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AIMS AND SCOPE

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Pharmata aims to contribute to the literature by publishing manuscripts at the highest scientific level. The journal publishes research articles, reviews and short communications that are prepared in accordance with ethical guidelines.

The scope of the journal includes but not limited to pharmaceutical analysis of complex system, quality control and methods of biotech drugs, action mechanism and their metabolisms in body, quantitative and qualitative analysis in the drug screening process, molecular pharmacology, biopharmaceutics, pharmacognosy, pharmaceutical botany, pharmaceutical technology studies, clinical laboratory and bioanalysis, the pharmacological studies, the toxicologic studies, analytical chemistry techniques and methods, new biochemistry methods for pharmaceutical analysis, rapid screening methods, new analytical techniques and methods, pharmaceutical chemistry, synthesis and analysis new drug molecules, others: pharmaceutical solid materials (including biomaterials, polymers and nanoparticles) biotechnology products (including genes, peptides, proteins and vaccines) engineered cells.

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Pharmata

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RESEARCH ARTICLES

REVIEW

ATATURK UNIVERSITY PUBLICATIONS

Research Article

DOI: 10.5152/Pharmata.2023.1270397

Development and In Vitro Characterization of Nanoemulsion Containing *Hippophaes rhamnoides* Aqueous Extract

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ABSTRACT

Objective: *Hippophaes rhamnoides* is a member of the Elaeagnaceae family and grows in many countries of the world such as Turkey, France, Germany, Norway, and Russia. *Hippophaes rhamnoides* extracts have antioxidant, anticancer, antidiabetic, antiviral, and cardiovascular protective effects. Nanoemulsions are suitable systems for formulations of herbal extracts. The aim of this study was to develop and *in vitro* characterize nanoemulsion formulation containing *Hippophaes rhamnoides* aqueous extract.

Methods: *Hippophaes rhamnoides* aqueous extract-containing nanoemulsion or blank nanoemulsion formulations were prepared using ethyl oleate, lipoid S100, Kolliphor RH40, Pluronic F68, Dimethyl sulfoxide (DMSO), and ultrapure water. The droplet size, polydispersity index, and zeta potential values of nanoemulsions were determined, and pH measurement, Fourier-transform infrared spectroscopy (FT-IR), and rheological analyses were also performed.

Results: The droplet size and polydispersity index values of nanoemulsions were found to be <200 nm and <0.3, respectively, which indicates monodispersity. The zeta potential values of blank nanoemulsion and *Hippophaes rhamnoides* aqueous extract-containing nanoemulsion were determined as (–)28.51 ± 2.61 and (–)30.11 ± 2.02 mV, respectively. FT-IR results showed that the extract is completely dissolved in the oil phase of formulations.

Conclusion: The nanoemulsion formulation containing *Hippophaes rhamnoides* aqueous extract was successfully prepared and characterized. The formulation may be beneficial for the usage of *Hippophaes rhamnoides* aqueous extract.

Keywords: Hippophaes rhamnoides, in vitro characterization, nanoemulsion

INTRODUCTION

Emulsion is defined as heterogeneous systems in which 2 immiscible liquids are dispersed as droplets within each other.[1] Nanoemulsion (NE), which is a subtype of emulsion systems, has a droplet size below 1000 nm and can be prepared as oil in water or water in oil. Nanoemulsions generally have droplet size in the range of 100-500 nm.[2,3] They are kinetically stable systems and can be prepared by high-energy methods (such as high-pressure homogenization, ultrasonication, and microfluidization) and low-energy methods (such as phase inversion and self-emulsification methods).[2] They can be administered via different routes (e.g., oral, topical, and parenteral). Nanoemulsions are preferred systems because they increase the solubility and bioavailability of active compounds and provide reproducible bioavailability.[4,5] Nanoemulsions can be prepared as dosage forms such as spray, cream, gel, foam, and aerosol.[3]

Hippophaes rhamnoides is a member of the Elaeagnaceae family and grows in many countries of world such as Turkey, France, Germany, Norway, and Russia. It is a valuable plant and has gained attention worldwide because of its medicinal and nutritional properties.[6] Moreover, it has been reported to have antioxidant activity, cardioprotective activity, antitumor effect, antidiabetic effect, wound healing properties, and antimicrobial and antiviral activities.[7–12] Bioactive compounds of *H. rhamnoides* are flavonoids, phenols, carotenoids, vitamins, fatty acids, terpenes, and sterols.[13,14]

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METHODS

Materials

In this study, ethyl oleate (Merck, Germany), lipoid S100 (Lipoid, Germany), Kolliphor RH40 (Sigma, Germany), Pluronic F68 (BASF, Germany), DMSO (Lab-Scan, Ireland) were used. The water was purified by Direct-Q®3 UV water purification system (Millipore, USA).

Preparation of Hippophaes rhamnoides Aqueous Extract

First, the aerial parts of *H. rhamnoides* were pulverized using a laboratory blender. About 250 mL of ultrapure water was added to 10 g powder and kept in a horizontally shaking water bath for 48 hours at 50°C. It was filtered every 24 hours, and fresh ultrapure water was added to enable the removal of plant material. After 48 hours, the filtrates were collected and evaporated to almost dryness (50°C, 60 rpm). The concentrated filtrate was frozen at −20°C and then lyophilized at −55°C for 24 hours. The dry *Hippophaes rhamnoides* aqueous extract (AE-Hr) was stored in airtight containers in a refrigerator (2-8°C), protected from light, for further studies.

Preparation of Nanoemulsion Formulations

Hippophaes rhamnoides aqueous extract-containing nanoemulsion (AE-Hr-NE) and blank nanoemulsion (B-NE) formulations were prepared using a high-energy method. First, the oil [ethyl oleate (5%) and lipoid S100 (1%)] and water [Kolliphor RH40 (1%), Pluronic F68 (1%), and ultrapure water] were prepared separately. The extract (10 mg) was added to the oil phase after dissolving in DMSO. Under magnetic stirring (750 rpm), the water phase was added to the oil phase, and coarse emulsion was prepared. The coarse emulsion was first homogenized with a high-speed mixer (Ultraturrax T-10; 25000 rpm, 5 minutes), and then ultrasonication (40% power, 15 minutes) was performed to ensure nanosized droplets. Blank nanoemulsions were prepared by the same procedure but without the addition of the extract.

Formulation Characterization

Droplet Size, Polydispersity Index, Zeta Potential, and Morphological Analysis

Zetasizer Nano ZSP (Malvern Ins., Ltd, UK) was used to determine the mean droplet size, polydispersity index (PDI), and zeta potential values of the NE formulations. In addition, the AE-Hr-NE was imaged using transmission electron microscopy (TEM) (Hitachi HighTech HT7700, Japan). After dilution for 100 times, the AE-Hr-NE was placed on a copper grid and dried at room temperature over 24 hours. Images of the grids were then obtained at 120 kV.

pH

The pH values of NEs were determined at room temperature using a pH meter (Thermo Scientific, Orion 3 Star, USA).

Rheology

A Brookfield RV DV2T cone and plate viscometer were used to measure the viscosity of the NEs at room temperature.

FT-IR Analysis

FT-IR analyses (4000-400 cm^{-1}) of the extract and NEs were performed using Fourier transform infrared spectroscopy (Shimadzu IRSprit-T).

Statistical Analyses

Statistical analyses were performed using Statistical Package for the Social Sciences Version 22.0 (IBM SPSS Corp., Armonk, NY, USA) software. The "independent *t*-test" was used to compare the differences between 2 independent samples. The significance of the difference between test results was determined, and the difference was accepted to be significant if $P < .05$.

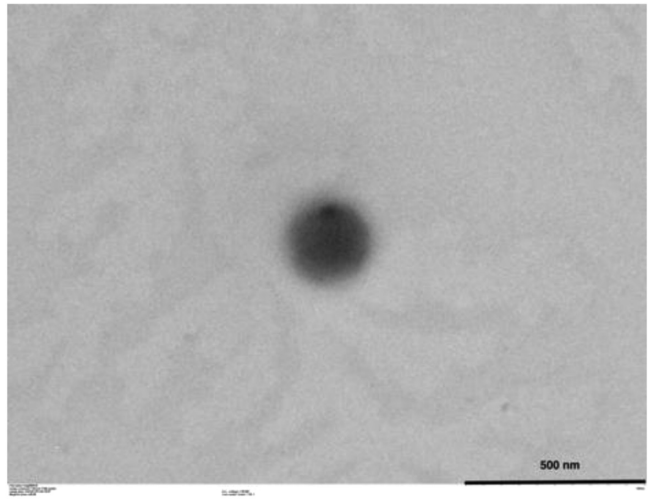

Figure 1. The transmission electron microscopy image of *Hippophaes rhamnoides* aqueous extract-containing nanoemulsion.

RESULTS

Droplet Size, Polydispersity Index, Zeta Potential, and Morphological Analysis

The droplet size, PDI, and zeta potential values of the prepared NE formulations are given in Table 1. The TEM image of AE-Hr-NE is shown in Figure 1.

pH Determination

The pH values of the NE formulations are given in Table 2.

FT-IR Analysis

FT-IR spectra of AE-Hr and the prepared NE formulations are shown in Figure 2.

Rheological Analysis

Rheograms of the prepared NE formulations are given in Figure 3.

DISCUSSION

Droplet Size, Polydispersity Index, Zeta Potential, and Morphological Analysis

Droplet size and droplet size distribution are parameters that affect bioavailability and physical and chemical stability and are very important for the characterization of NE formulations.[15–18] The average droplet sizes and PDI values of the prepared NE formulations are given in Table 1. When evaluated statistically, it was

Table 1. The Mean Droplet Size, PDI, and Zeta Potential Values of NEs ($X \pm SD$, n = 9)

Formulation	Droplet Size (nm)	PDI	Zeta Potential (mV)
B-NE	176.89 ± 4.79	0.257 ± 0.019	−28.51 ± 2.61
E-NE	183.07 ± 9.53	0.295 ± 0.045	−30.11 ± 2.02

B-NE, blank nanoemulsion; E-NE, extract-containing nanoemulsion; NE, nanoemulsion; PDI, polydispersity index; *X*, mean.

Table 2. pH Values of NE Formulations ($X \pm SD$; n = 9)

Formulation	pH
B-NE	5.19 ± 0.06
AE-Hr-NE	5.15 ± 0.01

AE-Hr-NE, *Hippophaes rhamnoides* aqueous extract-containing nanoemulsion; B-NE, blank nanoemulsion; *X*, mean.

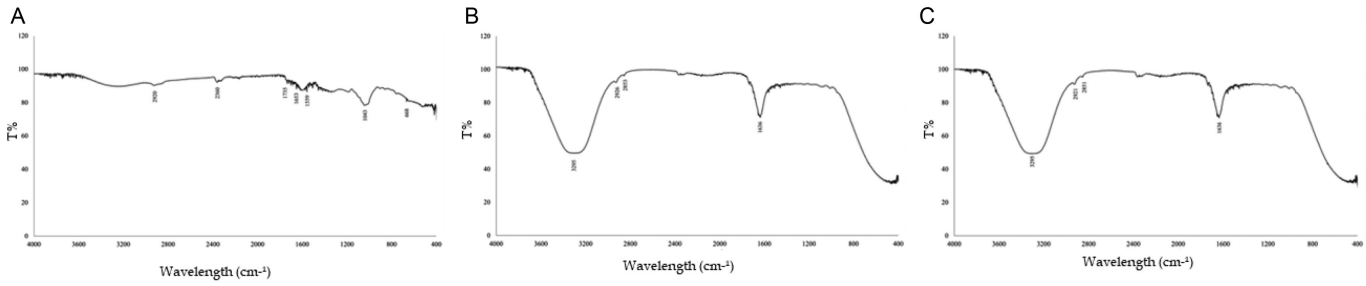

Figure 2. FT-IR spectra of *Hippophaes rhamnoides* aqueous extract (A), blank nanoemulsion (B), and *Hippophaes rhamnoides* aqueous extract-containing nanoemulsion (C).

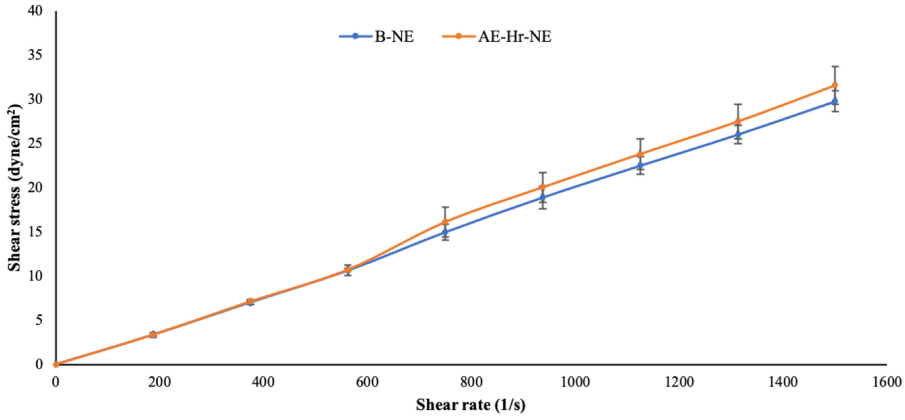

Figure 3. Flow rheograms of blank nanoemulsion and *Hippophaes rhamnoides* aqueous extract-containing nanoemulsion formulations ($X \pm SD$; n = 3).

determined that the increase in droplet size observed with the addition of the extract was not significant ($P > .05$). The PDI value expresses the uniformity of the droplet size of the formulation. In our study, it was observed that the PDI values of the NEs were <0.3 and the droplet size distribution was found to be in a narrow range. A PDI value <0.3 indicates good homogeneity.[19,20] Zeta potential is a term that describes the electrokinetic potentials of droplets and is very important in the evaluation of physical stability of colloidal dispersions. As a general rule, zeta potential values above ±30 mV are indicative of good stability. However, zeta potential values above ±20 mV can also provide sufficient stabilization in cases where high-molecular-weight surfactants and steric stabilization are also concerned. Non-ionic surfactants contribute to stability by forming a steric barrier between droplets. [21,22]

The TEM image of AE-Hr-NE is shown in Figure 1. It was observed that the droplets were nanosized and very close to spherical shape.

pH Determination

The presence of ions or the change in pH in an NE formulation is of great importance in formulation design and subsequent stability studies.[23] The pH values of the NEs are given in Table 2. There was no change in the pH value with the addition of extract to the formulations ($P > .05$).

FT-IR Analysis

In our study, FT-IR analysis was performed in order to obtain information about the functional groups in the structure of the formulation components and to determine whether there is an interaction between the components of the formulations and the extract. When the FT-IR spectra of B-NE and AE-Hr-NE are examined, it is seen that both spectra are quite similar, and the characteristic peaks of the extract are not in the spectrum of AE-Hr-NE (Figure 2). This indicates that the extract is dispersed at the molecular level in the NE formulation.[24]

Rheological Analysis

The relationship between shear rate and shear stress was determined in order to evaluate the flow properties of the prepared NEs (Figure 3). The viscosity values for B-NE and AE-Hr-NE were found to be 1.98 ± 0.08 and 2.10 ± 0.14, respectively, at a shear rate of 1500 s^{-1} at room temperature.

Ethics Committee Approval: Ethics committee approval was not required as no in vivo studies were included in this study.

Informed Consent: Since there was no in vivo study in this study, no patient consent was required.

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Author Contributions: Concept – Y.F.K., A.B.U.K., M.C.; Design – Y.F.K., A.B.U.K., M.C.; Supervision – A.B.U.K., M.C.; Resources – A.B.U.K., M.C.; Materials – A.B.U.K., M.C.; Data Collection and/or Processing – Y.F.K., S.C.; Analysis and/or Interpretation – S.C., Y.F.K., A.B.U.K., M.C.; Literature Search – S.C., Y.F.K., A.B.U.K., M.C.; Writing Manuscript – Y.F.K., A.B.U.K., M.C.; Critical Review – A.B.U.K., M.C.

Declaration of Interests: The authors declare that they have no competing interest.

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ATATURK UNIVERSITY PUBLICATIONS

Research Article

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Evaluation of Excipient Effects on Desloratadine Syrup formulation as Impurity and pH

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ABSTRACT

Objective: The aim of the research study is to compare the effects of excipient and pH and to develop a stable pharmaceutical product of desloratadine syrup formulation. For this stability study, 7 different formulation trial studies were prepared. In this study, different excipients were used and in this way different pH values were obtained to improve the product.

Methods: To improve the stability of desloratadine, 7 different formulations (F1-F7) were evaluated as different excipients, pH and impurities of desloratadine during the stability period. Each trial formulation was compared with pH and impurity results at different stability conditions at initial, 3rd month, 6th month.

Results: The most stable formulation study was evaluated within the pharmacopeia limits determined by comparing the stability results with each other in 7 different trial studies. According to the evaluation results, the F7 formulation was chosen as the most stable and best formulation in the studies.

Conclusion: According to the evaluation results, the F7 formulation was chosen as the most stable and best formulation in the studies.

Keywords: Drug formulation, desloratadine, syrup, excipient, pH, stability study, impurity, HPLC

INTRODUCTION

Desloratadine is a second-generation, tricyclic antihistamine which has a selective and peripheral H1-antagonist action. It is an effective descarboethoxy metabolite of loratadine (a second-generation histamine). Receptor-binding data specify that at a concentration of 2-3 mg/mL (7 nanomolars), desloratadine indicates significant interaction with the human histamine H1 receptor.[1] Desloratadines have a long-lasting effect and do not cause drowsiness because they do not readily enter into the central nervous system. Desloratadine belongs to the class of organic compounds known as benzocyclo-hepta-pyridines. These are aromatic compounds comprising a benzene ring and a pyridine ring fused to a 7-membered carbocycle. In general, many physical and chemical factors can have a negative effect on the stability of carbonic inhibitor.[2]

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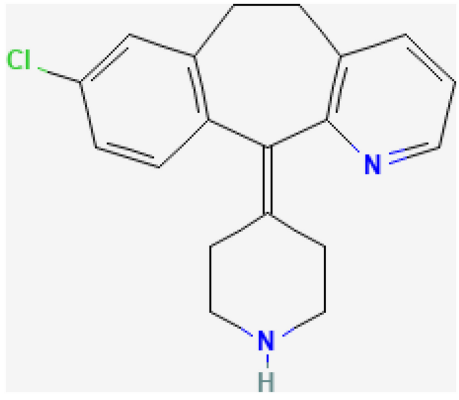

Figure 1. Desloratadine molecular structure[1]

[1] https://pubchem.ncbi.nlm.nih.gov/compound/Desloratadine#section=2D-Structure

The chemical designation of desloratadine is 8-chloro-6,11-dihydr o-11-(4-piperdinylidene)-5H-benzo[5,6]cyclohepta[1,2-b]pyridine. It is available as tablets and also available as syrup. It is a white to off-white powder that is slightly soluble in water but very soluble in ethanol and propylene glycol. The molecular formula of desloratadine is $C_{19}H_{19}ClN_2$ and the molecular weight is 310.8 g/mol. Desloratadine has a pyridine ring and a piperidine ring. A pyridine ring is a ring suitable for chemical interaction with ketone and aldehyde groups (Figure 1). Desloratadine molecular structure can be separated by proton donor. Because these groups are present in the molecule, Ethylenediamine Tetraacetic Acid (EDTA) should be used as the chelating aid. Due to its resistance to hydrolysis, EDTA can dissolve in water over a wide pH range with metal ions, which are not affected by temperature much, to form complexes. These complexes are particularly stable in the alkali environment and at high temperatures. EDTA provides the ligand function against the salts and ions in the raw materials in the product. Desloratadine and its compositions are prone to decomposition and oxidation by acidic excipients to form impurities such as deschlorodesloratadine, dehydrodesloratadine, and *N*-formyldesloratadine.[3] The desloratadine undergoes widespread degradation in the presence of common excipients such as lactose and stearic acid to form *N*-formyl desloratadine as a main degradation product. The basic salts of magnesium, calcium, or aluminum, plus avoidance of lactose and stearic acid, are used to control the degradation of desloratadine in liquid pharmaceutical compositions. Because of the widespread degradation of desloratadine in the presence of excipients such as lactose to form *N*-formyl desloratadine as a significant degradation product, the use of an effective stabilizer to increase the stability of the composition would be an important improvement in the field of therapeutic compositions.[4] The amount of EDTA to be used in the product is determined in the experiments for optimum impurity values. The buffering agents used for adjusting the product pH were quantified for experiments with minimum impurity. In addition, the pH of the product is investigated in the experiments made with the change of impurity.

METHODS

All trials are produced by using desloratadine of Vasudha Pharma Chem Ltd, Hindistan. Physical and chemical tests are made to justify the suitability of active substance to its specifications. As excipients, sodium methyl parahydroxybenzoate and sodium propyl parahydroxybenzoate are produced by Clarient, Switzerland, glycerol by Vance Bioenergy, Malezya, propylene glycol by Dow, United States, citric acid monohydrate by Jungbunzlauer, Basel Switzerland, sucrose by Çallı Gıda, Ataşehir/İstanbul, and orange flavor by Firmenich, Switzerland.

The syrup production method generally consists of a raw material weighing process, heating–cooling, solution preparation, mixing, and analytical analysis. A mechanical mixer, heater, and weighing device were used during the production of the product.[5] All production steps are detailed below. All excipients used in the trials are conventional raw materials used in pharmaceutical syrup formulations. In the stability analysis, the batch size is 150 mL for each trial. At the end of these trials, the product is placed in a 150 mL amber-colored glass bottle.

General Description of Production Process

1. Pure water is taken 40% of the total volume and to the main production tank and heated at 75-80°C.
2. Antimicrobial agents and sweeteners are added to the main production beaker of the heated 40% water. It is stirred until it becomes a clear solution.
3. In another preparation beaker, the stabilizer is heated at 40-50°C. Desloratadine raw material is added to the solution in the another production beaker in heated water.
4. The main production tank containing the solution obtained in step 2 is cooled to 40-50°C. Buffering and stabilizing agents are added to the solution obtained. It is stirred until it becomes a clear solution. The solution obtained in step 3 is added to the main solution by constant stirring. It is stirred until it becomes a clear solution.
5. A lubricant is added to the solution obtained in step 4, and with stirring, the solution is cooled to 30 ± 2°C.
6. Flavor is added to the solution obtained in step 5. It is stirred until it becomes a clear solution. A coloring agent is added to the solution obtained. The resulting solution is made up to volume and mixed for 10 minutes.
7. The solution obtained is filtered through 20 µm filters and filtered into the storage bottle.

Formulation trials from the desloratadine syrup F1-F7 are shown in Table 1. The combination of trials for minimum impurity with different excipients is presented below.

Through this experiment, we find that by adding a pH regulatoragent, a stable buffer system is formed. The pH value of the syrup and the kind of buffer system all can have an influence on the stability of formulation.[6] In these studies, all trials are prepared at optimum pH values and filtered. The prepared syrup is in the process of long-term stability. A significant change in pH and results above the pharmakope limits as impurities mean that the experiment is inappropriate..[7]

Trials are performed at different pH values. Various trials are performed with buffering agents (sodium citrate dihydrate and citric

Table 1. Formulation Trials

Function	Ingredients	F1 (g/150 mL)	F2 (g/150 mL)	F3 (g/150 mL)	F4 (g/150 mL)	F5 (g/150 mL)	F6 (g/150 mL)	F7 (g/150 mL)
API	Desloratadine	0.075	0.075	0.075	0.075	0.075	0.075	0.075
Sweet agent	Sucrose	90.00	90.00	90.00	90.00	90.00	90.00	90.00
Lubricant	Glycerol	7.50	7.50	7.50	7.50	7.50	7.50	7.50
Preservative	Sodium methyl hydroxybenzoate	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Preservative	Sodium propyl hydroxybenzoate	0.015	0.015	0.015	0.015	0.015	0.015	0.015
Antimicrobial agent	Propylene glycol	12.60	12.60	12.60	12.60	12.60	12.60	12.60
Buffering agent	Sodium citrate dihydrate	–	–	–	–	0.189	0.189	0.189
Buffering agent	Citric acid monohydrate	0.35	0.35	0.35	0.75	–	0.350	0.150
Chelating agent	EDTA	0.0375	0.075	0.150	0.0375	0.0375	0.0375	0.0375
Flavoring agent	Orange flavor	0.0075	0.0075	0.0075	0.0075	0.0075	0.0075	0.0075
Coloring agent	Sunset yellow	0.0012	0.0012	0.0012	0.0012	0.0012	0.0012	0.0012
Solvent	Purified water	To 150 mL	To 150 mL	To 150 mL	To 150 mL	To 150 mL	To 150 mL	To 150 mL

Table 2. The pH Values of the Finished Product Trials

	F1	F2	F3	F4	F5	F6	F7
pH	3.8	3.8	3.8	3.0	8.9	4.6	5.7

acid monohydrate) and chelating agent (EDTA). Since the amount of citric acid is 0.35 g/150 mL in the first 3 trials, the product is measured at acidic values. Sodium citrate dihydrate was used instead of citric acid in the F5 experiment for more basic pH results, whereby pH 8.9 was measured (Table 2). In the F6 trial, citric acid monohydrate and sodium citrate dihydrate were used together and the pH measured is 4.6. In the last trial, it is aimed to increase the pH to neutral level by reducing the amount of citric acid according to F6.

Preparation of Formulations for Stability Test

Product stability studies have been tested at 3 different temperature and humidity values. These values were taken according to the ICH guide. Long-term period (25°C ± 2°C; 60% ± 5% Relative humidity (RH)), Intermediate period (30°C ± 2°C; 65% BN ± 5% RH) Accelerated stability (40°C ± 2°C; 75% RH ± 5% RH). Samples were tested at the beginning of the stability, at the third month, and at the sixth month by impurity and pH tests.

Analytical Methods

Chemicals and Reagents

The pharmaceutical grade sample of desloratadine, impurity-A (methyl loratadine), impurity B (loratadine), and impurity-C (n-formyl loratadine) is purchased from Vasudha Pharma Chem Ltd. The injection water (0.05 µc) is produced by the Sartorius system as High Performance Liquid Chromatography (HPLC) grade.

HPLC Chromatographic Conditions of Desloratadine-Related Substances

HPLC, Av. Headlight. According to the current publication, monograph 2.2.29 limit is below.

Impurity A: 1.0%
Impurity B: 1.0%
Impurity C: 1.0%
Unknown impurity: 0.4%
Total impurity: 3.0%

Column	Inertsil ODS-2 5µm 150 mm × 3.0 mm or equivalent
Flow rate	0.7 mL/min
Dedector	UV, 278 nm
Injection volume	20 µL
Column temperature	40°C
Autosampler temperature	25°C
Filter	0.45 µm Polytetrafluoroethylene (PTFE)
Dilution solution	Moving phase B

Mobile phase A: 1.36 g KH_2PO_4 and 0.96 g pentane-1-sulfonic acid sodium salt are weighed and dissolved in 1000 mL distilled water. The pH was adjusted to 3.0 ± 0.05 with 2M phosphoric acid. It is mixed and degasified.

Mobile phase B: 0.5 mL trifluoroacetic acid is taken into a 1 L flask and filled with pure water.

The solution is mixed with methanol at a ratio of 90 : 10 (v/v). It is degassed.

Mobile phase C: Acetonitrile[8]

Preparations of a Standard Solution

About 25 mg desloratadine Working standart (WS) is exactly weighed and transferred into a 100 mL volumetric flask. It is dissolved in an ultrasonic bath for 5 minutes by adding 70.0 mL of solvent to it. This is made up to 100 mL volume with solvent. About 1 mL of this solution is taken and transferred to a 100 mL volumetric flask. The flask is made up to the volume by filling with the solvent. It is thoroughly mixed. About 4 mL of this solution is transferred to a 10 mL volumetric flask and filled with solvent in volume. It is mixed well. It is filtered through 0.45 µm PTFE filter and taken into the HPLC vial ($C_{Desloratadine}$: 0.001mg/mL).[9]

Sample Solution (2 Parallel sample solution prepared)

The syrup sample equivalent to 2.50 mg desloratadine is exactly weighed and transferred to a 10 mL volumetric flask. It is dissolved in an ultrasonic bath for 5 minutes by adding dilution solution to it. Make up to its volume with dilution solution. It is filtered through 0.45 µm PTFE filter and taken into the HPLC vial ($C_{Desloratadine}$: 0.25 mg/mL).

Procedure

After the column is equilibrated, dilution solution (1 time), standard solution (6 times), placebo solution (1 time), and sample solutions (once) are injected.

System Compatibility

"The area relative standard deviation (RSD) of the desloratadine peak in the standard solution of 6 consecutive injections should not be more than 5.0%."

The theoretical plate number for desloratadine should be at least 2000.

About Relative Retention Time (RRT)

- Desloratadine (approximately RT 11.5) 1.0
- Impurity A: (methyl loratadine) 1.2
- Impurity B (loratadine) 2.6
- Impurity C (*N*-formyl loratadine) 1.6
- The peaks from the placebo and blank chromatograms and those below 0.05% are ignored.

Calculation:

% Known and unknown impurity:

$$\frac{A_n}{A_s}\times\frac{W_s}{100}\times\frac{1}{100}\times\frac{4}{10}\times\frac{P}{100}\times\frac{100-WC}{100}\times\frac{10}{W_n}\times\frac{d}{L}\times 100$$

An, area of known and unknown impurity peak in sample solution; As desloratadine mean peak area in standard solution; Ws, desloratadine required for standard solution WS weight, mg; Wn the weight of the sample required for the sample solution, g; P, desloratadine WS dry potency, %; WC, desloratadine WS water amount, %; d, density of the sample, g/mL; Label value, 2.5 mg/5 mL.

Validation of Related Substance Method by HPLC

The optimized HPLC method is validated according to guideline vspecificity, linearity, accuracy, precision (system, method, and intermediate precision), and robustness. It's shown in Table 6.

Table 3. Initial Column Area, Second Column Area, and Percent Change

	Initial Column Area	Second Column Area	% Change
Desloratadine	47 616	48 506	1.87
İmpurity A	112 024	111 688	0.30
İmpurity C	124 900	123 118	1.43
İmpurity B	110 344	108 590	1.59

Table 4. This Solution Is Analyzed After Changing the Initial pH of Desloratadine Mobile Phase to pH 2.95

	Initial Area (pH:3.00)	After change Area (pH:2.95)	% Change
Desloratadine	47 616	48 528	1.92
Impurity A	112 024	111 410	0.55
Impurity C	124 900	123 173	1.38
Impurity B	110 344	108 597	1.58

Table 5. This Solution Is analyzed After Changing the Initial pH of Desloratadine Mobile Phase to pH 3.05

	Initial Area (pH:3.00)	After change Area (pH:2.95)	% Change
Desloratadine	47 616	48 841	2.57
Impurity A	112 024	111 095	0.83
Impurity C	124 900	123 312	1.27
Impurity B	110 344	108 592	1.59

Table 6. Relative Standard Deviation (RSD) Parameters and Results

Parameter		Result
Linearity	Range	LOQ-140.0%
	Equation	$y = 12\ 117\ 723.7107x\ 333.0610$
	Correlation coefficient	$r^2 = 1.0000$
Accuracy	Range average	80.0%-120.0%
	95% confidence interval limits	99.29-100.15
Precision	System precision	RSD = 0.31%
	Method precision	Total impurity RSD = 0.95%
	Intermediate precision	Total impurity RSD = 7.02%

LOQ, Limit of Quantification.

Column

The standard solution is prepared as specified in the desloratadine method.

This solution is analyzed in the starting column and after column change. Results are compared to baseline and the percent change was calculated. It's shown in Tables 3, 4 and 5.

pH of Mobile Phase

The standard solution is prepared as specified in the method.

System precision is conducted with 6 repeated injections of stock solutions prepared at 100% concentration, and the RSD of peak areas is found to be below 10%. Method precision is performed by preparing 6 sample solutions described in validation of Related Substance Method by HPLC part. Relative standard deviations were calculated, and the results were found to be below 10.0%. Intermediate precision is studied by different analysts and with different devices. Each analyst prepared 1 standard solution and 6 sample solutions. All the results were compared and RSD is calculated (Table 6).

To validate the sturdiness of the developed method, system parameters were certified, and standard solutions were tested. Hplc column temperature was changed to 40°C ± 2°C and mobile phase ratio was changed to 100 ± 5.0 mL and different lot numbered columns were used. Percent variations were calculated, and no important difference was found between initial and changed conditions.

Table 8. pH of Desloratadine Syrup Formulations During the Initial Stability Period

	F1	F2	F3	F4	F5	F6	F7
pH	3.8	3.8	3.8	3.0	8.9	4.6	5.7

Table 9. Impurity of Desloratadine Syrup Formulations During (40°C ± 2°C; 75% + 5 RH) the Third-Month Stability Period

	ICH Limits	F1 (g/150 mL)	F2 (g/ 150 mL)	F3 (g/150 mL)	F4 (g/150 mL)	F5 (g/150 mL)	F6 (g/150 mL)	F7 (g/150 mL)
Impurity A	1.0%	ND	ND	ND	ND	ND	ND	ND
Impurity B	1.0%	ND	ND	ND	ND	ND	ND	ND
Impurity C	1.0%	ND	ND	ND	ND	ND	ND	ND
Unknown impurity	0.4%	1.62%	1.56%	1.50%	2.23%	0.38%	0.63%	0.05%
Total impurity	3.0%	1.72%	1.66%	1.60%	2.34%	1.28%	0.73%	0.05%

ND, not detected.

Table 10. pH of Desloratadine Syrup Formulations During (40°C ± 2°C; 75% + 5 RH) the Third-Month Stability Period

	F1	F2	F3	F4	F5	F6	F7
pH	3.9	3.8	3.9	3.1	8.8	4.6	5.7

Also, solution stability is evaluated by pursuing the peak area response. Sample and standard solutions were analyzed shortly after their preparation 12, 24, and 48 hours after at room temperature. Results were compared and percent variations were calculated, and all results were below 10%.

RESULTS

Our studies are formulated with different excipients from F1 to F7. The syrup is made as described in the description of production process in general. Initial impurity and pH results in desloratadine syrup formulation trials are shown in Tables 7 and 8.

Product stability studies were performed on the stability batch according to the ICH requirements. Samples are stored at 40°C/75% RH for 3 and 6 months. No trends are observed during storage, and the results were not affected by the fill volume or storage position. Trials are stored at 40°C and 75% RH relative humidity for 3 and 6 months. The bottles are in an upright position during storage.[10]

Desloratadine syrup trials are carried out in amber-colored glass bottles.

In the production of Trials F1, F2 and F3, edta, whose chelator and antioxidant synergist properties were used in the product composition, was used as a multiplexed dose in these 3 formulations. By catalyzing the autoxidation reactions, it is thought to reduce the oxidation of desloratadine during stability by forming chelates with trace amounts of metal ions. In these 3 trials, the pH value was targeted to an acidic pH of 3.8 with Citric acid monohydrate and 0.35 g/150 mL was used in the F1, F2, F3 trials.

The effect of the amount of citric acid and sodium citrate dihydrate used as buffering agent on the changes of impurity and pH is investigated by keeping the EDTA amount constant in F4, F5,

Table 7. Impurity of Desloratadine Syrup Formulations During the Initial Stability Period

	ICH Limits	F1 (g/150 mL)	F2 (g/150 mL)	F3 (g/150 mL)	F4 (g/150 mL)	F5 (g/150 mL)	F6 (g/150 mL)	F7 (g/150 mL)
Impurity A	1.0%	ND	ND	ND	ND	ND	ND	ND
Impurity B	1.0%	ND	ND	ND	ND	ND	ND	ND
Impurity C	1.0%	ND	ND	ND	ND	ND	ND	ND
Unknown impurity	0.4%	0.05%	0.05%	0.05%	0.05%	0.05%	0.03%	0.06%
Total impurity	3.0%	0.05%	0.05%	0.05%	0.05%	0.05%	0.03%	0.06%

ND, not detected.

Table 11. Impurity of Desloratadine Syrup Formulations During (40°C ± 2°C; 75% RH ± 5% RH) the Sixth-Month Stability Period

	ICH Limits	F1 (g/150 mL)	F2 (g/150 mL)	F3 (g/150 mL)	F4 (g/150 mL)	F5 (g/150 mL)	F6 (g/150 mL)	F7 (g/150 mL)
Impurity A	1.0%	ND	ND	ND	ND	ND	ND	ND
Impurity B	1.0%	ND	ND	ND	ND	ND	ND	ND
Impurity C	1.0%	ND	ND	ND	ND	ND	ND	ND
Unknown impurity	0.4%	5.1%	5.4%	5.3%	5.6%	0.5%	3.0%	0.16%
Total impurity	3.0%	5.1%	5.4%	5.3%	5.6%	1.5%	3.0%	0.27%

Out-of-limit results are shown in red.

Table 12. pH of Desloratadine Syrup Formulations During (40°C±2° C; 75%+5 RH) the Sixth-Month Stability Period

	F1	F2	F3	F4	F5	F6	F7
pH	3.9	3.7	4.0	3.1	8.8	4.7	5.7

and F6. In these trials, pH 3.8 in F4, pH 8.9 in F5, and pH 4.6 in F6 were measured.

In F7 trial, impurity results at different pH values were evaluated over F6 formulation. Compared to the F6 trial, citric acid monohydrate was used to make the pH 5.7 in the F7 trial. Initial, 3rd month and 6th month stability impurity and pH results of all trials are shown in the Tables 8-12.

As shown in Table 7 of the initial impurity results, all results are within the limit. The initial results were analyzed on the day of production. The assays were tested at room temperature. When the results are examined, it is seen that other experiments give similar results. In the initial impurity results, the unknown impurity disregard value is 0.05%, which can be considered due to contamination during production.

According to the results of the third month analysis, edta was used in amounts of F1 (0.0375 g/150 mL), F2 (0.075 g/150 mL) and F3 (0.150 g/150 mL). Increasing edta concentration in the trials did not significantly change the desloratadine impurty results. Increasing the amount of citric acid in F4 caused impurity increase. In the first 4 trials, the unknown impregnation of desloratadine at 40°C±2°C 75% RH is beyond the limit. In these 3 trials, the product is degraded by the reaction of amine groups in the molecule due to the acidic pH value of the product to the highly unknown impurity. Citric acid monohydrate was not used in experiment F5 because it was desired to evaluate the impurity performance of alkaline solution as opposed to acidic solution in this trial. High pH value caused impurity C increase. In the F6 trial using citric acid monohydrate and sodium citrate dihydrate, the unknown impurity limit is within the total impurity limit. In the last trial, the level of unknown and total impurity disregard is 0.05%.

Basing on the results of sixth-month analysis, in F1, F2, F3, and F4 trials, unknown impurity and total impurity values are above ICH limits. This is due to the degradation of the pyridine and piperidine groups which exhibit basic character in the acidic product. This situation has increased the unknown impurity not called impurity A, impurity B, and impurity C.

In the F5 trial, the unknown impurity decreased by 90% compared to the F1, F2, F3, and F4 trials. However, the unknown impurity is 40% above the ICH limits. Total impurity is within the limit. Such a reduction in the unknown impurity depends on the alkaline pH of the product.

In the F6 trial, the product is used as much as citric acid monohydrate used in F1, F2, and F3 trials. In this experiment, unlike F1, F2, and F3, the buffering agent sodium citrate dihydrate is used, and the pH is around 4.6. This is 750% higher than the upper limit of unknown impurity. Factors causing such a high impregnation caused degradation of desloratadine by oxidation of the pyridine group.

Since the product is known to increase impurity when it is acidic in the F7 trial, the product is made at 5.7—a pH close to neutral pH. Citric acid monohydrate amount has been reduced in order to provide this pH value in F7. Thus, unknown impurity and total impurity are within the limit and at minimum values. In all experiments, unknown impregnation problem in desloratadine syrup formulation is completed with trial studies. As a result of the experiments, the optimum pH value of the product is found.

The research study is aimed at developing a syrup form of a desloratadine formulation during the stability impurity period. Based on the physicochemical properties of desloratadine and buffering and chelating excipients, the impurity parameter is optimized combination F7 formulation. The F7 formulation was selected for stability studies (40°C±2°C; 75%+5RH) for a time period of 3 and 6 months.[11] The formulations were appraised for the pH and related substances. From 3. month stability data, F7 formulation was found to be more stable than other trials in Impurity A, B, C, unknown Impurity and Total Impurity parameter. As shown in the tables, the stability data of the F7 formulation are suitable for ICH specifications. With the amount of excipients and formulation trials, impurity has been improved and the pH value has been brought to pH 5.5-6.0 values, and product safety and stability have been increased.

Ethics Committee Approval: This study has not been tested on a patient for whom an indication study has not been performed. only as a formulation, it has been made more stable with certain parameter changes.

Informed Consent: This study has not been tested on a patient for whom an indication study has not been performed. only as a formulation, it has been made more stable with certain parameter changes. therefore, patient consent is not required.

Peer-review: Externally peer-reviewed.

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ATATURK UNIVERSITY PUBLICATIONS

Research Article

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Determining the Relationship Between Distinct Obesity Prevalence Groups and Risk Factors By Panel Random-Effect Ordered Probit Model in Turkey

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ABSTRACT

Objective: The aim of this study was to analyze the sociodemographic and economic characteristics of families or individuals that can affect the normal weight, overweight, obesity, and severe obesity of the individual determined by the body mass index using the panel random-effect ordered probit model in Turkey.

Methods: The analysis used the survey data from the 2016 Turkey Health Research created by the Turkish Statistical Institute. The choice of the random-effect model was confirmed by a statistical test.

Results: We found that many sociodemographic and economic factors of family members or family significantly affect the probability of obesity groups. For example, considering the change in the age scale of obesity, the probability of being at normal weight decreases as the ages of family members increase, and this condition triggers being overweight, obese, and severe obese as the age progresses. As the family income increases, the individuals become more obese, while the education level of the individuals and the time devoted to walking for more than an hour a day show that the individuals are successful in their maintaining a normal weight.

Conclusion: Obesity is an important problem in Turkey and should be tackled effectively. It has been determined that the sociodemographic and economic factors of the individual significantly affect the probability of obesity. Social risk groups that are positively associated with obesity should be identified, and awareness training should be given to each group with appropriate methods.

Keywords: Obesity, risk factors, TUIK, Turkey

INTRODUCTION

Obesity is generally defined as the excessive increase in the proportion of the fat mass of the body to lean mass as a result of excessive increase in body weight over the desired level. Fatty tissue constitutes 15%-18% of the body weight in adult men and 20%-25% of the body weight in women. Obesity occurs when this rate exceeds 25% in men and 30% in women.[1] According to the World Health Organization data, worldwide obesity has increased nearly 3 times since 1975. In 2016, more than 1.9 billion adults over the age of 18 and above were overweight (OW), accounting for 39% of the world's population, while more than 650 million of them were identified as obese (O), which corresponds to 13% of the global population.[2] Also, in 2016, 41 million children under the age of 5 and 340 million children between the ages of 5 and 19 were categorized in the OW or O class. In this context, while OW and obesity are an increasing threat to both adults and children worldwide, this upward trend is more pronounced especially in children.[2,3] On the other hand, while the country with the highest obesity rate was the USA, the highest OW population is found in Albania, Bosnia, and Herzegovina and England (Scotland region), respectively. In contrast, Turkmenistan and Uzbekistan are lucky countries in which the prevalence of obesity ranges as low as 5%-23% in men and 7%-36% in women.[1] On the one hand, concerns about the early prevention of OW and obesity epidemic in low- and middle-income countries, essentially malnutrition, morbidity, mortality, and impaired child development, remain persistent[3]; on the other hand, the double burden of malnutrition, OW, and obesity has also been a subject of concern in high-income countries.[4]

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Over the past 50 years, obesity is not only a global public health threat that affects the quality of life, increases the risk of disease, and triggers healthcare spending in countries,[5] but it is also a fatal risk factor leading to the development of many diseases.[6] Overweight and obesity are often reported as the main cause of various chronic diseases such as heart disease, stroke, diabetes, and cancers.[6] For example, though preventable, obesity is the second leading cause of death in the USA. Another frightening figure is that obesity has doubled the risk of death since 1980, and the number of people who died from obesity is higher than the number of people who died of starvation.[7] It is suggested that 5% of deaths in the world are associated with obesity, and in 2030, 1 out of every 2 people is expected to be O.[8] While obesity is content with its threatening feature to human health, if proper measures are not taken in place and on time, it will remain the driving force of a significant part of deaths in the world.

While physical inactivity is undoubtedly the main factor among the reasons for the increase in obesity in developed and developing countries,[9] other driving forces triggering OW and obesity include the way people eat, the increase in the participation of the women in the labor pool, the socioeconomic factors such as increasing urbanization, education, occupation, and income, and the increased time spent on television, video games, and the internet.[10] For example, it is emphasized that fast-food consumption has a strong relationship with weight gain and insulin resistance and also triggers the risk of type 2 diabetes,[11] while the increase in body mass index (BMI) in regions with high fruit and vegetable prices is higher compared to regions with low fruit and vegetable prices.[12] While access to physical activity is also limited in regions with low-income levels[13] and nexus between poverty and obesity remain on the agenda,[14] the prevalence of OW is also common in high-income families, given the food purchasing capacities (e.g., power).[4] At the same time, the individual's self-anxiety, avoidance of peer relationship, introversion, exclusion, and even depression and anxiety are associated with obesity.[1,15] Contrary to these triggering factors, smoking decreases obesity in individuals, and it has been reported that a 10% increase in taxes on cigarettes causes a 4%-5% increase in O population while decreasing cigarette consumption.[16]

Many obesity-related diseases such as heart disease, cancer, hypertension, diabetes, and hyperlipidemia have a significant economic burden on both states and individuals.[17] It is well known that obesity affects economies directly and indirectly through cost channels. For example, loss of productivity in labor force caused by obesity, social and psychological constraints caused by the disease, and exclusion from certain job opportunities constitute indirect costs, while patient care fee, physician fee, and medical expenses are among the direct costs due to obesity.[18] The share of the fight against obesity in health expenditures in European Union countries can reach up to 6%.[19] In the USA, the medical costs of obesity of pediatric outpatients and inpatients are approximately $14.1 billion and $238 million, respectively, while the expenditure on combating obesity and OW was $147 billion in 2009,[12] reaching $1.42 trillion in 2014.[20] Besides, obesity is estimated to account for 2.8% of the global gross domestic product.[8]

Not only adult and childhood obesity have become an important and worrisome health problem in Turkey as in the world, but also the rapid increase in the obesity prevalence throughout the country in the last 3 years has caused policymakers to worry and start searching for remedies.[1] An O individual has 41.5% more health spending per capita than a normal weight (NW) adult, accounting for 16.5% of Turkey's health expenditures.[8] While scanning the studies in the literature in detail, it has been found that obesity not only poses a significant threat to human health but also imposes a huge financial burden on the economies of the country. However, studies on obesity in Turkey in which the heterogeneity of individuals within the family was ruled out have remained scant and unfortunately have not brought any novelty beyond simple analysis.[1,21] In this regard, it is of great importance to provide a more robust foresight to the health sector and policymakers in the country on the subject, by conceptualizing the behavioral responses of families toward the prevalence of obesity with controlling heterogeneity among family members in the light of the up-to-date data set and rich factors. In this study, the relationship between the sociodemographic and economic factors of family and family members and the prevalence of obesity [NW, OW, O, and severe obese (SO)] are analyzed using the panel random-effect ordered probit model. As it is known, the effect of obesity on explicit groups (e.g., intra-family heterogeneity), through perceptions about behavioral responses to food culture among family members, is known to be distinct and larger than the effect of differentiation between families on obesity. Accordingly, the effects of an inter-family factor on obesity prevalence in this study were performed by controlling behavioral heterogeneity among family members. Also, the unitary (marginal) effects in the sociodemographic and economic factors of family members and family are determined by analyzing them on each obesity group.

This study consists of 4 chapters, including the introduction. The second part introduces the empirical methods and data sets applied to the variables. The following part reports the empirical results of the study. The last part presents policy suggestions from the study.

METHODS

The analysis used the survey data from the 2016 Turkey Health Research (THR) created by the Turkish Statistical Institute (TSI) with the official permission of the institution (application number: 01202000000001B24). The data from Turkey Health Interview Surveys conducted in households in Turkey between January 1 and December 31, 2016, by the TSI were used in the study. The sample size included 8325 families and 17 242 individuals after the exclusion of missing observations and outliers. Descriptive definitions and statistical values of sociodemographic and economic characteristics of family members and family are given in Table 1.

In the present study, it is determined that 43% of individuals are NW, 35.2% are OW, 15.8% are O, and 6% are SO. Also, 44.5% of individuals are male, 69.1% are married, 34.5% are primary school graduates, 10.2% are university graduates, 10.1% have a green card, and 16.8% are retired. The average monthly income of 16.1% of families is more than Turkish lira (TL) 2540, and 53.1% and 61.8% of individuals eat fruits and vegetables at 1 or more meals per day, respectively. About 43.6% of individuals smoke cigarettes and 5.2% of them drink alcohol. It is found that 13.1% of individuals walk more than an hour a day. The variance inflation factor reveals that there is no multicollinearity problem among the independent variables. In addition, other descriptive statistics will not be given here, considering the page limit.

Table 1. Descriptive Statistics of Variables

Variable	Descriptive	Mean	SD	VIF
Normal weight	1 if BMI ≤ 25 (RG)	0.430	0.495	–
Overweight	1 if BMI > 25 and BMI ≤ 30, 0 otherwise	0.352	0.478	–
Obese	1 if BMI > 30 and BMI ≤ 35, 0 otherwise	0.158	0.365	–
Severe obese	1 if BMI > 35, 0 otherwise	0.060	0.237	–
Gender	1 if the individual is male, 0 otherwise	0.445	0.497	0.000
Age <30	1 if the individual is less than 30 years old, 0 otherwise (RG)	0.250	0.433	–
Age 30-44	1 if the individual is between 30 and 44 years old, 0 otherwise	0.293	0.455	1.588
Age 45-64	1 if the individual is between 45 and 64 years old, 0 otherwise	0.312	0.463	2.301
Age >64	1 if the individual is older than 64 years, 0 otherwise	0.146	0.353	2.908
Marital status	1 if the individual is married, 0 otherwise	0.691	0.462	2.601
Employment	1 if the individual is currently employed, 0 otherwise	0.464	0.499	1.496
No school	1 if the individual has no school diploma, 0 otherwise (RG)	0.154	0.361	–
Elementary school	1 if the individual has an elementary school diploma, 0 otherwise	0.345	0.475	2.661
Secondary school	1 if the individual has a secondary school diploma, 0 otherwise	0.173	0.378	2.582
High school	1 if the individual has a high school diploma, 0 otherwise	0.227	0.419	3.027
College	1 if the individual has a college degree including master and doctorate, 0 otherwise	0.102	0.302	2.383
Green card	1 if the individual has green-card health insurance, 0 otherwise	0.101	0.301	1.262
Entrepreneurial income	1 if the individual is an entrepreneurial income, 0 otherwise	0.078	0.268	1.272
Real estate	1 if the individual has an income from securities and real estate assets, 0 otherwise	0.035	0.184	1.098
Pension income	1 if the individual receives a pension income, 0 otherwise	0.168	0.373	1.533
Income group 1	1 if the household income is less than 1814 Turkish lira (TL), 0 otherwise (RG)	0.486	0.500	–
Income group 2	1 if the household income is between 1814 and 2540 TL, 0 otherwise	0.354	0.478	1.294
Income group 3	1 if the household income is greater than 2540 TL, 0 otherwise	0.161	0.367	1.521
Tobacco	1 if the individual smokes, 0 otherwise	0.436	0.496	1.336
Alcohol	1 if the individual smokes, 0 otherwise	0.052	0.222	1.038
Walking time	1 if the individual walks more than an hour a day, 0 otherwise	0.131	0.337	1.054
Sports	The time an individual devotes to sports in a week (minutes)	0.218	1.206	1.052
Heavy work	1 if the individual works in heavy duty, 0 otherwise	0.053	0.225	1.080
Fruit consumption	1 if eating 1 or more servings of fruit a day, 0 otherwise	0.531	0.499	1.482
Vegetable consumption	1 if eating 1 or more servings of vegetables a day, 0 otherwise	0.618	0.486	1.447
Depression	1 if the individual is diagnosed with depression, 0 otherwise	0.082	0.274	1.050
Physician	Number of physician visits in the last 12 months	0.920	2.090	1.047
Household size	Household size	3.349	1.686	1.367
Northeastern Anatolia	1 if residing in the northeastern Anatolia region, 0 otherwise (RG)	0.022	0.148	–
Istanbul	1 if the individual resides in Istanbul, 0 otherwise	0.133	0.340	2.480
Western Marmara	1 if residing in the western Marmara region, 0 otherwise	0.103	0.304	2.196
Aegean	1 if residing in the Aegean region, 0 otherwise	0.058	0.233	1.706
Eastern Marmara	1 if residing in the eastern Marmara region, 0 otherwise	0.044	0.204	1.558
Middle east Anatolia	1 if residing in the middle east Anatolia region, 0 otherwise	0.023	0.151	1.296
Mediterranean	1 if residing in the Mediterranean region, 0 otherwise	0.097	0.296	2.129
Central Anatolia	1 if residing in the central Anatolia region, 0 otherwise	0.145	0.352	2.596
Western Black Sea	1 if residing in the western Black Sea region, 0 otherwise	0.078	0.268	1.995
Eastern Black Sea	1 if residing in the eastern Black Sea region, 0 otherwise	0.203	0.402	3.108
Southeastern Anatolia	1 if residing in the southeastern Anatolia region, 0 otherwise	0.043	0.202	1.548
Number of households sampled			8325	
Number of total observations			17 242	

BMI, body mass index; RG, reference group; TL, Turkish lira; VIF, variance inflation factor.

Econometric Method

Consider the latent variable y_{ij}^* for individual *j* in family cluster *i* and the observed ordinal categorical variable y_{ij} corresponding to its latent response variable y_{ij}^*. The ordinal categorical models can be shown in terms of the latent response variable y_{ij}^* as follows:

$$y_{ij}^* = \theta_{ij} + \varepsilon_{ij}, y_{ij} = \kappa_{ik} \text{ if } \psi^{m_{ik-1}} < y_{ij}^* < \psi^{m_{ik}}$$
$$\text{where} \theta_{ij} = \beta_0 + \sum_{p=1}^{P} \beta_p x_{pij} \text{ and } \varepsilon_{ij} = \eta_i + v_{ij} \quad (1)$$

where x_{pij} represents a vector of explanatory variables, including household income and individual characteristics, β_p is the vector of parameter estimates corresponding to these variables, and ψ is the vector of all threshold parameters (e.g., $\psi^0 < \psi^1 < \psi^2 < \ldots < \psi^K, \psi^0 = -\infty, \psi^1 = 0, \psi^K = +\infty$). ε_{ij} is a standard normal error term uncorrelated across families *i*, but it may be correlated across individuals within a family *i* for which we assume the error term, ε_{ij}, can be written as the sum of family effect η_i and an idiosyncratic term v_{ij}, where $\eta_{ij} | x_{ij} \psi N(0, \sigma^2)$. As it is indicated above, observations across families are not correlated, while 2 observations for the same family *i* are then correlated because of the common term η_i.[22]

The maximum simulated likelihood estimation of the random effects can be constructed as follows. Let the probability of the observed vector κ_i of the sequence of ordinal choices ($\kappa_{i1}, \kappa_{i2}, \kappa_{i3}, \ldots, \kappa_{iK}$) for a family *i* conditional on the heterogeneity term η_i be:

$$\text{Prob}(\kappa_i) | \eta_i = \prod_{k=1}^{K} \left\{ f\left(\psi^{\kappa_{ik}} - \theta_{ij} - \eta_{ij}\right) - f\left(\psi^{\kappa_{ik-1}} - \theta_{ij} - \eta_{ij}\right) \right\} \quad (2)$$

The unconditional likelihood of the observed choice sequence can be then obtained by integrating out the heterogeneity term, η_i :

$$L_i(\psi,\beta,\sigma)=\int_{v=-\infty}^{\infty}\left[\prod_{k=1}^{K}\left\{f\left(\psi^{\kappa_{ik}}-\theta_{ij}-\eta_{ij}\right)-f\left(\psi^{\kappa_{ik-1}}-\theta_{ij}-\eta_{ij}\right)\right\}\phi(v)dv\right. \quad (3)$$

where $v=\eta_i/\sigma$ and f and are univariate standard normal cumulative and density function, respectively. The corresponding log-likelihood function can be written as:

$$\log L(\psi,\beta,\sigma)=\sum_i \log L_i(\psi,\beta,\sigma) \quad (4)$$

This log-likelihood function can be maximized either using Gauss–Hermite quadrature or using a simulated method. Here, we used the Gauss–Hermite quadrature method to obtain relevant parameter estimates of the log-likelihood function. Also, by taking the derivatives of Equation (2) with respect to the independent variables, the unitary (marginal) effects on the ordinal categorical probabilities were then achieved. Delta method was used to obtain the standard error of the abovementioned marginal effects.

RESULTS

Parameter estimates of the maximum likelihood panel random-effect ordered probit model are given in Table 2. The choice of the independent variables used in the ordered probit model has been confirmed (=242.838 and P=.000). Along with the constant coefficient, the other 2 threshold parameters (and) were found to be statistically significant, remarking that the transitions between obesity groups had profound distinct features. The fact that the parameter of the heterogeneous factor (e.g., σ) is statistically significant confirms our choice of the random effect and reflects a superiority against the pool model. On the other hand, unitary (marginal) effects of family members and family's sociodemographic and economic factors derived from the panel random-effect ordered probit model are given in Table 3. The next discussion will take place on these marginal effects.

Table 2. Maximum Likelihood Estimates of the Panel Random-Effects Ordered Probit Model

Variable	Parameters	SE	*z*-Value	95% Lower CI	95% Upper CI
Constant	−0.318***	0.061	−5.190	−0.439	−0.198
Gender	−0.030	0.024	−1.230	−0.077	0.018
Age 30-44	0.717***	0.032	22.180	0.654	0.781
Age 45-64	1.066***	0.035	30.220	0.997	1.135
Age >64	0.813***	0.043	18.820	0.729	0.898
Marital status	0.253***	0.025	10.060	0.204	0.303
Employment	−0.047**	0.023	−2.030	−0.093	−0.002
Elementary school	−0.138***	0.031	−4.420	−0.200	−0.077
Secondary school	−0.383***	0.041	−9.450	−0.463	−0.304
High school	−0.428***	0.040	−10.720	−0.506	−0.350
College	−0.540***	0.050	−10.880	−0.637	−0.443
Green card	−0.164***	0.038	−4.280	−0.239	−0.089
Entrepreneurial income	0.032	0.038	0.830	−0.043	0.107
Real estate	0.036	0.056	0.640	−0.074	0.146
Pension income	−0.055*	0.030	−1.820	−0.115	0.004
Income group 2	.087***	0.025	3.500	0.038	0.135
Income group 3	0.012	0.036	0.330	−0.058	0.081
Tobacco	−0.068***	0.022	−3.050	−0.112	−0.024
Alcohol	−0.092**	0.044	−2.120	−0.178	−0.007
Walking time	−0.159***	0.031	−5.120	−0.220	−0.098
Sports	−0.003	0.009	−0.370	−0.021	0.014
Heavy work	−0.010	0.045	−0.210	−0.097	0.078
Fruit consumption	0.102***	0.024	4.290	0.055	0.149
Vegetable consumption	−0.033	0.025	−1.340	−0.081	0.015
Depression	0.126***	0.034	3.690	0.059	0.192
Physician	0.019***	0.004	4.360	0.010	0.027
Household size	−0.022***	0.008	−2.800	−0.037	−0.007
Istanbul	0.065	0.049	1.320	−0.032	0.162
Western Marmara	0.068	0.051	1.330	−0.032	0.168
Aegean	0.229***	0.057	4.000	0.117	0.341
Eastern Marmara	.110*	0.062	1.780	−0.011	0.232
Middle east Anatolia	0.014	0.082	0.170	−0.148	0.175
Mediterranean	0.049	0.051	0.950	−0.052	0.149
Central Anatolia	0.050	0.047	1.060	−0.043	0.143
Western Black Sea	−0.050	0.057	−0.870	−0.163	0.062
Eastern Black Sea	0.055	0.046	1.190	−0.036	0.145
Southeastern Anatolia	−0.109	0.067	−1.620	−0.241	0.023
ψ_1			1.079*** (0.011)		
ψ_2			1.927*** (0.016)		
Sigma (σ)			0.478*** (0.020)		
Log-likelihood function			−18 730.285		
Restricted log-likelihood			−18 851.704		
χ^2_{36}			242.838***		
AIC			37 540.600		

***, **, and * indicate significance at 1%, 5%, and 10% levels, respectively. AIC: Akaike Information Criterion; CI: Confidence Interval.

Table 3. Marginal Effects of Exogenous Variables on the Obesity Groups

	Normal Weight		Overweight		Obese		Severe Obese	
Variable	Partial Effect	*z*-Value	Partial Effect	*z*-Value	Partial Effect	*z*-Value	Partial Effect	*z*-Value
Gender	0.011	1.220	−0.003	−1.220	−0.005	−1.220	−0.002	−1.230
Age 30-44	−0.241***	−24.220	0.049***	19.650	0.119***	22.080	0.073***	17.290
Age 45-64	−0.347***	−35.530	0.055***	15.730	0.173***	31.200	0.120***	21.760
Age > 64	−0.260***	−22.330	0.024***	6.480	0.136***	20.090	0.100***	13.290
Marital status	−0.090***	−10.080	0.031***	9.060	0.040***	10.290	0.019***	10.630
Employment	0.017**	2.030	−0.005**	−2.020	−0.008**	−2.030	−0.004**	−2.030
Elementary school	0.049***	4.420	−0.016***	−4.210	−0.022***	−4.470	−0.011***	−4.560
Secondary school	0.137***	9.500	−0.054***	−7.980	−0.058***	−10.220	−0.025***	−11.380
High school	0.153***	10.830	−0.059***	−9.170	−0.065***	−11.510	−0.028***	−12.570
College	0.193***	11.140	−0.084***	−8.900	−0.077***	−12.700	−0.031***	−15.270
Green card	0.058***	4.270	−0.021***	−3.830	−0.026***	−4.440	−0.012***	−4.760
Entrepreneurial income	−0.011	−0.830	0.003	0.860	0.005	0.830	0.002	0.810
Real estate	−0.013	−0.640	0.004	0.660	0.006	0.630	0.003	0.620
Pension income	0.020*	1.810	−0.006*	−1.750	−0.009*	−1.830	−0.004*	−1.870
Income group 2	−0.031***	−3.510	0.009***	3.610	0.014***	3.480	0.007***	3.420
Income group 3	−0.004	−0.330	0.001	0.330	0.002	0.330	0.001	0.330
Tobacco	0.024***	3.060	−0.008***	−3.020	−0.011***	−3.060	−0.005***	−3.070
Alcohol	0.033**	2.110	−0.011**	−1.960	−0.015**	−2.160	−0.007**	−2.270
Walking time	0.057***	5.100	−0.020***	−4.610	−0.025***	−5.270	−0.012***	−5.610
Sports	0.001	0.370	−0.001	−0.370	−0.001	−0.370	−0.001	−0.370
Heavy work	0.003	0.210	−0.001	−0.210	−0.002	−0.220	−0.001	−0.220
Fruit consumption	−0.036***	−4.290	0.011***	4.260	0.017***	4.290	0.008***	4.280
Vegetable consumption	0.012	1.340	−0.004	−1.350	−0.006	−1.340	−0.003	−1.330
Depression	−0.044***	−3.740	0.012***	4.270	0.021***	3.620	0.011***	3.410
Physician	−0.007***	−4.360	0.002***	4.330	0.003***	4.350	0.002***	4.350
Household size	0.008***	2.800	−0.002***	−2.770	−0.004***	−2.800	−0.002***	−2.810
Istanbul	−0.020	−1.330	0.007	1.410	0.011	1.310	0.005	1.270
Western Marmara	−0.024	−1.330	0.007	1.420	0.011	1.310	0.006	1.270
Aegean	−0.079***	−4.120	0.019***	5.890	0.039***	3.900	0.021***	3.460
Eastern Marmara	−0.039*	−1.800	0.011**	2.060	0.018*	1.750	0.009*	1.650
Middle east Anatolia	−0.005	−0.170	0.002	0.170	0.002	0.170	0.002	0.160
Mediterranean	−0.017	−0.950	0.005	1.000	0.008	0.940	0.004	0.920
Central Anatolia	−0.018	−1.060	0.005	1.110	0.008	1.050	0.004	1.030
Western Black Sea	0.018	0.870	−0.006	−0.840	−0.008	−0.880	−0.004	−0.900
Eastern Black Sea	−0.019	−1.190	0.006	1.230	0.009	1.180	0.005	1.160
Southeastern Anatolia	0.039	1.610	−0.014	−1.470	−0.017*	−1.660	−0.008*	−1.750

***, **, and * show significance at 1%, 5%, and 10% levels, respectively.

According to the results we obtained, it has been found that sociodemographic and economic factors of family members and family affect the probability of obesity. The study conducted in Serbia reported that increased age, being male, living in the countryside, being married, having a low level of education, and having high income were more likely to be associated with obesity. In the study, they emphasized that age and income groups, education level, and smoking were significantly related to body weight.[23,24] They also reported that lifestyle, diet behavior, social status, and other sociodemographic factors affected BMI differently in distinct weight categories. In the same study, it was reported that education, employment, and income variables had a strong effect on the possibility of being OW and O. Individuals with low education, profession, or income levels tend to have more obesity than individuals with very high social welfare.[25]

Considering the change in the age scale of obesity, the probability of being NW within a family decreases as the ages of family members increase, and this condition triggers being OW, O, and SO as the age progresses. There was a similar relationship between individuals' age and social status and being OW in the literature.[26] Another important result is that this situation is riskier, especially in middle-aged individuals (the probability of NW decreases by 34.7 points, while the probability of being OW, O, and SO increases 5.5, 17.3, and 12 points, respectively). On the other hand, we found that individuals with NW (individuals aged 30-44, 45-64, and over 64 were negatively affected by 24.1, 34.7, and 26 points, respectively) were most affected by the age factor. This result is expected to have a tendency from NW to O with the decrease in physical activity and energy needs of the body as the age progresses, overlapping with the results we previously obtained. Our results are also compatible with international findings.[21,27]

Married individuals are less likely to be of NW than single individuals within a family, and this increases the likelihood of married people falling into the category of OW, O, and SO. It has been observed that married individuals are responsive to a NW of approximately 2.5-5 times more than single individuals. Besides, it has been found that married individuals are the least responsive group to becoming SO (1.9 points) compared to single individuals. This situation can be explained by the fact that married individuals have more regular and monotonous lives than single individuals. While the risk of married individuals being O compared to singles was suggested to be 2 times more in 1 study,[21] it was stated that in another study, they tended to be 0.8% more O than single or widowed individuals.[1]

The fact that individuals work in any job increases the probability of individuals being at NW by 1.7 points, while the probability of being OW, O, and SO is decreased by 0.5, 0.8, and 0.4 points, respectively. While the most responsive group in working individuals within a family was the normal group with 1.7 points, the least responsive group was observed to be the SO group with 0.4 points. Based on these results, it could be emphasized that there is a negative relationship between the working status of individuals and OW, O, and SO. Since the working individuals being physically active increases mobility, the emergence of such a result is

in line with the expectations. Based on all these results, the existence of healthier generations can be ensured by expanding more business areas throughout the country.

It is observed that there is a negative relationship between the education level of individuals and being OW, O, and SO within a family. In addition, as the education levels of individuals increase, the risk of obesity decreases, while the probability of being at NW increases. People with education and higher education are less likely to be O or OW.[1,28] Another result obtained especially with the individuals who have university-level education indicates that the risk of obesity is much lower than individuals with other education levels (primary school). It has been found that individuals who are university graduates are approximately 4 times more responsive to obesity than individuals who graduated from other education groups. Kuntz and Lampert[25] reported that men in the lowest education level were 1.5 times more likely to be O than men in the highest education level group. Having a university or higher education degree reduces the likelihood of obesity by 7.4%.[1] In most of the studies, it was suggested that women without university education were OW and O than women with a university degree.[29] On the other hand, it was found that the group that was the least responsive to the education level was an SO group and the group that was the most responsive was the NW group. For example, individuals with a university degree decreased their probability of obesity by 3.1 points compared to individuals who were primary school graduates, while the probability of staying at NW increased by 19.3 points. This can be explained by the more conscious and healthy nutrition of people as the level of education advances. Education increases people's awareness of health and nutrition and their attention to health and consequently reduces the incidence of obesity.[30,31] In similar studies, researchers obtained evidence of a negative relationship between individuals' education levels and obesity.[21,26,27]

While individuals who have a green card are less likely to suffer from obesity, they are more likely to be at a NW. The individuals having a green card are likely to boost the probability of NW by 5.8 points, while the probability of being OW, O, and SO is decreased by 2.1, 2.6, and 1.2 points, respectively. The probability of O individuals with green-card health insurance decreases by 8.3%.[1] Also, individuals with green cards are responsive to the NW group with a maximum of 5.8 points, while they are responsive to an SO group with a minimum of 1.2 points. It could be noted that positive features of having a green-card assurance such as not having any obstacle to go to the doctor and receiving the necessary treatments reduce the likelihood of individuals being O in cases of illness caused by some environmental and hereditary factors (such as unhealthy diet, depression, and congenital obesity). Meanwhile, since green-card ownership is an indicator of poverty, it could be noted that individuals holding green cards are in the low-income group. Therefore, green-card ownership reduces the likelihood of these individuals becoming O by limiting both fast-food and other consumption expenditures. People with higher incomes are at higher risk for obesity. This might be due to higher energy foods as well as greater socioeconomic access to food.[28,32] Although this seems like a desirable outcome, it should not be overlooked that these individuals face unbalanced nutrition because they can live on a very low income. An additional nutrition assistance program such as the food stamp program in the USA that provides balanced nutrition by purchasing healthy food should be implemented in Turkey, too.

While the probability of individuals receiving a pension to be at NW increases, the probability of being OW, O, and SO decreases. Besides, the most responsive group among individuals who receive a pension is the NW group with 2 points, while the least responsive group is observed to be the SO group with 0.4 points. Income level is an important factor affecting consumer behavior. The prevalence of obesity is directly related to consumption habits and behaviors. Although individuals who receive pensions descend from a certain income level to a lower income level (there is a loss of income due to retirement), it is an expected result that their probability of becoming O will decrease as a result of the decrease in consumption expenditures.

Having a monthly income between 1814 and 2540 TL decreases the probability of being a NW in individuals by 3.1 points while increasing the probability of being OW, O, and SO, respectively, by 0.9, 1.4, and 0.7 points when compared to the families with a monthly income of 1814 TL. Among the groups, the normal response group gives the highest response to the income group in question (3.1 points), while the least response is given by the O group (0.7 points). It is seen that obesity is most responsive to individuals with middle-income levels among income groups.

It is emphasized that the probability of becoming O increases with an increase in the income of individuals.[21] As the income level of individuals increases, total energy and total fat intake increase, leading to the risk of weight and obesity.[33] In the study conducted in the USA, it has been found that consumers with an average annual income of $40 000 and above spend 28% higher for fresh fruit and 25% higher for fresh vegetable compared to consumers with an annual income of $20 000-29 999.[34] A study conducted in Adana, Turkey, has revealed that households spend more for fresh fruits and vegetables as their income increases; another study from Nigeria[35] has reported that higher income status makes consuming fresh fruit and vegetables more possible, while studies from Canada,[36,37] Australia,[38] and Gana[39] have shown that income increases fruit and vegetable consumption. Based on these results, a possible increase in income may increase the risk of obesity as individuals tend to consume more food (total energy and total fat intake increases). On the other hand, some studies evaluating the high-income level as a protective shield for obesity consider the existence of an inverse relationship between these 2 variables. They interpret it as an increasing incidence of economic access to healthy foods, thereby preventing obesity.[25,40,41] In the emergence of these different results in the literature, it has been observed that studies in developed countries find that an increase in income provides more access to healthy foods, while studies in developing countries find that an increase in income increases total energy and total fat intake.

One of the important results of our study is that fruit consumption has a positive relationship with obesity. Results indicated that individuals who consume more than one serving of fruit per day reduce the probability of being at NW by 4 points while increasing the likelihood of being OW, O, and SO. The OW and O groups are especially affected more than the SO group. It is underlined that eating fruit more than 4 times a week increases the risk of obesity in individuals.[28] Similarly, it is reported that those who eat fruit twice or more a day are 3.6% more likely to be O.[1] A fructose intake >10-15 g/day is predicted to be harmful to the proper functioning of glucose metabolism and increased weight with calories may become inevitable as the fructose content of a fruit increases. The 2018-2022 strategic plan of the Ministry of

Health, Turkey, states that consuming fruit more than once per day increases obesity.[1]

There is a negative relationship between smoking individuals and being O. While the probability of smokers within a family to be at NW increases by 2.4 points, the probability of being OW, O, and SO decreases by 0.8, 1.1, and 0.5 points, respectively. It has been determined that individuals with NW are approximately 5 times more responsive to smoking than individuals who are SO. Similar results were obtained for alcohol consumption. Smokers are up to 6.6% less likely to be O.[1] This can be explained by the decrease in regular eating habits that starts with the loss of appetite in smokers and alcohol drinkers. The results of this study coincide with findings from similar studies, indicating that smokers are less likely to have higher body weight than non-smokers.[23,42,43]

The walking variable that was included in the model as a physical activity affecting obesity prevalence was found statistically significant. People who walk more than an hour a day are more likely to be at a NW, while they are 5 times less likely to be OW, O, and SO. It is suggested that one of the most important variables in reducing obesity is physical activity.[21,24,25] Individuals who walk more than an hour a day increase their probability of being at NW by 5.7 points, while the probability of being O decreased by 2.5 points. Our results are in line with expectations. Individuals who regularly walk are less likely to become O by 0.3%.[1] The prevalence of obesity or OW is lower in individuals who exercise more than 5 times per week than others.[28] In this context, regardless of gender and age group, such activities should be encouraged by the relevant health institutions through visual and written media, including views of healthcare professionals about the positive effects of daily walking on human health. Also, local governments should offer public walking areas.

While the probability of individuals diagnosed with depression to be at NW decreases, they are more likely to be OW, O, and SO. In other words, it has been observed that there is a similar relationship between individuals diagnosed with depression and obesity. Among the groups, individuals at a NW are the most responsive to depression with 4.4 points, while those in the SO group are the least responsive with 1.1 points. Based on these results, the depression effect of the individuals in the NW group will be 4 times more pronounced than the individuals in the SO group. The increased probability of being O is an expected result in the individuals diagnosed with depression because they both tend to consume more food and are more passive in physical activity. The most important psychological problem with obesity is depression. It is stated that O people overeat in response to anxiety and depression, and about 50% of those with an eating disorder such as obesity have clinical depression.[44] In addition to medical problems, many psychological and social problems have also been associated with obesity.[45] Self-esteem, avoidance of peer relationships, introversion, exclusion, and depression are associated with obesity.[15]

While the probability of individuals working physically in heavy jobs to be at NW decreases by 0.7 points, the probability of being OW, O, and SO is increased by 0.2, 0.3, and 0.2 points, respectively. Individuals with NW within the groups are the most responsive to heavy work with 0.7 points, while individuals in the OW and O group are the least responsive with 0.2 points. Individuals in the OW and O group will have an effect of working in heavy jobs approximately 3 times less than those in the NW group. Since individuals working in heavy jobs mostly work with muscle strength, their physical needs increase accordingly. Therefore, the probability of individuals who work in heavy jobs becoming O increases in line with expectations and overlaps with our study findings since these individuals both consume more food and are more passive in terms of physical activity.

As the number of individuals in the household increases, the probability of individuals being at NW increases by 0.8 points, while the probability of being OW, O, and SO decreases. Obesity or OW has a significant and negative relationship with the size of the family. It is indicated that people with more family members have less risk of obesity.[28] Since the amount of food consumption per individual will decrease as the number of individuals in the family increases, this result is in line with expectations and overlaps with our findings.

When families in the North-eastern Anatolia region are taken as reference, the probability of people living in the Aegean and eastern Marmara regions to be at NW decreases while they are more likely to get O. On the other hand, it has been observed that individuals living in the southeastern Anatolia region are less likely to become O and SO. While the probability of individuals living in the Aegean region to be at NW decreases by 7.9 points, the probability of being OW, O, and SO increases by 1.9, 3.9, and 2.1 points, respectively. The effect of living in the Aegean region will be about 4 times more pronounced in individuals at a NW within the groups compared to individuals in the OW and SO groups. On the other hand, the probability of individuals living in the eastern Marmara region to be at NW decreases by 3.9 points, while those in the OW, O, and SO groups have an increased likelihood. The individuals who are most responsive to living in the eastern Marmara region are individuals in the NW group with 3.9 points, while individuals in the SO group are the least responsive group with 0.9 points. Considering the response of individuals in NW, OW, O, and SO groups to living in the regions, it is observed that they are most responsive to living in the Aegean region. The fact that the probability of the individuals living in the southeastern Anatolian region being O is in contrast to those living in the Aegean and eastern Marmara regions can be explained by the fact that individuals living in southeastern Anatolia are employed less at desk jobs and employed more at jobs with higher physical mobility (jobs are very common in agricultural areas).

DISCUSSION

This study analyzes the sociodemographic and economic characteristics of families or individuals that can affect the NW, OW, O, and SO determined by the BMI using the panel random-effect ordered probit model. Since the panel random-effect model provides intra-family heterogeneity, it is superior to pooled data models and produces unbiased, consistent, and ultimately efficient parameter estimates.

We found that sociodemographic and economic factors of family members or family significantly affect the probability of obesity groups. It is observed that married individuals are 5 times more likely to be O than singles within a family. Strong negative relationships are found between the probability of being OW, O, and SO, and the individual's level of education, employment status, green-card ownership, pension, smoking and alcohol consumption, household size, and physical activity status. Especially the individual being a university graduate and walking more than an hour a day reduces the chance of obesity significantly. Household

income, which is one of the economic status variables of individuals, increases the probability of individuals becoming OW, O, and SO.

It is suggested that the risk of obesity will be greatly reduced with awareness-raising activities to be conducted on obesity in Turkey, and significant policies must be identified and implemented accordingly. It is assumed that obesity will greatly reduce among the individuals who walk over an hour a day, and individuals must especially be informed in this regard in the country. Another important consideration from the 2018-2022 strategic plans of the Ministry of Health of Turkey (MHT), which requires attention from policymakers, is identifying the regions which lack peripheral circumstances for walking (rural areas) and constructing walking routes in such regions or increasing the facilities for the individuals to perform physical activities. The 2018-2022 strategic plans of MHT also require special packaging on fast-food-style foods which may increase obesity prevalence to discourage the habit of consuming such foods and to stress the importance of health and warn the people that obesity is lethal. For the Aegean and eastern Marmara regions, the MHT should conduct more inspections in the enterprises using materials that probably trigger obesity. The state should impose a new obesity tax (in line with the policies implemented by countries that have greatly reduced obesity) to significantly reduce the obesity rate, which is almost 30% across the country. Accordingly, studies available in the literature predict that when the tax burden on all foods triggering obesity is increased, it will prevent unhealthy nutrition of the consumer in the country as in other countries. It could also be highlighted that if policymakers encourage the working situation of individuals, it will positively affect both the country's economy and the health status of individuals.

On the other hand, obesity is an important problem in Turkey and must be combated effectively. The social risk groups that are positively associated with obesity should be identified, and awareness training should be provided to each group using appropriate methods. The objective of such a program is to encourage individuals to have adequate and balanced nutrition and regular physical exercise and to inform them about the negative effects of obesity on health (e.g., cardiovascular disease, diabetes, some types of cancer, and hypertension).

Considering the limitations of the present study, the sociodemographic and economic factors of the family and family members should also cover the children, who are also at risk of OW, O, and SO. However, since the BMI is not calculated for the people under the age of 15 due to the data set used in the analysis, it is recommended to research children in future studies to determine the sociodemographic and economic characteristics that lead to OW, O, and SO.

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ATATURK UNIVERSITY PUBLICATIONS

Review

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In Vitro Cytotoxicity Test Methods: MTT and Neutral Red Uptake

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ABSTRACT

The use of in vitro cytotoxicity tests is increasing day by day with the increase in human exposure to chemicals because these tests involve less cost and less time than in vivo methods. The purpose of in vitro cytotoxicity tests is to detect cell viability. The toxicity of the xenobiotic applied to the cell should be determined. In vitro cytotoxicity tests are frequently used in analyses to detect cell viability, such as drug development and cancer research. This review focuses on MTT and Neutral Red Uptake analyses, which are the most commonly used in vitro cytotoxicity test methods. At the same time, how in vitro cytotoxicity tests are performed, where they are used, and what are the advantages and disadvantages of these tests are also reviewed in this article. The purpose of this article is to help researchers who will use the information about these frequently used tests by compiling them.

Keywords: Cytotoxicity, cell viability, in vitro tests, MTT, NRU

INTRODUCTION

Developing a new therapeutic agent is a long and costly process. Preclinical studies is an important step to eliminate unsuitable candidates and reduce costs before clinical research is undertaken.[1] Therefore, the use of in vitro tests by pharmaceutical companies has increased in recent years.[2] Several methods are needed to ensure the safety of new agents in drug development. For this purpose, in vitro cytotoxicity test methods have been developed.[3]

Cytotoxicity is the inhibition of the synthesis of certain macromolecules in the cell as a result of various events and damage to the structure of the cell. Cytotoxicity tests are in vitro test methods that determine the extent of this damage to cells.[2] In vitro cytotoxicity tests also measure the ability of a compound to cause cell damage.[4] In in vitro cytotoxicity tests, cells are cultured in one microtiter well plate. In direct proportion to the proliferation and growth rate of the cells, the viability of the cell is indirectly measured with the dye used in vitro cytotoxicity test methods. Such tests determine whether the investigated chemical has a cytostatic effect as well as a cytocidal effect that causes the death of cells.[5] In vitro cytotoxicity test methods are important for preclinical studies because the accuracy of the data obtained as a result of these methods will affect the success of the drug candidate to continue.[1,6] With in vitro cytotoxicity tests, the use of animals to find the LD50 values of xenobiotics has been reduced, and xenobiotics have been rapidly screened. At the same time, these tests are simple, repeatable, and economical.[7] Many methods are used to detect cell viability or cytotoxicity in vitro.[8] In this review article, MTT and Neutral Red Uptake (NRU) analyses, which are the most commonly used cytotoxicity test methods to measure cell viability, are reviewed.

MTT

This test method is a colorimetric method introduced by Mossman in 1983 to determine the viability of cells after chemical, physical, and biological processes are applied to cells.[9,10] This method is used to determine the growth and viability of adherent cells. Recently, it has also been used to determine antimicrobial activity and microbial growth.[11-13]

MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) is a yellow dye that forms positively charged, mono-tetrazolium salts surrounded by 4 nitrogen atoms.[14,15] Since the MTT reagent is positively charged, it can pass through the cell membrane.[16] Thus, tetrazolium salts are converted into purple-colored formazan crystals by the mitochondrial dehydrogenase enzyme in the cell. The amount of formazan crystal increases in direct proportion to the number of viable cells.[17] After the crystals formed are dissolved in a suitable solvent (DMSO or isopropyl alcohol), reading is made by spectrophotometric or microplate reader method.[18] Cell viability is calculated by the following equation:

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Relative cell viability (%) = 100 × (*A*/*B*)

Here, *A* indicates the viable cells in the experimental well and *B* indicates the viable cells in the control.[18]

The MTT test is done as follows:

1. After applying drugs to the adherent cells and keeping them in the CO_2 incubator for a sufficient time, the cells are ready for MTT application.[19]
2. A stock solution of MTT tetrazolium salt is prepared at 5 mg/mL in PBS and stored at +4°C in the dark.[20]
3. Drugs applied at different concentrations to the wells of the 96-well plate and media in the control groups are removed before MTT application.[17,21]
4. 0.1 mL of medium containing MTT is added to each well of a 96-well plate.[22]
5. After the plates are wrapped with aluminum foil, they are incubated for 1-4 hours in a CO_2 incubator at 37°C.[23]
6. At the end of this period, the liquid parts in the wells are carefully removed and discarded.[24]
7. DMSO is added to dissolve the crystals in the wells. After waiting for 10-15 minutes and after a short shaking on the microtiter plate shaker, the reading is taken in the ELISA at 570 nm.[25]

Advantages and Disadvantages of MTT Analysis

Advantages

I. When compared with other in vitro cytotoxicity test methods, MTT analysis is a sensitive test that detects the dose-dependent degradation of xenobiotics in cell function.[26] In addition, MTT offers significant advantages over other viability experiments in terms of speed, simplicity, and precise quantitation.
II. During MTT analysis, the removal of the medium before formazan crystals are formed is the only process. Other than that, it does not require any washing process. This provides an advantage for the use of MTT analysis in non-adherent cell lines.[27]
III. MTT analysis analyzes more samples in less time without exposure to radiation.[28,29]

Disadvantages

I. DMSO or isopropyl alcohol used in MTT analysis can affect the structure and physicochemical properties of the bacterial cell membrane. Therefore, when used in bacterial growth analysis, it may cause erroneous results.[11]
II. In MTT analysis, crystals are formed in the cell.[30] Since these are insoluble in water, these crystals must be well dissolved before measuring because there are differences in absorbance between the wells.[31]
III. The sensitivity of MTT analysis may differ according to cell types.[24]
IV. MTT analysis is based on the conversion of tetrazolium dye to formazan. The rate of conversion to formazan is dependent on metabolic activity and the number of mitochondria. This can cause many interactions.[1]

Neutral Red Uptake Test

Neutral Red Uptake, one of the in vitro cytotoxicity test methods, is used to evaluate the cytotoxicity of various chemical agents such as pharmaceuticals and cosmetics.[32] It is also used to detect the toxicity and phototoxicity of physical agents.[33] Borenfreund and Puerner[34] established the standard protocol for the NRU test in 1984. Then, the In Vitro 3T3 NRU Phototoxicity Test, which was conducted to evaluate phototoxicity in 2000, was accepted in EU member states. It was accepted as an OECD test guideline in 2004.[35] Neutral red (3-amino-7-dimethyl-amino -2-methylphenazine hydrochloride) is a weak cationic dye that dissolves in water and gives a deep red color at slightly acidic pH.[34] The NRU cytotoxicity test procedure is a viability test based on the capacity of living cells to bind neutral red weak cationic dye in their lysosomes.[36,37] It is known that the amount of neutral red dye taken up by the cells is directly proportional to the total number of viable cells.[37]

The construction of the NRU test is as follows:

1. Adherent cells are kept in a CO_2 incubator for 24 or 72 hours after drug administration.[1,33]
2. About 0.04 mg/mL of neutral red working solution is prepared. It is incubated overnight at the same temperature as the cells.[33,38]
3. At the end of the waiting period in the incubator, the condition of the cells to which the drug was applied is checked with a microscope.[33]
4. Neutral red medium was centrifuged for 10 minutes at 1800 rpm to dissolve the precipitated dye crystals.[33]
5. The medium containing the cells is aspirated.[33]
6. 0.1 mL of neutral red medium is added to each well of the 96-well plate.[33]
7. The 96-well plate is left in the incubator for 2-4 hours.[33]
8. At the end of the period, the neutral red medium is removed.[33]
9. The wells are washed with 0.15 mL of PBS.[33]
10. 0.15 mL of neutral red destain solution per well is added.[33]
11. The plate is left on the microtiter plate shaker for approximately 10 minutes until the solution is homogeneous.[33]
12. The plate is measured at 540 nm in the spectrophotometer.[38]

Advantages and Disadvantages of NRU Analysis

Advantages

I. NRU analysis is easy. It provides fast and reliable analysis of large amounts of chemicals in a short time.[32,39]
II. NRU analysis is inexpensive, sensitive, and offers less interference than other tests.[33,34] It also does not use the labile reagents required for cell viability tests using tetrazolium salts (MTT, MTS, XTS, etc.).[33]

Disadvantages

I. NRU analysis is generally not affected by temperature; however, this analysis is affected by contaminants.[40]

DISCUSSION

Today, cytotoxicity test methods are used to determine the toxicological properties of any chemical agent.[31] The use of in vitro cytotoxicity tests has increased in recent years due to the simplicity of their methods and correlation with in vivo cytotoxicity test data,[41] as an alternative to animal testing.[2] In vitro cytotoxicity tests provide important tools for improving human in vitro to in vivo extrapolation.[4] In cell culture, several methods have been developed to study cell viability and cell proliferation. Thus, it is ensured that many samples can be analyzed quickly at the same time.[42] MTT analysis is the most widely used cytotoxicity test among tetrazolium salts.[13] MTT analysis is read using a plate

reader or by spectrophotometric measurement to quantify the number of metabolically active cells.[42,43] NRU analysis is another method. The basis of this method is based on the principle that the neutral red dye, which passes through the cell membrane by diffusion, accumulates in the lysosomes and measures the viability of the cell by spectrophotometric method. At the same time, this method can be used to detect whether there is damage to the cell membrane.[44]

In summary, the advantages and disadvantages of MTT and NRU analyses are mentioned in this review article. The above-mentioned in vitro cytotoxicity tests are colorimetric methods that measure cell viability, which are still used effectively today, despite their disadvantages.

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