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Contents

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Research Article

Physiological responses to excess boron in wheat cultivars

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ABSTRACT

This study investigates the response of two wheat cultivars to boron toxicity stress. Plants were cultivated in sand culture and boron was applied to the culture for 10-day. Symptoms, tiller number, boron concentration, soluble sugars, proteins and other free amino acids than proline were studied. The differences between the cultivars were apparent from higher boron and the chlorosis in tolerant cultivar was about 7% compared to the sensitive one 70%. Tiller number gradual decreased in tolerant-cultivar, while in sensitive one a dramatic reduction was exhibited by increasing boron level in culture media. In most boron levels, although the accumulation of soluble carbohydrates was significantly stimulated in shoot of B-sensitive cultivar (Gemmeza 9; S), there were no appreciable differences in the production of carbohydrates in shoot of B-tolerant cultivar (Sakha 93; T). However, the soluble proteins production did not affect by most boron levels in both cultivars. The presence of boron at various concentrations induced a production of free amino acids in shoots of each of the two test cultivars. Tiller number (yield index) decreased in the two test cultivars and was in range 50-59 and 84-92% less than control plants for tolerant and sensitive cultivar, respectively.

Keywords: Amino acids; Boron; Pigments; Soluble carbohydrates; Soluble proteins.

1. INTRODUCTION

Boron (B) is well documented as an essential micronutrient for optimum growth of vascular plants. However, when B is present above the permissible limit in the soil or ground water, plant growth and reproduction can be affected, limiting crop productivity throughout the world [1, 2]. Boron toxicity is extensively located in the agricultural areas of Australia, North Africa, and West Asia characterized by alkaline and saline soils together with a low rainfall and very scare leaching. In addition to this, B-rich soils also occur as a consequence of over fertigation and/or irrigation with water containing high levels of B [3, 4].

Negative impacts of excess B involves many developmental/biochemical processes in plants such as altered metabolism [5, 6], reduced activity in photosynthetic process [7], reduced root cell division [8], reduced shoot cell wall expansion [5] and generation of reactive oxygen species (ROS) followed by oxidative damage [9, 10]. Reid et al. [2] also demonstrated that excess B impairs the tolerance to photo-oxidative stress.

Boron is unique as a micronutrient: it has restricted mobility in many plant species while it is freely mobile in others [11]. Boron mobility within

plant parts determines the visible symptoms of B excess: in plants with low B mobility, the typical symptoms are chlorotic and/or necrotic patches (burn) of the older leaves where B tends to accumulate [12]. Differently, in plants with high B mobility the symptoms of B toxicity firstly appear in meristematic regions and in fruits, but not in mature leaves [13]. Moreover, B toxicity can affect crop productions through the reduction of leaf expansion, photosynthetic efficiency and fruit set [12]. The ability to restrict B uptake into the plants can minimize the physiological impairments caused by B toxicity [12, 14]. On the other hand, an inherent ability to tolerate excessive B concentration in plant tissues [15] or the differential antioxidant response that may reduce B-toxicity damage in some species [10] was suggested.

We are studying the toxicity effects of B on wheat cultivars and have earlier shown that Sakha 93 the most B-tolerant, out of five, test cultivars and Gemmeza 9 as the most B-sensitive one [16]. Thus, in this investigation it seemed necessary to consider some physiological and biochemical responses of the selected cultivars and how far these responses are correlated with the B-tolerance mechanisms at different B levels. Particular attention was focused to investigate the correlations among the B, toxicity symptoms, yield index, soluble carbohydrates, soluble proteins and other free amino acids than proline concentrations in the plant tissues of both the cultivars.

2. MATERIALS AND METHODS

2.1. Plant material, growth conditions, and treatments

Seeds of B-sensitive (Gemmeza 9; S) and B-tolerant (Sakha 93; T) cultivars of wheat (*Triticum aestivum* L.) were sterilized and grown in sand culture in 10 cm diameter plastic pots lined with polyethylene bags [16]. Fifteen grains were grown in 0.7 kg air-dried and cleaned quartz sand, which was kept at approximately 100% of the field capacity by watering with B-free distilled water and left for germination in a greenhouse under natural light. After 10 days, 5 seedlings were selected on the basis of vigor and uniformity, the undesired seedlings were removed. Then, boron stress treatment was initiated by applying Nable's solution [17] containing boric acid (H_3BO_3) to the seedlings, the pH was buffered to pH 5.7. The seedlings were grown in final B concentration of: 1, 3, 6, 8 and 10 mg kg^{-1} soil for ten days for vegetative growth and 35 days for tiller stage. Each pot represents as experimental unit with 5 plants per treatment; each treatment was replicated six times. The samples were collected: roots and shoots separated, washed with deionized water, weighed, frozen in liquid nitrogen, and stored at -80^{o}C and some samples were oven-dried at 70^{o}C for 48 hours.

2.2. Plant extraction

Shoot extractions were prepared using 50 mM potassium phosphate buffer (pH 7.0) containing 0.1 g polyvinylpyrrolidone (PVP) and used for the determination of soluble carbohydrates, soluble protein and other free amino acids than proline.

2.3. Soluble carbohydrates

Phosphate buffer extraction was mixed with anthrone reagent [18, 19]. The samples were placed in a boiling water bath for 10 min. The light absorption of the samples was determined spectrophotometrically at 625 nm. A calibration curve using pure glucose was constructed.

2.4. Soluble proteins

Proteins in the extract were estimated by Folin Ciocalteau's reagent [20]. The absorbance of color was measured using a spectrophotometer at 750 nm. A calibration curve was constructed using bovine serum albumin (BSA).

2.5. Amino acids

Other free amino acids than proline were determined using ninhydrin [21] and were measured using a spectrophotometer at 570 nm. A calibration curve was constructed using glycine.

2.6. Boron concentration

For boron concentration measurement, 0.01 g (DW) shoot samples were dry ashed in a muffle

furnace at 500ºC for 6 h. The ash was then dissolved in 0.1 N HCl and B was determined colorimetrically at 540 nm by the curcumin method [22].

2.7. Statistical analysis

All measurements were taken in independent 6 replications. The data given in all figures represent means ± SE. Statistical assays were carried out by using ANOVA (completely randomized) to determine if significant differences were present among means. Duncan's test was carried out to determine if mean difference significant at $P \leq 0.05$ (SPSS-11).

3. RESULTS

The response of plants to toxic levels of B has received renewed interest of late. There is a wealth of information about the effects of B toxicity on the biomass parameters and the metabolic response of plants corresponding to respective B concentrations in the growth medium. This large set of data was difficult to treat and present in full and thus simple two-dimensional parameter correlations are presented and discussed in the text below.

The main concern is that B is mainly transported *via* the transpiration stream and accumulated in leaves, whereas B cannot be remobilized in wheat plants. Therefore, all the studied parameters have done on leaves of two wheat cultivars (Gemmeza 9 and Sakha 93, the B-sensitive and B-tolerant cultivars, respectively).

3.1. Correlation between B and toxicity symptom

The symptoms reflect the distribution of B in most species with B accumulating at the end of the transpiration stream. The current study showed that wheat is very sensitive to excess B and has a relatively low B-demand during vegetative growth, accompanied by a high susceptibility to B toxicity. When the B concentration in soil was exceeded 1 mg B kg^{-1} soil characteristic symptoms of B toxicity appeared (Fig. 1). The first sign of B toxicity was yellow-green chlorosis, which first developed on the oldest leaves and progressed to the youngest. Later, small patches of necrotic tissue appeared. In Gemmeza 9, the B-sensitive cultivar, the chlorotic symptoms appeared 3 days after treatments, while in Sakha 93, the B-tolerant cultivar; symptoms appeared 7 days after treatments.

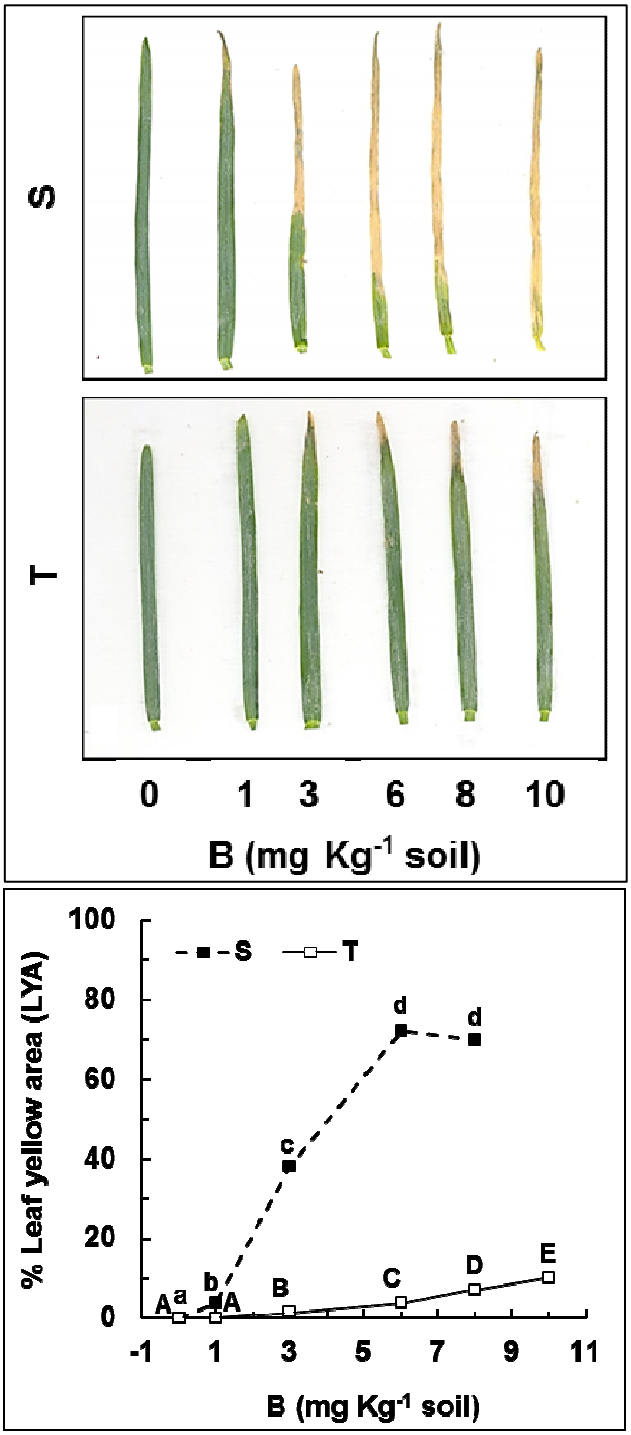

Figure 1. Dependence of leaf symptoms of 20-day-old Gemmeza 9 (S) and Sakha 93 (T), the B-sensitive and B-tolerant wheat cultivars, respectively, as affected by different boron levels in sand soil for 10 days. Yellow areas are expressed in terms of % of the leaf area. Data points represent mean ± standard error (n = 6).

3.2. Correlation between B content and tiller number

Special emphasis was laid on the influence of B toxicity stress on tiller number (yield index) of the two test wheat cultivars. In this respect, considerable differences were induced by the various levels of B (Figs. 2 and 3). The results presented in Figure 2 reveal that the tiller number of 35-day-old plants was markedly affected by B level. Tiller number decreased in the two test wheat cultivars (sensitive and tolerant) as B level increased. At 1, 3, and 6 mg B kg^{-1} soil the reduction in tiller number of Gemmeza 9 was quit pronounced (50%, 84%, and 92%, respectively) as compared with control. However, the decrease in tiller number of Sakha 93, the B-tolerant cultivar, at 3 and 6 mg B kg^{-1} soil B level amounted to about 50% and 59%, respectively as compared with control.

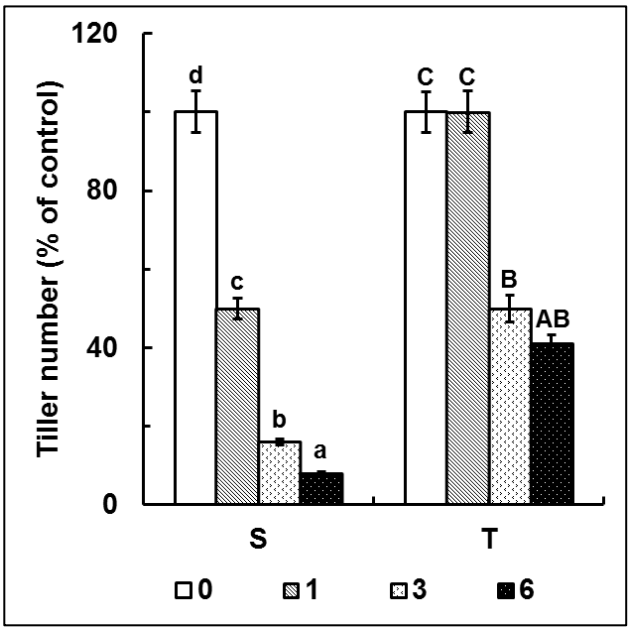

Figure 2. Tiller number (% of control) of 35-day-old of Gemmeza 9 (S) and Sakha 93 (T), the B-sensitive and B-tolerant wheat cultivars respectively, supplemented with different boron concentrations. Data points represent mean ± standard error (n = 6).

The data in Fig. 4 clearly demonstrated that B concentration in shoots of Sakha 93 and Gemmeza 9 increased gradually with the rise of B level in the soil. It is worth to mention that significant differences in B concentrations in shoots of Sakha 93 and Gemmeza 9 cultivars were manifested. At low B concentrations (1, 3 mg B kg^{-1} soil) Sakha 93 had B concentrations in shoots from 25-to 29% greater than in sensitive cultivar Gemmeza 9, while at higher levels (6, 8 mg B kg^{-1} soil) Gemmeza 9 had B concentrations in shoots from 33.5 to 55% greater than in Sakha 93.

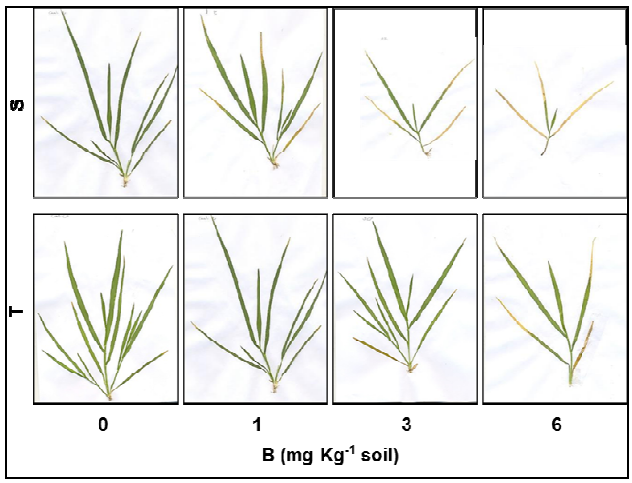

Figure 3. Tillering of 35-day-old of Gemmeza 9 (S) and Sakha 93 (T), the B-sensitive and B-tolerant wheat cultivars respectively, supplemented with different boron concentrations.

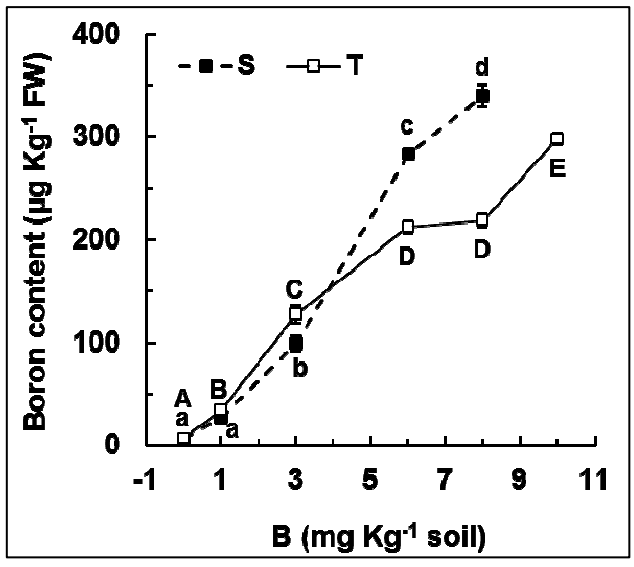

Figure 4. Shoot boron contents of 20-days-old Gemmeza 9 (S) and Sakha 93 (T), the B-sensitive and B-tolerant wheat cultivars respectively, as affected by different boron levels in sand soil for 10 days. Data points represent mean ± standard error (n = 6).

3.3. Correlation between B and soluble metabolites

The response of the tissue soluble sugars, proteins and amino acids concentrations toward B accumulation was variable in shoots of the tolerant cultivar and sensitive one (Fig. 5).

Although the accumulation of soluble carbohydrates was significantly stimulated in shoot of Gemmeza 9 with the increase of most B level, there were no appreciable differences in the production of carbohydrates in shoot of Sakha 93 at most B levels (Fig. 5A).

The successive increase in B concentration did not induce a stimulatory effect on the accumulation of soluble proteins in the shoots of the two test cultivars, except at level 6 mg B kg^{-1} soil, which was of a stimulatory effect on the synthesis of proteins in shoots of Sakha 93 (Fig. 5B).

The results presented in Figure 5C clearly demonstrate that the presence of B in the culture media at various concentrations induced appreciable induction in the production of other free amino acids than proline in shoots of each of the two test cultivars.

4. DISCUSSION

4.1. Correlation between B and toxicity symptom

The common feature of tolerant cultivars was that the B concentrations in their tissues were lower than in sensitive cultivars. From this, it was hypothesized that the tolerance trait was associated with an ability to restrict B uptake from the soil into the roots, thereby reducing transfer to the shoot [23]. The first visible result obtained in this experiment was the different sensitivity to B stress shown by the two wheat cultivars evaluated. In fact, marginal portion of leaves exhibited evident and marked symptoms of damage in Gemmeza 9, whereas these were scarce in Sakha 93. These symptoms which represent the general symptoms of B toxicity reflect the distribution of B in most species, with B accumulating at the end of the transpiration stream [24, 25]. Kohl and Oertli [26] demonstrated that B uptake followed the passive water flux from roots to leaves accumulated especially where termination of leaf veins terminate; these tissues show more evident symptoms of B toxicity such as chlorosis and necrosis. According to Shelp [27] higher B concentrations were found in leaf tissues than in phloem sap.

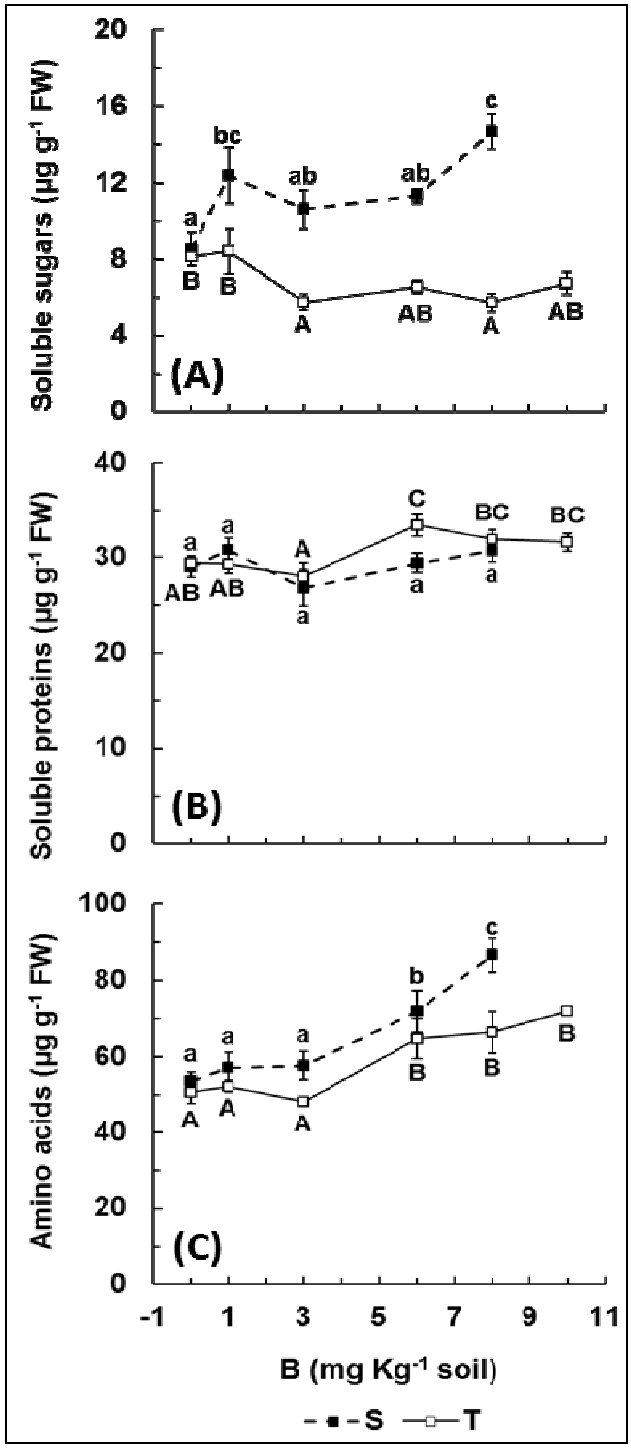

Figure 5. Soluble sugars (A), soluble proteins (B) and amino acids (C) concentration in shoot of 20-day-old Gemmeza 9 (S) and Sakha 93 (T), the B-sensitive and B-tolerant wheat cultivars respectively, as affected by different boron levels in sand soil for 10 days. Data points represent mean ± standard error (n = 6).

4.2. Correlation between B content and tiller number

Growth and yield were reported to be limited in all cases where plants were grown under root zone conditions of high B [28]. It is known that presence of high amount of B in irrigation water and soil adversely affects plant growth and yield production in different cereal plants [16, 29] due to its ease in absorption and mobility within plant cell/tissues [12, 30].

Tillering or vegetative branching is one of the most important components of shoot architecture in cereals because it contributes directly to grain yield [31, 32] and is involved in plant plasticity in response to environmental cues and stresses [33, 34]. In this study, the tiller number of both test cultivars decreased with increasing B level in the soil. This decrease was more evident in the sensitive cultivar where the B of only 1 mg kg^{-1} soil decreased the tiller number to about 50% of the control weight. B toxicity impacts heavily on wheat production in Australia (up to 11% yield reduction in affected areas [35], and breeding for tolerance in wheat is of high importance across southern Australia.

Species and genotypes susceptible to B toxicity generally have higher concentrations of B in leaves and shoots than do tolerant genotypes [36]. Gemmeza 9 is more susceptible to B toxicity and accumulates more B in its shoots than Sakha 93, the B tolerant cultivar. Working on several barley and wheat genotypes, Nable [17] reported that the most susceptible genotypes to excess B accumulate more B than tolerant genotypes. Some authors recorded a range of genotypic variation in response to B toxicity with mechanisms including B exclusion [37, 38] and an inherent ability to tolerate excessive B concentration in plant tissues [15]. It was observed that the B-tolerant barley cultivar Sahara 3771 has the capacity to maintain much lower B concentrations in roots as well as in xylem and leaves [14], for which the authors propose a mechanism of active efflux of the borate anion.

4.3. Correlation between B and soluble metabolites

Boron plays a key role in sugar transport and carbohydrate metabolism [39]. Our results demonstrate that B toxicity resulted in increased soluble carbohydrates in the shoot of Gemmeza 9. Under similar conditions there was no appreciable change in soluble carbohydrates in Sakha 93. The carbohydrate accumulation seems to be related to limitation of its use rather than increase in its synthesis. Cervilla et al. [10] reported that B-toxicity increased glucose, fructose and sucrose contents in the leaves of two tomato (*Lycopersicon esculentum*) cultivars (‘Josefina’ and ‘Kosaco’). Pérez-López et al. [40] reported that the accumulation of sugars in plants under stress conditions might be involved in the osmotic adjustment. However, the protective role of sucrose could be explained as a compatible solute, protecting structure of membranes [41].

The results of the present work demonstrate that B failed to induce appreciable variations in the production of soluble proteins in shoots of the two test cultivars. This result agree with the results reported by Reid et al. [2] who demonstrated that neither photosynthesis, respiration nor protein synthesis was particularly sensitive to B. Also, Uluisik et al. [42] showed that boron treatment does not change the expression pattern of most of the ribosomal protein genes.

Amino acids acts as a putative osmo-protective solute leading to lowering osmotic potential in several tissues exposed to stress [43]. In our experiment, both wheat cultivars subjected to high B toxicity (up 3 mg/kg soil) showed a significant increase in the content of amino acids. This observation is in accordance with that of Gopal [44] who indicated that application of 10 ppm B in sand culture was highly injurious to groundnut plant. The chlorotic leaves showed decreases in protein-nitrogen and considerable increase in soluble-nitrogen, contents of aspartic acid, glutamic acid, glycine and alanine. Also, Kaya et al. [45] suggested that B stress induces amino acid synthesis or activates the general amino acid control mechanism.

5. CONCLUSIONS

In conclusion, *T. aestivum* accumulates B in the oldest leaves and progresses to the youngest. The differences between the cultivars were statistically apparent only at 3 mg kg^{-1} soil levels of B

accumulation. Tiller number decreased in the two test cultivars and was found in the range from 50-59 and 84-92% less than control plants at 3 and 6 mg B kg^{-1} soil for tolerant and sensitive cultivars, respectively. The soluble carbohydrates and proteins concentrations did not affect by B accumulation, except for the carbohydrates of the sensitive cultivar where the concentration was stimulated. Both wheat cultivars subjected to B up to 3 mg kg^{-1} soil showed a significant increase in the content of amino acids.

AUTHORS' CONTRIBUTIONS

AMM and AMH conceived and designed research. AMM and RME conducted experiments. AMM, RME and AMH analysed data and wrote the manuscript. All authors read and approved the final manuscript.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

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Research Article

Nodulation and nitrogen fixation of some wild legumes from differing habitats in Egypt

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ABSTRACT

This study was devoted to exploring the natural nodulation and nitrogen fixation of wild legumes grown in different Egyptian habitats. These habitats are representative to four phytogeographical regions. Sites that inhabited by *Melilotus indicus*, *Medicago polymorpha*, *Trifolium resupinatum*, *Trigonella hamosa* and *Vicia sativa* in each region were selected for study. High nodulation, nitrogen fixation and plant biomass were recorded in plants grown at Nile region and Oases compared with those at Mediterranean region and Sinai. The inhibition in nodulation and potential of nitrogen fixation in legumes at MR and S were attributed to drought and low soil fertility. Differences in species, regions or their interaction have significant effect on nodulation, legheamoglobin, nitroginase activity and biomass of nodules, shoots and roots; the magnitude of effect due to different species was the greatest. Five rhizobial isolates (*Sinorhizobium fredii*, *Rhizobium mesosinicum*, *Rhizobium daejeonense*, *Rhizobium huautlense*, *Rhizobium alamii*) recovered from root nodules of the five species were identified by 16S rRNA gene sequence. The indigenous rhizobia of legumes grown at MR and S expected to be exhibit higher tolerance to the existing harsh environmental conditions. These rhizobia can be used as inoculants for crop legumes under unfavorable environmental conditions of agroecosystems or recently reclaimed desert.

Keywords: Nodulation; Nitrogen fixation; Wild legumes; Legheamoglobin; Rhizobia.

1. INTRODUCTION

The leguminous plants constitute one of the largest families of the flowering plants, consisting of ca. 730 genera and ca. 19,400 species [1]. It is an extremely diverse family with worldwide distribution, encompassing a wide range of life forms, from arctic alpine herbs and temperate or tropical perennial shrubs to annual xerophytes and equatorial giant trees [2].

Legumes play a vital role in agro-ecosystems based on their ability to form a symbiosis with soil rhizobia that fix atmospheric nitrogen [3, 4]. Biologically fixed N_2, either asymbiotic, associative, or symbiotic, is considered a renewable resource that should constitute an integral part of sustainable agro-ecosystems globally [5, 6]. *Rhizobium* spp. are Gram-negative soil bacteria that have a profound scientific and agronomic significance due to their

ability to establish nitrogen-fixing symbiosis with leguminous plants, which is of major importance for the maintenance of soil fertility [7, 8]. Nitrogen fixation by legumes, play an important role in sustaining crop productivity and soil reclamation of the semi-arid areas [9, 10].

It is well documented that wild leguminous plants inhabiting any region show adaptability to the environment and fix the atmospheric nitrogen more efficient than the cultivated legumes in that region [11-13]. Little information has been reported on natural nodulation of wild legumes [14-16]. One fascinating application of rhizobia of wild legumes is using it as inoculums for crop legumes. Many reports proved that some rhizobia of wild legume are more efficient in nitrogen fixation activity with other hosts than their compatible hosts [17, 18]. It has been reported that cross inoculation of crop legumes with rhizobia isolated from wild non-crop legumes enhanced nodulation and nitrogen fixation [19].

Nodule formation and nitrogen fixation of legumes are strongly affected by sub-optimal soil conditions, such as temperature extremes, salt stress, high or low soil pH, low water content, pesticide application and nutrient deficiency [20]. Environmental differences also strongly affect demographic processes like germination and seedling recruitment, which in turn affect genetic differentiation among plant populations [21], often due to random processes such as founder effects or genetic drift [22]. Hence, the hereditary structure of any plant species reflects its interaction with the environment [23]. Genetic diversity is strongly influenced by reproductive mode and mating system [24, 25]. In addition, genetic diversity is assumed to increase with abiotic and biotic heterogeneity and in stressful environments [26, 27]. Thus, ecological components such as temperature and precipitation also affect genetic diversity [28, 29]. The ability of species to respond to changes in the environment will ultimately determine survival in a particular habitat [30, 31].

Egypt extends across large areas of land comprising several geographical regions differ topographically, climatically and environmentally in general. Egypt is the meeting point of floristic elements belonging to at least four different regions: the African Sudano-Zambezian, the Asiatic Irano-Turanian, the Afro-Asiatic Saharo-Arabian, and the Euro-Afro-Asiatic Mediterranean [32]. Egyptian farmers facing a problem in providing crops with required nutrients, especially nitrogen, due to inadequate supply of mineral nitrogen fertilizers or the costs of these fertilizers. However, there is an urgent need to find alternatives on the base that soil fertility strongly depends on metabolic activities of microbes. Efficient symbiotic nitrogen fixation reduces the level of the requirement for external input of mineral nitrogen fertilizers. As nodule activity is known to vary diurnally and seasonally, also nodulation and nitrogen fixation of wild legumes could be varied depending on their habitat. Hence, improving our knowledge around the ecological distribution of wild legumes is a topic of utmost importance to better understand how to preserve it, increase their import and select the most efficient nitrogen-fixing wild legumes. Therefore, the present research aimed to study the biodiversity and biogeography of rhizobia associated with some wild legumes and their ability to fix atmospheric nitrogen.

2. MATERIALS AND METHODS

2. 1. Soil sampling and analysis

Soil samples from each site at four phytogeographical regions were collected for the physical and chemical analyses. Three soil samples were collected from profiles of 0-50 cm depth, pooled together to form a single composite sample, and carried to the laboratory in plastic bags. The samples were then spread over sheets of paper and left to dry in the air. Dried soils were passed through a 2 mm sieve and packed into paper bags for analysis. Soil texture was determined according to Allen et al. [33], organic matter according to Walkley and Black [34], soil sodium and potassium according to Williams and Twine [35], calcium and magnesium according to Johnson and Ulrich [36], chlorides according to Hazen [37], sulphates according to Black et al. [38], phosphates according to Woods and Mellon [39], bicarbonates according to Piper [40], nitrate according to Markus, McKinnon and Buccafuri [41], electric conductivity according to Jackson [42]. The pH value and soil water content also were determined.

2.2. Vegetation sampling and preparation

The current study was carried out along two successive years 2010-2011. The studied stands were chosen at locations inhabited by five wild nitrogen fixing legumes namely: *Melilotus indicus* (L.) All., *Medicago polymorpha* L., *Trifolium resupinatum* L., *Trigonella hamosa* L. and *Vicia sativa* L. One site at each of four Egyptian phytogeographical regions was chosen for this study: Assiut site in NR (27º 08' N, 31º 20' E) Al-Kharga site representing Oases (25º 32' N, 30º 37' E), Burg Al-Arab site in MR (30º 57' N, 29º 37' E) and Saint Katherine site in South Sinai (28º 33' N, 33º 56' E). At each site three individuals (as replicates) from the same population of every plant species were collected, and separated into roots and shoots. The shoots and roots were washed several times with distilled water, blotted gently with filter paper and were quickly weighted for fresh biomass (FW) determination and oven-dried at 70 ^{o}C for 48 hour to determine the dry biomass (DW).

2.3. Assessment of nodulation

Nodulation was assessed by up-rooting the plant, washing away adhering soil particles, and counting the number of nodules present. Nodules fresh and dry biomasses were determined. Some nodules from another individuals were also detached to check for the presence of red pigment (leghaemoglobin).

2.4. Determination of leghaemoglobin in nodule cytosol

One gram of fresh nodules was rinsed thoroughly with distilled water and immediately hand ground in an ice chilled mortar with 5 ml of distilled water. Nodule homogenates were filtered through four layers of cheesecloth and the filtrate was centrifuged at 500 x g for 2 min to remove nodule debris. The resulting supernatant was centrifuged at 12,000 x g for 15 min to sediment the bacteroids. Leghaemoglobin levels in the supernatant, the 'nodule cytosol', were determined colorimetrically as described by LaRue and Child [43], using Unico UV-2100 spectrophotometer. The colorimetric assay was standardized using freshly preapaerd Hemetrol reagent (solution of cyanmethemoglobin titrated exactly according to recommendations of BioMerieux, Marcyletoile, 69260 Carbon nieres les Bains, France)

2.5. Determination of nitrogenase activity

Nitrogenase activity was determined in detached roots, using gas chromatograph as described by Abd-Alla [44], (Thermo Scientific TRACE GC Ultraequipped with FID detector and Capillary column CP-PoraBOND Ufused silica plot 25 m × 0.32 mm, df = 7 m). The excised nodulated roots were placed in 500 ml bottles sealed with a rubber septum. 50 ml of air were taken and the same volume of acetylene gas introduced into the bottle, incubated at 37 $^{\circ}$C then samples from root atmosphere in bottles were with-drawn and injected to the gas chromatograph. Afterwards nodules of each individual root were counted and nodules fresh and dry mass were estimated. A calibration curve was constructed using pure ethylene.

2.6. Determination of proline content

Free proline was determined in fresh tissues according to method of Bates, Waldren and Teare [45], shoots samples (30 mg) were homogenized in 6 ml 3% sulfosalicylic acid, then filtered through filter paper. After addition of acid ninhydrin and glacial acetic acid, the resulting mixture was heated for 1 h in water bath at 100 $^{\circ}$C. The reaction was stopped by using ice bath. The mixture was extracted with toluene and mixed vigorously. The chromophore containing toluene was aspired from the aqueous phase and the absorbance measured at 520 nm. Proline concentration was determined using calibration curve.

2.7. Isolation of rhizobial strains

Twenty representative sites inhabeted by the studied species (one site for each species at each of the four phytogeographical regions) were chosen for rhizobial isolation. The strains of rhizobia were isolated from root nodules of *M. indicus, M. polymorpha, T. resupinatum, T. hamosa and V. sativa.* The isolates were grown on yeast extract mannitol

agar (YEMA) medium and incubated at 28 °C [46] on an orbital shaker at 120 rev per min for three days.

2.8. Molecular identification of rhizobia

From bacterial cultures using SDS/CTAB lysis and phenol/chloroform extraction method Ausubel et al. [47], DNA extracted sent to South Korea, Solgent Co., Ltd Bio industry Development for PCR-amplified at using primer pairs 16S (1492R 5' TACGGYTACCTTGTTACGACTT 3' and 27F 5' AGAGTTTGATCMTGGCTCAG 3'). The sequence reads were edited and assembled using BioEdit version 7.0.4 (www.mbio.ncsu.edu/ BioEdit/bioedit.html) and clustal W version 1.83 (http://clustalw.ddbj.nig.ac.jp/top-e.html). BLAST searches were done using the NCBI server at www.ncbi.nlm.nih.gov/blast/Blast.cgi. The rhizobial isolates identified and recoded in GenBank.

2.9. Nucleotide sequence accession numbers

The nucleotide sequences of the rhizobial isolates namely, *Sinorhizobium fredii, Rhizobium huautlense, Rhizobium daejeonense, Rhizobium alamii, Rhizobium mesosinicum* were deposited in the GenBank nucleotide sequence database under accession number [GenBank: KF879914.1, KF87 9916.1, KF879917.1, KF879920.1, KF879921.1, respectively].

2.10. Statistical analysis

Data were subjected to statistical analysis using SPSS package (version 19). One-way ANOVA, followed by Duncan multiple range test were employed and the differences between means deemed to be significant at $p < 0.05$. Correlation analyses were carried between soil variables and some parameters estimated in plants. Factorial ANOVA was carried to achieve the effect of species, regions and their interaction on different parameters estimated in plants and η^2 was calculated as: $\eta^2 = SS_{between} / SS_{total}$.

3. RESULTS

3.1. Soil

The data recorded in Table 1 revealed that there are significant differences between the physical and chemical properties of soils of the four phytogeographical regions. Soil of NR was characterized by the highest values of water content, K^+ and organic matter (OM), while soil of MR was characterized by higher content of HCO_3^- . Compare to other regions, soil of Saint Katherine (S) have high concentrations of Na^+, Mg^{+2} and SO_4^{-2} , and hence the TSS in the soil was high. The soil of the NR and O were rich in NO_3^-, while the soil of the NR and MR were rich in PO_4^{-3}. The pH value of MR and S soil was high as compared with the other regions.

3.2. Nodulation

The present study proved that the nodulation differ significantly among the studied plant species and as affected by the habitats at the four studied phytogeographical regions. Nodules number in all legumes inhabiting NR, O and MR increased significantly compared with those grown at Sinai (Fig. 1). Amongst the studied species, *T. resupinatum* showed the highest nodules number averaging about 223 nodule/individual plant compared with less than 70 in the four other legumes. The highest fresh and dry weight of root nodules was recorded in most species grown at the MR. Across all species, the fresh weight of nodules for plants sampled from MR was more than 1.5-fold of those sampled from other regions. The maximum fresh and dry weight of nodules was recorded in *M. indicus* (non-significant increase) (Fig. 2). Although there was a significant positive correlation between nodules fresh and dry biomass (r-value= 0.891**), both did not depend on the nodules number. Variations between species exerted the greatest magnitude of effect on the number of nodules where $\eta^2 = 0.904$ compared with $\eta^2 = 0.064$ for the effect of regions. Nodules fresh and dry biomass affected by differences between habitats more than their number (Table 3).

3.3. Leghaemoglobin content and nitrogenase activity

Leghaemoglobin content of root nodules and nitrogenase activity (acetylene reduction) differed significantly among the studied plant species as affected by the phytogeographical regions. The significant increases in leghaemoglobin content were recorded in legumes grown at S and NR (Fig. 3). The highest leghaemoglobin content and nitrogen-fixing activity were estimated in *T. resupinatum* (Figs. 3, 4). Despite there are non-significant differences in the content of leghaemoglobin between *T. resupinatum*, *T. hamosa* and *V. sativa*, interestingly the nitrogenase activity in *T. resupinatum* was about 10-fold of the activity in the both other species. Legumes grown at NR were characterized by a significant increase in nitrogenase activity compared with those at other regions. In all studied species, the content of leghaemoglobin was strongly correlated with the concentration of PO_4^{3-} and water content (WC%) of soil, while it negatively correlated with SO_4^{2-}, NO_3^-, TSS, pH, Na^+, Ca^{2+} and Mg^{2+} (Table 2). Nitroginase activity was positively correlated with the concentration of Cl^-, PO_4^{3-} and WC% of soil, while the activity negatively correlated with SO_4^{2-} (weak –ve r-values), NO_3^-, TSS, HCO_3^-, pH, K^+, Ca^{2+} and Mg^{2+}. As shown in Table 3, variation in species have the greatest magnitude of effect on legheamoglobin content ($\eta^2 = 0.878$), while its effect on nitrogenase activity reduced ($\eta^2 = 0.487$) in favor of differences in habitats or regions ($\eta^2 = 0.299$).

Table 1. Some physical and chemical properties of soils at different studied habitats. NR, Nile region (Assiut); O, Al-Kharga Oases; MR, Mediterranean region (Burg Al-Arab); S, South Sinai (Saint Katherine); TSS, Total soluble salts; OM, Organic matter. Values are means± SD, n=5.

Regions					
Parameter		**NR**	**O**	**MR**	**S**
Soil texture		Clay	Clay loam	Loam	Loam
Water content %		30.82±2.29 ^{d}	20.80±0.76 ^{c}	13.73±0.88 ^{b}	8.47±0.70 ^{a}
pH (in 1:5 extract)		7.68±0.13 ^{a}	7.78±0.09 ^{a}	7.98±0.02 ^{b}	8.09±0.02 ^{b}
E.C. (mS/cm)		0.365±0.06 ^{ab}	0.45±0.07 ^{b}	0.33±0.06 ^{a}	0.57±0.09 ^{c}
TSS%		0.117±0.02 ^{ab}	0.144±0.02 ^{b}	0.107±0.02 ^{a}	0.182±0.03 ^{c}
OM %		1.61±0.08 ^{c}	1.23±0.11 ^{bc}	0.82±0.16^{b}	0.47±0.05 ^{a}
Na^+	**mg/g soil**	0.13±0.01 ^{a}	0.15±0.03 ^{a}	0.13±0.01 ^{a}	0.19±0.02 ^{b}
K^+		0.10±0.02 ^{c}	0.01±0.00 ^{a}	0.04±0.00 ^{b}	0.04±0.01 ^{b}
Ca^{+2}		0.34±0.04 ^{a}	0.45±0.03 ^{b}	0.48±0.01 ^{b}	0.48±0.03 ^{b}
Mg^{+2}		0.09±0.04 ^{a}	0.09±0.04 ^{a}	0.12±0.02 ^{a}	0.29±0.04 ^{b}
Cl^-		0.83±0.20 ^{a}	0.89±0.18 ^{a}	1.14±0.18 ^{b}	1.12±0.14 ^{b}
HCO_3^-		2.44±0.30 ^{a}	2.44±0.31 ^{a}	3.25±0.32 ^{c}	2.75±0.26 ^{ab}
NO_3^-		0.37±0.02 ^{c}	0.35±0.06^{c}	0.22±0.02 ^{b}	0.08±0.01^{a}
SO_4^{-2}		0.43±0.05 ^{a}	0.38±0.04 ^{a}	0.34±0.01 ^{a}	0.85±0.04 ^{b}
PO_4^{-3}		0.06±0.001^{b}	0.043±0.001^{a}	0.063±0.004 ^{b}	0.039±0.002 ^{a}

Means with different letters are significantly different according to Duncan comparisons ($P < 0.05$).

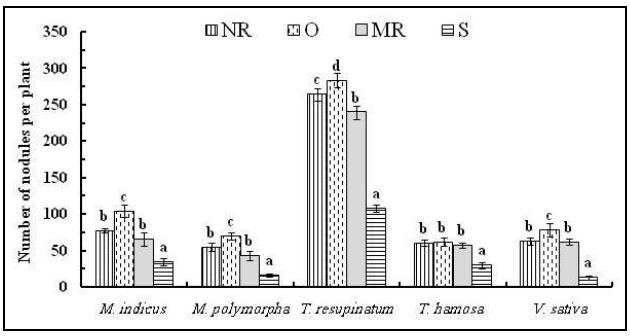

Figure 1. Nodule number (nodule/plant) of the legumes *M. indicus, M. polymorpha, T. resupinatum, T. hamosa* and *V. Sativa* inhabiting different phytogeographical regions of Egypt (NR= Nile region; O = Oases; MR = Mediterranean region; S = Sinai). The values are mean ± SD, n = 3, means of each species with different letters are significantly different at $P< 0.05$.

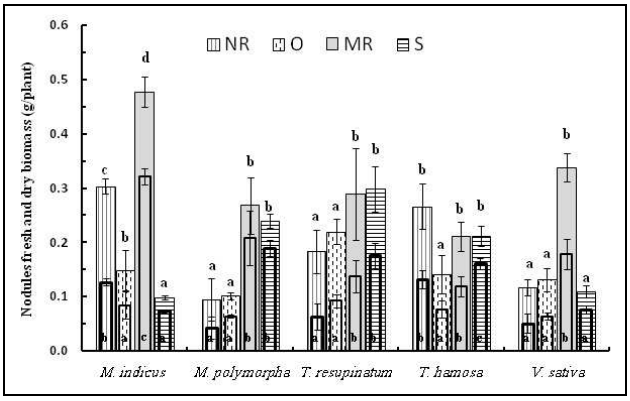

Figure 2. Nodule fresh and dry biomass (g/plant) of five legumes inhabiting different phytogeographical regions of Egypt. Statistics as in Fig. 1.

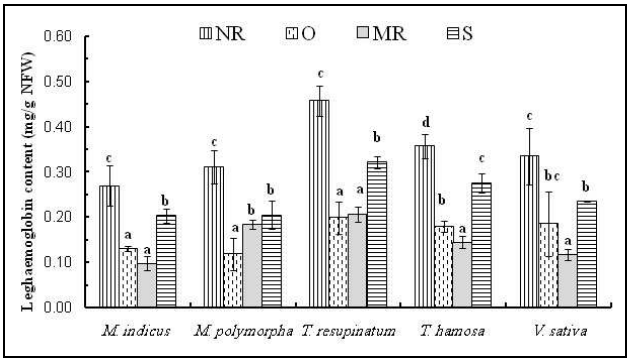

Figure 3. Leghaemoglobin content (mg g^{-1} nodule FW) in five legumes inhabiting different phytogeographical regions of Egypt. Statistics as in Fig. 1.

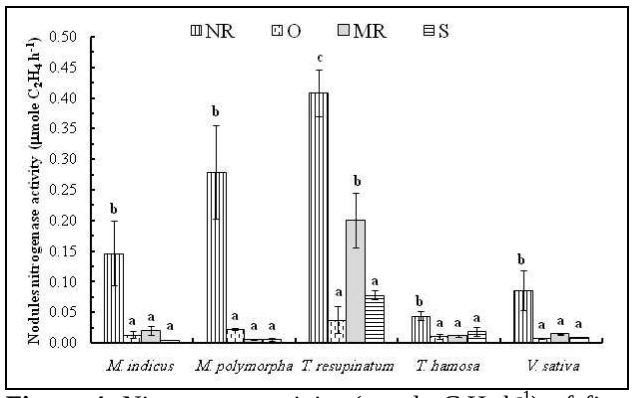

Figure 4. Nitrogenase activity (µmole C_2H_4 h^{-1}) of five legumes inhabiting different phytogeographical regions of Egypt. Statistics as in Fig. 1.

3.4. Proline content

The data recorded in Figure 5 shows that differences in habitat conditions have a significant effect on the proline content in the five wild leguminous plants. High proline content was recorded in plants grown at MR and S compared to those grown at the NR and O. It is clear that *M. indicus* and *T. hamosa* contain high proline content compared with *M. polymorpha*, *T. resupinatum* and *V. sativa*. On the bases of pooled data, proline content in *M. indicus, T. hamosa* and *V. sativa* was significantly higher than that in *M. polymorpha* and *T. resupinatum*. Also, the proline content in plants at MR and S was significantly higher than that in plants at NR and O. Correlation analyses of proline content in shoots of leguminous plants with soil variables showed undefined trend. Proline content negatively correlated with soil K^+ (r-values between -0.796 and -0.870), while it positively and weakly correlated with Na^+. A significant positive correlation resulted between proline content in *M. indica* and concentration of NO_3^- in the soil, but a significant r-value resulted in case of *M. polymorpha*. Against what was expected, a very weak correlation has been found between proline content in all studied plants and TSS in the soil (Table 2). Also, differences between species have the major magnitude of effect on proline content of shoots (η^2 = 0.77) rather than changing habitat conditions (Table 3).

Table 2. r-values of linear correlation analyses between some parameters estimated in different leguminous plants and soil variables (WC = soil water content; TSS = total soluble salts).

Para-meter	Plant species	Soil variables											
		Cl^-	SO_4^{--}	PO_4^{--}	HCO_3^-	NO_3^-	WC	TSS	pH	Na^+	K^+	Ca^{++}	Mg^{++}
Nodule fresh biomass	*M. indicus*	-0.060	-0.511	0.253	0.544	0.313	0.249	-0.310	-0.900*	-0.268	-0.624	-0.166	-0.573
	M. polymorpha	0.984**	-0.596	0.964*	-0.579	-0.999**	0.975*	-0.115	-0.767	-0.430	-0.680	-0.365	-0.255
	T. resupinatum	0.792	-0.289	0.975*	0.155	-0.847	0.720	-0.555	-0.262	-0.047	-0.645	0.160	-0.578
	T. hamosa	0.853	-0.175	0.968*	-0.454	-0.622	0.774	-0.547	-0.148	0.008	-0.588	-0.668	0.005
	V. sativa	0.997**	-0.465	0.993**	0.553	-0.798	0.840	-0.397	-0.690	-0.105	-0.613	-0.252	-0.172
Nodule dry biomass	*M. indicus*	-0.312	-0.303	-0.062	0.677	0.544	-0.056	-0.332	0.670	-0.463	-0.032	0.335	-0.247
	M. polymorpha	-0.896	0.957*	-0.618	0.950*	0.781	-0.915*	-0.847	0.091	-0.358	0.038	0.545	-0.503
	T. resupinatum	-0.374	0.482	-0.598	0.667	0.851	-0.986**	-0.641	0.198	-0.479	-0.109	0.360	-0.368
	T. hamosa	0.187	0.020	0.447	0.643	-0.350	0.108	-0.223	0.956*	-0.624	-0.029	-0.274	-0.003
	V. sativa	-0.347	-0.210	-0.180	0.501	-0.084	-0.266	-0.728	0.292	-0.551	0.016	0.677	-0.588
Leghemoglobin	*M. indicus*	0.964*	0.122	0.808	-0.172	-0.348	0.361	-0.953*	-0.369	-0.937*	0.074	-0.631	-1.000**
	M. polymorpha	0.996**	-0.785	0.862	-0.772	-0.955*	0.999**	-0.178	-0.940*	-0.984**	0.041	-0.588	-0.928*
	T. resupinatum	-0.049	-0.925*	0.644	-0.333	-0.884	0.817	-0.692	-0.769	-0.840	-0.008	-0.357	-0.985**
	T. hamosa	-0.372	-0.982**	0.393	-0.089	-0.869	0.690	-0.998**	0.121	-0.798	0.123	-0.190	-0.699
	V. sativa	0.572	-0.986**	0.660	0.272	-0.954*	0.855	-0.786	-0.861	-0.865	0.118	-0.399	-0.885
Nitroginase activity	*M. indicus*	0.840	-0.495	0.990**	-0.174	-0.495	0.798	-0.099	-0.950*	0.025	-0.174	-0.495	-0.272
	M. polymorpha	0.874	-0.303	0.998**	-0.283	-0.957*	0.851	0.588	-0.594	-0.129	-0.258	-0.721	0.063
	T. resupinatum	0.985**	0.249	0.672	-0.039	-0.525	0.574	0.132	-0.456	0.141	-0.141	-0.327	-0.173
	T. hamosa	0.977*	0.143	0.767	-0.626	-0.267	0.554	-0.282	-0.783	0.287	-0.151	-0.067	-0.183
	V. sativa	0.780	-0.259	0.669	-0.133	-0.468	0.736	0.232	-0.706	0.172	-0.196	-0.736	0.172
Proline	*M. indicus*	-0.193	0.706	-0.409	0.911*	0.995**	-0.863	0.267	-0.849	0.306	-0.825	0.236	-0.025
	M. polymorpha	0.981**	-0.848	0.802	-0.836	-0.917*	0.989**	-0.036	-0.300	0.139	-0.870	-0.036	0.315
	T. resupinatum	0.296	-0.347	0.704	0.811	-0.409	0.045	-0.222	0.210	0.513	-0.796	0.452	-0.043
	T. hamosa	0.600	0.933*	-0.044	0.195	0.612	-0.482	0.007	-0.250	0.548	-0.815	-0.702	0.489
	V. sativa	0.834	0.100	0.771	0.507	-0.332	0.446	0.037	-0.255	0.457	-0.841	-0.025	0.386

**:Correlation is significant at $P< 0.01$.

*:Correlation is significant at $P< 0.05$.

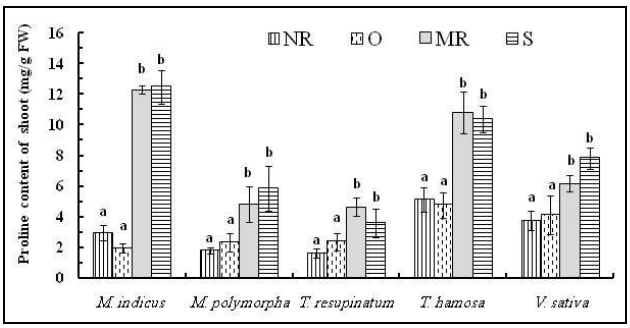

Figure 5. Proline content (mg/g FW) in shoots of the five studied legumes. Statistics as in Fig. 1.

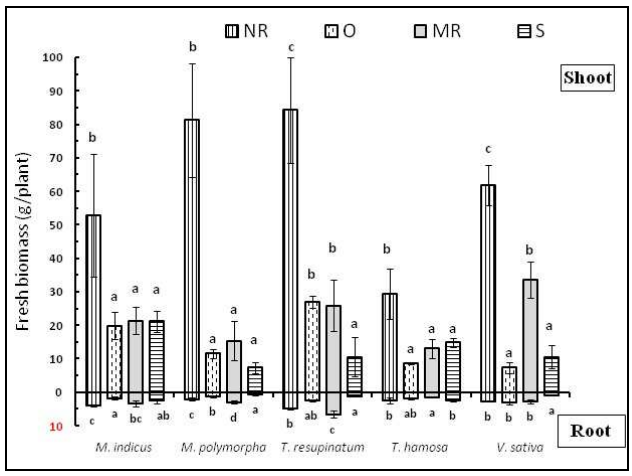

Figure 6. Shoot and root fresh biomass (g/plant) of the five studied legumes. Statistics as in Fig. 1.

Table 3. F-values of factorial ANOVA for the effect of species, regions and their interaction; and eta-square (η^2) calculated for each factor.

	Species		Region		Species*Region	
Parameter	**F-value**	η^2	**F-value**	η^2	**F-value**	η^2
Nodule number/ plant	3848.442	0.904	456.853	0.064	52.825	0.030
Nodule fresh biomass	385.634	0.835	63.956	0.083	12.414	0.065
Nodule dry biomass	421.846	0.776	106.467	0.117	20.856	0.092
Leghemoglobin	700.039	0.878	126.794	0.095	5.310	0.016
Nitroginase activity	178.100	0.487	182.088	0.299	29.166	0.192
Proline	649.648	0.770	218.976	0.156	22.627	0.064
Shoot fresh biomass	212.259	0.634	137.211	0.246	13.410	0.096
Shoot dry biomass	231.130	0.666	135.545	0.235	10.976	0.076
Root fresh biomass	196.687	0.805	28.338	0.070	9.493	0.093
Root dry biomass	136.973	0.770	29.391	0.099	6.414	0.086

All F-values are significant at $p < 0.001$.

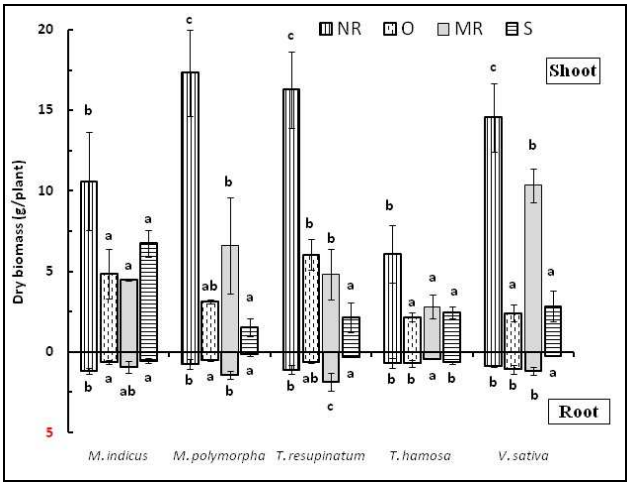

Figure 7. Shoot and root dry biomass (g/plant) of the five studied legumes. Statistics as in Fig. 1.

3.5. Plant biomass

The fresh and dry biomass of collected legumes was affected by phytogeographical regions. The highest fresh and dry biomass of the shoot and root system was recorded in the wild plants collected from NR followed by O, MR and S. Amongst species, *T. resupinatum* grown at NR attained the highest fresh biomass while *M. polymorpha* attained the highest dry biomass (Fig. 6, 7). Calculating the ratio of $root_{dry\ biomass}$: $shoot_{dry\ biomass}$ indicated that *M. indicus* (0.202), *M. polymorpha* (0.214) and *T. resupinatum* (0.384) inhabiting MR and *T. hamosa* (0.323) and *V. sativa* (0.446) inhabiting O have the highest ratios. The lowest root/shoot ratios were recorded for all studied species inhabiting NR (0.042 – 0.113). Averaging across all habitats, *T. hamosa* has the highest ratio (0.211), while *M. indicus* (0.129) and *M. polymorpha* (0.130) have the lowest ratios. As indicated by η^2 in Table 3, the plant, especially root, biomass is greatly related to the variations between species rather than regions or species*regions.

3.6. Rhizobial isolates

The rhizobial isolates recovered from different wild legumes collected from different habitats were identified by 16S rRNA gene sequence. The root-nodule bacteria that was isolated from *M. indicus, M. polymorpha, T. resupinatum, T. hamosa, V. sativa* and had been classified into five species namely: *Sinorhizobium fredii, Rhizobium mesosinicum, Rhizobium daejeonense, Rhizobium huautlense, Rhizobium alamii*, respectively.

4. DISCUSION

The present study clearly indicates that there is a critical role of the habitat conditions on nodulation and nitrogen fixation of wild legumes as indicated by the significant differences in number and biomass of the nodules between individuals of each studied species at different regions. Generally, soils of all habitats at the four phytogeographical regions where the wild legumes studied were not saline and the TSS were less than 0.2% (concentration of Na^+ was less than 0.02%). Soil at Assiut (NR) and for some extent at Kharga (O), with high water content, organic matter, and nutrients such as K^+, Ca^{+2} and PO_4^{-3} represents the best habitat for nodulation, nitrogen fixation and growth of plants. This could be attributes to the clay or clay loamy soil at NR and Oases, respectively. The desert soils of Egypt are inherently low in organic matter due to the arid or hyper-arid climate and historically low vegetation cover, and at all regions the OM were ranging from 0.5-1.6%. The fine textured soils at NR and O were characterized by relatively high level of organic matter, and hence increasing water retention capacity, K^+, Ca^{+2} and Mg^{2+}. Such conditions are essential for survival of rhizobia in the soil and support the process of root hair infection and nodule development. This study supports what have been found by Rao and Venkateswarlu [48] that not the high Indian desert soil temperature but the low organic matter and poor soil moisture were the major factors that reduced the numbers of different micro-organisms.

The poor nodulation and nitrogen fixation of wild legumes grown in sandy soils of MR and S could be attributed to the coarse soil, decreasing content of organic matter and water shortage. However, the nodule fresh biomass positively correlated, while its dry biomass negatively correlated with the soil water content; but both of the fresh and dry biomass negatively correlated with TSS. Another an important factor affecting nitrogen fixation is the temperature. The studied five legumes are annual herbs and their height ranging from 10 to 60 cm [49]; so they complete their life cycle nearly through the winter (the samples collected on April). The average maximum temperature of five months prior to sampling (from December to April) at Assiut, Kharga, Burg Al-Arab and Saint Katherine was 23 ± 2, 23 ± 2, 19 ± 1 and 15 ± 3 °C; while the average minimum temperature was 8 ± 2, 10 ± 2, 10 ± 1 and 4 ± 2 °C, respectively. As Abdel Gadir and Alexander [50] reported in Egyptian sandy soils, the temperature near the soil surface was 59°C when the air temperature was 39°C. However, the soil temperature decreased rapidly with depth, being moderate 35 °C, at 15 cm. Every bacterium has its own optimum conditions, under which it grows at its best. For most rhizobia, the optimum temperature range for growth is 28-31 °C, and many are unable to grow at 37°C [12]. Also, temperature plays an essential role on the exchange of molecular signals between rhizobia and their partners, thus reducing nodulation [51]. However, low temperature may be critical factor reducing nodulation and nitrogen fixation activity in all species at Saint Katherine.

Water, and its availability, is one of the most critical environmental factors that affect the growth and survival of micro-organisms. Drought is one of the most common stresses soil microorganisms have to face. The responses of bacterial cells to drought can be: shrinkage of the bacterial cytoplasm and capsular layers, increase in intracellular salt levels, crowding of macromolecules, damage to external layers (pili, membranes), changes in ribosome structure, and decrease in growth [52]. A shortage of water supply can slow the growth of the nodule and accelerate its senescence. So these results compatible with Ralston and Imsande [53] who reported that nodules in dry soils lose water faster than the vascular system can supply it and hence suffer water stress. Shortage in water supply to the nodules may result in collapse of cells near the surface creating impaired diffusion which reduce the adverse effects of drought. This also reflects why the water content of nodules is too low in plants inhabiting the MR sandy formations and South Sinai. Previous study indicated that harmful effects of water deficit can be alleviated by increasing K^{2+} supplementation [54]. Water stress is quickly reflected as changes in hormonal content [55]. The nodules are an active site of synthesis of auxins and cytokinins. Therefore, it is likely that nodules, besides the supply of organic N, are a source of cytokinins that makes the plant more tolerant to water stress [56]. The results indicated that there is a significant variation in rooting development

between the studied species as reflected on the root: shoot ratios. Plants inhabiting the NR, with the highest soil water content, have the lowest root: shoot ratios and vice versa for those inhabiting Sinai where the soil is relatively dry. However, in dry soil the plants tend to increase the extinction of lateral and sinker roots for mineral uptake and water absorption; and translocation of photosynthats for this vital purpose will affect negatively on that transported to nodules.

Nitrogenase activity is decreased significantly, accompanied by the decrease in respiratory activity of the root nodules [57, 58]. A limitation in metabolic capacity of bacteroids and oxidative damage of cellular components are contributing factors to the inhibition of nitrogenase activity in alfalfa nodules [59]. In addition, the transport of fixed nitrogen out of the nodule is decreased possibly due to an insufficient supply of photosynthates from stems and leaves under stress [60].

Leghaemoglobin content of nodule cytosol was also severely inhibited by drought stress so the leghaemoglobin content in this study was high in NR followed by O and low in MR and S. This decline was attributed to the induction of protease activity [54]. Legumes tend to maintain a level of O_2 within their nodules that can support respiration but is sufficiently low to avoid inactivation of nitroginase [61]. Despite leghaemoglobin act as a buffer for nodule O_2, Denison and Harter [62] adduced that it stores only enough O_2 to support nodule respiration for a few seconds. Gas permeability in nodules decreases under drought or upon exposure to nitrate, and as the permeability decreases may be there is no need to further leghaemoglobin. This may explain why the content of leghaemoglobin increased significantly in nodules of all studied species inhabiting NR, while it negatively correlated (significant –ve r-values in *M. polymorpha* and *V. sativa*) with the concentrations of NO_3^- in the soil.

Proline considered as an indicator for response to environmental stresses and it accumulates in relatively large quantities under stress conditions [63, 64]. Proline content was relatively high in shoots of wild legumes grown at MR and S, and this may be attributed to the low soil water content found at these habitats. Also, this proved that the plants at MR and S, at period of sampling, were more exposed to environmental stresses. The strong –ve correlation between contents of proline in shoots of all studied legumes and K^+ concentrations in the soil, have lead to a suggestion that availability of K^+ may suppress proline synthesis.

Rhizobial isolates recovered from wild legume plants grown in S and MR such as *Medicago polymorpha* (*Rhizobium mesosinicum* ASU8) and *Trigonella hamosa* (*Rhizobium huautlense* ASU3), are of good traits, such as tolerant to high salt, drought and temperature level. Isolation of root-nodules bacteria of wild legumes growing in arid region is very attractive and promising. These indigenous rhizobia are characterized by wide host ranges that offer these legumes ecological benefit. Therefore, successful isolation of rhizobia from such environment will definitely result in obtaining good rhizobia candidates for establishing successful symbioses in extreme environments useful for production of crop legumes. It is well documented that native rhizobia can form nodules with other wild or cultivated crop legumes, and can be utilized for genetic manipulation to improve and perform of symbiotic characters of other root nodule bacteria with crop legumes [19, 65]

5. CONCLUSION

The present investigation revealed that the natural nodulation and nitrogen fixation of wild legumes are drastically affected by habitat. In contrast to Mediterranean region and Sinai, wild legumes inhabiting Nile region and Oases were characterized by high nodulation and nitrogen fixation. *Rhizobium mesosinicum* ASU8 and *Rhizobium huautlense* ASU3 isolated from root nodules of *Medicago polymorpha* and *Trigonella hamosa* grown at Sinai and Mediterranean region are expected to be more tolerant to harsh environmental conditions than rhizobia from cultivated legumes. These rhizobia could be valuable in agricultural practice, specifically in the inoculation of crop legumes grown under unfavorable conditions or in the new reclaimed soil.

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AUTHORS' CONTRIBUTION

MHA-A: Conception, design of the work and Critical revision of the article; AEE: Data collection and revised the manuscript; TRM: Data analysis and interpretation the experimental; ME: Drafting the article; IMN: carried out the practical experiments. The final manuscript has been read and approved by all authors.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

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Research Article

Optimization of lactic acid production by a novel strain, *Enterococcus faecalis* KY072975 isolated from infants stool in Egypt

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ABSTRACT

Production of lactic acid using a novel strain of lactic acid bacteria isolated from infants stool was investigated in the present study. Out of ten isolates, a total of five bacterial isolates were found as positive in lactic acid production. The tested bacterial isolate W7 was observed as the potent strain in lactic acid production that exhibited a halo zone of 8 mm. The bacterial isolate W7 was identified phenotypically and genotypically as *Enterococcus faecalis* and was deposited in GenBank with accession number KY072975. The effect of different process parameters such as initial pH of the medium, incubation temperature, inoculum size and incubation time was also monitored to enhance lactic acid production and resulted in optimum lactic acid value of 0.72 mg/mL. The salted whey was the most applicable fermentation medium for production of lactic acid by *Enterococcus faecalis* KY072975 and recorded 2.07 ± 0.1 mg/mL.

Keywords: Optimization; Lactic acid; Fermentation; Skimmed milk; Whey; *Enterococcus*.

1. INTRODUCTION

Lactic Acid Bacteria (LAB) are a group of diverse Gram-positive bacteria commonly used in the food industry and used in making starter culture for dairy products. Bacterial fermentation has the advantage that by choosing a strain of lactic acid bacteria (LAB) producing only one of the isomers, an optically pure product can be obtained, whereas synthetic production always results in a racemic mixture of lactic acid. Lactic acid is an organic acid with a wide range of applications in the food, pharmaceutical and cosmetics industries [1, 2]. Lactic acid fermentation has gained increased attention in the recent years, mainly due to its importance as building block in the manufacture of biodegradable plastics [3-5]. In addition, it has recently been studied with great interest as a biodegradable polylactic acid (PLA) that can be used to improve physical properties in the production of food packaging, plastic utensils, garbage bags and agricultural plastic sheeting, thereby replacing products made from petroleum [6-7]. Recently, lactic acid is widely used as a food additive in food industry, where it serves in a

wide range of functions, such as flavouring, pH regulation, improved microbial quality and mineral fortification. Moreover, lactic acid is used commercially in the processed meat and poultry industries, to provide products with an increased shelf life, enhanced flavour and better control of food-borne pathogens. Due to the mild acidic taste of lactic acid, it is also used as an acidulant in salads and dressings, baked goods, pickled vegetables, and beverages etc. [1, 8].

Lactic acid can be produced either through microbial fermentation or chemical synthesis. Of the total lactic acid produced worldwide every year, about 90% are made by lactic acid microbial fermentation and the rest is produced synthetically by the hydrolysis of lactonitrile [9, 10]. Although most investigations of lactic acid production were carried out with lactic acid bacteria (LAB), some filamentous fungi may be used such as *Rhizopus* which utilizes glucose aerobically to produce lactic acid [11]. However, the yield and productivities of fungal and yeast strains are very low compared with lactic acid bacteria. The production of optically pure lactic acid is essential for the polymer synthesis in which lactic acid is used [12, 13]. In addition, optically pure L(+) lactic acid is polymerized to a high crystal polymer suitable for fiber and oriented film production and is expected to be useful in the production of liquid crystal as well [14]. Moreover, L (+) lactic acid is used by human metabolism due to the presence of L-lactate dehydrogenase and is preferred in foods as preservative as well as emulsifier [12, 15]. For the industrial production of L-lactic acid, it is necessary to provide cheap carbon sources that can be easily metabolized by lactic acid bacteria. A number of industrial by-products or wastes have been evaluated as substrates for lactic acid production with the aim of decreasing the cost of the process, such as sugarcane [16], molasses [17] and whey [18] as carbon sources, and to obtain the optimal conditions of fermentation with higher yields and production rates [19]. In the recent years, there have been various attempts by researchers to produce lactic acid efficiently from inexpensive raw materials by novel lactic acid bacterial isolates able to tolerate high salinity of the substrate and high temperatures. Therefore, the present investigation was aimed to evaluate and optimize lactic acid production by a novel strain of lactic acid bacteria tolerant to saline fermentation medium.

2. MATERIALS AND METHODS

2.1. Source of microorganisms

A total of 10 isolates of lactic acid bacteria previously recovered from infants stool on MRS agar medium were used in the present investigation. Ten infants stool samples were collected from different baby centers and analyzed by the dilution pour plate method. For this purpose, 10 grams of each sample were weighed aseptically and homogenized in 90 ml of sterile saline solution. Then, sequential decimal dilutions of the homogenate were obtained. One ml aliquot of the 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} dilutions was transferred to plates and the melted MRS agar medium was poured. MRS plates were incubated for 3 days at 30°C in anaerobic conditions. The separated colonies were picked, subcultured, maintained on MRS slants and stored at 4°C for further experiments.

2.2. Preparation of bacterial inocula

A loopful of refreshed bacterial culture was inoculated and grown in 50 mL bottles contained 20 mL of MRS broth medium and incubated for 24 h at 37°C under anaerobic conditions (GasPak System - Oxoid, Basingstoke Hampshire, England). The MRS medium [20] contained (g/l distilled water): glucose 10, peptone 10, beef extract 10, yeast extract 5, K_2HPO_4 2, sodium acetate 5, tri-ammonium citrate 2, $MgSO_4 \cdot 7H_2O$ 0.2, $MnSO_4 \cdot 4H_2O$ 0.2 and Tween 80 (1 ml).

2.3. Detection of lactic acid production on agar medium

Production of lactic acid by the tested isolates was determined on MRS-agar plates supplemented by 1% $CaCO_3$ [21]. The plates were inoculated with 10 µl starter culture by spotting technique and incubated under anaerobic conditions (GasPak System - Oxoid, Basingstoke Hampshire, England) at 37°C for 5 days. Lactic acid production was detected by formation of a clear zone around each culture. The diameter of each clear zone

was measured in millimeters and recorded.

2.4. Characterization and identification of selected bacterial isolate W7

2.4.1. Morphological characterization

The morphological growth characters of the selected isolate W7 were conducted on MRS agar medium and the colony color, shape and texture were recorded. The cell shape and arrangement were determined by microscopic examination after Gram staining technique in accordance with Collins and Lyne [22].

2.4.2. Physiological and biochemical characterization

The physiological and biochemical characteristics were estimated according to the standard methods. Catalase production [23], carbohydrate utilization [24], growth at 6.5 % NaCl, growth at different temperatures (15 and 45 °C), production of CO_2 from glucose and production of NH_3 from arginine were tested on the selected bacterial isolate W7.

2.4.3. Genotypic characterization

2.4.3.1. DNA extraction

DNA extraction was done using Genomic DNA preparation kit (Jena Bioscience) according to Kozaki et al. [25].

2.4.3.2. PCR amplification

The 16S ribosomal genes were amplified using standard PCR protocol and 16S primers: 16S F: 5′-GAGTTTGATCCTGGCTTAG-3′ and 16S R: 5′GGTTACCTTGTTACGACTT-3′. The PCR amplification was performed using Qiagen Proof-Start Tag Polymerase Kit (Qiagen, Hilden, Germany). The following substrates were combined in a total volume of 25 µl including about 20 ng of template DNA, 12.5 µl PCR Master Mix, 20 pmol (2µl) each of forward and reverse primers and the total reaction volume was completed by 8.5 µl of deionized water. The reaction conditions were: an initial denaturation at 94 °C for 5min, 37 cycles of denaturation at 94 °C for 30 s, annealing at 51°C for 30 s, and extension at 72 °C for 30 s. A final extension was conducted at 72°C for 5 min. PCR products were analyzed by electrophoresis on 1.5 % agarose gel in 1X TAE buffer and the gels were visualized and pictured under UV light. PCR products were purified from gel with the QIAquick gel extraction kit (Qiagen, Hilden, Germany).

2.4.3.3. DNA Sequencing

Sequence similarity was estimated by searching the homology in the Genbank DNA database using BLAST (https://blast.ncbi.nlm. nih.gov/Blast.cgi). Multiple sequence alignment and molecular phylogeny was evaluated using CLUSTALW program (http:// clustalw.ddbj. nig. ac. Jp/top- ehtml). The phylogenetic tree was displayed using the tree view program.

2.5. Monitoring lactic acid production

After bacterial culture was inoculated and grown in 50 mL bottles contained 20 mL of MRS broth medium and incubated for 24 h at 37◦C in carbon dioxide incubator, 1-2 drops of phenol phethalin solution were added as indicator onto the 2 mL of aliquots. Samples were titrated using standardized 0.1N NaOH solutions. When the first trace of pink color observed, titration was terminated. Consumption of the 0.1N NaOH was recorded. Each ml of 0.1N NaOH equals to 9.008 mg of lactic acid. Finally, the results were expressed in mg/mL.

2.6. Production of lactic acid in different raw materials

20 mL of different fermentation media including skimmed milk (nonfat dry milk made from pasteurized milk in U. S. A by dairy America company), salted whey (6.5 % salt) collected from Dairy processed center in faculty of agriculture at Suez Canal University and whey were prepared and sterilized in autoclave. After sterilization each bottle was inoculated with 1% culture bacteria then incubated at 37°C for 24 h under static conditions.

2.7. Optimization of process parameters

Effect of different process parameters such as

pH (3, 4.5, 5, 5.5, 6, 6.5, 7 and 8), inoculum size (0.5, 1, 1.5, 2, 3, 4 and 5%), incubation temperature (15, 20, 30, 37 and 45°C), and incubation period (0, 3, 6, 9, 24, 40, 44 and 48 h) on lactic acid production by the tested bacterial isolate was studied by varying the respective parameters to enhance lactic acid production from MRS broth medium.

3. RESULTS AND DISCUSSION

3.1. Screening of lactic acid production on agar medium

A total of ten isolates were screened on MRS - agar plate supplemented with $CaCO_3$ to study the ability of bacteria to produce lactic acid. The result was obtained as halo zone (index which consider primer survey for lactic acid production) around the inoculum. These halo zones forming colonies guaranteed to be LAB due to their lactic acid producing properties. The most active bacterial culture W7 showed clear zone of 8 mm was selected for further experiments. Other bacterial cultures (H3, W2, W4 and W6) gives index ranging from 6-7.4 mm while the others tested five isolates showed no clear zone (Table 1). The results were similar to the study conducted by Yi-sheng et al. [21], where a total of 88 acid-producing bacterial strains were isolated from the samples collected in mulberry farms of Taiwan.

3.2. Phenotypic characterization

The results of physiological and biochemical characterization of the isolate W7 were shown in Table 2. The obtained result revealed that the bacterial isolate W7 is Gram positive bacterium but was negative for catalase reaction. This isolate was able to tolerate salinity and grow at 6.5 % NaCl. Also, the bacterial isolate W7 was able to grow at different low temperature levels (15-45 ˚C). The results also indicated that the isolate was unable to produce CO_2 from glucose and NH_3 from arginine. The tested isolate has the ability to exploit glucose, mannose, galactose, xylose, maltose, mannitol, lactose and arabinose but unable to use sucrose and glycerol as a carbon source. Based on the taxonomic characteristics described above, the isolate W7 was assigned to the genus *Enterococcus*.

Table 1. Lactic acid production detected by halo zones (mm ± SE) on MRS - agar plate supplemented with $CaCO_3$.

Isolate number	Halo zone (mm ± SE)
H3	7 ± 0.1
W2	7 ± 0.4
W4	7.4 ± 0.2
W6	6 ± 0.2
W7	8 ± 0.4
Y1, W1, Yf-b, Yf-g & Mix	Negative

Table 2. Morphological and biochemical characterization of the selected bacterial isolate W7.

Test	Observation
Colony morphology	creamy, circle, entire, convex
Gram stain	+
Cells shape	cocci
Catalase production	-
Growth at 6.5 % NaCl	+
Growth at 15 °C	+
Growth at 45 °C	+
Production of CO_2 from glucose	-
NH_3 from arginine	-
Fermentation of:	
Xylose	+
Galactose	+
Arabinose	+
Maltose	+
Mannitol	+
Sucrose	-
Lactose	+
Gycerol	-
Mannose	+
Glucose	+

3.3. Phyllogenetic identification

The selected bacterial isolate W7 was identified using phylogenetic analysis of 16S rRNA gene sequences. The partial 16S rRNA gene sequences of tested isolate was matched with previously published bacterial 16S rRNA gene sequences available in National Center for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool (BLAST). Sequence analysis results revealed that the isolate W7 isolate have a sequence with 99% similarity to *Enterococcus faecalis*. A phylogenetic tree was constructed from a multiple sequences alignment of 16S rRNA gene sequences (Figure 1). The nucleotide sequences of the isolate W7 was deposited in the Gen-Bank nucleotide sequence database under new accession number KY072975.

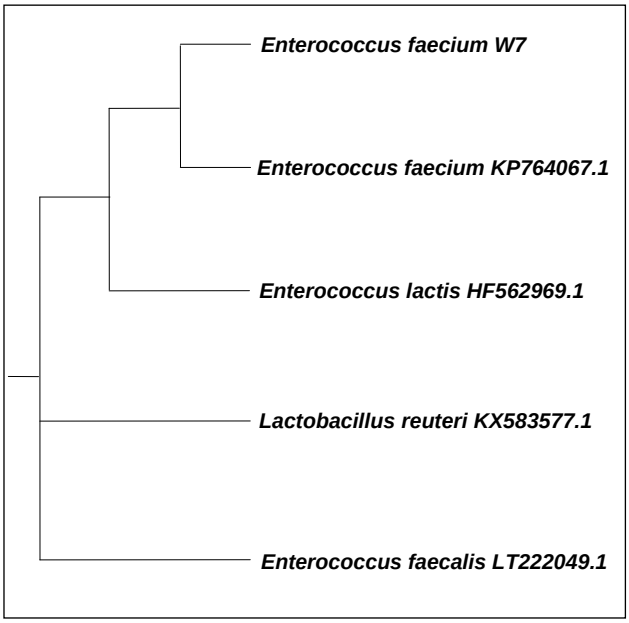

Figure 1. The neighbor-joining tree based on 16SrRNA gene sequences showing the positions of *Enterococcus faecalis* W7 and related strains in GenBank.

3.4. Effect of pH

The effect of pH on lactic acid production was evaluated by using skimmed milk as fermentation medium having a pH range of 3.0-6.8 (Figure 2). The maximum production of lactic acid was detected at pH 6.5, (0.58 mg/ml). While, the lactic acid production decreased at both higher and lower pH. A pH 6.5 was reported optimum for lactic acid production by *Lactobacillus casei* NBIMCC 1013 [26]. However, pH 5.5 has been used for lactic acid production using *L. helveticus* [27]. Lactococci have been found to withstand extracellular pH values down to 5.7 [28] or 5.0 [29]. Under these conditions, both the intracellular accumulation of the lactate anion [30] and the uncoupling of ATP synthesis [28] have been claimed to inhibit growth. From the above observations, a pH 6.5 was considered optimal for maximum lactic acid production. In the subsequent experiments, the pH of the fermentation medium was adjusted to 6.5.

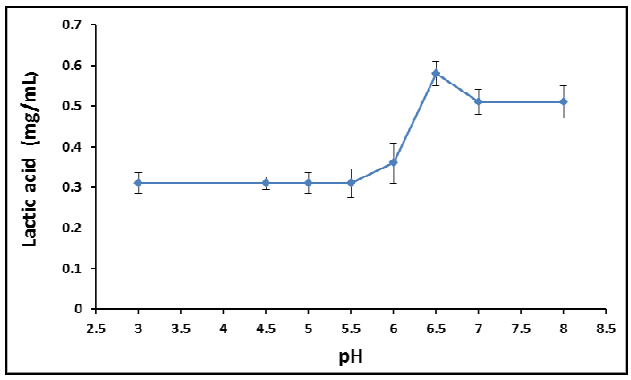

Figure 2. Effect of pH on lactic acid production by *Enterococcus faecalis* W7.

3.5. Effect of temperature

To study the effect of temperature on lactic acid production, skimmed milk medium after inoculation was incubated at a temperature range of 15-45°C. The lactic acid production increase gradually with increasing temperature to reach optimum lactic acid value (0.575 mg/mL) at 37°C then decreased at 45°C (Figure 3). The optimal temperature for growth of lactic acid bacteria varies between the genera from 20 to 45°C [31, 32]. In fermentations using *L. delbrueckii*, and *L. bulgaricus* a temperature of 45°C, or higher may be maintained [33]. *L. helveticus*, and *L. acidophilus* can be used in a temperature range of 37-45°C. Krischke et al. [34] and Panesar et al. [26] used 37°C temperature for lactic acid production using *L. casei*. However, a temperature of 28°C has also been reported optimal for *L. casei* in a separate

study [35]. The temperature is also one of the important factors, which influences the activity of metabolic cell enzymes. Enzymes are most active at optimum temperature and enzymatic reaction proceeds at maximum rate. Wouters et al. [36] noted reduced glycolytic activity leading to reduced production of lactic acid in *L. lactis* at low temperature. The ability to grow at high temperature is a desirable trait as it could translate to increased rate of growth and lactic acid production. At the same time, a high fermentation temperature reduces contamination by other microorganisms. So it was concluded that the optimum temperature for lactic acid production was 37°C and consequently a temperature of 37°C was selected for further experimentation.

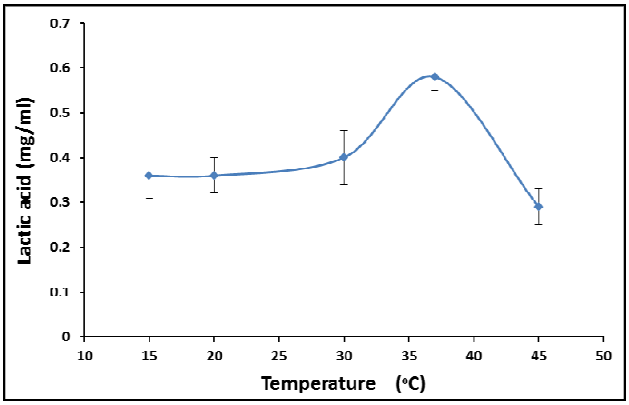

Figure 3. Effect of temperature on lactic acid production by *Enterococcus faecalis* W7.

3.6. Effect of inoculum size

Different inoculum levels (0.5-5%, v/v) were added to the fermentation medium (Figure 4). The lactic acid production increased gradually with increasing inoculum size to reach maximum value (0.72 mg/mL) at 5% inoculum size. This indicates that the lactic acid production increase with increasing density of starter culture. The low lactic acid production at 1% (v/v) inoculum level could be attributed to the low density of starter culture. The use of 2% (v/v) inoculum for the lactic acid production has been reported in earlier studies [37, 38]. 3%, v/v inoculum has also been used for lactic acid production [39]. Guha et al. [40] observed maximum lactic acid production of 2.52 gm/L with 4% (v/v) inoculum of bacterial culture. Panesar et al. [26] observed maximum lactic acid production of 33.72 gm/L when the fermentation medium was inoculated with 2-4% (v/v) inoculum of the tested bacterial culture.

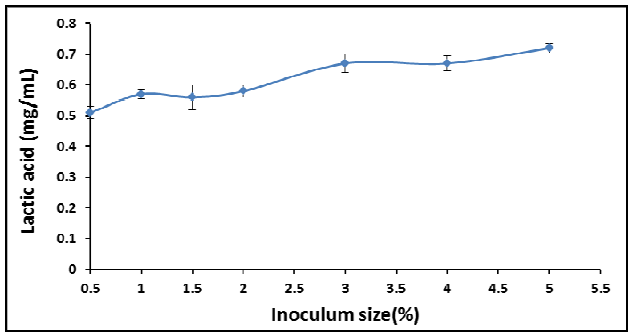

Figure 4. Effect of inoculum size on lactic acid production by *Enterococcus faecalis* W7.

3.7. Effect of incubation time

To find out the optimal incubation time for the maximal lactic acid production, the skimmed milk medium inoculated with bacterial culture was incubated for 48 h under the above optimized conditions. The samples were drawn at specified time intervals and the results obtained are presented in (Figure 5). As evident from the results, an increase in lactic acid production was found up to 24 h and thereafter no improvement was observed. A maximum lactic acid production of (0.582 mg/mL was recorded at 24 h of incubation. This could be attributed to the growth of the culture reached to the stationary phase and as a consequence of metabolism, microorganisms continuously change the characteristics of the medium and the environment. The incubation period of 48 h has been generally used for lactic acid production using different lactobacilli cultures [37, 39, 41]. Panesar et al. [26] observed maximum lactic acid production of 33.73 gm/L after 36 h of incubation. Guha et al. [40] observed maximum lactic acid production of 2.58 gm/L was observed after 48 h of incubation. The reduction in fermentation period is additionally advantageous to improve the economics of the process. The short incubation time is additionally advantageous to improve the economics of the process. Therefore, an incubation time of 24 h was considered optimal for maximum lactic acid production.

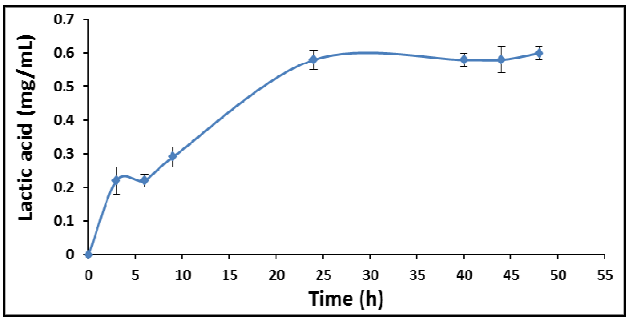

Figure 5. Effect of incubation time on lactic acid production by *Enterococcus faecalis* W7.

3.8. Lactic acid production in different fermentation media

The amount of lactic acid production was evaluated using skimmed milk, whey, salted whey and MRS media under the above optimized conditions. The results indicated that the higher amount of lactic acid in salted whey than MRS (Figure 6). However, the lactic acid produced in skimmed milk and whey was slightly the same and relatively low. The results showed that higher yield of lactic acid by cultural bacteria grown in salted whey will be valuable for future application in industry for producing large amount of lactic acid using cultural bacteria and low cost fermentation media. This test gave an indication of the osmotolerance level of a LAB strain. Bacterial cells cultivated in high salt whey would experience a loss of turgor pressure, which would then affect the physiology, enzyme activity, water activity and metabolism of the cells [42]. Some cells overcome this effect by regulating the osmotic pressure between the inside and outside of the cell [28]. There are reports describing strains of lactococci [43] and lactobacilli [44, 45] showing decreased growth rate with increasing osmolarity of the medium. The bacterial isolate W7 could be similarly protected to be able to grow at higher NaCl concentration during industrial fermentation, as lactic acid is being produced by the cells, alkali would be pumped into the broth to prevent excessive reduction in pH. Thus, the free acid would be converted to its salt form which would in turn increase the osmotic pressure on the cells. Therefore, a LAB strain with high osmotolerance would be desirable as an industrial strain.

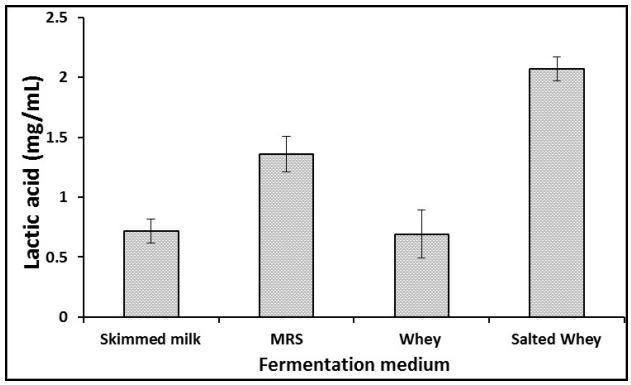

Figure 6. Effect of different fermentation media on lactic acid production by *Enterococcus faecalis* W7.

4. CONCLUSION

Lactic acid is one of the different organic acids produced by bacteria and has numerous uses in food biotechnology especially in dairy products. So, the present study was aimed to isolate a novel strain of lactic acid bacteria applicable for lactic acid production from different inexpensive substrates. The isolated bacterium *Enterococcus faecalis* W7 was the most active isolate for lactic acid production and deposited in GenBank by accession number KY072975.

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AUTHORS' CONTRIBUTION

AAA: Critical revision of the article; A-HMR: Conception, design of the work, carried out the practical experiments, data collection, data analysis and interpretation; MMO: Critical revision of the article; NK: carried out the practical experiments; SGD: Drafting the article. The final manuscript has been read and approved by all authors.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

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Research Article

Effects of cholecystokinin-octapeptide and cerulein on ovine digestive motility under cholinergic blockade

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ABSTRACT

In sheep, contribution of cholinergic system to the control of gastrointestinal motility by cholecystokinin is unknown. Accordingly, in six non-fasted rams chronic experiments were conducted and the myoelectrical activity of abomasal antrum, duodenum and jejunum was recorded before and after injection of atropine (two doses), pirenzepine (two doses), hexamethonium or atropine plus hexamethonium followed or not by injection of three doses of cholecystokinin octapeptide or cerulein. In the course of the experiments performed, the anticholinergic drugs and hormones suppressed spike burst activity both in abomasal antrum and small bowel and inhibited the migrating myoelectric complex and 'minute rhythm'. When the hormones were injected after cholinergic blockade, they induced longer inhibitory effects than cholinergic blockade alone. In the small bowel, some stimulatory effects were observed as well. The higher dose of pirenzepine and remaining anticholinergics induced rebound excitation in the small bowel, but when followed by cholecystokinin peptide administration, no rebound effect was denoted. Hexamethonium given alone or in combination with atropine followed by cholecystokinin peptide caused stronger inhibitory effect than that of atropine or pirenzepine. It is concluded that cooperation exists between the cholinergic system and cholecystokinin in the control of gastrointestinal motility in sheep and the role of nicotinic mechanisms is greater than that of muscarinic mechanisms.

Keywords: Ram; Abomasal antrum; Small intestine; Electromyography; Cholecystokinin octapeptide; Cerulein; Anticholinergic drug.

1. INTRODUCTION

Cholecystokinin (CCK) represents the meaningful peptide hormone and neuromodulator produced by endocrine cells in the gastrointestinal mucosa and by neurons in both central and peripheral nervous system [1, 2]. The hormone modulates motor function both in the stomach and small bowel and the character of motility alterations mostly depends upon the animal species and gastrointestinal segment [3]. CCK, as gastrin, its closely related peptide, can inhibit the abomasal motility and gastric emptying in the ruminants [4, 5]. It was reported that in sheep, CCK inhibits the arrival of the migrating motor complex (MMC) and accelerates small intestinal transit [6, 7]. Cerulein, the amphibian CCK, depresses abomasal motility, stimulates small intestinal contractions and disrupts the MMC in this species [8-13]. Both these peptides seem to be able to modulate also the 'minute rhythm' (MR) in the ovine small bowel [12, 14].

Most of these effects are similar to those observed in monogastric species [15-17]. There is also the increasing evidence that the nervous system strongly contributes to the action of CCK upon the gastrointestinal motility and that the action of the hormone is largely neuronal, both central and peripheral [2, 18]. When CCK was injected intracerebroventricularly, it disrupted the MMC pattern in the dog and rat [19, 20]. Thus, the mechanism of CCK action on gastrointestinal motility is composed. In sheep, CCK evoked central effect on forestomach motility suggesting that in this species CCK can indirectly modulate the gastrointestinal motor function [21]. It has also been reported that the vagus nerve participates in the control of gastrointestinal motility by CCK and the central effects are thus possible to occur [6, 22-24]. Peripheral administration of CCK does not seem to exert central effect directly since CCK probably cannot cross the blood-brain barrier [25]. This does not exclude the possibility of the involvement of peripheral neurons in CCK action upon the gastrointestinal motility. The cholinergic system could be the first candidate for such cooperation. It is well known, also in sheep, that the cholinergic system controls effciently the gastrointestinal motility and the cholinergic blockade can inhibit contractions and disrupt both the MMC and MR [26-28]. Several reports indicate that peripheral cholinergic system interferes in the actions of CCK upon the gastrointestinal motility while the problem has not yet been satisfactorily explored [29-31]. However, nothing is known about these mechanisms in sheep. Thus, the aim of this work was to demonstrate the modulatory role of cholinergic mechanisms in the action of CCK octapeptide (CCK-OP) and cerulein upon the antral, duodenal and jejunal motility in conscious rams. It is hypothesized that obtained results can embrace the question how does CCK cooperate with the cholinergic system in the control of ovine gastrointestinal motility.

2. MATERIALS AND METHODS

2.1. Animal preparation

Six healthy, adult, non-fasted rams, each weighing 38-44 kg, were used in the chronic experiments performed in the study. Animals were kept in cages with normal light rhythm. Before and after surgery, they were habituated for the experiments. Under general and local anaesthesia, right lateral laparotomy was performed and five platinum bipolar electrodes and one strain gauge force transducer (RP Products, Madison) were sewn onto the gastrointestinal serosa of each ram. The electrode localization was as follows: 1 - the abomasal antrum, 4 cm before the pyloric ring, 2 - the duodenal bulb, 6 cm below the pyloric ring, 3 - the duodenum, 56 cm distally to the pyloric ring, 4 - the first jejunal electrode, 256 cm distally to the pyloric ring, 5 - the second jejunal electrode, 356 cm distally from the pyloric ring.

The strain gauge force transducers, calibrated individually, were attached onto the duodenal serosa nearby the third electrode in four of these rams. Marked electrode and transducer wires were exteriorized over the skin, soldered to the plug in the designed order and fixed onto the integument. Within 2-3 days following the surgery, animals gradually returned to normal feeding and then the fodder (good quality hay and the grain mixture) was not restricted, except in the course of the experiment. The drinking water was restricted only during the experiment. The postsurgical recovery period lasted at least 10 days and thereafter the skin sutures were removed. Other details of the experimental model applied in this study were reported elsewhere [13, 32].

2.2. Experimental design

The total of 252 randomized experiments, each lasting 5-8 h, were performed. While the experiment was performed in one ram, the second ram was also present in the experimental room for company. Just before motility recording, the silastic cathether was introduced into the left jugular vein of each ram for intravenous drug and hormone administration. The myoelectric and motor activity was recorded throughout the experiments using the multichannel electroencephalograph (Reega, Alvar Electronic, Paris), also adapted for mechanical recordings. Before the experiments, the efficacy of the cholinergic blockade was checked in three rams with the use of bethanechol preceding atropine or pirenzepine administration and DMPP preceding

hexamethonium administration. During the first part of the experiment (i.e. before drug and hormone administration), the gastrointestinal electromyography and motility recordings were conducted. The normal motility patterns, namely the MMC and the MR were identified. All the MMC phases, including phase 2a and 2b, were regularly identified during this initial control period according to the appropriate criteria [10, 33-35]. 5 ml of 0.15 M NaCl was slowly administered intravenously during early phase 2b of the MMC. During this part of the experiment, at least one full MMC cycle was recorded. In the course of the second part of the experiment, drugs were given intravenously during phase 2b of the next MMC cycle at the doses tested previously. The various doses of cholecystokinin octapeptide (CCK-OP, Sincalide, Squibb Inst., Princeton) and cerulein (Takus, Farmitalia Carlo Erba, Milan) were injected after cholinergic blockade. Following drug administration, each lasting 30 s, the myoelectrical recordings were continued until the normal motility was restored, especially till the arrival of the normal, non-ectopic phase 3 of the MMC. The reference experiments (first series) with cholinergic blockade applied alone were conducted during which the following anticholinergic drugs were injected: atropine sulfate (At, Sigma, St. Louis) at the doses 0.02 and 0.1 mg/kg, each dose given in separate experiment, (2) pirenzepine dihydrochloride (Pi, Sigma, St. Louis) 0.02 and 0.1 mg/kg, each dose given in separate experiment, (3) hexamethonium bromide (Hx, Sigma, St. Louis) 2.0 mg/kg, (4) At 0.1 in combination with Hx 2.0 mg/kg given also in separate experiments. In the course of the proper experiments (second series), one of two CCK peptides was administered following cholinergic blockade. Each dose of CCK-OP (20, 200 or 2000 ng/kg) and cerulein (1, 10 or 100 ng/kg) was preceded by administration of the same anticholinergic drug and dose during separate experiments. The time lag between the smaller doses of Pi or At administration and CCK peptide administration was not longer that one min. In the case of the remaining types of cholinergic blockade, CCK peptides were given 1-2 min after the anticholinergic drug. At least two days overpassed between two consecutive experiments while after the experiments with Hx, duration of the break lasted at least three days.

2.3. Analysis of data

All the recordings were visually analysed in order to identify the motility patterns and to evaluate the intensity and arrangement of the spike bursts and contractions. During the initial part of the experiments, i.e. before cholinergic blockade, the correctness of motility recordings, mainly the occurrence of the normal motility patterns, was confirmed. In the abomasal antrum, duration of spike burst inhibition was calculated not only when complete lack of the spike bursts was present, but also comprised the periods in which the inhibition reached at least 70% of the maximal spike burst amplitude. In the small bowel, duration of the spike burst inhibition (regardless of the arrival of stimulatory events, i.e. the phase 3 of the MMC, premature phase 3, MR and rebound excitation) was calculated following the anticholinergic drug administration (results treated as the reference values) and following CCK peptide administration always preceded by cholinergic blockade. Duration of MMC disruption was measured from the end of anticholinergic drug administration till the arrival of the first phase 3 of the MMC at the given recording channel (the reference value). The time lags from the end of CCK peptide administration (after cholinergic blockade) until the onset of the first phase 3 of the MMC were measured as well. Finally, duration of MR inhibition, from the end of the anticholinergic drug administration till the arrival of the first MR episode in the given recording channel and the time lag from the end of CCK peptide injection (administered after cholinergic blockade) till the arrival of the first MR episode were measured. After stimulatory effects, evoked during the inhibitory period, the spike burst inhibition was still present for some time in almost all cases. These periods were also taken into account during calculations. After termination of the whole inhibitory period, the normal gastrointestinal motility reappeared.

2.4. Statistical elaboration of data

All the data were collected, analysed and grouped, and the mean values with standard deviations (±S.D.) were calculated. All the data were rounded and presented as the whole numbers.

The normality of data distribution was checked and the appropriate comparisons were performed using the variance analysis followed by the Student *t*-test for paired values [36].

2.5. Ethical approval

Protocol of study and informed consent were in compliance with the Helsinki convention and were approved by Local Ethics Committee.

3. RESULTS

During control parts of the experiments, saline injections did not evoke any effect upon the gastrointestinal motility.

Among the anticholinergic substances, Hx was the strongest inhibitory drug as to the antral myoelectrical activity although the spike burst inhibition was complete only in two of six experiments and lasted 2-3 min. Partial inhibition (less than 70% of the maximal spike burst amplitude) was approximately 2-3 times longer in this region than the periods of complete inhibition. Similar situation was observed following the combined cholinergic blockade, i.e. At plus Hx (At+Hx) administration (Table 1). After the smallest dose of CCK-OP administration preceded by Hx or by At + Hx, duration of the inhibitiory periods was slightly but significantly shorter than that after the relevant anticholinergic drug dose given alone. When the highest doses of CCK-OP and cerulein were applied after cholinergic blockade, the inhibitory periods were significantly longer as compared with the higher doses of At or Pi and with Hx or At + Hx administration (Figure 1). Cerulein induced more pronounced effect than CCK-OP (Table 1). Following the higher dose of At, cerulein exerted dose-dependent inhibitory effect upon the antral spike burst amplitude. The inhibitory effect evoked by the maximal doses of both CCK peptides, given after Hx, lasted longer than in response to Hx applied alone. After Pi and At, the effect of CCK peptide was slightly shorter than that after Hx (Table 1).

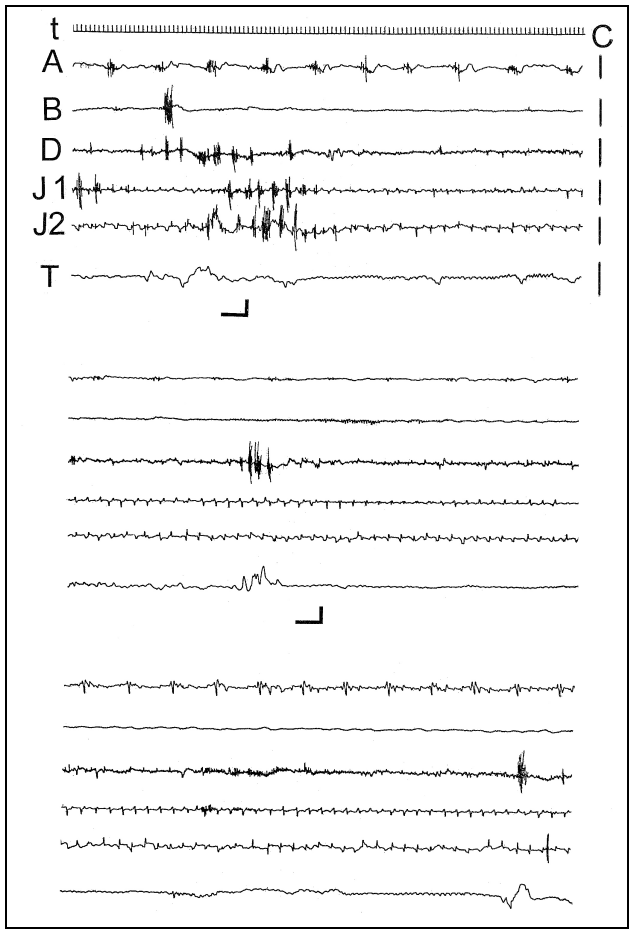

Figure 1. The effects of muscarinic blockade followed by cerulein administration upon the myoelectrical and motor activity of ovine abomasal antrum, duodenum and jejunum. Upper panel: administration of atropine at the dose 0.1 mg/kg (marked). Middle panel: continued recording, administration of cerulein at the dose 100 ng/kg (marked). Lower panel: next two minutes following cerulein administration. Note partial inhibition of the antral spike bursts after atropine and complete inhibition after cerulein administration. Complete inhibition of the intestinal motility in response to cholinergic blockade followed by cerulein administration with lack of the rebound effect, except the presence of single spike burst in the duodenum resembling the residual 'minute rhythm' during cerulein administration is also visible.

Explanations: t - time in seconds; A - electromyographical recording from the abomasal antrum; B - the duodenal bulb, D - the duodenum; J1 - proximal jejunum; J2 - recording from the the second jejunal electrode; T - mechanical recording from the duodenal strain gauge force transducer; C - electrode and transducer calibration, 100 μV and 5g, respectively; ┘(the bent bar) - termination of drug or hormone administration. Other explanations are as in the chapter Materials and Methods.

Table 1. Duration of the spike burst inhibition in the abomasal antrum by the cholinergic blockade applied alone and by the cholinergic blockade followed by cholecystokinin peptide administration in rams.

		Atropine		Pirenzepine		Hexam. 2.0	Atropine 0.1 + Hexam. 2.0
		0.02	0.1	0.02	0.1		
No CCK peptide	Mean	0	1.0	0	0	4	5
	±S.D.	0	0.0	0	0	2	2
CCK-OP 20.0	Mean	0	0^{c}	0	0	1^{a}	2^{a}
	±S.D.	0	0	0	0	0	1
CCK+OP 200.0	Mean	0	0^{c}	0	0	3^{y}	4
	±S.D.	0	0	0	0	1	2
CCK+OP 2000.0	Mean	2^{cz}	2^{az}	4^{cz}	6^{cz}	8^{az}	9^{y}
	±S.D.	1	1	1	3	2	4
Cerulein 1.0	Mean	0	0^{c}	0	1^{b}	2	4^{x}
	±S.D.	0	0	0	0	1	1
Cerulein 10.0	Mean	0	1^{c}	0	0	3	5
	±S.D.	0	0	0	0	1	2
Cerulein 100.0	Mean	0	5^{cz}	4^{cz}	7^{cz}	11^{cz}	10^{x}
	±S.D.	0	2	1	3	5	4

Explanations: doses of the anticholinergic drugs expressed in mg/kg, doses of CCK peptides expressed in ng/kg. Statistical significances: n=6; $^{a}P<0.05$, $^{b}P<0.01$, ^{c} $P<0.001$ vs. reference value (no CCK peptide administration); $^{x}P<0.05$, $^{y}P<0.01$, $^{z}P<0.001$ vs. the relevant value obtained in response to the lowest dose of CCK peptide. Other explanations as in the chapter Material and methods.

Table 2. Partial excitatory events observed during inhibitory periods evoked by the cholinergic blockade applied alone and by the cholinergic blockade followed by cholecystokinin peptide administration in rams.

	Duodenal bulb						Duodenum						Jejunum 1						Jejunum 2					
	At		Pi		Hx	At+ Hx	At		Pi		Hx	At+ Hx	At		Pi		Hx	At+ Hx	At		Pi		Hx	At+ Hx
	l	h	l	h			l	h	l	h			l	h	l	h			l	h	l	h		
No CCK	0	0	0	0	4	3	1	3	2	2	6	3	2	4	4	5	1	2	0	2	4	4	0	2
OP 20.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	2	0	0	0
OP 200.0	1	0	0	0	0	0	3	0	3	0	1	1	0	1	5	1	1	0	0	0	5	1	0	0
OP 2000.0	5	1	0	1	0	0	4	1	4	6	0	2	2	3	1	1	0	1	0	0	0	4	0	2
Cer 1.0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	0	0	0	0	1	1	0	0
Cer 10.0	2	0	1	0	0	0	3	2	2	0	0	2	1	0	0	0	0	0	0	0	0	0	0	0
Cer 100.0	6	0	3	3	0	0	5	4	5	4	0	1	4	0	5	6	3	1	3	0	0	5	0	0

Values represent numbers of the experiments in which the excitatory event arrived. The excitatory events comprised three types of episodes. The premature phase 3 was observed in response to Pi administration at the lower dose. The rebound excitation was seen in the experiments with remaining types of the cholinergic blockade and after the lower dose of Pi followed by CCK peptide administration. The presence of single spike bursts was denoted once after the moderate dose of CCK peptide or often 2-3 times following its highest dose. l. - lower dose (0.02 mg/kg), h. higher dose (0.1 mg/kg); OP - cholecystokinin octateptide; Cer - cerulein. Other explanations as in Table 1.

In the small intestine, unlike in antrum, administration of the anticholinergic drugs followed or not by CCK peptides, induced various stimulatory effects that arrived during the inhibitory periods. The premature phase 3 was evoked in the most cases only by Pi given alone at the lower dose (as shown in Table 2). Administration of At, the higher dose of Pi, Hx and At + Hx, not followed by CCK peptide, evoked clear rebound excitation exhibiting stationary character. When the animals were treated by the lower dose of Pi and then by CCK peptide, no premature phase 3 arrived and instead, the rebound excitation was observed, but not in all the animals studied (Figure 2). When CCK peptide followed the administration of At, the higher dose of Pi, Hx and At + Hx, no rebound excitation was observed although the spike burst inhibition was incomplete (Figure 3). Following the cholinergic blockade, the arrival of usually one or two separate stronger spike bursts was often observed in the duodenum during or just after CCK peptide injection at the moderate or high dose (Table 2, Figure 1). These single spike bursts resembled the MR-forming spike bursts. Sometimes, following the moderate dose of the peptide, more than one isolated spike burst was observed. These effects are also presented in Table 2.

Duration of the spike burst inhibition was different in the various small intestinal segments. When the cholinergic blockade was applied, the spike burst inhibition (calculated including periods when the excitatory effects occurred during the inhibitory response, namely the premature phase 3, rebound excitation or the isolated spike burst) lasted longer in the duodeno-jejunum than in the duodenal bulb (Table 3A, B). Among the anticholinergic drugs, Hx exerted the strongest inhibitory effect, especially in the jejunum, where the Hx-evoked rebound excitation was usually absent (Figure 4). At induced rebound excitation mostly in the duodeno-jejunum and rather not in the duodenal bulb. When CCK peptides were given after cholinergic blockade, they often exerted significant, dose-related effect. Following the highest dose of both CCK peptides, the inhibitory period lasted much longer than after both lower doses. The effect of cerulein was often more pronounced that the relevant effect of CCK-OP. It was seen mostly in the jejunum. Introduction of the lower dose of At followed by cerulein, inhibited the spike bursts for the period longer than in the experiments in which the same dose of cerulein injection was preceded by the higher dose of At (Table 3A, B). Similar observation concerned also Pi. In all the regions examined, administration of Hx or At + Hx combined with both CCK peptides evoked significantly longer inhibitory effects than those of At and Pi when injected before CCK peptide, regardless of their doses (Table 3A,B, see also Figure 5). Cerulein, given at the lowest dose and preceded by the both doses of Pi, produced significantly shorter inhibitory response in the duodenum than Pi given alone. CCK-OP, used at the lowest dose and preceded by the lower dose of Pi, Hx or by higher dose of At, inhibited spike burst activity in the jejunum for significantly shorter time than the relevant anticholinergic drug given alone (Table 3A, B).

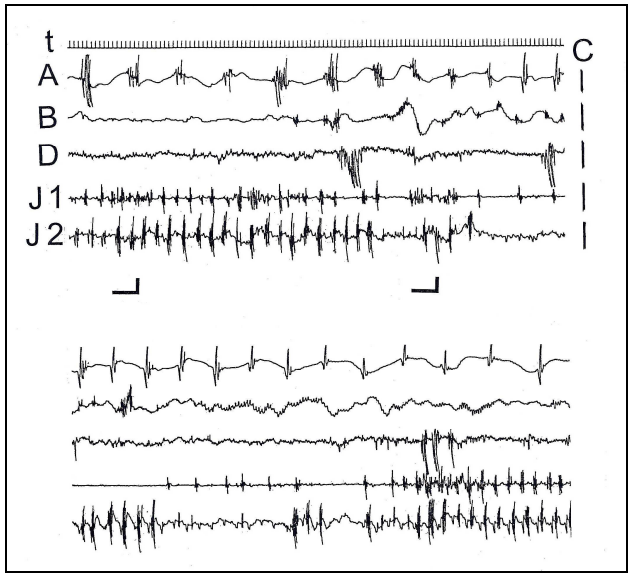

Figure 2. The effects of muscarinic blockade followed by cholecystokinin octapeptide (CCK-OP) administration upon the myoelectrical activity of ovine abomasal antrum, duodenum and jejunum after muscarinic blockade. Upper panel: administration of pirenzepine (Pi) at the dose 0.02 mg/kg (left bar) followed by CCK-OP at the dose 200 ng/kg (right bar). Lower panel: continued recording after OP-CCK administration. Note the stronger inhibitory effect on antral spike burst after OP-CCK than after Pi. Pi did not inhibit the jejunal myoelectric activity and OP-CCK inhibited it in part. Symbol explanations as in Figure 1.

Table 3A. Duration of the spike burst inhibition in the duodenal bulb and the duodenum in response to the cholinergic blockade applied alone and to the cholinergic blockade followed by cholecystokinin peptide administration in rams.

		Duodenal bulb						**Duodenum**					
		Atropine		**Pirenzep.**		**Hx**	**At 0.1 + Hx 2.0**	**Atropine**		**Pirenzep.**		**Hx**	**At 0.1 + Hx 2.0**
		0.02	**0.1**	**0.02**	**0.1**	**2.0**		**0.02**	**0.1**	**0.02**	**0.1**	**2.0**	
No CCK peptide	Mean	4	3	2	4	7	7	6	10	14	15	11	12
	±S.D.	1	1	1	2	2	3	3	4	6	5	4	6
CCK-OP 20.0	Mean	10^{b}	17^{c}	6^{a}	7	16^{a}	29^{c}	8	19	15	12	9	26^{a}
	±S.D.	2	7	2	3	7	7	4	8	6	4	4	7
CCK OP 200.0	Mean	16^{cx}	24^{c}	16^{cz}	5	33^{cx}	42^{c}	13	25^{a}	24	8	26^{ax}	61^{cz}
	±S.D.	4	9	3	2	11	9	5	10	9	3	11	19
CCK OP 2000.0	Mean	13^{b}	39^{cx}	19^{cz}	18^{cx}	48^{cz}	61^{cz}	12	32^{c}	17	19	34^{cz}	93^{cz}
	±S.D.	6	14	5	7	19	15	6	11	8	5	13	27
Cerulein 1.0	Mean	11^{a}	8^{a}	5^{a}	12^{a}	54^{c}	33^{c}	12	9	2^{c}	5^{b}	32^{c}	28^{b}
	±S.D.	5	3	2	5	16	12	4	3	1	2	11	7
Cerulein 10.0	Mean	19^{c}	11^{b}	17^{cy}	46^{cz}	60^{c}	52^{c}	14^{a}	13	12^{z}	9	35^{c}	46^{cx}
	±S.D.	9	4	7	14	17	11	5	5	5	4	14	10
Cerulein 100.0	Mean	23^{cz}	28^{cz}	21^{cz}	16^{b}	66^{c}	58^{c}	18^{b}	25^{ax}	18^{z}	14^{y}	55^{c}	49^{cx}
	±S.D.	7	12	6	7	20	17	6	11	7	4	19	12

Explanations as in Table 1.

Table 3B. Duration of the spike burst inhibition in the upper and more distal jejunum by the cholinergic blockade applied alone and by the cholinergic blockade followed by cholecystokinin peptide administration in rams.

		Jejunum 1						**Jejunum 2**					
		Atropine		**Pirenzep.**		**Hx**	**At 0.1 + Hx 2.0**	**Atropine**		**Pirenzep.**		**Hx**	**At 0.1 + Hx 2.0**
		0.02	**0.1**	**0.02**	**0.1**	**2.0**		**0.02**	**0.1**	**0.02**	**0.1**	**2.0**	
No CCK peptide	Mean	8	14	7	9	12	13	3	15	3	6	16	12
	±S.D.	3	7	2	3	5	6	1	7	1	3	6	6
CCK-OP 20.0	Mean	6	12	2^{a}	11	7	24	12^{b}	8	2	5	6^{a}	32^{c}
	±S.D.	1	5	1	4	3	7	4	2	1	2	2	8
CCK-OP 200.0	Mean	9^{x}	20	14^{az}	13	8	38^{c}	17^{c}	19^{z}	15^{cz}	7	14	41^{c}
	±S.D.	2	8	5	5	3	8	6	4	6	2	7	12
CCK OP 2000.0	Mean	18^{ax}	26	23^{cz}	22^{bx}	24^{az}	57^{cz}	14^{a}	25^{z}	23^{cz}	12^{x}	16^{x}	54^{c}
	±S.D.	5	11	9	6	7	18	8	6	8	5	6	16
Cerulein 1.0	Mean	11	12	6	7	53^{c}	21	18^{c}	14	9^{a}	3	30	38^{c}
	±S.D.	5	5	2	2	16	8	6	6	3	1	12	13
Cerulein 10.0	Mean	17^{a}	7	11	5	78^{c}	35^{cx}	25^{c}	6^{ax}	14^{c}	6	45^{c}	52^{c}
	±S.D.	6	2	4	2	24	6	11	2	5	3	16	19
Cerulein 100.0	Mean	24^{bx}	8	18^{bz}	6	96^{c}	49^{cz}	28^{c}	7	36^{cz}	8^{x}	63^{cx}	86^{cz}
	±S.D.	9	3	5	2	31	9	11	3	11	3	21	24

Explanations as in Table 1.

Duration of inhibition of phase 3 of the MMC was often long and dependent upon the intestinal segment examined. In the most distal recording channel (jejunum 2), these periods were usually shorter than in the proximal sites since the first phase 3 of the MMC, which arrived after cholinergic blockade applied alone and also after the combination of anticholinergic drugs with CCK peptides, was ectopic. It was started most often just from this distal region (Table 4A, B). Duration of

phase 3 inhibition was longer after Hx or after At + Hx administration than after At or Pi. Despite of the arrival of premature phase 3 following the lower dose of Pi, no inhibitory effect on the regular phase 3 was denoted and the arrived regular phase 3 of the MMC was not ectopic. The premature phase 3 was often ectopic and abortive. When At or Pi were injected, duration of the subsequent phase 3 inhibitory periods was related to the drug dose. When CCK peptide administration followed the cholinergic blockade, the time lags, measured from CCK administration until the appearance of the regular ectopic phase 3, were significantly longer than after cholinergic blockade alone (Table 4A, B). Following the highest doses of CCK peptides, these periods were relatively very long. In the most experiments, the effect of CCK-OP administration was more pronounced than the effect of relevant dose of cerulein, at least in the duodenum and upper jejunum (Table 4A, B).

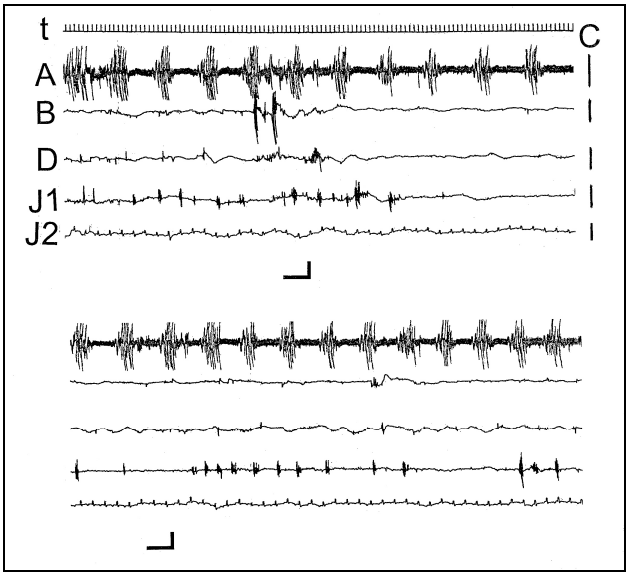

Figure 3. The effects of muscarinic blockade followed by cholecystokinin octapeptide (CCK-OP) administration upon the myoelectrical activity of ovine abomasal antrum, duodenum and jejunum.
Upper panel: administration of pirenzepine (Pi) at the dose 0.1 mg/kg (marked). Lower panel: continued recording and administration of CCK-OP at the dose 200 ng/kg (marked). Note the inhibition of intestinal motility by pirenzepine and lack of rebound effect. CCK-OP exerted slight stimulatory effect in the upper jejunum. No clear inhibition of antral myoelectrical activity is also visible. Symbol explanations as in Figure 1.

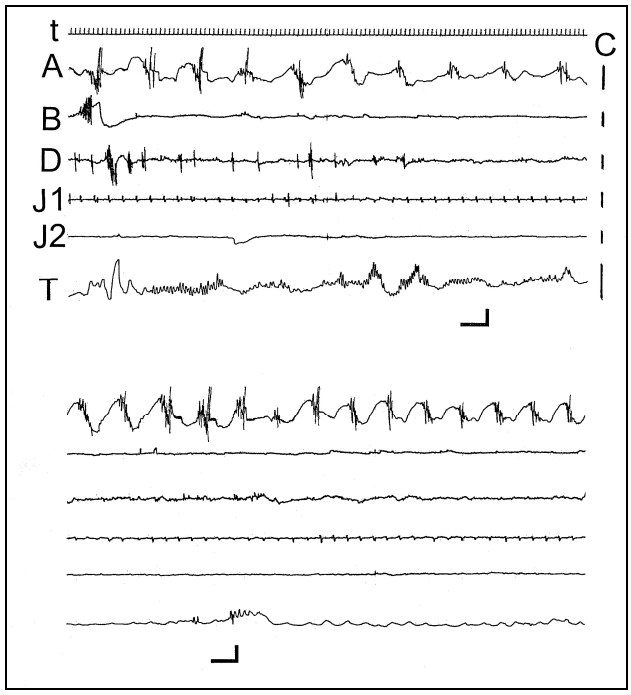

Figure 4. The effects of nicotinic blockade followed by cerulein administration upon the myoelectrical and motor activities in the ovine abomasal antrum, duodenum and jejunum. Upper panel: adninistration of hexamethonium (Hx) at the dose 2.0 mg/kg (marked). Lower panel: continued recording and administration of cerulein at the dose 100 ng/kg (marked). Note the partial inhibition of antral spike bursts by Hx and complete inhibition by cerulein. The electrical and mechanical activity of the small intestine is also inhibited by both of these drugs.
Symbol explanations as in Figure 1.

The time lags between cholinergic blockade and arrival of the first MR episode were usually shorter than phase 3 disruption periods in all the small intestinal segments examined (Tables 4A, B, 5A, B). In the most experiments, duration of MR inhibition was longer following Hx or At + Hx administration than that after At or Pi (Table 5 A, B). Following At injection, this effect was dose-related in all segments examined while after Pi it was rather dose-independent and relatively short. Duration of MR inhibition following CCK-OP and cerulein application after cholinergic blockade exhibited dose-related character, especially in the jejunum. Administration of both CCK peptides, at least at two highest doses, often delayed MR arrival for significantly longer periods than the cholinergic blockade applied alone. These periods were the longest when Hx or At+Hx was followed by CCK peptide administration, especially at its highest dose.

Table 4A. Duration of inhibition of the phase 3 of the migrating myoelectric complex in the duodenal bulb and the duodenum by the cholinergic blockade applied alone and by the cholinergic blockade followed by cholecystokinin peptide administration in rams.

		Duodenal bulb						**Duodenum**					
		Atropine		**Pirenzep.**		**Hx**	**At 0.1 + Hx 2.0**	**Atropine**		**Pirenzep.**		**Hx**	**At 0.1 + Hx 2.0**
		0.02	**0.1**	**0.02**	**0.1**	**2.0**		**0.02**	**0.1**	**0.02**	**0.1**	**2.0**	
No CCK peptide	Mean	26	62	29	34	73	64	17	63	21	67	56	66
	±S.D.	11	18	8	14	22	19	8	19	9	22	11	17
CCK-OP 20.0	Mean	49[a]	76	45	31	83	54	42[c]	64	46[a]	75	63	52
	±S.D.	10	18	17	10	24	14	6	21	15	24	21	15
CCK-OP 200.0	Mean	68[c]	61	71[c]	117[cz]	131[cy]	87	58[c]	71	61[c]	119	116[c]	89[z]
	±S.D.	21	21	23	38	21	21	20	22	20	39	38	17
CCK-OP 2000.0	Mean	96[cz]	116[c]	74[c]	124[cz]	186[cz]	158[cz]	104[cz]	117[cx]	83[c]	178[cz]	166[cz]	155[cz]
	±S.D.	27	28	14	35	45	39	32	30	37	41	39	37
Cerulein 1.0	Mean	47	56	41	62[a]	78	66	34	58	43	94	81	59
	±S.D.	12	17	18	14	16	17	16	17	16	19	19	16
Cerulein 10.0	Mean	56[c]	70	79[c]	68[c]	148[cz]	121[cy]	65[c]	104[cx]	65[c]	88	146[cz]	120[cz]
	±S.D.	11	21	24	12	35	34	22	29	18	23	34	32
Cerulein 100.0	Mean	87[cz]	98[x]	86[cx]	73[c]	197[cz]	176[cz]	85[cz]	107[cz]	79[c]	74	198[cz]	174[cx]
	±S.D.	20	24	26	19	46	48	18	27	25	21	48	47

Explanations as in Table 1.

Table 4B. Duration of inhibition of the phase 3 of the migrating myoelectric complex in the upper and more distal jejunum by the cholinergic blockade applied alone and by the cholinergic blockade followed by cholecystokinin peptide administration in rams.

		Jejunum 1						**Jejunum 2**					
		Atropine		**Pirenzep.**		**Hx**	**At 0.1 + Hx 2.0**	**Atropine**		**Pirenzep.**		**Hx**	**At 0.1 + Hx 2.0**
		0.02	**0.1**	**0.02**	**0.1**	**2.0**		**0.02**	**0.1**	**0.02**	**0.1**	**2.0**	
No CCK peptide	Mean	18	38	19	34	45	37	17	37	20	35	35	38
	±S.D.	4	16	7	13	9	14	5	15	5	13	12	16
CCK-OP 20.0	Mean	33[b]	49	48[a]	47	38	53	28	43	27	23	45	37
	±S.D.	8	22	21	13	17	12	11	18	11	8	14	12
CCK-OP 200.0	Mean	57[c]	73	52[c]	120[cz]	62	76[c]	39[a]	54	22	34	59	49
	±S.D.	18	25	16	36	26	19	15	24	7	11	18	11
CCK-OP 2000.0	Mean	102[cz]	86[c]	67[c]	134[cz]	139[cz]	153[cz]	87[cz]	58	66[cz]	67[az]	118[cz]	76[cz]
	±S.D.	30	24	28	35	28	38	21	16	20	17	32	13
Cerulein 1.0	Mean	28	42	54[c]	29	62	60	21	41	18	27	43	31
	±S.D.	12	11	19	12	24	14	8	10	4	11	12	9
Cerulein 10.0	Mean	54[c]	48	58[c]	45	111[c]	122[cz]	39[c]	47	29	39	76[cx]	78[bz]
	±S.D.	16	16	18	21	44	33	11	14	10	17	21	20
Cerulein 100.0	Mean	69[cz]	52	61[c]	48	176[cz]	156[cz]	53[cz]	53	38[az]	54	109[cz]	135[cz]
	±S.D.	19	14	17	19	51	54	17	13	11	21	26	33

Explanations as in Table 1.

These effects were most pronounced in more distal jejunum (Table 5A, B). In the most experiments, initial administration of lower doses of At and Pi potentiated the MR inhibition by CCK peptide even more than pretreatment with their higher doses. When cerulein administration at the

lowest dose was preceded by the higher dose of At, MR inhibition was significantly shortened as compared with the experiments with At alone (Table 5A, B).

Table 5A. Duration of the 'minute rhythm' inhibition in the duodenal bulb and the duodenum by the cholinergic blockade applied alone and by the cholinergic blockade followed by cholecystokinin peptide administration in rams.

		Duodenal bulb						**Duodenum**					
		Atropine		**Pirenzep.**		**Hx**	**At 0.1 + Hx 2.0**	**Atropine**		**Pirenzep.**		**Hx**	**At 0.1 + Hx 2.0**
		0.02	**0.1**	**0.02**	**0.1**	**2.0**		**0.02**	**0.1**	**0.02**	**0.1**	**2.0**	
No CCK peptide	Mean	14	28	8	7	41	38	8	23	9	11	45	46
	±S.D.	5	7	3	3	10	11	3	10	4	5	11	12
CCK-OP 20.0	Mean	15	31	15	9	52	44	11	32	16	12	45	38
	±S.D.	4	9	5	3	18	13	4	10	5	4	21	11
CCK-OP 200.0	Mean	39^{bz}	48	17^{a}	12	97^{cz}	46	28^{cz}	46	18	13	32	64
	±S.D.	16	19	6	4	18	17	8	17	7	4	8	18
CCK+OP 2000.0	Mean	58^{cz}	79^{cz}	33^{cy}	22^{ax}	146^{cz}	68^{a}	42^{cz}	80^{cz}	26^{b}	22^{ax}	96^{cx}	97^{cz}
	±S.D.	18	22	9	10	38	17	14	23	9	6	31	24
Cerulein 1.0	Mean	18	8^{c}	9	6	56	52	24^{c}	8^{a}	17^{a}	14	36	49
	±S.D.	7	2	3	2	12	16	4	3	4	6	9	17
Cerulein 10.0	Mean	48^{cz}	12^{c}	16	15^{ay}	63	76^{b}	18^{a}	12	26^{c}	18	32	56
	±S.D.	12	4	6	4	19	21	6	5	7	8	12	18
Cerulein 100.0	Mean	10	22^{y}	7	24^{cz}	68^{a}	64	9^{z}	23^{y}	11^{x}	25^{a}	57	55
	±S.D.	4	10	3	7	16	19	2	8	3	8	18	21

Explanations as in Table 1.

Table 5B. Duration of the 'minute rhythm' inhibition in the upper and more distal jejunum by the cholinergic blockade applied alone and by the cholinergic blockade followed by cholecystokinin peptide administration in rams.

		Jejunum 1						**Jejunum 2**					
		Atropine		**Pirenzep.**		**Hx**	**At 0.1 + Hx 2.0**	**Atropine**		**Pirenzep.**		**Hx 2.0**	**At 0.1 + Hx 2.0**
		0.02	**0.1**	**0.02**	**0.1**	**2.0**		**0.02**	**0.1**	**0.02**	**0.1**		
No CCK peptide	Mean	9	19	16	10	42	45	13	23	14	16	54	36
	±S.D.	3	6	5	4	14	12	5	11	7	7	10	11
CCK-OP 20.0	Mean	10	31	16	12	33	26	34^{c}	54^{a}	24	17	44	43
	±S.D.	3	10	6	3	11	7	6	16	9	6	18	11
CCK-OP 200.0	Mean	29^{cz}	47^{b}	19	24^{cz}	48	42	29^{b}	66^{c}	29	30^{ax}	66	66
	±S.D.	11	18	6	4	18	14	8	20	12	7	15	21
CCK-OP 2000.0	Mean	43^{cz}	80^{cz}	25	46^{cz}	96^{cz}	62^{z}	47^{c}	84^{c}	26	45^{cz}	105^{ax}	106^{cz}
	±S.D.	15	21	8	12	24	17	16	25	8	13	41	32
Cerulein 1.0	Mean	33^{c}	9^{a}	11	9	34	28	38^{c}	10	16	22	54	52
	±S.D.	12	3	3	3	9	9	10	3	5	8	17	12
Cerulein 10.0	Mean	30^{c}	13	25^{z}	18^{x}	61^{x}	61^{z}	27^{a}	15	36^{a}	39^{b}	84^{a}	87^{cx}
	±S.D.	11	5	6	6	19	21	9	5	14	13	21	22
Cerulein 100.0	Mean	26^{c}	24^{y}	46^{cz}	25^{ay}	86^{cz}	99^{cz}	31^{a}	24^{y}	42^{by}	38^{b}	119^{cz}	106^{cz}
	±S.D.	8	8	14	9	14	28	11	7	18	12	26	33

Explanations as in Table 1.

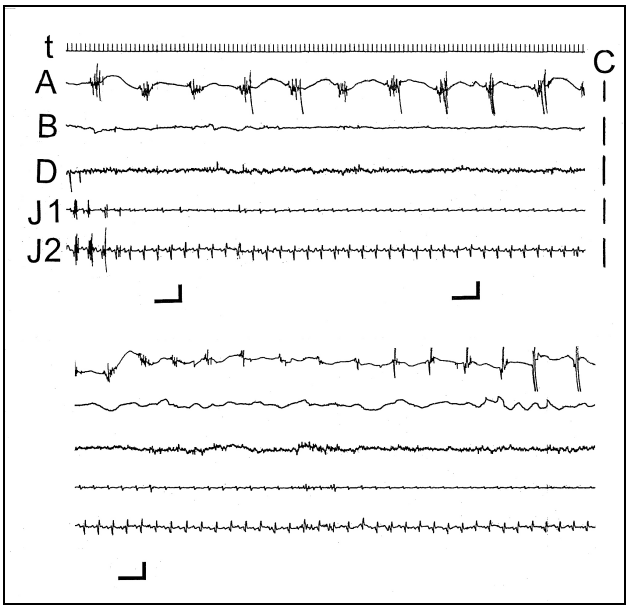

Figure 5. The effects of combined muscarinic-nicotinic blockade followed by cholecystokinin octapeptide (CCK-OP) administration upon the myoelectrical activity of the ovine abomasal antrum, duodenum and jejunum.
Upper panel: adminstration of atropine at the dose 0.1 mg/kg (left bar) and hexamethonium at the dose 2.0 mg/kg (right bar). Lower panel: administration of CCK-OP at the dose 2000 ng/kg (marked). Note the partial inhibition of antral spike burst by anticholinergic drugs and complete inhibition by CCK-OP. The inhibition of the intestinal myoelectrical activity is seen both after cholinergic blockade and CCK-OP administration.
Symbol explanations as in Figure 1.

4. DISCUSSION

The results indicate that CCK profoundly contributes to the control of motility of the ovine abomasal antrum and upper small bowel, and its effects can be efficiently mediated by the cholinergic system. In the abomasal antrum, inhibitory effects were evoked primarily by cholinergic blockade. In sheep, the influence of At and other anticholinergic drugs on the antral spike bursts and contractions is limited as it was observed in the present and previous study [35]. Similar observations were reported in man and dog [30, 37]. Wong and McLeay [38], in the *in vitro* study on ovine antral smooth muscle preparations, did not observe any influences of At or Hx upon the spontaneous contractions. As it was found in the present study, when the anticholinergic drug administration was followed by CCK injection, inhibition of antral spike bursts was much longer than after cholinergic blockade applied without subsequent CCK administration. These effects were also more distinct than the effects of both CCK peptides administered without cholinergic blockade although they were also inhibitory [9, 39]. Thus it is clear that in ovine abomasal antrum, CCK exerts inhibitory effect what was observed also by others [7]. Antral response to CCK is not the same in sheep and dog in which it can be stimulatory [29, 40]. Other studies confirmed further the presence of marked species differences. When CCK was given intraarterially *in vivo* or during *in vitro* studies with the canine antral muscle, it also exerted stimulatory effect [37, 41]. In man, the reported effects of CCK on antral motility are controversial. Its stimulatory effect *in vitro* was confirmed *in vivo* by the inhibitory action of loxiglumide, the CCK receptor antagonist, although the suppressive action of CCK on human antral motility was observed as well [30, 42, 43]. In rats, stimulatory, inhibitory or the lack of the effect was denoted [44, 45]. In the guinea pig, stimulatory action of CCK seems to predominate although the presence of dual effect was also described [46-48]. The effect of CCK on antral motility is, thus, distinct in sheep what suggests that the mechanism of CCK action might be somehow different from that observed in other species. Moreover, the obtained results show that in sheep CCK amplified inhibitory effect evoked by the cholinergic blockade. This effect of CCK was dose-dependent, at least in part, and it also seems to be additive to the effect induced by cholinergic blockade. The existence of cooperation of CCK with acetylcholine has been described [1], but it seems improbable during the efficient cholinergic blockade. This cooperation may concern rather stimulatory than inhibitory action of CCK. The effect of CCK on the ovine gastrointestinal motility can be dual [13, 49], thus it is possible that in the present study the anticholinergic drugs hampered exclusively the stimulatory component of CCK action prolonging the inhibitory effect. At least three pathways of CCK action on antral motility under cholinergic blockade can be considered, however. CCK might be able to evoke the inhibitory effect rather independently of the cholinergic system and this effect could be local and direct on the smooth muscle that represents first possibility.

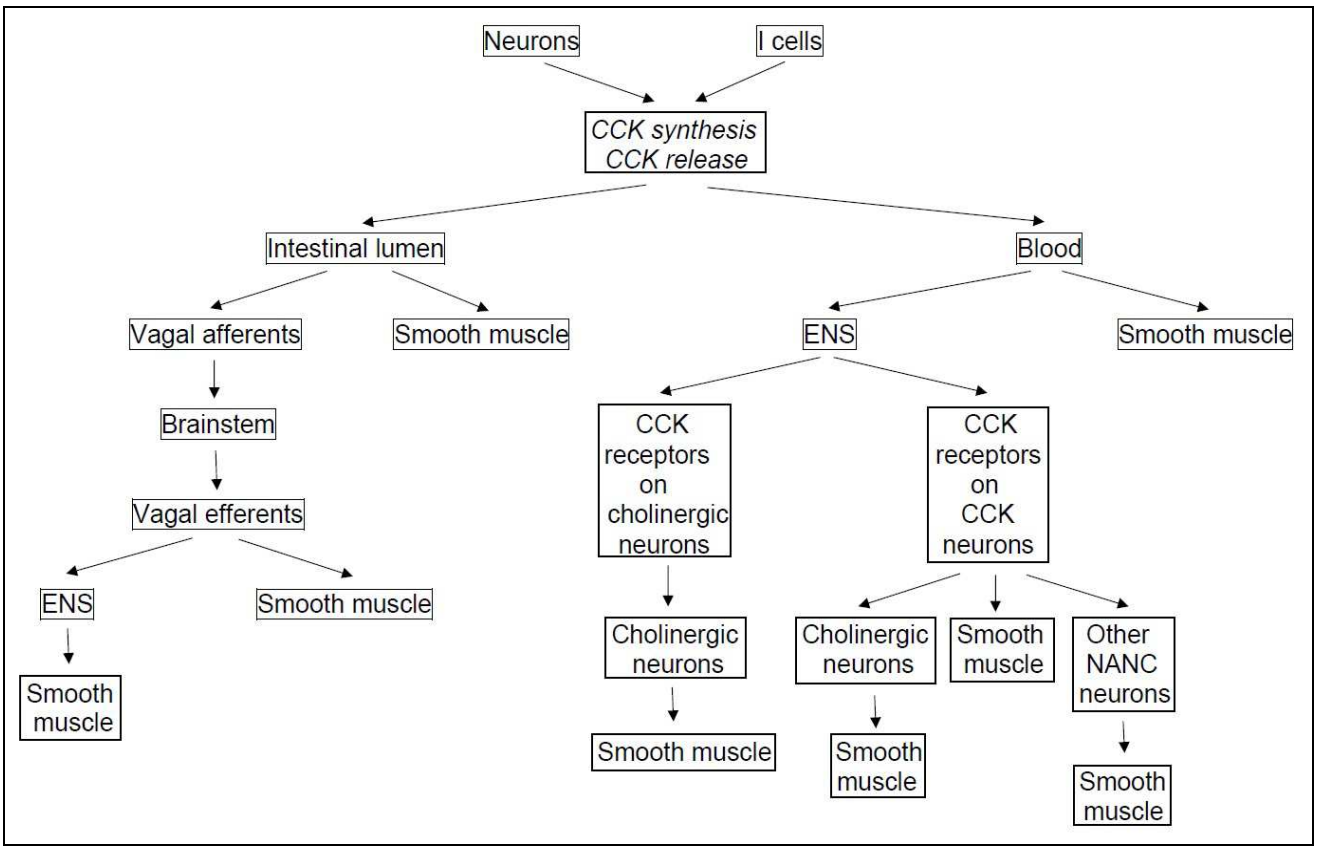

Figure 6. Proposed mechanisms of CCK actions on gastrointestinal motility. Other explanations see text.

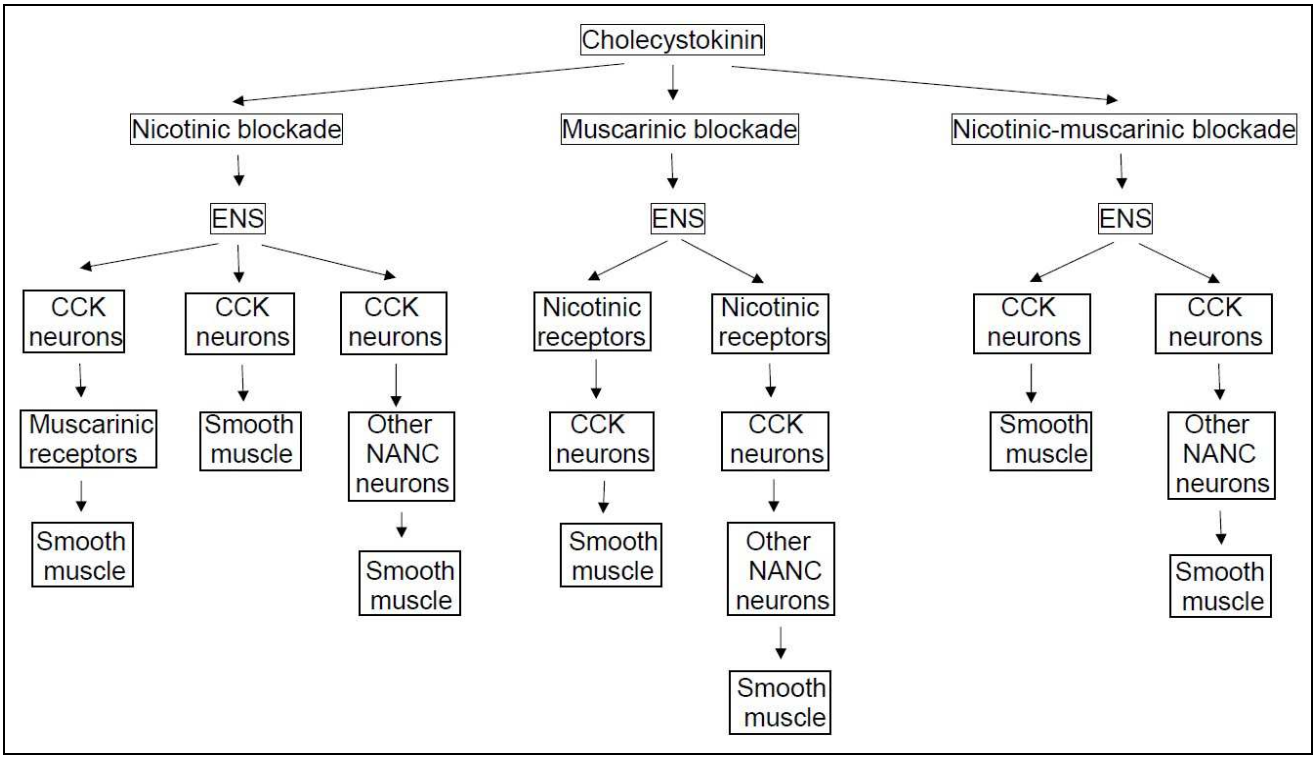

Figure 7. Proposed neuronal CCK actions on gastrointestinal motility during various types of cholinergic blockade. Other explanations see the text.

CCK can also act as a neuromodulator what represents second possibility [28, 50]. Third possibility occurs when the action of CCK could be amplified by inhibitory effect of such hormone as somatostatin released by CCK from the ovine antrum [51]. Some possible mechanisms of CCK action on gastrointestinal motility are also summarized in Figure 6. Similarly to CCK-OP, the inhibitory effect can also be triggered by cerulein confirming the involvement of the same or similar evoking mechanisms [9, 52].

CCK exhibits high affinity to both CCK receptors, CCK-1 and CCK-2 [2]. CCK engages CCK-1 receptor acting centrally on gastric motility in sheep [53]. While in ovine omasum, CCK-1 receptor mediates the action of CCK, in the abomasal antrum, CCK-1 receptor antagonist did not alter the myoelectrical activity [54, 55]. It is thus likely that CCK action in antrum involves CCK-2 receptor and is local. This receptor can be present in antrum since pentagastrin, acting principally via CCK-2 receptor, inhibited antral motility in sheep and also in calves [56, 57, see also 2]. However, it remains unclear whether CCK acted as the gut hormone and/or as neuromodulator.

In the small intestine, Pi used at the lower dose, evoked stimulatory effect, i.e. the premature phase 3, which arrived during short inhibitory period. This finding was also reported in sheep earlier [58]. Since Pi used at the smaller dose evoked the premature phase 3 only in some of the animals studied, it seems likely that its action *via* M_1 cholinergic receptor subtype is not entirely specific comparing with the actions of another selective anticholinergic drug, telenzepine [59-61]. The action of the smaller dose of Pi upon the MMC was stimulatory, but Pi at the higher dose and other anticholinergics inhibited the MMC in sheep and in other species including dog that was reported also by others [26, 27, 62, 63]. Both CCK and cerulein inhibited phase 3 and the whole MMC pattern in sheep and similar effect was observed in other species like dog in response to CCK administration [7, 10, 11,15]. The CCK peptide, applied even at the smallest dose after Pi, converted the premature phase 3 to rebound excitation. Both the mechanism and physiological meaning of this event are unknown. After At, Hx and the higher dose of Pi given alone, the rebound excitation was observed and also described earlier [32, 64]. When CCK peptide administration was followed by the cholinergic blockade, no rebound excitation was observed. Thus, even the small doses of the hormone can prevent undesired (stimulatory) actions of atropine when used, for example, during the intestinal surgery. Since the rebound excitation was regularly evoked during cholinergic blockade, it appears that the non-adrenergic non-cholinergic (NANC) stimulatory neurons underlie its triggering mechanism. In the course of the cholinergic blockade, the vagus-dependent inhibition may be alternated by the stimulation *via* vagal efferent NANC nerves or by other NANC neurons located in the enteric nervous system [65, 66]. Therefore, CCK might be able to exert central, but also peripheral neuronal stimulatory action upon the gastrointestinal tract when the cholinergic receptors are blocked. Stimulatory effect of CCK on duodenal motility in sheep is also consistent with the observations in other ruminant species like calves, in which the CCK receptor antagonist, tarazepide, depressed the duodenal myoelectrical activity [67]. This also concerns the dog, cat and guinea pig [41, 68-70]. It was found in the present study that CCK evokes biphasic or other inconsistent effects upon the ovine small bowel motility what was also observed previously in the rat and sheep [13, 44, 49, 71, 72]. In man, the results are also contradictory. While the *in vivo* study revealed the inhibitory influence of CCK-8 on duodenal motility, administration of CCK antagonist, loxiglumide, decreased the total number of duodenal contractions [30, 43]. In the jejunum, CCK is stimulatory in man, dog and rat [29, 73, 74]. Stimulatory effect could be exerted by direct action of CCK on the small intestinal smooth muscle. It was found that during luminal perfusion of the small bowel by decanoic acid, the CCK-releasing factor, the segmental-type of motor activity was induced [75]. When, during *in vitro* study, CCK was applied, it evoked the ejective pattern [76]. After CCK, jejunal segment ejected fluid bidirectionally, thus the motility pattern evoked by CCK exhibited rather stationary character. After the cholinergic blockade, stimulatory effect of CCK in the small bowel was greatly reduced as compared with the experiments engaging CCK alone, what was observed both in the present and previous studies [11, 13].

The highest dose of CCK peptide, preceded by Hx administration, produced considerably longer spike burst inhibition than Hx given alone. This suggests that the efficient cooperation between CCK and nicotinic cholinergic receptors exists in the gut. Since duration of the spike burst inhibition in the duodeno-jejunum after combined nicotinic-muscarinic blockade followed by CCK administration was the longest, the effects might be additive, at least in part. It has been established that the nicotinic receptors are located in the intramural ganglia of the gastrointestinal tract while the muscarinic receptors are located more distally, mainly on the smooth muscle cells [66]. Therefore, the muscarinic cholinergic blockade inhibited this regulatory pathway although the nicotinic blockade was more effcient since it could block also the non-cholinergic stimulatory neurons. The concept of command (cholinergic) neurons can illustrate this phenomenon further [66].

It was found herein that cholinergic blockade delayed the appearance of phase 3 of the MMC in the small bowel. First phase 3 that arrived afterwards was ectopic and originated from the jejunum. These effects were also earlier described in sheep [63, 64]. The cholinergic blockade is more efficient than vagotomy in the MMC inhibition [22, 77, 78]. CCK is known to exert similar effect, not only in sheep [11, 15]. When CCK was administered after cholinergic blockade, the inhibition of phase 3 of the MMC was prolonged. Therefore, in the small bowel CCK amplified the effect evoked by cholinergic blockade and the question arises whether this effect is additive or synergistic, at least in part. Duration of the inhibitory period was related to the CCK dose, type of cholinergic blockade and region examined. It was reported that CCK and acetylcholine potentiate mutually their effects both in the stomach and in small bowel [3, 79]. When CCK, given under cholinergic blockade inhibited the gastrointestinal motility, especially of phase 3 of the MMC, its action was rather independent of the direct acetylcholine influences. It cannot negate the presence of cooperation between cholinergic and CCK-related mechanisms in the control of gastrointestinal motility, however. In monogastrics, CCK may inhibit contractions in the duodenum acting simultaneously *via* CCK-1 and CCK-2 receptors [18]. It is uncertain whether the same may also occur in sheep. It seems likely that the long inhibition of phase 3 in the duodenal bulb, observed in the present study, occurred because phase 3 in the duodenal bulb of sheep is often absent or reduced. This was also observed previously [80]. The normal (non-ectopic) phase 3 of the MMC originates in ewes most frequently from the duodenum [81]. The duodenal bulb represents the region distinct from the remaining part of the duodenum in sheep [80, 82]. When in sheep, CCK was given alone, it inhibited phase 3 of the MMC for the period shorter than that after the combination of CCK with the anticholinergic drug [63]. First phase 3 of the MMC that arrived following CCK, administered after cholinergic blockade, was also ectopic (it started from the jejunum). Therefore, the CCK-dependent inhibitory mechanisms may cooperate with cholinergic mechanisms, perhaps also during partial cholinergic blockade. Duration of phase 3 inhibition in the jejunum was shorter than that in the duodenum. Most pronounced effects were observed in the jejunum when application of the highest dose of CCK peptide was preceded by nicotinic or nicotinic-muscarinic blockade. Thus the effect of CCK upon the MMC appears to be evoked principally *via* CCK receptors located within the enteric nervous system (both CCK 1 and CCK 2 receptors, see [2]), possibly in the cooperation with other (maybe central) neurons. The direct action of CCK on the small intestinal smooth muscle also cannot be excluded although it appears more feasible in the control of the spike bursts than in the control of the MMC. CCK can be released from I cells located in the duodenum and jejunum [1]. In ruminants, presence of I cells in the small bowel is questionable although CCK can be released from this region as CCK-OP [83]. During the cholinergic blockade, circulating CCK was probably unable to act *via* the central nervous system. It was reported that CCK is not able to cross the blood-brain barrier [25] thus it seems likely that peripheral CCK cannot act centrally. Whether this is really true or not in various animal species is not known since it was demonstrated in rats that peripherally administered CCK acted on the brain stem neurons [84]. However, it has been recognized that CCK, most probably released from the peripheral neurons and/or from the I cells, can evoke the discharge of

vagal neurons acting through CCK-2 receptors located on vagal afferents, while both CCK receptors are present in vagus nerve [85]. Stimulation of vagal afferents enhances neuronal transmission in the nucleus of the solitary tract, activates central CCK-1 receptor pathway and possibly also acts in other centers of the brain. These actions may disrupt the MMC [86]. It was also reported that capsaicin affected CCK action on gastrointestinal motility in rats that confirms further that this mechanism exists [72, 87]. Therefore, peripheral CCK may act centrally omitting the blood-brain barrier at least in some species (see Figure 6).

The inhibition of the MR in the duodenal bulb was longer than in the duodenum suggesting that the latter region represents the main site of MR initiation. Although the MR undergoes cholinergic influences what was found in this and previous studies [23, 28], almost nothing is known as to the localization and contribution of the cholinergic receptor subtypes involved in the control of the pattern. At given intracerebroventricularly in rats remained without effect upon the MR evoked centrally by naloxone [88]. Thus, the character of central control of the MR remains unclear. When CCK peptide injection followed the nicotinic blockade, the highest dose of CCK-OP was the most effective in the MR inhibition observed in the duodeno-jejunum. Cerulein often exerted more pronounced effect in the jejunum than in the duodenum. These differences between the effects evoked by both CCK peptides suggest that the mechanism of action of these CCK peptides in the gut may be similar, but not be the same. Presented results indicate that CCK, exerting its action under cholinergic blockade, is able to inhibit the MR appearance while the nicotinic blockade is more efficient than the muscarinic blockade (see Figure 7). It seems likely that the mechanisms controlling the MR in the small bowel can be similar to those controlling the arrival of the spontaneous spike bursts.

Both lower doses of CCK-OP used in the study were physiological. The highest dose also appeared to remain within the physiological range, perhaps at its upper border [39]. When CCK exerts the inhibitory action on the gastrointestinal motility *via* neuronal pathway, the greater dose of exogenous hormone may be required. Therefore, the highest dose of CCK could be treated as the physiological one. This may also depend upon the site of CCK action. When CCK acts as a gut hormone its physiological dose can be greater than when it acts as a neuromodulator. In sheep it is an unexplored question while it appears that both these pathways can be taken into account. Cerulein doses used in this study, i.e. 20 times lower than that of CCK-OP, appeared to be relevant to the doses of CCK-OP, although it was suggested that cerulein is only 8-15 times times stronger than CCK-OP [see 14]. The long inhibition of phase 3 of the MMC by combined actions of both the anticholinergic drugs and CCK may result also from the cooperation with other regulators like gastrin and somatostatin acting centrally or peripherally [19, 89, 90]. Both these hormones inhibit the arrival of phase 3 of the MMC [56]. The release of somatostatin from the upper gastrointestinal segments is possible in this situation, since it may be independent of the cholinergic system. This view is based upon the observation of Bell et al. [4] that in the calf somatostatin secretion was not blocked by vagotomy. Furthermore, the cooperation of CCK with other inhibitory regulators as opioids and with some other, like secretin, glucagon, VIP and GIP cannot be excluded [79, 91].

5. CONCLUSIONS

It is concluded that in sheep:

1) cholinergic system modulates CCK action upon the gastro-intestinal motility,
2) inhibitory actions of CCK upon the gastro-intestinal motility, observed after cholinergic blockade, were dose-dependent,
3) CCK, acting under cholinergic blockade, prevents the arrival of normal and premature phase 3, ‘minute rhythm and rebound excitation in the gut,
4) cooperation between the cholinergic system and CCK, regarding the inhibition of the gastrointestinal motility, is most efficient when the nicotinic receptors are involved,
5) following the application of cholinergic blockade the effects of cerulein upon the gastrointestinal motility were comparable with those of CCK-OP,
6) mechanism of pirenzepine action on gastro-intestinal motility is dose-related,

7) the question whether CCK acts as a hormone or as neuromodulator still remains unclear.

TRANSPARENCY DECLARATION

The author declares no conflicts of interest.

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Research Article

Leaf biomass and leaf area equations for three planted trees in Iran

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ABSTRACT

Leaf area (LA) and leaf biomass (LB) are important variables for most physiological, horticultural and agronomic studies involving plant growth, development rate, radiation use efficiency, and water or nutrient use. Measuring these variables need destructive and aggressive sampling. Fortunately, evolving allometric equations can help for low cost and non-destructive estimation of such variables. The aims of this study are Estimate, compare and develop allometric models of LA and LB per tree and per stand for *Alnus subcordata* (AS), *Populus deltoides* (PD) and *Taxodium distichum* (TD) plantations. We selected 12 sample trees in each stand. Leaf Fresh weight of randomly selected branches was weighted in the field. Branch-level LA was modeled as a function of branch diameter ($R^2 > 0.8$) and total fresh weight of LB has been calculated for each sampled tree. For each species, 100 leaves from all canopy directions of trees were randomly selected and transported to the laboratory. At the lab, leaf area has been measured using leaf area meter. Allometric equations were derived using regression analysis. For all species, derived equations showed high accuracy (R^2 ranged from 0.837 to 0.947). However, with respect to mean square error, power regression equations (individual leaf area = a(L×W)b and LA or LB = a DBH^b) are best models to estimate Individual Leaf Area, LA, and LB of AS, PD, and PD. The highest LAI was in the order of 16.9 > 5.5 > 4.5 for AS, PD, and TD, respectively.

Keywords: Non-destructive sampling; Regression analysis; Broad-leaved; Needle-leaved.

1. INTRODUCTION

Forests play a major role in the flow energy and material exchange between the land and atmosphere [1]. Leaf surface, as the main exchange surface, is a key important indicator in biological studies [2]. Leaf surface area and leaf weights are two important factors that affect many tree and stand-level processes and functions (including photosynthesis, gas exchange, growth, development rate, radiation use efficiency, water and nutrient use, nutrient cycling stand productivity and canopy dynamics [3, 4-6].

Leaf Area Index (LAI) is one of the most important parameters for analyzing the structure of the canopy; because it explains the response of plants to the environmental condition of sites, whether these changes are natural or anthropogenic [7, 8]. LAI is defined as the total area of leaves per

unit area of land, leaf area (LA) is defined as the one-sided projected surface area [3, 4, 9], and leaf biomass (LB) is the total dry weight of leaves of a tree. LA and LB estimation methods are divided into two direct and indirect categories [7]. In the direct method, the leaves surface and dry weight are measured directly. The major limitations of these methods are being costly and destructive. The fact that makes indirect methods more popular among scientists.

Indirect method models the relationship of these attributes to those which are readily measurable. This approach models LA or LB based on one or more easily measured dimensions of the tree, which makes the method cheap, rapid, reliable and non-destructive [10]. There is a general relationship between the amount of LA, diameter at breast height (DBH), height (H), and biomass production. DBH is the most commonly used variable for modeling LA and LB [11], while some researchers reported that LB and LA estimation has been significantly improved by adding variable relating to the crown structure to the models [12].

Adl [13] estimated LB and LAI of *Quercus brantii* and *Pistacia mutica* in Zagros forests. LB for *Quercus brantii* and *Pistacia mutica* were 1317.3 and 57.2 (kg ha^{-1}), respectively and mean LAI was 1.20. Babaei Kafaki et al. [14] estimated LAI and LB (3.33 and 1864 kg ha^{-1}, respectively) for *Quercus macranthera* coppice stand at the northeast of Khalkhal in Iran. Pourhashemi et al. [15] estimated LB and LAI (37 kg and 3.7, respectively) for *Celtis Caucasica* using direct method (leaf gathering from the crown) in the oldest urban forests of Sanandaj city in Iran. Also, the compound variable, $DBH^2 \times H$, was the most efficient factor to estimate the LB (R^2=0.69), but in LAI equation, the root of DBH was the best variable (R^2=0.72). Kumar Sarker et al. [16] developed and compared 16 different allometric equations based tree DBH and H for predicting LA and LB of *Artocarpus chaplasha* in Bangladesh. Their result showed that the models based on only single predictor of tree DBH have more statistical accuracy among all models. Rance et al. [17] reported that the LA and LB are related to stem cross-sectional area. However studies about LA and LB are scarce, and model quantification of LA and LB for fast-growing trees is even rarer. The aims of this study are Estimate, compare and develop allometric models of LB and LA per tree and per stand for three native and introduced tree species in northern Iran.

2. MATERIAL AND METHODS

2.1. Site description

The study site is located on the southern coast of the Caspian Sea, 10 Km from Amol city, north of Iran 36˚35′N″, 52˚10′E″, 5 m above sea level. Rainfall with wetter months occurs between September and March, and a dry season occurs from April to August. The climate is temperate, with a mean annual temperature of 16.9 ˚C and a mean annual precipitation of 823.5 mm. The soil of plantations is poor drainage and has a silty loam texture with pH 7.6-8.1.

2.2. Stand description

The study site was established in 1992 with the aim of wood production using three rapid growth species including alder (*Alnus subcordata* C.A. Mey) (AS), eastern cottonwood (*Populus deltoides* Bartr. Ex Marsh) (PD) and bald cypress (*Taxodium distichum* L.C. Rich) (TD). The site where previously covered by natural stands dominated by *Carpinus betulus* and *Parrotia perssica* [10]. Tree spacing in all stands is 4×4 m. No thinning operations were made in these plantations.

2.3. Sampling design

36 sampling plots with dimensions of 16×16 m were selected randomly in each of the three stands [18]. In each plot, the DBH of individual trees was measured with calipers, the crown width (CW) measured with a tape, height (H) and the crown height (CH) was measured with a Haglöf-VERTEX IV clinometer. The average values of tree DBH, H, CH, and CW of stands are summarized in Table 1.

The diameter range of all trees of each species was divided into 12-diameter size classes (class size was 3 cm for all species). From every size class, one representative tree was selected and destructively sampled. In total, 36 trees were sampled; 12 trees per species. The trees were felled down with a

chainsaw. Prior to branch removal, the diameter of each branch was measured and five representative branches from the smallest to the largest branch throughout the crown were sampled. All branches were then clipped from the tree, and the leaves were collected in order to obtain total fresh weight at the site using a hanging scale to the nearest 0.1 kg. Three samples of 200 g of leafs were collected from each tree and taken to the laboratory in sealed plastic bags, dried at 70 ºC until achieving a stable weight. Additionally, 100 leaves per species were collected in randomly and were transferred to the laboratory and individual LA, leaf length (LL) and leaf width (LW) were measured with a leaf area meter (type CI-202, USA; see Table 2). Subsequently, the leaves were dried in an oven at 65 ºC until they reached a constant dry weight and then weighed on a scale with a precision of three digits. All measurements were made in late of the growing season.

Table 1. Summary characteristic of Stands, Diameter at Breast Height (DBH), Height (H), Crown Height (CH), Crown Width (CW).

Species	Variable	Mean	Standard Deviation	Minimum	Maximum	Skewness	Kurtosis
AS n/ha = 459±51	DBH (Cm)	31.32	7.70	15.92	50.96	0.39	-0.32
	H (m)	21.99	4.40	10.10	31.80	-0.16	-0.35
	CH (m)	8.91	2.70	2.90	16.40	0.37	-0.28
	CW (m)	4.02	1.39	1.20	7.35	0.21	-0.65
PD n/ha = 557±38	DBH (Cm)	27.43	6.36	12.42	43.95	0.00	-0.25
	H (m)	29.96	4.96	16.90	44.20	-0.21	0.34
	CH (m)	11.54	3.41	4.00	20.90	0.14	-0.35
	CW (m)	3.145	1.22	1.00	5.850	0.15	-0.79
TD n/ha = 557±48	DBH (Cm)	28.45	6.86	11.27	47.05	0.01	-0.43
	H (m)	17.74	2.67	9.80	24.10	-0.33	0.48
	CH (m)	6.67	1.95	1.10	14.90	0.24	1.58
	CW (m)	4.25	0.96	1.48	6.40	-0.24	0.13

Table 2. Means ± standard error (SE), minimum (min) and maximum (max) values for the leaf length (L), width (W), leaf area (LA) and length (L) × width (W) of the AS, PD, and TD.

Species	L (Cm)			W (Cm)			LA (Cm^2)		
	Mean±SE	Min	Max	Mean±SE	Min	Max	Mean±SE	Min	Max
AS	9.0±0.3	2.3	19.9	7.8±0.2	4.5	12.3	54.9±3	12.7	188.9
PD	10.6±0.2	4.2	15.4	10.4±0.2	4.6	13.8	73.8±3	12.8	130.7
TD	4.1±0.1	2.1	7.9	1.9±0.1	0.7	3.6	3.3±0.2	0.5	8.9

To estimate LA and LB per hectare, we first calculated the LA (m^2) and LB (Kg) by applying the corresponding adjusted allometric equation to all the trees in the plot area. Considering a sampling plot with an area of 256 m^2, the LA and LB per hectare was obtained for every species.

2.4. Statistical analysis

DBH of the harvested trees was used to prepare a simple linear and power function fitted to the data using least square regression. The model with the best goodness of fit was selected based on by SE (standard error) and adjusted R^2 (coefficient

of multiple determination). For comparing LB and LA of different plantations, one-way ANOVA was applied. The ANOVA was followed by a Dunnet-test to separate the species and the different leaf characteristics. All differences were considered significant at $P<0.05$. All statistical analysis were conducted on SPSS software, ver. 19.0 (SPSS Inc., Chicago, II., USA).

3. RESULTS AND DISCUSSION

3.1. Predict leaf area

The regression analysis showed that most of the variation in LA values was explained by "length × width" as the predictor variable (Table 3). The best fitting equations (showing the highest coefficient of determination, and the lowest standard error) were obtained for the individual leaf area by power model within all planted trees.

Figure 1 presents the relationships between LA (cm^2), L×W and distribution of the residuals for each species. Visually, there is a good correlation between predictors and dependent variable for all species. For all species, based on the distribution of residuals, heteroscedasticity of residuals is obvious.

Estimating LA through measuring leaf dimensions has been an interesting subject for many researchers. Most of these researchers used leaf length, leaf width, or combinations of these variables as predictors in allometric models [19, 20]. In some scientific work, it is not possible to measure these variables destructively, because the research needs to be continued while the plant is performing its functions. In this study, we used leaf length and leaf width combinations to establish allometric models for predicting leaves variables. Results from the present study were in accordance with some of the previous studies (including ref. [13, 19, 22]) on establishing reliable equations for predicting LA through measuring leaf dimensions. Some researches, as well as our research, showed that LA and LB of leaves can be predicted using leaves dimensions with high accuracy.

3.2. Allometric equations for tree leaf area and LB

The average values for LA and LB of AS, PD, and TD trees are summarized in Table 4. The LA was 312.3 m^2 for AS, 85.3 m^2 for TD and 46.8 m^2 for PD. In addition, The LB was 30.89, 13.94, and 5.09 Kg for AS, PD, and TD, respectively (Table 4).

Table 3. Fitted coefficient (b) and constant (a) values of the models used to estimate the individual leaf area (LA in cm^2) of single leaves from the length (L) and width (W) measurements. A coefficient of determination (R^2), mean square errors (MSE), and F of the various models are also given. L and W are in cm.

Species	Model	*a*	*b*	R^2	MSE	F_{calc}
AS	LA= a(L×W)+b	0.746	-0.319	0.977	4.289	4071.25^{***}
	$LA = a(L\times W)^b$	0.778	0.988	0.982	3.117	5253.99^{***}
PD	LA= a(L×W)+b	0.638	1.715	0.975	4.052	3891.84^{***}
	$LA = a(L\times W)^b$	0.636	1.005	0.972	3.873	3435.64^{***}
TD	LA= a(L×W)+b	0.329	0.661	0.876	0.560	686.50^{***}
	$LA = a(L\times W)^b$	0.561	0.855	0.923	0.243	1153.89^{***}

Table 4. Means ± standard error (SE), minimum (min) and maximum (max) values for LA and LB of the AS, PD, and TD.

Species	LA (m^2)			LB (Kg)		
	Mean±SE	Min	Max	Mean±SE	Min	Max
AS	312.3+21	51.5	750	30.89+2.1	5.19	75.71
PD	46.8+3.4	4.6	97.3	5.09+0.31	0.52	11.15
TD	85.3+7.1	19.5	263.3	13.94+1.2	2.19	46.31

Table 5 summarizes the results of modeling LA and LB based on power equations. For all species and variables, the models were highly significant (*P-value* < 0.001). Coefficients of determination showed a strong relationship between tree DBH and LB (ranges from 0.837 to 0.947) for all species. Figure 2 presents the relation ships between individual leaf area (cm^2), L×W (Cm), and distribution of the residuals for each species.

Based on multiple means comparison results, there were significant differences between LAI of the different plantation. Mean LAI of AS, PD and TD are 16.9 > 5.5 > and 4.5, respectively (Table 6). LB was also different significantly between species and for AS, PD, and TD and was 12032.5 > 2490.6 > and 6504.6 Kg ha^{-1}, respectively.

Table 5. Fitted coefficient (a and b) of the models used to estimate LA (m^2) and LB (kg) of a single tree from DBH (Cm). Coefficient of determination (R^2), mean square errors (MSE), and F (F_{calc}) of the various models are also given.

Species	Variable	*a*	*b*	R^2	MSE	F_{calc}
AS	LA	0.029	2.610	0.947	0.223	161.00^{***}
	LB	0.003	2.610	0.947	0.223	161.00^{***}
PD	LA	0.008	2.509	0.905	0.344	85.76^{***}
	LB	0.001	2.509	0.905	0.344	85.76^{***}
TD	LA	0.157	1.798	0.849	0.347	50.48^{***}
	LB	0.017	1.943	0.837	0.406	46.33^{***}

Table 6. Means ± Standard error (SE) values for LAI and LB of the AS, PD, and TD.

Species	LAI	LB (Kg/ha)
AS	16.95+7.65 a	12032.55±2235.84 a
PD	5.52+2.64 b	2490.62±201.02 b
TD	4.46+2.33 b	6506.6±686.7 c

The lowercase letter shows a significant difference at 0.05 significant level.

It has been proved that using allometric equation is a better alternative for estimating those variables which need to be destructively measured; because this method is not only environmentally friendly but also cost and time effective [23]. The results of this study showed that LA and LB can be estimated using easily measured tree variables such as diameter at the breast; all equations were highly significant (R^2 = 0.837 - 0.947). The proposed models accounted for more than 80% of the variation based on DBH in the LA and LB models (Table 4 and 5) and provided a sound, nondestructive means to predict these canopy properties in fast growing trees. Therefore, a regression model can be a good alternative method for determining LA instead of devices of LA meter which is consistent with the findings of Calvo-Alvarado et al. [24], and Pokorný and Tomášková [25]. Socha and Wezyk [26] found that the diameter at breast height (DBH), explain more than 80% of the variation in LA and LB of Scots pine (*P. sylvestris* L.). Based on finding that reported by Grace et al. [27] and Vertessy et al. [28] DBH could explain 91% of the variation in LA of *Acacia koa* and *Eucalyptus regnans*, respectively.

Unfortunately, we could not compare our results with other similar works in similar forests because allometric equations on canopy properties are unavailable in northern Iran. However, further development of these equations through destructive methods and increased sample sizes would facilitate the development of regional estimates of LA and

LB. Finally, the results of this study could serve as a basis for more precise quantification of fast growing tree physiological and environmental processes in the plantation of northern Iran, mostly for the areas being planted by AS, PD, and TD. The founding of this study is important for the ecological purpose (including transpiration rate, biomass estimation, light interception, and carbon storage). In addition, this study is important for tree growth model of these fast growing species.

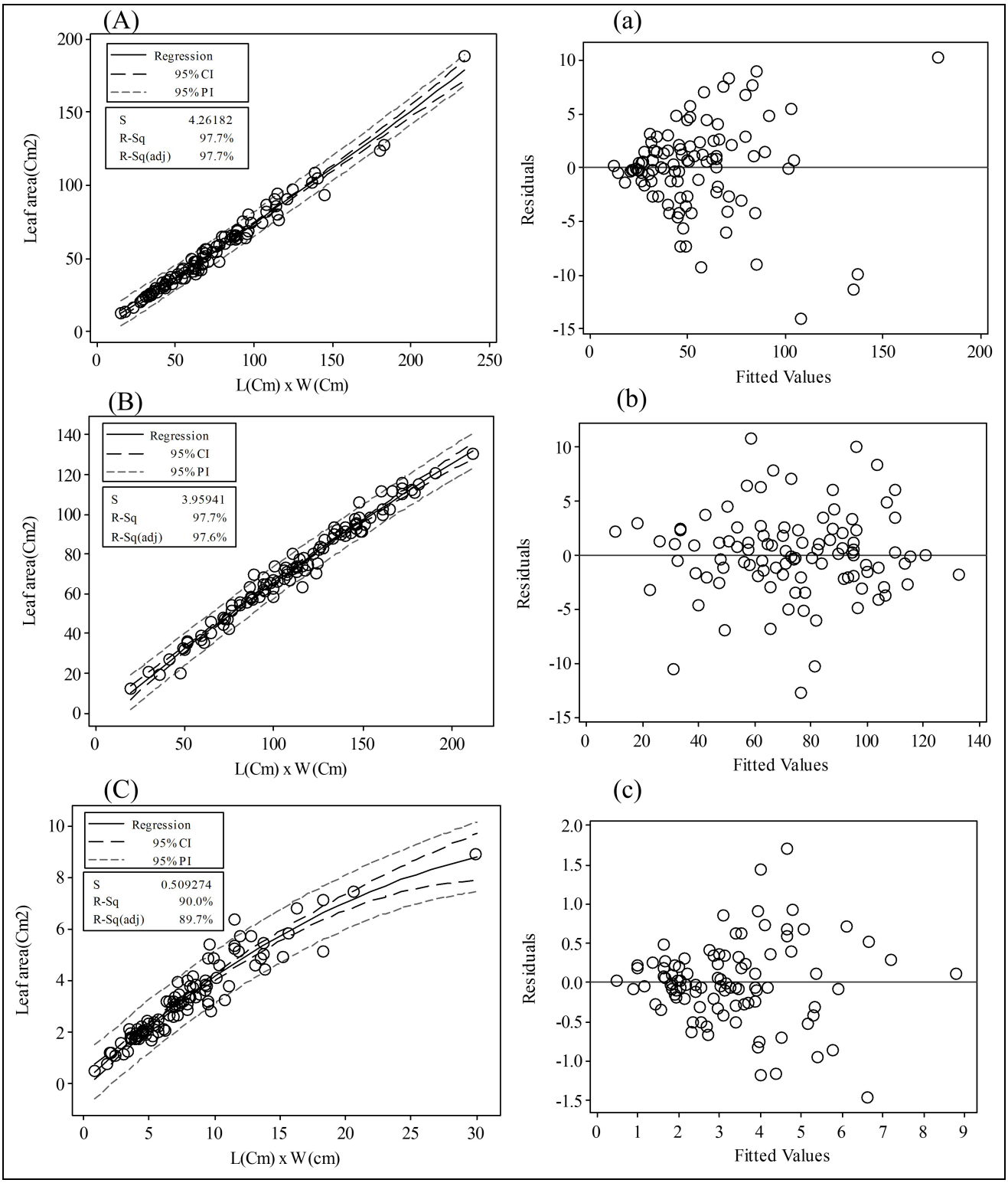

Figure 1. Relationship between individual leaf area (cm^2), L(Cm) ×W(Cm), and L×W residuals against individual leaf area (A, B, and C are *A. subcordata*, *P. deltoides*, and *T. distichum*, respectively) (n = 100).

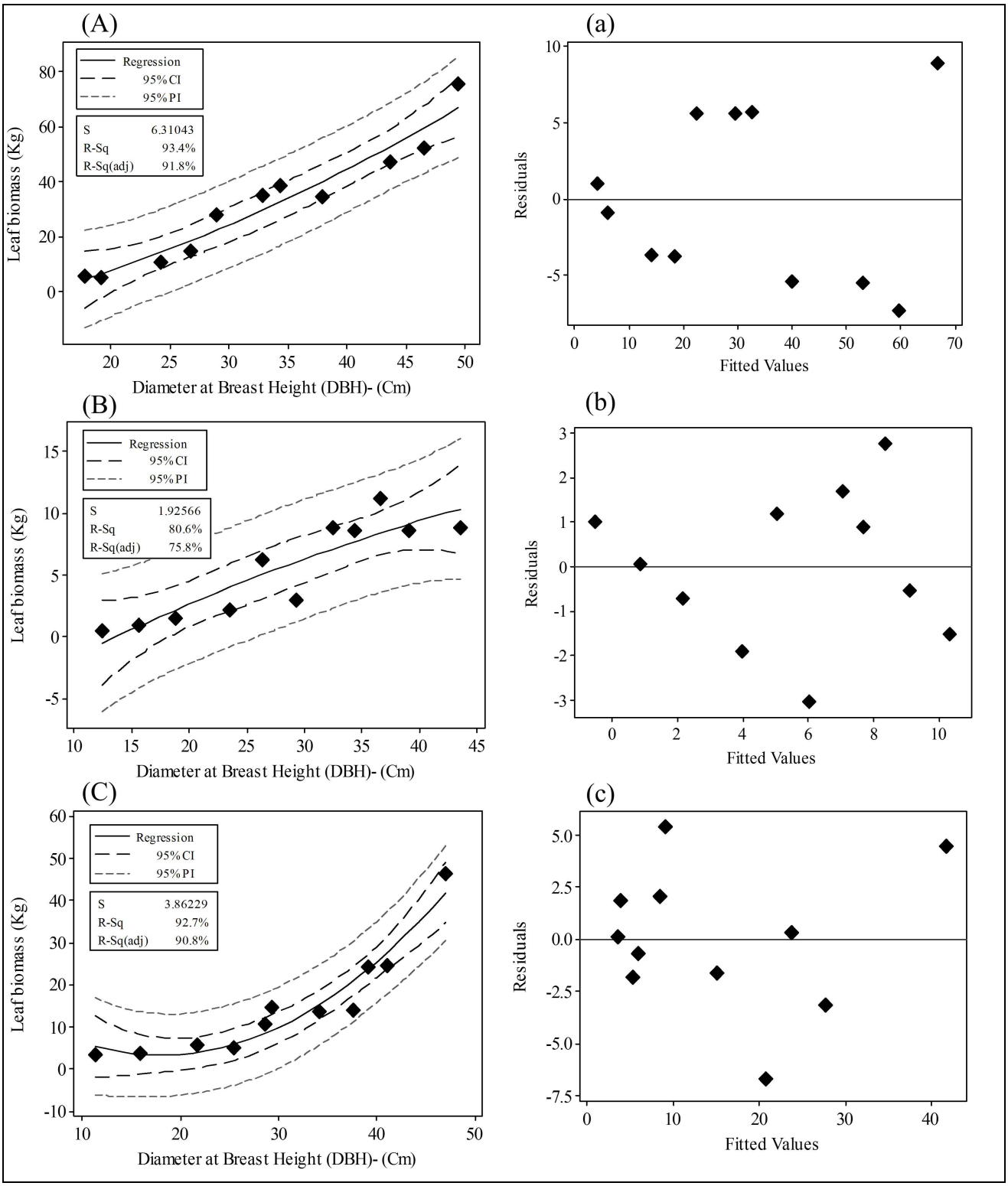

Figure 2. Relationship and residuals between DBH (X axis) and total tree LB in Kg for 12 harvested trees (Y axis) (A, B, and C are *A. subcordata*, *P. deltoides*, and *T. distichum*, respectively).

4. CONCLUSIONS

This study is one of the few reports on the allometric relationship for estimate individual leaf area from length × width (L×W) and estimate LA and LB of plantation trees in Iran. Through regression analysis, it was found that there was a strong power relationship (coefficient of determination > 0.8) between LA, LB, and DBH within each of the planted trees, with a level of

significant relationship. The analysis of mean square error between in linear and power models in three planted species show that the power regression equation (individual leaf area = a(L×W)b - LA or LB = a DBH^b) can best estimate of Individual Leaf Area, LA and LB for *Alnus subcordata* and *Populus deltoides*, and well for *Taxodium distichum*.

AUTHORS' CONTRIBUTION

JE: Field works and collecting the data, the laboratory analysis, running the data analysis, and writing the paper; HS: Designing the experiment, supervising the work, and writing the paper; SMH: Designing the experiment and supervising the work; BN: Supervising the field works and collecting the data. The final manuscript has been read and approved by all authors.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

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Research Article

Moringa oleifera Lam extract attenuates gastric ulcerations in high salt loaded rats

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ABSTRACT

Moringa oleifera Lam is a plant used extensively both in traditional and orthodox medicine to treat myriad ailments, including gastrointestinal disorders. This study was carried out to investigate the effect of leaf extract of *M. oleifera* some gastrointestinal function parameters in high salt loaded rats. Acute toxicity study was done using 70 male white mice (18-20 g) were used for the study. They were randomly selected and assigned to 7 cages of 10 animals per cage. Percentage mortalities were converted to probits and plotted against the $\log_{10}$ of the dose of the extract from which the LD_{50} value was calculated. Fresh leaf extract of *M. oleifera* was Soxhlet extracted. 24 albino Wistar rats were randomly assigned into 4 main groups of 6 rats each. Fed on normal rat chow, high salt (8% NaCl) diet + 1% NaCl drinking water and/or *M. oleifera* extract (600 mg/kg bw). The feeding regimens lasted for 42 days. Results obtained revealed that the extract had an LD_{50} value of 1,872.22 mg/kg from which a test dose of 600 mg/kg was derived for the feeding regimen. The salt fed rats had significantly ($p<005$) raised basal gastric acid output (9.03 ± 0.17 mmol/L/hr) compared with control (7.27 ± 0.17 mmol/L/hr), but had blunted response to administered histamine and cimetidine, while treatment with the extract enhanced the sensitivity of histamine in high salt loaded rats. Gastric mucus concentration was significantly ($p<0.05$) higher in the salt untreated group (0.25 ± 0.004 g) compared with other groups. The salt fed untreated group also had significantly ($p<0.05$) raised gastric ulcers (10.83 ± 0.70) compared with other groups, these were reversed following *Moringa* treatment. In conclusion, *Moringa oleifera* extract reverses gastric ulcers and blunted histaminergic receptors in high salt fed rats. The mechanism by which high salt increases gastric secretion is independent of the histaminergic mechanism.

Keywords: *Moringa oleifera* Lam; Gastric acid secretion; Ulcers; Mucus; Rats.

1. INTRODUCTION

In spite of tremendous development in the field of orthodox medicines during the 20th century, plants still remain the first line of medication in modern and traditional system of medicine [1-4]. Among these medicinal plants is *Moringa oleifera* Lam. *Moringa oleifera* has been utilized to manage variety of ailments for many centuries [5-7].

The plant is known by common names like Miracle Tree, Horseradish tree, drumstick tree, never die tree, kelormarango, moonga etc., it local names include Zogalegandi in Hausa, Eweigbale in Youruba and Okweyibo in Igbo indicating its world-

wide significance. The trees originated from North Western region of India [8, 9].

M. oleifera leaves are edible and of high nutritive value and possesses analgesic, anti-diabetic, anti-hypertensive and anti-inflammatory effects [10-14]. This plant also has biological effects on the thyroid hormone, central nervous system and digestive system. The leaves of *M. oleifera* are also used traditionally to treat hepatotoxicity, rheumatism, venomous bites, and wounds; influenza, fever, nervous weakness, hysteria, pains, bowel disorders and worms [15]. Phytochemicals screening of the leaves of *M. oleifera* reveals that it contains some active ingredients like flavonoid, alkaloid, glycoside, niazirin, niazirinin, 4-benzl isothiocynate, benzyl glucosinolate, and carotenoids [16, 17].

Obviously, the first contact of ingested drugs and other substances in the body is the digestive system [18], different substances ingested into the body affect the functions of the digestive system in different ways. The digestive system is made up of the gastrointestinal tract (GIT) or alimentary canal and the accessory organs like the liver and pancreas, which help in the process of digestion, absorption, motility, secretion and excretion [19-21]. These parameters like gastrointestinal motility, gastric acid secretion, mucous output etc. are used to assess gastrointestinal function and could be deranged or enhanced by ingested substances.

Gastrointestinal functions have been shown to be altered by high salt intake in both man and experimental animals, high salt loading damages the lining of the GIT most especially the stomach. It decreases jejunal sodium reabsorption in young rats, and impairs intestinal Na^+/K^+ ATPase activity [22]. High salt diets interfere with normal food digestion (especially protein) by its ability to reduce the production of pepsin that enhance protein digestion. High salt diet also enhances vasoconstriction of mesenteric arteries, contributing to elevated blood pressure in rats [23].

There is paucity in scientific literature on the effect of *M. oleifera* extract on gastric ulcers, gastric acid and mucus secretion. The study is therefore aimed to investigate the effect of leaf extract of *M. oleifera* on gastric acid output, ulceration and mucus secretion in high salt loaded rats.

2. MATERIALS AND METHODS

2.1. Experimental animals

Forty-eight (48) male albino Wistar rats weighing initially between 160 to 200g obtained from the animal house of the Department of Physiology, University of Calabar, Nigeria were employed for this study for 6 weeks. The animals were allowed free access to their feed and drinking water. The rats were weighed before commencement of the feeding experiment and thereafter were weighed daily. Ethical approval was obtained from the Ethics Committee of the Faculty of Basic Medical Sciences, University of Calabar, Nigeria. They were nursed under control of environmental conditions in accordance with international standard [24].

2.2. Experimental plant

Fresh leaves of *Moringa oleifera* Lam (gene code number JX091931: Encycl. were purchased from the Botanical Garden of Calabar Municipality, Cross River State, Nigeria during the rainy season and were identified as authenticated by a botanist (Mr. Frank Adepoju) in the Department of Biological Sciences, University of Calabar, Calabar, where a voucher specimen was deposited with voucher number ERU/2011/345.

2.3. Preparation of plant extract

Fresh leaves of *M. oleifera* first washed free of sand and debris. Wash water was blotted off and the leaves ground to paste. A quantity of the ground sample (50 g) was weighed and Soxhlet extracted with 150 mL distilled water at 100°C for 9 h. Where larger ground samples were used, extraction was done under reflux with an appropriate volume of distilled water. The extract was slowly evaporated to dryness in vacuo at 40°C using a rotary evaporator. A total yield of 31% was obtained. Weighed samples of the extract were then used to prepare the stock solution [25].

2.4. Preparation of high salt diet

High salt diet containing 8% of sodium chlo-

ride was prepared using a standard diet containing 0.3% sodium chloride after the method of Obiefuna and Obiefuna [26].

2.5. Experimental protocol

The forty-eight (48) male albino Wistar rats were divided into 2 batches of 24 rats each. Batch 1 was used for gastric acid secretion and mucus secretion studies, while batch 2 was used for the ulcer study. Each batch was further sub-divided into 4 groups of 6 rats each. They were fed as follows: The group 1 (served as control) was fed on normal rat pellet + drinking water. The group 2 (NT) was fed on normal rat pellet + drinking water + 600 mg/kg b.wt. of *M. oleifera* extract orally once daily. The group 3 (SF) was placed on high salt diet (8% sodium chloride) + 1% sodium chloride drinking water. The group 4 (ST) received same as the third group + *M. oleifera* extract (600 mg/kg b.wt.) orally once daily. The feeding regimens lasted for six weeks. The animals were weighed daily.

2.6. Measurement of gastric acid secretion

Measurement of gastric acid secretion was done by the continuous perfusion method of Ghosh and Schild [27], modified by Osim et al. [28]. Rats from the control and test groups were fasted for 18-24 hours before the start of the experiment. The rats were anaesthetized with 0.6 ml/100 g body weight of 25% (wt/v) solution of urethane (Sigma, UK) given intraperitoneally. The trachea was exposed and cannulated. An infusion tube 75 cm length and 3mm diameter connected to 60 ml syringe carried by a pump was passed to the stomach through mouth and oesophagus. A ligature to stop back flow was made around the oesophagus in the neck. The abdomen was opened along the *linea alba* to minimise bleeding. The small intestine was reached and a semi-transection of 1-2 cm away from the pylorus was made and a fistula 8 cm long passed gently into the stomach through the pyloric sphincter and knotted.

Normal saline solution pH 7.00 placed in the pump was perfused through the stomach at 1 ml/minute via a perfusor. After an initial wash, the perfusate collected every 10 minutes interval and was titrated with 0.01 N NaOH solution in a 25 ml burette using phenolphthalein as indicator with pink coloration indicating the end point.

The pH of the saline was maintained by passing the perfusion tube through a water bath maintained at temperature of 37^{o}C. Also a low wattage bulb was placed above the animal to warm it and the body temperature monitored. A rectal thermometer was inserted via the anus to ensure that the body temperature was at 37^{o}C, care had to be taken not to ligate the vagus nerve or other blood vessels. To each 10 minute perfusate was added 2 drops of phenolphthalein indicator before titration against 0.01 N NaOH (Analar BOH, England) to determine total acidity.

2.7. Effect of histamine and cimetidine on gastric acid secretion

Upon collection of the basal gastric acid output using the normal saline for one hour (i.e. 6 aliquots were collections at 10 minutes interval), histamine (100 mg/kg) was thereafter injected into the rats subcutaneously, and the perfusate collected for another one hour. Thereafter, cimetidine (11.3 mg/kg) was injected intramuscularly, followed immediately with histamine (100 mg/kg). and the perfusate collected for one hour.

A total of 18 aliquots were collected at 10 minutes each, the time for each collections in 10 minutes was converted into 1 hour (by multiplying 10 minutes by 6).

2.8. Analysis of gastric acid

Gastric acid output was measured by titrimetric analysis. The calculation of acid in millimole per litre per hour (Mol/L/hr) follows the principle that states that a gram equivalent of acid balances a gram equivalent of the base at neutralization point. This means that:

Normality (N) of Acid x Volume (V) of Acid = Normality of Base x Volume of Base

i.e. $N_A \times V_A = N_B \times V_B$

From the above equation since Normality (N) of base is known i.e. 0.01 N and the volume of base needed for neutralization is known, the gram equivalent can be calculated thus: $N_B \times V_B$. This at the end points to the gram equivalent of the acid. If the volume is in mls, the acidity end point is in

milli-equivalent of acid. For a small animal like the rat milliequivalent will be too small and is always converted to µeq or µmol.

2.9. Ulcer studies

Gastric ulceration was induced in rats as described by Tekeuchi et al. [29], by oral instillation of 1 ml of 0.1 N HCl + 70% ethanol through intubation after an over night fast. One hour later, the animals were sacrificed using over dose of diethyl ether/chloroform and the stomachs were removed and opened along the greater curvature. Haemorrhagic lesions were examined microscopically using a hand lens (x18) and scored with a Vanier calliper as described by Elegbe [30].

Ulcer scoring:

Score	Description
0	Plan
0.5	0-6 mm
1	2-3 mm
2	>3 mm

2.10. Determination mucous secretion

The adherent gastric mucous was determined by the method described by Ettarh and Okwari [31]. The stomach was removed and washed in normal saline and then opened along the greater curvature. It was again rinsed in saline and pinned to a cork board with dissecting pins. Mucus was extracted using a spatula from the spread stomach into a known weight of beaker containing 4 ml of water. The weight of mucus was derived from the difference in the initial and final weights of beaker + 4 ml of water as follows:

Wt of beaker + 4 ml of water = x

Wt of beaker + 4 ml of water + mucus = y

Weight of Mucus = (y-x) g

This procedure has also been described by Tanet al. [32].

2.11. Statistical Analysis

Data are presented as mean ± SEM. Data were analysed using a one way analysis of variance (ANOVA) then followed with post hoc test (Least Significant Difference). P value of less than 0.05, 0.01 or 0.001 were declared as significant statistically.

3. RESULTS

3.1. Comparison of mean basal gastric acid output in control and tests groups

The mean basal acid output (BAO) in the control (group 1) was 7.27 ± 0.16 mmol/L/hr. The BAO was significantly ($p<0.001$) increased in salt fed untreated (group 3) and salt treated group (group 4) with basal acid output of 9.03 ± 0.17 mmol/L/hr and 10.10 ± 0.27 mmol/L/hr respectively compared with control and normal + extract (group 2). The BAO in salt treated was in turn significantly ($p<0.001$) higher compared with salt fed untreated group (Figures 1 and 2).

3.2. Comparison of the effect of histamine on gastric acid secretion in control and tests groups

Administration of histamine in the control group increased mean BAO significantly ($p<0.01$) from 7.27 ± 0.16 mmol/L/hr to 11.4 ± 1.44 mmol/ L/hr (producing about 58.66% increase in gastric acid output). In normal + extract group the increase was 47.66%. In groups 3 and 4, their mean gastric output changed from basal levels of 9.03 ± 0.17 mmol/L/hr to 9.27 ± 0.88 mmol/L/hr (2.12% increase) and from 9.87 ± 1.00 mmol/L/hr to 10.10 ± 0.27 mmol/L/hr (2.52% decrease) respectively following histamine administration. Showing significant ($p<0.01$) decreases in groups 3 and 4 compared to control and normal + extract treated groups (Figures 1 and 2).

3.3. Comparison of the effect of cimetidine on histamine-induced gastric acid secretion in control and tests groups

Administration of cimetidine attenuated the effects of histamine in all the groups. But the attenuation was marked in salt fed groups compared with the normal rats. In the control and normal + extract groups, administration of histamine + cimetidine decreased mean gastric output by 16.35% and 14.48% respectively. While in the salt fed untreated and treated groups the reductions

were 43.13% and 46.80% respectively. Showing significant ($p<0.001$) reductions in the salt groups compared with the normal rats (Figures 2 and 3).

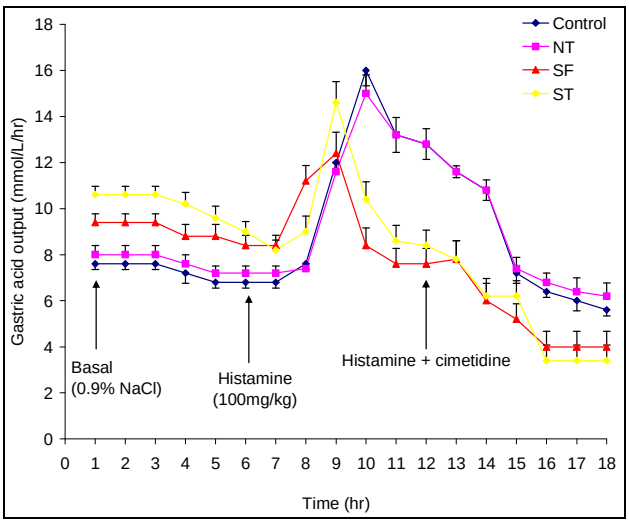

Figure 1. Basal gastric acid output and induced secretion to histamine and cimetidine in the different experimental groups.Values are expressed as mean ± SEM, n = 6.

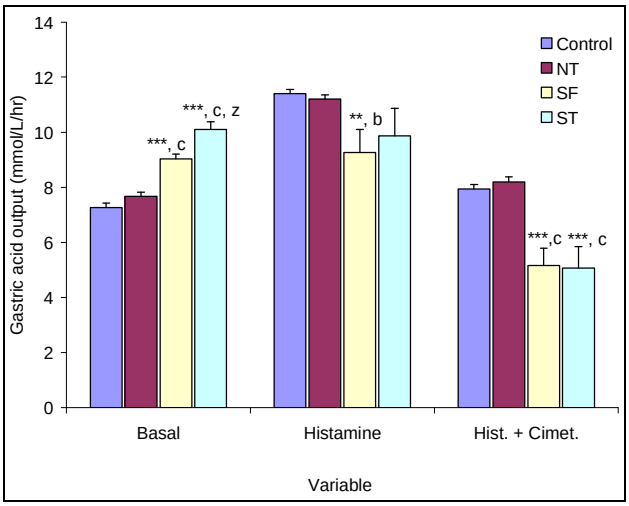

Figure 2. Comparison of basal, histamine and histamine + cimetidine induced gastric acid secretion in the different groups.
Values are mean ± SEM, n = 6. ** = $p<0.01$, *** = $p<0.001$ vs. control; b = $p<0.01$; c = $p<0.001$ vs. NT; z = $p<0.001$ vs. SF.

3.4. Comparison of mean gastric mucus levels in control and tests groups

The salt fed untreated group had significant ($p<0.001$) increase in mean gastric mucus output compared with other groups. The mean gastric mucus output for the different experimental groups were 0.13 ± 0.02 g in the control group, 0.12 ± 0.01 g for normal treated group, 0.25 ± 0.004 g in the salt fed untreated group and 0.16 ± 0.003 g in the salt treated group (Fig. 3).

3.5. Comparison of ulcer scores in control and tests groups

As shown in Fig. 4, the mean gastric ulcers in the salt fed untreated group (10.83 ± 0.70) was significantly ($p<0.001$) higher compared with the control (6.42 ± 0.48) and normal + extract (5.83 ± 0.48) and (7.92 ± 0.88) groups.

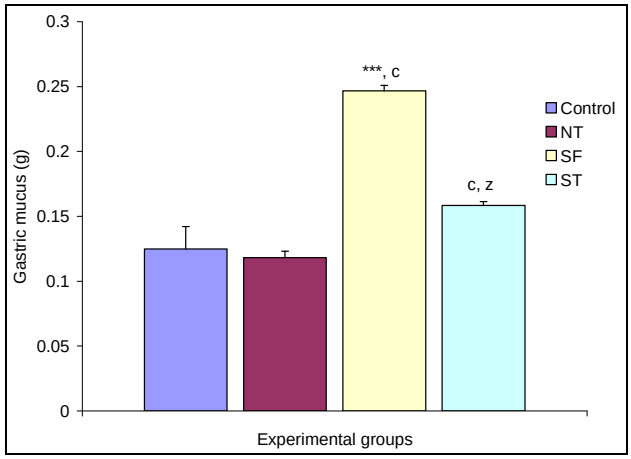

Figure 3. Comparison of mean gastric mucus in the different experimental groups.
Values are mean ± SEM, n = 6. *** = $p<0.001$ vs. control; c = $p<0.001$ vs. NT; z = $p<0.001$ vs. SF.

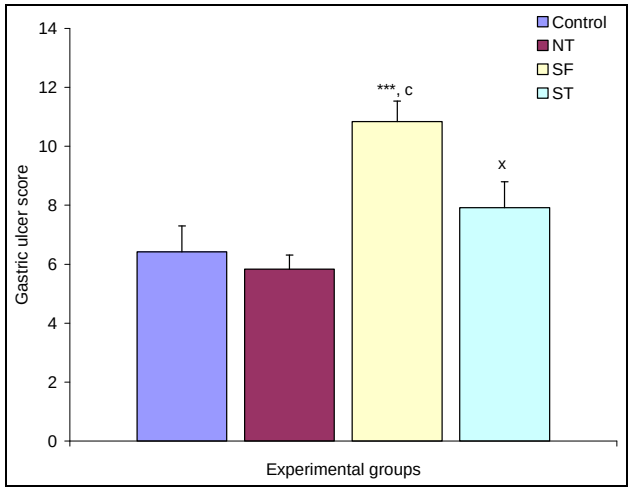

Figure 4. Comparison of mean gastric ulcer score in the intestinum in the different experimental groups.
Values are mean ± SEM, n = 6. *** = $p<0.001$ vs. control; c = $p<0.001$ vs. NT; z = $p<0.001$ vs. SF.

4. DISCUSSION

The effect of aqueous extract of *Moringa oleifera* Lam leaf on some gastrointestinal function in high salt loaded rats was investigated in this study. Gastrointestinal function indices studied included changes in gastric acid secretion, gastric mucus secretion and gastric ulcers. The results obtained from this study has strong indication that the aqueous leaf extract of *M. oleifera* leaf has tremendous effect on the gastrointestinal function following high salt loading.

Gastric acid output is an eminent parameter for assessing gastrointestinal function. This study revealed significant increase in basal gastric acid output of rats placed on high salt diet. The direct effect of high salt on the parietal cells could be a possible explanation for this elevated gastric acid output in high salt fed rats. Because one of the mechanisms earlier postulated for gastric acid secretion is the combination of $NaCl + H_2O + CO_2$ in the parietal cells, with a forward reaction of $HCl + NaHCO_3$ being produced. Hence, the increase availability of NaCl, the ultimate source of chloride ions for HCl formation, can step up the speed of reaction and increase gastric acid secretion [33], while salt withdrawal has been shown to depress gastric acidity, solely due to alteration in the rate of ionic transfer in the parietal cells.

This effect was not possibly via the histaminergic H_2 receptors, since gastric acid output in the salt fed rats was depressed following histamine administration. High salt loading is directly correlated with the incidence of *Helicobacter pylori*. *H. pylori* is responsible for the many gastric acidity, ulcerations and cancers in people [34, 35]. *H. pylori* gastritis, which is confined to the antrum and unaccompanied by atrophy, results in hyper secretion of acid. The increased acid secretion in subjects with antral predominant non-atrophic gastritis is mainly due to the *H. pylori* gastritis stimulating increased release of the hormone gastrin which circulates and stimulates the body of the stomach to secrete acid. Subjects with *H. pylori* antral gastritis have increased basal, meal stimulated and gastrin releasing peptide (GRP) stimulated serum gastrin concentrations [36-38]. The increased circulating gastrin associated with *H. pylori* is mainly due to an increase in gastrin-17 [39]. This form of gastrin originates mainly from the antral mucosa and increase after meals. The increase in gastric acidity observed in the high salt loading in this present study could probably be due to increase in *H. pylori*.

The above explanation suffices for the increase in gastric ulcerations evidenced in high salt loaded rats recorded in this study. Besides solubilisation of mucus constituents could be a possible reason, as earlier noted by Glavin and Szabo et al. [40]. *M. oleifera* extract was effective in reducing the ulcers but not the gastric acidity in this study, possibly by enhancing the protective mechanism of the stomach in the presence of gastric acidity. Gastric ulceration involves breaking the mucosal barrier and exposing the underlying tissue of the stomach or duodenal lining to corrosive action of acid and pepsin or gastrin [40]. Among the factor proposed for the pathogenesis of peptic ulceration are increase gastric acidity and pepsin secretion, decreased in mucosal resistance and mucosal blood flow and increase in free radical generation and inhibition of somatostatin, some of these may be acquired during life, while some are predetermined [5]. *M. oleifera* is rich in anti-oxidant activity due to the present of phytochemicals like flavonoids, tannins, vitamins A, E and C in it, these are known protective chemicals to the stomach, thereby reducing gastric lesion and ulcers [41].

In the high salt loaded rats, gastric mucus concentration was elevated compared to other groups. Earlier report endorses high mucus secretion following high salt loading due to release of prostaglandins which stimulates mucus secretion and that the mucus can be degraded by proteases originating from enteric parasite [41]. However, one would have expected that the increase in mucus secretion in the salt fed untreated group would protect the gastric mucosal from injury and ulcerations. Previous reports has shown that, in-spite of raised gastric mucosa, the presence of a high concentration of sodium chloride damages to the gastric mucosa, leading to cell death and consequent regenerative cell proliferation, while in the longer term high NaCl concentration leads to inflammation and diffuse erosion of gastric mucosa [42]. Also, it has been observed from previous study that gastric mucus consists of two histo-chemically different kinds of mucin, surface mucous cell mucin (SMCM)

and gland mucous cell mucin (GMCM), salt loading shift gastric mucosa from the glandular to cell surface, where *H. pylori* strive most, leading to ulcerations [43, 44]. It is possible that the loosely adherent mucus that can be easily excised was produced following high salt loading and not the firmly adherent mucus that anchor firmly to the epithelium thereby preventing erosion by gastric acidity [45]. Research shows that the layer of mucus closest to the epithelium (firmly adherent mucus) is responsible for maintaining the integrity of the gastric mucosa [46].

5. CONCLUSION

High salt loading increases gastric acidity and ulcerations in rats despite the elevated gastric mucus following high salt loading. *Moringa oleifera* Lam extract prevented the ulcerogenic effect of high salt loading, and enhanced the sensitivity of the histaminergic receptors blunted in high salt loaded rats.

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AUTHORS' CONTRIBUTION

EEI wrote the initial draft of the manuscript; OEO designed the study and did the statistical analysis; AAN proof read, and edited the word. All authors were involved in the execution of the research plan. The final manuscript has been read and approved by all authors.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

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Research Article

Antioxidant response of vitamin A during the exposure of blood platelets to electromagnetic radiation generated by LCD monitors - in vitro study

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ABSTRACT

The article presents the results of in vitro studies aimed at identifying changes in activity of the enzyme superoxide dismutase (SOD-1) as a parameter of oxidative stress and protective antioxidant role of vitamin A during the exposure of blood platelets to electromagnetic radiation (EMR) generated by LCD monitors. Blood platelets were exposed to an electromagnetic radiation for 30 min. and 60 min. generated by monitors, which is characterized by parameters: 1 kHz frequency and 220 V/m intensity. The enzymatic activity of SOD-1 increases significantly compared to control values after 30 min. of exposure to EMR (from 2523.39 U/g protein to 3896.15 U/g protein), and decreases after 60 min (to 2846.58 U/g protein). A significant decrease in enzyme activity after the addition of vitamin A was noticed (to 1569.54 U/g protein). In samples exposed for 30 min. the SOD activity was significantly increased by addition of vitamin A and decreases after 60 min. Changes in enzymatic activity of SOD-1 dependent on exposure time and application of vitamin A suggest an important preventive role of vitamin A to protect against the effects of EMR which we are exposed to in everyday life.

Keywords: Electromagnetic radiation; LCD monitors; Superoxide dismutase; Vitamin A; Antioxidants; Oxidative stress.

1. INTRODUCTION

For several dozen years, power tools have become an integral part of life for most societies. Any such device emits electromagnetic radiation that as a new environmental factor drew researchers' attention starting with the 1960s. After years numerous reports about its harmfulness to living organisms appeared and attempts to limit the negative consequences of its effects were made. Prophylaxis for electromagnetic radiation (EMR) can rely on the norm creation in the particularly

dangerous places and antioxidant prevention aimed at alleviating the effects of oxidative stress, one of the most dangerous effects of electromagnetic radiation.

Oxidative stress is a state of disturbed balance between oxidative processes that induce the formation of reactive oxygen species (ROS) and counteracting antioxidant defense system. ROS oxidizing proteins, lipids, DNA contribute to cellular damage and consequently to apoptosis. The state of pro-oxidant-antioxidant balance is maintained by the activity of enzymes such as superoxide dismutase, catalase, glutathione peroxidase, and other low molecular weight substances, for example melatonin, vitamin A (used in medicine as tretinoin), C (ascorbic acid) and E (tocopherol) [1]. The reactions of the low molecular weight antioxidants with ROS are less specific than the antioxidant enzymes, causing these compounds more universal protectors, performimg several functions. They act as a second line of defense degrading ROS, which are not removed by superoxide dismutase and catalase. The biological role of superoxide dismutase (SOD-1) (EC 1.15.1.1) consist in removal of superoxide anion radical by dismutation into oxygen and hydrogen peroxide:

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$$

In turn, the enzyme catalase (EC 1.11.1.6) prevents the build-up of hydrogen peroxide catalyzing the disproportionation reaction of this compound.

Oxidative stress underlies many pathological conditions and diseases. The pathological implications of the reaction of reactive oxygen species and oxidative stress include, inter alia, multiple sclerosis, atherosclerosis, rheumatoid arthritis, Parkinson's disease [2], Alzheimer's disease [3], diabetes, in which increased ROS production by phagocytes and elevated plasma MDA level were observed [4]. Other studies also proved that ROS and antioxidants stimulate HIV replication in an organism [5].

There is also evidence that ROS, produced mainly by activated neutrophils infiltrating the wound or to the locus in which the inflammation occurred, increased expression of some proto-oncogenes and may also be mediators of inflammation cocarcinogenic action [6].

In addition, studies have shown that tumors are often characterized by decreased activity of superoxide dismutase Cu, ZnSOD, where the activity of another kind of dismutase - MnSOD lowers as a rule [1].

Studies carried out on the molecular level are justified by the fact that changes taking place in cells are responsible for the response of the organism as a whole. There are many reports focused on the influence of electromagnetic radiation on the oxidative metabolism of cells: increased lipid peroxidation [7, 8] and changes in the activity of antioxidant enzymes in various cells and tissues [9, 10]. Additionally, the effect of oxidative stress caused by electromagnetic radiation is confirmed by numerous studies [11, 12].

Study of Agarwal et al. reported that chronic exposure to electromagnetic radiation reduced the enzymatic activity of superoxide dismutase, glutathione peroxidase and catalase, and increased the lipid peroxidation [13]. The harmfulness of electromagnetic radiation emitted by the LCD monitors was showed by the epidemiological studies of Korpinen et al.

The respondents had skin symptoms when they stayed in front of a computer screen for a long period [14]. Results of other studies indicate that computer users more often complained of headaches, bones and joint pain, hearing loss, vertigo/ dizziness, tension - anxiety symptoms depending on the time of daily usage [15].

Among the biochemical studies conducted on the effects of electromagnetic field (EMF) emitted by monitors, Balci's et al. experiments conducted on corneal and lens tissue of rats reported harmful effects. The results of these studies indicated that this factor may induce oxidative stress manifested in an increase of the MDA concentration and activity of antioxidant enzymes [16].

Considering the above data, the authors of this study attempted to determine the effect of vitamin A on the oxidation - reduction reaction occurring in the blood platelets under the influence of electromagnetic radiation generated by LCD monitors. The aim of this study was to determine the applicability of this antioxidant vitamin as prophylactic action, shielding the body from the harmful effects of EMF.

2. MATERIALS AND METHODS

2.1. Sample preparation

Pork blood was collected from a slaughterhouses during the exsanguinations of animals. It was taken to 1% ethylenediaminetetraacetic acid (EDTA). Platelets were obtained by fractionated centrifugation at 1200 rpm x g for 10 min. at room temperature. As a result of the centrifugation platelet rich plasma (PRP) was obtained from the whole blood, which was carefully pulled by plastic pipette from the deposited layer of erythrocytes and transferred into polyethylene tubes. Then the obtained platelet rich plasma was centrifuged at 3000 rpm x g for 15 min. The precipitated platelets were suspended in 0.2 ml of 0.9% NaCl. The obtained suspension of blood platelets was an input research model.

2.2. Incubation of platelets with vitamin A

An ethanolic solution of vitamin A containing 3 mg of retinol (cat. no. R7632-25MG) in a volume of 10 ml was used in this study. 2 µl of this solution was added to 0.2 ml of a suspension of blood platelets, avoiding bright light. The sample was incubated in a dark place for 30 min., and then subjected to a further procedure.

2.3. Exposure condition setting and instruments

In a laboratory stand designed for reconstruction of the parameters of electromagnetic radiation generated by display screens (1 kHz, 220 V/m), a flat capacitor was the source of electromagnetic field. Requirements of the TCO (The Swedish Confederation of Professional Employees) and MPR (National Board for Measurement and Testing) specifies strict conditions for the measurement of exposure. Authors measured the field by the measurement procedure on the location of points placed in front of the monitor.

When electromagnetic radiation of low frequency is tested the electric and magnetic components should be investigated independently. Monitors with the liquid crystal screens produce non-sinusoidal electromagnetic fields, with the dominant electric component, due to control of power semiconductor chips. Significant fields are fields with frequency the lower power consumption and voltage switching power supply, with superimposed oscillations dampened RLC circuits, which act as voltage ripple smoothing filters. The source of the signal simulating shape of the field generated by the LCD was a programmable generator Hameg 8010, which is amplified by the measuring amplifier W-320, and the source of the electric field was a flat capacitor arrangement. The capacitor was formed by two circular copper plates positioned over and under a plastic support in which 8 polyethylene tubes containing the tested preparation were inserted into holes made symmetrically on the circumference of the circle the diameter of which was smaller than that of the capacitor plates so that the electrical component of the field acting on the tubes was homogeneous in nature. The tested preparation was placed in polyethylene tubes, each containing 0.2 ml of the preparation. The temperature in the laboratory stand was on the same level all the time and it was $+24/+25^0$C. Preserving constant conditions of the environment the preparation was exposed to the activity of the electromagnetic field of 1 kHz frequency and 220 V/m intensity (corresponding to a distance of 15 cm from the monitor) for 30 and 60 min. The exposure of the platelets to the radiation was done on the day they were collected from the slaughterhouses.

2.4. Measurement of antioxidant activity of superoxide dismutase (Cu, Zn-SOD) (SOD-1) (EC. 1. 15. 1. 1.)

This parameter of oxidative stress were measured before and immediately after the exposure. The study samples were obtained by adding 0.2 cm^3 of platelet suspension at the concentration of $1x10^9/cm^3$, 0.8 cm^3 redestilled water cooled to $+4^0$C and 0.5 cm^3 of 96% C_2H_5OH and 0.25 cm^3 chloroform. The obtained mixture was shaken for 4 min. and then centrifuged at 4200 x g at $+4^0$C for 10 min. After centrifugation, the enzyme remained in the upper layer of the suspension. Then 0.2 cm^3 of supernatant was transferred into glass tubes together with 2.6 cm^3 0,05M carbonate buffer of pH 10.2 and 0.2 cm^3 of adrenaline.

Table 1. The values of enzymatic activity of SOD.

Individuals	Control (I)	Control + vit. A (II)	Exposure to EMR, 30 min. (III)	Exposure to EMR, 30 min. + vit. A (IV)	Exposure to EMR, 60 min. (V)	Exposure to EMR, 60 min. + vit. A (VI)
SOD (U/g protein)	2523,39 ±1268,1	1569,54 ±663,7	3896,15 ±1409,02	7442,87 ±4538,61	2846,58 ±1218,95	2166,25 ±1091,61

The blind test did not contain supernatant, the carbonate buffer was used instead. The values were presented in U/g of platelet protein.

The amount of enzyme which causes a 50% inhibition at the maximal increase of absorbance by 0.025 of unit/min on a rectilinear segment of adrenochrome formation at $+25^0$C at 480 nm is defined as a unit of SOD activity [17]. It was used 30 control and exposed samples.

Spectrophotometer T60 VIS firmy OMC Envag was used for the measurement of superoxide dismutase activity at 480 nm wavelength. Absorbance in the control and study samples was measured every minute at $+25^0$C for 10 min.

2.5. Statistical analysis

The following statistical parameters were determined for each characteristics in the study groups: arithmetic mean, standard deviation, median, minimum, maximum, skewness coefficient. All data were presented as median ± SD. The obtained results were analyzed using a nonparametric Kruskal-Wallis Anova rank test equivalent to analysis of variance and Mann-Whitney U Test to compare the variables between the groups. The value of $p < 0.05$ was considered the level of confidence. Calculations were made using the program STATISTICA PL (Table 2).

3. RESULTS

Each of 30 sample blood was divided into 6 fractions, each of them distributed in a different experimental group: unexposed to radiation, unexposed + vitamin A, exposed for 30 min., exposed for 30 min + vitamin A, exposed for 60 min., exposed for 60 min. + vit. A. In each sample the level of SOD-1 activity were determined. In the in vitro studies the enzymatic activity of superoxide dismutase in blood platelets increases significantly ($p < 0.05$) compared to control values after 30 minutes of exposure to EMF of 220 V/m intensity and 1 kV/m frequency (from 2523.39 U/g protein to 3896.15), and then the activity decreases (measured after 60 min.), being higher (not statistically significant $p > 0.05$) compared to initial values (from 2523.39 U/g protein to 2846.58 U/g protein). The activity of SOD significantly decreases ($p < 0.05$) in the blood sample unexposed to EMF with vitamin A in comparison with the unexposed sample (from 2523.39 U/g protein to 1569.54 U/g protein).

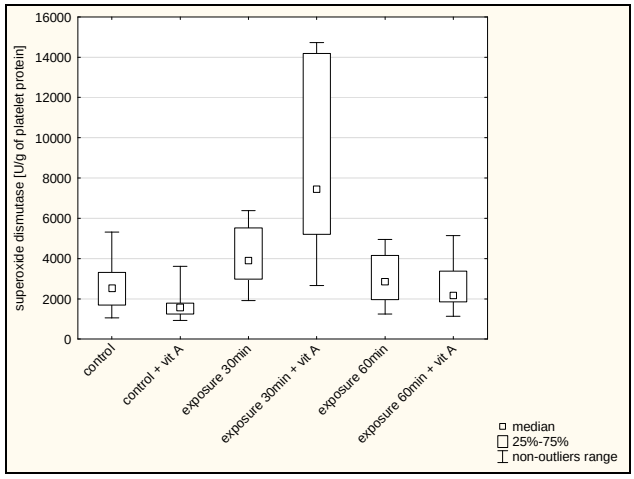

Figure 1. Enzymatic activity of superoxide dismutase (SOD-1) in blood platelets exposed to electromagnetic field dependent on exposure time and application of vitamin A (n = 30).

The activity of SOD significantly increases ($p < 0.05$) in the blood sample exposed to EMF for 30 min., to which vitamin A was added as compared with the sample exposed for the same period of time without vitamin A (from 3896.15 U/g protein to 7442.87 U/g protein). The activity of superoxide dismutase decreases (not statistically significant) in the blood sample exposed to the EMF for 60 min., to which vitamin A was added as compared with the

sample exposed for the same period of time without vitamin A (from 2846.58 U/g protein to 2166.24 U/g protein) (Table 1, Figure 1).

Table 2. Statistical analysis of the enzyme activity of superoxide dismutase (SOD-1) in blood platelets treated with electromagnetic radiation dependent on exposure time and application of vitamin A (n = 30).

Kruskal-Wallis Anova rank test	H = 91,0042 p<0.05
[1)] Test $Z^{I,III}_{Mann-Whitney}$	Z = -3,72 p<0.05
[2)] Test $Z^{I,V}_{Mann-Whitney}$	Z = -1,06 p>0.05
[3)] Test $Z^{I,II}_{Mann-Whitney}$	Z = 3,34 p<0.05
[4)] Test $Z^{III,IV}_{Mann-Whitney}$	Z = -3,91 p<0.05
[5)] Test $Z^{V,VI}_{Mann-Whitney}$	Z = 1,69 p>0.05

H - value of the Kruskal-Wallis Test; Z - value for pair of variables; [1)] correlation between control (I) and exposure to EMR, 30 min. (III); [2)] correlation between control (I) and exposure to EMR, 60min. (V); [3)] correlation between control (I) and control + vit. A (II); [4)] correlation between exposure to EMR, 30 min. (III) and exposure to EMR, 30 min. + vit. A (IV); [5)]correlation between exposure to EMR, 60 min. (V) and exposure to EMR, 60 min. + vit. A (VI).

4. DISCUSSION

Despite numerous inconsistencies, decades of research on the effects of electromagnetic radiation proved the negative effect of this factor on the health of living organisms e.g., on the cardiovascular system [18], nervous system [19], as well as the formation of tumors [20]. Studies conducted at the cellular level focused on the analysis of individual parameters of oxidative stress, ie. free radicals generation, the enzymatic activity of superoxide dismutase, catalase, glutathione peroxidase, or a concentration of malondialdehyde - a marker of lipid peroxidation also indicate a negative impact of EMF.

Research on the effects of electromagnetic field of 1000 Hz frequency, and a magnetic induction of 0.5 mT on the enzymes antioxidant defense of platelets also showed reduction of superoxide dismutase activity after both the 30- and 60- and 90-minute exposure [21].

In another study, authors have found that vertical and horizontal application of ELF electric fields in the range of 1.35, 1.5, and 1.8 kV/m increased SOD levels as compared to the controls ($p<0.05$) and to applied electric fields of 0.3, 0.6, 0.8, and 1 kV/m [10].

Our study demonstrated that the exposure to EMF emitted by LCD monitors changes the activity of the superoxide dismutase enzyme in blood platelets. After 30-minute irradiation of field of 220 V/m intensity the enzyme activity increases relatively to the control value, and then decreases (measured after 60 min.).

As a result of EMFs effect, an increase in generation of free radicals both in the cell membrane platelet blood cells and organelles is induced - as confirmed by the above-mentioned research, including their own authors [22]. This can cause changes in SOD enzyme activity due to the increased concentration of free radical substrates.

Vitamin A is a general term that refers to fat-soluble compounds from the group of retinoids. The active form of vitamin A is retinol that is similar in structure and biologic activity. The carotenoids (most commonly beta-carotene) are the precursors of vitamin A (retinol). The role of vitamin A as an antioxidant is debatable. The carotenoids such as beta-carotene have in recent years received more attention from the scientific community because of the harmful role they may play as pro-oxidants [23]. Studies have shown that high dose of beta-carotene increases the incidence of lung cancer and increases mortality among smokers [24].

Additionally, the results of large, controlled trials of an intervention of beta-carotene supplementation did not support the detected beneficial associations or a role for supplemental beta-carotene in lung cancer prevention; instead, they provided striking evidence for its adverse effects among smokers [25]. Despite these discrepancies, vitamin A is known to help repair damaged tissue and therefore may be beneficial in counter-acting free radical damage [26].

The conclusion is that beta-carotene may serve as an antioxidant or as a prooxidant, depending on its intrinsic properties as well as on the redox potential of the biological environment in which it acts.

Among the many studies in the field of antioxidant role leveling effects of electromagnetic radiation of vitamins those that relate to vitamins C and E are the most numerous.

The results of the study of Jelodar et al. suggest that radio waves lead to oxidative stress in testis tissue and vitamin C via antioxidant role improved antioxidant enzymes level and decreased lipid peroxidation [27].

Results of Al-Damegh study indicate that the electromagnetic radiation from conventional cellular phone had a negative impact on the oxidant and antioxidant status in rat blood and testicular tissue. This finding also indicated the possible role of vitamins C and E in mitigating the oxidative stress imposed on the testes and restoring normality to the testes [28].

Karsiloglu et al. examining the protective effect of vitamin E on the occurrence of gamma irradiation-induced cataract in rats lens, showed that this vitamin's antioxidant role is to act by reducing oxidative stress and thus, the incidence of cataracts. It has been shown among others that vitamin E increases enzymatic activity of superoxide dismutase and glutathione peroxidase [29].

Studying the available literature, we can find publications about the antioxidant role of carotenoids, especially beta-carotene (a precursor of vitamin A), but little is related to vitamin A. Moreover, the available publications relate mainly to the protective role of these compounds in UV radiation protection.

A study by Stahl et al. investigated the antioxidant effect of carotenoids and tocopherols based on their ability to scavenge ROS generated during photooxidative stress. The antioxidants used in this study provided protection against erythema in humans and may be useful for diminishing the sensitivity to ultraviolet light [30].

The changes in the activity of superoxide dismutase in the pork blood samples after the addition of vitamin A were observed in the present study. When comparing samples of control material - not exposed to EMF (control vs control + vit. A) a significant decrease in enzyme activity after the addition of this vitamin was noticed. Vitamin A acts as an antioxidant by scavenging existing free radicals (arising due to natural metabolism), which probably contributes to the decrease in the amount of free radical substrates for the operation of SOD, causing a decrease in its activity.

In the blood samples exposed to EMF for 30 minutes the SOD activity was significantly increased by addition of vitamin A. In this case it seems that vitamin A as an auxiliary antioxidant action of cellular enzymes contributes to increasing their activity.

Whereas, after the 60-minute exposure to EMF, SOD activity decreases after adding vitamin A (exposed for 60 min. vs. exposed for 60 min. + vit. A). In this case, after prolonged exposure, a depletion of the enzymatic activity of SOD follows and thus the antioxidant activity of vitamin A also decreases. As a result the generation of free radicals may increase leading to cellular damage, for example in the intensified process of lipid peroxidation in cell membranes, which can be expressed by the above-mentioned increase of malondialdehyde (MDA) concentrations - marker of peroxidation changes.

The changes of enzymatic activity of superoxide dismutase in our study may indicate the negative effect of the used radiation and the protective antioxidant role of vitamin A.

The presented results suggest an important preventive role of vitamins A, C and E to protect against the effects of electromagnetic radiation.

AUTHORS' CONTRIBUTION

Conception and design, Study supervision: AB; Development of methodology: MR, KP; Acquisition of data: ML, GH; Analysis and interpretation of data: ML, MZ; Writing, review and/or revision of the manuscript: ML; Administrative, technical, or material support: MR. The final manuscript has been read and approved by all authors.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

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Research Article

Antioxidant potentialities of some strains belonging to endophytic, entomopathogenic and saprophytic fungi

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ABSTRACT

Antioxidants have recently become the topic of interest as radical scavengers, which inhibit the free radical mediated processes. This study was carried out to investigate the antioxidant activity of 100 fungal strains (26 endophytes, 42 entomopathogens and 32 saprophytes). Three different assays (reducing power, total phenolic contents and flavonoid contents) were determined and used to evaluate the antioxidant potential of the fungal ethanolic extracts. The results revealed that all fungal strains under study showed antioxidant activity up to varying extent. A total of 21, 35 and 19 out of the tested endophytic, entomopathogenic and saprophytic strains, respectively, had a reducing power activity. High reducing power activities (≥ 0.6 mg/ml) were recorded by 9, 20 and 14 strains of the three tested groups, respectively. All tested strains have the ability to produce phenolic compounds with levels ranged from 0.92 to 63.44 mg/ml. The highest levels of total phenolic contents (≥ 40 mg/ml) observed in the extract of 12, 28 and 18 strains of endophytes, entomopathogens and saprophytes, respectively. Finally, all tested strains produced flavonoids with levels of 0.166 to 68.806 mg/ml. The highest flavonoid producers (formed ≥ 35 mg/ml) were only one strain of each of the endophytic and entomopathogenic fungi and three strains of saprophytic fungal group. The obtained results suggest that the tested strains, especially those of endophytes, had the potentiality as sources of strong natural and safe antioxidants for application in food and cosmetics industries.

Keywords: Antioxidant; Fungi; Flavonoids; Phenols.

1. INTRODUCTION

For thousands of years, fungi have been recognized as nutritious, highly palatable functional foods in many societies and are now accepted as a valuable source for the development of medicines and nutraceuticals [1, 2]. Fungi have proven to be a rich source of bioactive and novel organic compounds with interesting biological activities and a high level of biodiversity [3, 4]. Fungi produce a diverse array of secondary metabolites. Secondary metabolites have a tremendous impact on society and are exploited for their antibiotics and pharmaceuticals activities such as anticancer, antitumor, immuno-stimulatory, and antioxidants [5]. It is clear that, fungi represent a largely untapped source of potentially powerful new pharmaceutical products [2, 6].

Endophytes are microorganisms that colonize internal plant tissue and can live there for all or part

of their life cycle without causing any apparent damage or disease [7]. Entomopathogenic fungi are ecologically classified as fungi that grow either inside of insect bodies or on the surface of their exoskeleton, which eventually causes the death of the host insect [8]. Fungi play an important role in the research for antitumor compounds and might also represent an alternative source for the production of therapeutic agents that are not easily obtained by chemical synthesis. Generally these fungi are a store house of novel secondary metabolites including antibiotics, antioxidants, anticancer and immunosuppressant compounds [9-11].

Antioxidants are critical for the maintenance of normal cell function, health and well-being. They are compounds which prevent the initiation or propagation of oxidizing chain reactions which in turn inhibits or delays oxidative damage related to aging and disease. Although have developed natural mechanisms to protect cells from free radical damage by neutralizing them, the amount of antioxidant produced under normal conditions is not always sufficient. Fungi are a well-known source of antioxidants which can be used to prevent oxidative damage and as such, can limit their deleterious effects in humans and animals alike.

Antioxidants are the molecules, which prevent cellular damage by reducing the oxidative stress and therefore have a beneficial effect on human health [12]. Antioxidants may be characterized by their mode of action in preventing oxidative damage, being classified as preventative, scavenging, and repair or *de novo* antioxidants [13]. Antioxidants prevent the formation of reactive oxygen and nitrogen species ROS/RNS by reducing hydrogen peroxide and lipid hydro peroxidases, respectively, or by sequestering metal ions such as iron and copper [14].

Phenolic compounds are aromatic hydroxylase compounds possessing at least one aromatic ring with one or more hydroxyl groups [15]. By this means, a structure-function relationship exists between phenolic compounds; with their antioxidant activity depending on the number and position of the hydroxyl groups and the nature of substitutions on the aromatic rings [16]. These compounds are common in fungi and are important sources of bioactive substances [15]. Generally, antioxidants are obtained from fungal sources include phenolic compounds (tocopherol, flavonoids, and phenolic acids), nitrogen compounds (alkaloids, amino acids, and amines) or carotenoids as well as ascorbic acid [17, 18].

The main cause of mortality and morbidity in the world is atherosclerosis, the accumulation of oxysterol, cholesterol, and peroxide lipids in arteries, generated by free radicals which lead to heart attack. Hence, there has been an increased interest in the application of antioxidants [19].

Natural compounds such as ascorbic acid, vitamin E, carotenoids, flavones and phenolic acids which are common to fungi possess the ability to scavenge free radicals in the human body. They play a key role in health maintenance and prevention of chronic and degenerative diseases such as atherosclerosis, carcinogenesis, neurodegenerative diseases, DNA damage and aging [20]. Antioxidants serve as the defensive factor against free radicals in the body. Synthetic antioxidants such as butylated hydroxyl anisole (BHA), butylated hydroxyl toluene (BHT) and tert-butylhydroquinone (TBHQ) are usually used as food additives by the food industry to prevent lipid peroxidation. However, their application has been limited because of possible toxic and carcinogenic components formed during their degradation. In view of these health concerns finding safer, more effective and economic natural antioxidants is highly desirable [21]. A number of plants and mushrooms are commonly known to produce antioxidants but there are few reports on lower fungi [22]. These include *Penicillium roquefortii*, *Aspergillus candidus*, *Emericella falconensis*, *Acremonium* sp., *Colletotrichum gloeosporioides* [22], *Chaetomium* sp., *Cladosporium* sp., *Phoma* sp. etc. [23]. A lot more fungi still needs to study. Keeping above in mind the present study was aimed to determine the total phenolic, flavonoid content and reducing power of ethanolic extracts of a total 100 strain of endophytic (26 strains), saprophytic (32) and entomopathogenic (42) fungi collected from different sources.

2. MATERIALS AND METHODS

2.1. Collection of fungal strains

A total of 100 fungal strains (26 endophytic, 32 saprophytic and 42 entomopathogenic fungal

strains) isolated from different sources were kindly provided by the Assiut University Mycological Centre (AUMC), Assiut University, Assiut, Egypt.

2.2. Preparation of fungal inoculum

Inocula of tested fungi were prepared by suspended 1cm from seven day-old culture of each organism on potato dextrose agar (PDA) in 5 ml of sterile distilled water supplemented with 0.01% of Tween 80 and suspending the spores with a sterile loop [24]. This spore suspension was used as inoculum for cultivation of each organism.

2.3. Preparation of crude fungal extracts

Fungal strains were grown on potato dextrose broth (PDB) in 250 ml Erlenmeyer flasks. Cultures were incubated for 10 days at 28 ± 2 ^{0}C. The mycelia and the fermentation broth of each fungal strain were blended with 150 ml ethanol in electric blender; the extracts were filtered using filter paper to remove the mycelia. Mislabel extracts were individually transferred into rotatory evaporator under reduced pressure at 35 ^{0}C till semisolid residue was obtained.

2.4. Antioxidant assays

Three different assays including reducing power, phenolic content and flavonoids were used to evaluate the antioxidant potential of fungal extracts. Each experiment was done in triplicate and mean values were taken.

2.4.1. Determination of antioxidant activity by reducing power measurement

The reducing power of the extract was determined according to Chang et al. [25] with slight modification as follows: an aliquot of 0.5 ml extract was added to 0.1 ml of 1% (w/v) potassium ferricyanide. After incubating the mixture at 50 ^{0}C for 30 min, during which the ferricyanide was reduced to ferrocyanide, it was supplemented with 0.1 ml of 1% (w/v) trichloroacetic acid and 0.1% $FeCl_3$ and left for 20 min. Absorbance was read at 700 nm to determine the amount of ferric ferro-cyanide (Prussian blue) formed. Higher absorbance of the reaction mixture indicates higher reducing power of the sample. Ascorbic acid concentrated of 10 to 200 µg/ml was used as standard.

2.4.2. Determination of total phenolic content

The total phenolic content (TPC) was determined according to the Folin-Ciocalteu (F-C) colorimetric method [26]. Briefly, 50 µl of sample and 50 µl of F-C reagent were pipetted into an eppendorf tube. The contents were vortexed for 10 second and then left to stand at room temperature for 2 min before the reaction was stopped by adding 500 µl of 5% (w/v) sodium carbonate solution and 400 µl of distilled water, and the volume was adjusted to 1 ml. The mixture was then vortexed and incubated at 45°C for 30 min before cooling rapidly with ice. The absorbance of the solution was measured at 760 nm. Gallic acid concentrations ranging from 10 to 300 µg/ml were prepared and a calibration curve was obtained using a linear fit.

2.4.3. Determination of flavonoid content

The total flavonoid content was determined according to the aluminum chloride method [27]. Briefly, 0.5 ml of sample and 300 µl of $NaNO_2$ (1:20 w/v) were pipetted into a test tube and the contents were vortexed for 10 second and left to stand at room temperature for 5 min. After standing, 300 µl of $AlCl_3$ (1:10 w/v), 2 ml of NaOH (1 M) and 1.9 ml of distilled water were added to the reaction mixture, which was then vortexes for 10 s, and the absorbance was measured at 510 nm. Narnginine concentrations ranging from 10 to 800 µg/ml were prepared and a standard calibration curve was obtained using a linear fit.

3. RESULTS AND DISCUSSION

Epidemiological studies show that human body is damaged by reactive oxygen and nitrogen species. Thus, it is considered important to increase the intake of antioxidants in the human diet. Some synthetic antioxidants may exhibit toxicity with carcinogenic potential, show lower efficiency than natural antioxidants, and require high manufacturing costs. Thus, there is a need to identify natural and possibly more economic and effective antioxidants

[21, 28, 29]. So, in present study, ethanolic extracts of a total 100 fungal strains belonging to endophytic (26), saprophytic (32) and entomopathogenic (42) strains were investigated for antioxidant potential by using three different methods, all extracts showed antioxidant activity up to varying extent.

3.1. Reducing power

The reducing power evaluation of the fungal extracts is an important parameter related to assessing the antioxidant activity. Reducing power measure the ability of a sample to act as an electron donor and therefore, reacts with free radicals converting them to more stable products and thereby terminate radical chain reaction. In order to examine the reducing power of fungal extracts, the reaction of Fe^{3+} was observed. The reducing power of the extracts was determined according to Change et al. [25]. Increase absorbance of the reaction mixture indicated increased reducing power of the sample. Absorbance was read to determine the amount of ferric Ferro cyanide (Prussian blue) formed. Ascorbic acid was taken as the standard. The results revealed that a total of 21, 35 and 19 out of 26, 42 and 32 strains of endophytes, entomopathogens and saprophytes, respectively, had a reducing power activity and formed ferric ferro cyanide with activities ranged between 0.01 and 1.116 mg/ml fungal extract (Tables 1-3). These producer strains can be classified according to their reducing power activities into three groups. The highest reducing power activities (which formed ≥ 0.6 mg/ml) were represented by 9, 20 and 14 strains of the three tested groups of fungi, respectively (Tables 1-3). The highest one of endophytic fungi was *Emericella nidulans* AUMC 8854 (recorded 0.972 ± 0.04 mg/ml) followed by *Aspergillus versicolor* AUMC 6872 (formed 0.942 ± 0.001 mg/ml) (Table 1). *Beauveria bassiana* AUMC 3873 was the greatest strain of entomopathogenic group and recorded 1.042 ± 0.01 mg/ml followed by *Aspergillus niger* AUMC 9890 which yield 1 ± 0.005 mg/ml (Table 2). On the other side, the best strain of saprophytic fungi was *Phoma herbarum* AUMC 3509 (formed 1.116 ± 0.01 mg/ml) followed by *Aspergillus terreus* AUMC 3101 and *Botryotrichum piluliferum* AUMC 6467, they yield ferric ferro cyanide with activities equal to 1.022 ± 0.003 and 1.020 ± 0.003 mg/ml, respectively (Table 3).

Moderate activities of reducing power (from 0.4 to > 0.6 mg/ml) were observed in the extract of 4, 3 and 2 strains of endophytes, entomopathogens and saprophytes, respectively. On the other hand, 8, 12 and 3 strains of endophytic, entomopathogenic and saprophytic fungi under study, respectively, were recorded as lower producers for reducing power compounds with activities less than 0.4 mg/ml (Tables 1-3). Only 5, 7 and 13 out of the tested strains of endophytic, entomopathogenic and saprophytic fungi, respectively, could not to produce any detectable amounts of reducing power compounds (Tables 1-3).

These results are in agreement with those obtained by Chandra and Arora [29]. They determined the reducing power of 51 strains of fungi isolated from different area of Indian soil and recorded that only 32 fungal strains showed reducing power ranged from 0.115 to 1.6 mg/ml. Recently, Kumaresan et al. [30] reported that the reducing power of the extracts of four endophytic fungi *Chaetomium* sp., *Curvularia* sp., *Colletotrichum* sp. and *Trichoderma* sp. were ranged between 0.935 and 1.241 mg/ml extract. They found that *Chaetomium* sp. exhibited maximum reducing power (1.241 mg/ml) and *Colletotrichum* sp. showed the lowest reducing power (0.935 mg/ml).

3.2. Total phenolic content (TPC)

The total phenolic content was determined according to Folin-Ciocalteu (F-C) colorimetric method as described by Cicco et al. [26]. The TPC of ethanolic fungal extracts have been expressed as gallic acid equivalent (GAE). TPCs are known to be responsible for antioxidant activity and the high TPC is positively correlated with the antioxidant potential of an organism. All tested strains could be producing TPCs with levels ranged from 0.92 to 63.44 mg/ml fungal extract. Total phenolic compounds produced by endophytic fungi were ranged from 18.3 to 63.44 mg/ml while those produced by saprophytic and entomopathogenic fungi fluctuated between 0.92 to 60.54 and 6.6 to 61.72 mg/ml, respectively (Tables 1-3).

Most of research achieved on detected the total phenolic compounds produced by fungi using endophytic fungi [11, 31]. In this study out of the

total 26 tested endophytic strains, 12 strains (46.15%) were recorded as highly producer strains which secreted total phenolic compounds with activities equal to or more than 40 mg/ml. The superior endophytic fungal strain was *Emericella nidulans* AUMC 8854 which formed TPCs with activity reached to 63.44 ± 0.001 mg/ml, followed by *Aspergillus oryzae* AUMC 8863 which formed activity reached to 52.72 ± 0.008 mg/ml (Table 1). On the other side, 28 and 18 fungal strains represented 66.67% and 56.25% out of the tested strains of entomopathogenic and saprophytic fungi were recorded as highly producers for TPC and secreted total phenolic compounds with activities equal to or more than 40 mg/ml in (Tables 2, 3). The greatest strain of entomopathogenic fungi was *Epicoccum nigrum* AUMC 3148 which yield 61.72 ± 0.06 mg/ml extract in (Table 2). While the greatest two strains of saprophytic fungi were *Paecilomyces lilacinus* AUMC 6499 and *Penicillium roquefortii* AUMC 6398 which recorded 61.6 ± 0.03 mg/ml and 60.54 ± 0.01 mg/ml, respectively (Table 3).

Table 1. Total phenolic compounds, flavonoids and reducing power as antioxidant activities of some endophytic fungi recorded as mg/ml fungal extracts.

Fungal strains	Reducing power	Level	Total phenolic	Level	Flavonoids	Level
Alternaria						
A. alternata AUMC 6836	0.71 ± 0.02	H	19.82 ± 0.01	L	0.586 ± 0.001	L
A. alternata AUMC 8840	0.712 ± 0.05	H	39.2 ± 0.001	M	7.726 ± 0.02	L
A. alternata AUMC 8841	0.388 ± 0.001	L	40.32 ± 0.1	H	8.046 ± 0.04	L
Aspergillus						
A. awamori AUMC 8855	0.762 ± 0.01	H	44.4 ± 0.05	H	9.626 ± 0.001	L
A. fumigatus AUMC 8872	0.74 ± 0.5	H	42.3 ± 0.2	H	12.686 ± 0.2	L
A. niger AUMC 8852	L.D.	N	40.82 ± 0.06	H	9.486 ± 0.03	L
A. niger AUMC 8856	0.348 ± 0.001	L	48.64 ± 0.002	H	19.086 ± 0.03	M
A. oryzae AUMC 8863	0.224 ± 0.007	L	52.72 ± 0.008	H	19.346 ± 0.01	M
A. versicolor AUMC 6872	0.942 ± 0.001	H	37.34 ± 0.009	M	0.406 ± 0.003	L
Circinella muscae AUMC 8861	0.078 ± 0.001	L	38.2 ± 0.1	M	6.946 ± 0.05	L
Chaetomium globosum AUMC 8862	L.D.	N	33.94 ± 0.09	M	7.846 ± 0.004	L
Fusarium						
F. lateritium AUMC 6833	0.592 ± 0.001	M	40.4 ± 0.02	H	9.986 ± 0.002	L
F. oxysporum AUMC 6827	0.552 ± 0.02	M	48.28± 0.001	H	9.926 ± 0.03	L
F. semitectum AUMC 6816	L.D.	N	19.06 ± 0.01	L	1.106 ± 0.02	L
F. scirpi AUMC 8858	0.744 ± 0.05	H	49.72 ± 0.005	H	3.746 ± 0.001	L
F. subglutinans AUMC 8839	L.D.	N	33.54 ± 0.008	M	3.506 ± 0.001	L
Gliocladium solani AUMC 6802	0.102 ± 0.002	L	18.3 ± 0.005	L	4.086 ± 0.001	L
Emericella						
E. nidulans AUMC 8854	0.972 ± 0.04	H	63.44 ± 0.001	H	10.286 ± 0.01	L
E. rugulosa AUMC 8867	L.D.	N	41.54 ± 0.2	H	12.106 ± 0.004	L
Exophiala costellanii AUMC 8865	0.264 ± 0.2	L	37 ± 0.003	M	7.366 ± 0.05	L
Papulaspora irregularis AUMC 8843	0.58 ± 0.002	M	23.08 ± 0.03	M	0.466 ± 0.05	L
Penicillium						
P. aurantiogriseum AUMC 8847	0.35 ± 0.03	L	38.86 ± 0.001	M	4.826 ± 0.002	L
P. funiculosum AUMC 8850	0.762 ± 0.01	H	44.4 ± 0.05	H	9.626 ± 0.001	L
P. raistrickii AUMC 7265	0.45 ± 0.01	M	36.72 ± 0.01	M	13.346 ± 0.05	L
Penicillium sp. AUMC 8859	0.392 ± 0.03	L	22.7 ± 0.03	M	5.866 ± 0.003	L
Pleospora tarda AUMC 8871	0.862 ± 0.3	H	24.9 ± 0.03	M	61.826 ± 0.003	H

Table 2. Total phenolic compounds, flavonoids and reducing power as antioxidant activities of some entomopathogenic fungi recorded as mg/ml fungal extracts.

Fungal strains	Reducing power	Level	Total phenolic	Level	Flavonoids	Level
Aspergillus						
A. flavus AUMC 9881	L.D.	N	49.98 ± 0.006	H	29.406 ± 0.04	M
A. flavus AUMC 9885	L.D.	N	58.74 ± 0.03	H	6.086 ± 0.001	L
A. flavus AUMC 9903	0.834 ± 01	H	39.72 ± 0.4	M	6.226 ± 0.001	L
A. flavus AUMC 9904	0.682 ± 0.006	H	48.6 ± 0.07	H	14.986 ± 0.09	L
A. niger AUMC 9882	0.292 ± 0.1	L	42.64 ± 0.01	H	24.806 ± 0.05	M
A. niger AUMC 9890	1 ± 0.005	H	44.26 ± 0.08	H	18.106 ± 0.03	M
A. sydowii AUMC 9888	0.814 ± 0.09	H	41.2 ± 0.01	H	12.406 ± 0.07	L
A. tamarii AUMC 9902	0.27 ± 0.05	L	47 ± 0.08	H	25.086 ± 0.3	M
Beauveria						
B. bassiana AUMC 3847	0.76 ± 0.1	H	18.46 ± 0.00	L	5.906 ± 0.01	L
B. bassiana AUMC 3848	0.712 ± 0.001	H	44.2 ± 0.1	H	5.743 ± 0.03	L
B. bassiana AUMC 3849	0.646 ± 0.01	H	47.34 ± 0.09	H	7.746 ± 0.006	L
B. bassiana AUMC 3850	0.858 ± 0.03	H	55.4 ± 0.04	H	5.266 ± 0.05	L
B. bassiana AUMC 3852	0.486 ± 0.04	M	35.94 ± 0.1	M	2.706 ± 0.002	L
B. bassiana AUMC 3853	0.81 ± 0.1	H	41.6 ± 0.006	H	5.906 ± 0.1	L
B. bassiana AUMC 3854	0.058 ± 0.01	L	19.42 ± 0.001	L	1.986 ± 0.001	L
B. bassiana AUMC 3855	0.306 ± 0.002	L	44.26 ± 0.03	H	5.926 ± 0.05	L
B. bassiana AUMC 3856	0.75 ± 0.1	H	39.54 ± 0.4	M	7.726 ± 0.06	L
B. bassiana AUMC 3858	0.74 ± 0.001	H	16.22 ± 0.008	L	0.606 ± 0.003	L
B. bassiana AUMC 3859	0.374 ± 0.02	L	11.86 ± 0.07	L	16.686 ± 0.01	M
B. bassiana AUMC 3860	0.516 ± 0.06	M	55.28 ± 0.2	H	11.906 ± 0.04	L
B. bassiana AUMC 3862	0.598 ± 0.04	M	53.16 ± 0.09	H	8.626 ± 0.03	L
B. bassiana AUMC 3864	0.72 ± 0.01	H	48.22 ± 0.001	H	5.786 ± 0.07	L
B. bassiana AUMC 3866	0.764 ± 0.001	H	41.24 ± 0.004	H	15.526 ± 0.06	M
B. bassiana AUMC 3867	0.39± 0.07	L	60.83 ± 0.3	H	12.666 ± 0.03	L
B. bassiana AUMC 3869	0.182 ± 0.01	L	11.58 ± 0.3	L	0.806 ± 0.04	L
B. bassiana AUMC 3870	0.726 ± 0.002	H	40.9 ± 0.03	H	7.586 ± 0.001	L
B. bassiana AUMC 3873	1.042 ± 0.01	H	45 ± 0.005	H	10.626 ± 0.03	L
B. bassiana AUMC 9894	0.33 ± 0.06	L	41 ± 0.02	H	9.446 ± 0.1	L
B. bassiana AUMC 9895	0.09 ± 0.001	L	42.96 ± 0.2	H	19.126 ± 0.05	M
B. bassiana AUMC 9896	0.266 ± 0.06	L	37.74 ± 0.003	M	10.246 ± 0.4	L
B. bassiana AUMC 9908	0.91 ± 0.08	H	6.6 ± 0.02	L	6.413 ± 0.003	L
Epicoccum						
E. nigrum AUMC 3148	0.804 ± 0.01	H	61.72 ± 0.06	H	36.626 ± 0.07	H
E. nigrum AUMC 3149	0.348 ± 0.003	L	47.2 ± 0.11	H	12.206 ± 0.03	L
Fusarium						
F. nygamai AUMC 9891	L.D.	N	41 ± 0.3	H	18.506 ± 0.1	M
F. nygamai AUMC 9892	L.D.	N	54 ± 0.007	H	10.566 ± 0.02	L
F. pseudocircinatum AUMC 9899	L.D.	N	30 ± 0.09	M	4.166 ± 0.03	L
F. solani AUMC 9893	0.254 ± 0.3	L	33.4 ± 0.001	M	6.086 ± 0.006	L
F. verticillioides AUMC 9889	L.D.	N	45.58 ± 0.005	H	11.126 ± 0.01	L
Metarhizium anisoplea AUMC 5130	L.D.	N	32.6 ± 0.02	M	8.446 ± 0.05	L
Penicillium						
P. corylophilum AUMC 9900	0.838 ± 0.1	H	33 ± 0.008	M	7.666 ± 0.004	L
P. oxalicum AUMC 9898	0.79 ± 0.003	H	52.92 ± 0.1	H	16.086 ± 0.05	M
Penicillium sp. AUMC 9901	0.834 ± 0.07	H	42.6 ± 0.005	H	7.226 ± 0.04	L

Table 3. Total phenolic compounds, flavonoids and reducing power as antioxidant activities of some saprophytic fungi recorded as mg/ml fungal extracts.

Fungal strains	Reducing power	Level	Total phenolic	Level	Flavonoids	Level
Alternaria						
A. alternata AUMC 3128	0.926 ± 0.05	H	24.98 ± 0.1	M	9.726 ± 0.007	L
A. alternata AUMC 3131	0.026 ± 0.001	L	40.98 ± 0.006	H	8.126 ± 0.08	L
Aspergillus						
A. flavus AUMC 3200	L.D.	N	42.98 ± 0.1	H	9.886 ± 0.002	L
A. fumigatus AUMC 48	0.978 ± 0.09	H	13.04 ± 0.01	L	1.066 ± 0.001	L
A. terreus AUMC 3101	1.022 ± 0.003	H	16 ± 0.2	L	2.326 ± 0.004	L
A. terreus AUMC 3102	L.D.	N	59.8 ± 0.08	H	45.606 ± 0.06	H
Botryotrichum piluliferum AUMC 6467	1.02 ± 0.003	H	49.84 ± 0.2	H	14.086 ± 0.01	L
Chaetomium						
C. globosum AUMC 113	0.636 ± 0.01	H	41.6 ± 0.03	H	16.006 ± 0.02	M
C. globosum AUMC 114	0.652 ± 0.04	H	48.72 ± 0.01	H	11.886 ± 0.001	L
Cladosporium						
C. cladosporioides AUMC 132	0.432 ± 0.08	M	39.56 ± 0.02	M	16.746 ± 0.003	M
C. cladosporioides AUMC 133	0.708 ± 0.001	H	41.24 ± 0.01	H	11.906 ± 0.01	L
C. cladosporioides AUMC 3111	L.D.	N	41.6 ± 0.3	H	7.006 ± 0.007	L
C. cladosporioides AUMC 6091	0.946 ± 0.01	H	13.12 ± 0.09	L	8.386 ± 0.003	L
Fusarium						
F. oxysporum AUMC 3224	0.846 ± 0.03	H	5.28 ± 0.005	L	0.166 ± 0.07	L
F. proliferatum AUMC 3190	L.D.	N	40.46 ± 0.07	H	8.626 ± 0.1	L
F. solani AUMC 222	L.D.	N	21.62 ± 0.002	M	3.546 ± 0.006	L
F. solani AUMC 223	0.988 ± 0.02	H	15.36 ± 0.03	L	3.986 ± 0.05	L
Gliocladium						
G. catenulatum AUMC 6103	0.908 ± 0.05	H	11.06 ± 0.001	L	2.826 ± 0.3	L
G. roseum AUMC 3763	0.952 ± 0.003	H	51.04 ± 0.02	H	25.426 ± 0.01	M
Lecanicillium antillanum AUMC 9905	0.01 ± 0.006	L	45.8 ± 0.001	H	45.686 ± 0.2	H
Paecilomyces						
P. lilacinus AUMC 6275	L.D.	N	45.8 ± 0.01	H	14.126 ± 0.01	L
P. lilacinus AUMC 6499	L.D.	N	61.6 ± 0.03	H	10.606 ± 0.007	L
P. variotii AUMC 3112	0.514 ± 0.03	M	39.38 ± 0.05	M	8.546 ± 0.01	L
Papulaspora irregularis AUMC 3107	L.D.	N	49.4 ± 0.4	H	11.566 ± 0.03	L
Penicillium						
P. roquefortii AUMC 6397	L.D.	N	36.18 ± 0.06	M	11.906 ± 0.03	L
P. roquefortii AUMC 6398	L.D.	N	60.54 ± 0.01	H	64.806 ± 0.08	H
Periconia digitata AUMC 6235	0.956 ± 0.1	H	0.92 ± 0.03	L	0.746 ± 0.1	L
Phoma herbarum AUMC 3509	1.116 ± 0.001	H	54.36 ± 0.04	H	13.326 ± 0.01	L
Trichoderma						
T. longibranchiatum AUMC 3113	L.D.	N	35.16 ± 0.04	M	6.846 ± 0.09	L
T. pseudokoningii AUMC 6430	L.D.	N	50.1 ± 0.02	H	11.466 ± 0.003	L
Rhizopus stolonifer AUMC 9906	0.238 ± 0.01	L	30.56 ± 0.05	M	4.726 ± 0.05	L
Stachybotrys chartarum AUMC 1661	L.D.	N	44.4 ± 0.006	H	5.306 ± 0.008	L

Several previous studies have proved that the phenolic substances are considerably more potent antioxidants than vitamin C and vitamin E. These compounds have also been found to exhibit many other health related properties because of their antioxidant activities [32]. The interest in the phenolic compounds has increased tremendously due to their prominent free radical scavenging activity [33], attributed to their redox properties, which allow them to act as reducing agents or hydrogen atom donor [34].

The results in this study link with some previous finding specially those of endophytic fungi and their antioxidant activity. *Chaetomium* sp. isolated from *Nerium oleander* possessed the highest antioxidant capacity with phenolic content reached to 13.95 ± 0.11 mg/ml [35]. Chandra and Arora [29] examined 51 strains of soil fungi for TPCs production and found that all strains had the ability to produce TPC with levels ranged from 1.01 and 20.59 mg/ml extract. Also, the same authors in another paperArora and Chandra [36] examined four fungal isolates: *Aspergillus wentii* 1, *A. wentii* 2, *Penicillium citrinum* and *P. granulatum* for their antioxidant potential and detected 20.6, 12.1, 12.03 and 7.2 mg/ml extract of the four fungal isolates, respectively. Ruma et al. [31] reported that the TPC in different selected six endophytic fungal extracts ranged from 4 to 144 mg/ml extract. *Aspergillus terreus* isolated from *Ocimum sanctum* exhibited antioxidant activity with 14.96 ± 0.07 mg/g dry weight [37].

Yadav et al. [11] isolated 21 endophytic fungal isolates from *Eugenia jambolana* Lam in India and screened their ability to produce TPC. They found that their TPC varied from 4.20 to 60.13 mg/g of dry weight. Also, they observed the highest level of TPC was in the extract of *Chaetomium* sp. (60.13 mg) followed by *Aspergillus niger* (58.46 mg). Recently, Kumaresan et al. [30] studies the antioxidant potential of four endophytic fungi and reported that the TPCs in the extract of *Chaetomium* sp., *Curvularia* sp. *Colletotrichum* sp. and *Trichoderma* sp. were 28.5, 9.82, 10.63, and 7.51 mg/g dry weight, respectively. Sugiharto et al. [38] reported that the TPCs of *Acremonium charticola* and *Rhizopus oryzae* isolated from the Indonesian fermented dry cassava were 26.25 ± 0.39 and 16.08 ± 0.16 mg/100 g, respectively.

3.3. Flavonoids

The amount of total flavonoids compounds was determined as the naringenin equivalent using an equation obtained from a standard naringenin graph. The results in this study appeared that the tested fungal strains have the ability to produce flavonoids with levels of 0.166 to 68.806 mg/ml (Tables 1-3).

The highest flavonoid producers (formed ≥ 35 mg/ml) were only five of tested fungal strains of endophytes (1), entomopathogens (1) and saprophytes (3) shows in (Tables 1-3). Out of the 26 tested strains of endophytic fungi, only *Pleospora tarda* AUMC 8871 was recorded as highly producer strain and formed 61.826 ± 0.003 mg/ml followed by *Aspergillus oryzae* AUMC 8863 formed 19.346 ± 0.01 mg/ml as in (Table 1). The highest entomopathogenic strain was *Epicoccum nigrum* AUMC 3148 (recorded 36.626 ± 0.07 mg/ml) flowed by *Aspergillus flavus* AUMC 9881 (formed 29.406 ± 0.04 mg/ml) in (Table 2), while the highest three strains of saprophytic fungi were *Penicillium roquefortii* AUMC 6398, *Lecanicillium antillanum* AUMC 9905 and *Aspergillus terrus* AUMC 3102 which recorded 64.806 ± 0.08 mg/ml 45.686 ± 0.2 mg/ml and 45.606 ± 0.06 mg/ml, respectively (Table 3).

Similar results were obtained by several researchers. Kumaresan et al. [30] reported that all tested endophytic fungi had the ability to produce flavonoids in varied quantity ranged from 3.08 and 11.83 mg/g dry weight. Smith et al. [39] examined 10 species of filamentous fungi for their antioxidant capacity and reported that the total flavonoid compounds of the ten species were in the range of 0.02-3.90 mg/g. Sugiharto et al. [38] found that the total flavonoids of *Acremonium charticola* and *Rhizopus oryzae* isolated from the Indonesian fermented dried cassava were 2.92 ± 0.15 and 10.87 ± 0.37 mg/100 g dry weight, respectively.

4. CONCLUSION

The present study demonstrates the ability of fungi belonging to endophytes, entomopathogenes as well as saprophytes to produce compounds having significant antioxidant activities. High levels of total phenolic and flavonoid compounds were

recorded in the extracts of most tested fungal strains specially those belonging to endophytes. Production of these compounds by fungi will be helpful in the biotechnological mass production of safe alternative sources of antioxidants for incorporation into some food products and supplements preventing many free radical mediated diseases and healthy cosmetics.

AUTHORS' CONTRIBUTION

All authors contributed in design and execution the research plan point to point. Also, they contributed in writing, read and approved the final manuscript.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

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