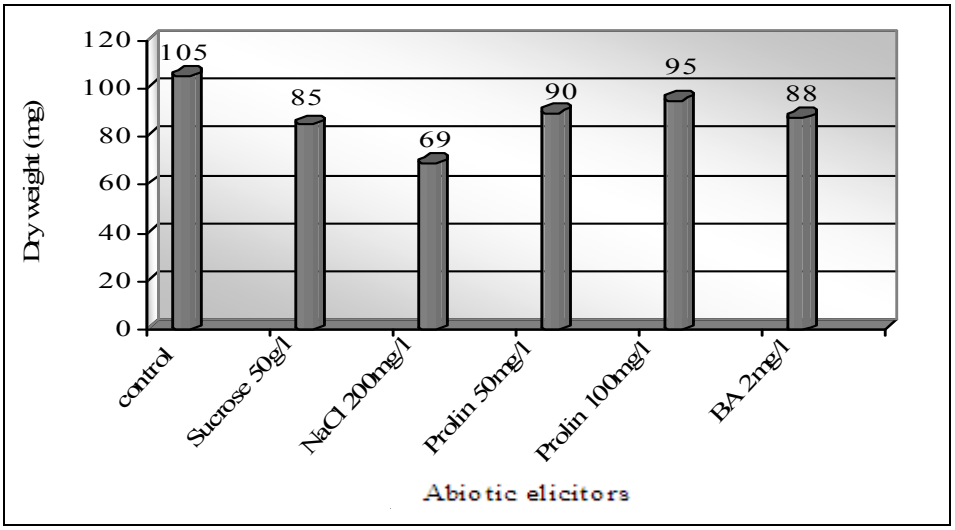

Fig. 6 Influence of abiotic elicitors on dry weight of callus growth of *Hyoscyamus niger* L. grown on MS solid medium for 6 weeks.

LSD at (0.05) level for dry weight: 14.55.

that at control treatment the highest average of fresh and dry callus weight were produced and reached 1,958 and 105 mg respectively, which are significantly difference with supplemented 100 mg/L proline. Supplementation with 200 mg/L NaCl produced the lowest average callus weight. No significant differences were detected in the dry weight between treatments of supplementation of sucrose (50 g/L), proline (50 and 100 mg/L) and BA (2 mg/L) which produced the amounts of 85, 90, 95 and 88 mg of dry callus respectively. The reduction in fresh and dry weight averages of the callus after using abiotic elicitors might be attributed to the stress in the medium inflected by these agents who reflected negatively on cells growth and division, then upon callus growth and suspended cells.

These results are in agreement with what Ajungla et al. [23] were found after using biotic and abiotic elicitors in cell cultures of *Datura metel*, and also with the results of Pinol et al. [24] where they used calcium ion to enhance tropane alkaloid production in cell cultures of *Datura stracionium*.

3.2 Effect of Abiotic Elicitors on Alkaloids Production

The results illustrated in Tables 1 and 2 indicated the presence of significant differences between concentrations of tropane alkaloids (Hyoscyamine and scopolamine) produced depending on type of elicitor used and its concentration (Fig. 7). Concentration of Hyoscyamine was found to increase in the medium supplemented with proline (50 and 100 mg/L) and reached to level of 128.40 and 99.62 ppm with percentage increase of 58.03 and 21.37% respectively, compared with the control (81.25 ppm). Supplementation with other abiotic elicitors, such as sucrose (50 g/L), NaCl (200 mg/L) and BA (2 mg/L) caused reduction in hyoscyamine concentrations to percentage of 30.97, 95.66 and 38.60% respectively, compared with the control. The results of Table 1 indicated that Hyoscyamine did not appear in *H. niger* leave extract of three months of age. This is due to that alkaloids produced at early ages in plant roots transformed after a year to other plant parts such as leaves [25]. Table 2 represents the effect of abiotic

Table 1 Influence of abiotic elicitors on hyoscyamine content in the callus culture of *Hyoscyamus Niger* L..

Type of elicitors	Hyoscyamine con. (ppm)	Percentage of control	
		+	-
Control	81.25		
50 g/L sucrose	56.08		30.97
200 mg/L NaCl	3.72		95.66
50 mg/L proline	128.40	58.03	
100 mg/L proline	99.62	21.37	
2 mg/L BA	48.88	-	-
Dry leaves	-	-	-

Table 2 Influence of abiotic elicitors scopolamine content in the callus culture of *Hyoscyamus niger* L..

Type of elicitors	Scopolamine con. (ppm)	Percentage of control	
		+	-
Control	56.93		
50 g/L sucrose	130.39	+ 129.03	
200 mg/L NaCl	151.97	+ 166.94	
50 mg/L proline	174.00	+ 205.51	
100 mg/L proline	141.84	+ 149.20	
2 mg/L BA	-		
Dry leaves	-		

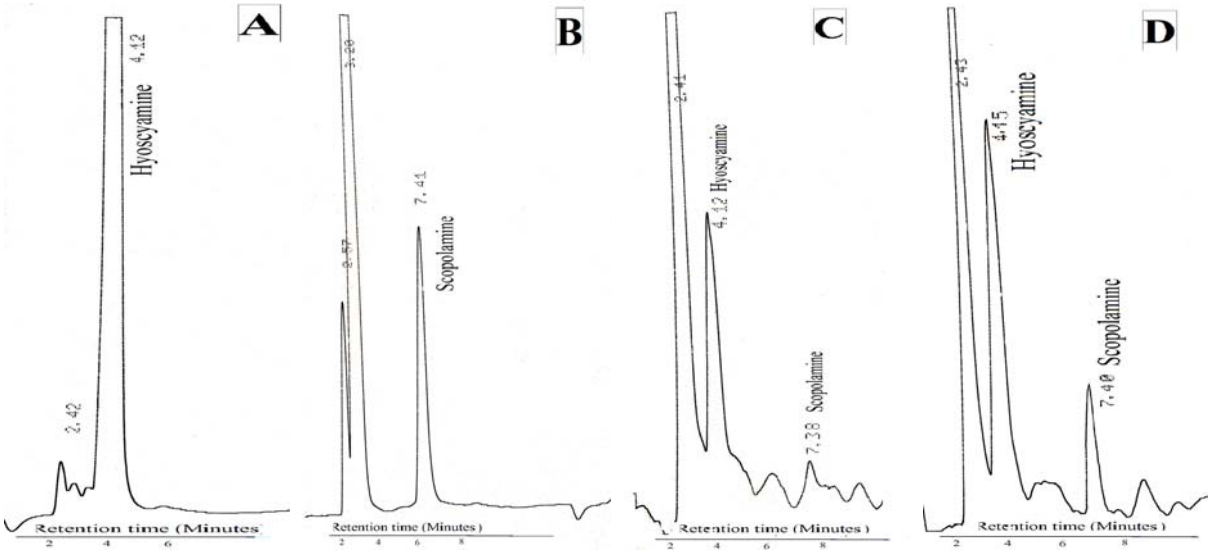

Fig. 7 HPLC chromatograms for: A, standard hyoscyamine; B, standard scopolamine; C, control (without elicitors); D, elicitor treatment (50 mg/L proline).

elicitors in scopolamine concentration extract from callus of *Hyoscyamus niger*. The results showed the positive effect of all elicitors added to the medium. This phenomenon was reflected in the increased amounts of scopolamine compound in callus extract, except with BA which lead to complete disappearance of scopolamine from callus extract. The highest amount (194.00 ppm) of scopolamine was obtained when the callus medium supplemented with 50 mg/L proline in comparison with 56.93 ppm of the control which represent percentage increase of 205.51%. Percentages increase of scopolamine were found to be 166.94, 149.20 and 124.03 in medium supplemented with NaCl (200 mg/L) , proline (100 mg/L) and sucrose (50 mg/L) respectively compared with the control.

Previous investigations indicated that supplementation with abiotic and biotic elicitors to medium cultures lead to either positive or negative development cells or tissues cultures, and the process depend on type and concentration of elicitors, type and source of cultured plant part and the incubation conditions. Ajungla et al. [23] found that adding of abiotic elicitors such as salicylic acid, Na_2SO_4, NaCl, $CaCl_2$ and $AlCl_3$, with different concentrations caused an increase in the amount of tropane alkaloids (Hyoscyamine and scopolamine) produced by *Datura metel*. Supplementation with the abiotic elicitors SiO_2 and jasmonic acid, increased the production amounts of secondary metabolic compound of *Ammi majus* callus cultures [26]. Sharma et al. [27] found that exposure of *H. niger* to low dose of gamma radiation enhance the plant to double the amount of alkaloids. The results depicted in this article are found to be in harmony with that of Ibrahim et al. [18], which stated that the increase in sucrose concentration lead to reduction in alkaloids cumulated, by the callus of *H. muticus*. Yamada and Hashimot [10] found that incubation of *H. niger* callus in darkness, increased the amount of scopolamine and decreased Hyoscyamine compound with 32.9% in callus incubated in light. The results illustrated in this investigation indicated that the increase amounts of scopolamine produced by calls of *H. niger* were come out from the effect of abiotic elicitors supplemented to the medium, in addition to the dark condition used for callus incubation.

As conclusions, the results indicated the possibility of using tissue culture technique in callus initiation of *H. niger* from young leaves. Secondary increasing amounts of secondary metabolic compounds could be achieved by supplementation of abiotic elicitors and incubation of callus medium under dark condition. The percentage increase of scopolamine compound

ranged between 129-204% except of using BA as abiotic elicitor. Thus could satisfy the increasing demand for this compound in the field of medicine.

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HPLC-UV Analysis and Antioxidant Potential of Phenolic Compounds from Endemic Shrub of Arid Environment *Tamarix pauciovulata* J. Gay

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Abstract: This research presents complete phenolic compounds and biological activity of *Tamarix pauciovulata* J. Gay, an endemic Saharan species. The antioxidant assays revealed that crude extract showed strong DPPH scavenging activity (IC50 = 49.357 μg/mL) but in reducing power and hydrogen peroxide scavenging activity, butanolic and ethyl acetate fractions have a potent ferrous ion-chelating ability in particularly the butanolic fraction (63.18% reduced power at 50 μg/mL) and a powerful scavenging activity on hydrogen peroxide in particularly ethyl acetate fraction (IC50 = 0.205 μg/mL). The phenolic compounds of *Tamarix pauciovulata* leaves were analyzed by HPLC-UV. The major phenolic of leaf extracts are syringic acid (1.07 g/100g), quercetin (34.1 mg/100g), kaempferol (5.77 mg/100g), isorhamnetin (5 mg/100g). Others phenols were identified such as isoquercetin, catechin, epicatechin and its derivatives. Results indicated that the leaves extracts from *Tamarix pauciovulata* have great capacities to prevent diseases caused by the overproduction of radicals and can become important source of dietary compounds with health protective potential.

Key words: *Tamarisk pauciovulata*, biological activity, polyphenols, syringic acid, quercetin.

1. Introduction

Tamarix species (Tamaricaceae Family) are bushes or trees, mostly evergreen and pink or white blossoms. They are relatively long-lived plants that can tolerate a wide range of environmental conditions and resist abiotic stresses [1]. *Tamarix* species, commonly known as tamarisk or salt cedar are widely distributed on a large area from Morocco to India. It is an introduced tree in southwestern United States [2]. Tolerant of alkaline and saline soils [3, 4], *Tamarix* withstands saline soils by regulating its salt balance via excretion of excess salts through foliar glands and consuming large quantities of water from underground sources [1]. *Tamarix* species are looked upon with favor as a check to erosion, as a windbreak and are also employed in traditional medicine as astringent, aperitif, stimulus of perspiration and diuretic [5], their fruit and leaves good astringents, are used for the treatment of dysentery and chronic diarrhea and are also considered to be effective in the treatment of leucoderma [6]. In traditional Egyptian medicine, extracts of *Tamarix* have been used especially as antiseptic agents, and also used for tanning and dyeing purposes [7]; they are useful again in spleen trouble and eye diseases [8]. Furthermore, several researches proved antioxidant and antimicrobial activities of some *Tamarix* species such as *Tamarix ramosissima* [9], *Tamarix hispida* [10], *Tamarix aphylla* [11]. Usually, *Tamarix* wood is used for fuel because it produces a fragrant odor when burned [12].

Chemopreventive activity of *Tamarix* species was

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due to their content on secondary metabolites, in particularly on their phenolic content, such as flavonoids, which are known to possess potent antioxidant properties. The reduction activity of phenolic and flavonoids compounds depends on the number of free hydroxyl groups in the molecular structure [13]. Several studies show that phenolic can directly scavenge molecular species of active oxygen, regulate nitric oxide, stabilize membranes by decreasing membrane fluidity and inhibit lipid peroxidation by trapping the lipid alkoxyl radical. Phenolic compounds, especially flavonoids, possess ideal structural chemistry for this activity and have shown to be more effective in vitro than vitamins E and C [14]. For example, The ortho 3',4'-dihydroxy structure in the B ring is the important determinant for the antioxidant potential of quercetin and catechin [15] and appears to cause many beneficial effects on human health. As antioxidants, phenolic compounds may protect cell constituents against oxidative damage and limit the risk of various degenerative disease associated to oxidative stress [16]. Their antioxidant capacity confers a therapeutic potential in cardiovascular diseases, gastric or duodenal ulcers, cancer or hepatic pathologies. Also important are their antiviral and their antiallergic actions, antithrombotic and anti-inflammatory properties [17].

Tamarix pauciovulata J. Gay is endemic to south area of Algeria, is a Saharan shrub, recommended to treat internal hematomas and inflammation. *Tamarix pauciovulata* has not been studied and no report in literature has done phytochemical and biological activity. For this reason, the aim of this work was to study several antioxidant activities and to analyze the phenolic constituents of *Tamarix pauciovulata* leaves.

2. Material and Methods

2.1 Source of Plant

Tamarix pauciovulata J. Gay collected from *Adrar* (arid area in south Algeria). The leaves was dried and ground finely to a powder in an electric mill and stored at 4 °C until use.

2.2 Extraction of Bioactive Compounds

About 100 g of the powdered leaves was extracted for 48 h with 70% methanol at room temperature. The mixture was filtered through whatman paper N°1 and filter paper of 0.45 µm porosity, then filtrate was dried using Laborota 4000 rotary evaporator at 50 °C. The residue was dissolved in sterile distilled water and freeze-dried using CHRIST-ALPHA 1-4 Lyophilizator.

For antioxidant activity, a part of the extract (CME) was dissolved in distilled water and partitioned with ethyl acetate and butanol successively to afford a butanol soluble fraction (BF), ethyl acetate fraction (EAF) and an aqueous fraction (QF).

2.3 Phytochemical Analysis

To detect the presence of various chemical constituents, The crude extract was qualitatively analyzed for the presence of alkaloids, saponin, flavonoids, tannins, cardiac glycosides, steroids and triterpenes, reducing sugars and cyanogen compounds [18-22] and each of the tests was qualitatively expressed as negative (–) or positive (+). The phenolic constituents of the leaves were analyzed by method CATHPLC: HPLC-UV (method Cofrac, Agrobio, Rennes).

2.4 Reducing Power Activity

The ferric reducing power assay was carried out by the method of Oyaizu [23]. Each concentration of aliquot of extract (1 mL) was mixed with 2.5 mL sodium phosphate buffer (200 mM; pH 6.6) and 2.5 mL potassium ferricyanide (1%). After incubation at 50 °C for 20 min; 2.5 mL TCA (10%) was added to the mixture and then centrifuged at 3000 rpm for 30 min. A 2.5 mL of upper layer was mixed with 2.5 mL distilled water and 0.5 mL ferric chloride (0.1%) was added. The green color developed was measured at 700 nm with UV-visible spectrophotometer

(Shimadzu UV mini 1240). A higher absorbance indicated the higher reducing power.

2.5 Scavenging of Hydrogen Peroxide

A solution of hydrogen peroxide (20 mM) was prepared in phosphate buffered saline (PBS, pH 7.4). Various concentrations of 1 mL of the extracts or standards in methanol were added to 2 mL of hydrogen peroxide solution in PBS. The absorbance was measured at 230 nm after 10 min against a blank solution contained extracts in PBS without hydrogen peroxide [24]. The percentage inhibition was calculated by using the following formula:

$$\text{Scavenging activity (\%)} = [(\text{Abs control} - \text{Abs sample})/\text{Abs control}] * 100 \quad (1)$$

IC50 value is the concentration of the sample required to scavenge 50% free radical. Experiments were performed in triplicate.

2.6 DPPH Antioxidant Activity

The free radical scavenging activity was determined by the method described by Brand-Williams et al. [25]. Leaves extract (0.1 mL) at different concentration was added to 3.9 mL of a 6×10^{-5} M DPPH solution in methanol. Absorbance at 515 nm was determined after 30 min and the percentage inhibition activity was calculated from {[(Ac-At/Ac] × 100}, where Ac, absorbance of the negative control (solution of DPPH without extract) and At is the absorbance of the extract.

2.7 Statistical Analysis

All the experimental results were mean ± SD of three measurements and data were evaluated by using student's *t*-test. $P < 0.05$ was regarded as significant.

3. Results and Discussion

3.1 Phytochemical and Phenolic Compounds of Tamarix pauciovulata

The percentage yield of crude extract and fractions was found to be 285.2 mg/g leaves powder for crude methanolic extract (CME), 94.05 mg/g CME for ethyl acetate fraction, 67.857 mg/g CME for butanolic fraction and 534.528 mg/g CME for aqueous fraction.

Preliminary phytochemical screening of crude methanolic leaves extract of *Tamarix pauciovulata* revealed the presence of the following classes of chemical compounds such as saponins, favonoids, condensed and hydrolysable tanins, steroids and tritterpenes, cardiotonic glycosids and reducing glucids. We note that alkaloids and cyanogenic components were absents.

Phenolic composition, and quantification analysis with HPLC-UV showed that syringic acid was the major component (1.07 g/100g), naturally occurring 4-hydroxy-3,5-dimethoxybenzoic acid, a type of phenol acid C6-C1. Four flavonols (C6-C3-C6) were identified and quantified, quercetin, isoquercetin, kaempferol and isorhamnetin (Table 1). Plants need

Table 1 HPLC-UV analysis: chemical composition and quantification of *Tamarix pauciovulata* leaves.

Analysis method	mg/100g Leaves powder
Phenolic acids: HPLC-UV	
Syringic acid	= 1070
Flavonols: HPLC-UV	
Quercetin	= 34.1
Isoquercetin	< 5
Kaempferol	= 5.77
Isorhamnetin	= 5.00
Total	≤ 49.9
Catechins: CATHPLC: HPLC-UV	
(+)-Catechin	< 0.1
(−)-Epicatechin	< 0.1
(−)-Epicatechin gallate	< 0.1
(−)-Epigallocatechin	< 0.1
(−)-Epigallocatechin gallate	< 0.1

phenolic compounds for pigmentation, growth, reproduction, resistance to pathogens and for many other functions [26]. Plants are exposed to ambient solar ultraviolet-B (UV-B) radiation (280-320 nm) that is an environmental challenge negatively affecting DNA, proteins and membranes, thus leading to altered metabolism through the generation of reactive oxygen species (ROS). Plants protect themselves from this harmful radiation by synthesizing phenolic compounds [27, 28]. It has been proposed that flavonoids with their high absorptivity at 250-270 and 335-360 nm act as good UV screens [29]. Quantitatively, the important component of these flavonoids identified is quercetin: 34.1 mg/100g. Quercetin is one of the most active antioxidants found in medicinal plant. In human, it has known anti-inflammatory effects and it also helps to prevent cancer, prostatitis, heart disease, cataracts, allergies, bronchitis, and asthma.

3.2 Reducing Power Assay

The reducing power assay serves as a significant indicator of potential antioxidant proposed for the antioxidant activity such as prevention of chain initiation, binding of transition-metal ion catalysts, decomposition of peroxides, and prevention of continued hydrogen abstraction, reductive capacity and radical scavenging [30]. Crude extract and fractions showed concentration dependent reductive effects.

The data in Fig. 1(A, B, C, D, E) show the reducing power (as indicated by the absorbance at 700 nm) of the crude extract and its derived fractions compared to ascorbic acid (e), their ranking order (as indicated by EC50) was as follows: butanolic fraction (0.037 mg/mL ± 0.007) > ethyl acetate fraction (0.098 mg/mL ± 0.009) > aqueous fraction (0.580 mg/mL ± 0.08) > ascorbic acid (0.812 mg/mL ± 0.062 ± 0.072) > crude extract (16.458 mg/mL ± 1.005).

The reducing power of three fractions of *Tamarix* was remarkably stronger than that of pure antioxidant standard ascorbic acid. The highest reducing activity was again observed for butanolic fraction. The reducing properties are generally associated with the presence of different reductants [31]. The antioxidant action of reductants is based on the breaking of the free radical chain by donating an hydrogen atom. Reductones also react with certain precursors of peroxide, thus preventing peroxide formation [32].

3.3 Hydrogen Peroxide Assay

Hydrogen peroxide can cross cell membrane [33]. It is not very reactive for itself, but sometimes is toxic to cell because it may give rise to hydroxyl radical in the cells [34] and can inactive a few enzymes directly, usually by oxidation of essential thiol (-SH) groups [33].

The scavenging abilities of crude extract and fractions with hydrogen peroxide were shown in Fig. 2(A, B, C, D) and compared with ascorbic acid (E). It was noticed that all the samples were able to scavenge hydrogen peroxide. In order the IC50, the hydrogen peroxide scavenging activity were: ethyl acetate fraction (0.20 ± 0.00161 µg/mL), > butanolic fraction (1.51 ± 0.0181 µg/mL) > ascorbic acid (4.77 ± 0.12056 µg/mL) > aqueous fraction (13.83 ± 0.1775 µg/mL > crude extract (40.057 ± 2.5866 µg/mL). According to these results, we can note that ethyl acetate fraction from *Tamarix pauciovulata* showed stronger hydrogen peroxide inhibition than the standard ascorbic acid.

3.4 DPPH Antioxidant Activity

Radical scavenging activities of crude extract, fractions and standard (Ascorbic acid) at different concentrations were tested by the DPPH method and the results are shown in Fig. 3(A, B, C, D, E). DPPH is relatively stable nitrogen centered free radical that can accept an electron or hydrogen radical to become a stable diamagnetic molecule [35], when DPPH expose to antioxidant compounds, its purple color

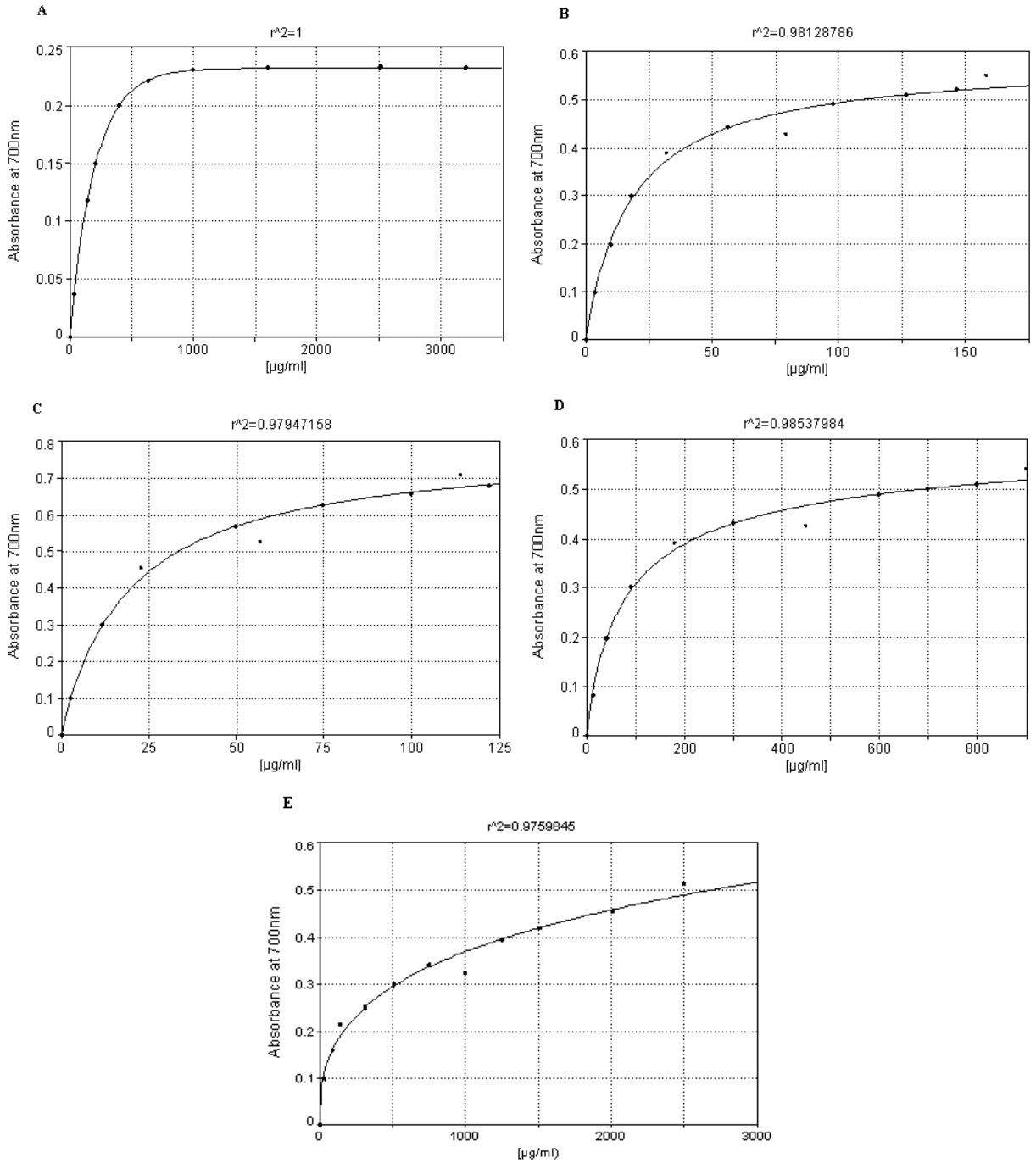

Fig. 1 Reducing power of crude extract and fractions compared to antioxidant standard; ascorbic acid.

changes to yellow. Antioxidant reacts with DPPH, which is a stable free radical and converts it to α-α-diphenyl-β-picrylhydrazine. The degree of discoloration indicates the scavenging potential of the antioxidant extract [36]. From result, it may be postulated that crude extract and different fractions have hydrogen donors, thus scavenge free radical DPPH but the best DPPH scavenging activity was exerted by crude methanolic extract and ascorbic acid as reference.

The quality of the antioxidants in the extracts was determined by the IC_{50} values, a low IC_{50} value indicates strong antioxidant activity. In the present study, the radical scavenging activity of crude

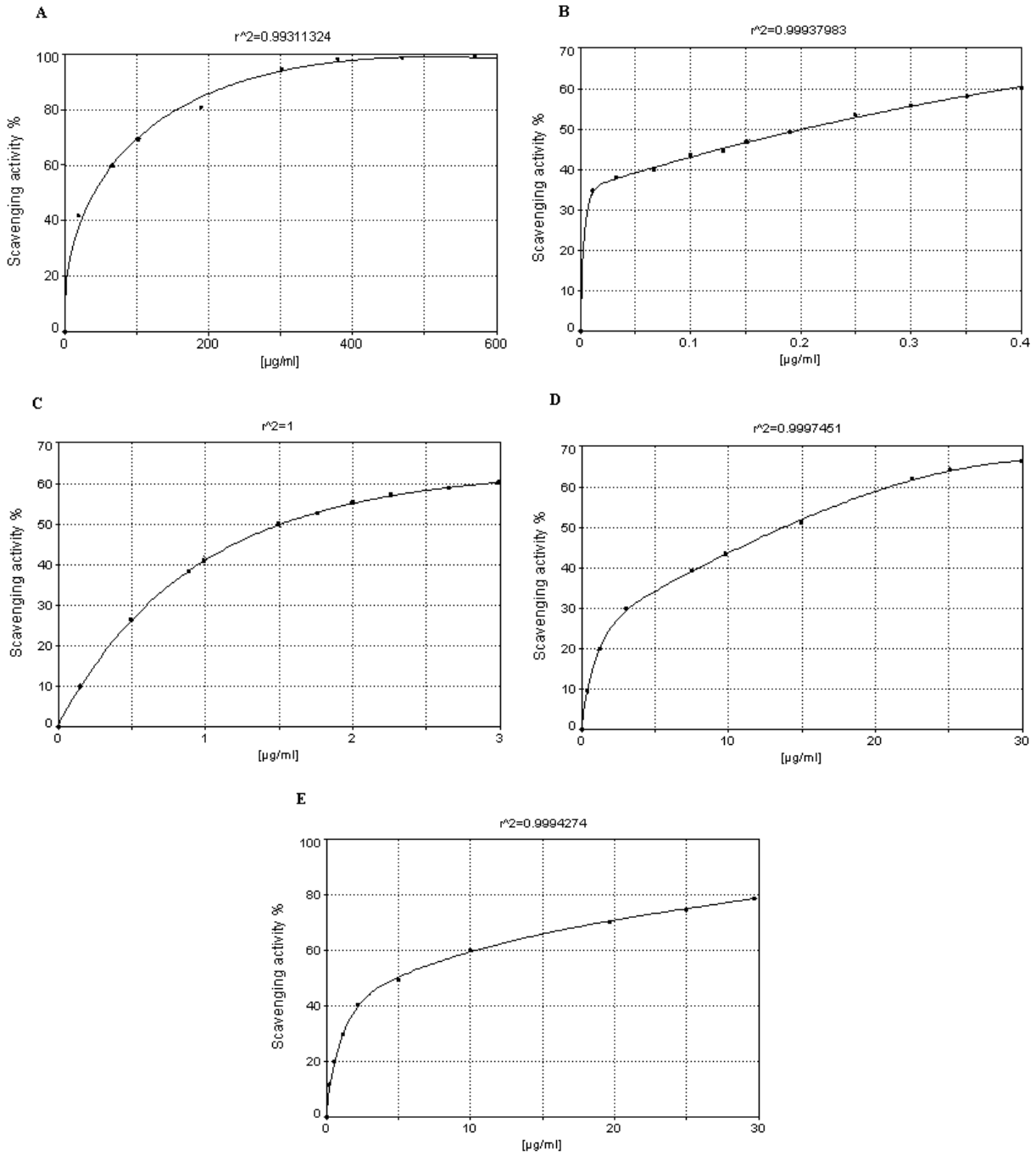

Fig. 2 Hydrogen peroxide scavenging activity of crude extract, fractions and ascorbic acid.

methanolic extract was more than that of the tree fractions. It means that the IC_{50} of crude methanolic extract was less than those of others but the standard have a high antioxidant power. IC_{50} for ascorbic acid, crude extract, aqueous fraction, butanolic fraction and ethyl acetate fraction were 4.498 ± 0.75095 µg/mL, 49.357 ± 2.42679 µg/mL, 892.03 ± 11.60786 µg/mL, 1.002 ± 0.07071 mg/mL, 2.653 ± 0.40164 mg/mL respectively.

It has been mentioned that the antioxidant activity of plants might be due to their phenolic compounds [37] and correlated with the content of this groups of compounds [38]. The leaves of *Tamarix pauciovulata* are rich on phenolic compounds and present a good

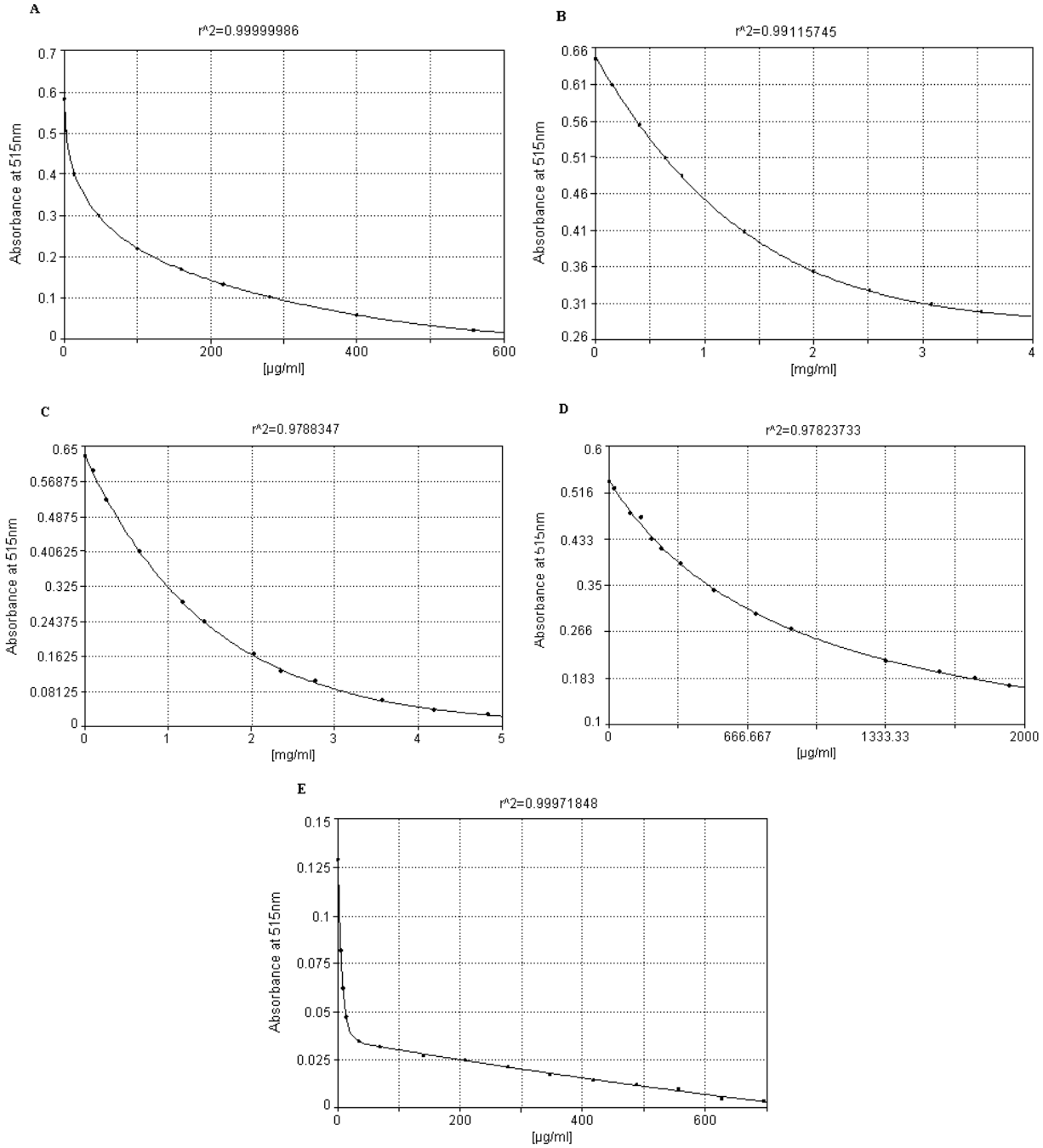

Fig. 3 DPPH scavenging activity of crude extract, fractions and ascorbic acid.

source of natural antioxidants. Free radicals cause autoxidation of unsaturated lipids in food [39] and Antioxidants cease the free radical chain of oxidation and to donate hydrogen from the phenolic hydroxyl groups. Therefore formed stable end-product does not permit further oxidation of the lipid [40]. *In vitro* antioxidant activity of quercetin was tested for DPPH free radical, superoxide anions, hydrogen peroxide and hydroxyl radical. It scavenges oxygen radicals, inhibits xanthine oxidase, protects against lipid peroxidation, chelates metal ions and forms inert complexes that can't take part in the conversion of superoxide radicals and hydrogen peroxide into hydroxyl radicals [41].

4. Conclusions

In conclusion, the leaves extract and fractions showed antioxidant activity, by measuring their capacity to scavenge the DPPH and the hydrogen peroxide and to reduce iron (III) to iron (II). The results of the present study indicate that leaves from *Tamarix pauciovulata* can be used as easily accessible source of natural antioxidants. The high antioxidant activity showed may be directly correlated to the high phenolic contents of the leaves in particularly of a high content on synergic acid and quercetin.

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Investigation of Argentinean Plant Extracts for Their Antibacterial Activity

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Abstract: Plants of *Baccharis* (Asteraceae) genus are commonly known in Argentina as "carqueja". The antimicrobial activity and minimal inhibitory concentration of *B. articulata*, *B. trimera* and *B. crispa* aqueous and ethanolic extracts were evaluated by using the micro-well dilution method. Previously, the components of extracts were analyzed by spectroscopial means. Gram-positive bacteria were more sensitive to *Baccharis* species extracts than Gram-negative bacteria. Out of 3 plant species, *B. trimera* showed significant antibacterial activity and aqueous and ethanolic extracts were active against *Staphylococcus aureus* (MIC = 2,500 μg/mL and 1,250 μg/mL, respectively) and *Listeria monocytogenes* (MIC = 625 μg/mL and 625 μg/mL, respectively). All ethanolic extracts inhibited the growth of the selected Gram-positive (MIC values ranged between 625 μg/mL and 1,250 μg/mL). Therefore, all Gram-negative bacteria were resistant to the ethanolic and aqueous extracts tested. One flavone, genkawanin, was identified from the three ethanolic extracts as the responsible of antibacterial activity. Two terpenes, hawtriwaic acid and bacrispine, were identified from ethanolic extract of *B. crispa* and *B. trimera* as the responsibles of antibacterial activity. These preliminary studies corroborated the antimicrobial activity of the selected plants used in folklore medicine. Therefore, they could be potential sources of new antimicrobial agents used in treatment of infectious diseases.

Key words: *Baccharis articulata*, *Baccharis crispa*, *Baccharis trimera*, ethanolic extract, aqueous extract.

1. Introduction

Many natural compounds isolated from plants have demonstrated a wide spectrum of biological activities [1].

Among these various kinds of natural substances, medicinal plants have received particular attention as a novel way to reduce the proliferation of microorganisms [2].

The American genus *Baccharis* (Asteraceae) consists of approximately 500 species [3, 4], near 100 of them are present in Argentina.

Plants of the Baccharis genus are very rich in secondary metabolites as sesquiterpenes, clerodan type diterpenes, triterpenes, and phenylpropanoids [5, 6]. It is very frequent the isolation of several types of flavones, usually with a high degree of oxygenation. Some of the aforementioned metabolites are described as interesting chemotaxonomic markers.

Some metabolites such as diterpenoids, flavonoids and volatile terpenoids have been earlier determined by spectroscopical means in *Baccharis* sp. ethanolic extracts tested in the present study (Table 1) [7, 8].

Several *Baccharis* species are commercially used in folk medicine as antiseptics and anti-inflammatory agents, and to treat both gastric ulcers and skin sores [6]. For example, *Baccharis incarum* phytopreparation applied topically could be used to treat skin and soft tissues infection produced by methicillin-resistant *Staphylococcus aureus* [9]. *Baccharis dracunculifolia*

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Table 1 Characteristics of *Baccharis* species used against pathogenic bacteria isolated in San Luis (Argentina).

Specie	Chemical composition	Traditional uses
Baccharis articulate (Lam.) Persoon	Genkwanin 7-4'-di-O-metilapigenin Acacetin Circimaritin Salvigenin Barticulidiol malonate Bacchotricuneatin A	Decoctions from leaves are used for diarrhoea and respiratory and urinary infections
B. trimera (Less) DC.	Genkwanin 7-4'-di-O-metilapigenin Hawtriwaic acid Hawtriwaic acid lactone 1-desoxibacrispine Bacrispine	Infusion, decoctions or tintures of the aerial parts for liver disease, wound, diarrhea, angina, rheumatism, renal disorders, diabetes
B. crispa Sprengel	Genkwanin 7-4'-di-O-methylapigenin Hawtriwaic acid Hawtriwaic acid lactone 1-desoxibacrispine Bacrispine Butenolids	Infusion, decoctions of the aerial parts are used for digestive disorders and ulcers

leaves extracts present antifungal and antibacterial activity [10]. Hexane and dichloromethane extracts of *B. grisebacchii* are active on methicillin-resistant and methicillin sensitive *S. aureus* [11].

Nevertheless, to our knowledge there are few reports available in the literature on the biologically active constituents of *Baccharis articulata, B. trimera* and *B. crispa*, and their biological properties against pathogenic bacteria.

Therefore, our purpose in the present study was evaluated the antibacterial activity of aqueous and organic extracts from these three native plants against Gram positive and Gram negative pathogenic bacteria species. The results should be provided scientific evidences about the efficacy of their use in folklore medicine.

2. Materials and Methods

2.1 Plant Collection and Identification

Baccharis articulata (Lam.) Persoon., *B. trimera* (Less) DC. and *B. crispa* Sprengel, aerial parts were collected in Departamento La Capital, San Luis Province, Argentina, between November 2009 and February 2010. Voucher specimens were identified by Ing. Luis del Vitto and Dr. Elisa Petenatti and lodged in the University of San Luis (Argentina) Herbarium (Nº 7366, 6709, 376, respectively).

2.2 Preparation of Extracts

Ground plant materials (5 g) were extracted with 100 mL of 70% ethanol (EtOH) in a sonication bath at room temperature for 1 h. The extracts were then filtered under vacuum through Whatman Nº1 filter paper. They were concentrated *in vacuo* using a rotary evaporator at 30 ºC. The resultant extracts were air-dried at room temperature [12]. 5 g of the ground material were soaked in 100 mL of sterile water and allowed to stand for 72 h. Then, the crude extracts were obtained by filtration [13]. Extracts were dissolved in small drops of dimethylsulfoxide (DMSO) and toped up with physiological saline and the stock solution kept at 4 ºC.

2.3 Microorganisms

The following bacteria were selected for this study. One strain of *Listeria monocytogenes* was obtained from the Pasteur Institute, France, culture collection: CLIP 74910. It was chosen in order to represent any specie responsible of food-borne disease.

Staphylococcus aureus ATCC 43300 was used as a wound/skin pathogen. *Escherichia coli* (isolated in UNSL laboratory), was used to represent pathogens that cause gastro enteritis while *Pseudomonas aeruginosa* ATCC 27853 was used as an environmental pathogen.

2.4 Antimicrobial Activity

2.4.1 Determination of Minimum Inhibitory Concentration (MIC)

The MIC of *Baccharis articulata*, *B. trimera* and *B. crispa* extracts were determined by microplate method (micro-well dilution) [14], in MH broth (Britania, Argentina) pH 7.2 supplemented with 0.01% (w/v) of 2,3,5-triphenyltetrazolium chloride (TTC) as visual indicator of bacterial growth. The inoculum of each strain was prepared from 18 h broth culture and adjusted to the tube 0.5 of Mc Farland scale (10^8 bacterial cells). Then, they were diluted 10 times. The extracts were disolved in 20% Tween-80 and then diluted with phosphate buffer saline (PBS) to the highest concentration to be tested (5,000 μg/mL), and then serial two-fold dilutions were made in concentration ranges from 5,000 to 78 μg/mL. The 96-well plates were prepared by dispensing into each well 95 μL of nutrient broth and 5 μL of the inoculum. 100 μL aliquot from the stock solutions of the extracts and their serial dilutions initially prepared was transferred into seven consecutive wells. The final volume in each well was 200 μL. The plates were covered with sterile plate sealer and then incubated at 37 °C for 24 h. MIC was defined as the lowest concentration of the extract in the medium in which there was no visible growth after incubation (no red colour signifying live growth). It is established that TTC, a water-insoluble, colorless compound, can be reduced to water-insoluble red formazan by a variety of organisms. TTC reduction is used as a quantitative method in the evaluation of tissue viability. The experiments were replicated at least twice.

2.4.2 Determination of Minimal Bactericidal Concentration (MBC)

Extracts that showed inhibitory activity in the preliminary broth assay were submitted to a subculture on the surface of the tripticase soya agar plates, in order to evaluate bacterial growth. MBC was determined as the lowest concentration that showed no bacterial growth in the subcultures after 24 h of aerobic incubation at 37 ºC.

2.5 Thin-Layer Chromatography (TLC)

Merck F_{254} plates, 10 × 10 cm, 1 mm thick were used. *B. articulata*, *B. trimera* and *B. crispa* aqueous and ethanolic extracts were applied and the chromatogram was developed using chloroform-methanol (9:1, v/v) and chloroform-ethanol (7:1, v/v) as solvent systems, respectively. TLC plates were run in duplicate. Spots and bands were visualized using the following spray reagents *p*-anisaldehyde : acetic acid : sulfuric acid (1:97:2; v/v) and sulfuric acid : acetic acid : water (16:80:4; v/v), followed by heating in an oven at 150 °C for 5 min. The diterpenes bacchotricuneatin A, hautriwaic acid, and bacrispine as well as the flavone genkwanin were used as standards. The set of TLC plates for the bioautography assays were dried overnight in a sterile room for complete removal of solvent and were used unrevealed.

2.6 Bioautography

Plates TLC were covered with 1-2 mm layer of soft medium (BHI with 0.6% agar) containing 0.1% (w/v) TTC and an aliquot of an overnight culture of *S. aureus* ATCC 43300 (10^8 CFU/mL) and *L. monocytogenes* CLIP 74910 (10^8 CFU/mL), respectively. The plates were placed in a sterile tray, sealed to prevent the thin agar layer from drying, and incubated at 37 °C for 24 h. Where microbial growth has been inhibited an uncoloured area can be seen on the deep pink-red background. The plates were run in duplicate.

3. Results and Discussion

Extracts of regional *Baccharis* species from semi-arid western region Argentina, were screened for their antibacterial activity against *S. aureus*, *L. monoytogenes*, *E. coli* and *P. aeruginosa*.

Previously, the chemical composition of *B. articulata*, *B. crispa*, and *B. trimera* extracts were investigated by spectroscopical means [7, 8]. A total of 12 components were identified (Table 1).

The extracts of *B. crispa* and *B. trimera* resulted in the identification of the same diterpenoids and flavonoids but butenolids were only present in the extract of *B. crispa*. *B. crispa* and *B. trimera* yielded extracts rich in hautriwaic acid and their lactone as well as the neoclerodane diterpenes 1-desoxibacrispine and bacrispine. From *B. articulata* the neoclerodanes barticulidiol malonate and bacchotricuneatin A were the most important metabolites (Table 1).

There are few data in the literature on the compounds with antibacterial activity from the species included in this study, whereas data have been reported on the antimicrobial activity of terpenoids and flavonoids isolated from other *Baccharis* species such as *B. incarum* [15-18] and *B. boliviensis* [15]. The antibacterial activity of terpenoids is generally believed to involve actions at phospholipids membranes, where partitioning results in destabilisation and disorder culminating in ion leakage in bacteria and disruption of membrane dependent energy generating processes in eukaryotic microorganisms [19].

Traditional healers use primarily water as the solvent but in our studies we found that plant extracts in organic solvent (ethanol) provided more consistent antimicrobial activity compared to those extracted in water. These observations can be rationalized in terms of the polarity of the compounds being extracted by each solvent and, in addition to their intrinsic bioactivity, by their ability to dissolve or diffuse in the different media used in the assay.

In agreement with previous reports, gram-positive bacteria were more sensitive to *Baccharis* species extracts than Gram-negative bacteria [20, 21]. The minor susceptibility of Gram-negative bacteria may be attributed to an outer membrane surrounding the cell wall which restricts diffusion of hydrophobic compounds through the lipopolysaccharide. Moreover, the periplasmic space contains enzymes, which are able of breaking down foreign molecules introduced from outside [22].

Out of 3 plant species, *B. trimera* showed significant antibacterial activity and both the extracts (aqueous and ethanolic) were active against the Gram-positive investigated bacteria.

All aqueous extracts inhibited *S. aureus* but only *B. trimera* aqueous extract was active against *L. monocytogenes* (Table 2).

The TLC plates corresponding to the *B. trímera* aqueous extract showing an intense spot in the polar region of the plate. This result probably is due to the presence of some glicoside type compound (Fig. 1a).

Table 2 Antibacterial activity of aqueous extracts against Gram-positive and Gram-negative bacteria.

	B. articulata		*B. trimera*		*B. crispa*	
	[2]MIC	[3] MBC	MIC	MBC	MIC	MBC
S. aureus ATCC 43300	1,250	2,500	1,250	2,500	625	1,250
L. monocytogenes [1]CLIP 74910	[4]NA	NA	625	1,250	NA	NA
E. coli	NA	NA	NA	NA	NA	NA
P. aeruginosa ATCC 27853	NA	NA	NA	NA	NA	NA

[1]CLIP: *Listeria* Collection of the Pasteur Institute; [2]MIC: Minimum inhibitory concentration (μg/mL); [3]MBC: Minimun bactericidal concentration (μg/mL); [4]NA: no activity.

All ethanolic extracts inhibited the growth of the selected Gram-positive bacteria (Table 3).

Therefore, all Gram-negative bacteria were resistant to the ethanolic and aqueous extracts tested (Tables 2, 3).

The MICs of extracts determined by microplate method (micro-well dilution) ranged from 625 to 2,500 μg/mL. The most sensitive microorganism to extracts from *B. trimera* and *B. crispa* was *L. monocytogenes*, with MIC of 625 μg/mL (Tables 2, 3). Similary, the ethanolic extract of *B. crispa* was active against *S. aureus* with MIC of 625 μg/mL.

S. aureus was inhibited by *B. articulata*, *B. trimera* and *B. crispa* aqueous extracts at the highest MIC (2,500 μg/mL) (Table 2).

MBC values were one or two fold higher than the corresponding MIC values in both extracts (Tables 2, 3).

To obtain some information on the active components, the extracts were analyzed by TLC on silica gel and assayed for bioautography. This assay for qualitative antibacterial activity detection demonstrated well-defined inhibition zones against *S. aureus* (Fig. 1) in correspondence with those flavonoids and sapogenines bands.

Fig. 2 shows the appearance of the chromatogram after treatment with *L. monocytogenes*, indicating the localization of bacterial inhibition zone.

One flavone, genkawanin, was identified from the three ethanolic extracts as the responsible of antibacterial activity (Fig. 1). Two terpenes, hawtriwaic acid and bacrispine, were identified from ethanolic extract of *B.crispa* and *B. trimera* as the responsibles of antibacterial activity (Fig. 2).

4. Conclusions

The results of the present study support the folkloric usage of the studied plants and suggest that

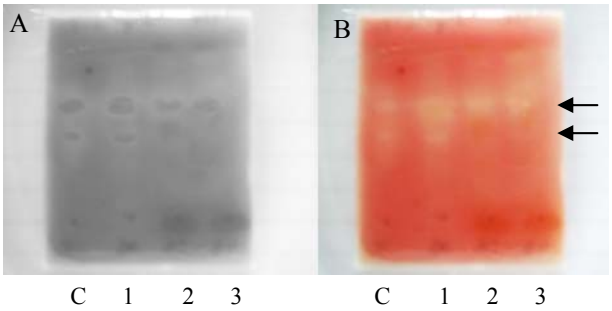

Fig. 1 Thin layer chromatography plate of (1) *B. articulata*, (2) *B. crispa* and (3) *B. trimera* ethanolic extracts. A: visual appearance. B: *S. aureus* bioautography overlay. Arrows indicate regions of inhibition growth visualized wih tetrazolium red. C: Flavones (standard).

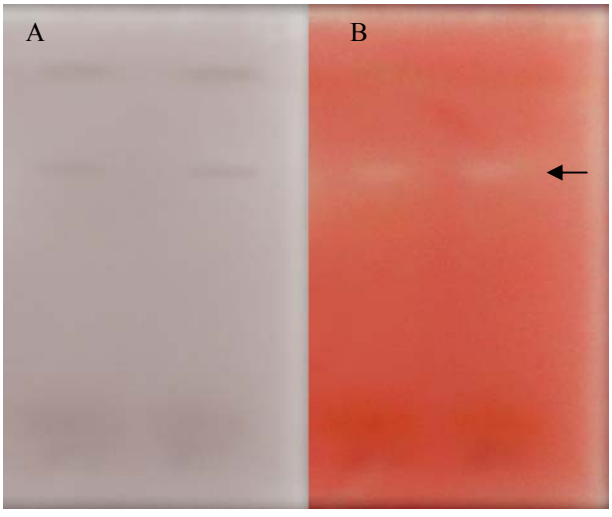

Fig. 2 Thin layer chromatography plate of (1) *B. trimera* aqueous extract. A: visual appearance. B: *L. monocytogenes* bioautography overlay. Arrow indicate regions of inhibition growth visualized wih tetrazolium red. C: Glycoside (standard).

Table 3 Antibacterial activity of ethanolic extracts against Gram-positive and Gram-negative bacteria.

	B. articulata		*B. trimera*		*B. crispa*	
	[2]MIC	[3] MBC	MIC	MBC	MIC	MBC
S. aureus ATCC 43300	1,250	2,500	1,250	2,500	625	1,250
L. monocytogenes [1]CLIP 74910	1,250	2,500	625	1,250	625	1,250
E. coli	[4]NA	NA	NA	NA	NA	NA
P. aeruginosa ATCC 27853	NA	NA	NA	NA	NA	NA

[1] CLIP: *Listeria* Collection of the Pasteur Institute; [2] MIC: Minimum inhibitory concentration (μg/mL); [3] MBC: Minimun bactericidal concentration (μg/mL); [4]NA: no activity.

some of the plant extracts possess compounds with antibacterial properties that can be used in new drugs for the therapy of infectious diseases caused by pathogens.

Acknowledgments

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Antibacterial Effect of the Essential Oils Extracted From *Ruta chalepensis* L. and *Ruta montana* (L.) L.

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Abstract: *Ruta* genus is a member of the family *Rutaceae* that has been cultivated widely in many regions of the world because of its medicinal properties. In Tessala Mountain (Sidi Bel Abbes Country, North-Western of Algeria), the *Ruta* genus commonly known by "fidjel" is represented by two species: *Ruta Chalepensis* L. and *Ruta Montana* (L.) L. frequently used by local population in a traditional treatment. The water-distilled essential oils from this species yielded 7.23% and 6.104% for *Ruta chalepensis* L. and *Ruta montana* (L.) L. respectively. The essential oil of *Ruta montana* (L.) L. showed a strong antibacterial activity against all bacterial strains tested (*Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Mycobacterium kansasii* ATCC 12478 and *Mycobacterium vaccae* ATCC 1548314) compared to *Ruta chalepensis* L. oils which have a moderate effect only on *Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 27853. Therefor, the antibacterial properties of the essential oils of *Ruta Chalepensis* L. and *Ruta Montana* (L.) L. are now well established through this study and therefore could justify their future uses in the treatment of nosocomial infections.

Key words: Tessala mountain, *Ruta chalepensis* L., *Ruta montana* (L.) L., essential oils, antibacterial effect.

1. Introduction

The region of Tessala (country of Sidi Bel Abbes), mountain area with particular characteristics in western of Algeria, is known by its rich flora used by the local population. Its potential plant resources and the valorization of its species have only been partially studied [1-4] and remains to be evaluated.

In this context, the present work will undoubtedly contribute to the valorization of the local flora of Tessala Mountain. The *Ruta* genus with its two species (*Ruta chalepensis* L. and *Ruta montana* (L.) L.) will be the subject of our investigation.

The objective of our study was to extract the essential oils of the *Ruta* genus species, and highlighting their biological activities towards *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Mycobacterium kansasii* ATCC 12478 and *Mycobacterium vaccae* ATCC 1548314 isolated from a microbiological analyses hospital laboratory of Sidi bel Abbes country.

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2. Materials and Methods

2.1 Presentation of Sampling Sites

The species were collected in June 2011 in the Tessala Mountain (Algerian North-West). *R. chalepensis* L. was collected at a station whose latitudinal coordinates 35°16.125' N, and longitudinal 0°46.283' W located at altitude 797 m. The soil type in this station was loam, deep, with a slightly faster

drainage and a cover feature of scrubland.

While *R. montana* (L.) L. was harvested in a station with a southeast exposure, located at latitude 35°16.126' N and longitude 0°46.714' W with an altitude of 894 m. This station is characterized by a steep slope with a bare soil.

Specimens of the both species were deposited in the herbarium of the faculty of sciences of Djillali Liabes university of Sidi Bel Abbes.

2.2 Extraction of Essential Oils

After being collected, the aerial parts of several individuals of the species of the *Ruta* genus were weighed for measure their fresh weights. Then, they were dried for a period of fifteen days in the shade. After that, they were weighed to determine their dry weight. The difference between the two weights gives the water content estimates in percentage.

The dried aerial part of *R. chalepensis* L. and *R. montana* (L.) L. were water-distilled for 5 h using the Clevenger-type apparatus [5]. The oils were extracted from distilled water with diethyl ether, dried over anhydrous sodium sulphate, filtered and the solvent was removed at room temperature under reduced pressure on a rotary evaporator yielding the oils. The oils obtained were stored under nitrogen in a sealed vial in the dark at 4 °C.

2.3 Microbiological Study

The technique used in our study was the Vincent method (Aromatogram) that can permit to study in a reliable and reproducible way the sensitivity and the resistance of the essential oils' germs [6].

The highlighting of the sensitivity and the resistance of the microbial agents consist to put them on a solid agar culture (in our case Mueller-Hinton agar culture for *S. aureus* and *P. aeruginosa*, Sauton agar culture for *M. kansasii* and *M. vaccae* [7]) in contact with the essential oils, in order to assess their antibacterial effect.

The sensitivity of the germs tested towards the essential oils was characterized by the formation of a clear circle (inhibition zone) around the disks containing these oils. For this purpose, the inhibitory power of the essential oils was evaluated by the determination of the magnitude of the diameter of the inhibition zone formed [6, 8].

First, the essential oils extracted from the species of *Ruta* genus were diluted in dimethyl sulfoxide (DMSO). For this, 100 μL of oil were diluted in 200 μL of DMSO (the resulting solution was called solution 1/2). From the latter were taken 100 μL of their turn in 200 μL of DMSO (the resulting solution was called solution 1/4). Similarly, the solution 1/8 was obtained [9].

Then, the bacterial strains were inoculated in nutritive broth and incubated for 18 h at 37 °C. For the Aromato gram test, the dilutions of the bacteria were prepared from the pre-cultures. The culture mediums are inoculated with an optical density between 0.08 and 1 with a wavelength λ= 600 nm.

The disks in filter paper; sterilized and impregnated by the essential oils at different concentrations, were deposited on the surface of Meuller-Hinton and Sauton agars culture which inoculated with *S. aureus*, *P. aeruginosa*, *M. vaccae* incubated at 37 °C for 48 h and *M. kansasii* incubated for 21 days at the same temperature reason of it tardy growth. Subsequently, the antibacterial activity was evidenced by the appearance of inhibition zones around the disks.

3. Results and Discussion

3.1 Determination of the Water Content

The values of the water content for *R. chalepensis* L. and *R. montana* (L.) L. was respectively 19.01% and 41.99%.

Lower content of water for *R. chalepensis* L. and for *R. montana* (L.) L. can be explained by the adaptive strategy of the both species. Indeed, these plants are xerophytes which grown in the semi-arid climates making their water's economies through their water evaporating surface. The reduced leaves for *R. chalepensis* L. and more smaller for *R. montana* (L.) L.

explained the difference in water content between them [10].

3.2 Yield of the Essential Oils

The extracted oils appear as liquid, with an oily aspect, transparent, with a yellow color, characterized by strong odor.

For *R. chalepensis* L. the obtained mass of the essential oil after water-distilled was 7.23 g for an initial mass of 100 g of vegetable drugs, so we have a performance of 7.23%. For *R. montana* (L.) L., the oil mass obtained from 100 g of vegetable drugs is 6.104 g with a performance of 6.104%.

This difference in the essential oils performance can be explained by the phonological stage of each species in fact that *R. chalepensis* L. has been gathered during the inflorescence, so a high content of the oils is contained in the flowers. While *R. montana* (L.) L. has been during the elongation stage.

Similar works on *R. chalepensis* L. mentioned yields of 1.22%, 0.31% and 2% in essential oil [11-13]. The difference between this yields and our result can be explained by the ecological factors that involved in the development of the specie (altitude, climate, the soil …), the harvest period, the harvested portion, the extraction technique and also its duration.

However, for *R. montana* (L.) L. any work on the essential oils has been found.

3.3 Study of the Biological Activity

For clarification, we had performed the test six times for each bacterial strain so we will have six Petri dishes with the average in the inhibition zone will be more meaningful. Consequently, our results showed in Table 1 were presented in means ± Standard Deviation.

The results of the antibacterial effect of *R. chalepensis* L. oil on *M. kansasii* and *M. vaccae* indicate that this bacterium presents a resistance even in higher concentration the fact that the diameter of the inhibition zone was zero in all Petri dishes inoculated by these strains. *P. aeruginosa* presents a resistance to low concentrations (1/8) of the same oil. We had found among six, two Petri dishes without inhibition zone. Against *S. aureus*, the result shown that the *R. chalepensis* L. oil was effective at all concentrations.

On the other hand, *R. montana* (L.) L. oil was effective against all the test strains even in weak concentration. Thus, the inhibition zone diameter increases gradually when the concentration increases.

Essential oils may possess bacterial activity and can be exploited as an ideal treatment for future to eliminating strain spread. Suppression on bacterial production by oil treatment could make a major contribution to limiting the spread of the pathogen by lowering the strain load in the storage atmosphere and on surfaces [14].

By comparison between the average diameters of the inhibition zones of the growth of the bacterial strains that were the subject of our investigation, the results of the Aromato gram test showed that *R. montana* (L.) L. oil present effective treatment toward the bacterial strains tested which manifested by the formation of inhibition zone diameters more important even at weak concentration (Table 1) than *R.chalepensis*

Table 1 Diameter of the inhibition zone (mm) of the different bacterial strains in the presence of different concentration of the essential oils of *Ruta* genus species.

	R. chalepensis L.			*R. montana* (L.) L.		
	1/2*	1/4*	1/8*	1/2*	1/4*	1/8*
S. aureus	2.6 ± 0.21^{a}	1.7 ± 0.13^{a}	1.6 ± 0.12^{a}	4.3 ± 0.73^{a}	3.1 ± 0.62^{a}	2.1 ± 0.23^{a}
P. aeruginosa	0.18 ± 0.01^{b}	0.13 ± 0.01^{b}	0.08 ± 0.01^{b}	5.8 ± 0.75^{a}	3.5 ± 0.81^{a}	1.8 ± 0.25^{a}
M. kansasii	-	-	-	3.2 ± 0.33^{b}	1.8 ± 0.33^{b}	0.2 ± 0.13^{a}
M. vaccae	-	-	-	2.6 ± 0.83^{b}	1.2 ± 0.83^{b}	0.6 ± 0.13^{a}

Same letter in the same column are not significantly different at $P < 0.05$ level mean followed by ± S.D.

- : resistance; *: essential oil dilution.

L. oil which was ineffective against the mycobacterium. This behavior is not surprising because it has an intrinsic resistance to a wide range of biocide [15].

Moreover, previous studies signaled an efficiency of infusion and decoction of *R. montana* (L.) L. leaves toward mycobacterium [15, 16]. The magnitude of the diameters formed by *R. montana* (L.) L. oil can be explained by the high content of active molecules that make up the essential oil, which led to the blocking of the growth and proliferation of bacterial strain.

Indeed, essential oils are complex volatile compounds produced in different plant parts, which are known to have various functions in plants including conferring pest and disease resistance. The complexity in essential oils is due to terpene hydrocarbons as well as their oxygenated derivatives, such as alcohols, aldehydes, ketones, acids and esters [17-20].

Indeed, this difference in resistance between the oils of both species towards bacterial strain can be directly related to the chemical nature of the outer membrane of each strain, composed of polysaccharides forming an impermeable barrier to hydrophobic compounds [9].

4. Conclusions

The essential oils as antimicrobial agents present two main characters: the first is their natural origin which means more safety to the people and the environment, the second is that they have been considered at low risk for resistance development by pathogenic microorganisms.

The work that we have done concerning *R. chalepensis* L. and *R. montana* (L.) L. aims to get more knowledge about the value of the species of the *Ruta* genus in Tessala Mountain which have economic and ecological interests and which bring to the landscape a particular imprint.

The extraction of the essential oils of both species by water-distilled revealed a performance of 6.104% and of 7.23% for *R. montana* (L.) L. and *R. chalepensis* L. respectively.

The antibacterial effect study by the Aromato gram technique has shown that *R. montana* (L.) L. oil was found to be active against all the bacterial strains studies compared to *R. chalepensis* L. oil in which there was no effect on *M. kansasii* and *M. vaccae*.

For this, we can say that *Ruta* genus species essential oils could constitute a good basis for the elaboration of new antibacterial substances, whose main advantages are their natural origin, and their complexity makes impossible the development of the pathogens' resistance.

Acknowledgments

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Microanatomy of *Moina eugeniae* (Branchiopoda, Cladocera)

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Abstract: *Moina eugeniae* is the most abundant species in the southwest lagoons of Buenos Aires province. The aim of this work is to study the histology of this cladoceran by light microscopy so as to expand the knowledge of this species. The parthenogenetic females were fixed in formaldehyde 4%. Sections were cut 3 µm thick and stained with Hematoxylin and Eosin. The intestine has simple cuboidal epithelium with apical projections and the cells have 1 or 2 nucleoli in their nuclei. Two compound eyes were found and no naupliar ocelum. The ovary is saccular and it is presented in a pair at both sides of the intestine with follicles in different states of development. The striated muscle with notorious microfibrills is recognized in antennas, antennules and trunk appendices. This article may represent the first detailed description of the histology of this species.

Key words: Light microscopy, cladocera, histology, *Moina eugeniae*.

1. Introduction

Moina eugeniae belongs to a group of crustacea commonly called “water fleas”, which are distributed in permanent or temporary water ponds or lagoons that can be slightly salted, located in arid regions with varying ranges of rains [1, 2]. The common name is referred not only to the tiny shape but to the typical movement which these animals display when they swim.

M. eugeniae, considered as part of the zooplankton of coastal lagoons, is found in the Salada Grande lagoon (Buenos Aires province, Argentina) [3] and in La Pampa and Río Negro Provinces [4]. And most of the data from this species are bio-ecological related to environmental parameters [5, 6].

Since late 1960s few morphological descriptions were carried out [3, 7, 8] and less evolved crustacean histology data are scarce if they are compared with those concerned to Decapoda.

This is the reason we propose that this may be a seminal contribution to the morpho-histological study of *Moina eugeniae*.

The body of this species consists of a head and a trunk. The antennas are the main means of locomotion. Two big eyes are covered by a layer, located at both sides of the head. The trunk is surrounded by the caparace which is periodically replaced. There is an incubating chamber on the dorsal side of the body where eggs and embryos are developed and it opens to the exterior by a unique pore [9].

The present study is part of a program of research dealing with zooplanktonic communities found in Buenos Aires Province’ lagoons. The objective of this work is to analyze this species by light microscopy and may also contribute to a better understanding of the Cladoceran’s biology and acquacultural management aspects due to the potential value of *Moina eugeniae* as food for fish.

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2. Materials and Methods

The samples were taken during Spring months in 2006 from the Calderón lagoon, Buenos Aires, Argentina (38°43'27.9" S-62°2'26.1" W). Methods published by Rennella and Quirós [10] were used. The animals were fixed "*in situ*" in formaldehyde 4%. At the lab, the specimens were identified and classified by microscope following the Olivier [11], Goulden [3] and Lopretto and Tell's criteria [12]. Only parthenogenetic females were found. The protocol consisted of dehydration through a series of alcohol from 70% to absolute ethanol, clearing with xylene and impregnation and embedding in paraffin blocks. These blocks were then sliced, in sections of 3 µm thickness using a sliding microtome. The samples were stained with Hematoxylin and Eosin. Microphotographs were taken with a C7070 Olympus camera attached to the Olympus BX51 microscope.

3. Results

Normally, the reproductive cycle of *Moina eugeniae* is by parthenogenesis. They produce eggs in number of four to six that are developed in a dorsal chamber (Fig 1). Only parthenogenetic females were found in the samples collected. The analyzed specimens had a compressed body formed by a head and a trunk rounded by a pair of ovoid valves.

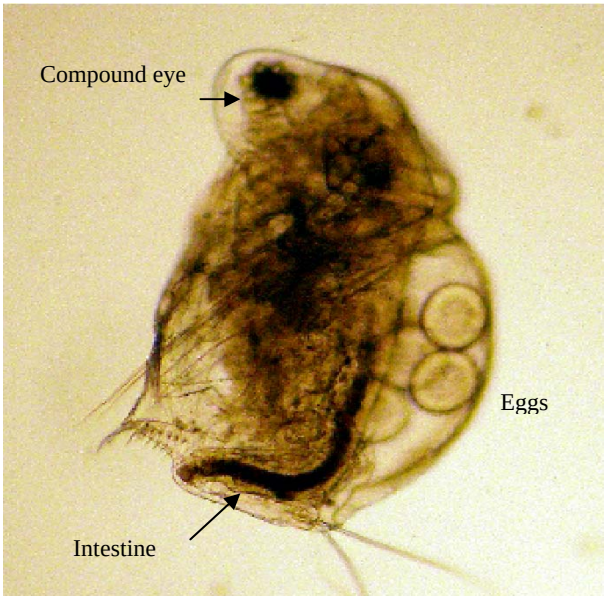

Fig. 1 Microphotograph of *Moina eugeniae* with eggs in its chamber.

Appendages

The antennules, antennas and maxillae appeared in the anterior part of the head. The antennules were long and show characteristic sensitive extensions (aestheteascs). The two branched antennae were well developed and display typical setae which belong to Moina's genus. In the trunk, five pairs of toracopods were observed, which filtrate and are used for locomotion (Fig. 2). It was found striated muscle in the appendences (Fig. 3). Muscle cells had notorious striation.

Eyes

There was a pair of compound eyes located laterally in the head (Fig. 4a). Light microscopy revealed that these eyes were compound by ommatidia which were connected by muscle and neuronal fibers (Fig. 4b). No nauplius eyes were found.

Digestive tube

The digestive tube was represented by a long cylinder form by a simple epithelium with cuboidal cells (Fig. 5). The epithelial cells showed microvilli in the apical surface and conspicuous nuclei with one or two nucleoli. No zonification along the digestive tube was observed.

Ovaries

Two dorsal and sacular ovaries appeared in *Moina eugeniae* in parallel with the digestive tube (Fig. 6).

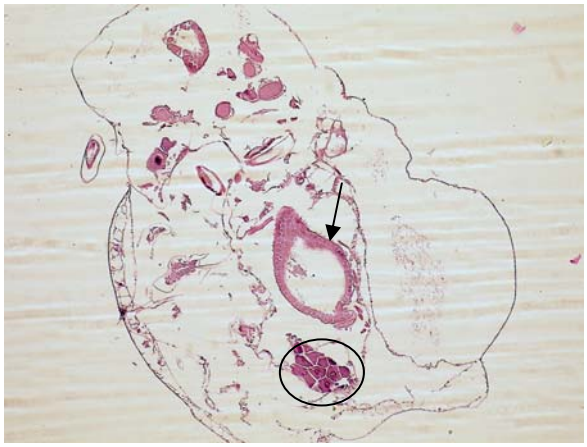

Fig. 2 Sagital cut of the whole body where the intestine (arrow) and ovary (circle) is observed. ×10. H & E.

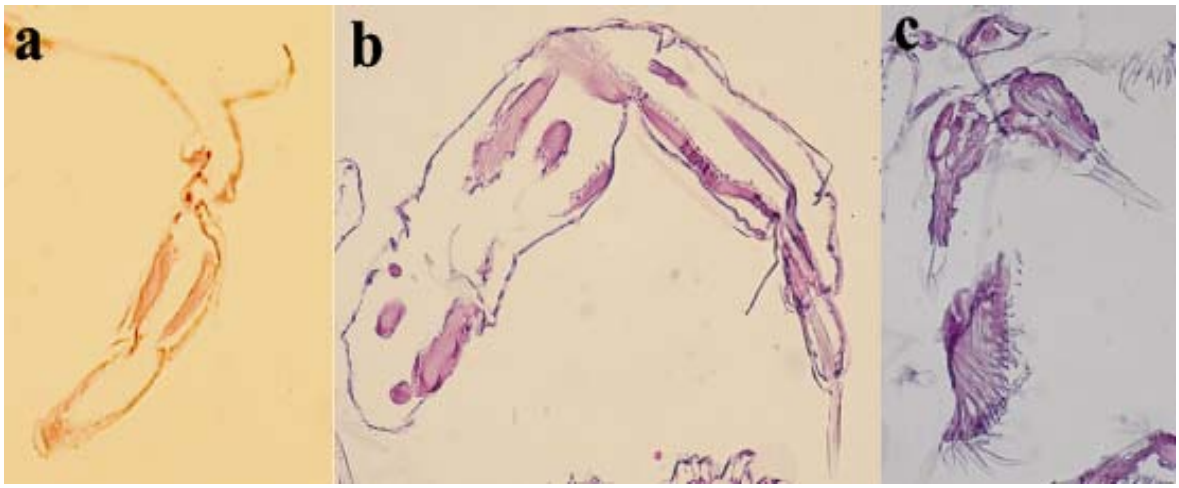

Fig. 3 a and b- Micrographs showing striated muscle fibers that are insert inside the appendences. c- Sagital sections of filopods and thoracic appendences. ×20. H & E.

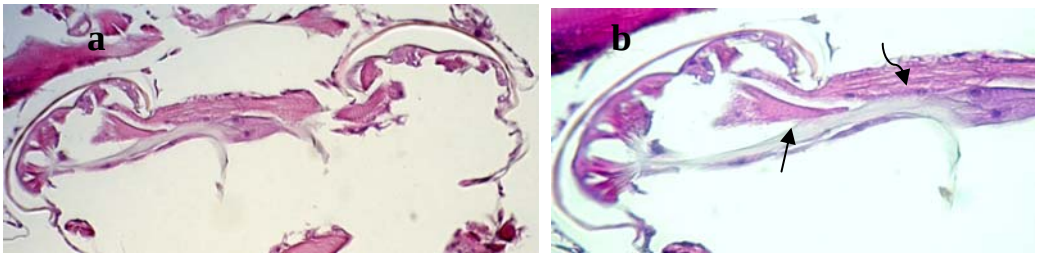

Fig. 4 a General view of both eyes. ×10. H & E. b- Compound eyes connected by neuronal (straight arrow) and muscle (squiggly arrow) fibers. ×40. H & E.

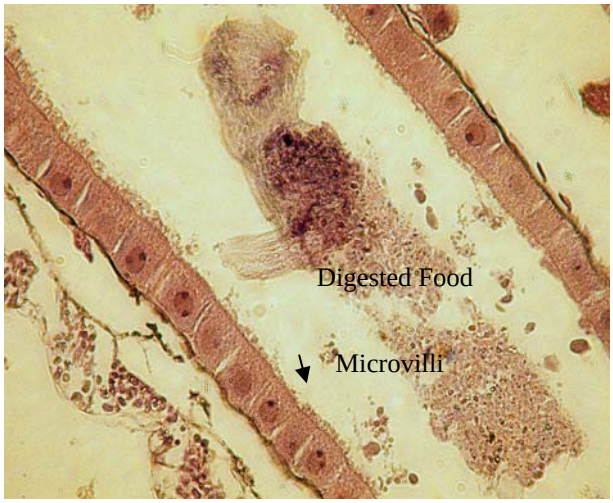

Fig. 5 Detail of the intestinal epithelium where microvilli in its apical surface is observed. ×40. H & E.

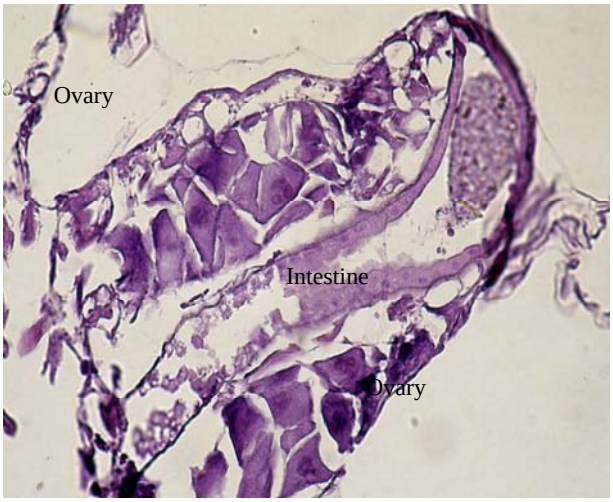

Fig. 6 Ovaries in pair surrounding the intestine. The eggs are in different stages of folliculogenesis. ×10. H & E.

Inside the ovary, different stages of folliculogenesis were observed. The eggs were classified in vitellogenic and mature. In the first case, the previtellogenic ones were 10 µm large with no vitellogenin in the cytoplasm and the nucleous was dense chromatin. The vitellogenic eggs were 50 µm and the nucleous was full of gross chromatin granules. The yolk was widely dispersed in the cytoplasm. And, in the mature ones (100 µm) the nucleous reached a larger size with notorious nucleoli and lipidic vacuoles in the cytoplasm. In any of the stages a pellucid zone was found.

4. Conclusions

One of the most characteristic groups of the zooplankton is Cladocera and *Moina eugeniae* belongs to this suborder of Crustaceans. This genus is quite tolerant to poor quality water and resists great variation in dissolved oxygen. Also, this invertebrate, has a high rate of reproduction which gives the species a rapid adjustment in face of environmental changes.

Since there is scarce published data about microscrustacean's histology we propose that anatomical descriptions can provide additional information for aquaculture management.

The compound eyes are common in the Crustacea group except for copepods. Compund eyes' publications of less evolved crustaceans are useful as evidence for a monofiletic origin in Arthropods [13, 14]. The eyes of *M. eugeniae* appeared connected with muscle fibers, this should indicate that the ocular organs can be moved in some directions to detect preys or mates.

On the other hand, the digestive tube runs along the body. Most of the time, this is fill with food. Pennak [15] describes no specializations in cladocera's digestive tract. In *M. eugeniae*, this tube shows a simple cubic epithelium with microvilli which are in charge of water and food absorption. Cladocera's digestive tube cells are different from the others arthropods because of their simplicity in the basal membrane (few folds). This fact leads to conclude that water absorption is poor which is primordial for aquatic animals [16]. TEM's studies of *Daphnia pulex*, reveals a zonification in the intestine epithelium but this was not the same in *M. eugeniae*. Noteworthy, the present work is based on light microscopy while the other is electronic microscopy. Both studies have coincidence in the surrounded musculature and the apical microvilli of the digestive cells [17].

We published recently [18] the female reproductive tract of *M. eugeiae* which consist of a pair of ovaries with polyedric eggs in different stages of maturation and absence of pellucid zone. This latter anatomic structure is in concordance with the reproductive mode: parthenogenesis. Normally, there is no sperm to recognize, so this layer does not exist.

Usually, the filopods are schematized in morphology books but in the present work sagital sections of these appendences are showed (Fig. 3). In the micrographs, the musculature insertions are observed in the different filopods and indicates its function in locomotion and feeding [19].

The present work represents a first contribution in *Moina eugeniae*'s histology so as to expand knowledge in Moinids morphology in particular and, in little invertebrates, in general. Besides, contributions like this would enhance the potentiality of this species as food fish in aquaculture.

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The Relationships Between Milk Constituents and Various Milk Properties in Anatolian Buffaloes

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Abstract: The objectives of this study were to investigate the relationships among milk composition, renneting time, urea concentration, acidity, density and pH of Anatolian Buffaloes' milk. As a total of 115 individual milk samples from 53 Anatolian buffalo cows that calved in 2004 and 2005 on days of their lactations 30 ± 15, 60 ± 15, 90 ± 15, 120 ± 15, 150 ± 15, 180 ± 15, 210 ± 15, 240 ± 15 and 270 ± 15 in 8 units of Ilıkpınar village were collected in morning milkings in June, September, December and March. Samples were analysed for total dry matter (TDM), fat, protein, ash, density, pH, acidity, renneting time and urea content. Data were classified according to the following environmental factors: lactation stages: 1 (30 ± 15, 60 ± 15, 90 ± 15 days): 2 (120 ± 15, 150 ± 15, 180 ± 15 days): 3 (210 ± 15, 240 ± 15, 270 ± 15 days); calving year: 1 (2004), 2 (2005); calving season: 1 (January-May), 2 (September and October); month of samples collection: 1 (June), 2 (September), 3 (December), 4 (March); lactation order: 1 and 2:1, 3 and 4:2, 5 and 6:3. Means and correlation coefficients for the characteristics investigated were calculated. There were negative significant correlations between daily milk yield with TDM, fat and protein percentages, and between pH and all of the milk constituents. Density reduces as TDM, fat and protein contents increase. Relationships between density and coagulation time with milk yield and pH were not significant. Relationships between milk urea concentration with none of milk constituents, milk yield, density, pH and titratable acidity were not significant statistically. It was concluded that genetic selection has to be directed towards increasing fat, protein and total not fat dry matter yields. Under selection programs in which milk yield is taken into consideration, fat and protein yields also increase, but fat and protein concentrations decrease.

Key words: Anatolian buffalo, milk, coagulation, renneting, urea, pH.

1. Introduction

It is a well established fact that reducing protein concentration (80 gr/kg FCM and lower) diminishes milk yield and its fat percentage [1, 2] and that increasing milk yield leads to a decrease in milk fat and protein concentrations [3, 4]. Milk coagulation properties (rennet coagulation time, firming time and firmness of clot) are well known important criteria for cheese production. These properties (rennettability) can be affected by genotype [5, 6], season, lactation order, lactation stage and feeding [7]. Moreover, they change throughout the lactation depending on milk yield, protein and fat concentrations. These properties are found best at the beginning and the end of lactation. Piironeen et al. [8] reported that protein content affected milk coagulation considerably, which increased as the lactation stages progressed, and that any negative alterations in milk composition had a clear effect on milk coagulation time. Milk coagulation properties also differ significantly from one unit to another. It is most likely that differences are due to feeding and management factors [5]. Povinelli et al. [6] found that titratable acidity and protein content had a significant effect on milk coagulation ability. pH has a negative influence on milk coagulation ability and the effect increases to a significant degree as lactation progresses [8].

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Milk urea concentration can be used as a tool to monitor crude protein and energy intake [9]. It is related to the rate of protein-energy in ration and crude protein intake [10, 11]. In order to use milk urea concentration as a tool to identify any imbalances related to feeding, food intake and ration composition together with other factors and levels of their effect have to be determined and taken into consideration while interpreting urea concentration [12]. These factors can be ordered as follows: sample collection season, analyze method used, live weight of animal, parity and milk yield of cow [13]. Roy et al. [14] reported that milk urea concentration increased significantly in Murrah Buffaloes as the control day milk yield increased. As the lactation number increased, a significant reduction occurred in milk urea concentration. However, lactation stage did not have significant effects on urea and protein concentrations of milk.

The objectives of this study were to investigate relationships among milk composition, renneting time, urea concentration, acidity, density and pH of Anatolian Buffaloes milk.

2. Materials and Methods

The material of the study were formed by 115 milk samples from 53 Anatolian buffalo cows of Ilıkpınar Village of Kırıkhan District of Hatay Province in 8 units that calved in 2004 and 2005. Milk samples were collected from the morning milkings for June, September, December and March. The cows were on 30 ± 15, 60 ± 15, 90 ± 15, 120 ± 15, 150 ± 15, 180 ± 15, 210 ± 15, 240 ± 15 and 270 ± 15 days of their lactations. Samples were analysed for total dry matter (TDM), fat, protein, ash contents, pH, density, rennetting time and milk urea content. Protein and fat contents were determined by Formol Titration [15] and Gerber Methods [16] respectively. Rennet coagulation time was determined by recording time from the addition of enzyme to milk to appeareance of first clot using Berridge Method [17]. Milk urea content determined with diacetyl monoxime by Photometric Method, as described in Merck handbook [18].

The means and correlation coefficients of the characteristics were calculated. SPSS programme [19] were used in the statistical analysis.

3. Results and Discussion

Correlation coefficients between milk yield and milk constituents contents are given in Table 1. Relationships among the rennet coagulation time with composition, pH, density, titratable acidity and urea content of milk are shown in Table 2a and Table 2b.

Table 1 Correlation coefficients between milk yield and milk constituent contents.

Variables measured		Correlation coefficient (r)
Morning milk yield	Daily milk yield	0.737**
TDM %	Morning milk yield	-0.030
	Daily milk yield	-0.232*
	Fat %	0.675**
	Protein %	0.660**
	Ash %	-0.408**
Fat %	Morning milk yield	-0.028
	Daily milk yield	-0.202*
	Protein %	0.596**
	Ash %	-0.338**
Protein %	Morning milk yield	0.052
	Daily milk yield	-0.204*
	Ash %	-0.495**
Ash %	Morning milk yield	-0.104
	Daily milk yield	0.084

*P <0.05, **P < 0.01.

Table 2a Relationships between various variables.

Coagulation time		Urea content		Density	
Variables	Correlation coefficient	Variables	Correlation coefficient	Variables	Correlation coefficient
Morning milk yield	0.238*	Morning milk yield	-0.069	Morning milk yield	-0.138
Daily milk yield	0.038	Daily milk yield	-0.118	Daily milk yield	-0.165
TDM %	0.320**	TDM %	0.084	TDM %	-0.247*
Fat %	0.293**	Fat %	-0.046	Fat %	-0.247*
Protein %	0.447**	Protein %	-0.058	Protein %	-0.256*
Ash%	-0.273**	Ash %	-0.143	Ash %	0.210*
Density	-0.049	Density	-0.015	pH	0.027
pH	-0.022	pH	0.050	Titratable acidity	0.367**
Urea	0.035	Titratable acidity	0.002		
Titratable acidity	0.094				

*$P < 0.05$, **$P < 0.01$.

Table 2b Relationships between various variables.

Titratable acidity		pH	
Variables	Correlation coefficient	Variables	Correlation coefficient
Morning milk yield	-0.159	Morning milk yield	-0.055
Daily milk yield	-0.323**	Daily milk yield	0.127
TDM %	0.171	TDM %	-0.339**
Fat %	0.205*	Fat %	-0.358**
Protein %	-0.029	Protein %	-0.291**
Ash %	0.098	Ash %	-0.280**
pH	-0.394**		

*$P < 0.05$, **$P < 0.01$.

There was a significant relationship between morning and daily milk yields in Table 1. There are negative significant correlations between daily milk yield with TDM, fat and protein percentages. These result were confirmed by the following literature [protein [20-22], fat [21]]. There were negative relationships between TDM with fat and ash contents and positive relationships between fat with protein concentrations and TDM with fat and protein contents. In other words, as ash content increased, TDM content decreased. Fat content was adversely affected by the increase in ash content and the increase in TDM and protein contents positively. Protein content increased as fat and TDM contents increased. However, Roy et al. [14] reported that protein concentration did not change significantly. Milk component concentrations have negative relationships with production characteristics, and changing component contents only by genetic selection is not possible. However, there are significant correlations between milk yield and fat, protein and TDM yields. It suggests that genetic selection has to be directed towards increasing fat, protein and total not fat dry matter yields. Under selection programs in which milk yield is taken into consideration, fat and protein yields also increase, but fat and protein concentrations decrease.

As daily milk yield and pH increase, titratable acidity is affected negatively in Table 2a and Table 2b. In parallel to increase in fat rate, titratable acidity rises. In the literature it is reporeted that titratable acidity rises together with a decrease in urea content of milk [23]. Whereas feeding level is influencial on the urea content of milk [24, 25].

There were significant negative relationships between pH and all of the milk constituents. As pH increased, the amount of milk constituents decreased.

Relationship between milk yield and pH was found insignificant. Piironen et al. [8] reported that protein percentage had a positive effect on pH, and the effect enhanced as lactation stage progressed.

Density reduces as TDM, fat and protein contents increase. Similarly, as ash content rises density also increases. Relationships between density with milk yield and pH were not significant. Despite the fact that there is positive correlation between TDM content and density of milk, the negative correlation found in the study was due to increase in fat percentage of TDM content.

Time laps from the addition of rennet to the appearance of first clot get longer as TDM, fat and protein percentages increase, whereas as ash content increases it becomes shorter. Likewise, the studies [5-7] supported that there were positive relationships between rennet coagulation time with protein and fat contents. Negative alterations related to milk composition were reported to have clear effects on milk coagulation properties and alterations in protein content related to production season result in rennet coagulation properties of milk [8].

In this study, relationship between milk coagulation time and pH was not significant. The study [8] supported this findings and state that as lactation stage progressed the effect increased significantly. Relationships between milk urea concentration with none of milk constituents, milk yield, density, pH and titratable acidity were not significant statistically. Correlation between urea content and milk yield was found to be negative and not significant as opposed to the literature [13, 14].

4. Conclusions

Milk component concentrations have negative relationships with production characteristics, and changing component contents only by genetic selection is not possible. However, there are significant correlations between milk yield and fat, protein and TDM yields. It suggests that genetic selection has to be directed towards increasing fat, protein and total not fat dry matter yields. Under selection programs in which milk yield is taken into consideration, fat and protein yields also increase, but fat and protein concentrations decrease.

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Some Fishery Biology of Molluscivorous Catfish, *Helicophagus leptorhynchus* in Thailand

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Abstract: The molluscivorous catfish, *Helicophagus leptorhynchus* is an economic fish in Thailand, but the fish is poorly know. This paper is a review of some biology support data for aquaculture and fisheries. The fish distributed in Mekong-Chaophraya Basin. The spawners migrate to upstream in early flood season for spawning. After hatching, the fish larva migrating in downstream and the juvenile fish move in deep pool of Mekong Basin on dry season. Adult fish live in downstream and large tributary. The fish is omnivorous and bottom feeder. Spawning season of the fish is May-July. Gillnetting and beach seine fisheries are main the catfish's fisheries in Thailand and the catfish's fisheries season in Mekong River began on January-July.

Key words: Biology, Molluscivorous catfish, *Helicophagus leptorhynchus*, Mekong Basin.

1. Introduction

The molluscivorous catfish is a freshwater fish species that belongs to the order Siluriformes and family Pangasiidae. This fish is indigenous species in Mekong-Chaophraya Basin, and economically importance fish in Thailand. The wild catch of this fish have required in Indo-China Region markets which a large demand. Now, the fish is never reported about mass production and less data for aquaculture. Therefore, this paper is a review of some biology support data for aquaculture of molluscivorous catfish in Thailand.

2. Taxonomic Status

The catfish in family Pangasiidae have three genera *Pangasianodon, Pangasius and Helicophagus*, with 28 species [1, 2]. The molluscivorous catfish refer to fish in genus *Helicophagus* have three species worldwide namely *H. typus, H. waandersii* and *H. leptorhynchus* [3, 4]. In Thailand, the molluscivorous catfish that mean *H. leptorhynchus*. The fish is found in Mekong-Chaophraya Basin [3, 5-7]. In worldwide, the fish is distributing in Indo-China Region [3, 8]. *H. typus* and *H. waandersii* are finding in Malay Peninsula and Indonesia [3, 9, 10].

3. Migration Behavior

In flood season (May-June), juvenile of molluscivorous catfish occurred in Mekong mainstream and large tributary such as Mun River and Songkram River. The fish larva, after hatched will not migrate into the flood area, but it migrate to nursery ground in Mekong downstream. In early dry season, the juvenile fish will migrate into deep pools of Mekong River and the maturity fish will migrate into Mekong upstream for mating and spawning on April to June of every year [8]. Warren et al. reported that

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molluscivorous catfish migrated into Mekong upstream (Thailand and Lao PDR) two times of each year, December-April and April-June [11, 12]. According to the report of Jutagate and others, they reported that the molluscivorous catfish migrated into the Mun River on March-June and the fish go back into Mekong River on September-December [13]. Nongkhai Fishery Station found the maturity fish in Mekong upstream on January; they presume that the fish migrate into upstream for feeding and spawning. The factor that affect to fish migration behavior (e.g. temperature, water level and turbidity) [12].

4. Food and Feeding Habit

Stomach content analysis of molluscivorous catfish in mainstream of Mekong River, Nongkhai Province, Thailand found food items namely bivalves, freshwater sponges, shrimps, plants, planktons, oligochaete and aquatic insects respectively [14]. Bivale (*Corbicula tenuis*) is a main food item, according to report of Roberts and Vidthayanon, who study feeding habit of this fish in Mun River, Thailand [4, 14]. In 2001, Ng and Kottelat reported that molluscivorous catfish in Thailand feeds dominantly on bivalves two genera; Corbicular (Corbiculidae) and Physunio (Amblemidae) [3]. In others Indo-China Region (Cambodia, Myanmar and Lao PDR) bivalve is main food of adult molluscivorous catfish and feeding area of adult fish is main stream or large tributary. Feeding habit of Juvenile fish presume that similarly of others juvenile fish in family Pangasiidae namely invertebrate, zooplankton and organic matter [2, 8]. The mouth of molluscivorous catfish is inferior position and small. It has upper jaw teeth, lower jaw teeth and palatine teeth. The molluscivorous catfish teeth are a molariform and the intestinal length is 1-1.5 times of standard length. The all data indicated that, the fish is bottom feeder. According to the stomach content data, the fish can be considered as omnivorous [14-16].

5. Reproductive Biology

The maturity molluscivorous catfish will migrate from Mekong River in Cambodia to Mekong River in Thailand and Southern Laos for mating and spawning on each year, especially April to May [8]. According to report of Nongkhai Fishery Station, they found the maturity fish (Standard length about 35 cm or more) on January to April and it was most migrated in March. Therefore, optimal time range for spawner accumulation in Thailand is March-May [16].

In 2008, Thapanand-Chaidee reported that 42.01 cm TL was the female length at 50% maturity in a Mun River's population [12]. The minimum maturity female length was 33 cm TL and the maximum maturity female length was 54 cm TL. The fecundity of maturity female in a Mun River's population was 21,547-191,539 eggs and the average fecundity ranged was 85,174 ± 43,206 eggs per maturity female [12]. In upstream of Mekong Basin, spawning season of the fish begin in June-July. The fish will migrate from downstream to upstream [17, 18]. During migratory time range, Mekong River and tributary are the high water [19] and water quality of the fish's habitat show in Table 1. According to report of Nongkhai Fishery Station, they reported time range of spawning of the

Table 1 Water quality of the fish's habitat [10].

Parameter	Dry season	Wet season
Temperature (°C)	29.8-31.7	28.0-28.5
DO (mg/L)	7.2-8.8	7.8-8.9
pH	6.8-7.1	6.0-6.5
COD (mg/L)	5.1-6.4	4.4-6.4
Salinity (ppt)	0	0
Transparency (cm)	73-112	12-16
Depth (m)	10-30	No data

fish in Thailand was March to May [16]. The estimation of Gonadostomatic Index of the Mun River's population showed that high value in May-June and the maximum value were found in June [12].

6. Note on Fisheries

Gillnet and beach seine are main fishing gears in the catfish's fishery in Mekong River, Thailand [20]. In 1985, Nongkhai Fishery Station collected the spawner in Mekong River, Nongkhai Province, Thailand by the gillnetting. After, they were began artificial breeding of the fish. The spawner female (average 3,750 gBW) were injected with Hormone 1 + 1,000 IU (Dose 1 + CG) at 1st injection and Hormone 2.5 + 2,000 IU (Dose 2 + CG) at 2nd injection. All spawner female were die after the injection, the artificial breeding in this time is not succeeded [16].

7. Conclusions

The molluscivorous catfish, *H. leptorhychus*, is popular fish in Thailand. It is riverine fish that belongs to family Pangasiidae. The fish distributed in Mekong-Chaophraya Basin. This fish is migratory species that behavior of migration depends on water temperature, water level, turbidity and so on. In Thailand, the molluscivorous migration differ in each area such as Mekong upstream, this fish migrates two times of each year (December-April and April-June), Mun River, it migrates on March-June. The molluscivorous is omnivorous. But, the main food is bivalves in adult stage. The spawning behavior of this fish will migrate to upstream in early flood season. The optimal spawning time in Thailand is March-May. The length at 50% maturity in female was 42.01 cm TL and average fecundity ranged was 85,173 ± 43,206 eggs per maturity female.

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Diversity and Florogenesis of Subnival Flora of the Caucasus

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Abstract: This paper presents the results of systematic, ecopathological, and chorological studies of the diversity of the subnival belt (zone) flora of the Caucasus Mountains, peculiarity of altitudinal distribution, endemism and florogenesis. Comparative analysis of the diversity of the subnival flora on different types of stone and at different altitudes in various parts of the Caucasus has been made. It is based on field investigation and on literature research. 226 species, 96 genera and 35 families were recorded in the subnival belt of the Caucasus within a range of 2,800 (2,900)-4,000 m a.s.l. Among these 117 species or 51% are common endemics of the Greater Caucasus and Caucasus. It is proved that floristic elements of different origin (authochronic and aloctonic) and age (Miocene-Pliocene and Pleistocene) contributed to the florogenesis of the subnival belt of the Caucasus. The Caucasian, the Eu-Caucasian, the Eastern Asian, the Minor Asian, the Dagestan-Iranian, the Caucasia-European groups plaied an important role in the florogenesis. Criophilic evolution on the of the some plants was related to oreophytizacion during formation of the Caucasus mountains (in the second half of the Tertiary), as well as the glaciations scale. Species composition and coenotic role are different in various parts of the Caucasus and within each part. This is conditioned by the different hypsometry of various parts of the Caucasus, the character of glatiations, edaphic and climatic conditions, lythological diversity. Compared with the Greater Caucasus, the relative floristic poverty of the Lesser Caucasus is due to low elevations and extensive rather recent vulcanism.

Key words: Caucasus mountain, subnival flora, geographycal isolation, endemic, glatiation, volcanogenic rock-screes.

1. Introduction

Different data have been provided on the diversity of the subnival flora of different parts of the Caucasus. 80-230 plant species were recorded within the boundaries of the Greater Caucasus [1-9] and 60-100 species in the Lesser Caucasus [10-12]. In part these differences are caused by inclusion of species more comon in the subalpine and alpine belts in floristic lists of the subnival zone. For example, A. Dolukhanov [3] indicated 191 species for the volcanic plateau of the river mouth Didi Liakhvi (in the central Greater Caucasus) at 3,300 m a.s.l. This number seems unlikely high for the subnival zone and was increased due to inclusion of subalpine and alpine species (*Anemone fasciculata* L., *Geranium ibericum* Cav., *Delphinium speciosum* M. Bieb., *Inula orientalis* Lam., *Polygonum carneum* C. Koch (= *Bistorta carnea* (C. Koch) Kom.), *Cardamine uliginosa* Bieb., *Chaerophyllum roseum* Bieb., *Swertia iberica* Fisch. & C.A. Mey., *Macrotomia echinoides* (L.) Boiss. (= *Huynchia pulchra* (Roem. & Schult.) Greuter & Burdet), *Gentiana septemfida* Pall., *Betonica grandiflora* Willd. (= *B. macrantha* C. Koch), and *Centaurea cheiranthifolia* Willd., etc.). These plants are typical species of the meadows (subalpine and alpine) of the glacially formed Caucasian high mountains [6, 7] and create diverse phytocenoces. The studies conducted by A. Kharadze [1, 2], B. Zurebiani [4], and B. Prima [5] reflect better the diversity of typical subnival flora of the Caucasus. The subnival zone represents the upper level of the alpine belt,

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almost devoid of soil cover. Therefore, it seems unreasonable to include here the vegetation of the nival belt as it is given in some works [8, 9]. Only a few plants are found here and respectively only mono- or bidominant plant communities [2]. In the European Alps the upper limit of the closed vegetation distribution is mainly at 2,800 m a.s.l. [13-16]. At the same time, it is also worth noting that systematic and geographical diversity of the flora in the subnival belt, this inique, comparatively young climatic zone, fails to reflect its coenotic and landscape diversity fully i.e., the so-called beta and gamma diversity [17].

The reason for data differences is insufficient knowledge of high mountain areas, different definitions of the subnival zone, and more or less different high montane relief, climate, hypsometry and history of peri-glacial flora formation process in various parts of the Caucasus. In this context formation process of high mountain flora was influenced by a different extent of glaciation in the western, central and eastern parts of the Greater Caucasus, and the Lesser Caucasus in the Quaternary. It is known that during the Pleistocene glaciers descended to 1,000-1,200 m in the western Greater Caucasus, to 680-700 m in the central Greater Caucasus (Svaneti, gorges of the rivers Mulkhura, Dolra, Nenskra), to 1,700-1,800 m in the eastern Greater Caucasus (Tusheti, Pirikiti Khevsureti, gorges of the rivers Pirikiti Alazani, Asa and Arghuni) and to 2,200-2,300 m in the Lesser Caucasus [18-20]. At present the subnival belt in the Greater Caucasus covers altitudes of 2,900-3,800 (-4,000) m. Its lower boundary is a transition strip and “intersection” between upper alpine and subnival belts. Complex eco- and coenotones of these two belts frequently occur in this strip.

2. Description of the Study Area

2.1 Orography and Geology

The Great Caucasus stretches for about 1,500 km from the north-west to the south-east, between the Taman Peninsula to the Apsheron Peninsula. According to the absolute altitudes and other physico-geographical peculiarities it is traditionally divided into three parts: west, central and east Caucasus. The borders between these sections are Mt. Elbrus and Mt. Kazbeg. The highest peaks surpass an elevation of 5,000-5,600 m and are located in the Central Caucasus (Fig 1). Of these 15 summits are permanently covered by snow and glaciers, and exceed the Alps in altitude, among them Montblanc. The highest peaks, Elbrus (5,642 m) and Kazbegi (5,033 m), are situated on the lateral ridge and represent cones. The highest summit of the southern macroridges is Shkhara (5,624 m).

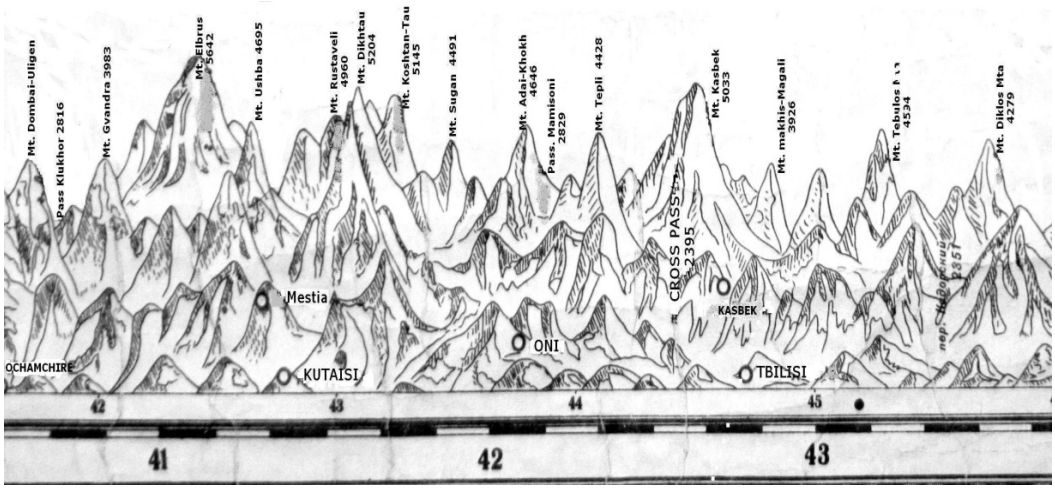

Fig. 1 Geographysical profile of Caucasus.

The Caucasus Mountains differ from the Alps by minor length and width, greater absolute and relative heights of the peaks, comparatively simple orographic height and existence of young (Pliocene-Quaternary) volcanoes.

Upper Proterozoic and Paleozoic crystal slates play a prominent role in the geological structure of the western region of the Caucasus. The main watershed ridge from the mouth of the Belaya River to the Mamisson Pass is built of granites, gneisses, metamorphic and crystal rocks. Jurassic slates of the Mesozoic period, sandstones and Cretaceous limestones are spread as stripes on both sides of this crystal axis.

In the geological structure of the East Caucasus intrusive and metamorphic rocks almost do not take part. An exception is the barren granite massif in the Dariali Canyon (gorge of the river Tergi). The eastern part of the Caucasus is mainly built with Jurassic sediments. Rocks of the Cretaceous are represented only sporadically (north-east Daghestan and north Azerbaijan). Unlike in the western part of the Caucasus, young effusive rocks of which the volcanic relief of the Tergi, Ksani-Aragvi (East Georgia) is built, are widespread in the east. They are of Quaternary origin, but in some places Pliocene volcanic remnants are preserved. The largest volcanic cones in the Greater Caucasus are Elbrus, Mkinvaltsveri (Kazbegi), Kabarjini, and the Keli Plateau.

Compared to the West Caucasus, due to the dry continental climate in the East Caucasus the lower limit of permanent glaciers is 150-200 m higher at 3,600-3,700 m a.s.l. [18-20]. These conditions cause differences in altitudinal distribution and partially in spatial distribution of plants between these two parts of the Caucasus.

2.2 Climate

The peculiarity of the complex mountain relief determines the climatic diversity of the Caucasus. The Caucasus range is located at the junction of the temperate and subtropical climatic zone. This border is caused by the Greater Caucasus mountain range which blocks the intrusion of cold air masses from the north to the south and warm masses from Transcaucasia to the north. The northern part of the Caucasus belongs to the temperate zone and Transcaucasia belongs to subtropical zone. A main difference between both is air temperature. The average annual temperature is 10 °C on the northern slopes and in the south—16 °C. The average January temperature in Transcaucasia is 5 °C, in west Transcaucasia—6 °C, and in the eastern Caucasus—3 °C. In summer there is minor difference between the north and south, but on the other hand remarkable contrast of temperatures between the west and east part exists. In the west Caucasus the average temperature in July is 23-24 °C and in the east—25-29 °C.

Climatic peculiarity of the Caucasus is also conditioned by the fact that it is subject of movements of Atlantic and Mediterranean humid air masses on the one hand and of dry Eurasian continental masses on the other hand. Stavropol Upland and Likhi Ridge play an important role for climate peculiarities of various parts of the Caucasus. Annual rainfall varies between 100 mm and 3,682 mm. Climatic conditions are subject of vertical zoning and at the same time, fluctuate in horizontal direction too. Atmospheric precipitations decrease from the west to the east.

2.3 Soils

Over 50 soil types which comprise more than one half of the soil diversity of the former Soviet Union, have been described on the territory of the Caucasus. This diversity is conditioned by lithological and geomorphological peculiarities, mountain rocks, age of relief and history [21, 22].

The main soil types of the Caucasus are: moderate belt steppe soils, forest soils, dry and humid subtropical soils, mountain meadow-steppe soils, mountain-meadow chernozem, meadow soils, swamp

soils, grove soils and numerous other types of soils. The highest diversity of the Caucasus soils can be found in the lowlands, and it decreases in the mountains.

High-mountain soil types of the Caucasus change with the vertical belts. Brown forest soils predominate in the forest belt. On the north and northwest facing slopes, where subalpine crooked-stemmed birch forests and thickets of *Rhododendron caucasicum* Pall. are common, peat-humus soils are presented that frequently extend up to 2,500 m. Soil cover on the limestone territory of the Caucasus is made up by calcareous humus soils.

Mountain meadow soils are presented at the elevation from 1,800-2,100 m to 2,700-3,100 m a.s.l. The soils of the subalpine belt include forest brown soils, average depth skeletal forest brown soils, soddy mountain meadow soils, and so on. The soils of the alpine include soddy mountain-meadow soils, underdeveloped skeletal mountain meadow soils, soddy-skeletal and primitive soils of medium and small depth [21, 22] The mentioned soils are frequently located in a complex in the Greater Caucasus.

3. Materials and Methods

The present work summarizes long-term research results carried out by us on the Caucasus and literature study. The research is based on the analysis of systematic and geographical structure of the flora of the subnival zone, ecotopology, peculiarities of hypsometric distribution, coenosis diversity of various floristic complexes, endemism and florogenesis issues. Atypical plant species indicated for this zone by some researchers were excluded from the general list of flora. Aspects and inclination degrees of slopes were taken into account; botanical-geographical profiles and floristic lists compiled at every 100 (200) m a.s.l upwards.

Types of the distribution ranges of the high mountain plant species are given according to the typological system of the distribution ranges elaborated for the Caucasus by Gagnidze and Ivanishvili [23, 24]. The system of Grossheim [25] was also used with some amendments. The classification of distribution range types is based on the so-called “center of gravity” of species distribution, as well as their common distribution. Within each type of distribution range groups of distribution ranges that show local or comparatively wide distribution of taxa are singled out.

Plant taxonomy and nomenclature follows Ketskhoveli et al. [26], Czerepanov [27], Gagnidze [28], and Takhtajan et al. [29].

4. Results and Discussion

4.1 Systematical Structure

The subnival belt flora of the Greater Caucasus comprises 226 species, 96 genera and 35 families. It includes common plants typical only for the subnival zone as well as those spread on the limits between this zone and upper alpine belt.

The leading families are *Asteraceae* (25 species), *Caryophyllaceae* (25 species), *Brassicaceae* (23 species), *Poaceae* (14 species), *Fabaceae* (12 species), *Campanulaceae*, *Scrophulariaceae*, *Ranunculaceae*, *Rosaceae* (each 11 species), *Saxifragaceae* (10 species), *Primulaceae* (7 species), *Apiaceae* (6 species), *Lamiaceae* (5 species), *Crassulaceae*, *Gentianaceae*, *Cyperaceae*, *Liliaceae* (each 4 species). The remaining families are presented by 1-3 species. The leading genera are *Campanula* (11 species), *Saxifraga*, *Minuartia* (each 10 species), *Ranunculus* (9 species), *Draba* (8 species), *Cerastium* (7 species), *Alchemilla* (5 species), *Silene*, *Primula*, *Sedum*, *Anthemis*, *Gentiana*, *Alchemilla*, *Carex* (each 4 species). *Campanulaceae* and *Saxifragaceae* are represented only by a single genus in the subnival zone (Fig. 2).

Species composition and coenotic role are different both in various parts of the Caucasus and within each part. This is conditioned by the above mentioned

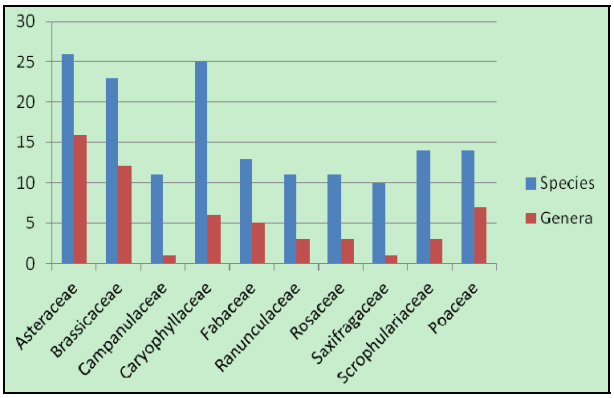

Fig. 2 The families with the largest number of taxa and genera.

circumstances—different hypsometry of various parts of the Caucasus, the character of glaciations, edaphic and climatic conditions, lithological diversity, history of flora formation, and so on.

In the massive mountain system of the Greater Caucasus the volcanic cones of the Elbrus and Kazbegi yield to other mountain parts by floristic diversity. This fact is conditioned by the juvenility of soil cover in this place which is formed by lavas of the Quarternary period and also strong Quarternary glaciations in the Central Caucasus [2]. For example, the petrophile flora of volcanic relief of the subnival belt in the Mount Kazbegi environs is rather species poor. At 3,200-3,400 m a.s.l. only *Saxifraga sibirica* L., *S. flagellaris* Willd., *S. moschata* Wulfen, *Delphinium caucasicum* C.A. Mey., *Omalotheca supina* (L.) Cass., *Ranunculus caucasicus* M.Bieb., *Myosotis alpestris* F.W. Schmidt, *Veronica gentianoides* Vahl, *Campanula tridentata* Schreb., *Cerastium polymorphum* Rupr., *Minuartia imbricata* Woron., *Sedum tenellum* M. Bieb., *Epilobium anagallidifolium* Lam., and *Senecio taraxacifolius* (M. Bieb.) DC., are found; *Senecio sosnovskyi* Sof., inhabits areas at glacier ends between skeleton substrates in silver carpets of mosses (*Racomitrium canescens*) and *Tephroseris karyaginii* (Sof.) Holub., grows up to 3,050 m. These species are widespread almost everywhere in the Greater Caucasus.

In the Kazbegi region (east part of the Central Caucasus) the floristically most interesting part are the watershed between the Aragvi and Tergi rivers (in mt. Chaukhebi massif) and surrounding shale screes. At 3,349 m a.s.l. all the above listed plants and also *Lamium tomentosum* Willd., *Jurinella subacaulis* Iljin, *Chaerophyllum humile* Stev., *Cerastium kasbek* Parrot, *Symphyoloma graveolens* C.A. Mey., *Primula bayernii* Rupr., *Cruciata coronata* (Sibth. & Sm.) Ehrend., *Scrophularia minima* M. Bieb., and *Viola minuta* M. Bieb., are found. Of interest is also Kuro massif and especially Khde shale gorge (gorge of the Kistinka River).

Volcanogenic rock-screes of the Mt. Fidar in the eastern part of the Central Caucasus are floristically richer. In spite of the fact that there the subnival zone is only fragmented and lower (3,000-3,200 m), rare species like *Dentaria microphylla* Willd., *Corydalis alpestris* C.A. Mey., *Cerastium polymorphum* Rupr., *Jurinella subacaulis* Iljin, *Ranunculus lojkae* Sommier & Levier, *Veronica schistosa* E.A. Busch, *V. telephiifolia* Vahl, *Apterigia pumila* Galushko, *Eunomia rotundifolia* C.A. Mey., *Oxytropis cyanea* M. Bieb., *Pedicularis armena* Boiss. and Huet, *Polygonum viviparum* L., etc. are found. The mentioned species diversity is caused by the fact that due to the comparatively low altitude the Keli volcanic plateau was less affected by glaciation than other parts of the Central Caucasus. Floristically the eastern part of the Keli volcanic plateau—Arkhi ridge (the mouth of the Ksani River) is similar to Mt. Fidar although *Eunomia rotundifolia* C.A. Mey. is absent.

Floristically, the East Caucasus, especially its NE part, is rather different. For example, in the Atsunta-Kvakhidi massif at 2,999-3,200 m a.s.l. (ca. 42º N; ca. 45º E), both local endemics of the East Caucasus—*Ranunculus tebulossicus* Prima, *Vicia larissae* Prima, *Erisimum subnivale* Prima, *Alopecurus tuscheticus* Trautv., *Silene caucasica* (Bunge) Boiss., *Veronica petraea* Steven, *Arabis farinacea* Rupr., *Podospermum grigoraschvili* Sosn., *Pyrethrum aromaticum* Tzvelev, and common endemics of the Central and East Caucasus like

Campanula petrophila Rupr., *Cerastium multiflorum* C.A. Mey., *Silene humilis* C.A. Mey., *Primula bayernii* Rupr., *Saxifraga ruprechtiana* Manden., *Campanula argunensis* Rupr., and *Jurinea filicifolia* Boiss. are present. Autochthonous development of the high mountain flora of the East Caucasus in the first place is associated with other parts of the Caucasus on its long-term isolation. At present this isolation is caused by the depression between Cross pass and the Bezhtin ridge [17]. In general the vegetation of the Kazbegi region reveals similarities with the vegetation of the East Caucasus but the spectrum of Caucasian endemic species of the subnival zone is rather different between both regions.

The major reason for this difference is the fact that in the East Caucasus, due to dry continental climate, the lower border of everlasting snowcaps and glaciers is elevated to 3,700 (3,800) m. On the relief released from the glaciers, closed phytocenoses are relevantly lifted up with edificators are *Festuca woronowii* Hack., and other grasses of the so-called "antagonistic" communities (*Poa alpina* L., *Colpodium variegatum* (Boiss.) Griseb., *Alopecurus dasyanthus* Trautv., *Festuca supina* Schur). In this high-altitude areas of cryogenic relief the occurrence of *Sibbaldia parviflora* Willd., *Chamaesciadum acaule* (M. Bieb.) Boiss., *Campanula tridentata* Schreb., *Minuartia inamoena* Woron., and *Cerastium polymorphum* Rupr., is also important.

If we take global climate models into account altitudinal distribution of the high mountain and among them subnival belt plants will probably significantly change in the nearest future. These models indicate striking changes for the Caucasus region caused by a rise of the yearly global mean temperature by about 3 degrees until the end of this century (2071-2100) compared to 1961-1990 (Fig. 3) Due to this the upper distribution border of some plant species is supposed to shift upwards approximately by 100-150 m. At the same time mesophilous communities will be replaced by xeromesophylic communities. The tendencies of such changes are already more or less observable in the East Caucasus. An example of this can be the rise of alpine belt plants *(Aconitum tuscheticum* N. Busch, *Heracleum osseticum* Manden., *Pseudomuscari pallens* (M. Bieb.) Garbari, *Aster alpinus* L. etc.) in the East Caucasus up to the lower border of the subnival belt [30].

4.2 Geographycal Analysis and Florogenesis

The subnival belt flora of the Caucasus includes 7 types and 16 subgroups of the distribution range (Table 1, Fig. 4). The Eucaucasian type (EUCAUC) includes 84 species. Subgroups are: proper Caucasian

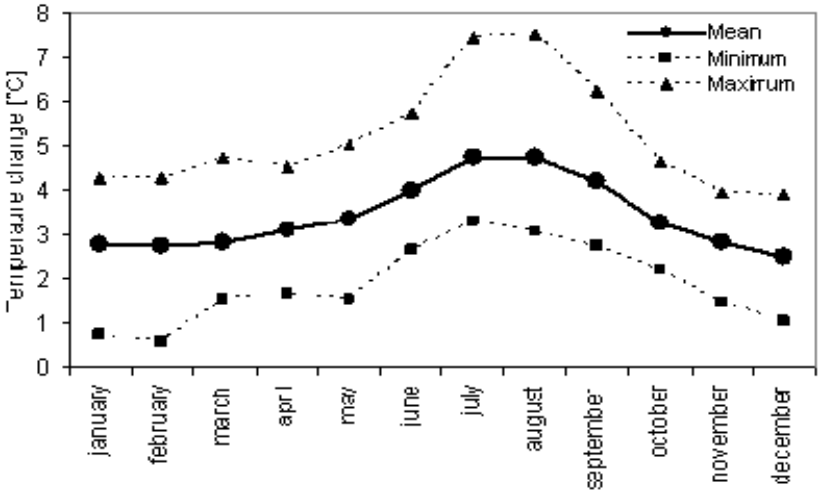

Fig. 3 Temperature projection 2071-2100 vs. 1961-1990 Ensemble-range of 20 global climate models, Scenario A1B (www.cenn.org. Newsletter, November, 2011).

Table 1 Chorological and ecotopological spectrum of the subnival flora.

Types	Groups	Ecotopes					Species
		var.r	var.s-sts	lr	ls-sts	alp.car	
Eucauc	eucauc	10	13	-	-	2	25
	eucauc: w. gr. cauc.	2	2	5	2	4	15
	eucauc: centr. gr. cauc.	2	2	-	-	-	4
	eucauc: o. gr. cauc.	6	13	-	-	3	22
	eucauc: w. centr. gr. cauc.	1	2	-	-	1	4
	eucauc: centr. o gr. cauc.	6	4	-	-	-	10
	eucauc-cauc. min.	-	3	1	-	-	4
Cauc	cauc: lat. cauc	10	18	-	-	5	33
	cauc.- as. min	5	20	-	-	14	39
	cauc- as. anter	7	17	-	-	8	32
	cauc-europ	-	1	-	-	-	1
	cauc- medit	1	-	-	-	-	1
Europ-Medit	europ-medit.	1	1	-	-	1	3
Medit-As. min	medit-as. min.	-	1	-	-	1	2
Palearkt	palearkt.	8	2	-	-	4	14
Holarkt	holarkt.	6	5	-	-	5	16
Pancont	pancont.	-	-	-	-	1	1
Total: 7	17	75	94	6	2	49	226

Abbreviations and symbols used:

Cauc	Caucasian	lat. Cauc	Common Caucasian
Eucauc	Eucaucasian or Greater Caucasian	centr. gr.cauc	Central and Eastern Greater Caucasian
o. gr. cauc	Eastern Greater Caucasian	centr et o.gr. cauc	Central and Eastern Greater Caucasian
w. gr. cauc.	Western Caucasian group	w.centr.gr.cauc	Western Central Caucasian
As Min	Asia Minor	West Asian	Asia Anterior
Medit	Mediteranian	Europ	European
Holarkt	Holarktic	Paleark	Palearktic
Pancont	Pancontinetal	var. r	Various rock
Lr	Limestone rock	ls-sts	Limestone screes and stone sceletion substrate
alp.car	Alpine carpet	var.s-sts	Various lithological screes and stone sceletion substrate

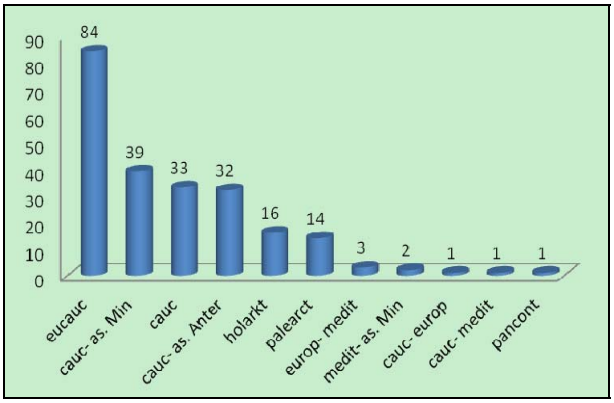

Fig. 4 Distribution of the species to phytogeographic region.

(EUCAUC) like *Campanula circassica* Fomin, *Valeriana saxicola* C.A. Mey., *Pseudobetckea caucasica* (Boiss.) Lincz., *Scrophularia minima* M. Bieb., *Delphinium caucasicum* C.A. Mey., *Saxifraga pseudolaevis* Oetting., *Anthemis sosnovskyana* Fed., and *Cerastium polymorphum* Rupr., West Caucasian (w.gr.cauc) like *Campanula circassica* Fomin., *Ranunculus lojkae* Sommier & Levier, *Minuartia trautvetteriana* Sosn. & Charadze, and *Saxifraga pontica* Albov, East Caucasian (o.gr.cauc) like *Trigonocaryum involucratum* Kusn., and *Valeriana daghestanica* Rupr. ex Boiss., West and Central Caucasian (w.gr.cauc.) like *Barbarea ketzkhoveli* Mardalejschvili, *Charesia akinfievii* (Scmalh.) E.A. Busch, and *Cerastium undulatifolium* Sommier & Levier, and Central and East Caucasian (centr.w.gr.cauc.) like *Tripleurospermum caucasicum* Hayek, *Teprhoseris karjaginii* Holub, *Primula bayernii* Rupr., *Draba siliquosa* M. Bieb., *Cerastium kasbek* Parrot, and *Minuartia inamoena* Woron. It should be mentioned that the above listed species are endemic

species of the Greater Caucasus and their distribution is restricted to the Greater Caucasus or parts of it. This fact indicates once more that the Caucasus is a mountain system of distinct geographical isolation for the origination and distribution of endemic species [1-5, 7, 10, 12, 31-34].

The Caucasian type (CAUC) includes 106 species. Among these, 33 species, like *Eunomia rotundifolia* C.A. Mey., *Pseudovesicaria digitata* Rupr., *Scrophularia ruprechti* Boiss., *Carex meinshauseniana* V.I. Krecz., and *C. medwedewii* Leskov belong to the common Caucasia group. These species are endemic or subendemic to the Caucasus.

32 species belong to the Caucasus-Anterior Asian type (CAUC-AS.ANTER): *Minuartia aizoides* Bornm., *Didimophysa aucheri* Boiss., *Draba hispida* Willd., *Sedum tenellum* M. Bieb., *Alchemilla sericea* Willd., *A. retinervis* Buser, *Pedicularis crassirostris* Bunge, *Veronica gentianoides* Vahl, *V. minuta* C.A. Mey., *V. telephiifolia* Vahl., *Senecio taraxacifolius* (M. Bieb.) DC., *Poa caucasica* Trin., etc. This group also includes the monotypic genus *Vavilovia* Fed., which rarely occurs in the Caucasus and is mainly distributed in Anterior Asia and also in Armenia.

39 species belong to the Caucasus-Minor Asian type (CAUC-AS.MIN): *Carum caucasicum* Boiss., *Nepeta supina* Stev., *Thlaspi huetii* Boiss., *Minuartia colchica* Charadze, *Rhododendron caucasicum* Pall., *Vavilovia formosa* Fed., *Geranium gymnocaulon* DC., *Epilobium algidum* M. Bieb., *Pedicularis nordmanniana* Bunge, *Luzula pseudosudetica* V.I. Krecz., *Alopecurus glacialis* C. Koch, *A. dasyanthus* Trautv., etc., 1 species to the Caucasus-European type (CAUC-EUROP) (*Saxifraga exarata* Vill., *S. moschata* Wulfen); 3 species to the Euro-Mediterranean type (EUROP-MEDIT) (*Festuca* woronowii Hack., *Gentiana pyrenaica* L., *Plantago saxatilis* M. Bieb.); 12 species to the Palearctic type (PALEARKT) 14 species (*Erigeron uniflorus* L., *Taraxacum porphyranthum* Boiss., *Lloydia serotina* (L.) Salisb. ex Rchb., *Potentilla crantzii* (Crantz) Beck ex Fritsch, *P. gelida* C.A. Mey., *Poa alpina* L., *Oxyria elatior* R. Br. ex Meissn., etc.), and 16 species to the holarctic type (HOLARKT) (*Myosotis alpestris* F. W. Schmidt, *Cerastium cerastioides* (L.) Britton, *C. polymorphum* Rupr., *Epilobium anagallidifolium* Lam., *Vaccinium myrtillus* L., *V. vitis-idaea* L., *Trisetum spicatum* (L.) K. Richt., etc.).

Of the total number of subnival belt flora 117 species or 51% are endemic. Among these, 80 species are endemics of various parts of the Greater Caucasus and 33 species are common endemics of the Caucasus. In the subnival belt of lateral ridges of the East Caucasus local steno-endemic species occur like *Ranunculus tebulossicus* Prima, *Veronica bogosensis* Tumadzhanov, *Pyrethrum aromaticum* Tzvelev, *Didymophysa aucheri* Boiss., *Silene humilis* C.A. Mey., *Saxifraga ruprechtiana* Manden.. *Vicia larissae* Prima, *Pseudobeckea caucasica* (Boiss.) Lincz., and *Alopecurus tuscheticus* Trautv. Very sporadic distribution is characteristic for the Caucasus-Anterior Asian oligotypic genus *Vavilovia* Fed. (*V. formosa* (Steven) Fed.). This plant, with *Silene humilis* C.A. Mey., *Pseudobetckea caucasica* (Boiss.) Lincz., *Veronica bogosensis* Tumadzhanov, and *Ranunculus tebulossicus* Prima, is among the higher plants within the Greater Caucasus. Floristically, slate screes and rocky habitats are most diverse (Fig. 4).

In the subnival belt 5 endemic genera of the Caucasus are widespread. As a result of morphological and geographical study it has been established that *Symphyoloma* C.A. Mey., is the oldest endemic genus; it is a diploid plant (2n = 22) [35]. Its origin is connected with adaptation to cold environments which resulted in a specialized life form [33, 36, 37]. The monotypic *Pseudobetckea* (Hock.) Lincz. of the subnival belt is also considered to be one an old species. It is related to *Valerianella* Hill and the Andean *Betckea* DC. As a result of high altitude adaptation, probably during the orogenesis of the Caucasus, its fruits and flowers has been reducing. The formation of this plant as high montane life form must have happened also as a result of cryophilization by means of oreophitization due to orogenesis of the

Caucasus [33, 37].

The endemic genus *Pseudovesicaria* (C.A. Mey.) Rupr., of the Great (Central and East Caucasus) and Lesser Caucasus, is also restricted to the subnival belt. It is an orophyte with rosette habit and is characterized by succulent leaves. This plant is also characterized by Anterior-Asian-Mediterranean related links. It is related with the old xerophytic, Anterior-Asian monotypic *Elburzia* Hedge (*E. fenestrata* (Boiss.) Hedge) and *Coluteocarpus* Boiss., and with the Anterior -Asian-Central Asian genus *Didymophysa* Boiss.

An endemic monotypic genus of the Central Caucasus (N Ossetia, Svanetia) is *Charesia* E.A. Busch (*Ch. akinfievii* (Schmalh.) E.A. Busch.). It is related to *Silene* L., especially to some of its Anterior Asian species but sharply differs from it by box structure, as well as pollen morphology [33]. An endemic genus of the NE part of the Caucasus is *Trigonocaryum* Trautv. (*T. involucratum* Kusn.). It is related to *Myosotis* L., differing from it in chromosome number (2n = 14). Their main ranges are the subalpine and alpine belts, and it reaches the subnival belt only in Daghestan.

In spite of a significant number of apomictic and clonally expanded species in the highlands genetic diversity is rather high [38-41]. However, an exception are the so-called refugium species [41]. In the Caucasus subnival belt some of the above listed plants can be considered as refugium species, like *Delphinium caucasicum* C.A. Mey., *Symphyolma graveolens* C.A. Mey., *Ranunculus helenae* Albov, and *Pseudobetckea caucasica* (Boiss.) Lincz.

Caucasian and Anterior Asian species distributed in the subnival belt are *Eunomia rotundifolia* C.A. Mey., *Saxifraga cartilaginea* Willd., *Chaerophyllum humile* (M. Bieb.) Boiss., *Valeriana saxicola* C.A. Mey., *Senecio taraxacifolius* (M. Bieb.) DC., *Draba bryoides* DC., *Pedicularis crassirostris* Bunge, *Lamium tomentosum* Willd., *Primula amoena* M. Bieb., etc. [1, 3, 11].

Species belonging to the Daghestan-Iranian are *Didymophysa aucheri* Boiss., *Cicer minutum* Boiss. & Hohen., and *Dracocephalum botryoides* Steven. These are rare species occurring only in Daghestan and north Iran [42, 43]. It should be noted that Didymophysa aucheri is also distributed in NE Iraq and N Anatolia. This species and *D. fedchenkoana* Regel (from Afghanistan) are characterized by limited distribution. They are typical petrophytes, sometimes reaching as high as 4,000 m a.s.l. [44]. Narrowly limited distribution within the Greater Caucasus is characteristic to *Cicer minutum* Boiss. & Hohen. (NE Caucasus, Bazar-Diuzi mountain system) which was collected there by Prima for the first time. The distribution of the mentioned plant proves once more that floristic links (“intermontane links”) existed between the Caucasus and Minor Asia in Pliocene and Pleistocene [44].

In the subnival belt a rather small number of Arctic mountain species *(Poa alpina* L., *Trisetum spicatum* (L.) K. Richt., *Lloydia serotina* (L.) Salisb. ex Rchb., *Cerastium cerastioides* (L.) Britton, *Minuartia verna* (L.) Hiern, *Saxifraga flagellaris* Willd., *Omalotheca supina* (L.) DC., *Potentilla crantzii* (Crantz) Beck ex Fritsch, *Thalictrum alpinum* L.), Caucasian-European (*Corydalis alpestris* C.A. Mey., *Myosotis alpestris* F. W. Schmidt, *Saxifraga moschata* Wulfen, *S. exarata* Vill.), and Caucasian-Central Asian (*Primula algida* Adams, *Herniaria caucasica* Rupr., *Oxyria elatior* R. Br. ex Meissn., *Potentilla gelida* C.A. Mey) are present.

Different data have been obtained as a result of karyosystematic study of species of the alpine and subnival belts. Karyological studies have established that in the Caucasus subnival belt diploid species [35, 45] but in the Swiss Alps and Arctic polyploid species dominate [46-49]. The question arises again as to what stimulates polyploidy in plants. Are there severe climatic conditions, assimilation of new territories by plants or both of them? This question is again disputable. To solve the orogenesis within the compared regions and peculiarities of glaciations and

floristic history should be considered. The important features chaping the complex evolutionary history of alpine plants include narow ecological adaptation to severe habitats, abrubt altitudinal gradients of well-difined ecological riches [50, 51]. In high mountains each florocoenosic complex (high mountain meadows, tall herbaceous, alpine carpets, petrophytes, and so on.) represents the community of ecologically and coenotically different species formed during long period. This opinion is also supported by the fact that species of meadows and rocks are karyologically remarkably different. The latter is characterized by rich endemism and accordingly, a major number of diploid species. We totally agree with Sokolovskaya and Strelkova [45] who explained the low percentage of polyploids in the Caucasus by a great number of endemic species which origin is connected with the Caucasian orogenesis. The distribution success of polyploid cytotipes is probably more due to the benefits of apomictic reproduction than to genetic consequences of polyploidization [52]. In the same way, the small number of polyploid species in the subnival belt can be explained by the high number of old authochronic oreophites [35]. Although there is evidence that plant ploidy does not always depend on the altitude we consider that this factor plays a determining role in the Caucasus as an isolated mountain system. This opinion is also supported by the fact that in the Caucasus despite decrease of species number with altitude, the relative number of endemic species increases [1-7, 31-33]. This especially concerns the subnival belt with dominating rock-scree and skeletal substrate ecotopes. Compared with these the alpine carpets and comparatively “closed” coenoses are characterized with realtively low endemism.

There are different points of view on the origin and age of the high mountain flora of the Caucasus. Such disagreement always focused on the alpine (subnival) belt. As Korner [15] indicated, the origin of present-day high mountain taxa is a heredy mainly of Tertiary elements, consisting of a mixture of immigrants from various initial floras and new evolutionary lines [53-56].

Some authors consider that formation of the core of the Caucasus alpine flora is connected with the Tertiary [57-61]. Other botanists believe a considerable role is played by processes within the Quaternary [25, 37, 62-64]. Migration processes also played important role in this process. Aspects of florogenesis of the subnival belt are especially concerned by Kharadze [1, 2, 35, 37], who states that the species of early as well as comparatively recent origin played an important role in the formation of the core of the subnival belt flora. To the first belong Caucasus-Anterior Asian species which root back to more low-altitude plants of the Tertiary. Due to gradual adaptation towards existing ecological conditions they started to inhabit cooler areas. This view is evidenced by links between species of some genera (*Campanula* L., *Cerastium* L., *Silene* L., *Saxifraga* L., *Scrophularia* L., *Erysimum* L., *Pedicularis* L., *Delphinium* L., *Jurinea* Cass.) of the middle and subnival zones of the Caucasus mountains, geographical and hypsometric vicariance. Another group developed as a result of oreophytization of the Caucasian-Anterior Asian or authochtonously from Caucasian ancestor species (*Cerastium kasbek* Parrot., *Saxifraga scleropoda* Sommier & Levier).

Morphological isolation of the high mountain endemic genera *Pseudobetckea* (Hock.) Lincz., *Symphyoloma* C.A. Mey., and *Pseudovesicaria* Rupr., disjunct ranges and geographic variability evidence their early origin [35, 36]. Geographic isolation, tectonic movement, climatic changes, glaciation, high mountain habitat differentiation and variation in history of migration lead to high taxonomic richness [1-17, 31-37, 44-56, 65, 74]. Cold-climate evolution of the genera was related to oreophytization during formation of the Caucasus Mountains (in the second half of the Tertiary) as well as the glaciation scale. In Pleistocene their ranges must have been fragmented

resulting in ecological differentiation of different populations [35, 36]. During the Pleistocene glacial periods between the Himalaya and Central Asia with the Iranian, Caucasian and some European mountains facilitated a long-distance migration of several cryophilic species [43]. As Elenevskii [75] indicated, also the high mountain flora of the Lesser Caucasus has different origins and correspondingly different plant species. Alpine carpets are settled by allochthonous species that invaded during the Pleistocene period and High Mountain rock communities by autochthonous species from the Miocene-Pliocene authochtone group. Botanical investigations conducted on the Mt. Aragaz and Javakheti Plateau (Lesser Caucasus) also evidenced different origins and age of the species of the subnival belt [10, 12].

The floristic composition of the subnival belt in the Lesser Caucasus, i.e. Didi Abuli (ca. 60 species), is similar to that of the subnival belt flora of the Greater Caucasus. In the massif of Didi Abuli (Lesser Caucasus) the subnival belt is situated above 2,920 m a.s.l., with small populations of *Pedicularis armena* Boiss. & A. Huet., *Draba hispida* Willd., *Erysimum krynitzkii* Bordz., *Senecio taraxacifolius* (M. Bieb.) DC., *Potentilla gelida* C.A. Mey., *Aster alpinus* L., *Plantago saxatilis* M. Bieb., *Murbeckiella huetii* (Boiss.) Rothm., *Myosotis alpestris* F.W. Schmidt, and *Luzula pseudosudetica* V.I. Krecz., Near the summit (above 3,250 m) only several ultra-oreophytes occur, like *Chamaesciadum acaule* (M. Bieb.) Boiss., *Cerastium pseudokasbek* Vysokoostr., *Tripleurospermum caucasicum* Hayek, *Jurinella subacaulis* Iljin, *Eunomia rotundifolia* C.A. Mey., *Minuartia woronowii* Schischk., *Pedicularis armena* Boiss. & A. Huet, *Erysimum krynitzkii* Bordz., *Astragalus vavilovi* Fedorov & Tamamsch., *Crepis wildenowii* Czerep., and the grasses *Poa alpina* L., *Festuca supina* Schur, and *Alopecurus dasyanthus* Trautv. During florogenesis of the Lesser Caucasus subnival belt a decisive role was played by the floristic centre of Anterior Asia (*Chamaesciadum acaule* (M. Bieb.) Boiss., *Veronica gentianoides* Vahl, *Carum caucasicum* (M. Bieb.) Boiss., *Saxifraga sibirica* L., *Pedicularis crassirostris* Bunge, *Draba hispida* Willd., *Luzula pseudosudetica* V.I. Krecz.), and a minor role by the floristic center of the Greater Caucasus (*Eunomia rotundifolia* C.A. Mey., *Tripleurospermum caucasicum* (Hayek.) and arcto-alpine floristic centers (*Myosotis alpestris* F.W. Schmidt) [10-12, 76].

A floristic link of the Greater and Lesser Caucasus is also provided by several ultra-oreophytes vicariant species in these two mountain systems differing in altitude and petrology. Compared with the Greater Caucasus, the relative floristic poverty of the Lesser Caucasus is due to low elevations and extensive rather recent vulcanism. Volcanic rocks (andesites, basalts) are less inhabited by plants than clay, slaty, and marl soils. The low level of endemism is also conditioned by weak geographical isolation.

5. Conclusions

An in-depth botanicalgeographic analysis of subnival flora of the Greater Caucasus proves that both autochthonous and allochthonous plant groups participated simultaneously in the florogenesis of the high mountains. To the first group belong *Scrophularia minima* M. Bieb., *Delphinium caucasicum* C.A. Mey., and the monotypic endemic genera *Symphyoloma* C.A. Mey., *Pseudovesicaria* (C.A. Mey.) Rupr., and *Pseudobetckaea* (Hock) Lincz. For cold climate adaptation of these plants oreophytization processes have more importance than glaciation phases. Their actual distribution and polymorphism took place rather recently in the Pleistocene [37, 63]. Plants of the second group are *Pedicularis crassirostris* Bunge, *Veronica gentianoides* Vahl, *V. minuta* C.A. Mey., *V. telephiifolia* Vahl, and *Senecio taraxacifolius* (M. Bieb.) DC. It should be noted that in the formation of the subnival belt species a significant role was played by orogenesis processes of the Caucasus, which is caused extinction of ancestral species at lower altitudes and resulted in their geographical isolation. This is proved by geographical and hypsometric

vicarism of some species of *Campanula* L., *Cerastium* L., *Silene* L., *Erysimum* L., *Pedicularis* L., *Delphinium* L., and *Jurinea* Cass. Not only are the western, central and eastern parts of the Caucasus floristically different from each other, but also from neighboring mountain massifs. The above mentioned is conditioned by distinct geographic isolation pronounced in the mountain system of the Caucasus, different altitudes, petrology, and glaciogenic relief. Floristically, slate screes and rocky habitats are most diverse.

Along with elevation the proportion of autochthonous species is increased. In the subnival belt the role of arcto-alpine elements is relatively insignificant. Floristic similarity between the Great and Lesser Caucasus is mainly by allochthonous species.

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Botanical Assessment of Forest Genetic Resources Used in Traditional Cosmetic in Togo (West Africa)

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Abstract: In the current context of REDD+ opportunities, it is important to evaluate forest genetic resources for local communities' benefits. The aim of this ethnobotanical survey with an emphasis in cosmetopoeia—by referring to the word pharmacopoeia—was to explore, investigate, collect and identify natural resources used in traditional cosmetic in Togo for that purpose. The specific objectives were (i) to inventory plant species used as cosmetic in Togolese ethnocultural groups, and (ii) to describe their biological forms for their sustainable use. Based on ethnobotanical approach, this survey identified through multistage sampling design and semi-structured interview, 177 plant species belonging to 167 genera and 59 families with 82.45% dicotyledonous and 17.55% monocotyledonous species. According to life forms, these species were distributed as ligneous (56.50%) and herbaceous (43.50%). The computation of Whittaker's Index of Association led to three communities of ethnic groups. The explanatory effect of the ethnic based-tradition was significant and confirmed by Monte Carlo permutation test (P = 0.0020) after 499 permutations under split-plot constraints. This first outline confirmed ethnobotany as a viable tool in search for plant genetic resources in cosmetic industries. These findings could be incorporated into future conservation management plans of forest genetic resources in Togo and other tropical countries.

Key words: Cosmetic, ethnobotany, forest genetic resources, Togo.

1. Introduction

The biodiversity of the tropical world contributes to local communities' survival and wellbeing [1-4]. The researches of new active substances for cosmetic industries through natural resources is more and more increasing in recent years [5]. However in spite of the numerous ethnobotanical inventories carried out in the African countries [6-8], very few investigating have taken into account the skin care and cosmetic plant species. A recently publishing data on 380 principal plant species of trees, bushes and lianas in the dry zones of West Africa flora, enumerates about ten species only as cosmetic plant species [9].

Moreover, chemical analysis of some plant of these forest resources showed the presence of protein, vitamin C, D, E and K justifying their use as anti-ageing, anti-wrinkle, skin lightening, hair softness and buttocks care's lotions [10].

In the same time, the tropical zones would contain cosmetic value safeguarded by the traditional beauty knowledge [11]. So, cosmetic plant species can be found in tropical forest ecosystems, and be valorized industrially by the cosmetic firms [5, 12].

It is obvious that the lack of data on these forest resources constitutes a shortfall especially for local communities [13].

Surprisingly, little research has been done on plant

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species used in traditional cosmetic and quantitative information related to these kinds use of forest resources is poorly documented. They are not including into efficient strategy of biodiversity valorization and rational management programs [13]. In the current context of REDD+ opportunities, it is therefore important to evaluate cosmetic plant species both for sustenance of skin health care and local communities' benefits.

The aim of the present ethnobotanical survey with an emphasis in cosmetopoeia—by referring to the word pharmacopoeia [5]—was to explore, investigate, collect and identify natural resources used in traditional cosmetic in Togo for future biodiversity valorization and sustainable management.

The specific objectives were (i) to inventory plant species used as cosmetic in Togolese ethnocultural groups, and (ii) to describe their biological forms for their sustainable use.

This knowledge is crucial to enhance the tropical world biodiversity uses which requires regular updating.

2. Materials and Methods

2.1 Study Area

The survey area covered all Togo, a country of West Africa belonging to five ecological zones [14]. It is lying between 0° to 1° west longitudes, and 7° to 8° of north latitudes (Fig. 1). The vegetation is mainly characterized by fragments of forests, savanna and fallows. The 4th General Census of the Population and the Habitat (RPGH4) announced recently a human population of about 6,191,155 inhabitants [15]. This population is heterogeneous with 38 cultural groups [16]. The most represented are Ewe, Kabye, and Tem.

2.2 Data Collection

In this research, field surveys were conducted through multistage sampling design [17]. The five ecological zones of the country [14] were considered as a first level of stratification. Within these zones, the various ethnocultural groups are considered as a second level from which representative localities are randomly selected based on PHARMEL model database [6].

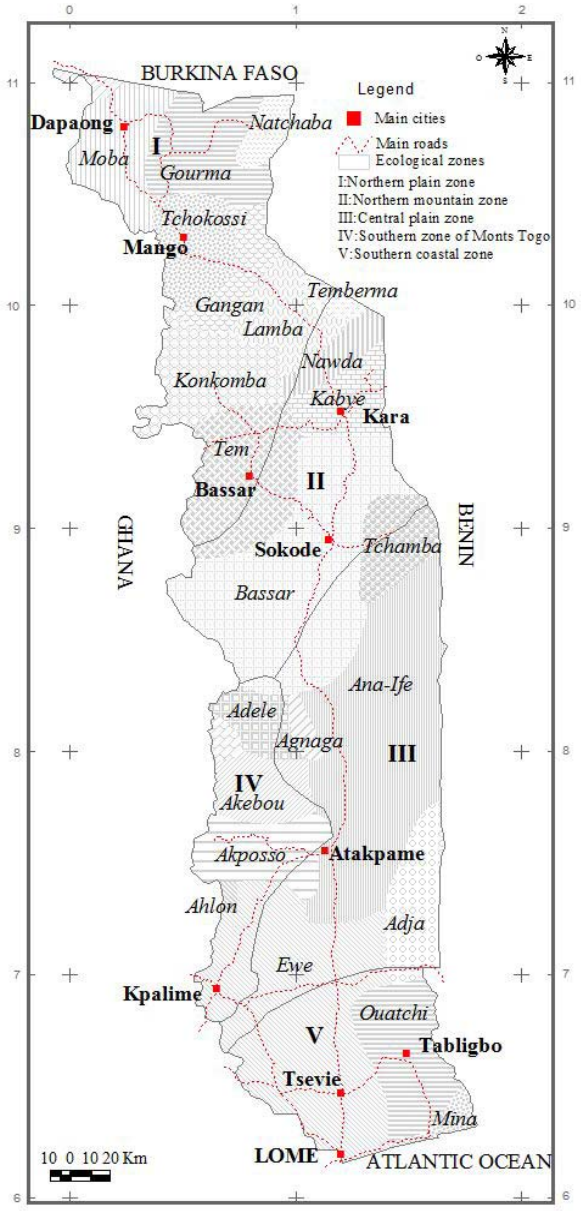

Fig. 1 Location of survey area: Ecological zone and ethnic groups.

Questionnaires were used during field surveys to gather information on cosmetic plants used by local communities. Vernacular names of plant species were collected. A total of 148 informants were interviewed, of which 63 were men (age 25-78 years), 85 women (age 17-83 years) in 96 localities. Repeated queries

were made to get the data conformed.

Regarding to taxonomy and phytogeographical types, plant species were systematically identified by the help of Benin analytic flora [18]. The plant species were classified into life form based on Raunkier definition [19]. And the specimens recorded have been compared with the reference voucher specimens of herbarium of University of Lome.

The geographical coordinates of the prospected localities were recorded by GPS in order to map the study area.

2.3 Data Analysis

Through the cosmetic plant species list established on the basis of the information gathered, the binary code was affected to each ethnic group. The information occurrence in the pivot table "plant species" *versus* "ethnic groups" permitted firstly to calculate frequencies, and secondly to identify the ethnic groups who used similar cosmetic plant species.

The similarity between these ethnocultural was performed by Gradient Analysis Method of direct Canonical Correspondence Analysis (CCA) in Canoco® 4.51. The unimodal method applied was focused on inter-species distances [20].

Other sophistical analysis based on Whittaker's Index of Association (WIA) adjusted the similarity results [21, 22].

The WIA is expressed as the fraction of the total number of individuals in the sample to measure similarity distance and computed by:

$$\frac{1}{2}\sum_{i=1}^{n}\left|\frac{s_{i1}}{\sum_{i=1}^{i=n} s_{i1}} - \frac{s_{i2}}{\sum_{i=1}^{i=n} s_{i2}}\right|$$

where i is the number of samples holding species i and S is the total number of columns (samples).

3. Results and Discussion

3.1 Floristic Assessment and Specific Richness

Through this survey, 177 plant species belonging to 167 genera and 59 families were identified with 82.45% dicotyledonous and 17.55% monocotyledonous species. Family Poaceae had highest number of cosmetic species (15 species). This number is very high compared to a similar study carried out in Pakistan which found 78 plant species [23]. Several environmental and ecological factors between the two study areas can explain this fact.

The botanical investigation showed that the six most represented families (58.33%) are Poaceae (15 species), Euphorbiaceae (12 species), Asteraceae (9 species), Combretaceae (8 species), followed by three families (Fabaceae, Moraceae, and Rubiaceae) containing seven species each of cosmetic plant species. Several species of indigenous flora which are most commonly used for cosmetic purpose and recognized by local communities are reported in Table 1.

3.2 Biological Forms, Life Forms and Phytogeographic Spectra

The plants species recorded have different growth habits which include herbs, shrubs and trees. According to biological forms, ligneous species were more involved (56.50%) in cosmetic preparation. The savannas ecosystems provided (57.05%) are these species followed by introduced species (22.82%) and forest species (20.13%).

The results showed that the spectrum of life forms is dominated by phanerophytes (70.12%) with a low representation of therophytes (10%), hemicryptophytes (9.37%), geophytes (8.12%), and chamephytes (1.87%). The phytogeographical spectrum is dominated by the Guinean-Congolese species (29.90%) and Sudano-Zambezian (28.40%). Further investigations of the present research revealed that although these savannas and forests plant species are highly valued for other purpose as their medicinal importance, they have not been so cultivated.

Table 1 Some plants used as cosmetic.

Family	Scientific name	Local name	Parts used	Ethnic groups
Fabaceae	*Abr*	Kas	Sed	Bag
Malvaceae	*Ada*	Gla	Bat	Aya
Apocynaceae	*Ade*	Nab	Bat	Tco
Sapindaceae	*Bli*	Kpe	Baf	Lam
Burseraceae	*Can*	Zan	Res	Ewe
Rutaceae	*Cla*	Afl	Fdl	Ewe
Euphorbiaceae	*Eup*	Bom	Lat	Mob
Moraceae	*Fic*	Kpr	Bat	Ake
Rubiaceae	*Gar*	Kew	Sed	Tem
Euphorbiaceae	*Hym*	Nak	Frl	Tco
Bignoniaceae	*Kig*	Gna	Fru	Ewe
Lythraceae	*Law*	Lal	Frl	Tca
Cucurbitaceae	*Luf*	Egb	Fru	Ewe
Rubiaceae	*Mit*	Gbe	Sap	Mob
Cucurbitaceae	*Mom*	Kpa	Wpl	Akp
Clusiaceae	*Pent*	Agb	Sed	Ani
Mimosaceae	*Pros*	Tch	Bat	Tem
Fabaceae	*Pte*	Oss	Hea	Ani
Polygalaceae	*Sec*	Djo	Rot	Tco
Poaceae	*Sor*	Kpd	Sed	Kab
Apocynaceae	*Tab*	Kpg	Sed	Ewe
Asteraceae	*Ver*	Sow	Frl	Ade

Scientific name: *Abr* = *Abrus precatorius* Linn. ssp. *africanus* Verdc., *Ada* = *Adansonia digitata* Linn, *Ade* = *Adenium obesum* (Forssk.) Roem & Schult.. *Bli* = *Blighia sapida* C.König, *Can* = *Canarium schweinfurthii* Engl., *Cla* = *Clausena anisata* (Willd.) Hook. f. ex Benth., *Eup* = *Euphorbia thymifolia* Linn., *Fic* = *Ficus gnaphalocarpa* Miq., *Gar* = *Gardenia aquala* Stapf & Hutch, *Hym* = *Hymenocardia acida* Tul., *Kig* = *Kigelia africana* DC., *Law* = *Lawsonia inermis* Linn., *Luf* = *Luffa aegyptiaca* Mill., *Mit* = *Mitracarpus villosus* Zucc, *Mom* = *Momordica charantia* Linn., *Pen* = *Pentadesma butyracea* Sabine, *Pro* = *Prosopis africana* (Guill. & Perr.) Taub., *Pte* = *Pterocarpus erinaceus* Poir., *Sec* = *Securidaca longepedunculata* Fres, *Sor* = *Sorghum bicolor* (Linn.) Moench, *Tab* = *Tabernaemontana pachysiphon* Linn. *Ver* = *Vernonia colorata* (Willd.) Drake.

Local name: Afl = *Aflafleti*, Agb = *Agbipkanya*, Bom = *Bombomkou-yo'fin'nin*, Djo = *Djoro*, Egb = *Egbekoutsa*, Gbe = *Gbengbanlan*, Gla = *Gl'al*, Gna = *Gnakpekpe*, Kas = *Kasikisikiya*, Kew = *Kewonye*, Kpa = *Kpakle*, Kpd = *Kpandjin*, Kpag = *Kpangboe*, Kpe = *Kpessire*, Kpr = *Kpre'ndjo*, Lal = *Lali*, Nab = *Nabiak*, Nak = *Nakpable*, Oss = *Ossoun*, Sow = *Sowaka*, Tch = *Tchalo*, Zan = *Zanti*.

Parts used: Baf = Bark of fruits, Bat = Bark of twigs, Fdl = Fresh and dried leaves, Frl = Fresh leaves, Fru = Fruit, Hea = Heartwood, Lat = Latex, Res = Resin, Rot = Roots, Sed = Seeds, Wpl = Whole plant.

Ethnic groups: Ade = Adja-Ewe, Ake = Akebou, Akp = Akposso, Ani = Ana-Ife, Aya = Ayanga, Bag = Bago, Kab = Kabye, Lam = Lamba, Mob = Moba, Tca = Tchamba, Tco = Tchokossi.

3.3 Plant Part Used in Traditional Cosmetic

The herbaceous are used in all their parts. However, the organs most used from ligneous families are the leaves (32%), fruits (18%), bark of the trunk (14%), followed by roots (11%) and stems (9%). The latex resins and flowers (4%) are very few used.

Out of the 75% species for which only one organ were used, this study detected 17% and 8% species used respectively for two and three organs. The leaves, bark and roots of *Ipomoea pes-caprae* (L.) R Br., *Mitracarpus scaber* Zucc and *Newbouldia laevis* (P. Beauv.) Seemann ex Bureau were used as cosmetic ingredients. These species were under intense exploitation and others purposes but with no attention of preparedness for its replacement. It confirms that the use of plants in cosmetic and different skin care

product by local communities was passed down from generation to generation [11].

Most of the plants, which are enlisted in Table 1, are widely used in the other parts of the world in similar or different ways. For example, *Cocos nucifera* L. [12] and *Hibiscus sabdariffa* L. [23] are widely used for cosmetic purposes. Some cosmetic properties of the plants recorded are unveiled by scientific findings and justifying their traditional uses [10, 23]. Many of the plants identified in this study are widely used as cosmetic and marketed internationally. Some examples include Anogeline from *Anogeissus leiocarpa* (DC.) Guill. & Perr., and Kigeline from *Kigelia africana* (Lam.) Benth.

3.4 Cosmetic Species Distribution in the Ethnocultural Groups and Ecological Zones of Togo

The spatial distribution of the cosmetic plant species according to the five ecological zones showed that zones I, II and III recorded the greatest number of species with respectively 73, 69 and 53 quotations. The number of quotations is very weak in the zone V with 19 quotations (Fig. 2). According to other authors, rapid modernization is causing old traditions to vanish [24-26].

These utilizations for cosmetic purpose in almost all the prospected ethnic groups are a fact of heritage knowledge and plant species availability around communities.

Certain species as *Elaeis guineensis* Jacq., *Vitellaria paradoxa* C.F. Gaertner and *Pterocarpus erinaceus* Poir. were recorded in at least four ecological zones. This fact attesting the majority uses into skin care product practices by people but also indicated that the cosmetic practices can be acquired by cultural knowledge mixing caused by promiscuity and contact between people. The CCA results confirmed that ethnic groups (A, B, C, D and E) who have similar traditional manner used practically the same plant species in cosmetics preparation (Fig. 3). The sophistical adjustment of the CCA findings by

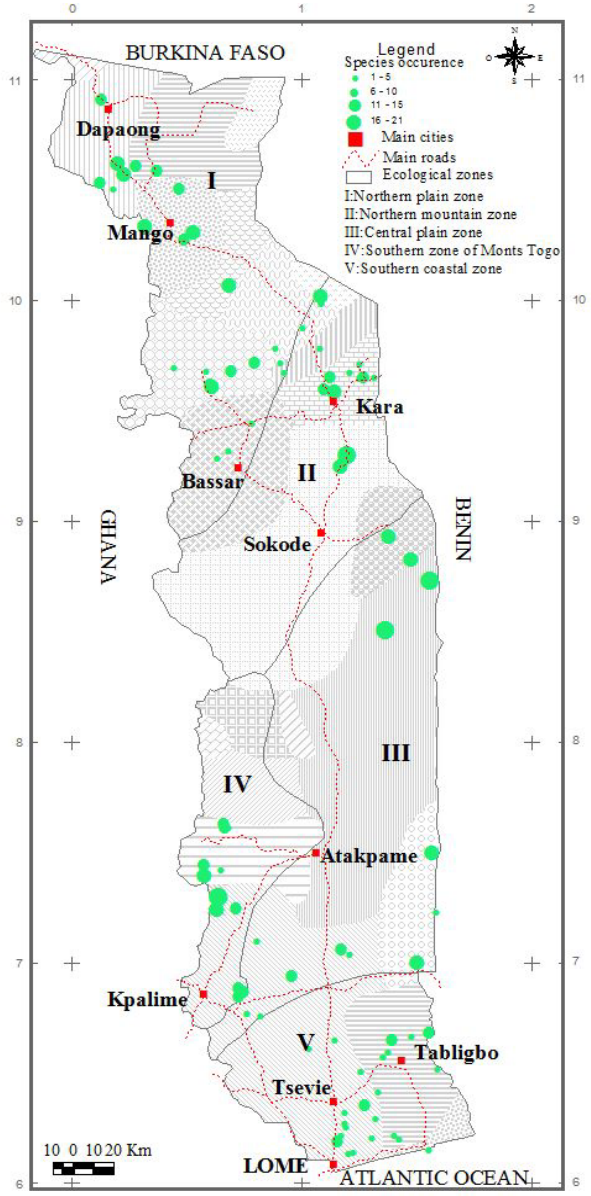

Fig. 2 Spatial occurrence of plant species in ethnic groups and ecological zones.

WAI based on plant species abundance in each ethnocultural groups classify three groups (A', B' and C') in Fig. 4. The canonical axis 1 explained 60.6% of the total variability in the species data than the canonical axis (43.8% for axis 2, and 43.2% for axis 3). The explanatory effect of the ethnic based-tradition was significant. It was confirmed by Monte Carlo permutation test estimating the significance level (P = 0.0020) after 499 permutations under split-plot constraints.

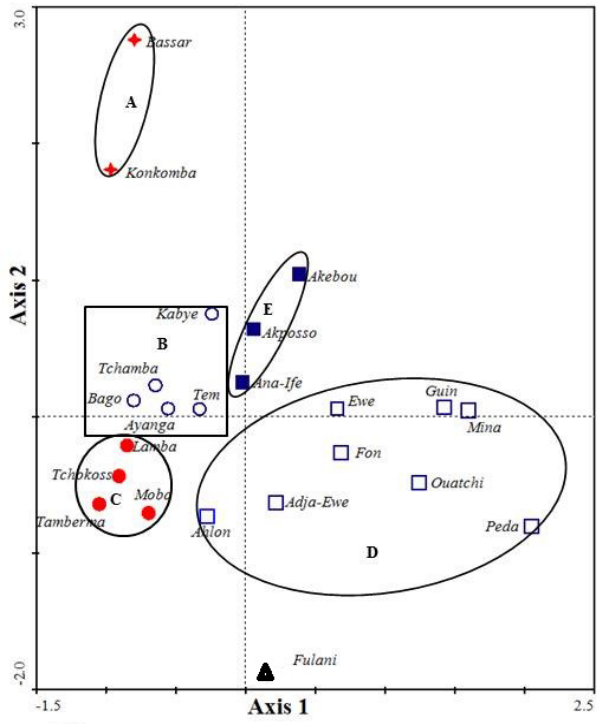

Fig. 3 Similarity between ethnic groups through Canonical Correspondence Analysis.

On the other hand, others plant species were relatively specific to only one ecological zone. It was the case of *Kigelia africana* (Lam.) Benth. in the zone V, *Harungana madagascariensis* Lam. Ex Poir. in zone IV, *Crateva adansonii* DC ssp. *adansonii* in zone III, *Colocynthis citrullus* (Flax.) O. Ktze. in zone II and of *Adenium obesum* (Forssk.) Roem & Schult., *Borassus aethiopum* Mart., *Detarium senegalense* J.F. Gmel. in zone I. Certain cosmetic plant species wer not used in some ethnic groups, for example in Konkomba and Moba groups. The evoked reasons were the totemic and magic considerations attributed to these species plant. Specially, *Sterculia setigera* Delile is considered by Moba and Konkomba groups as “devils tree”.

4. Conclusions

The study draws up the preliminary list of the plant species used in traditional cosmetic in Togo. It

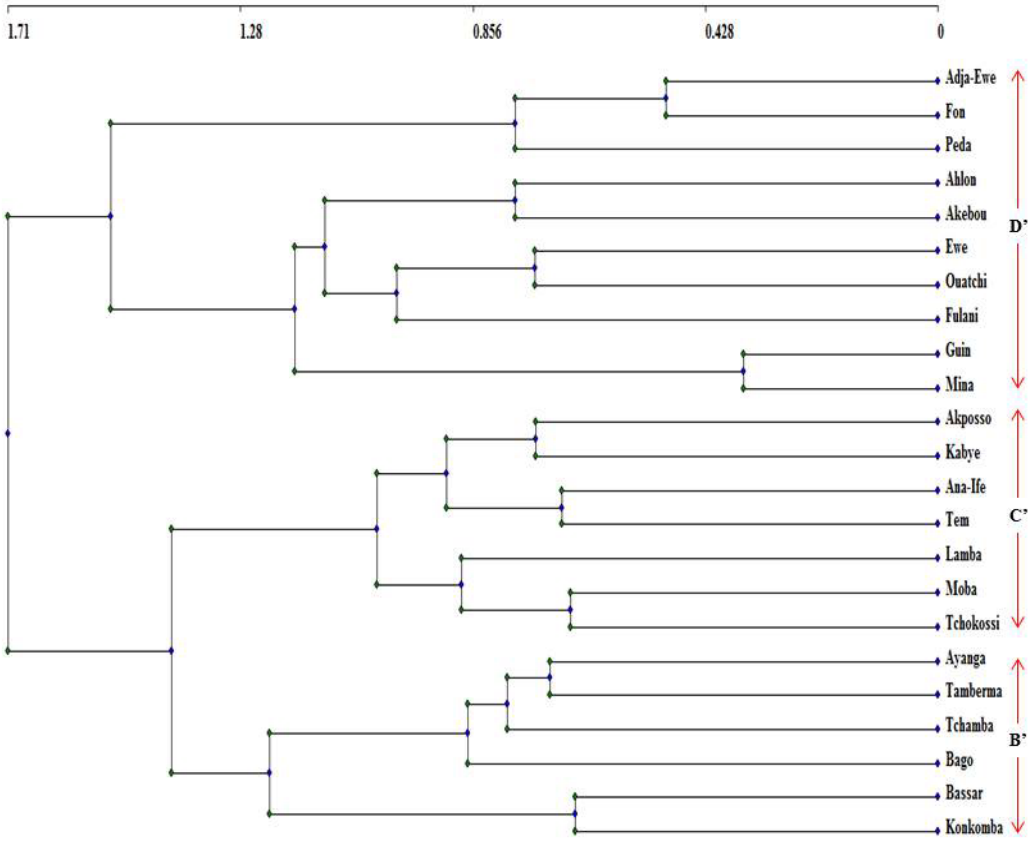

Fig. 4 Dendrogram adjusted by Whittaker’s Index of Association explaining similarity between ethnic groups.

indicated the interrelation between communities and forest genetic resources by associating traditional knowledge. This research unveils some new aspects of plant-based cosmetic. Undoubtedly forest resources in Togo can play significant contribution but effort should be made to inventory completely these resources which are rapidly decreasing.

It was found that cosmetic plant species tend to vary among different cultural groups for many reasons such as indigenous knowledge loss and intermingling factors. People are practicing skin care based on what they currently understand about the modern cosmetic products. Many plant species have become threatened due to habitat loss as result rapid urbanization and other anthropogenic factors. It suggests that these findings be incorporated into future biodiversity management and valorization plans.

However, in the context of biocultural heritage and the high interest of new cosmetic ingredients of industries, it is urgent to prevent biopiracy by associating local communities in problem formulation and decision making process. Then at local community level, these cosmetic plant species through capacity building can generate potential benefit.

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Agronomic Evaluation of New Cassava Varieties Introduced to Farmers in Nigeria

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Abstract: In 2005, 10 new cassava varieties were officially selected and released to farmers in Nigeria for high root yield, high dry matter content and acceptability for food. This work compared in three on farm locations, the root and stem yield of these varieties together with an old improved variety currently in farmers' field. In all the locations, root yield of all the new materials were significantly higher ($P < 0.05$) than the old improved variety. The root yields of the new varieties were between 40%-50% higher than the old improved varieties. Overall yield advantages of the new materials over the old improved material ranged between 40%-150%. Stem yield figures showed significant variations with few of the new varieties producing higher stem yield in comparison with the old improved variety due to inherent growth pattern. Figures for tuber girth and node numbers per unit of stem were similar. The high yield levels of the new varieties might lead to high demand for stems indicating the likely wider spread and higher demand for varieties with high stem yield potentials.

Key words: Cassava varieties, stem yield, root yield.

1. Introduction

The need to increase food production is always a priority in Africa. To feed the ever increasing urban population, food supply from every farm household has to increase by at least 63% in 10 years [1]. Cassava is a security food crop [2] because of its ability to grow under a wide range of conditions, some of which are quite unsuitable for other crops. In recent years, cassava production has doubled and tripled with nearly 90% of production when small holder farms cropped with earlier released improved varieties (TMS 30572,TMS 4(2)1425, TMS 30555) and other traditional low yielding (average of 11t/ha) cuitivars such as *odongbo, Ege dudu, idi leruwa* and *Isunikan kanniyan*. Farmers prefer the improved varieties because of their higher yields, earlier maturity and higher suppression of weed and greater resistance to diverse diseases and pests [3-5]. The increase in yield obtained from the earlier released improved varieties together with the recent cassava opportunities in the industries and new markets have led to expansion of cultivation under corresponding poor farm resource base with yields still remaining 60-70% below the potential of the crop. One of the ways of improving the productivity of primary production with no or low cash input within the same farm size is by introducing superior varieties with multiple advantages of higher yield, resistance and lower chance of having disease. In response to the renewed interest in cassava cultivation and entrepreneurship, ten new varieties of cassava were recently introduced to farmers after several on-farms testing in several locations [6]. While an improved cassava may perform excellently well in well managed on-farm trials, yield performance and other attributes may differ on farmer's field. The

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cultivation of improved cassava cultivars in Nigeria has been unequal, principally because of their levels of yield performance and age of maturity [3, 7].

Information on agronomic performance of most recently released varieties is needed to guide farmers and distribution agencies in improving productivity of cassava farms. Oto and Ham [7] compared the performance of early and recently improved cassava varieties and concluded that most recent releases such as TMS 50395 and TMS 30572 were doing much better in farmer's fields than earlier releases such as 60506 and TMS 30001. The extents of performance of the newly released varieties on farmer's field have not being fully documented. The agronomic performances of ten recently released cassava varieties in Nigeria in three farm locations were compared in this work.

2. Materials and Methods

2.1 Experimental Sites

Field equipments were carried out during 2007 and 2008 in Akure in the rainforest zone of south western Nigeria (latitude 7°30' N and longitude 3°52' E). There are two rainy seasons; one from April to July (early season) and the other from mid-August to November (late season.). December through February constitutes the major dry season. Average annual rainfall ranges between 1,000 mm and 1,200 mm. Annual average minimum and maximum temperature are 24.80 °C and 28.10 °C respectively. The mean relative humidity is 75%. The sites for the three experiments have been under continuous rotated cultivation for about 5 years. The soil is sandy loam, skeletal, kaolinitic oxic paluestalf.

2.2 Soil Sampling and Analysis

Soil samples were collected at 0-15 cm depth in each treatment plot one week after land preparation. Soil samples were air-dried and sieved using 2 mm sieve. The samples were subjected to routine chemical analysis as described by Tel (1984). Organic matter was determined using dichromate oxidation method. Available P was determined calorimetrically after Bray-P1 extraction. Exchangeable K, Ca, and Mg were extracted with ammonium acerate and determined using flame photometer and atomic absorption spec photometer respectively. Total N was determined by kjeldahl method [8].

2.3 Crop Establishment and Experimental Design

Ten newly released cassava varieties (treatments) in addition to an old improved variety (TMS 30572) were replicated three times in a randomized complete block experimental design with three replications. The compared varieties are TMS 98/0581, 99/0505, 98/0510, 92/0326, M98/0040, 95/0166, 95/0289 98/0002, 95/0166, 30572 and TME 419. The land was ploughed, harrowed and ridged mechanically. Freshly cut stems (20 cm) from a first ratoon crop from a multiplication farm were established 1 m × 0.8 m densities (12,500 plants/ha). Pre-emergence herbicide: (*Metalachor* 5 L/ha and *Glyphosate* 2 L/ha) was applied second day after planting. Two subsequent hand weeding were carried out at 14 and 28 WAP. Mature stems were harvested at 12 months after planting, 1 m long, and tied in bundles of 50 stems each. Roots were harvested at 12 months after planting.

2.4 Measurements

Initial plant height was determined at harvest on samples of ten uniform plants selected from three central row of each plot. Leaf area per plant was determined as described by Boardman [9]. Measurements were taken on number of 1 m length stakes per plot, number of node per 25 cm cutting, and number of main branches/plant. At harvest, tuber number per stand, tuber girth, single root weight and root yield (t/ha) were estimated. Harvest index (the efficiency with which assimilates are partitioned into the economically useful part of the plant) was estimated by the formula proposed by Butterworth (1994):

$$\frac{Economic\ yield}{Total\ yield} \times 100$$

2.5 Statistical Analysis

The data were analysed by separate two-way analysis of variance test. Means of the treatments were statistically compared by the least significant difference technique [11] at $P = 0.05$ levels.

3. Results and Discussion

3.1 Growth Parameters

Variability in most growth parameters figures (Table 2) of the cassava varieties were significant ($P = 0.05$). Taller varieties (TME = 419, 974763, 98/51, 98/0289 and 95/0166) had less branches than varieties with low plant height figures (98/0505, 98/0002, 92/0326). The number of nodes per 25 cm cutting was highest in shorter varieties.

The old improved variety (TMS 30572) had shorter internodes length and lower number of nodes per stem than most recently released varieties thus making up for height advantages of the new materials which are expected to translate more efficiently useful availability of stems in propagation. Internode lengths vary considerably depending on varieties and environmental conditions [12]. In all locations, attainable height of the new varieties were slightly lower than those described by IITA [6] as attainable varietal height for reasons attributable to possible difference in growth environment at various on farm trials. Various workers [13, 14] have reported varying growth figures from cassava grown in varying growth environment. The highly branched varieties (98/0505 and 95/0166) were comparable in branching with the old improved varieties. Varieties 98/0510, 98/0581 were moderately branching while TME419, 97/4763 were non-branching. Branching is a factor in varietal choice for crop mixtures; the non branching types (TME 419, 96/1642, 98/0581) are the most suitable for intercropping. Non-branching can also confer commercial advantage over some varieties which may be mechanical planter friendly being straight stems and well suitable for large scale pure stands. Though leaf area figures were similar among varieties, consistent lower figures were recorded for the reference

Table 1 Pre-planting soil properties in the three locations.

	Oba-ile	Ijapo	Ado-north
pH 1:2 (H_2O)	6.07	6.05	6.06
%N	0.08	0.06	0.06
%OM	1.24	1.22	1.21
%C	0.69	0.68	0.66
Av. P (mg/kg)	6.01	6.0	6.00
Na (cmol/kg)	0.07	0.07	0.17
K (cmol/kg)	0.30	0.32	0.40
Ca (cmol/kg)	1.10	1.09	1.10
Mg (cmol/kg)	0.71	0.72	0.70
H^+	0.11	0.12	0.10

Table 2 Growth parameters of newly released cassava varieties.

Varieties	Plant height (cm)			Length of Internodes(cm)			Number of nodes 25 cm cutting			Number of main branches			Leaf area (cm^2)		
	O	I	A	O	I	A	O	I	A	0	I	A	0	I	A
98/0581	230	225	220	2.3	2.0	2.5	12	13	13	0	0	0	32	33	31
98/0505	150	160	155	2.0	2.3	3.0	18	15	16	2.4	2.6	2.3	28	29	27
98/0510	198	190	190	3.0	3.1	3.0	10.5	11.8	11	2.8	2.4	2.6	42	41	40
92/0326	197	140	191	2.5	2.1	2.2	12	12	12.5	1.6	1.5	1.4	39	38	39
M/98/0040	199	190	254	2.2	2.2	3.2	18	16.2	17.2	0	0	0	39	36	43
95/0166	210	215	262	1.6	3.2	3.3	12	11	13.2	2.5	3.2	4.3	41	40	34
98/0002	189	193	191	2.3	3.2	2.1	13	14	12.2	0	0	0	42	39	41
95/0289	214	214	185	3.1	2.1	2.2	11.2	11.5	11.3	2.2	1.8	1.6	40	42	41
TME 419	316	318	310	5.0	5.1	5.5	8.0	9.7	9.0	0	0	0	39	38	37
96/1642	202	210	220	3.2	2.1	2.2	12	12	13	0	0	0	36	36	39
TMS 30572	160	188	194	1.6	2.2	1.8	12	12	12	3.2	2.5	2.8	31	30	33
LSD (5%)	18.9	21.3	18.2	0.4	1.2	0.8	6.5	4.2	6.1	1.3	1.05	1.4	ns	ns	ns

O = Oba-Ile, I = Ijapo, A = Ado North.

(TMS 30572) treatment.

3.1 Stem Yield

There were significant (P = 0.05) varietal response to stem yield parameters (Table 3) in all the three locations. Figures for number of 1 m long stem per crop stand were similar to the response obtained for plant height. Highest stem yield were obtained from TME 419, 97/4763, 95/0166, 95/0289 and 95/0510. TMS 30572, 98/0002, 93/0326 and 98/0040 produced relatively low stem yield. Though response pattern was inter location similar, stem yield was particularly higher for all varieties in Ijapo and Ado north thereby suggesting possible site controlled variability in stem quantity per unit farm land. IITA [6] reported an expectation of 350 bundles per hectare of stem yield. Eke-Okoro et al. [15] put expected stem yield per varying plant populations per hectare at between 400 bundles and 600 bundles. Generally, the new materials produced significantly more stem cuttings than the old improved variety.

3.2 Yield Parameters

In all the three locations, all recently released varieties had a significant number of tubers/plant, higher single root weight and total root yield than the

Table 3 Stem yield of newly released cassava varieties.

Varieties	Number of 1 m length cutting/stand			Cutting yield/ha (bundles)		
	O	I	A	O	I	A
98/0581	2.2	2.0	2.9	240	200	280
98/0505	2.0	2.3	2.8	180	160	160
98/0510	2.3	2.3	2.5	260	260	200
92/0326	1.6	2.0	1.8	120	200	260
M/98/0040	1.6	2.2	3.1	120	240	220
95/0166	1.8	1.9	2.1	260	180	220
98/0002	1.6	1.8	1.1	190	160	220
95/0289	2.5	3.4	2.3	300	380	260
TME 419	3.5	2.8	2.2	300	260	340
97/4763	3.1	3.2	3.4	320	340	380
TMS 30572	1.8	1.7	1.0	160	140	109
LSD (5%)	0.80	0.35	0.20	51.2	93.4	68.7

Table 4 Yield and yield parameters of newly released cassava varieties.

Varieties	Number of tubers/stand			Tuber girth(cm)			Single root weight(kg)			Tuber weight (t/ha)			Harvest index		
	O	I	A	O	I	A	O	I	A	0	I	A	0	I	A
98/0581	6.0	5.0	4.3	30	30	30	0.8	0.8	1.0	48	40	43	65	64	63
98/0505	6.4	6.5	7.0	30	30	30	0.8	0.7	0.9	51	46	63	63	63	63
98/0510	5.4	6.0	5.5	35	31	32	0.7	0.9	1.0	54	54	55	62	62	64
92/0326	5.2	3.0	4.0	30	31	31	0.6	0.8	0.6	31	27	24	63	63	62
M98/0040	4.0	3.0	4.0	31	31	32	0.8	0.9	0.9	32	27	36	63	62	63
95/0166	3.0	4.0	3.8	21	22	31	1.62	1.4	0.9	49	56	34	63	62	62
98/0002	3.0	4.0	3.0	21	25	32	0.9	1.0	0.6	27	40	24	63	63	63
95/0289	6.0	5.0	7.0	31	31	31	1.0	0.9	0.8	60	60	45	64	62	64
TME 419	3.0	5.0	3.6	31	28	25	1.9	0.8	1.7	56	40	40	62	63	63
96/1642	6.0	4.2	3.3	25	21	31	1.5	0.4	0.8	30	18	26	63	63	63
TMS 30572	4.0	3.8	3.2	28	30	32	0.6	0.6	0.8	24	23	26	64	64	63
LSD (5%)	2.2	1.6	1.5	ns	ns	ns	0.32	0.4	0.6	8.2	5.4	6.0	ns	ns	ns

old improved variety (Table 3). Among the new materials, 98/0581, 98/0505, 98/0510, 98/0166 and TME 419 produced tuber yield figure higher (between 40-60) than other newly released materials. The old improved variety (TMS 30572) ranked with 97/4673 and 98/0002 in performance. 98/0581 and 98/0510 were the most stable in consistency of higher yield in all the three on farm locations. In an on farm multi-locational trials, IITA [6] obtained yield levels reported in this study only in about 10% of the locations. Other locations recorded yield levels lower than the one reported in this study. This finding suggests that these varieties are likely to have varying adaptability strength to various ecologies and management. The old improved variety (30572) has been reported [16] to be the most stable high yielding varieties in Africa. More than 50% yield increase obtained from newer releases over the widely trusted old improved variety in unfertilised soils will push up demand for these varieties. Adekunle et al. [17] estimated the yield of 37 new varieties (including the varieties evaluated in this study) to be between 25-45 t/ha with 90% falling between the upper yield level.

Regardless of the yield levels of these varieties, final adoption by farmers depends on many other factors such as availability of stems, local presence and capacity of distributing agencies as a consistent source of planting materials to small holder farmers [3, 18]. The farmer's perception of benefits is not only based on superior yield figures alone but also on general economies of the varieties and local conditions [5]. The farmer has to make a personal choice of replacing old inferior varieties or to adopt a gradual supplementation (with new variety) strategy. This will be possible if intervention strategies are sustainable at all levels.

4. Conclusions

New cassava varieties can improve productivity of cassava farms by increasing root and stem yield with little cash and labour inputs. The wide range of materials is suitable for different cropping plans and different growth environments.

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Measuring Telomere Length in Proliferating Cells by Flow-FISH Method

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Abstract: The purpose of present work is a measurement of telomere length dynamic in proliferating cells *in vitro* by modified flow-FISH method. This method is a combination of two modifications: telomere length measurement in differentiated cells by surface antigen and analysis of cells divisions' number by vital dye dilution. Lymphocytes were activated by anti-CD3 Abs with IL-2 presents and grown *in vitro* for 7 days. Cells division's number was measured by dilution of CFSE vital dye which cells were stained previously activation. For telomere length measurement we used flow-FISH method with Cy3 labeled telomere PNH probe. Using this method we evaluated the dynamic of telomere length in $CD4^+$ and $CD8^+$ T-cells after 7 days culturing *in vitro* and revealed the difference in telomere lengthening and shortening versus division rounds in cell subsets. In $CD8^+$ cells telomeres start lengthen on a second division with the maximum on 4th division round becoming more that 20% longer compared with undividing cells. In $CD4^+$ cells telomeres did not have any length peculiarities through all division rounds demonstrating different telomere regulation in subsets. This probably occurs due to the higher level of hTERT protein expression in $CD8^+$ than $CD4^+$ cells do.

Key words: Cell senescence, telomere length, flow-FISH, CDSE, lymphocytes.

1. Introduction

Antigen-presenting cells process and present antigens to lymphocytes with specific T-cell receptors that are complementary to the antigen structure. During the immune response, T-lymphocytes undergo 15-20 division cycles that result in a 10^5- to 10^6-fold increase in antigen-specific lymphocyte circulating pool [1]. This proliferative "explosion" lasts for several days and should lead to rapid telomere contraction.

Telomeres are terminal chromosomal structures consisting of hexameric repeats (TTAGGG) that progressively contract in every round of DNA replication protecting chromosome ends from fusion and degradation. When telomeres reach a critical length the replicative senescence occurs and the cells lack the possibility to divide. Thus, telomere length is a marker of proliferative history that can be used to determine cell replicative history and proliferative potential [2]. Telomerase is the enzyme which synthesizes telomere repeats and counteracts telomere shortening. It is known that after *in vitro* activation the telomerase activity rises with a maximum activity level on a third day followed by the decline [3]. In some types of cells the telomerase acts as a reverse transcriptase [4, 5]. Previously we showed that the level of hTERT in T-cells subsets is associated with diversity in telomere elongation [6]. In the present work, we investigated changes in telomere length in lymphocytes during several cell divisions *in vitro* in response to polyclonal stimulation. It has been demonstrated that a modified flow-FISH method is useful for determining telomere length in cells with known number of cell divisions.

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2. Materials and Methods

2.1 Cell Isolation, Culture and CFSE Staining of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMCs) were isolated using LSM® Lymphocyte Separation Medium (MP Biomedicals LLC, UK) from 7 health human subjects. Then cells were washed in sterile phosphate-buffered saline (PBS) and then stained with carboxyfluorescein diacetate succinimidyl ester vital dye (CFDA-SE, CFSE) (Invitrogen, USA) according to manufacturer's protocol. Briefly, PBMCs were suspended in RPMI 1640 at a final concentration of 1×10^6 cell/mL and incubated with 2 μM CFSE for 15 min at 37 °C. Then cells were washed two times in RPMI with 10% FCS (HyClone, UK). CFSE-stained cells were plated at 1×10^6 cells/mL in a round-bottom 16-well plate in RPMI 1640 medium containing 10% FCS , 2 mM L-glutamine, 50 μg/mL Gentamicin, 25 μg/mL Tienam in the presence of plate-bound purified anti-CD3 mAb (BD Bioscience, USA) and 100 U/mL IL-2 (R&D systems, USA). PBMCs were collected after 7 days culture for further staining and flow cytometry analysis.

2.2 Murine Immature T Cells Isolation

Murine splenocytes were obtained from C57/Black mice ("Rassvet" mice breeding facility, Tomsk, Russia). These cells were used as an internal control and as a standard for hybridization experiments. Splenocytes suspensions were washed twice in PBS, 0.1% BSA (Sigma, USA), 0.01% EDTA (Sigma, USA), 0.1% NaN_3 (ICN Biomedicals Inc., USA) (PBSA), centrifuged and resuspended in FCS, 10% DMSO (ICN Biomedicals Inc., USA) and then was kept at – 80 ºC in small aliquots before being used.

2.3 $CD4^+$ and $CD8^+$ Lymphocytes Immunostaining

Collected 4×10^6 cells of cultured PBMCs were divided into two tubes, washed one time with PBS and resuspended in 100 μL of staining buffer. Murine anti-human CD4-biotin antibodies (Becton Dickinson, USA) were added to one of the tubes and anti-human CD8-biotin were added to another one. After 20 min of incubation cells were washed in PBS-A and stained with streptavidin-Cy5 (Amersham Bioscience, UK) for 20 min. After cells were washed one time and resuspended in 4 mM BS3 (Pierce, USA) and incubated for 30 minutes at 37 ºC. After the incubation 1 M Tris was added to stop reaction in a final concentration 20 mM for 15 minutes and cells were washed again in PBS-A.

2.4 Telomere Length and CFSE Fluorescence Measurement by Flow-FISH

Telomere length measurement was made by flow-FISH protocol described earlier [7, 8]. After immunostaining equal amounts of murine splenocytes (1×10^6) C57B/6 line were added in each tube with CSFC labeled PBMCs and precipitated. Then cells were resuspended in 600 μL of hybridization solution containing 70% formamide (Sigma, USA), 20 mM Tris (pH = 7.1) (Sigma, USA) and 1% BSA. Cell suspension was divided into two 1.5 mL tubes of 300 μL. One tube contained cells without PNA-probe (control tube) and one with Cy3-labled (CCCTAA)3 Peptide Nucleic Acid (PNA) (Eurogentec Ltd., Belgium), complementary to telomere sequence of DNA probe. The PNA probe was added in a final concentration 0.3 μg/mL. Samples were mixed and incubated at 80 ºC for 10 min. After that cells were incubated at RT for 3 hours in a dark place. After incubation the cells were transferred to flow cytometry tubes and were washed first in 70% formamide containing 0.1% BSA, 0.1% Tween-20 (Sigma, USA) and 10 mM Tris (pH = 7.1) and after by PBS-A containing 0.1% Tween-20. Then cell pellets were resuspended in 0.5 mL of PBS-A. Four-color flow cytometry was performed on FACS Calibur (Becton Dickinson, USA) using Cell QuestPRO software (Becton Dickinson, USA). Cell populations were gated first by morphology from side vs. forward

scatter distribution with followed Cy5-conjugated $CD4^+$ ($CD8^+$) FL4 channel discrimination (Fig. 1A and B). The number of cell divisions was detected by CFSE florescence on FL1 channel (Fig. 1C). After the gating on CFSE peak of interest the signal of telomere fluorescence Cy3-probe (FL2 channel) was determined as mean fluorescence intensity (MFI) of cells with PNA by substracting of the background MFI of cells without PNA. Relative telomere length was determined as a ratio of MFIs of a sample cells and murine splenocytes (internal standard) (Fig. 1D). To "normalize" the number of telomere ends per cell between human and murine cells containing a different number of chromosomes the normalization coefficient was used (0.913).

2.5 Intracellular hTERT Protein Level Measurement

The level of hTERT protein was measured according to Ased Ali [9]. PBMCs from donors were isolated and activated as described above. Every day a little quantity of cells was transferred from plate into tubes and washed with PBS-EDTA. Then 1×10^6 cells were stained in flow cytometry tube with mouse antihuman R-PE-labeled anti-CD4 or CD8 antibodies (Becton Dickinson, USA) and mouse anti-human APC-labeled anti-CD3 antibodies (Becton Dickinson, USA) for 20 min, at RT in the dark. Then cells were washed with PBS-EDTA and fixed with 1 mL 1%

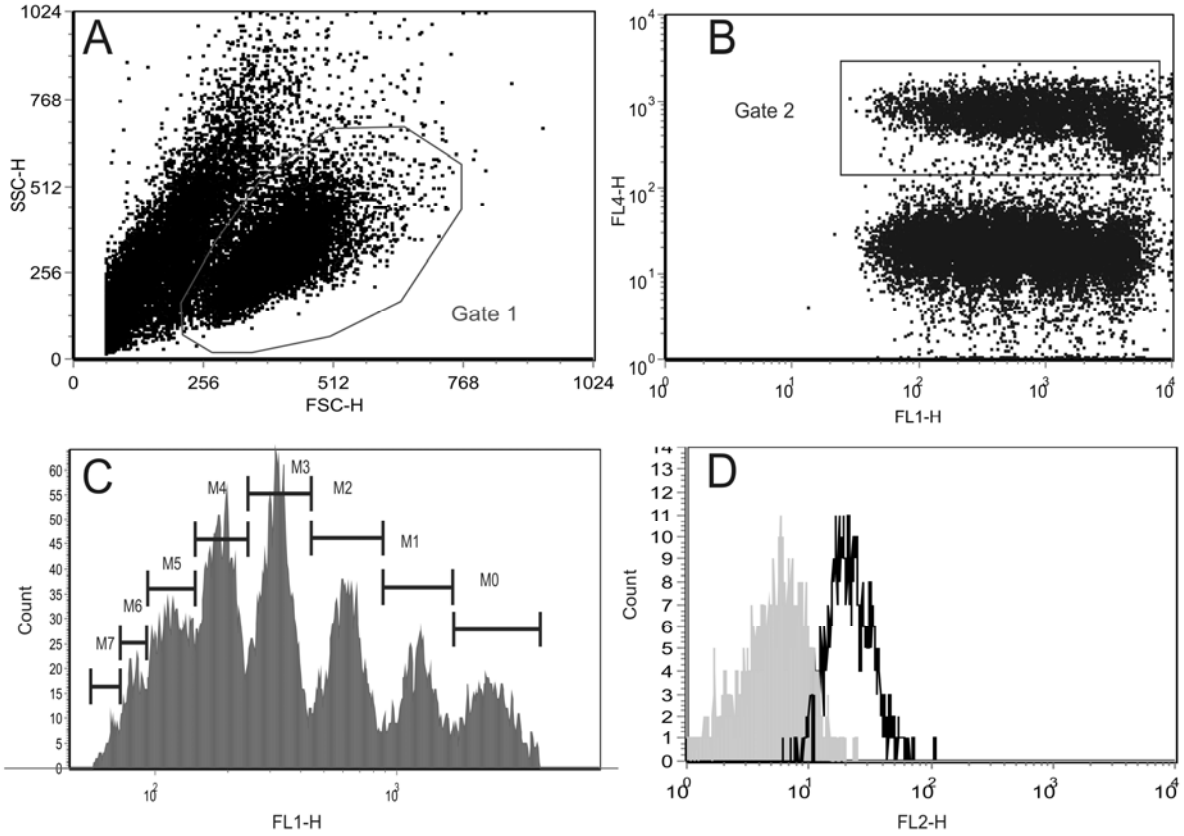

Fig. 1 The gating strategy for the measurement of telomere length in cells with known number of divisions. The analysis starts from side vs. forward scatter dotplot distribution of leucocytes where the lymphoid cells region is outlined and marked as Gate 1 (Fig. 1A). Then the dotplot distribution of CFSE fluorescence (FL1-H) of cells differentiated by surface antigen labeled anti-CD4 or CD8 antibodies-APC (FL4-H) is gated from Gate 1. In Picture C there is a histogram of CFSE fluorescence distribution gated from Gate 2. Each marker from M0 to M7 restricts cells with 0 to 7 rounds of made cells divisions correspondingly with followed gated fluorescence distribution of cells by Cy3 dye (FL2-H) (Fig. 1D). The filled gray histogram is the fluorescence distribution of cells without PNA telomere-specific probe; the empty black histogram is the distribution of cells with PNA-probe. Flow cytometry diagrams from one representative experiment are presented.

PFA 10 min in the dark. Permeabilization was performed with 1 mL of 0.2% Tween-20 in PBS for 10 min, at RT, in the dark. After the centrifugation cells were stained with first intracellular rabbit monoclonal antibodies anti-hTERT (Epitomics, #1531-1) according to the manufacturer's protocol for 20 min in the dark and washed with PBS-EDTA. Then cells were incubated with second goat FITC-labeled polyclonal anti-rabbit antibodies (Abcam, ab6717) for 20 min in the dark and then washed again with PBS-EDTA. After washing, PBS-EDTA was added in 0.5 mL volume and samples were analyzed by flow cytometry (FACSCalibur, Becton Dickinson, USA). Mean sample fluorescence was measured by subtraction the geometric mean fluorescence of cells incubated with second antibodies from the geometric mean fluorescence of the same cells incubated with both first and second antibodies. Results are presented in a format Mean ± SD.

2.6 Statistical Data Analysis

Differences between groups were analyzed with either Wilcoxon matched pairs test or Mann-Whitney U Test. $P < 0.05$ were considered as statistically significant. SPSS 13.0 (SPSS Inc., USA) and Microsoft Excel (Microsoft, USA) software packages were used for statistical calculation and data presentation.

3. Results and Discussions

In many studies it was shown that telomeres shortening occurs in somatic cells during cultivation in vitro. However a couple of original studies showed the telomere elongation in first week of cultivation [6, 10]. We decided to evaluate how telomere length is changing with each cell division round in T-cells subsets.

3.1 Modification of Flow-FISH Method

Flow-FISH technic is a generally used method for mean telomere length measurement and was described originally in several articles [7, 11]. Later this method was modified with surface antigen labeling to distinguish proper cells subpopulation [8, 12]. The cells proliferative activity by cells divisions' number was measured using vital dye dilution [13]. CFSE is one of the most popular cell tracers allows separate cells by the number of divisions undergone. Later CFSE was used in studying telomere length changes in dynamic of cell divisions *in vitro* [14]. We have jointed all these separate modifications in one flow cytometry analytical panel.

Standard flow-FISH protocol includes cells staining with DNA-specific dye. It is necessary to exclude cell aggregates and separate out cells in G0/G1-phase of cell cycle for analysis [11, 14]. But common used DNA dyes in flow-FISH protocol for telomere length measurement such as 7-aminoactinomycin D (Emission 647 nm), Ethidium Bromide (Emission 620 nm) and Propidium Iodide (Emisson 617 nm) are detected on both FL2 (filter: 585/42) and FL3 (filter: 670 fluorochrom LP) channels on FACSCalibure, BD. The fluorescence signal from telomere probe labeled Cy3 fluorochrom with emission 570 nm (615 nm) is detected on FL2 channel simultaneously with all mentioned DNA dyes so we cannot use ones in this protocol. Whereas we studied quite homogeneous population as lymphocytes the cells aggregates could be enough effective separated on forward vs. side scatter dot plot after is situ hybridization that consist of less than 1% (data not shown). Cells doublets give a double high level of telomere fluorescence and could be separated on histogram of telomere probe fluorescence. In regard to cells in S-phase which have a double high level of telomere fluorescence as well that was clearly shown by Hultdin et al., they could be discriminated on telomere probe histogram too [11]. It gives a possibility not to use DNA dye in our panel.

3.2 Proliferation Activity of T-Cells Subpopulations

All steps are described above in "Material and Methods" section. The vital dye was stable and it was

possible to identify CFSE proliferation peaks after 80 °C heating (Fig. 1C).

Before the measurement of telomere length we have found that the $CD4^+/CD8^+$ cell ratio has changed seriously from 1,085 before activation to 0.545 after one ($P < 0.01$, Wilcoxon matched pairs test). Using the CFSE cell tracer assay we found that after 7 days culture *in vitro* the cells of both $CD4^+$ and $CD8^+$ subsets underwent for up to 7 divisions (Fig. 1C). The detected proliferation intensity for $CD4^+$ and $CD8^+$ T-cells was different. $CD8^+$ T lymphocytes proliferated faster, and by day 7, most of cells had undergone at least 5 divisions (mitotic index 20.6% of all $CD8^+$ cells), whereas $CD4^+$ cells had undergone 3 divisions only (mitotic index 23.4% of all $CD4^+$ cells). These results confirm that $CD8^+$ T cells have faster proliferative response and it is the reason for $CD4^+/CD8^+$ ratio changes. For example, after bone marrow transplantation the recovery of T cell pool starts from $CD8^+$ T cells, which number is restored in several months, whereas the number of $CD4^+$ T cells reaches the normal level in 1-2 years [15].

3.3 Mean Telomere Length Measurement

When the mean telomere length was measured after one week cultivation the telomere elongation in both subsets was revealed. Before the activation, telomeres were 3.7% longer in $CD4^+$ cells than in $CD8^+$ cells, but the difference was not significant. Usually, the normal length of telomeres in the $CD4^+$ lymphocytes of humans is larger than in $CD8^+$ cells [16, 17]. In the present study, after 7 days of cultivation, this relationship was switched reversely: the average telomere length was 2.2% higher in $CD8^+$ cells than in $CD4^+$ cells. Notably lengthened telomeres were in both subsets and in $CD8^+$ cells it was significant.

3.4 Telomere Length Dynamic by Cell Divisions

Further analysis revealed that changes in telomere length started after the second cell division in both subsets (Fig. 2A). The maximum telomere length was detected after the third division for $CD4^+$ cells and after the fourth division for $CD8^+$ cells with following gradually contraction in both subsets. In $CD4^+$ cells the change in telomere length was not significant and did

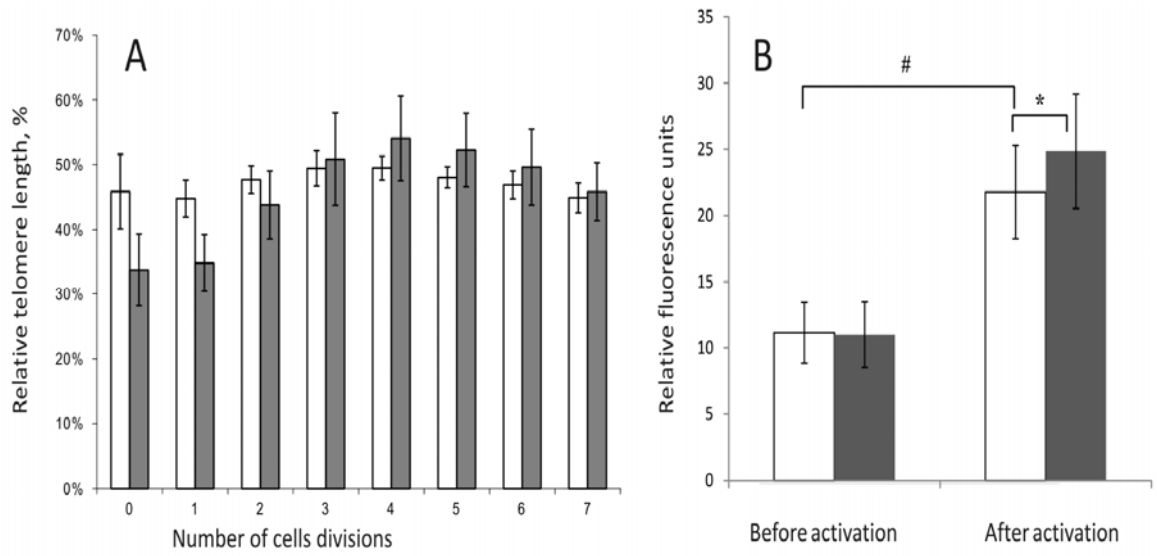

Fig. 2 Telomere length and hTERT protein level in lymphocytes before and after 7 activation. Picture A. The dynamics of telomere length in T-cell subsets by cell divisions number. White bars represents $CD4^+$ and gray bars $CD8^+$ T cells. Whiskers indicate standard error of mean. Mean data from 7 representative experiments is shown. Picture B. The rate of hTERT intracellular protein before and after activation *in vitro*. #: $P < 0.05$ (Mann-Whitney U Test) between hTERT level before and after activation in both subsets, *: $P < 0.05$ (Wilcoxon matched pairs test) between $CD4^+$ and $CD8^+$ subsets after activation.

not differ statistically between any divisions. In $CD8^+$ subset the cells with shortest telomeres were found in both group of undivided cells and cells undergone for just 1 division. $CD8^+$ cells undergone 2 or more divisions had significantly longer telomeres than $CD4^+$ cells even after 7 rounds of division.

3.5 hTERT Protein Level

To determine the telomerase activity which causes of telomere elongation, we measured the level of hTERT protein expression [18]. The expression increased with a maximum level on the 3rd day with further decrease each day thereafter. Therefore levels of hTERT protein in both subsets were measured before activation in vitro and on the third day after one. Without activation the detected hTERT expression level was the same in both subsets (Fig. 2B).

On the third day of cultivation the hTERT expression levels have been increased and it was substantially higher in $CD8^+$ cells than in $CD4^+$ cells, (before activation $CD4^+$ 11.12 ± 7.27 MFI, $CD8^+$ 10.97 ± 7.83 MIF and after one 21.77 ± 11.15 MIF and 24.85 ± 13.63 MIF, respectively, $P < 0.05$). It explains why the telomere elongation in $CD8^+$ cells is more intensive than in $CD4^+$ cells as was observed in our experiments after 7 days cultivation by flow-FISH.

4. Conclusions

Using the multi-color approach combining the CFSE-based proliferation assay together with flow-FISH method provides an opportunity to study telomere length dynamic in target cell subset. In present study we have showed different intensity of telomere elongation in $CD4^+$ and $CD8^+$ subsets owing to distinct telomerase activation level after polyclonal activation *in vitro*.

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Stability and Refolding of Prophenol Oxidase Protein with 2-Propanol in *Drosophila melanogaster*

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Abstract: Phenol oxidase in *Drosophila melanogaster* occurs as folded phase precursors designated as prophenol oxidase A_1 and A_3, and prophenol oxidase is activated with alcohol, especially 2-propanol, within a few minutes as unfolded-phase *in vitro*. To clarify a common effect of alcohols on proteins and peptides, the extract containing prophenol oxidase protein was prepared. Phenol oxidase activity activated with 2-propanol has been maintained stable at least 24 hours remains as it is. Protein of prophenol oxidase was not denatured opposite hypnoses known as the instability of protein with alcohol. Activated prophenol oxidase with 2-propanol remain enzyme activity with no aggregation, stable, renaturation, and the refolding phenomena occurred around the active phase within the catalytic active center of prophenol oxidase protein in *Drosophila melanogaster*. This study is important to induce the wide range applications of the effect in many fields for rational drag design.

Key words: Stability, 2-propanol, refolding, prophenol oxidase, *Drosophila melanogaster*.

1. Introduction

Insect phenol oxidase (designated tyrosinase in mammals) is a copper-containing enzyme that catalyzes two reactions: oxygenation of monophenol to *o*-diphenol and oxidation of diphenol to *o*-quinone using catechol and catecholamine as the substrates. These two reactions are key steps to synthesis of black pigment melanin. Detailed analyses has been performed extensively on the activation systems of prophenol oxidase (folding phase) with innate activating system, designated AMM-1, *in vivo* (unfolding phase) [1] and with organic cationic and anionic detergents, chymotrypsin, fatty acid, and various kinds of alcohol including 2-propanol as the induced fit type (refolding phase) *in vitro* [2]. Phenol oxidase activity (unfolding phase) was applicable to the Ping-pong model in *Drosophila melanogaster*. Purified fragment of prophenol oxidase shows homodimer *in vivo* and binds to di-copper bonds at the active center of the prophenol oxidase protein. The copper-binding active center with a short Cu(II)-Cu(II) distance of 2.9 Å is coordinated by the evolutionary highly conserved in the given prophenol oxidase protein in invertebrate species [3].

Effects of alcohols on proteins and peptides of prophenol oxidase have been studied [2], since these studies are important on the wide range applications of the alcohol effects on many fields [4, 5]. This application implies, focus, and new hypothesis on that a conformational change and refolding phase of prophenol oxidase protein without the previous showing danaturation of rigid native state proteins. This article shows and discusses the properties and effects on that prophenol oxidase became active and stable state in presence of the alcohol, especially 2-propanol, in *Drosophila melanogaster*.

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2. Materials and Methods

2.1 Flies and Chemicals

Late third instar larvae of the *Drosophila melanogaster* were reared on a standard cornmeal yeast medium at 25 °C. Laboratory strain, Oregon-R, was served as the standard. Pefabloc (4-(2-aminoethyl)-benzenesulfonyl fluoride, hydrochloride) SC (AEBSF) was purchased from Roche Diagnostics GmbH, (Mannheim, Germany), dopamine was from Nakarai Tesque Inc., (Kyoto, Japan). Pefabloc SC from Boehringer Mannheim Biochemicals, GmbH (Mannheim, Germany), Protein Assay Kit was from Bio-Rad Laboratory (Hercules, CA), FPLC system from Pharmacia LKB Biotechnology (Uppsala, Sweden).

2.2 Preparation of Prophenol Oxidase

The following procedures were performed at 0-4 °C, unless otherwise specified. Centrifugation was performed at 30,000 *g* (16,000 rpm), for 2 min using a Sakuma M-150 (Tokyo, Japan). Prophenol oxidase was collected in the supernatant after centrifugation and used as the starting material. The larvae were homogenized with sample buffer containing 25 mM Tris-HCl, 5 mL 2-mercaptoethanol (Nakarai Tesque, Inc., Kyoto, Japan), 10 mL glycine, 2 g sodium dodecyl sulfate, bromophenol blue (Nakarai Tesque, Inc., Kyoto, Japan), powder and distilled water up to 50 mL by glass bar. After centrifugation at 16,000 rpm, for 2 minutes, at 4 °C using a Sakuma Model M-150 (Tokyo, Japan), supernatant preparation including prophenol oxidase A_1 and A_3 was collected for characterization.

2.3 Activation of Prophenol Oxidase, Phenol Oxidase Activity, and Circular Dichrois Spectroscopy

Activation of prophenol oxidase with 50% 2-propanol and determination of the phenol oxidase activity were performed as described in our previous report [1]. Dopamine in 200 mM Tris-maleate buffer, pH 6.0 at room temperature was used as the substrate of phenol oxidase. Circular dichroism (CD) spectra of prophenol oxidase protein with 50% 2-propanol had been measured in the range of 195-245 nm as the previous paper [6]. Distilled water against 2-propanol was used as the control. Five times were run and calculate the average values.

2.4 Refolding Reaction

Refolding reaction of prophenol oxidase protein was performed after our previous paper [2]. 50% 2-propanol, dopamine in 200 mM Tris-maleate buffer, pH 6.0 at room temperature, Spectrophotometer using a Hitachi U-2000 (Tokyo, Japan), block-heater, using a Dry Thermo Unit, Taitec (Tokyo, Japan) were used for this research.

3. Results and Discussion

3.1 Stability of Prophenol Oxidase Protein with 2-Propanol

Suspend including prophenol oxidase was activated with 50% 2-propanol with small amounts of Pefabloc powder to inhibit the activation of prophenol oxidase with innate activating factor, AMM-1. Phenol oxidase activities were detected at least 24 hours after addition of 50% 2-propanol to prophenol oxidase protein at both temperatures (Fig. 1). After incubated these samples at 4 °C or 30 °C, value of phenol oxidase activity was measured at both temperatures. Special phenomenon was that no agglutination and polymerization was occurred against the commonsense protein agglutination with alcohol [4]. These data will be applied to make new medicine prevent amiroid-agglutination in the Altsheimer disease.

Values of phenol oxidase activity calculated by spectrophotometer showed approximately same as 0.20 between 4 °C and 30 °C, or without 2-propanol (control) and with 2-propanol. The values of the phenol oxidase activity tend to present higher with time, however, no statistical differences with or without 50% 2-propanol were shown. These chain

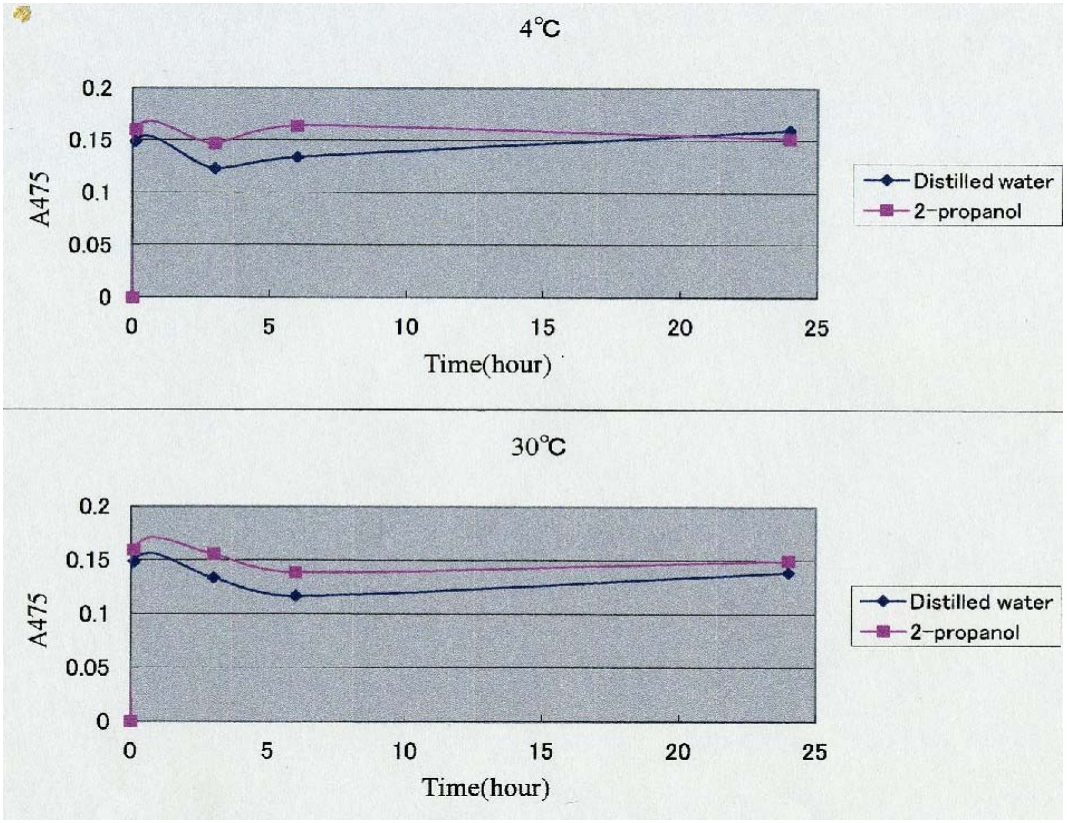

Fig. 1 Change in rate in activation of prophenol oxidase (folding phase) with 50% 2-propanol and phenol oxidase activity was determined after incubation with 10 mM dopamine as the substrate in *Drosophila melanogaster*. Distilled water against 2-propanol was used as the control. Activation velocity is fast (unfolding phase), maintained at least 24 hours the enzyme activity showing the prophenol oxidase protein is stable. Data point are at 4 °C (above) and 30 °C (below). Dots indicate the average values (n = 5).

reactions can be attached enzyme to the Ping-pong Bi Bi reaction. The reason why the effect is no statistical difference between distilled water and 2-propanol is not clear. The data shows that prophenol oxidase protein is not unstable but stable with alcohol, especially 2-propanol. Since the authors' previous study presented activation of prophenol oxidase with alcohols, methanol, ethanol, 1-butanol, 2-butanol, 1-propanol, 2-propanol, and glycerol unless melanization in the solution [1, 6], the stability of prophenol oxidase protein with 2-propanol is gives emphasis. No significant evidence was detected showing the oxygenase activity during time in this study.

3.2 Structural Redundancy at the Active Center of Prophenol Oxidase Protein

Depend on the Ribbon-model of higher-order structure and CD spectra of prophenol oxidase cleared the secondary structure at the active center of prophenol oxidase protein, that is, the active center was located almost not at the peripheral site of the protein, but at the central site of the protein molecule of prophenol oxidase. The significance of the presence of di-copper was estimated as the strong magnetic binding with the two oxygen-binding Cu(II) atoms separation by 2.9 Å [3] surrounded by α-helices and three His residues per single Cu(II) atoms (total six His residues, Fig. 2). After addition of 2-propanol to prophenol oxidase protein, redundancy of the secondary structure, especially α-helix, was occurred (unfolding phase) immediately, substrate can enter from outside to the active center by the narrow canal of prophenol oxidase protein. The chain reaction can be attached enzyme to the Ping-pong Bi Bi model in enzyme reaction.

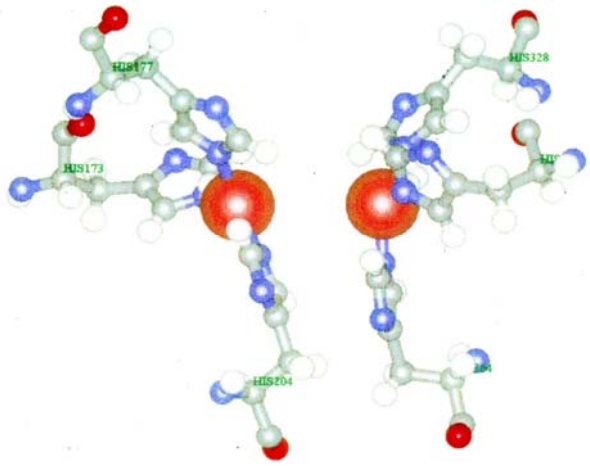

Fig. 2 Refolding phenomena of prophenol oxidase protein activated with 2-propanol. Activation velocity of prophenol oxidase (unfolding phase) is fast, however, reactivation velocity after dilution is slow like the Origami model.

3.3 Refolding of Prophenol Oxidase Protein with 2-propanol

After our previous study [7], effect of several ionic concentrations from 0 to 200 mM KCl on the specific activity of phenol oxidase was examined using FPLC gel filtration chromatography. Considering the salt concentration in *Drosophila* hemolymph, the results indicate that prophenol oxidase exists as a homodimer *in vivo*, and the higher-order structure (folding phase) of prophenol oxidase can be activated and changed the structure fast velocity (unfolding phase), then reversibly slow velocity (refolding phase) by change salt concentration used *in vitro*.

At a temperature of 80 °C, prophenol oxidase was partially denatured, however, it showed reversible recovery (refolding phase) by renaturation (refolding phase) at 30 °C. The prophenol oxidase protein involved in 2-propanol would offer an ideal system for studying the difference of higher-order structures between active and inactive states in the insect phenol oxidase. The diversification of melanin particle process in *Drosophila melanogaster* reflects evolutionary change, biodiversity, is primarily driven by effect of mutations and stochasticity [8] (Fig. 3).

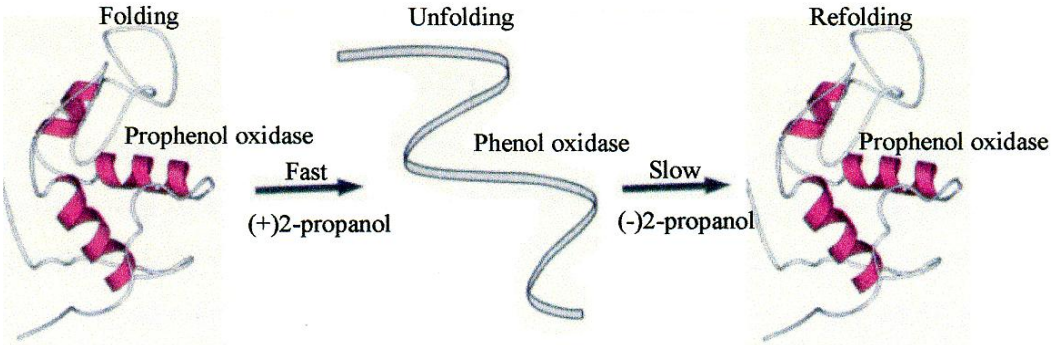

Fig. 3 Ribbon model diagram at the active center of prophenol oxidase A_1 isoform in *Drosophila melanogaster*. Dicopper, Cu(II), domain of prophenol oxidase is shown to be sorrowed by six His residues at the center. Helical region is omitted in this figure.

The protein of prophenol oxidase shows reversible dissociation and reassociation in the higher-order structure. These reactions can be applied the Origami (Japanese typical paper craft) model and the Anfinsen dogma on behalf of β-lacto albumin to prophenol oxidase, and of 2-mercaptoethanol to 2-propanol. In this study, velocity dimension was first discussed on refolding of the protein.

4. Conclusions

After prophenol oxidase activated with 2-propanol, the protein was not unstable, but stable until 24 hours and maintained the phenol oxidase activity. These findings are consistent with the Origami model and useful in designing new medicines to prevent protein agglutination in Alzheimer's disease.

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Evaluation of Native Strains of *Isaria fumosorosea* (Wize) Against *Anastrepha ludens* (Loew) (Diptera: Tephritidae)

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Abstract: We evaluated the pathogenicity of six strains *I. fumosorosea* to determine their potential as biological control agents of *A. ludens* by performing bioassays under laboratory conditions (25 ± 2 °C, 60 ± 5% R.H and 12:12 h L: D) exposing larvae and pupae to a concentration of 1×10^8 conidia per milliliter using three different methods: direct spraying, spray of soil and submerged for both instars. The results showed that direct spraying method Pfr-612 strain showed the highest mortality (47%) in pupae while for larvae was strain HIB-27 to 46%. Regarding spray of soil Pfr-612 strain showed the highest percentage of mortality for pupae and larvae with 45 and 57% mortality, respectively. Finally, for the submerged method in pupae HIB-27 strain showed 62% mortality whereas in larvae HIB-32 strain showed 46%. These results indicate that *A. ludens* is susceptible in at least two instars to *I. fumosorosea* and make this fungus a promising agent for control of the Mexican fruit fly.

Key words: *A. ludens*, biological control, entomopathogenic fungi, *I. fumosorosea*.

1. Introduction

The Mexican fruit fly *Anastrepha ludens* (Loew) (Diptera: Tephritidae) is a pest that seriously affect fruit production in Mexico and other countries Neotropical. This insect is considered one of the main pests that affect fruit crops worldwide [1]. EI damage is caused by larvae feeding on the pulp are deteriorating galleries fruit and allowing the invasion of other organisms such as fungi and bacteria that increase the damage by rot, which causes premature decline and loss of fruit. In Mexico, *A. ludens* is distributed mainly in the production areas of mango, orange, guava, apple and peach, covering 49.75% of the country [2].

A. *ludens* is capable of causing direct damage estimated at 30% loss in production, marketing and hinder due to the imposition of strict quarantine barrier [3]. The battle of the fruit flies in Mexico is conducted through various methods with a trend toward integrated pest management, with the primary methods of pest control are the legal, mechanical, cultural, insect technique sterile, chemical and biological [4]. Chemical control is based on the use of bait insecticides to control adults and land application of insecticides to kill larvae and newly emerged adults, but this type of control causes high pollution and mortality of beneficial insects [5-7].

Furthermore, the use of biological control offers alternatives to avoid side effects by use of chemical agents. Biological control of tephritid pest has been primarily through mass releases of parasitoids *Diachasmimorpha longicaudata* (Ashmead) (Hymenoptera: Braconidae) and *D. tryoni* (Cameron) (Hymenoptera: Braconidae) [8, 9]. So far, the documentation of the activity of entomopathogenic fungi against *A. ludens* in Mexico, little being reduced

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to the evaluation of *Metharizium anisopliae* strains (Metschnikoff) Sorokin [10, 11], however, there are no reports documenting the implementation of methods to assess the pathogenicity of isolates of other genera and/or species of entomopathogenic fungi against the different stages of *A. ludens* in the laboratory, therefore the objective of this study was to determine the toxic activity of native strains of *I. fumosorosea* in two stages of *A. ludens.*

2. Materials and Methods

Obtaining insects: The Mexican fruit fly, *Anastrepha ludens*, used for these bioassays was originated from our colony maintained in insect breeding area at the Institute of Biotechnology of the UANL, in Nuevo León, Mexico on artificial diet under laboratory Conditions at 25 ± 2 °C, 60 ± 5% R.H and 12:12 h L:D.

Activation of the strains: We used native strains of *I. fumosorosea* isolated from citrus-growing areas in Mexico (HIB-27, HIB-28, HIB-29, HIB and HIB-30-32) and Pfr-612 from the collection of the Institute of Biotechnology, FCB-UANL, stored in cryogenic state (10% glycerol at – 80 °C). The strains were thawed at room temperature were then inoculated as a striated in potato dextrose agar (PDA) and incubated at 28 °C for 14-21 days.

Preparation of conidial suspension: After incubation time for each strain was added 10 mL of bidistilled water with 0.01% Tween-80 and adjusted to a concentration of 1×10^8 conidia/mL in a Neubauer chamber.

Preparing the soil: Different soil samples were sieved and sterilized, placing 200 g in plastic containers of 1,000 mL with 75% humidity.

Bioassays: Three methods were used for the treatment of larvae and pupae:

Direct spray: We sprayed directly the pupae and larvae with the conidia solution (1×10^8) corresponding to each strain then larvae placed in containers with sterile soil.

Submerged: Consisted of immersing larvae and pupae for 30-35 seconds in the solution of conidia (1×10^8) then filtering the suspension with a thick paper towel, then placed in containers with sterile soil.

Spray of soil: Sterilized soil was sprayed with the conidia solution (1×10^8) corresponding to each strain. Then add larvae and pupae of *A. ludens* to each container with soil.

Incubation of bioassays: There were 15 repetitions for each of the treatments included a control with 0.01% Tween 80 and an absolute control. All containers, once covered were reversed, and remained at 25 ± 2 °C for three days [12] to allow *A. ludens* become infected with fungi [13, 14]. After this time, larvae and pupae were extracted and placed in Petri dishes (60 × 15 mm) in a chamber at 26-28 °C and a relative humidity of 60-65% for 7 days to encourage the development of mycosis and check the mortality due to fungus. Reported the final mortality rate in percentage for each of the entomopathogenic fungi.

3. Results and Discussion

3.1 Bioassay with Pupae

For mortality of pupae of *A. ludens* strain that produced the highest mortality was strain HIB-27 (62%) by the method of submerged, whereas the remaining strains obtained values ranging between 22% and 47%. For soil spraying method, the strain was highest death rate was the Pfr-612 strain (45%), while the other strains yielded rates below 50%. For the method of direct spray the strain obtained pupae increased mortality rate was Pfr-612 (47%), while the other strains ranged between 24% and 34% (Table 1). The minimum values for bioassays with pupae of *A. ludens* were recorded in controls (absolute = 0%, Tween 80 = 15%).

3.2 Bioassay with Larvae

In the bioassay by the method of spraying soil treatments evaluated caused an average mortality of 20-57% for larvae of *A. ludens* while Pfr-612 strain

Table 1 Mortality of pupae of *Anastrepha ludens* caused by strains of *Isaria fumosorosea* under laboratory conditions (25 ± 2 °C, 60 ± 5% R.H and 12:12 h L:D).

Strain code	Methods (percentage of final mortality)		
	Direct spray	Spray of soil	Submerged
HIB-27	34	44	62
HIB-28	30	32	31
HIB-29	29	35	33
HIB-30	24	34	22
HIB-32	30	26	22
Pfr-612	47	45	41
Absolute control	0	0	0
Tween 80 control	0	0	0

produced the highest mortality (57%). On the other hand in the bioassay with the direct spray method larvae HIB-27 strain caused higher mortality (46%), whereas the remaining strains showed lower values from 40 to 42%. Finally in the bioassay method of submerged larvae, HIB-32 strain showed higher mortality (46%) while the average mortality caused by the different treatments evaluated are presented in the range of 27-40% (Table 2). The minimum values for larval bioassays were recorded in controls (absolute = 0%, Tween 80 = 0%).

In the bioassays described in this paper the results show that *A. ludens* is susceptible in its larval stage to *I. fumosorosea*. A study reports that in evaluating seven different strains of entomopathogenic fungi on *Ceratitis capitata* Wiedemann (Mediterranean fruit fly) found that *M. anisopliae* and *Paecilomyces fumosoroseus* (= *I. fumosorosea*) were the most pathogenic fungi with LD_{50} values of 5.1 and 6.1×10^3 conidia/fly, respectively [15]. Also to evaluate extracts was reported that *M. anisopliae* was more toxic, with a 90% mortality at a concentration of 25 mg of diet and also fertility was reduced to 94 and 53% respectively. Furthermore in other study evaluated the susceptibility of *A. ludens* to *Beauveria bassiana*, *Metarhizium* spp. and *P. fumosoroseus* with conidial suspensions at a concentration of 1×10^8, finding that *Metarhizum* spp. 100% of the pupae subjected to bioassay showed invasion of the fungus, while in the adult stage bioassays was observed in individuals infected by hyphal growth of the fungus on the cuticle of the same [16] consistent with other similar studies who reported that at a concentration of 10^8 CFU/mL showed a cumulative mortality of larvae and pupae of *A. ludens* in a range of 37.5-98.75% [11].

Table 2 Mortality of larvae of *Anastrepha ludens* caused by strains of *Isaria fumosorosea* under laboratory conditions (25 ± 2 °C, 60 ± 5% R.H and 12:12 h L: D).

Strain code	Methods (percentage of final mortality)		
	Direct spray	Spray of soil	Submerged
HIB-27	46	42	33
HIB-28	27	38	33
HIB-29	42	34	37
HIB-30	42	20	27
HIB-32	40	38	46
Pfr-612	41	57	40
Absolute control	0	0	0
Tween 80 control	0	0	0

4. Conclusions

According to the results need to be continued at laboratory bioassays to determine the appropriate concentration that causes mortality of larvae and pupae of *A. ludens*, and the method of bioassay and the use of native isolates of entomopathogenic fungi for control of flies Mexican fruit.

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