Use of Aromatherapy in Recent History and Today

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Aromatherapy, as a special field of Phytotherapy, is accepted by scientific authorities as protecting health with the controlled use of essential oils, supporting treatment in some diseases (adjuvant), and generally as one of the fields of 'Complementary Medicine'. Aromatherapy was most accurately defined literally by the Frenchman René-Maurice Gattefossé in the early 20th century. In ancient Greek, he combined the words therapeia = care and aroma = scent substance, expressed it in a short and concise way, and ensured that this definition was adopted in a very short time. He shared his knowledge and experiences on certain essential oils that he studied sufficiently, with physicians because of the difficulty about finding or even unavailability of antibiotics during the World War I and II, led to the usage of essential oils for the treatment. His book 'Essential Antiseptics' in 1937 attracted physicians' interest in this field. From the same school, Dr. Jean Valnet published his book 'Aromathérapie -Traitment de maladies par essences des plantes' (1964), and due to the interest of physicians working in the field of Complementary Medicine in the medical community, it was translated into other languages. Dr. Paul Belaiche's three-volume work titled 'Traité de Phytothérapie et d'Aromathérapie' is mainly on the antibacterial and antiviral effects of essential oils (1979). In the following years, Dr Daniel Pénoel, who is also a homeopath, and Dr Pierre Franchomme published their standard, comprehensive and treatment-oriented book called 'L'Aromathérapie Exactament' in 1990. In Austria, Prof. Wofgang Steflitsch from the Chest Diseases Clinic of Otto Wagner Hospital, Vienna, published his comprehensive book titled 'Aromatherapie: Wissenschaft-Klinik-Praxis', which gives clinical practices together with case reports, in 2007. He is also the long-time president of the Austrian Aromatherapy Association.

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Aromatherapy, by making use of the effects of different active substance groups of essential oils, is used as a field that supports the treatment of physiological diseases and psychic problems, as well as in combating stress, anxiety, insomnia, hyperactivity and certain pediatric diseases. It is also used in bronchopulmonal diseases and as an immune system stimulant. In addition, it is also widely used in geriatrics for the treatment of certain dermatological problems such as acne and dermatitis. It is a part of modern phytotherapy, due to its antimicrobial effects, which is one of the main effects of essential oils. Aromatherapy has also been successfully applied during the COVID-19 pandemic.

There are professional associations and organizations, including physicians, pharmacists and academicians working in these fields, established for the purpose of carrying out scientific studies and practices in this field in an orderly and ethical manner. The top supervisory authority is the Ministry of Health.

SGMP (Swiss Association of Physicians Practicing Phytotherapy) (Swiss Medical Society for Phytotherapy)

GAMED (Austria-Vienna University, Faculty of Medicine Natural Medicine Association) (Vienna International Academy of Holistic Medicine)

ÖGwA -Österreichische Gesellschaft für wissenschaftliche Aromatherapie & Aromapflege- (Austrian Society for Scientific Aromatherapy and Aroma-nursing) can be given as an example of professional organizations established for this purpose in Central Europe.

In the treatment of symptomatic diseases, appropriate essential oils with monographs are selected for the treatment area to be used and they are prepared alone or in mixtures with other essential oils that have similar effects. For inhalation, in the diffuser, for application to the skin with a massage technique, use a compatible massage oil (fixed oils), or by dropping 1-2 drops of Tea Tree or Eucalyptus essential oil into half a glass of water, gargling, hot or cold compresses, or half/full baths. They are used in recommended doses and durations. Essential oils listed for use in essential oil monographs for children can be administered in low doses. Even in some difficult-to-treat diseases, it is used in a way that is compatible with classical medical units and supports each other in treatment, and in relevant clinics under the supervision of physicians who are trained in aromatherapy and are experienced and competent in their fields.

Administration and Route of Action of Essential Oils

Orally: The essential oil, which has a monograph and is free from mono-ketone components such as thujone and has been standardized, has been prepared

in hard or soft capsules as appropriate for the purpose and in the form of preparations of 80, 160, 300 and 500 mg. It is used according to the physicians' prescription until the symptoms disappear.

By massage: Essential oils have a lipophilic structure. Small molecules can easily penetrate the lower layers of the skin through hair follicles. Applying essential oils to the skin should be in fixed oils and in certain concentrations.

In normal skin, the essential oil spreads throughout the body in approximately 20-25 minutes, depending on the applied area and the structure of the essential oil and fixed oil used (small molecule size). As a result, essential oils are obtained completely naturally from botanically correctly identified medicinal plants. Nature-identical and synthetic substances are never used in aromatherapy applications and their use is strictly prohibited within the framework of the relevant regulations.

As a result, Aromatherapy is just the beginning to be recognized among Complementary Medical Practices in Türkiye. In addition to presentations at scientific congresses, clinical studies approved by ethics committees will ensure that this treatment field is recognized and implemented more quickly and according to scientific facts. It should not be forgotten that this field of treatment should be handled within the triangle of physician-pharmacist and patient and applied in accordance with ethical rules. It should also be known that the application of aromatherapy can be very risky and cannot be used in epilepsy, where drug interactions are common.

Keywords: Aromatheraphy, essential oil

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New timed-release tablets of montelukast sodium for the treatment of nocturnal bronchial asthma

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ABSTRACT

Montelukast compressed tablets were prepared by dry blending of excipients and direct compression. The inner layer containing 3 mg of Montelukast to provide a burst release at 6.5 h. A sustained-release layer consisting of 7 mg and different proportions of HPMC K4M was used to optimise the formulation. The different drug: layer ratio, from 3:7 to 5:5 used for F1 to F10 batch, but 20 % of HPMC K4M (F5) with the layer ratio of 3:7, gave the desired release profile within 8 h and achieved burst release from the immediate release layer in 6.5 h. The swelling and erosion properties of optimised formulation were investigated at different pH. *In vivo* pharmacokinetic studies in rabbits confirmed the sustained release with an average T max value at 10 h and C max value of 478.32 ng/mL compared to the conventional tablet, as C max was 490.10 ng/mL attained by 2 h (T max).

Keywords: Bronchial asthma, chronotherapy, Montelukast sodium, timed-release preparation

INTRODUCTION

Chronobiology is the science of biological rhythms and their functions[1-3]. Chronotherapy is a technique that alters the release of drugs according to the body's

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biological rhythms[4]. Chronopharmaceutical technologies currently available in the market are CONTIN®, which drug release depends on selective hydration of polymer. Chronotropic® and Pulsincaps®, in this device, in the form of capsule which release gel-layer either by diffusion and/or erosion. CEFORM®, prepared as microsphere which subjecting biodegradable polymers or bioactive agents to a combination of thermal gradients, mechanical forces, and flow-rates during processing. TIMERx® is the technology enables drugs to be delivered after a predetermined lag-phase that coincides with the circadian rhythm or allows drug to be delivered to various sites within the gastrointestinal tract. OROS®, a technology based on osmotic pump where the drug-loaded compartment, a push compartment and a semi permeable membrane. Egalet®, the drug-loaded core is then coated with a plasticized enteric coating and thereafter coated with a mixture of water insoluble and enteric polymers. Diffucaps®, uses erosion instead of diffusion to control drug release[5,6].

Chronopharmaceutics enables a new approach to drug delivery[7,8]. The sigmoidal release of drug materials is achieved by coating two different layers one after the other to release the drug with the desired release pattern. Thereby, drug's side effects minimized and therapeutic activity of drug molecules increased[9].This drug delivery has been used successfully for the treatment of ulcers and asthma. In the case of heart disease, if prolonged release delivery given at the time of 10 pm and chronotherapy to lower heart rate and increase blood pressure comfortably between 6 pm and 12 am[10,11].

Asthma is a worldwide disease affecting an average of 300 million people. The symptom of asthma mostly in Africa, Eastern Europe, Latin America, and Asia the no of active cases increases. Asthma symptoms are common with the episode of wheezing, chest congestion, continuous cough at night time and early morning as well[7]. Especially antiasthmatic drugs like leukotriene (LT) receptor antagonists (Montelukast, Pranlukast, and Zarfirlukast) and the 5-lipoxygenase inhibitor zileuton benefit to lower the leukotriene level and adjust the airway inflammation and bronchial hyper responsiveness. Thereby improves conditions for bronchial asthma. Montelukast sodium is the drug of choice and the only LT receptor antagonist prescribed at night for bronchial asthma[12,13]. Multi layered multi disc tablets of Theophylline and Diltiazem prepared and reported[14]. Correspondingly, chronotherapeutics Oral Drug Absorption System (CODAS) technology[15] is the multilayer coating technology developed with the concept of administering night time. In this technology, a non-enteric coating technique is applied to the beads to delay the release of the drug for up to 5 h. The drug release control depends on water-soluble and water-insoluble poly-

mers. The hydrophobic polymer act to resist and control the drug release for prolonged period of time[16,17]. The drug release rate independent of pH, posture, and food.

The goal of this study was to develop a once-daily sustained-release dosage form of Montelukast sodium containing an inner layer to provide burst release at the appropriate time (6.5 h). The matrix tablet formulated with Hydroxypropyl Methylcellulose (HPMC) K4M in varying proportions. This chronopharmaceutical dosage from evaluated and characterized by micrometrics, differential scanning calorimetry (DSC), swelling study, erosion rate study, *in-vitro* drug release study and stability study.

METHODOLOGY

Montelukast sodium was obtained from Orchid Health Care Ltd., (Chennai), Hydroxypropyl Methylcellulose purchased from DOW Chemical Company ((HPMC, Methocel® K4M, Midland MI) and Lactose Monohydrate was purchased from DFE Pharma (Borculo, Netherlands). Povidone K30 purchased from ISP Pharmaceuticals, (Plasdone K30 Povidone®, Wayne, NJ, USA) and Croscarmonellose sodium FMC Biopolymer, (Ac-di-sol®, Mandaue City, Philippines), and Sucrose obtained from Lantic Inc (Montréal, QC). Iron oxide red, purchased from BASF India Limited (Sicotrans®,iron oxide pigments, Katipalla, Mangalore), and Magnesium Stearate from Mallinckrodt's Pharmaceutical Lubricants (Coviden, Ireland). All other chemicals used were of analytical grade.

Method of calculation of timed-release dose of Montelukast sodium

Montelukast sodium 10 mg timed-release tablet administered at 10 pm, the formulation releases the drug slowly and elicits a pharmacological response for up to 24 h *in vivo*. Immediate-release layer releases the drug between 4 am and 5 am (6.5 h after the administration of tablet) to control nocturnal bronchial asthma. Therefore, in this study, 7 mg of Montelukast was proposed for slow, prolonged release, while 3 mg of drug was designed for burst release.

Differential Scanning Calorimetry (DSC)

The thermal melting endotherm of montelucast sodium and montelucast sodium with HPMC K4M, HPMC K100M matrix mixture was determined using a differential scanning colorimeter (DSC, Universal V4.7A TA instrument, Elstree, Hertfordshire, UK)[18]. The amount of samples 3-5 mg was scanned in crimped sealed and placed in perforated aluminium pans, under static air atmosphere. An empty pan was used as reference. The heating rate was 10 °C/min, and the temperature interval used was 30.0-250 °C.

Micrometrics properties

The angle of repose was measured by the fixed funnel method. Bulk and tap densities were determined by carefully pouring a pre-weighed powder into a 100 mL graduated cylinder and measuring the volume occupied by the powder. The tapped bulk density was determined by the volume of the powder bed after tapping the cylinder onto a wood surface fifty times from a height of about 2.5 cm at 2 sec intervals, and the ultimate tapped density was calculated after continued tapping caused no further reduction in volume. The compressibility index was calculated using the volume and final tapped bulk density[19].

Preparation of immediate release layer

The burst release tablets were prepared by direct compression method and prepared according to following procedure. All ingredients sieved by 60 mesh in order to get uniform sized particle. The amount of active ingredient as shown in Table 1 along with excipients except red iron oxide and magnesium stearate were accurately weighed and passed through 60 mesh. The above blend was mixed for 10 min. Magnesium stearate and red iron oxide were weighed and passed through 60 mesh. The above blend was mixed for 5 min and subjected to compression using 5.5 mm round flat face bevelled edge.

Table 1. Formulations component used to prepare montelukast sodium tablets (inner layer)

Batch / ingredients	**F1 (mg)**	**F2 (mg)**	**F3 (mg)**	**F4 (mg)**	**F5 (mg)**	**F6 (mg)**	**F7 (mg)**	**F8 (mg)**	**F9 (mg)**	**F10 (mg)**
Montelukast sodium	3	3	3	3	3	4	5	3	4	5
Lactose 11 SD	52.9	52.9	52.9	67.9	67.9	66.9	65.9	67.9	66.9	65.9
Red iron oxide	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Sucrose	15	15	15	-	-	-	-	-	-	-
Povidone K30	4	4	4	4	4	4	4	4	4	4
Acdisol	4	4	4	4	4	4	4	4	4	4
Magnesium stearate	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Total weight	80	80	80	80	80	80	80	80	80	80
Sustained Release Layer										
Montelukast sodium	7	7	7	7	7	6	5	7	6	5
Lactose 11 SD	175.4	191.4	207.4	191.4	207.4	208.4	209.4	223.4	224.4	225.4
HPMC K4M (methocel)	128	112	96	80	64	64	64	48	48	48
Sucrose	-	-	-	32	32	32	32	32	32	32
Povidone K30	6.4	6.4	6.4	6.4	6.4	6.4	6.4	6.4	6.4	6.4
Magnesium stearate	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2
Total weight (mg)	320	320	320	320	320	320	320	320	320	320

Preparation of sustained release layer

The amount of active ingredient along with excipients except magnesium stearate were accurately weighed and passed through 60 mesh (Table 1). The above blend was mixed for 10 min in a polyethylene bag. Magnesium stearate were weighed and passed through 60 mesh. The above blend was mixed for 5 min.

Preparation of Montelukast sodium sustained release tablets

The matrix tablets were prepared by direct compression method. Formulation was made in two parts, inner layer (containing immediate release) and outer layer (containing sustained release). Blend was prepared by direct mixing with previously shifted materials. Soft tablets were compressed with punches and dies of size 5.5 mm round flat faced bevelled edge. Outer layer was prepared with the punches and dies of the size 9.55 mm round flat faced bevelled edge. Soft tablets (80 mg) were placed in dies filled with half of outer layer (160 mg) and the final compression was made after filling the second half of outer layer (160 mg).

In vitro dissolution studies

The *in vitro* drug release studies were performed using a USP dissolution apparatus Il equipped with paddles. Since Montelukast sodium has a pH-dependent solubility[20], 0.5 % sodium dodecyl sulphate (SLS) was included in the medium to maintain sink conditions. Dissolution testing was done using paddle speed of 50 rpm, different dissolution media [0.1 N HCI, simulated gastric fluid, pH 4.0 acetate buffer, pH 6.8 phosphate buffer] with 900 mL were used. Sampling aliquots of 5.0 mL were withdrawn at 0, 1, 2, 4, 6, and 8 h and replaced with an equal volume of the fresh medium maintained at the same temperature. All media were maintained at 37±0.5°C. The amount of dissolved Montelukast sodium was determined by HPLC with UV-detection at the wavelength of 345 nm. Shimadzu prominence quaternary system with dual wavelength detector. The Column (Symmetry®) - C18 150 x 4.6 mm, 5µ operates at ambient temperature. The sample injection volume was 50 µl, the mobile phase was acetonitrile:0.1 M acetate buffer in the ration of 15:85 (v/v) respectively and its flow rate was 1.5 mL/min. Filtered through 0.45 µm membrane filter and degassed for about 10 min. The retention time of Montelukast was 5 min.

Swelling studies

The Previously weighed (W1) tablets were placed individually in the petri dish containing 10 mL of distilled water. The weight of the tablet (W2) was noted after 30 min after wiping out the excess water by filter paper[21]. The swelling index was calculated using the formula,

$$SI = \frac{W2 - W1}{W2} \times 100 \quad \ldots\ldots\ldots (1)$$

Erosion studies

The standard USP/NF dissolution apparatus I (ERWEKA DT800) was used for this purpose. The dry matrix weighed (Wi) into a dissolution basket and placed in the dissolution medium maintained at 37 ± 0.5 °C with the basket spinning at 100 rpm. Periodically basket-matrix assemblies were removed from the dissolution vessels, tablets were dried to a constant weight in a hot-air oven at 50 °C and reweighed (Wt). The separated samples were used for each interval[22]. The experiments were carried out in triplicate. The percentage matrix erosion (E) at time, t, was estimated from the following equation:

$$E = \frac{Wi - Wt}{Wt} \times 100 \quad \ldots\ldots\ldots (2)$$

Release kinetics

Several dissolution models were applied to study the release mechanism of the optimised formulation[23]. The models included zero order, first order, Higuchi's, and Korsmeyer-Peppas model. All formulations were tested with zero order release rate kinetics and then optimised formulations were selected.

Pre-clinical studies (preparation of sustained release (SR) layer for *in vivo* pharmacokinetic study)

The amounts of active ingredients and excipients other than magnesium stearate were accurately weighed and passed through a 60 mesh. The above blend was blended for 10 min. Magnesium stearate were weighed and passed through 60 mesh. The above blend was mixed and blended for 5 min[24]. The above blend was subjected to compression using 5.9 x 3.9 mm oval shaped. Table 2 shown the formula for preparation mini layer of Montelukast sodium for animal study.

Table 2. Formulation of SR layer for *in vivo* animal study

Ingredients	mg/unit
Montelukast sodium	0.517
Lactose 11 SD	26.28
Povidone K 30	0.8
HPMC K4M	8
Sugar	4
Magnesium stearate	0.4
Total weight	40

Pharmacokinetic study procedure

To assess the applicability of the method the study conducted with Male rabbit weigh 1kg. Institutional Ethical committee permission was obtained, IAEC No: IAEC/XLV1/03/CLBMCP/2015.

The animal was housed and free to assess the food and water. Rabbits in the group (N = 6) were orally administered the developed sustained-release tablet formulation (0.50 mg/tablet) by gavage. After single oral administration, 0.5 mL of the blood were collected from marginal ear vein for 0.5, 1, 2, 4, 6, 8, 10, 12, 18 and 24 h after the administration in to the tubes containing EDTA. Plasma concentration of Montelukast analysed by chromatographic technique (Thermo Scientific Accela LC Systems 1250, software versions; ChromQuest 4.2). The mobile phase consists of 250 volume of water, 200 volume of methanol 550 volume of Acetonitrile, and 3 volume of Acetic acid finally adjust the pH 5.5 with Triethylamine. The column was C18, 250 x 4.6 mm, 5µ (YMC-Pack HPLC column, Genetec). The mobile phase was delivered at a flow rate of 1.0 mL/min, the detection wavelength was 240 nm and the ratios of the peak height of Montelukast to that of the concentration of internal standard were calculated for each sample. The concentrations of Montelukast in the plasma samples were then determined from the calibration curve. The assay has been validated, and has a good linearity (-0.999 with slope of 28958 and y intercept of -4276) from 20 to 1000 ng/mL with acceptable reproducibility. All measurements were performed at ambient temperature[25].

RESULTS and DISCUSSION

Differential scanning calorimetry

Differential scanning calorimetry was used to elucidate the physical state of the drug in the system. A sharp melting transition of Montelukast sodium was observed at 140.8 °C (Figure 1) and thermal stability up to 180.3 °C. The mixture shows a broad melting transition with a peak maximum at 140.2 °C. However, reported values was 135.5 °C for Montelukast sodium[26]. From these observations, it can be concluded there was no interaction between the components during heating.

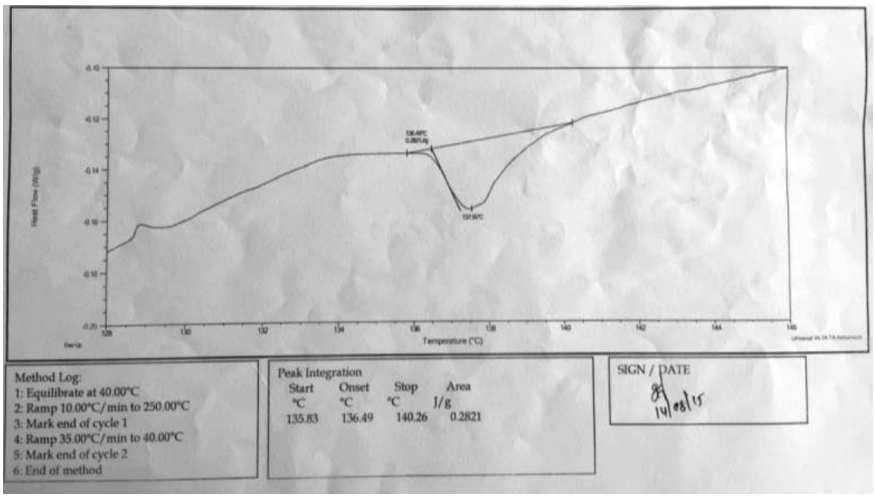

Figure 1. Differential scanning calorimetry of Montelukast:HPMC K4M (1:1) mixtures

Micrometrics

The tapped bulk density measurements of the Montelukast sodium granules for all batches of sustained release (SR) layers (F1-F10) were found to be relatively higher than those of the inner layers. The tapped bulk density of Polymer (HPMC K4M) 1.3, which was lower than that of the drug, and the interlayer density was poor (difference in the density). Further, this value indicating the presence of the comparatively higher number of enclosed voids space. The compressibility index used as an indicator of the change in filler arrangement when the powder was struck, and direct measure of the powder's tendency to consolidate as it undergoes vibration, transportation, and handling[27]. F10, which has the highest compressibility index, indicated diminished flow, as higher values tend to indicate less flow property of granules. The lowest compressibility index was 5-15 % which indicates excellent flow properties. The lo-

west compressibility index 5-15 %, which indicates excellent flow properties. It indicated that 38 % of drug having 400 µ sizes which retained in 40 mesh. Correspondingly, about 26 %, 5 % and 13 % of drug granules having size of 250 µ, 177 µ and 74.11µ which were retained on 60, 80 and 200 mesh respectively. However, it was reported that the spread in particle size had a significant and complex influence on the short-term post-compaction increase in tablet tensile strength[28].

Evaluation of formulated tablets

Montelukast sodium bilayer tablets of F1-F9 were evaluated for various parameters like as hardness, friability, weight variation for inner layer and SR layer, thickness and % drug content. The results of these parameters shown in Table 3. The results were confirming with the official and Organisation of Pharmaceutical Producers of India (OPPI) Standards for tablets[29-31].

In vitro dissolution studies

Effect of polymer concentration on burst release

All powder blends of the inner and polymer layers were successfully compressed into tablets with a total weight of 400 mg (F1-F10). The dissolution profile of all prepared formulation analysed by chromatographic technique. Instead of using calibration curve method, drug release was calculated by assay method using Montelukast standard drug. As Montelukast sodium soluble in water exhibits pH dependent solubility. 0.5% w/v of SLS was used in the dissolution media to maintain sink condition. Dissolution was performed simultaneously at 3 different physiological pH values (1.2, 4.5, and 6.8) without changing the liquid or tablet, and the cumulative drug release was calculated. To study the effect of various concentration of polymer (Methocel K4M), batch of Fl -F4 tested with 40 %, 35 %, 30 % and 25 % (where all polymer concentration calculated in percentage to the total weight of layer in SR layer) was used with the constant layer (inner:sustained) ratio of 3:7. Drug release studies indicated that F1, F2, F3, and F4 (Figure 2) were 6 %, 9 %, 11 %, and 13 % at 1 h, and 32 %, 36 %, 46 %, and 97 % of drug released at 8 h respectively for F7. This was due to the higher concentration of polymer closing the pores on the surface of the matrix tablet, resulting in less diffusion.

Table 3. Physical and chemical parameters of formulated Montelucast sodium compressed tablet formulations (F1 to F10)*

Test	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10
Thickness (mm)	4.26 ± 0.8	4.28 ± 0.9	4.22 ± 1.3	4.27 ± 1.5	4.25 ± 1.0	4.27 ± 1.2	4.35 ± 1.0	4.26 ± 0.9	4.27 ± 0.8	4.29 ± 1.5
Hardness (kp)	10.5 ± 0.8	10.8 ± 1.5	10.9 ± 1.2	11 ± 0.9	10.2 ± 0.8	10.4 ± 0.8	10.5 ± 1.5	10.1 ± 1.3	10.5 ± 1.2	10.2 ± 1.0
Friability (%)	0.54 ± 0.8	0.53 ± 1.2	0.50 ± 1.0	0.50 ± 0.8	0.57 ± 1.2	0.58 ± 1.5	0.58 ± 0.9	0.6 ± 1.6	0.54 ± 1.3	0.58 ± 1.4
Inner layer (mg) (IR)**	80 ± 0.8	79.5 ± 0.5	79.8 ± 0.7	80 ± 0.5	81 ± 0.4	80.5 ± 0.8	79.2 ± 0.7	80 ± 0.6	80 ± 0.7	80 ± 0.4
Outer layer (mg) (SR)***	320 ± 2.0	320 ± 2.5	320.4 ± 2.1	320.5 ± 2.1	320 ± 2.2	325 ± 2.5	320 ± 2.0	320.2 ± 2.5	320.2 ± 2.5	320 ± 2.3
Total weight (mg)	400 ± 2.5	400 ± 2.3	400.2 ± 1.5	400.5 ± 2.2	401 ± 2.5	401.5 ± 2.0	399.2 ± 2.6	400.2 ± 2.5	400.5 ± 2.5	399.2 ± 1.0
Drug content (%)	100.10	99.5	100.1	100.2	101.2	100.7	99.2	99.5	99.1	99.5

**All values are mean ± SD and % RSD*

*** Inner layer*

**** Sustained release layer*

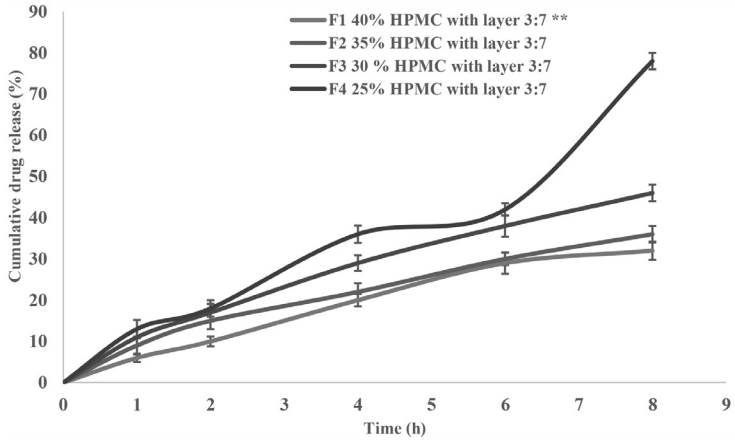

Figure 2. *In vitro* dissolution profile of Montelukast (F1-F4)*

**All values are mean±SD and % RSD for n=3*

*** Drug Inner layer: Sustained release layer (mg)*

As the polymer concentration was inversely proportional to the drug release found in that formulation, it was decided to use 20 %, polymer concentration with drug layer ratio of 3:7, 4:6, and 5:5 for F5, F6, and F7, which gave the release of 15 %, 17 %, and 23 % at 1 h; 90 %, 98 %, and 100 % release was obtained at 8 h (Figure 3). However, desired profile for the inner drug layer for burst release achieved only with F5 and F6 at 6.5 and 6 h. Where as in the formulation F7, burst release obtained at around 5-6 h. Two reasons postulated for this, firstly, the difference in the time for separation of the burst release was due to variations in the swelling index. Deviations of 15-20 % were calculated over the study period and represented as SD in the graph. Secondly, the release rate of the formulation was one of the important criteria for optimization.

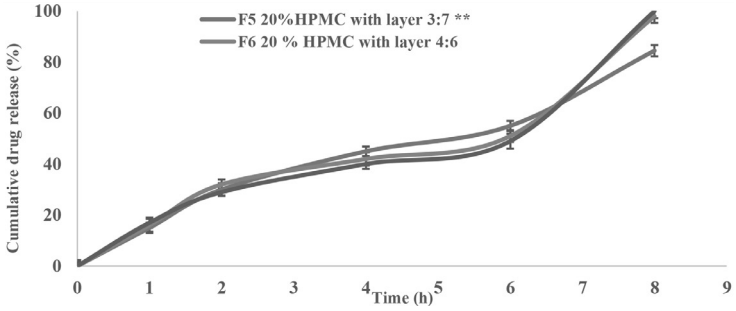

Figure 3. *In vitro* dissolution profile of Montelukast (F5-F7)*

**All values are mean±SD and % RSD for n=3*
*** Drug Inner layer: Sustained release layer (mg)*

Further reducing the polymer concentration to 10 % to maintain layer ratios for F8, F9 and F10 were 3:7, 4:6 and 5:5, respectively (Figure 4).

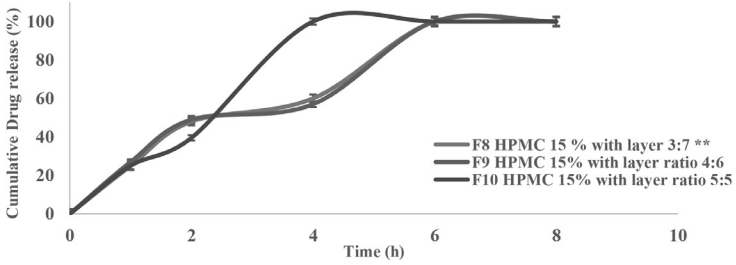

Figure 4. *In vitro* dissolution profile of Montelukast (F8-F10)*

**All values are mean±SD and % RSD for n=3*
*** Drug Inner layer: Sustained release layer (mg)*

The better dissolution profile obtained, where 25 % of the drug released at 1 h and inner layer separated out at around 3-4 h. This results were in agreement with published report where the author stated that increasing the concentration of the blends from 20 to 40 % each, showed a sustaining effect on drug release profile[32-34].

Effect of layer ratio on drug release

To study the effect of drug release on the layer ratio, formulation Fl-F5 was compressed at a ratio of 3:7. In all cases drug release was found to be affected by polymer concentration only where the inner layer was separated at 6.5 h with F5. From Fl-F4, inner layer did not separate until 8 h. When the ratio altered to 4:6 and 5:5 for formulation F6 and F7, inner layer separated out at

around 6 h which were not desirable. Further decreased polymer concentration only effected the drug release. Therefore, the layer ratio did not bring any significant changes to the drug release pattern except for the burst release. Since the inner layer is sandwiched between two SR layers, only changes in the weight ratio or layer pattern that affected the drug release profile[35,36].

Swelling and erosion studies

Measurements of the swelling behaviour of all formulations were carried out to find out their water uptake capacity. Initially, visual inspection of matrix tablets showed that swelling was dominant in all batches, as the tablet surface was smooth and slippery to the touch. This study was proposed to conduct until the sandwiched burst release layer separated from the tablet. Swelling behaviour studied at pH 1.2, 4.5, and 6.8. Figure 5 (X, Y, Z graph) shows the rate of swelling for matrix tablets. Swelling of the matrix, which was indicated by the transition of the polymer from the glassy to the rubbery state[37]. The highest degree of swelling was achieved by F1 to F5 contains 40 %, 35 %, 30 %, 25 %, and 20 % of HPMC K4M. There was about 227, 192, 165, 150 and 120 % weight gain at the end of 8 h due to swelling in these matrices. On the other hand, F6, F7, and F8 contains 20 %, 15 % and 15 % respectively, gave around 120 % only. These matrices could hydrate only up to 6 h (F6, F7) and up to 4 h (F8) after which, there was no further increase in the tablet weight due to water uptake. The F9 and F10 swelling studies could not be performed because the sandwiched inner layers separated at around 3 h. Analogous observations were reported by Nerurkar et al[38] for HPMC containing matrices regarding their inability to hydrate for longer time periods and percentage swelling was directly proportional to the polymer concentration. The swelling behaviour was not affected by any significant changes with respect to pH (1.2, 4.5, 6.8), but the separation of the immediate release (IR) layers was shown in Figure 6. This response was to be the physiochemical characteristic of the polymer especially HPMC K4M, lower Methoxy and Hydroxy propyl content as reported by J. Siepmann et al[39]. In hydrophilic polymeric matrix systems, the overall dissolution rate and, ultimately, drug availability were reported to be controlled by the rate of matrix swelling, and erosion of the outer gel layer[40]. The percent erosion found to be 22 % at the end of 8 h, for F1 whereas F2, F3, F4 and F5, it was found to be 28, 42, 53, and 68 % respectively at the end of the 8 and 6 h (F4, F5). At the end of the dissolution run, the matrix of F6 and F7 was completely eroded. In the case of the F9 and F10, no swelling and erosion studies were performed because of both swelling and erosion were shown to have been occurring at the same time as reported[41].

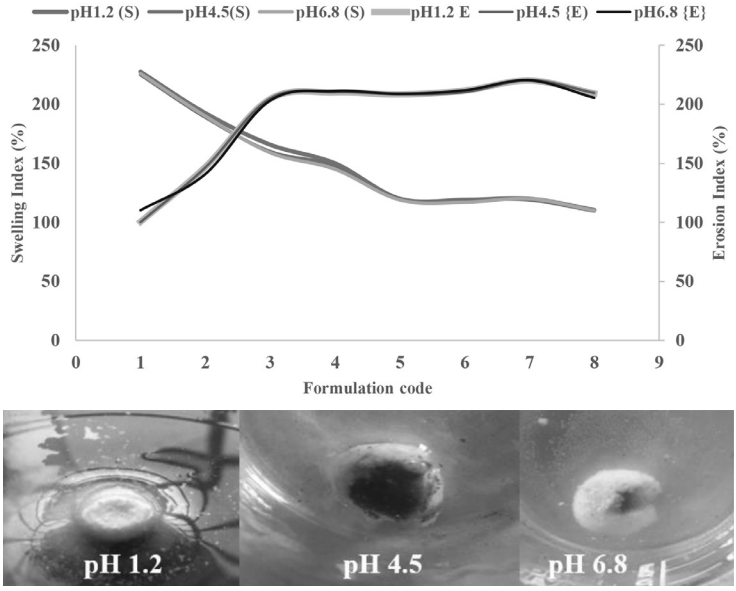

Figure 5. Swelling and Erosion profile of Montelukast formulation (optimized)

Table 4. Fitting with various kinetic model of optimizing formulation (F5)

Parameter	Zero order Release	Higuchi Kinetics	First order Kinetics	Weibull kinetics	Korsemeyer Peppas model
Slope	10.53	40.93	0.099	0.006	0.8335
R_obs-pre	0.9754	0.9619	0.9592	0.9688	0.9885
R^2	0.9482	0.9242	0.9187	0.9381	0.977
R^2adj	0.9482	0.9091	0.9024	0.9072	0.9617
MSE	59.0061	9.0	111.1414	105.6742	33.6876
WSSR	8.0	5.0	555.7069	422.6967	101.0629
AIC	43.0858	47.7452	48.2417	48.3266	33.6945
MSC	2.3106	1.645	1.574	1.5619	2.774
Rate (%/h)	10.5	41.0	0.22799	2.472	16.258
T25 (h)	2.201	1.504	1.6	2.186	0.538
T50 (h)	4.402	3.523	3.647	4.289	2.785
T90 (h)	7.924	9.554	11.772	7.141	7.628

coefficient of determination (R^2), adjusted R^2(R^2_{adj}), mean square error (MSE), Akaike Information criterion (AIC), weighed sum of square of residues (WSSR), model selection criterion (MSC). R-observed-predicted (R_obspre), Time in hours requied to release 90 % of the drug from tablet (T90).

Kinetics of drug release

In order to optimize the formulation, drug release from all batches fitted in to the zero order equation shown in **Table 4.** The *in vitro* release rate (K), which gave the same value for F4 and F5, was further found to separate the burst inner layer at exactly 6.5 h (F5) and was chosen as the optimised formula. Hence, formulation F5 fitted in to the various mathematical models especially using the Peppas and Korsmeyer[42]. The t 25, t 50 and t 90 values of the formulations tested were in the range of 2 to 7 h, indicating a time range for completely releasing the drug from the matrices and further burst release of the IR layer. The value of release exponent *n* was calculated with standing the burst release layer as well. Thus, F5 optimized formulation gave non-fickian release kinetics (*n*= 0.833).

Table 5. Comparison of *in vivo* data of formulation F-5 with market product

AUC summary	Optimized formulation (F5) ($ng*h\ mL^{-1}$)	Market product ($ng*h\ mL^{-1}$)	AUMC summary ($ng*h^2\ mL^{-1}$)	Optimized formulation (F5) ($ng*h^2\ mL^{-1}$)	Market product ($ng*h^2\ mL^{-1}$)
Last conc. value	20.0l	0.9	Last conc. time value	120	10.8
Cumulative observed AUC(0-t)	1628	1185	Cumulative observed AUMC(0-t)	3845	654
Remaining AUC (t-∞)	16	2	Remaining AUMC (t-∞)	110	28
AUC(0-∞)	1644	187	AUMC (0-∞)	3955	682
Cmax (ng/ml)	478.32	490	-	-	-
T max (h)	10	2	-	-	-

Area under plasma concentration curve (AUC), Area under the moment curve (AUMC), The peak time (Tmax), the peak concentration (Cmax)

Pre-clinical evaluation

In order to investigate the *in vivo* performance of the optimized formulation, 200 numbers of sustained release mini tablets with the total weight of 40 mg containing the 0.517 mg of Montelukast sodium was prepared. The *in vivo* study was carried out only for the SR layer due to the low dose of IR (around 0.2 mg). The animal dose calculated based on the surface area method and approved by the Institutional animal ethical committee. Singular tablets containing 10 mg of Montelukast sodium was used for the comparison. Plasma concentration-time curves of Montelukast sodium following a single oral administration of optimized formulation (F5) and Market sample in rabbits were compared Figure 7, and their pharmacokinetic parameters were summarized in Table 5.

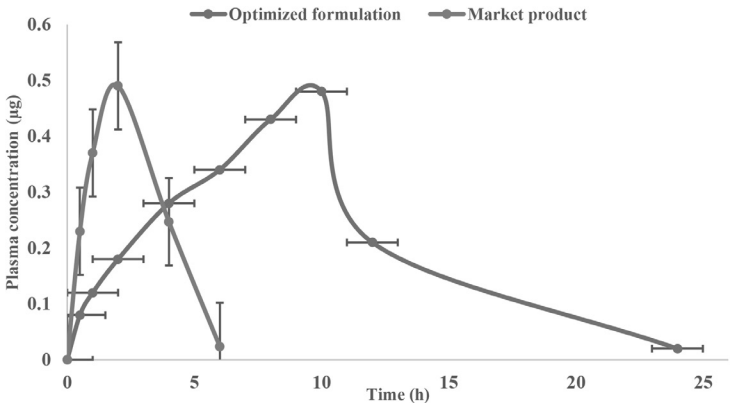

Figure 7. *In vivo* profile of Montelucast Sodium (Optimized F5)

The plasma concentration-time profile was different than expected. When the F5 tablet was administered to 6 rabbits, the time of the first appearance of Montelukast in plasma was 0.5 ± 0.2 h. The plasma concentration increased steadily after administration of the optimised formulation and continued until 24 h after administration. Although the plasma concentration was higher in the market product, it was not detectable after 6 h. The C max, 490.10 ng/mL attained by 2 h (T max) but 478.32 ng/mL of C max only reached by 10 h in an optimized formulation. However, the AUC and AUMC values were profoundly higher for the optimized F-5 formulation. Enrique Muñoz et al[43] reported. C max value of close to the 1 mcg after administering two 10 mg tablet of Montelukast sodium tablet to the human subjects. However, Zaid An et al[44] reported C max value was around 500 ng/mL. Therefore, the long lasting plasma concentration was likely caused from the matrix tablet throughout the gastrointes-

tinal (GI) tract, thereby suitable for chronopharmaceutical delivery.

In conclusion, the new Montelukast sodium timed-release tablet consists of two layers: (a) an inner layer with 3 mg as a burst release at 6.5 h after administration and (b) sustained release of 7 mg released up to 24 h, which could be fabricated as a bilayer tablet. The SR layer made of HPMC K4M was needed to control the release rate by swelling and burst release. Further, *in vivo* plasma concentration profile proved the release of drug over 24 h in comparison with conventional product, this novel bilayer tablet successful approach for Nocturnal Bronchial Asthma.

STATEMENTS OF ETHICS

Animal studies were approved by IAEC of C.L. Baid Metha COP, Chennai-97.

CONFLICT OF INTEREST STATEMENTS

No financial and non-financial competing interests with regards to the publication of this research work.

AUTHOR CONTRIBUTIONS

All authors contribute the work equally throughout.

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A cross-sectional study on the knowledge of pharmacists about Alzheimer's Disease: enhancing services for patients and caregivers

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ABSTRACT

Alzheimer's disease (AD) is a progressive neurodegenerative disorder impacting cognitive function and daily activities. Pharmacists' accessibility enables them to contribute to rational drug use, monitor interactions, and educate patients and caregivers, improving health outcomes. This study investigates pharmacists' knowledge of AD. A descriptive study in Türkiye utilized a web-based questionnaire with demographic questions and the Alzheimer's Disease Knowledge Scale (ADKS). 185 participants (72.43% female, mean age 36.9±11.9) exhibited significant knowledge of Alzheimer's disease (mean ADKS score: 7.48±1.18). Scores varied across factors like gender, age groups, professional experience, and geographical region. Statistically significant correlations were found between these factors and specific domains of AD knowledge. AD necessitates specialized care, and pharmacists' services improve health outcomes for patients and families. Cognitive pharmacy services for complex treatment regimens benefit AD patients and their relatives. A multidisciplinary approach is crucial in effectively addressing the impact of AD and providing optimal treatment.

Keywords: Alzheimer's disease, pharmacists, knowledge, ADKS, dementia

INTRODUCTION

Alzheimer's disease (AD), a neurodegenerative condition, is characterized by progressive deterioration of the brain over time. Dementia encompasses a range of specific symptoms, including memory and language decline, difficulties

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with problem-solving, and significant impact on daily functioning. While various factors can contribute to the development of dementia, AD is the prevailing form within this category [1,2]. The underlying cause of these symptoms is attributed to the impairment or destruction of neurons responsible for cognitive functions, including thinking, learning, and memory [2]. Due to the progressive nature of AD, the pathological changes extend to neurons in various regions of the brain. Consequently, individuals experience significant impairment in their ability to perform basic physical activities, such as walking and swallowing. In the advanced stages of AD, individuals typically become bedridden and require constant care and assistance throughout the day [1,2].

Pharmacists are readily accessible healthcare professionals, and as a result, many patients seek their assistance at pharmacies with the expectation of resolving their health concerns. Pharmacists have the capacity to contribute to multidisciplinary healthcare by promoting rational drug use, monitoring drug-drug interactions, and closely monitoring the adverse effects of medications [3–7]. A pharmacist possesses the ability to monitor the evolving symptoms in elderly patients, identify individuals at risk, and educate caregivers about the disease and available treatment options. By providing these comprehensive services, pharmacists not only enhance the overall health outcomes of AD patients but also contribute to the well-being of their caregivers, while promoting rational drug utilization [8–10]. Pharmacists have the capability to provide specialized monitoring and follow-up for patients with Alzheimer's disease, including the observation of specific signs and symptoms, assessment for early detection of Alzheimer's symptoms, identification of individuals who may be at risk, and the provision of education to both patients and their family members [9,11]. The aim of this study is to investigate pharmacists' knowledge about AD.

METHODOLOGY

Design, Sample Size and Participants

A descriptive, observational study was conducted in Türkiye between November 2020 and February 2021. Participants were limited to pharmacists who expressed their willingness to participate in the study. The study received ethical approval from the Bezmialem Vakif University local Ethics Committee, with a decision number of 18/346. The exponential non-discriminative snowball sampling method was utilized to select participants. The study adheres to the CROSS (A Consensus-Based Checklist for Reporting of Survey Studies) standards for reporting [12].

$$n = \frac{Z^2\, p(1-p)}{d2}$$

The formula described above is utilized to determine the sample size required for the study. In the formula, the variable "n" denotes the sample size, while a p-value of 0.2 is used to represent the proportion. A margin of error of 0.05 is set for sampling, and "Z" represents the standard normal value at a 95% confidence interval, which is equal to 1.96. Based on these parameters, the formula is employed to compute the sample size, resulting in a value of 245. However, for the final analyses, only 185 participants were included, as depicted in Figure 1. The study included participants who were pharmacists and individuals who voluntarily provided their consent by signing the consent form. Exclusions from the study comprised non-pharmacists who declined to participate, pharmacy faculty students, and individuals who lacked the required cognitive abilities.

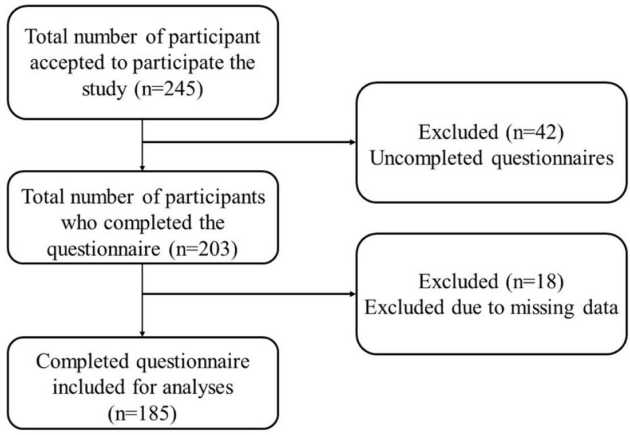

Figure 1. Flowchart CROSS guideline requirements

Questionnaire development

To assess the level of AD knowledge among participants, a web-based questionnaire was developed using Google Forms. The questionnaire was disseminated through various channels, including email, direct messages, and social media platforms, along with information about the study. To encourage participation, participants were encouraged to share the questionnaire link with their social networks. The questionnaire was divided into two sections with 16 items in total, the first focusing on demographics (age, gender, educational level, years of work experience, province), while the second section comprised the Alzheimer's Disease Knowledge Scale (ADKS). Participants were required

to provide an electronic approval as part of the written consent form, and each response was cross-checked to ensure no duplicate entries were made. Additionally, a hyperlink to the consent form was embedded in the online questionnaire for participants to access for personal reference. The option to fill out the Google form once was enabled.

ADKS as prepared with modifications of questionnaire prepared and validated by carpenter [13]. Yilmaz and Çolak 2019 conducted cross-cultural adaptation and assessed the validity and reliability of the Turkish version of the ADKS questionnaire[14]. The Turkish validation of the questionnaire modified by authors and the number of items has been reduced. The modified ADKS questionnaire is a self-administered survey that encompasses five subdomains. It comprises a total of ten items that require participants to select either "Yes" or "No" as their answer. Only one answer can be chosen per inquiry, and each correct response is assigned a score of one (1), while incorrect responses are assigned a score of zero (0). The participant's survey score can range from a minimum of zero (0) to a maximum of ten (10) points. The five domains of the modified ADKS questionnaire are as follows: risk factors (1 item), assessment and diagnosis (1 item), symptoms (2 item), life impact (1 item), treatment and management (5 item).

The questionnaires underwent a process of bilingual translation from English to Turkish, which included both forward and backward translation. The structured items within the questionnaires were carefully evaluated to ensure that they were appropriately rephrased, reformatted, and deemed relevant for use. A team of experts consisting of four professionals was consulted to provide feedback on the questionnaire's language design and the questions were revised based on their recommendations. Additionally, the questionnaire was pretested with two professionals who possess expertise in the related field. The pre-testing involved retrospective cognitive interviews with a focus on assessing the content, format, and wording of the questionnaire. Pilot research was conducted with a group of fifteen individuals who did not participate in the initial evaluation, and feedback obtained was used to improve the questionnaire's clarity and understandability. A group of twenty participants, who were not included in the original study data set, were asked to complete the questionnaire within a period of two weeks. The questionnaire takes approximately 5 to 10 minutes to complete and is designed for use with pharmacists.

To assess the test-retest reliability of the questionnaire, the Spearman correlation coefficient, Wilcoxon test, and intraclass correlation coefficient (ICC) were evaluated using a sample of 20 participants. The results indicated that

the questionnaire exhibited a statistically insignificant correlation of 0.723 ($p>0.05$) and an ICC of 0.718 (95%GA: 0.593-0.865, F: 4.39, $p<0.001$). The reliability of the ADKS scale was evaluated using Kuder-Richardson's formula, which yielded a value of 0.872 for the questionnaire tool used in this study.

Statistical Analysis

As descriptive statistics, mean, median, standard deviation, and interquartile range [IQR] or count and percentages are given for continuous variables. The frequency and percentage are given for categorical variables. The Kolmogorov-Smirnov, Shapiro-Wilk tests Q-Q plots, histogram and density analysis, skewness and kurtosis values was used to test for normality of continuous variables. The statistical analysis of the data was performed using SPSS version 26 and Jamovi software. The mean and standard deviation of the total score on the ADKS was calculated and then the difficulty and discrimination indexes were calculated for each item. The total score of the ADKS was determined by adding up the correct responses. The difficulty index (p) was calculated to show the percentage of people who answered the item correctly. The discrimination index (DI) was estimated (ideally between 0.3 and 0.7) to measure how well the items discriminated between people with high and low scores and was calculated by subtracting the number of correctly answered items from low scorers from the number of correctly answered items from high scorers, divided by the number of people in the sample. The internal consistency of the ADKS was determined using Kuder-Richardson's formula for items scored dichotomously.

RESULTS and DISCUSSION

A total of 245 participants were enrolled in the study, but 42 were excluded due to incomplete questionnaires, and an additional 18 were excluded due to missing data. The study's flowchart is depicted in Figure 1. Out of the initial 245 participants, a final analysis was conducted using 185 questionnaires. Table 1 provides an overview of the demographic characteristics of the study participants. Predominantly, the majority of participants were female (134, 72.43%), and the mean age of participants was 36.9 ± 11.9. Approximately one-third of the respondents are located in the Marmara region (54, 29.67%). This finding aligns with Türkiye's population density, supporting the outcomes of our study. Subsequently, the Central Anatolia region emerges as the second most prevalent geographical area where the participants reside (43, 23.63%). When examining the professional experience of the questionnaire participants, it becomes evident that there is a relatively balanced distribution. Out of the total

185 participants, 93 (50,27%) individuals had less than 10 years of professional experience, whereas 52 (28.11%) participants were pharmacists with over 20 years of professional experience.

The present study involved the participation of 185 pharmacists, and its primary objective was to assess the level of knowledge pertaining to AD using the ADKS. A multidisciplinary approach in AD care is recommended in many different studies in the literature. Pharmacist could take active part in multidisciplinary care teams by providing cognitive pharmacy services. The most important patient-oriented services that can be provided by pharmacists in free pharmacies are detailed drug examination, patient education, referral for early diagnosis and psychosocial support [15–20].

Table 1. Demographic characteristics of the study participants

Gender (n, %)	**n=185**
Male	51 (27.57%)
Female	134 (72.43%)
Age (Mean± SD)	**36.9 ± 11.9**
21 - 30	66 (35.68%)
31 - 50	85 (45.95%)
>51	34 (18.38%)
Years of work experience (Mean±SD)	**14.4±12.6**
0-5	60 (32.43%)
6-10	33 (17.84%)
11-20	40 (21.62%)
20+	52 (28.11%)
Region (n, %)	
Marmara	54 (29.67%)
Central Anatolia	43 (23.63%)
Aegean	29 (15.93%)
Black Sea	19 (10.44%)
Mediterranean	15 (8.24%)
Eastern Anatolia	14 (7.57%)
Southeastern Anatolia	11 (6.04%)

The responses provided by the pharmacists who took part in our study, as indicated by the ADKS questionnaire, demonstrated a significant level of knowledge regarding AD. the frequencies of participants' responses to the ADKS Items and Domains is given in Table 2. The mean ADKS score, reflecting the participants' knowledge, was calculated as 7.48±1.13 (Table 3). The participants demonstrated a notable level of accuracy in responding to the survey items, with three specific items garnering the highest number of correct answers. These items, respectively, are, *"Acetylcholinesterase inhibitors are effective in early to moderate stages of Alzheimer's Disease"* which received 177 correct responses (95.67%), *"Memantine is effective in moderate to severe Alzheimer's Disease"* with 174 correct responses (94.05%), and "*Patients with Alzheimer's are prone to depression*" which received 171 correct responses (92.43%). On the other hand, participants displayed a notable number of incorrect responses for three specific questions. These questions, respectively, are, *"It is proven that mental exercise can prevent a person from developing Alzheimer's Disease"* which received 172 incorrect responses (92.97%); "*It is highly recommended to use Benzodiazepines in Alzheimer's Disease patients who have also developed anxiety*" with 115 incorrect responses (62.16%), and *"Decline in cognitive functions will continue in patients with Alzheimer's after initiation of drug therapy"* which garnered 65 incorrect responses (35.14%). Mean ADKS rate of knowledge of AD among participants is given in Table 3. The participants' mean scores on the 5 domains of the modified ADKS questionnaire were as follows, Assessment and Diagnosis (0.89 ± 0.31, [1 item]), Symptoms (0.75 ± 0.44, [1 item]), Risk factors (1.59 ± 0.55, [2 items]), Life impact (0.93 ± 0.26 [1 item]), and Treatment and Management (3.74 ± 0.89 [5 items]).

Table 2. ADKS rate of knowledge of Alzheimer's disease among participants

ADKS Items and Domains	Correct Answers (n, %)	Wrong Answers (n, %)
Assessment and Diagnosis		
Alzheimer's is a type of Dementia	163 (88.11%)	22 (11.89%)
Symptoms		
Tremor and shaking hands or arms is a common symptom for Alzheimer's Disease	138 (74,59%)	47 (25,41%)
Risk factors		
High cholesterol and blood pressure levels increase the chances for an individual to develop Alzheimer's Disease	123 (66.49%)	62 (33.51%)
It is proven that mental exercise can prevent a person from developing Alzheimer's Disease	13 (7.03%)	172 (92.97%)
Life impact		
Patients with Alzheimer's Disease are prone to depression	171 (92.43%)	14 (7,57%)
Treatment and Management		
Acetylcholine esterase inhibitors are effective in early to moderate stages of Alzheimer's Disease	177 (95.67%)	8 (4.32%)
The side effects of Acetylcholine esterase inhibitors are Diarrhea, Nausea, Vomiting and Bradycardia	151 (81.62%)	34 (18.38%)
It is highly recommended to use Benzodiazepines in Alzheimer's patients who has also developed anxiety.	70 (37.38%)	115(62.16%)
Memantine is effective in moderate to severe Alzheimer's Disease	174 (94.05%)	11 (5,95%)
Decline in cognitive functions will continue in patients with Alzheimer's Disease after initiation of drug therapy	120 (65.86%)	65 (35.14%)

In order to improve the quality of services to be provided by pharmacists to AD patients, increasing the level of knowledge of community pharmacists about AD has been found useful in the literature in terms of early diagnosis, proper management of disease symptoms and improving the quality of life [21]. According to our results, Turkish pharmacists ADKS score was 7.48±1.13 which was equivalent of 74.8%. Based on existing literature, it is evident that the level of knowledge regarding AD among Turkish pharmacists is comparable to findings reported in similar studies [16,22–25]. Turkish pharmacists exhibited superior performance compared to a study conducted by Mat Nuri et al. in Malay-

sia [24]. However, Spanish pharmacists outperformed Turkish pharmacists, as evidenced by a study conducted by Alacreu et al. in Spain [23]. Furthermore, in a study conducted by Nordhus et al. in Norway involving psychologists, Turkish pharmacists demonstrated higher levels of proficiency compared to the psychologists in Norway [22].

Table 3. Mean ADKS rate of knowledge of Alzheimer's disease among participants

ADKS domains	Mean ADKS	SD
Total domains score	7.48	1.13
Assessment and Diagnosis	0.89	0.31
Symptoms	0.75	0.44
Risk factors	1.59	0.55
Life impact	0.93	0.26
Treatment and Management	3.74	0.89

According to our results, Turkish pharmacists received the highest score in the Treatment and Management domain (3.74±0.89, 93.52% correct). On the other hand, the lowest score was recorded in the Symptoms subdomain (0.75±0.44, 74.59% correct). These results are higher than the results obtained by Mat Nuri et al. in both domains [24]. In contrast, our study yielded a higher score in the Treatment and Management subheading when compared to the findings reported by Mónica Alacreu et al. [23]. However, Turkish pharmacists exhibited lower success rates in the Symptoms subheading compared to their Spanish colleagues. Existing literature indicates that Turkish pharmacists often seek counseling from their patients, as independent pharmacists serve as one of the readily accessible components of the healthcare system [26].

Table 4. Comparison of demographic data with ADKS Questionnaires scores among participants

	ADKS (Mean± SD)	Assessment and Diagnosis	Symptoms	Risk factors	Life impact	Treatment and Management	p
Gender (Mean± SD)							
Male	7.32 ± 1.27	0.84±0.36	0.70±0.46	1.13±0.50	0.88±0.32	2.07±1.74	>0.05*
Female	7.54 ± 1.07	0.91±0.29	0.76±0.43	1.04±0.51	0.95±0.22	1.71±1.39	
Age (Mean± SD)							
21 – 30	7.50 ± 1.17	0.86±0.35	0.61±0.49	1.11±0.29	0.95±0.29	2.04±1.61	<0.05#
31 – 50	7.56 ± 1.07	0.88±0.32	0.82±0.38	0.96±0.21	0.91±0.21	1.42±1.32	
>51	7.21 ± 1.21	0.94±0.24	0.82±0.39	1.22±0.28	0.91±0.29	2.31±1.51	
Years of work experience (Mean± SD)							
0-5	7.52 ± 1.17	0.90±0.30	0.63±0.48	1.13±0.55	0.88±0.32	2.04±1.63	<0.05#
6-10	7.55 ± 1.09	0.87±0.33	0.82±0.40	1.02±0.42	0.97±0.17	1.25±1.12	
11-20	7.62 ± 1.18	0.83±0.38	0.73±0.45	0.96±0.41	0.95±0.22	1.41±1.31	
20+	7.28 ± 1.08	0.92±0.27	0.85±0.36	1.11±0.57	0.94±0.23	2.20±1.54	
Region (Mean± SD)							
Marmara	7.62 ± 1.08	0.89±0.31	0.74±0.49	1.13±0.49	0.94±0.25	2.19±1.58	>0.05#
Central Anatolia	7.51 ± 1.03	0.86±0.35	0.70±0.55	1.05±0.55	0.97±0.315	1.76±1.47	
Aegean	7.25 ± 1.33	0.86±0.35	0.80±0.55	1.00±0.31	0.90±0.31	1.45±1.6	
Black Sea	7.47 ± 1.37	0.89±0.32	0.84±0.54	1.11±0.54	1.00±0.33	2.01±1.66	
Mediterranean	7.60 ± 0.91	0.94±0.25	0.69±0.43	0.87±0.42	1.00±0.30	1.37±1.36	
Eastern Anatolia	7.27 ± 1.42	0.91±0.30	0.73±0.20	0.91±0.47	0.73±0.46	1.25±1.15	
Southeastern Anatolia	7.11 ± 0.93	0.90±0.32	0.80±0.58	1.35±0.48	0.70±0.48	1.78±1.61	

*Mann-Whitney U test

#Kruskal-Wallis test

Another potential factor contributing to the higher results we obtained compared to the existing literature is the frequent utilization of free pharmacies in Türkiye for consultation by the population. Considering the demographic characteristics of Turkish society, it is apparent that individuals requiring long-term care are predominantly supported by their family members rather than being placed in nursing homes could be attained as another contributing factor. This can be attributed to the extensive experience of Turkish pharmacists in interacting not only with AD patients but also with their caregivers, including family members.

Upon comparing the mean ADKS scores across various factors such as gender, age groups, professional experience, and geographical region, it was observed that the highest scores were obtained by women (7.54 ± 1.07), individuals aged between 31-50 years (7.56 ± 1.07), those with professional experience ranging from 11-20 years (7.62 ± 1.18), and participants residing in the Marmara region (7.62 ± 1.08), respectively (Table 4). In terms of mean scores, female participants exhibited higher performance in the Assessment and Diagnosis domain (0.91 ± 0.29), Symptoms domain (0.76 ± 0.43), and Life impact domain (0.95 ± 0.22). On the other hand, male participants demonstrated greater proficiency in the Risk factors domain (1.13 ± 0.50) and Treatment and Management domain (2.07 ± 1.74) (Table 4).

When examining the ADKS domains across different age groups, participants aged 21-30 years attained the highest mean scores in the Life impact domain (0.95 ± 0.29), while those aged 31-50 years achieved the highest mean scores in the Symptoms domain (0.82 ± 0.38). Pharmacists aged 51 years and above demonstrated the highest mean scores in the Assessment and Diagnosis domain (0.94 ± 0.24), Risk factors domain (1.22 ± 0.28), and Treatment and Management domain (2.31 ± 1.51) (Table 4).

Unlike Mat Nuri, Zerafa, Scerri and Smyth et al., elder pharmacists with higher professional experience obtained higher scores in our sample [21,24,27]. In addition, a statistically significant correlation was found between increasing age and professional experience and symptom domain score ($r:0.215$, $p<0.005$ and $r: 0.169$, $p < 0.022$ respectively). In addition, pharmacist in charge had better score in comparison to other professional roles within the pharmacy and a statistically significant correlation has been obtained between professional role within the pharmacy, and the Symptoms domain. This observation can be attributed to the increasing number of encountered AD patients with advancing professional experience, as well as the continuous professional education offered by Turkish pharmacy authorities.

In terms of geographical regions, notable variations were observed. Participants residing in the Mediterranean region achieved the highest average score (0.94 ± 0.25) in the Assessment and Diagnosis domain. The Black Sea region attained the highest average score (0.84 ± 0.54) in the Symptoms domain. The Southeast Anatolia region obtained the highest average score (1.35 ± 0.48) in the Risk Factor domain. The Mediterranean region also secured the highest average score (1.00 ± 0.30) in the Life Impact domain. Lastly, the Marmara region obtained the highest average score (2.19 ± 1.58) in the Treatment and Management domain (Table 4).

Upon evaluating the ADKS scores of participants from various regions, it is evident that pharmacists in the Marmara region achieved the highest scores. It is hypothesized that the higher ADKS scores among independent pharmacists in the Marmara region may be attributed to factors such as enhanced accessibility to information and the region's high population density. The Marmara region's relatively smaller geographical area, coupled with its higher population density in comparison to other regions, potentially contributes to a greater number of AD patients being served by pharmacists in this area. This, in turn, may result in an elevated level of knowledge and experience regarding AD among pharmacists in the Marmara region. In addition, the presence of a significant number of pharmacy faculties in the Marmara region contributes to a higher frequency of continuing professional development opportunities available for pharmacists.

A statistically significant correlation was identified between professional experience and gender, professional role within the pharmacy, and the Symptoms domain (Spearman's rho: 0.175, $p < 0.017$; -0.591, $p < 0.001$; and 0.169, $p < 0.022$, respectively). Furthermore, a statistically significant correlation was observed between age groups and the Symptoms domain (Spearman's rho: 0.215, $p < 0.05$). Additionally, a statistically significant correlation was detected between geographical region and the Treatment and Management domain (Spearman's rho: -0.199, $p < 0.005$). Lastly, a statistically significant correlation was found between the Risk Factor and Treatment and Management domains (Spearman's rho: 0.463, $p < 0.001$).

This study has certain limitations that should be acknowledged. It is possible that some participants may have consulted external sources while responding to the questionnaire, introducing potential bias. Moreover, the assessment did not include an evaluation of participants' prior education and personal experiences related to AD (e.g., having a family member with AD), which could potentially influence the ADKS scores. While it would have been beneficial to

include a greater number of items for each domain, such an approach could have extended the completion time of the questionnaire, potentially reducing participant acceptability. Additionally, it is important to note that this study was conducted on a relatively small sample size therefore the generalizability of the findings to all pharmacists in Türkiye is limited.

AD is a progressive neurodegenerative condition characterized by an increasing prevalence worldwide, primarily affecting older individuals. The clinical manifestations of AD necessitate heightened care and have a direct impact on individuals and their families. Embracing a multidisciplinary approach is crucial in mitigating the detrimental effects of the disease, particularly in terms of treatment. Pharmacists, as healthcare professionals, possess the potential to make a significant contribution to the management of AD by providing specialized pharmacy services to both AD patients and their families. Cognitive pharmacy services, specifically designed for patients with complex treatment regimens and their relatives, contribute to the promotion of positive health outcomes.

STATEMENT OF ETHICS

The study received ethical approval from the Bezmialem Vakif University local Ethics Committee, with a decision number of 18/346.

CONFLICT OF INTEREST STATEMENT

The authors affirm that the research was carried out without any affiliations or financial associations that could be perceived as a possible conflict of interest.

AUTHOR CONTRIBUTIONS

Conceptualization: EZ and MYB; methodology: EZ and MYB; formal analysis and investigation: EZ and MYB; writing-original draft preparation: EZ and MYB; writing-review and editing: EZ and MYB; resources: EZ and MYB; supervision: EZ and MYB.

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Antibacterial and anthelmintic effect of the combination of pomegranate peel and olive leaf extracts

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ABSTRACT

Pomegranate peel and olive leaf are known to have antibacterial activity. These two plants have been studied in various studies. However, there is no study on the synergistic effect of these two plants. In this study, the antibacterial activity of a supplement containing pomegranate peel and olive leaf extracts against *S. mutans* and its antihelmintic activity against *C. elegans* were determined. Results obtained by the disk diffusion method and microdilution test are 12.5 mm and ≥ 1024 µg/mL, respectively. Anthelmintic activity experiments revealed that the lifespan of worms was shortened as a result of the synergistic effect of the extracts. Our results revealed the synergistic effect of these two extracts against the microbes and possible helminths in the oral flora. Increasing antibiotic resistance has led researchers to work on the detection of new plant extracts and substances. It is aimed that this study on plant extracts will help future studies.

Keywords: Pomegranate peel, olive leaves, antibacterial, antihelmintic, *C. elegans*

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INTRODUCTION

Various parts of plants are used to make medicines, cosmetics, and nutraceuticals. The contents of these parts have scientific indications depending on the method of obtaining them[1-3]. The places of plants that are not preferred as nutrients can show special effects and are newly discovered. The peel of pomegranate (*Punica granatum* L.) fruits, which are widely produced by Mediterranean countries including Tunisia, Turkey, Egypt, Spain, Morocco, and Italy, has recently been noticed for its rich and valuable content. Pomegranate peel consists of three parts; exocarp, mesocarp, and pericarp rich in polyphenols such as punicalagin and ellagitannins, gallic acid, and ellagic acid[4]. Punicalin also contains flavone-3-ols, gallotannins, hydroxycinnamic acids, hydroxybenzoic acids, and gallagil esters[5, 6]. It has been observed that these unique ingredients exhibit antioxidant, anti-inflammatory, antiatherogenic, antiangiogenic, antihyperglycemic, and anticarcinogenic effects. They also accelerate wound healing[7, 8]. Antibacterial and antiviral effects of pomegranate peel extracts were also observed[9-12]. It has been determined that pomegranate peel extracts have an antiviral effect against the influenza virus through the inhibition of viral absorption and RNA transcription. Studies show that antiviral activity can also be used against the SARS-CoV-2 virus[13, 14]. Phenolic compounds in the pomegranate peel show activity against Gram negative and Gram positive bacteria. Antimicrobial effects of phenolic compounds were observed to be comparable with those of a chemical antibacterial agent on the tooth[15]. It has been seen that pomegranate peel has important effects in the field of oral and dental health and is a good alternative natural resource to chemical-based applications[16, 17].

Olive leaf is a special medicinal product that is not consumed as a nutrient but contains specific secondary metabolites such as oleuropein and oleacein. Oleuropein, the main component of olive leaf, has anti-inflammatory, antiatherosclerotic, and anti-cancer properties, as well as a strong antioxidant effect with its ability to bind endogenous peptides[18, 19].

Olive leaf extract is a dark brown, bitter-tasting liquid obtained from the leaves of the olive tree (*Olea europaea* L., Oleaceae) native to the Mediterranean region. This leaf extract was found to have antioxidant activity as well as cardioprotective and chemopreventive properties. The main biophenol in the extract is oleuropein, and other biophenols such as verbascoside, apigenin-7-glucoside, and luteolin-7-glucoside are present in lower amounts[20, 21]. The antimicrobial effect of oleuropein has been studied and found to be effective against pathogenic bacteria. It has been found that the antimicrobial activity increases with the phenolic compounds accompanying oleuropein in olive leaf extract[22]. There is

no clear information about the efficacy dose when used in combination with olive leaf extract and pomegranate peel extract. In acute toxicity studies on rats with oleuropein, no deaths or adverse effects were observed despite the administration of a high dose of 1000 mg/kg[23].

Dental caries and dental plaque formation are caused by a mixture of microorganisms and food residues. *Streptococcus mutans* bacteria produce acid in the presence of fermentable carbohydrates such as sucrose and fructose. It especially reproduces on tooth surfaces and damages the hard tooth structure[24, 25]. It has been reported in various studies that *S. mutans*, one of the bacteria that plays a role in the deterioration of dental health, develops resistance to many antibiotics and antimicrobial agents. For these reasons, new drug candidate molecules should be investigated and defined, especially for the treatment of oral infections caused by *S. mutans*[26-28].

Soil-Borne Helminth (STH) infections are caused by intestinal nematodes. Unfortunately, one-fourth of the general population worldwide is infected with STH. These diseases are most common in places like tropical and subtropical regions where fresh water and sanitation are deficient. They cause malnutrition, anemia, retardation of development, and mental problems, especially among school-aged children. In addition to these diseases, oral helminth infestations, including those caused by roundworms, were reported[29]. Resistance to drugs used in the treatment of helminth infections is increasing day by day, as in bacterial infections. Therefore, the discovery of new and safe drugs against pathogenic worms is remarkably important for the pharmaceutical industry. *C. elegans* is a suitable roundworm model organism for preliminary *in vivo* studies due to its many benefits, including its ease of manipulation and cultivation, transparency, short life cycle (2-3 weeks)[30], generation time, tiny size, large hatching size, minimal maintenance costs, cryopreservation, and absence of ethical approval requirements[31]. Moreover, the life cycle of a worm is shortened at 35°C[32]. Due to the brief experimental duration, the thermotolerance assay at 35°C is favored as the initial screening protocol in research on aging and lifespan. Thermotolerance and life span traits in *C. elegans* have been shown to be related[33, 34]. Hence, *C. elegans* is a very useful model organism for discovering new anthelmintic compounds.

In this study, the effectiveness of a commercial dietary supplement, DOLEV Sprey, containing pomegranate peel extract and olive leaf extract, which can be easily used in mouthwash, was investigated against *Streptococcus mutans* and *C. elegans* for determining antibacterial and anthelminthic properties, respectively.

METHODOLOGY

Determination assay of ingredients

Olive Leaf Extract (Tabimer Türkiye) HPLC assay 5,36 % Oleuropein

Pomegranate peel extract (Türkiye)

Determination of Pomegranate Peel Extract

Thermo Orbitrap Q-EXACTIVE (USA)

Mobile Phase A % 1 Formic acid - H_2O

Mobile Phase B % 1 Formic acid – MeOH

Colon: Troyasil C18 HS – 150 x 3 mm 5 µ

Capillary temp. (°C): 320

100 mg/L internal standard solution was added to the extracted sample at a concentration of 3 ppm. The sample was taken through a 0.45 µ filter and taken into a vial. It was analyzed by giving it to the device.

Determination of Olive Leaf Extract

Oleuropein amount were determined in HPLC-PDA/PERKIN ELMER FLEXAR PDA Plus Detector agianst the oleuropein standart of FocusHerb with Purity 80 %. Instrumental analysis conditions were as follows.

Column:	PERKIN ELMER N9303514-ser13120620T COL-AnaIytical C18 Particle size:5µm; Column Length: 250 mm; Inside Diameter : 4,6 mm (5 µm, 250 x 4,6 mm)
Wavelength:	233 nm
Injection:	20 CL
Oven temperature:	2S'C
Time:	30 min
Flow:	1.00mL/min
Mobile phase:	Trifluoroacetic acid: Methanol: Water (1:400:600) Isocritic

Rest of the major ingredients in olive leaf extract were determined as follows.

1 mL of the extract was taken into a 5 mL flask and then a 50% Water - 50% MeOH mixture was added. The mixture was kept in an ultrasonic bath for 15 minutes. It was centrifuged for 5 minutes and the supernatant was taken. The

concentration was adjusted to 3 ppm by adding 100 mg/L of internal standard solution. The sample was taken through a 0.45 μ filter and taken into a vial. Given to device for analysis[35].

Mobile Phase A: %1 Formic Acide – H_2O

Mobil Phase B: %1 Formic Acide- MeOH

Column 3pm Fortis C18 — 150 x 3.0 mm

Commercial Final Formulation

The commercial product formulation contains 20% pomegranate peel extract and 2% olive leaf extract. It also contains xylitol as a stabilizer, benzoic acid and potassium sorbate as a preservative, sucralose as a sweetener, and a nature-identical flavor.

Macroscopic parameters were evaluated on behalf of appearance, final spray volume, pH, and density.

Each puff contains 29.38 mg of pomegranate peel extract and 1.469 mg of olive leaf extract.

Antibacterial activity

Bacterial Strains and Culture Conditions

Streptococcus mutans ATCC 25175 strain was obtained from Ege University, Faculty of Science, Basic and Industrial Microbiology Department. Bacteria were stored at -20 °C in Mueller Hinton Broth (MHB) (Biolife, Italy) supplemented with 20% glycerol.

Then, bacteria were reactivated from stock cultures stored at -20 °C by transferring to Petri dishes containing blood agar and incubating at 37 °C in microaerophilic conditions (95% air and 5% CO_2) for 24-48 hours before assay. The inoculum was prepared as recommended by the Clinical and Laboratory Standards Institute by direct colony suspension method (CLSI, 2012). Colonies of an overnight culture of *S. mutans* were suspended in sterile distilled water and adjusted to 0.5 McFarland standards to reach a final inoculum corresponding to approximately 1 x 10^8 CFU/ml.

Kirby-Bauer Disk Diffusion Test

The antibacterial activities of pomegranate peels and olive leaf extracts were determined by the disc diffusion assay according to the standard method[36, 37]. Briefly, fresh colonies were used to prepare an inoculum at 0.5 McFarland turbidity. The bacterial suspension was streaked using a sterile swab on Mueller

Hinton agar (MHA) plates (Neogen, USA). Paper discs (6 mm diameter) containing pomegranate peels and olive leaf extracts of known concentration (300 µl of 205.6 mg/ml extract) were placed on an MHA plate. Ampicillin discs (10 µg/disc) were used as positive controls and a blank disc (Thermo Fisher Scientific, USA) was used as negative control. Plates were incubated overnight at 37°C, and the antibacterial activity of the spray was expressed by measuring the diameter of the inhibition zone (mm).

Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) of pomegranate peels and olive leaf extracts was determined against *S. mutans* by using the Clinical Laboratory Standards Institute methods[38, 39]. Briefly, overnight-grown cultures of *S. mutans* were prepared in Brain Heart Infusion Broth (BHIB). The extract was solubilized to 2048 µg/mL in DMSO, and two-fold serial dilutions were prepared in a 96-well microplate (Sarstedt, Germany). Overnight cultures of *S. mutans* strains adjusted to 0.5 McFarland standards ($1x10^8$ CFU/ml) and diluted to 1:10 with BHIB ($1x10^7$ CFU/ml). 5 microliters of the dilution were added to each well to a final density of 5×10^5 CFU/well. A positive control test was performed without an antimicrobial agent and a negative control test was performed without bacteria. The plate was incubated at 37 °C for 24 hours. MIC was defined as the lowest concentration of antimicrobial agent that inhibited the visible growth of the test organism.

Caenorhabditis elegans Survival Assay under Heat Stress

The wild-type strain (N2) *of C. elegans* is provided by the *Caenorhabditis* Genetics Center (CGC), Minnesota. The worms were sustained at 22±2˚C on Nematode Growth Medium following standard procedures[40]. Nematodes were given an OP50-1 *E. coli* strain food source with an optical density (OD) of 0.5. Extracts were added to the L broth containing *E. coli* for the experimental groups. Control-1 contains the same amount of solvent as in the experiment groups, and Control-2 comprises only L broth cultured with *E. coli*. Three plates were prepared for each individual condition (Table 1).

Table 1. The experiment groups information

Groups	Content	Amount	Number of worms
Group A	Pomegranate Peel Extract, Olive Leaf Extract	20 uL in 980 uL E.coli	446
Group B	Pomegranate Peel Extract	20 uL in 980 uL E.coli	555
Group C	Glycerol (Solvent)	20 uL in 980 uL E.coli	462
Group D	-	1000 uL E.coli	320

The tests were conducted on populations of healthy, age-matched, and uncontaminated worms. At the end of the L4 larval stage, exposures were initiated. Prepared petri dishes containing the animals were exposed to room temperature for 24 hours. Subsequently, they were transferred to a pre-heated incubator at 35˚C, and monitored by taking images with a high-resolution scanner (Epson Perfection, V800 Photo) once every 20 minutes till all the worms died. Worms that remained stationary during two consecutive scans were considered to be dead. Utilizing the online application OASIS, survival analysis was performed[35].

RESULTS and DISCUSSION

Determination of Pomegranate Peel Extract

The four highest compounds determined as a result of the analysis are fumaric acid, gallic acid, ellagic acid, and ascorbic acid, respectively (375.02, 109.48, 21.70 and 19.35 mg/L). Uncertainty values are given with the results within the 95% confidence interval.

Determination of Olive Leaf Extract

The major component in olive leaf extract was oleuropein, and its amount was determined as 5,36 % ± 0,09.

Except for oleuropein, the three main compounds were determined as fumaric acid, hederagenin, and caffeic acid, respectively (140.72- 53.22- 21.59 mg/L).

Formulation

Macroscopic evaluation of the final product

Appearance:	Dark Red-brown liquid
Volume:	30 mL
pH (25 °C):	4,15
Density:	1,14 g/cm^3

C. elegans Assay

The combination of the extracts of pomegranate peel and olive leaf decreased nematode lifespan (Figure 1) at 35˚C. There were 320 living worms in Group D, the control group, that were not exposed to any substances. At the ninth scan (after 180 minutes) and under the parameters described, all the worms were dead. In Group C, there were 462 animals treated with extract solvent (glycerol) alone, and all warms were counted dead on the tenth scan (after 200

minutes). In Group B, with 555 live nematodes treated with only pomegranate peel extract, all worms lost their viability on the seventh scan, 140 minutes after treatment. Worms survived until the seventh scan, 140 minutes, in Group A, which contained 446 live worms treated with pomegranate peel and olive leaf extract. It was found that the death rate was higher when both extracts were used together than when only one extract was used.

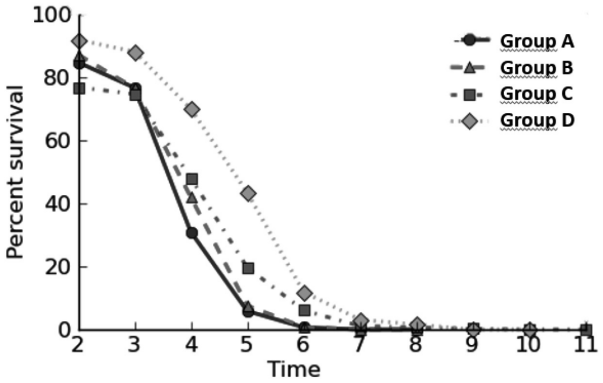

Figure 1. % Survival/Time Curve of experiment groups (1 time interval corresponds to 20 minutes.)

The online application for survival analysis (OASIS) was used to analyze the data[41]. Lifespan data are shown in Table 2. The mean, standard error of the mean, and p-values were determined using the log-rank (Mantel–Cox) method.

Table 2. Restricted mean and % mortality of experiment groups.

Groups	Restricted mean			Age in minutes at % mortality		
	Time intervals	Std. error	95% C.I	50%	75%	90%
Group A	3.99	0.05	3.89 ~ 4.09	100	120	140
Group B	4.14	0.05	4.05 ~ 4.23	100	120	140
Group C	4.27	0.07	4.13 ~ 4.41	80	100	120
Group D	5.10	0.08	4.94 ~ 5.26	100	120	140

The restricted mean is a clinically meaningful representation of average survival or life expectancy over a specific time span beginning at time zero[42]survival analysis has been increasingly used to evaluate prognostic outcomes [1]. Researchers may be familiar with the use of Cox proportional hazards (PH. When comparing the restricted mean values of the groups, it was discovered that

they corresponded with distinct time intervals. The compound with the longest expected lifespan belongs to Group D, which means that all of the formula's components, including the solvent, reduced the lifespan (Table 1). In contrast to the findings reported in the scientific literature[43, 44]. It has been discovered that extracts at these concentrations reduce the lifespan of the worms in our experimental conditions. The fact that the shortest lifespan was observed in the experimental group that received the extracts in combination shows that these extracts may act via distinct mechanisms. In terms of preclinical in vivo toxicity and anthelmintic activity, the findings of this study can help guide future studies and provide new approaches in our fight against oral helminth infestations, especially the ones caused by roundworms that effect people in endemic regions[29].

According to the p-values derived by the Log-Rank test, there was no significant difference between the experimental groups that received the extract combination and those that received simply the pomegranate peel extract. There was a statistically significant difference between all other groups.

Antibacterial Activity

In this study, antibacterial activity of pomegranate peel and olive leaf extract against *S. mutans* was investigated by disc diffusion and MIC tests. The inhibition zone obtained by the disk diffusion method is 12.5 mm (Figure 2). After confirmation of the antimicrobial activity, the minimum inhibition concentration was determined to be ≥ 1024 μg/mL by microdilution test (Figure 3).

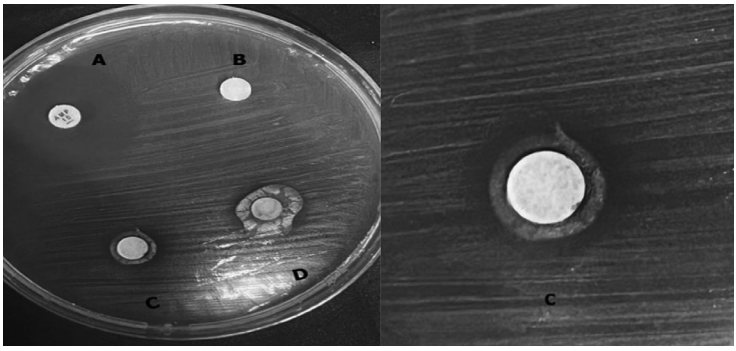

Figure 2. Antimicrobial activity of pomegranate peel and olive leaf extract against *S. mutans* estimated by the disc diffusion method. A=Positive control, B= Negative control, C= 300 ml of spray, D= 600 ml of spray

In another scientific study, the antimicrobial effect of 8 mg/ml and 12 mg/ml concentration of pomegranate peel extract against *S. mutans* was investigated and showed inhibition zone as 9.5 mm[45]. The antibacterial activity of polyphenolic extracts prepared from acacia honey, myrtle leaf and pomegranate peel against cariogenic bacteria in terms of single and synergistic effect was eveluated[46]. They reported that the pomegranate peel extract created an inhibition zone of 16.2 and 11.2 mm (2 mg/disc and 1 mg/disc, respectively) against *S. mutans* and the MIC value was 10 μg/μl. The antimicrobial activity of pomegranate peel against *S. mutans* was evaluated and determined the inhibition zone as 19.75 mm[47]. Pomegranate peel showed higher antimicrobial activity than flower, leaf and stem extracts. In line with these study, we observed remarkably meaningful antimicrobial activity against *S. mutans*.

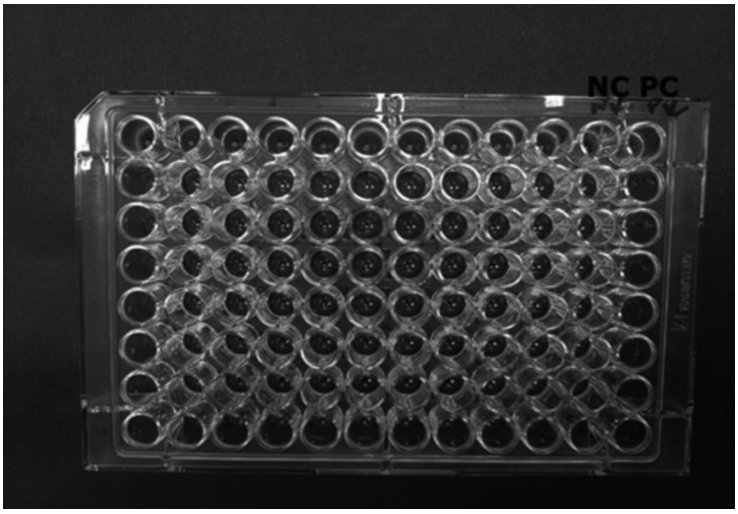

Figure 3. Minimum inhibitory concentrations (MIC) of pomegranate peel and olive leaf extract on S. mutans microbial growth. NC= Negative control, PC=Positive control.

The MICs of olive leaf extracts on *S. mutans* isolates was ranged between 55.80 to 106.8 mg/ml. In addition, the inhibition zone of *S. mutans* (30.3; 27.3; 30.3; 29.5 and 34.3) significantly increased when combined to silver nanoparticles (1:1). In this study, the minimum inhibition concentration (MIC) was determined to be ≥ 1024 μg/mL by microdilution test.

Oral infections have been known to be counterproductive to overall health for over 3000 years. Recent research has added to our understanding of the pathogenic mechanisms linking oral infections to mortality and morbidity. Poor oral health appears to be associated with all forms of mortality, especially among the elderly[48]. This study examines an oral spray containing two distinct plant

extracts; *Punica granatum* and *Olea europaea*. According to studies, pomegranate extract is efficient against pathogenic oral bacteria, gingivitis, plaque, and periodontal disease[49, 50]. The pomegranate peel is the most abundant part of the pomegranate fruit in terms of bioactive compounds[51]. Olive leaf containing phenolic compounds such as oleuropein and hydroxytyrosol has antimicrobial activity[52, 53]. However, there are few studies on the antimicrobial effect of olive leaf on *S. mutans* evaluated the antibacterial effects of olive leaf (aqueous extracts) on *S. mutans* isolates[54, 55]. As the second component of this study, anthelmintic activity is investigated with the help of the model organism, *C. elegans*. It is cost-effective, simple to maintain, and readily available, and has been utilized for research in diverse fields of medicine and biology. Given that it is a nematode, it is not surprising that it is used to find new anthelmintics[56, 57]. The combination of olive leaf and pomegranate peel was tested for the first time in this study and demonstrated significant antibacterial and antihelmintic activity against *S. mutans* and *C. elegans,* respectively.

CONFLICT OF INTEREST STATEMENT

The authors declare no competing financial interest.

AUTHOR CONTRIBUTIONS

Levent Alparslan: Formulation design, supplying of ingredients, evaluation of determination assays, revising manuscript. Betul Giray: Antibacterial activity, revising the manuscript. Meltem Gulec: Anthelmintic activity, revising the manuscript. Nil Kaya: Antibacterial activity, revising the manuscript. Dogan Uvey: Supplying of extracts, revising the manuscript. Abdullah Olgun: Evaluated the biological assay. All authors read and approved the final manuscript.

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DBeQ-mediated pharmacological modulation of the retrotranslocation step of ERAD may exhibit a potent therapeutic approach against colorectal cancer

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ABSTRACT

The incidence of colorectal cancer is 30% higher in men compared to women. Although there are different treatment options for colorectal cancer, including chemotherapy and immunotherapy, acquired drug resistance and some specific mutations substantially restrict the treatment options. N(2), N(4)-dibenzylquinazoline-2,4-diamine (DBeQ) is a selective and ATP-competitive inhibitor molecule of p97/Valosin-containing protein (VCP) protein. p97/VCP is a well-conserved and abundant hexameric type II ATPases associated with diverse cellular activities (AAA+) type ATPases protein. It functions as an ATP-dependent segregase and plays a role in various cellular processes, such as autophagy and endoplasmic reticulum-associated degradation (ERAD). Herein, we evaluated the therapeutic potential of DBeQ on colorectal cancer cells, Caco-2 and HT-29. Our data indicated that DBeQ treatment strongly reduced the proliferative capacity, colonial growth and anchorage-independent growth of colorectal cancer cells. Moreover, DBeQ strongly increased cytochrome-c and CCAAT-enhancer-binding protein homologous protein (CHOP) protein levels and also induced cleaved caspase-3 and caspase-7 levels. Present findings suggest that DBeQ may offer a potential therapeutic effect for colorectal cancer treatment.

Keywords: Anti-tumorigenic, colon cancer, DBeQ, ER-associated degradation, p97/VCP

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INTRODUCTION

Colorectal cancer begins to spread from the large intestine or the rectum. It is called colon or rectal cancer, depending on where it starts[1]. It can be caused by inherited or acquired mutations and certain types of diets that may lead to activating oncogenes or turning off tumor suppressor genes. The incidence rates of colorectal cancer are 30% higher in men than in women. The lifetime risk of developing colorectal cancer is about 4.3% for men and 4% for women. Although there are different treatment options for colorectal cancer, including radiation therapy, surgery, chemotherapy, targeted therapy and immunotherapy, acquired drug resistance and some specific mutations substantially restrict the treatment options for colorectal cancer [2, 3]. Therefore, there is a need to improve our understanding of the molecular mechanisms underlying colorectal cancer and the development of effective new treatment approaches.

N(2), N(4)-dibenzylquinazoline-2,4-diamine (DBeQ) is a selective, reversible and ATP-competitive inhibitor molecule of p97/Valosin-containing protein (VCP)[4]. DBeQ debilitates autophagy and the destruction of the endoplasmic reticulum-associated degradation (ERAD) substrates[5]. ERAD is a main quality-control mechanism responsible for targeting unfolded, misfolded or improper oligomerized endoplasmic reticulum (ER) proteins for proteasomal degradation[6]. Recent studies highlighted that mammalian ERAD has been associated with the progression of carcinogenesis, including prostate cancer[7]. ERAD is a sophisticatedly controlled physiological mechanism consisting of multiple steps, including recognition and ubiquitination of substrate molecules, retrotranslocation from ER lumen to cytosol and proteasomal-mediated degradation. A large number of proteins responsible for the functioning of these steps work simultaneously in a synchronized manner[5,6]. The retrotranslocation step, which is responsible for the transfer of substrate molecules from the ER lumen to the cytosol, is extremely critical and this function is mainly coordinated by the p97/VCP in yeast and mammalian[8]. p97/VCP is a well-conserved, ubiquitously localized and abundant expressed hexameric type II AAA+ (ATPases associated with diverse cellular activities) type ATPases protein[8]. It functions as an ATP-dependent segregase and also plays a crucial role in cellular processes, including transcriptional control, cell-cycle regulation, endosomal differentiation, homotypic membrane fusion, autophagy and ERAD[8,9].

In recent studies, it has been better understood that protein quality control mechanisms, such as ERAD supported the carcinogenesis process by exhibiting varying activities in many cancer types, such as breast and prostate cancer.

In the present study, we aimed to investigate the therapeutic potential of phar-

macological inhibition of p97/VCP on colorectal cancer cells by DBeQ. For this purpose, we used human epithelial colorectal adenocarcinoma cells Caco-2 and HT-29, which mimic colorectal cancer well *in vitro*. Our findings indicated that DBeQ treatment strongly reduced the proliferative capacity of colorectal cancer cells. Also, colony formation and anchorage-independent growth of colorectal cancer cells were significantly reduced by DBeQ in a dose-dependent manner. Lastly, we analyzed some cell death-related protein levels, including cytochrome-c, CHOP (CCAAT-enhancer-binding protein homologous protein), full- and cleaved caspase-3 and -7 levels by immunoblotting to understand the mechanism of anti-cancer effect of DBeQ on colorectal cancer cells at the molecular level. Our data indicated that DBeQ treatment remarkably increased the expression levels of cytochrome-c and CHOP and also induced cleavage of caspase-3 and caspase-7 proteins. These findings suggest that pharmacological modulation of protein quality control components may offer a potent therapeutic approach against colorectal carcinoma.

METHODOLOGY

Materials

Cell culture materials, including L-Glutamine, fetal bovine serum (FBS) and other cell culture grade requirements were obtained from Capricorn Scientific (Capricorn Scientific GmbH, Ebsdorfergrund, Germany). McCoy's 5a Medium and Eagle's Minimum Essential Medium (EMEM) were purchased from Biological Industries (Biological Industries, USA). DBeQ (sc-499943) was purchased from Santacruz Biotechnology (Santa Cruz Biotechnology, Inc. California, USA). Rabbit polyclonal antibodies caspase-3 (#9662)(1:1500), caspase-7 (#12827)(1:1500), cytochrome c (#11940)(1:2000) and mouse polyclonal CHOP (#2895)(1:2000) were obtained from Cell Signaling Technology (Cell Signaling Technology Inc., Danvers, Massachusetts, USA). Monoclonal mouse beta-actin antibody (#A5316)(1:10000) was obtained from Sigma-Aldrich (Sigma-Aldrich, St. Louis, USA). HRP-conjugated secondary goat anti-mouse antibody (#31430)(1:5000) and goat anti-rabbit antibody (#31460)(1:5000) were purchased from Thermo Scientific (Thermo-Scientific Pierce, Fisher Scientific, Dublin, Ireland). High pure Dimethyl Sulfoxide (DMSO) was obtained from Serva (SERVA Electrophoresis GmbH, Heidelberg, Germany).

Cell Culture

Human epithelial colorectal adenocarcinoma cell lines Caco-2 (HTB-37™) and HT-29 (HTB-38™) were obtained from American Type Culture Collection (ATCC) (ATCC, Rockville, MD). Caco-2 and HT-29 cells were routinely pro-

pagated in EMEM and McCoy's 5a Medium enriched with 10% FBS and 2 mM L-glutamine in a conventional cell culture condition, the humidified atmosphere of 5% CO_2 and 95% air at a constant temperature of 37°C, respectively. Identification of mycoplasma infection was evaluated by EZ-PCR Mycoplasma Test kit (Biological Industries, USA).

Cell viability test

The cell viability was measured by WST-1 assay according to the manufacturer's instructions (TaKaRa, Mountain View, CA, USA). The cells were seeded in 96-well plate (7500 cells/well) and growth in regular media for 24 h. Following cells were treated with DBeQ as indicated doses for 48 h. To determine the cell proliferation, 20 µL WST-1 was added per well and cells were incubated for 2 h under the conventional cell culture conditions at 37°. The absorbance values were read at 450nm, with 600nm set as the reference wavelength by using microplate reader (BioTek, Epoch 2).

Western-blotting

Immunoblotting analysis was performed as described before[7]. Cells were lysate by radioimmunoprecipitation assay (RIPA) buffer and then centrifugated at 14.000 rpm for 20 min at 4°C. The insoluble phase was removed and supernatant was collected. The total concentration was determined by bicinchoninic acid assay (BCA) kit (TaKaRa, Mountain View, CA, USA). Protein samples were mixed with 4x Laemmli buffer and boiled at 70°C for 15 min. Typically, 30 µg of total protein was loaded in the hand-cast sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and then transferred to an Immun-blot® polyvinylidene fluoride (PVDF) membrane for 24 h (Bio-Rad, Hercules, CA, USA). Target proteins were marked by specific primary antibodies and HRP-conjugated secondary antibodies and then visualized by using clarity western enhanced chemiluminescence (ECL) substrate in ChemiDoc XRS+ system (Bio-Rad, Hercules, CA, USA).

Colony formation assay

Cells were seeded in a 6-well cell culture plate (2000 cells/ml) and propagated at conventional cell culture conditions for 24 h and then cells were treated with a compound. Following 72 h, colonies were fixed and stained with %0.05 crystal violet solution (MERCK, Darmstadt, Germany). Quantification of growing colonies were analyzed by using ImageJ software (http://imagej.nih. gov/ij/).

3D cell culture

3D cell culture analysis was performed as described before[7]. Cells were embedded in low melting agar enriched with growth media and treated with compound after 24 h. Cells were propagated under conventional cell culture conditions for 14 days and then the images of growing colonies were taken using Sunny SopTop invert microscope and OD400UHW camera system. % colonial growth was calculated by counting the colonies in the photographs taken from 5 independent areas and comparing them between the groups.

Statistical Analysis

Results are presented as mean ± standard deviation and analyzed by using GraphPad Prism 5 software. The statistical significance of differences between groups was determined by a two-tailed equal variance Student's t-test with a minimum of 95% confidence interval. The significant level was set at 5% ($p<0.05$) for all tests.

RESULTS and DISCUSSION

The evaluation of the effect of DBeQ on cell viability of Caco-2 and HT-29 cells

To evaluate the effect of DBeQ on the cell-viability of Caco-2 and HT-29 cells, cells were treated with various doses of DBeQ, including 1, 2.5, 5, 7.5, 10, 12.5 and 15 μmol for 48 h and then cell viability was measured with WST-1 based cell viability assay. Our results indicated that DBeQ treatment significantly decreased cell viability for both cell lines in a dose-dependent manner (Figure 1). We also determined the IC_{50} value of DBeQ, 9.201 μmol for Caco-2 and 7.625 μmol for HT-29 cells (Figure 1).

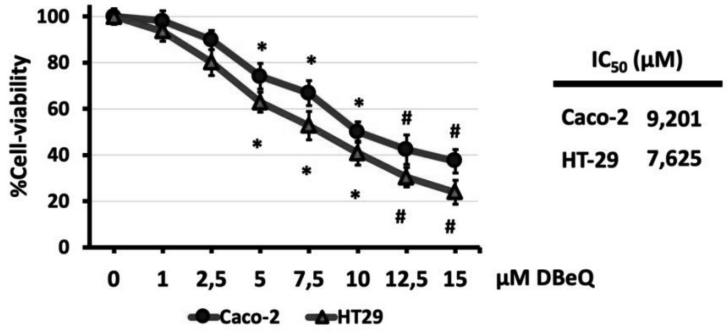

Figure 1. Evaluation of the effect of DBeQ on cell-viability in Caco-2 and HT-29 cells

Cells were treated with a vehicle or 1, 2.5, 5, 7.5, 10, 12.5 and 15 μmol DBeQ for 48 h. Cell viability was analyzed by WST-1 based assay. Data represented as mean ± SE of three independent experiments made in three replicates (*p<0.05, # p<0.001).

DBeQ remarkably reduced the colony formation ability of Caco-2 and HT-29 cells

To evaluate the effects of DBeQ on the tumorigenic capacity of colon cancer cells, we performed the 2D colony formation assay. Caco-2 and HT-29 cells were treated with IC_{50} and $^1/_2$ IC_{50} doses of DBeQ. Our findings indicated that DBeQ administration significantly reduced the colonial growth of both cell lines in a dose-dependent manner (Figure 2a, b). Moreover, we observed that HT-29 cells were more sensitive to the DBeQ than Caco-2 cells.

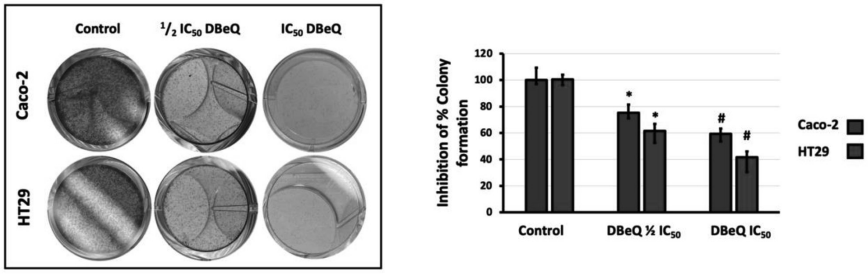

Figure 2. The effect of DBeQ on colony formation of Caco-2 and HT-29 cells

Cells were treated with vehicle or IC_{50} and $^1/_2$ IC_{50} doses of DBeQ for 72 h and the cells were fixed and stained with crystal violet solution. % inhibition of colonial growth was calculated by ImageJ software. Data represented as mean ± SE (n=3) (* p<0.05, # p<0.001).

3D tumor formation of Caco-2 and HT-29 cells is markedly reduced by DBeQ treatment

Anchorage-independent growth is considered one of the tumor hallmarks[13]. Our findings indicated that DBeQ administration significantly limited the 3D tumor formation of Caco-2 and HT-29 cells. Also, our data revealed that tumor volume was remarkably minimized by DBeQ treatment for both cell lines in a dose-dependent manner compared to the control group (Figure 3).

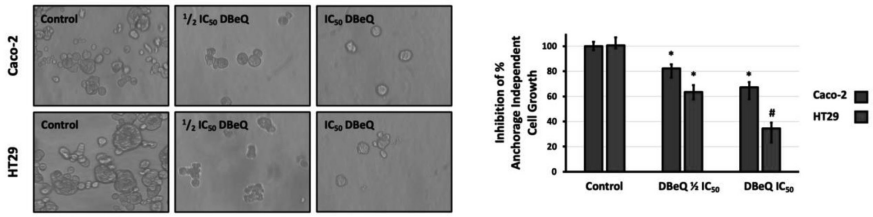

Figure 3. The effect of DBeQ on anchorage-independent cell growth in Caco-2 and HT-29 cells

3D cell culture protocol was performed as explained in the material-method section. Cells were treated with vehicle or IC_{50} and $^{1}/_{2}$ IC_{50} doses of DBeQ. Data represented as mean ± SE of three independent experiments made in three replicates (* $p<0.05$, # $p<0.001$).

Investigation of the effect of DBeQ on cell death-associated proteins in Caco-2 and HT-29 cells

To evaluate the impact of DBeQ on cell death-related proteins in Caco-2 and HT-29 cells, the protein levels of cytochrome-c, CHOP, full and cleaved forms of caspase-3 and caspase-7 were examined by immunoblotting assay. Our data indicated that DBeQ dose-dependently induced cleavage forms of caspase-3 and caspase-7 in both cell types (Figure 4). Although the expression level of CHOP protein was increased in both cell lines depending on the applied doses of DBeQ, it was more strongly induced in HT-29 compared to Caco-2 cells in a dose-dependent manner (Figure 4). Moreover, cytochrome-c levels were gradually increased depending on the dose of DBeQ in both cells.

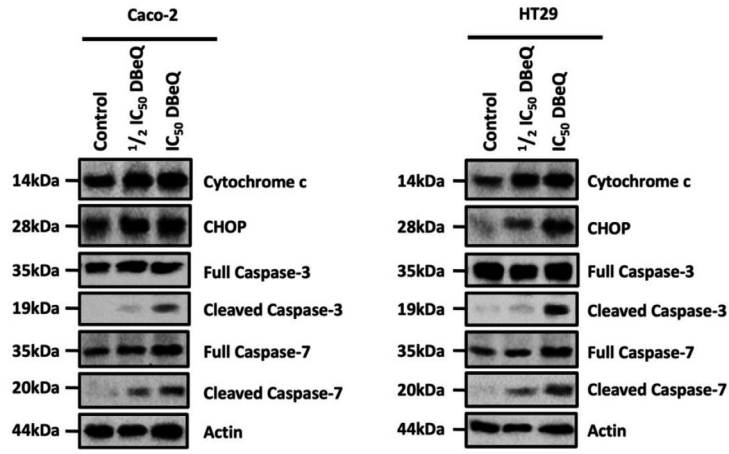

Figure 4. Investigation of the effects of DBeQ on cell death-related protein levels in Caco-2 and HT-29 cells

Cells were treated with vehicle or IC_{50} and $^1/_2$ IC_{50} doses of DBeQ for 24 h and then expression levels of CHOP, cytochrome-c, full and cleaved caspase-3 and caspase-7 were analyzed by immunoblotting assay. Beta-actin was used as a loading control.

Recent studies suggest that ER protein quality-control mechanism, ERAD is a potent therapeutic target for numerous cancer types, such as breast and prostate cancer[7,10]. ERAD is one of the most effective protein degradation systems in mammalian cells, which is specifically targeting the undesirable proteins to the 26S proteasome[10]. It effectively regulates the steady-state level of numerous physiologically crucial proteins, such as cholesterol biosynthesis rate-limiting enzyme 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductase, tumor suppressor Kangai-1 (KAI1)/Cluster of Differentiation 82 (CD82) and superoxide dismutase 1 (SOD1) and brain and muscle ARNT-like1 (Bmal1)[7,11,14]. Due to the increasing protein quality processes in cancer cells, the pharmacological targeting of ERAD makes it a therapeutically potent target in new treatment approaches.

Herein, we tested the anti-tumorigenic effect of DBeQ on colorectal cancer cells, Caco-2 and HT-29. Firstly, we examined the effect of DBeQ on cell viability and determined the IC_{50} doses for both cell types. Our results indicated that HT-29 cell was more sensitive to DBeQ than Caco-2 cells. IC_{50} value of DBeQ for Caco-2 cells is 9.201 μmol and for HT-29 cells is 7.625 μmol (Figure 1). IC_{50} is expressed as the half-maximum inhibitory concentration and is the most widely used and informative measure of the efficacy of a drug[12]. Therefore, in the next experimental steps, studies were continued with 1x and $^1/_2$ x IC_{50} doses of DBeQ.

Increased invasive and migration ability, colonial growth capacity and anchorage-independent cell growth are essential hallmarks of cancer progression[13]. Therefore, we aimed to evaluate the impact of DBeQ on these tumorigenic characteristics by 2D colony formation and 3D anchorage-independent cell growth assay. Our results indicated that DBeQ administration strongly limited the colonial growth of Caco-2 and HT-29 cells in a dose-dependent manner (Figure 2). 3D tumor formation of Caco-2 and HT-29 cells was significantly reduced by DBeQ administration and also tumor volume was importantly minimized for both cell types in a dose-dependent manner (Figure 2). Collectively these results suggest that DBeQ efficiently restricts the tumor progression of colon cancer cells by reducing the tumorigenic characteristics.

Lastly, we examined the anti-tumorigenic effect of DBeQ with studies at the protein level in colorectal cancer cells. For this aim, we evaluated some programmed cell death-related protein levels, including cytochrome-c, CHOP, full and cleaved caspase-3 and caspase-7. Cytochrome-c is localized in the inner membrane of mitochondria and is known as an electron-transporting protein. Also, it plays a pivotal role in oxidative phosphorylation-mediated ATP synthesis in eukaryotic cells[14]. Alteration in the levels of cytochrome-c is mostly used in investigating apoptotic cell death in cells. Upon apoptotic stimulation, cytochrome-c is released from mitochondria to the cytoplasm and causes activation of caspases[15]. Our data indicated that DBeQ treatment increased the cytochrome-c levels in Caco-2 and HT-29 cells in a dose-dependent manner (Figure 4). Consistent with these results, cleaved forms of caspase-3 and caspase-7 levels were markedly induced depending on the doses of DBeQ (Figure 4). Caspase-3 and caspase-7 are effector caspase proteins liable for cleaving downstream substrates, including Poly (ADP-ribose) polymerase 1 (PARP-1)[16–18]. Our results suggest that DBeQ promotes the programmed cell death of colorectal cancer cells by activating caspase-3 and caspase-7.

Additionally, we examined the effect of DBeQ on CHOP protein expression. CHOP is a pro-apoptotic transcription factor and is mainly induced under certain cellular stresses, including nutrient starvation and prolonged ER stress[19]. Our data indicated that DBeQ administration remarkably induced steady-state levels of CHOP protein in a dose-dependent manner in colorectal cancer cells (Figure 4). Together present findings suggest that DBeQ administration strongly induces programmed cell death in colorectal cancer cells. Considering the expanded metabolic requirements in cancer cells, including increased protein synthesis capacity, DBeQ-mediated disruption of ER-protein quality-control processes in colorectal cancer cells suggests the underlying cause of potent anti-cancer properties of DBeQ.

Herein, we showed that the reversible selective inhibitor of p97/VCP, DBeQ, significantly reduced the cell viability of colorectal cancer cells. Also, we found it remarkably decreased tumorigenic features of colorectal cancer cells, such as colony formation and anchorage-independent growth by inducing programmed cell death. Present findings suggest that pharmacologically targeting the ER protein quality-control mechanism components may present a promising therapeutic approach against colorectal cancer.

STATEMENT OF ETHICS

This study does not require any ethical permission.

CONFLICT OF INTEREST STATEMENT

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

Y. ERZURUMLU initiated, directed the project, designed and conducted the experiments, analyzed, interpreted the results and wrote the manuscript. H.K. DOGAN assisted experimental studies. All correspondence and requests for materials should be addressed to Y.E. All authors have read and approved the final version of the manuscript.

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Determination of quality of tablets containing irbesartan and hydrochlorothiazide via newly developed and validated simultaneous HPLC method

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ABSTRACT

Irbesartan and hydrochlorothiazide are common combination drugs used to treat hypertension. The goal of this study was to develop an HPLC method for simultaneous quantification of IRB and HCT and to use this method in tablet quality control tests. The mobile phase in gradient elution mode HPLC method was 30 mM sodium acetate buffer (pH:5.00): water: ACN (40:40:20, *v/v/v %*) at a flow rate of 0.6 mL/min and 230 nm and that employed avanafil as an internal standard. The ICHQ1 (R2) guideline was used to determine its applicability and capacity studies. The tablets were then subjected to weight variation, thickness-width-length tests, hardness tests, content uniformity, and dissolution tests as quality control tests. The mean recovery for hydrochlorothiazide in the accuracy study was 99.76% and 99.10% for irbesartan. The dissolution test results were discovered that 85% of both active substances were released into the dissolution medium within the first 15 minutes.

Keywords: Fixed dosage form, hydrochlorothiazide, irbesartan, tablet quality control tests

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INTRODUCTION

Hypertension is an important public health problem due to its widespread prevalence around the world and the increased risk of death it poses when combined with other diseases [1, 2]. Antihypertensive drugs are used not just to lower blood pressure, but also to eliminate the negative consequences of hypertension [3]. Many different drug classes are used to treat hypertension. Angiotensin-converting enzyme inhibitors (ACE-I), beta-blockers, calcium channel blockers (CCB), thiazide diuretics, and angiotensin receptor blockers (ARB) are the drugs used to treat hypertension [4-6]. If monotherapy with a single antihypertensive drug group fails, most guidelines advise going with a thiazide diuretic and an ARB [5, 7, 8]. In comparison to monotherapy, combined drug treatments use less active substance on the patient. In this way, using combined drugs instead of monotherapy in the treatment of hypertension provides a more effective treatment with fewer side effects [5].

Irbesartan (IRB) (2-butyl-3-[[4-[2-(2H- tetrazol-5-yl)phenyl]phenyl]methyl]-1,3-diazaspiro[4.4]non-1-en-4-one) is a non-peptide ARB [9, 10]. Hydrochlorothiazide (HCT) (6-chloro-1,1-dioxo-3,4-dihydro-2H- 1lambda6,2,4-benzothiadiazine-7-sulfonamide)is a thiazide class diuretic [11]. Avanafil (AVA) was used as an internal standard (IS). Figure 1 shows the chemical structures of IRB, HCT and AVA.

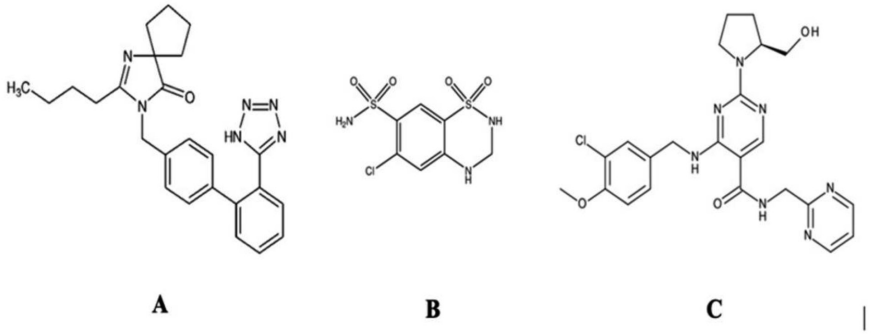

Figure 1. Molecular structure of Irbesartan (A), Hydrochlorothiazide (B), and Avanafil (C)

Various detection methods are still being developed in order to identify, quantify, or purify active substances. It is difficult and time-consuming to determine active substances simultaneously in combined preparations containing more than one active substance [12, 13]. HPLC is one of the most commonly used methods for the analysis of pharmaceutical formulations and body fluids due to its advantages, ease of application, and availability of low-cost instruments. Creating an effective method ensures that laboratory resources are optimized

while meeting the routine goals that must be met at each stage of drug development [13].

Our study aims to develop a validated High-performance liquid chromatography (HPLC) method for the simultaneous quantification of IRB and HCT, as well as to perform Quality Control (QC) tests on all combined IRB/HCT tablets on the market.

METHODOLOGY

Chemicals

All of the chemicals and solvents used were analytical reagent grade. Acetonitrile (ACN) and distilled water were purchased from Merck KGaA (Darmstadt, Germany). Milli-Q water is purified using Millipore SAS's Millipore Milli-Q Synthesis A10 system (Molsheim, France). Sodium acetate, acetic acid and sodium hydroxide was purchased from Sigma-Aldrich Chemie GmbH (Darmstadh Germany). Standards for IRB, HCT and AVA were obtained from Molekula GmbH (Munchen, Germany). IRB/HCT (300/25 mg) fixed-dose combination drug product was supplied from local pharmacies.

Preparation of the calibration standards

The standard stock solution of IRB (930 μg mL^{-1}) and HCT (540 μg mL^{-1}) were prepared in ACN. The working standard solutions (9.3, 27.9, 74.4, 148.8, 186, 223.2, 279, 334.8, 372 and 409.2 μg mL^{-1} for IRB) (5.9, 17.7, 47.2, 94.4, 118, 141.6, 177, 212.4, 236, 259.6 μg mL^{-1} for HCT) were prepared by diluting the stock solution in the ACN. The stock solution was kept at +4°C where it is stable for at least one week. Standard solutions were daily prepared by diluting the stock with ACN.

Preparation of the QC samples

Ten tablets were weighed and ground to determine the average weight. Amount of powder equivalent to average weight was transferred to a 250 mL volumetric flask, 200 mL of diluent (ACN %100) was added, and sonicated for 15 minutes. The volume was diluted to produce a solution containing 1200 μg mL^{-1} IRB and 100 μg mL^{-1} HCT. Before injecting the solution into the HPLC system, it was filtered through a 0.20 μm PTFE membrane filter and diluted with diluent to 300 μg.mL^{-1} IRB and 25 μg mL^{-1} HCT. The QC samples were kept frozen (at -18 °C) until used, and calibration samples were prepared fresh for each batch.

Preparation of the mobile phase

For use in the mobile phase, a 30 mM sodium acetate buffer solution (pH 5.00) was prepared. Acetic acid and sodium hydroxide were used to adjust the pH to 5.00. For 20 minutes, it was sonicated. It was then filtered with 0.45 µm non-sterile cellulose acetate membrane filter paper using a vacuum filtration equipment and degased before use.

Chromatic equipment and conditions

HPLC analysis was performed on a chromatographic system equipped with Nexera-i LC 2040C 3D device from Shimadzu (Japan). The chromatographic data were collected, integrated, and analyzed using a LabSolutions Software data system. PDA detection was performed with the wavelength set to 230 nm and the real-time spectra were recorded at 640 msec data sampling. C18 core-shell column (SUPELCO® Ascentis Express, 100 × 4.6 mm, 2.7 µm i.d.) was used for separation. AVA was used as an IS to quantify IRB and HCT since the AVA peak did not interfere HCT and IRB and the retention time was longer than the targeted compounds.

In gradient elution mode, the mobile phase was 30 mM sodium acetate buffer (pH:5.0) : water : ACN (40:40:20, *v/v/v %*) at a flow rate of 0.6 mL.min^{-1} and injection volume was 1 µL. The column ovent temperature was set 30 °C. Table 1 shows the gradient elution conditions.

Table 1. Gradient elution conditions

Time (min)	ACN (%)	Buffer (%)
0.00-3.50	20.0 → 60.0	40
3.50-4.00	60.0	40
4.00-4.50	60.0 → 20.0	40
4.50-8.00	20.0	40
8.00	Stop	

Method validation

To determine the applicability and capacity of the analytical method used, linearity, accuracy, precision, selectivity and specificity, sensitivity, and robustness tests were performed as part of the validation studies. Validation studies were conducted following ICH guidelines and published literature [14, 15].

Specificity

Method trials were carried out to ensure the specificity of the method and to avoid interference between the compound to be analyzed and other peaks in the medium.

Linearity and sensitivity

For the linearity study, triplicate measurements at 10 different concentrations (9.3, 27.9, 74.4, 148.8, 186, 223.2, 279, 334.8, 372 and 409.2 μg mL^{-1} for IRB) (5.9, 17.7, 47.2, 94.4, 118, 141.6, 177, 212.4, 236, 259.6 μg mL^{-1} for HCT) were plotted against the ratios of the peak areas of IRB and HCT to IS, and the calibration curves were obtained. The calibration curve's line equation and the regression coefficient (r^2) were calculated.

Limit of detection (LOD) and limit of quantification (LOQ) values were calculated to demonstrate the analytical sensitivity of the method. The ICH guidelines LOD and LOQ formulas were used for the calculation [14].

Accuracy and precision

The accuracy value expresses the relationship between the measured value and the values contained in the analyte. The fact that the measurements of the series obtained after sampling the same sample multiple times under the same conditions demonstrates the precision of the method. Intra-day and inter-day recovery studies were carried out to demonstrate the accuracy and precision of the method.

The assay accuracy of the method was determined for intra-day variations using ten times analysis and inter-day variations using twenty times analysis of samples containing 148.8, 186, 232.2 μg mL^{-1} IRB and 94.4, 118, 141.6 μg mL^{-1} HCT. Three different concentrations of standard solutions (within the linear range) were analyzed on three consecutive days (inter-day precision) and ten times within the same day (intra-day precision). The obtained values for relative standard deviation (RSD) and Bias of intra- and inter-day studies.

Robustness

The analytical method used expresses the robustness of the rate of being affected by small changes in the method's parameters. At the same time, it demonstrates how reliable the method is during application. The robustness of the analytical method was determined by making changes to the flow rate, the percentage of organic solvent in the mobile phase, the buffer capacity used in the mobile phase, the column temperature, and the wavelength.

Quality Control Tests

In the Turkish pharmaceutical market, QC tests were performed on fixed dose tablets of 300/25 mg (IRB/HCT). There were tablets from three different companies. These tablets were assigned codes A, B, and C at randomly. Weight variation, thickness-width-length test, hardness tests, content uniformity, and dissolution tests were used to demonstrate the quality control (QC) of the tablets. All tablet QC tests were performed in accordance with the guidelines and literature [16-18].

RESULTS and DISCUSSION

Method optimization

HPLC is one of the most widely used methods for drug analysis in pharmaceutical preparations and physiological fluids due to advantages such as simplicity of use and low cost [13, 19]. HCT, one of the pharmaceutical active substances quantified, is much more polar than IRB, another pharmaceutical active substance [12, 20, 21]. In our experiments, we noticed that HCT elutes rapidly. During the simultaneous determination of active substances, it was noticed that IRB eluted too late due to the polarity difference among IRB and HCT, while trying to remove HCT from dead time and obtain a capacity factor greater than one.

The outcomes of the preliminary study shows that gradient elution should be used. The gradient elution conditions were used after all optimization studies. Elution was finalized in less than 5 minutes using this method. The method required the use of an IS. IS has been tried with a variety of molecules. However, AVA was used as IS because it did not interact with IRB and HCT and eluted relatively late from HCT and IBR to an acceptable degree. Under optimized conditions, the required specificity for the HPLC methods HCT, IRB, and AVA was successfully accomplished. Figure 2 shows the chromatogram that was used to separate the obtained peaks.

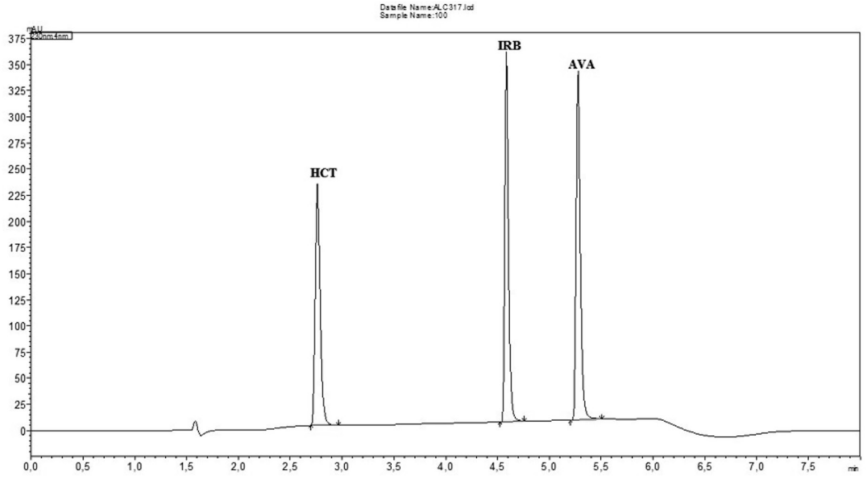

Figure 2. Chromatogram of the peaks of IRB, HCT, and AVA

Peaks from three different substances could be eluted separately, for a total analysis time of eight minutes. The wavelength used for analysis was 230 nm, and all three active substances produced strong signals at this wavelength. The injection volume was selected to be 1 µL. Under optimized experimental conditions and 1 µL injection volume, the symmetry of the peaks and theoretical layer number were calculated as 11817 for HCT and 63038 for IRB, and these results were within acceptable limits (N>2000). The analytical method's system suitability data were calculated, and the results are shown in Table 2. Our analytical method is successful, as demonstrated by the process parameter and chromatograms.

Table 2. The system-suitability data for HCT (118.0 µg mL-1) and IRB (186.0 µg mL-1)

Parameter	Obtained Value		Acceptance Criteria
	HCT	IRB	
Retention Time (min)	2.76	4.59	-
Relative Retention Time (min)	0.52	0.87	-
Standard Deviation (%) of Relative Retention Time	0.01	0.02	RSD[b] ≤1%
Precision for Relative Area	0.24	0.35	RSD[b] ≤ 1%
Injection Precision for Retention Time (min)	0.04	0.05	RSD[b] ≤ 1%
Theoretical Number of Plates (N)	11817	63038	N > 2000
Resolution (Rs)	13.91	20.87	>2
Tailing Factor (T)	1.29	1.39	≤2
USP Widtha	0.10	0.07	≤1
HETPc	12.69	2.38	-

[a] Calculated according to USP.

[b] RSD: Relative Standard Deviation (%).

[c] HETP: Height Equivalent to One Theoretical Plate.

Method validation

The calibration curve's was determined with peak normalization method and regression coefficients for both analyte were calculated 0.99 as a good linearity. Also; for n=10 and n=30, intraday and interday precision studies were carried out separately. The study results in Table 3 show that the analytical method provides precise results.

Table 3. Statistical data for the linearity, sensitivity, and precision studies of HCT and IRB

Parameter	HCT	IRB
Linearity Range (μg mL^{-1})	5.90 - 259.6	9.30 – 409.2
Slope (intraday, n=10)	12.766	0.9687
Intercept (intraday, n=10)	0.019	0.003
Regression Coefficient (intraday, n=10)	0.9980	0.9996
Standard Error of Slope (intraday, n=10)	0.020	0.007
Standard Error of Intercept (intraday, n=10)	0.016	0.009
Slope (interday, n=30)	1.277	0.974
Intercept (interday, n=30)	0.022	-0.002
Regression Coefficient (interday, n=30)	0.9979	0.9986
Standard Error of Slope (interday, n=30)	0.011	0.007
Standard Error of Intercept (interday, n=30)	0.009	0.009
LOD (ng/mL)	0.094	0.053
LOQ (ng/mL)	0.284	0.160
ANOVA	F (2.27)=0.0004 P=0.9996 (P>0.05)	F (2.27)=2.76×10^{-5} P=0.9999 (P>0.05)

The LOD and LOQ calculated using the ICH guideline equation were 0.094 μg mL^{-1} and 0.284 μg mL^{-1} for HCT, and 0.053 μg mL^{-1} and 0.160 μg mL^{-1} for IRB, respectively [14]. All calculated values were found to be significantly below the lowest concentration in the range, demonstrating that the analytical method developed sensitive results for both HCT and IRB.

Table 4 shows the outcomes of the analytical method's accuracy studies. The relative standard deviation (RSD) was found to be less than 2% in all of the results obtained. The mean recovery in the accuracy study was 99.76% for HCT and 99.10% for IRB. Because all of the results are within the range of 100%±2, it is assumed that the technique provides accurate results.

Table 4. Statistical evaluation of accuracy studies

Compound	Added Concentration (µg mL^{-1})	Measured Concentration (µg mL^{-1})	Recovery (%)	Standard Deviation	Relative Standard Deviation (%)	Recovery Error (%)	Mean Recovery (%)
HCT	94.4	95.3	100.9	1.15	0.41	0.91	99.76
	118.0	116.3	98.6	1.51	0.57	-1.45	
	141.6	141.8	100.2	0.79	0.42	-0.19	
IRB	148.8	146.3	98.3	0.47	0.88	-1.77	99.10
	186.0	185.9	99.9	1.28	0.33	-0.04	
	223.2	221.0	99.0	0.58	0.39	-0.10	

Considering the method robustness parameters, it was calculated that the most change was in the HCT peak area according to the detection wavelength. In the organic phase and flow rate changes, the retention time of AVA changed more. In the resolution change, both the analytes and the internal standard were less affected. The robustness date calculated for each compounds are as given in the Table 5.

Table 5. Robustness data *(n=3)*

		Retention time (min)		Peak area		Resolution	
		Observed value	Difference (%)	Observed value	Difference (%)	Observed value	Difference (%)
				HCT			
Column temperature °C	27	2.9	3.3	755548	-1.0	14.0	2.2
	33	2.7	-3.3	749505	-1.8	13.1	-4.3
Flow rate (mL/min)	0.54	3.1	10.7	842267	10.3	14.3	4.5
	0.66	2.5	-8.6	690832.7	-9.4	13.0	-5.2
Organic phase (%)	18	3.0	7.8	750093	-1.7	16.5	20.4
	22	2.6	2.6	758175	-0.7	11.6	-15.4
Buffer (%)	36	2.8	0.2	754176	-1.1	13.7	-1.2
	44	2.8	0.3	755485	-1.0	14.1	2.7
Detector wavelength (nm)	226	2.8	0.1	1115274	46.1	13.7	0.1
	234	2.8	0.1	306169	-59.8	14.2	3.8

	IRB						
Column temperature °C	27	4.6	0.1	866188	-0.8	19.9	-4.7
	33	4.6	0.4	860137	-1.5	21.8	4.5
Flow rate (mL/min)	0.54	4.9	6.8	969770	11.1	20.2	-3.3
	0.66	4.3	-5.2	793683	-9.1	22.0	5.6
Organic phase (%)	18	4.9	8.8	869550	-0.4	23.3	11.6
	22	4.3	-6.9	870331	-0.3	18.7	-10.3
Buffer (%)	36	4.6	0.2	855813	-2.0	21.0	0.6
	44	4.6	0.3	865022	-0.9	20.8	-0.5
Detector wavelength (nm)	226	4.6	-0.6	891133	2.1	20.9	0.0
	234 n	4.6	0.2	815615	-6.6	21.0	0.5
	AVA						
Column temperature °C	27	5.3	-0.3	947496	-1.3	8.5	-5.3
	33	5.3	0.4	944593	-1.6	9.2	2.9
Flow rate (mL/min)	0.54	5.6	6.3	1058900	10.3	8.7	-2.3
	0.66	5.0	-5.2	868366	-9.5	8.8	-2.0
Organic phase (%)	18	4.9	-6.9	952325	-0.8	9.0	1.0
	22	5.7	8.4	942416	-1.8	8.4	-5.7
Buffer (%)	36	5.3	0.1	951492	-0.8	8.7	-2.7
	44	5.3	0.1	948365	-1.2	8.7	-2.7
Detector wavelength (nm)	226 nm	5.3	0.0	903618	-5.8	9.0	0.0
	234 nm	5.3	0.0	1017867	6.1	9.0	0.0

Quality Control Tests

Table 6 and Table 7 show the calculated results of the tablet quality control tests. and obtained chromatogram was given Figure 3.

Table 6. Weight variation results (n=20)

Parameters	Drug A	Drug B	Drug C
Acceptable Range (±%5) (mg)	596.28 - 659.05	590.75 - 652.93	579.20 - 640.16
Minimum Tablet Weight (mg)	610.63	614.48	598.78
Maximum Tablet Weight (mg)	643.97	632.16	616.82
Average Tablet Weight (mg)	627.67	621.84	609.68
Standard Deviation	8.95	5.07	4.35

Table 7. Content uniformity results (Mean ± SD) (mg)

	HCT	IRB
Drug A	22.70 ± 0.17	293.15 ± 1.03
Drug B	22.66 ± 0.07	289.79 ± 0.30
Drug C	21.83 ± 0.08	286.66 ± 0.75

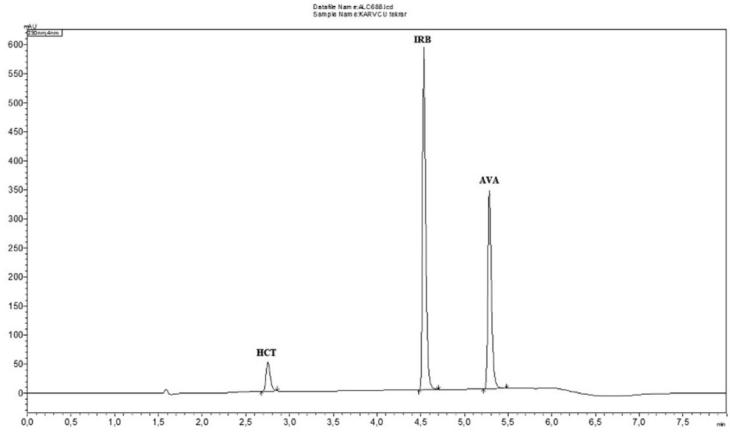

Figure 3. The obtained chromatogram of quality control test

The tablet weight variation was determined to be 610.63 – 643.97 mg for Drug A, 614.48 – 629.12 mg for Drug B, and 598.78 – 616.82 mg for Drug C. For tablets weighing more than 250 mg, the acceptable weight distribution is ±5%. A maximum of two tablets could be within the ±5% range on average among the weight measured values on 20 tablets. No tablet, however, should outweigh the ±10% range [14]. When the data was analyzed, all of the tablets from three different brands complied with the specifications.

The content uniformity test was carried out on six tablets. Each tablet was individually weighed and disintegrated in a volumetric flask with 250 mL of ACN in a sonicator for 20 minutes. Content uniformity test results shown in Table 7, RSD values in all companies were calculated to be less than 2%.

Table 8 shows the thickness, width, and length measurements. The obtained data show that the tablets are self-consistent.

Table 8. Thickness-Width-Length test and Hardness test results (n=10)

	Drug A	Drug B	Drug C
Thickness (Mean ± SD) (mm)	5.58 ± 0.09	5.95 ± 0.01	5.73 ± 0.03
Width (Mean ± SD) (mm)	9.39 ± 0.09	9.31 ± 0.02	9.32 ± 0.04
Length (Mean ± SD) (mm)	17.49 ± 0.13	17.54 ± 0.08	17.48 ± 0.12
Hardness (Mean ± SD) (Newton)	143.12 ± 33.15	118.57 ± 8.03	129.60 ± 12.21

In accordance with the pharmacopea dissolution study, 1000 mL of 0.1 N HCl was used for 45 minutes using apparatus 2 at a rotation speed of 50 rpm. The cumulative drug concentration (%) was calculated using samples taken at 5, 10, 15, 20, 30, and 45 minutes. Figure 4 and Figure 5 indicate the dissolution profiles.

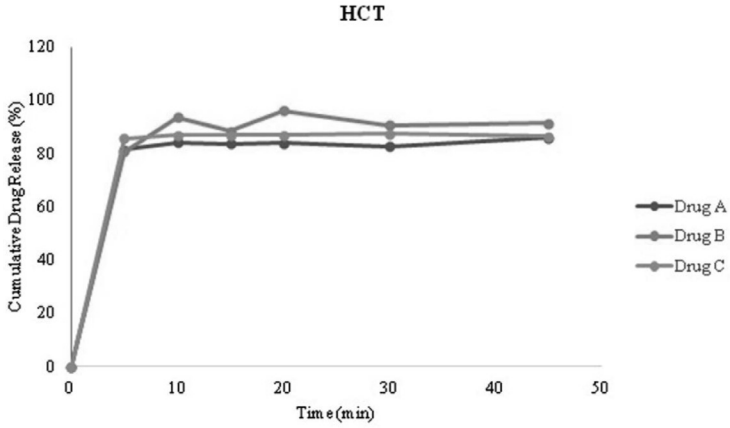

Figure 4. Dissolution profiles of HCT (The error bars indicate SD.) (n=6)

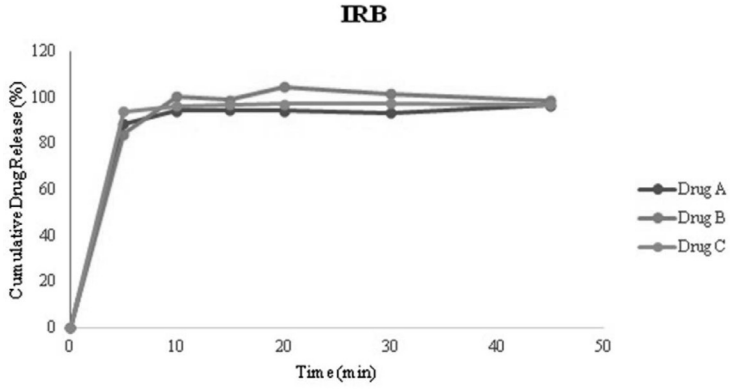

Figure 5. Dissolution profiles of IRB (The error bars indicate SD.) (n=6)

We worked with film-coated tablets that immediately released (IR). In the first 15 minutes, IR tablets should release 85% of the drug content [22]. When we evaluate the dissolution profiles, we show that 85% of the IRB is released within the first 10 minutes in each of Drug A, Drug B, and Drug C. HCT, on the other hand, reached 85% in Drug A before the 5th minute and within the first 10 minutes in Drugs B and C.

It is critical to meet the validation conditions so that the analytical methods planned for quantification can produce accurate, precise, and sensitive results. We developed a gradient elution HPLC method with AVA as an internal standard in this study. In addition, we have fully validated this method by the ICHQ2(R1) guideline.

This analytical method that we developed is a simple and low-cost method for simultaneously quantifying IRB and HCT.

We discovered that all 300/25 mg, IRB/HCT-containing tablets in the Turkish pharmaceutical market met all of the requirements when we examined the data from the weight variation, thickness-width-length test, hardness test, content uniformity, and dissolution tests, which we performed later.

STATEMENT OF ETHICS

Not applicable as no human or animal subjects were involved in the study.

CONFLICT OF INTEREST STATEMENT

The authors declare there is no conflict of interest associated with this study.

AUTHOR CONTRIBUTIONS

Concept: MSK (Mustafa Sinan Kaynak), SÖ (Saniye Özcan); Design: MSK, SÖ; Supervision: MSK, SÖ, MÇ (Mustafa Çelebier); Materials: BK (Berna Kaval), SÖ; Data collection and/or processing: BK, SÖ; Analysis and/or interpretation: BK, SÖ, MSK; Literature search: BK; Writing: BK, SÖ, MÇ; Critical reviews: SÖ, MSK, MÇ.

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Not Applicable

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Rapidly synthesized zinc oxide nanoparticles can increase the activity of antimicrobial drugs against clinical isolates of *Pseudomonas aeruginosa* and *Escherichia coli*

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ABSTRACT

This study aimed to investigate the effectiveness of combining zinc oxide nanoparticles (ZON) to antimicrobial drugs against clinical isolates of *Pseudomonas aeruginosa* and *Escherichia coli*. We explored two different methods to combine nitrofurantoin, cefepime, imipenem, azithromycin, gentamicin and sulfamethoxazole to ZON, using paper disks and 96 well plates. ZON was synthesized using the microwave-hydrothermal method and was characterized by UV-visible and Raman spectroscopy, X-ray diffraction, scanning electron microscopy and energy-dispersive X-ray spectroscopy. ZON cytotoxicity was tested against BGM cells, and its anti-inflammatory potential was also tested *in vitro*. The nanoparticles average size was of approximately 85 nm, and they decreased significantly the minimal inhibitory concentration of the tested antimicrobial drugs (ranging from 16 to more than 2000 times) when combined to them at the concentrations of 8 or 16 μg/mL - except for azithromycin against *E. coli* isolates. It also lacked cytotoxicity even at 1000 μg/mL. ZON were more effective than tenoxicam on the anti-inflammatory test. Further *in vivo* studies are necessary to set safe doses on living organisms.

Keywords: Antimicrobial, cytotoxicity, *Escherichia coli, Pseudomonas aeruginosa,* zinc oxide nanoparticles

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INTRODUCTION

Nanoparticles are fragments of materials such as metals, polymers and lipids, with sizes usually ranging from 1-500 nm, varied shapes, increased surface area and unique electromagnetic, optical and other physicochemical properties such as light absorption and emission[1, 2]. They are used as superconductors, semiconductors, for preparation of paints and coatings, and to increase the strength and durability of materials of varied interests, such as dentistry, orthopedics and textile industries[1, 2]. There is a growing interest in using nanoparticles for diagnostic and treatment of diseases, due to their possible interactions to molecular targets of interest. Such interactions may provide evidence of ongoing pathophysiological processes (in a more reliable and faster way compared to currently available methods) and/or change the course of diseases[2, 3]. Therapeutic benefits might be achieved using nanoparticles isolated or combined to clinically relevant drugs, increasing their pharmacological activity[2, 4].

Zinc oxide is used in several ways in its bulk form, such as in sunscreens and pharmaceutical anti-inflammatory formulations. Zinc oxide nanoparticles (ZON) are considered as safe for mammals as the bulk form, and have been explored in antimicrobial and antitumoral studies[3, 4]. Concerning their antimicrobial properties, ZON are mostly explored alone or functionalized with antimicrobial drugs[5], what usually requires complex reactions of organic synthesis. Combining nanoparticles to antimicrobial drugs is a simple strategy that can be useful to treat infectious diseases; however, this approach is poorly explored, mostly due to solubility and compatibility difficulties. In the current scenario of bacterial resistance, such combinations would be of interest if proven to be effective.

Bacterial resistance to antimicrobial drugs is a world health issue for which several economic and social problems have been predicted[6]. The global gross domestic product might fall around 4% by 2050 due to bacterial resistance impacts in people's work and academic productivity[7]. Mechanisms of bacterial resistance include molecular modification of drug targets, enzymatic inactivation or modification of drugs, efflux pumps, and biofilm formation[8-11]. A post-antibiotic scenario (i.e., a context in which infectious diseases of low clinical complexity - or even self-limiting - are not easily managed with the currently available antimicrobials) has been acknowledged, given the growing shortage of effective therapies[8]. Furthermore, new antimicrobials are not top interests of pharmaceutical industries[9]. Thus, strategies such as exploring nanoparticles to make the currently available drugs more effective are relevant.

This study aimed to describe the synergistic potential of ZON with relevant antimicrobial drugs against clinical isolates of *Pseudomonas aeruginosa* and *Escherichia coli,* which are relevant Gram-negative pathogens. Both are related to urinary and gastrointestinal infections, and reports on bacterial resistance to antimicrobials of these species are growing[10, 11]. ZON were synthesized using a rapid microwave-assisted method. We analyzed ZON using UV-visible and Raman spectroscopy, X-ray diffraction, scanning electron microscopy and energy-dispersive X-ray spectroscopy. We used an *in vitro* method to demonstrate ZON anti-inflammatory potential, and cytotoxicity assays suggested its safety. Our data open doors for further studies with the combinations using *in vivo* models of infectious diseases.

METHODOLOGY

Synthesis and characterization of zinc oxide nanoparticles (ZON)

Zinc acetate dihydrate was used to prepare ZON using the microwave-hydrothermal method described in detail by Marinho et al.[12], with reaction time of two minutes at 90 °C. ZON were collected by centrifugation, washed several times in distilled water and ethanol, and dried at 80 °C to obtain a fine powder. As they are poorly soluble in water, we tested different polar non-toxic solvents which would not hamper the biological assays.

ZON was characterized with different methods. Raman spectra was obtained at room temperature with an Ocean Optics portable spectrometer ($\lambda = 785$ nm, 499 mW), following the manufacturer instructions. X-ray diffraction (XRD) analyses were performed in a diffractometer (XRD-6000, Shimadzu, Japan), equipped with CuK radiation ($\lambda = 1.5406$ Å) in the 2θ range from 10° to 100° (0.02°/min scan increment, 2 s steps fixed time). Results were refined using the Rietveld's profile analysis method[13]. Scanning electron microscopy (SEM) was performed using the EVO MA 10 microscope (Zeiss, Germany), and energy-dispersive X-ray (EDX) spectra was obtained using an EDX analyzer operating at 200 kV (Oxford Instruments, UK).

Cytotoxicity assay

The cytotoxicity of ZON was assessed using BGM cells (American Type Cell Culture, USA), an immortalized fibroblast-like kidney cell line. ZON was tested at an initial concentration of 250 μg/mL. Culture and test protocols used in this study were conducted as described by our group [14]. Cells were cultured in RPMI 1640 media (Sigma, USA), supplemented with glutamine (0.3 mg/L), penicillin (200 IU/mL), streptomycin (100 μg/mL) and fetal bovine serum (10%). Plates were prepared with 180 μL/well, with an estimated counting of

$1x10^4$ cells each. The plates were incubated for 4 h, and the uptake of neutral red vital dye (50 μg/mL, 20 μL) was measured with a microplate reader (λ = 540 nm). Untreated cells prepared in ZON-free RPMI media were used as control. This test was performed in triplicate. The IC_{50} index was calculated using GraphPad Prism for Windows.

Bacterial strains

A total of 10 bacterial isolates of *E. coli* and 10 isolates of *P. aeruginosa* were used in this study. *E. coli* isolates are from urinary infections and *P. aeruginosa* isolates are from tracheal secretions. All strains are part of the microorganisms collection from Pitagoras College. Their identity was confirmed with VITEK 2 system version R04.02 (bioMérieux, France). Similarity indexes of 90% (or higher) were considered confirmative of the species of each isolate.

Minimal inhibitory concentration (MIC) assay

The MIC of ZON was determined in triplicate using untreated sterile 96-well polystyrene microtiter plates following CLSI standards and a protocol standardized by our group[14, 15]. A stock solution of ZON (5 mg/mL) was prepared using propylene glycol and water (4:1, previously sterilized in autoclave), which was diluted in sterile water for the tests (100 μL/well). The bacterial inoculum was primarily prepared at 0.5 MacFarland scale ($1.5x10^8$ CFU/mL) in sterile saline (0.9%), then diluted to $1x10^5$ CFU/mL in fresh sterile double concentration Mueller Hinton broth (Difco, Becton Dickinson, USA). The final concentration of ZON ranged from 1024 to 8 μg/mL, and the final concentration of the bacterial suspensions was of $5x10^4$ CFU/mL (final volume of the wells: 200 μL). MIC was established as the lowest concentration in which resazurine staining (0.1 g/L, 50 μL) resulted in no color modification from blue to pink in all strains. ZON at 1 mg/mL was used as a negative control.

This procedure was also performed to determine the MIC of azithromycin, gentamicin and sulfamethoxazole (all from Sigma, USA), for each species. Stock solutions of the drugs (4 mg/mL) were prepared in sterile water, and their final concentration of the drugs ranged from 1024 to 8 μg/mL. The final concentration of the bacterial suspensions was of $5x10^4$ CFU/mL (final volume of the wells: 200 μL). Resazurine staining (0.1 g/L, 50 μL) was used as described above.

Interference of ZON on antimicrobial drugs

The effects of combining ZON to antimicrobial drugs (synergism or antagonism) were assessed using two different methods, both performed in triplicate. First, we used an interference method standardized by our group[16] with three isolates of each species, as a preliminary assay. The selected disks were

nitrofurantoin 300 μg and cefepime 30 μg for *E. coli*, and imipenem 10 μg and azithromycin 15 μg for *P. aeruginosa* (all from Sensifar, Brazil), applied in petri dishes as for conventional susceptibility test. Following, briefly, 10 μL of the ZON solution at 1000 μg/mL was dispensed in each disk. Plates were incubated overnight at 37 °C, and the inhibition zone mean diameter was compared to control plates (untreated disks). Synergism and antagonism were inferred considering a 2 mm increase or decrease in the inhibition zone compared to the control, respectively.

We then used the checkerboard method[17] to test interactions of ZON and antimicrobials, with some modifications. Overnight-grown bacterial cultures were prepared in Mueller-Hinton broth as for the MIC assays. ZON was serially diluted vertically to reach final concentrations from 1024 to 8 μg/mL, and the antimicrobial drugs were serially diluted horizontally to reach final concentrations from 1024 to 0.5 μg/mL. Results were obtained using resazurine staining, as described at the MIC assay section.

In vitro anti-inflammatory potential of ZON

We used the bovine serum albumine (BSA - Thermo Fisher, USA) denaturation assay to investigate the anti-inflammatory potential of ZON (at 1000 μg/mL), as previously described[18], with slight modifications. BSA denaturation was conducted at 70 °C for 15 minutes, and Tenoxicam (Sigma, USA, 1000 μg/mL) was used as a positive control. BSA and Tenoxicam were prepared as aqueous solutions.

Statistics

Homocedacisty of data was checked by Bartlett's test, and normality was verified using Shapiro-Wilk test (square root transformation was performed when necessary). Differences on the activity of the antimicrobial drugs were analyzed using paired T-test. Calculated and observed XRD were analyzed using chi-square. The anti-inflammatory potential of ZON was analyzed using one-way ANOVA followed by Tukey test. All analyses were conducted using Bioestat 5.0 for Windows. Significant and highly significant levels were set as $p<0.05$ and $p<0.01$, respectively.

RESULTS and DISCUSSION

ZON characterization

XRD and Raman analyses indicated peaks that correspond to the hexagonal structure of wurtzite-like nanoparticles. For XRD, Rietveld's refinement of the results indicated that calculated and observed diffraction patterns are correlated (Fig 1), suggesting good long-range crystal ordering. The nanoparticles presented a single phase of wurtzite structure (JCPDS 36-1451), without the formation of secondary phases or impurities. Sharp and narrow diffraction peaks consistent with the zinc oxide wurtzite-type hexagonal structure were observed, indexed according to the JCPDS 36-1451 crystallographic record and $P6_3mc$ space group. The Rietveld refinement indexes, the lattice parameters and volume, obtained by Rietveld refinement for ZnO were a = b (Å) = 3.25014(5); c (Å) = 5.20715(9) and V (Å^3) = 47.636 (2); Rwp (%) = 4.86; Rp (%) = 3.68; Rbragg (%) = 1.59 and $\chi 2$ = 1.65. Raman spectra was more intense at E_{2H} at 438 cm^{-1} (Fig. 2). The characteristic bands are attributed to the active Raman modes of the zinc oxide wurtzite single crystal. These results are in good agreement with the work of Marinho et al.[12].

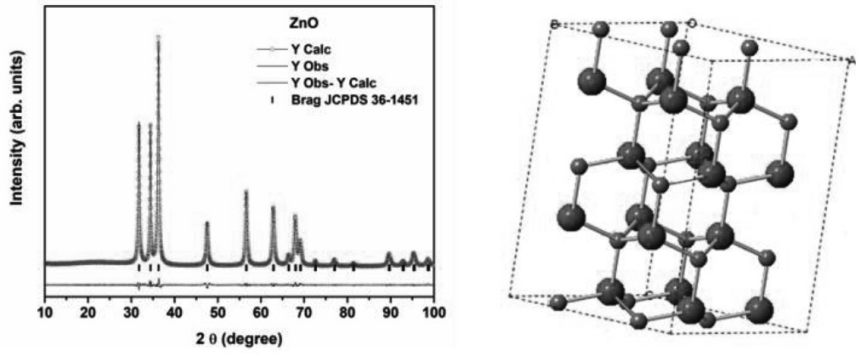

Figure 1. Results obtained by X-ray diffractogram of ZON after using Rietveld's refinement method.

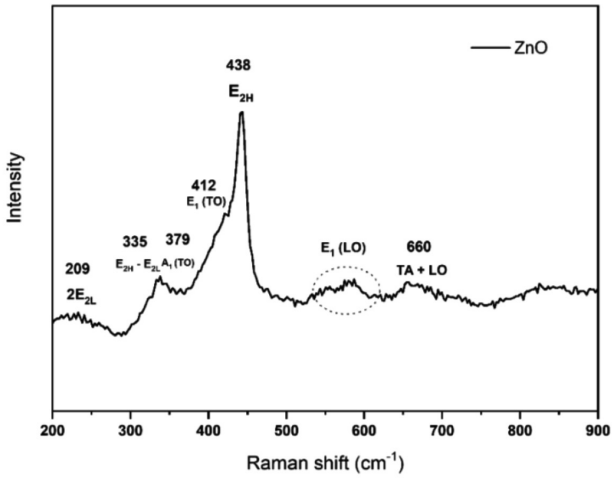

Figure 2. Raman spectra of ZON.

Scanning electron microscopy and EDX results are shown in figure 3. Approximately 100 particles were considered from the visual field images to determine average particle size. ZON presented regular and agglomerated shapes, with an average size of approximately 85 nm. Small, aggregated particles of approximately 30 nm were also observed. The peaks observed in EDX spectrum confirmed the pure composition of ZON.

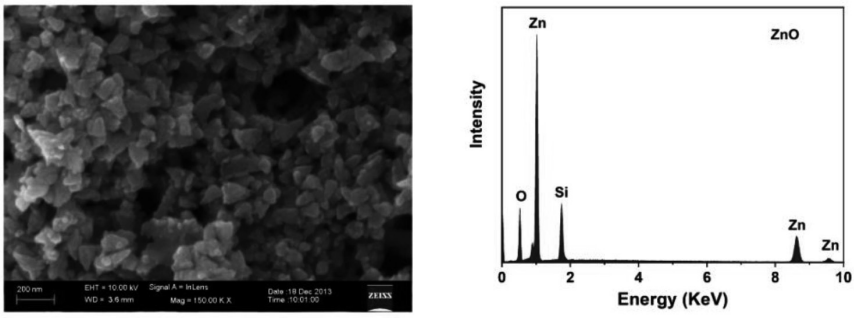

Figure 3. Scanning electron microscopy (left) and energy-dispersive X-ray spectra of ZON (right).

ZON solubility was tested with several possible solvents that would not interfere on the biological tests. A 4:1 blend of propylene glycol and water provided us the best solubility results.

MIC assays

The MIC values of the antimicrobial drugs are presented on table 1. Gentamicin was the most effective drug against *P. aeruginosa* isolates, as azithromycin was for *E. coli* isolates. ZON isolated presented no antimicrobial activity against any of the isolates of the tested species.

Table 1. MIC values of antimicrobial drugs

Species	Azithromycin	Gentamicin	Sulfamethoxazole
E. coli	16	128	>1024♦
P. aeruginosa	256	8	512

Data are expressed in μg/mL. Results are referent to all tested isolates.
♦ MIC was superior to the highest tested value.

Drug interaction assays

We used two different methods to check the possible effects of the combination of ZON at 1 mg/mL and antimicrobial drugs. Using the interference method[16], we detected significant increase of the inhibition zones of the antimicrobial drugs in disks (tables 2 and 3) upon the addition of ZON ($p<0.05$). The synergic effect was more evident for cefepime (against *E. coli*) and for azithromycin (against *P. aeruginosa*).

Table 2. Interference of ZON on the antimicrobial activity of drugs in disks for *E. coli*

Strain	Nitro	Nitro + ZON	Cef	Cef + ZON
E2	10	12*	27	34*
E5	17	19*	0	10*
E9	18	20*	0	12*

Nitro: Nitrofurantoin; Cef: cefepime; +ZON: addition of zinc oxide nanoparticles. Data are expressed as inhibition zone dimensions in millimeters. *All data with +ZON are significantly different of their ZON-free counterparts ($p<0.05$).

Table 3. Interference of ZON on the antimicrobial activity of drugs in disks for *P. aeruginosa*

Strain	Imip	Imip + ZON	Azit	Azit + ZON
P3	23	25*	17	19*
P6	21	23*	10	17*
P7	15	24*	0	20*

Imip: Imipenem; Azit: azithromycin; +ZON: addition of zinc oxide nanoparticles. Data are expressed as inhibition zone dimensions in millimeters. *All data with +ZON are significantly different of their ZON-free counterparts ($p<0.05$).

Given these results, we proceeded to the checkerboard method[17] to conduct a more detailed study on the effects of combining ZON to antimicrobial drugs. An adaptation on the interpretation of this method was necessary, as ZON presented no antimicrobial effect. Thus, we determined the new MIC of the drugs (table 4), instead of their fractional inhibitory concentration. For *P. aeruginosa* isolates, all new MIC values with addition of ZON were significantly lower than the MIC values obtained without ZON ($p<0.05$). For *E. coli,* the new MIC values for gentamicin and sulfamethoxazole with addition of ZON were significantly lower than the MIC values obtained without ZON ($p<0.05$). Surprisingly, the new MIC of azithromycin against *E. coli* increased significantly by the addition of ZON ($p<0.05$).

Table 4. New MIC values of antimicrobial drugs with the addition of ZON on checkerboard assay

Species	Azithromycin/ZON	Gentamicin/ZON	Sulfamethoxazole/ZON
E. coli	256 + 8	1 + 32	0.5 + 8
P. aeruginosa	0.5 + 8	0.5 + 8	8 + 16

Data are expressed as concentrations of drugs + ZON in μg/mL. Results are referent to all tested isolates.

Anti-inflammatory potential and cytotoxicity

ZON presented anti-inflammatory effect (Figure 4) and was more effective than tenoxicam at the highest concentration tested ($p<0.05$). ZON were not toxic to BGM cells even in the highest tested concentration (toxicity superior to 1000 μg/mL), suggesting their safety. IC_{50} could not be, therefore, calculated.

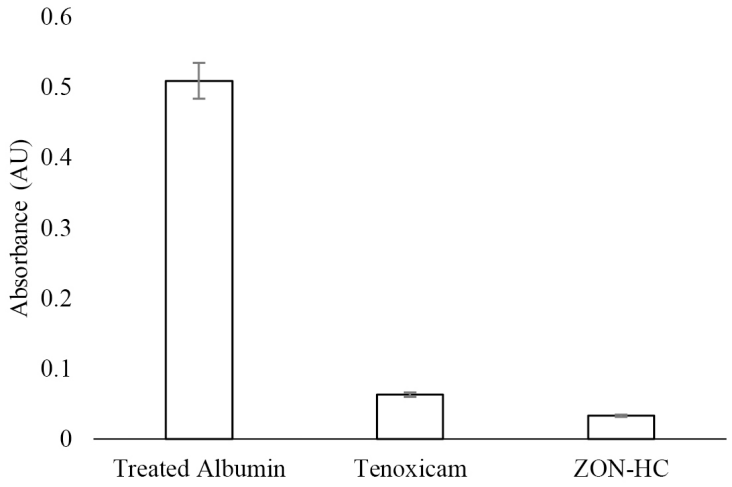

Figure 4. Comparative anti-inflammatory activities of ZON, tenoxicam and treated albumin (i.e. exposed to high temperature and free of ZON and tenoxicam). AU: arbitrary units, ZON-HC: ZON at its highest concentration (1024 µg/mL).

This study described that ZON synthesized by the microwave-hydrothermal method described by Marinho et al.[12] can increase the activity of antimicrobial drugs against Gram-negative pathogenic strains. ZON was synthesized without significant formation of impurities, and their nanoparticles features were confirmed by XRD and EDX spectrum peaks, Raman signals and scanning electron microscopy (Figs. 1-3). The antimicrobial mechanisms of action of ZON include cell disruption, induction of oxidative stress, and downregulation of several bacterial genes, without affecting human cells[19,20]. Generally, the cytotoxicity and poor water solubility of different nanoparticles hamper investigations on their possible biological properties. ZON lacked cytotoxicity at the tested concentrations (up to 1000 µg/mL), and we could overcome solubility issues using a blend of propylene glycol and water.

One may wonder why we tested the interactions of ZON with the antimicrobial drugs, as they were not active against bacteria. As previously described by our group, the activity of antimicrobial drugs might be increased or impaired by molecules with no antimicrobial properties[21-23]. Eventual synergism or antagonism resulting from such combinations are poorly predictable and require experimental evidence to be determined. In this context, our results are consistent with the observation of others that ZnO nanoparticles can present a synergistic behavior with antimicrobial drugs. A study described the combination of ZnO nanoparticles (average diameter of 20 nm) doped or not with Fe, Mn,

Cu and Co. The combination of doped ZnO nanoparticles to ciprofloxacin and ampicillin was synergic against *Bacillus subtilis, E. coli* and *S. aureus*, whilst combinations with pure ZnO nanoparticles resulted in additive effect[24]. ZnO nanoparticles (average diameter of 78 nm) in sub-inhibitory concentrations presented synergism when combined to ciprofloxacin and ceftazidime against *Acinetobacter baumannii*[25]. More recently, ZnO nanoparticles combined to fennel essential oil in a potato starch packing film were active against *S. aureus, E. coli* and *Aspergillus flavus*[26].

Among the drugs tested in the present study is azithromycin. Reports on the resistance of pathogenic bacterial species to this drug are increasing[27], especially due to its large use in patients with SARS-CoV-2 infections[28]. Azithromycin interaction with ZON resulted in decrease of MIC value for *P. aeruginosa* isolates from 256 to 0.5 μg/mL, whereas for *E. coli* isolates the MIC value increased. Similarly, a study reported an antagonistic behavior of ZnO nanoparticles (average diameter ranging from 10-30 nm) combined to β-lactams against *Yersinia intermedia*, whereas cephalosporins of second and third generations, tetracycline and nalidixic acid were not affected by the combination to the nanoparticle[29]. Recently, ZnO nanoparticles (average diameter of 50 nm) were combined to meropenem, ciprofloxacin and colistin against *P. aeruginosa*, and synergism was detected only for colistin[30].

The lack of antimicrobial activity of ZON alone might be at least partially explained by the method of synthesis, given that it influences morphological characteristics such as uniformity of size and contact surface. These properties may also interfere with their interactions to microbial targets[31]. Nevertheless, ZnO nanoparticles synthesized with other strategies might present antimicrobial activity. An investigation on the antimicrobial potential of ZnO nanoparticles (66 to 112 nm in diameter) prepared with *Albizia lebbeck* stem bark extract found that they were as effective as ciprofloxacin against *Bacillus cereus* and *Salmonella typhi,* using the agar diffusion method[32]. A study on ZnO nanoparticles prepared using the precipitation technique (232 to 692 nm in diameter) were shown to be active against *Aspergillus niger*[33].

The anti-inflammatory activity of ZON was expected, given that zinc oxide, in its bulk form, present this biological potential[34, 35]. We used an *in vitro* method based on the ability of a substance to inhibit BSA denaturation, exposure of its chromophore groups and protein aggregation, as observed in different inflammatory diseases[18]. ZON was more effective than tenoxicam in inhibiting BSA denaturation (Figure 4). The main known mechanisms that help to explain the anti-inflammatory potential of ZnO nanoparticles include suppression

of mRNA expression and protein levels of pro-inflammatory enzymes such as COX-2, and of cytokines such as TNF-α and IL-1β[36, 37].

Combinations of ZON and antimicrobial drugs were more effective than the drugs alone against clinical isolates of *E. coli* and *P. aeruginosa*. Furthermore, ZON lacked cytotoxicity and presented anti-inflammatory potential. The synthesis method is rapid, reproducible, and relatively simple, important aspects considering an eventual large-scale manufacturing. Although this study is not without limitations concerning the variety and number of bacterial strains, our data open doors for more studies exploring the combinations of antimicrobial drugs and ZON using *in vivo* models of infectious diseases.

STATEMENT OF ETHICS

All the necessary ethical rules were followed while performing research.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Conceptualization: MVDS

Methodology: MVDS, RCL

Validation: MVDS, RCL

Formal Analysis: LFCM, JZM, IPC, RCL, MVDS

Investigation: LFCM, JZM, IPC

Resources: MVDS, RCL, JZM

Writing - Original draft: LFCM, JZM

Writing - Review and editing: MVDS

Supervision and Project administration: MVDS

[†]Authors contributed equally to this work.

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Nutritional Scrutiny, Chemical Profiling and Antioxidant Potentials of Crude Extracts of *Moringa oleifera* Lam. (Moringaceae) from Kasur, Pakistan

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ABSTRACT

Moringa oleifera L. is a miraculous plant due to the presence of essential nutrients, phyto-constituents and natural antioxidants. This study evaluated the aqueous and methanol extracts of *M. oleifera* leaves for various nutritional parameters, phyto-constituents and antioxidant activities. Results indicated varying contents of moisture (78.0±2.2, 7.3±0.7%), ash (0.8±0.1, 1.50±0.8%), fat (0.9 ±0.2, 1.8±0.9%), crude fiber (2.1±0.3, 20.5±1.7%), crude protein (7.1±0.8, 22.7±1.9%) carbohydrates (11.1±1.3, 46.2±2.1%) and energy kcal/100g (81.0±2.5, 292±4.2). Proteins, carbohydrates, hydroxyl-anthraquinone, tannins, alkaloids, saponins, flavonoids, terpenoids and saponins were present in *Moringa* leaves powder except phytosterol and fixed oil. In the methanol extract of *Moringa* fresh leaves, TPC recorded was 76.7±2.5 mg GAE/g and TFC was 24.6±0.40 mg QE/g while in the dried leaves powder, TPC and TFC were 86.2±1.8 mg GAE/g and 29.8±0.4 mg QE/g which were higher than the aqueous extracts. Antioxidant activity of *Moringa* dried leaves methanol extract with DPPH displayed maximum percentage inhibition (92.5±3.2%) than aqueous extract (65.2±2.5%) and BHT (57.6±2.1%) at 100µg/ml. Same tendency was observed in the reducing power assay for *Moringa* dried leaves powder methanol extract Conclusively, *M. oleifera* possess a wealth of nutrients and bioactive compounds with potential antioxidant activity for extraordinary applications in the food and pharmaceutical industries.

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Keywords: *Moringa oleifera*, nutritional analysis, phyto-constituents, antioxidant activity, Pakistan

INTRODUCTION

Since the ancient time, plants are regarded as a significant source of medicine. According to the World Health Organization, up to 80% population of the world is still dependent on herbal preparations as medicine to treat different diseases[1]. The *Moringa oleifera* Lam. is a famine resistant plant from the Moringaceae family[2]. It is a plant of tropical forests whose all parts including the gum, seed, fruit, flowers, leaves, bark and roots are rich in proteins, vitamins and minerals like calcium, phosphorous, potassium, folic β-carotene, iron and acid. The fresh leaves or dried leaves powders of *Moringa* are employed for the development of food products to have improved nutritional quality and therapeutic effects[3]. In the ancient times, leaves of this plant were given to animals as feed[4] and were used in human diet for better health. As its popularity rises, its various parts like roots, pods and seeds were found to be nutritious and medicinally significant. That's why; this plant is taken as a food ingredient, nutraceutical and medicine due to the presence of essential phytochemicals. It has been said that the phytochemicals in different parts of a particular plant makes it very significant and versatile medicinally[5-7]. Numerous phytochemicals have been extracted and reported from *Moringa,* including phenolics, flavonoids, tannins, alkaloids, saponins and glucosides etc. Polyphenolic compounds like flavonoids and phenolic acids are abundantly present in the dried leaves of *Moringa*[9,10].

Previously, phytochemicals like carbonic acid, 2-Isopropoxyethyl propionate (16.87%), 1,3-dioxolan-2-one, 4,5-dimethyl- (6.16%), 4H-pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl- (8.98%), 1,3-dihydroxyacetone dimer (3.85%), 2-hydroxy-2-methyl- (3.14%), butyl 2-pentyl ester (20.64%), alpha-d-glucose (3.44%), azetidin-2-one 3,3-dimethyl-4-(1-aminoethyl)- (4.67%), tetra acetyl-d-xylonic nitrile (5.03%) and butanedioic acid, were documented in *M. oleifera* leaves aqueous extracts. Compounds like 2-ethyl-2-(hydroxymethyl)- (21.19%), 1,3-propanediol, 2-methyl-, octyl ester (15.02%), propionic acid, n-ethyl-n-nitroso- (5.21%), ethanamine, 9,12,15-octadecatrienoic acid,(Z,Z,Z)- (5.00%), 4H-pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl- (4.18%), benzeneacetonitrile, monomethyl malonate (2.56%), n-hexadecanoic acid (2.57%), 3-oeoxy-d-mannoic lactone (3.29%) were also recognized in the methanol extracts of *M. oleifera* leaves[11].

The phytochemicals from *Moringa* leaves and seeds have been reported with therapeutic potencies[12,13] against cardiovascular diseases[14], hypolipidemic disorders[15],

with biological activities including antispasmodic and diuretic[16], antiobesity[17], antiulcer[18], antihypertensive[19,20], antitumor and apoptotic[21,22], hepatoprotective[23,24], antidiabetic[25-27], antimicrobial[28], wound healing[29], analgesic[30], antipyretic[31], antiasthmatic[32], anti-inflammatory[33,34], antiurolithiatic[35] and antioxidant[36,37] activities. *Moringa* plant have also protective effects against immune disorders[38] and neurodegenerative diseases including Parkinson's[39] and Alzheimer's[40].

As there is emergent interest in the assessment of nutritional and therapeutic efficacies of natural compounds from plants origin with their utilization in the development of products and drugs in the food and pharmaceutical industries, this study further extents the nutritional parameters, phytochemical profiles and antioxidant potentials of aqueous and methanol extracts of *M. oleifera* fresh leaves and dried leaves powder from the Kasur district of Pakistan.

METHODOLOGY

Moringa sample preparation

Fresh leaves of *M. oleifera* were acquired from the Kasur district of Pakistan and the collected specimens were identified by experts from the Government College University, Lahore, Pakistan. Primarily, the leaves were cleaned up with distilled water and placed in aluminum trays at 27°C for 5 days to shade dry. The dried leaf specimen was then grinded (Grinding mill, Germany) and the obtained powder was sieved with a 2 mm pore size siever and stored at -4°C for further experimentation[41].

Nutritonal evaluation

For moisture analysis, *Moringa* fresh leaves and dried leaves powder were placed in oven at 105 to 110°C to a constant weight. Nutritional parameters like, crude lipid, crude protein, crude fiber and total ash content were estimated by adapting AOAC standard methods[42]. Estimation of total carbohydrates was done using the method described in literature[43,44]. The values of energy in Kcal/100 g were estimated by multiplying obtained values of carbohydrates, lipids and proteins by factors of 4 and 9 and the sum obtained was presented in kilocalories[45].

Solvent extraction and phytochemical analysis

A 1% (w/v) stock concentration of extract was obtained from fresh and dried leaves powder of *Moringa* using methanol and water as extraction solvents. Following standard procedures given by Harborne[46] and Kokate[47], the *Moringa* extracts were tested with positive and negative controls for qualitative testing of phytochemicals like amino acids, alkaloids, triterpenoids, tannins, phytosterols, anthroquinone, flavonoids, cardiac glycosides, carbohydrates, glycosides, saponins, fixed oils/fats and proteins.

Estimation of TPC and TFC

The total phenolic content (TPC) of *Moringa* fresh leaves and dried leaves powder were determined quantitatively by folin-ciocalteau reagent method at 760 nm[48] with some modifications[49] and the obtained results were presented in mg GAE/100g. For the total flavonoid content (TFC) estimation in *M. oleifera* fresh leaves and dried leaves powder, the aluminium chloride colorimetric method was used[50] with minor modifications[51]. Quercetin was used as a standard compound for flavonoids quantification and the obtained values were presented as mg QE/100g.

Antioxidant potentials of *Moringa*

The free radical scavenging potency of *M. oleifera* fresh leaves and dried leaves powder was assessed using DPPH method[52] with some modifications[53,54] where the antioxidants minimize the free radicals absorbing light at 517 nm. The ability of aqueous and methanol extracts of *Moringa* fresh leaves and dried leaves powder to reduce iron (III) to iron (II) (total reducing power) was evaluated following Oyaizu[55] with slight modification[53,54] and compared to a strong reducing agent BHT. The absorbance (700 nm) of the samples was plotted against each concentration taken.

Statistical analysis

Data was analyzed statistically and standard deviation (SD±) was estimated in the Microsoft excel program. Differences at $p < 0.05$ were considered significant[56].

RESULTS and DISCUSSION

Nutritional evaluation of *M. oleifera*

Nutritional analysis of *M. oleifera* fresh leaves and dried leaves powder performed using the proximate analysis is critical in determining the nutritional quality of *Moringa*. The results of nutritional parameters assessed in *M. oleifera* leaves are given in Table 1 where the fresh leaves and dried leaves powder exhibited varying contents of moisture (78.0 ± 2.2, 7.3 ± 0.7%), ash (0.9 ± 0.1, 6.50 ± 0.8%), fat (0.8 ±0.2, 1.8 ± 0.9%), crude fiber (2.1 ± 0.3, 20.5 ± 1.7%), crude protein (7.1 ± 0.8, 22.7 ± 1.9%), carbohydrates (11.1 ± 1.3, 46.2± 2.1%). The total energy estimated in *Moringa* fresh leaves and dried leaves powder were 81.0 ± 2.5, 272 ± 3.2 Kcal/100g.

These results regarding the moisture and ash contents recorded in *Moringa* dried leaf powder (7.3%, 6.5%) are in line with the data reported in previous studies on *Moringa* where mean contents of moisture recorded were between

5 -10% and ash between 6-11%[57,58]. In other investigations, elevated levels of moisture and slightly low ash contents were reported in *Moringa* on the basis of dryness of the original sample[59,60]. The ash of *Moringa* may possess inorganic minerals that are necessary for growth and development. It has been found that *Moringa* possess more Ca and iron as compared to spinach, that's why; the leaves powder of *Moringa* could be utilized as a substitute for iron medications for the treatment for anemia. It has been also found that *Moringa* leaves have around 25.5–31.03 mg of zinc/kg[61].

The mean protein contents of fresh leaves and dried leaves powder of *Moringa* were 7.1±0.8 and 22.7 ±1.9% respectively. These results regarding the protein contents are in accordance with the data reported by Valdez-Solana *et al.*[62] and Fejér *et al.*[63]. However, Moyo *et al.*[64], Castillo-López *et al.*[65] and Fokwen *et al.*[66] reported slightly lower (27.2%) protein contents in *Moringa*.

Other nutritional parameters assessed in fresh leaves and dried leaves powder of *Moringa* include fat (0.9±0.2, 1.8±0.9%), crude fiber (2.1±0.3, 20.5±1.7%), crude protein (7.1±0.8, 22.7±1.9%) and carbohydrates (11.1±1.3, 46.2±2.1%). These outcomes are consistent with the results of earlier inquiries of Yameogo *et al.*[67], Witt[68] and Isitua *et al.*[69]. The nutrient variation in *Moringa* could be due to the seasonal fluctuations such as climate, location, geography and some other environmental factors[70]. The physicochemical analysis confirmed that *M. oleifera* is a miraculous plant and its leaves are a high quality food source making itself a candidate plant to be utilized directly in human diet or in the improvement of balanced diets in animal nutrition[71]. In spite of this difference, proximate analysis displayed that the leaves of *M. oleifera* remained good sources of fats, fiber, carbohydrate and proteins which are the primary sources of energy and are used in food and medicinal products like bread, biscuits, drinks, cookies, soups and medicinally coated capsules.

Table 1. Results of nutritional parameters of *M. oleifera* fresh leaves and dried leaves powder

Parameters	Percentage values	
	***Moringa* leaves (Fresh)**	***Moringa* dried leaves (Powder)**
Moisture	78.0±2.2	7.3±0.7
Ash	0.9±0.1	6.5±0.8
Fat	0.8±0.1	1.8±0.9
Crude fiber	2.1±0.3	20.5±1.7
Crude protein	7.1±0.8	22.7±1.9
Carbohydrates	11.1±1.3	41.2±2.1
Energy (Kcal/100g)	81±2.5	272±3.2

Values are the mean (± SD) of three readings presented as (%) g/100 g

Phytochemical analysis of *M. oleifera*

Basic phytochemical analysis for confirming the presence of major phytocompounds is critical because many drugs' active principles are secondary metabolites present in plants. Findings showed that the aqueous and methanol extracts of *Moringa* leaves possess many phytochemical groups including tannins, alkaloids, saponins, flavonoids, protein, terpenoids, hydroxyl anthraquinone and carbohydrates, but no phytosterol or fixed oil was found in both the extracts (Table 2). These outcomes are in accordance with the results of Vergara-Jimenez *et al.*[72], Ayoade *et al.*[73], and Sudha *et al.*[74]. The utilization of methanol and water as extraction solvents indicated the presence of assorted active principles with solvents selective solubility with different polarities used in succession, inferring the solvents significance as a promising factor[75]. Furthermore, the evidence points to the significance of a particular test as a key factor in endorsing phytochemicals presence. Since *M. oleifera* leaves are rich in amino acids and carbohydrates, it is proposed that these nutrients are very beneficial and may be utilized as a growth promoters and nutritional supplements[76,77]. Phytochemical data of *M. oleifera* may be used to produce lead compounds in the quest for innovative herbal medications[78,79].

Table 2. Phytochemical profile of aqueous and methanol extracts of *M. oleifera* fresh leaves

Phytochemicals	Tests/ Experiments	Blank	Control	Aqueous extract	Methanol extract
Tannin	Ferric chloride	-	+++	+	++
Alkaloids	Dragendorff's test	-	++	+	++
Saponins	Foam test	-	++	-	+
Triterpenoids	Salkowski	-	+++	++	+++
Phytosterols	Liebermann-Burchard	-	+++	-	-
Cardiac glycoside	Keller killani	-	+++	-	+
Flavonoids	Lead acetate	-	+++	++	+++
Hydroxyanthraquinone	Potassium hydroxide	-	+++	++	+
Amino acid	Millon's test	-	++	+	++
Fixed oils and fats	Copper sulphate	-	++	-	-
Carbohydrates	Molisch's test	-	+++	++	+++
Proteins	Biuret test	-	++	+	++

++++ (Very high), +++ (High), ++ (Moderate), + (Low), - (Nil), Positive controls and blank (water)

Total phenolic content (TPC) in *M. oleifera*

Phenolic compounds are much prevalent in plants with potential antioxidant activities as they generate hydrogen ions that form stable intermediate radicals[80]. This study assessed the TPC with folin-ciocalteu method where the *Moringa* fresh leaves aqueous extract displayed lower (60.2±1.3 mg GAE/g) and the methanol extract showed higher TPC content (76.5±1.7 mg GAE/g). While, the aqueous extract of dried leaves powder contains 68.3±1.5 mg GAE/g and methanol extract has TPC of 86.2±1.8 mg GAE/g (Figure 1, Table 3). The TPC reported here are faintly lower as compared to the TPC content (9535.3±57.74 mg/100g) reported by Ilyas *et al.*[81] whereas; slightly higher values of TPC were described in *Moringa* leaves by Abdulkadir *et al.*[82]. The variations in TPC might be associated with the difference in polyphenolics extraction methods or solvents polarity and also the plants geographical distribution.

Previous data shows that the *Moringa* leaves are rich in valued compounds like vitamin, protein, calcium, iron and antioxidants including ascorbic acid, carotenoids, phenols and flavonoids[83]. Due to the polyphenols and other antioxidants, many researchers have claimed that the methanol extract of *M. oleifera* leaves exhibit a strong antioxidant action[84]. Many studies have also proposed that the *Moringa* leaves possess anti-diabetic, anti-inflammatory, anti-epileptic, anti-hypertensive, and antitumor activities and these are associated with the phenolic compounds present in the plant. Further, the phenolic compounds have strong antioxidant activities against tissue impairments instigated by the free radicals[85,86].

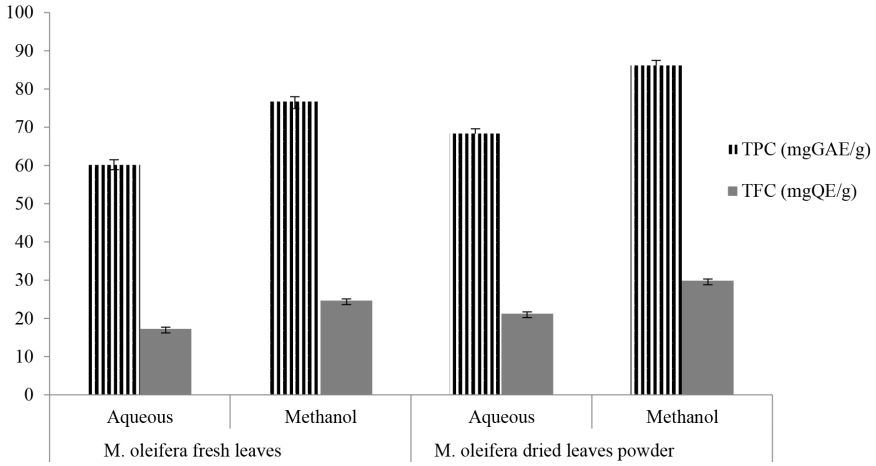

Figure 1. Total phenolic and flavonoid contents in methanol and aqueous extracts of *M. oleifera* fresh leaves and dried leaves powder

Table 3. Total phenolic and flavonoid contents in *M. oleifera* fresh leaves and dried leaves powder

Extracts	*M. oleifera* fresh leaves		*M. oleifera* dried leaves powder	
	TPC (mgGAE/g)	TFC (mgQE/g)	TPC (mgGAE/g)	TFC (mgQE/g)
Aqueous (H_2O)	60.2±1.3	17.2±0.2	68.3±1.5	21.2±0.3
Methanol (MeOH)	76.7±1.7	24.6±0.5	86.2±1.8	29.8±0.4

Data are represented as mean (± SD) of three readings

Total flavonoid content (TFC) in *M. oleifera*

It was found that the methanol extract of *Moringa* dried leaves powder has greater concentration of flavonoids (29.6±0.4 mg QE/g) than its aqueous extract (21.2±0.3 mg QE/g) (Figure 1, Table 3). Flavonoids were confirmed in both the aqueous and methanol extracts of *Moringa* fresh leaves and dried leaves powder. According to Lin *et al.*[87], flavonoids are polyphenolic compounds mostly found in dried leaves of *Moringa*. They are secondary metabolites which are most prevalent phytochemical group. According to Masood *et al.*[88], plant's antioxidant capacity is correlated with its level of TPC and TFC. Flavonoids have positive effects on the human body and provides protection against various diseases[89]. The leaves of *M. oleifera* contain variety of flavonoids; however the most prevalent flavonoids with significant pharmacological action are quercetin, apigenin, kaempferol and isorhamnetin[90]. Flavonoids also possess potential anti-microbial, anti-inflammatory, antioxidant, anti-allergic potentials and other significant biological activities[91,92].

Antioxidant activity of *M. oleifera* extracts

The free radical scavenging potentials of plant extracts improves with the increase in extract concentration. This pattern of radical scavenging was observed in the present inquiry where the percentage inhibitions recorded in methanol extract of *Moringa* dried leaves powder were 35.5, 50.2, 65.9, 78.1, and 92.5% at 20, 40, 60, 80, and 100 µg/ml concentrations , while the percentage inhibitions of the aqueous extract at same concentrations were 25.4, 34.1, 45.6, 55.3, and 65.2 respectively. Similarly, the percentage inhibitions of the standard synthetic antioxidant BHT at same concentrations recorded were 21.5, 30.2, 40.7, 48.3, and 57.6% (Figure 2). The aqueous and methanol extracts of *Moringa* dried leaves powder showed the similar patterns of DPPH scavenging activities which are higher than BHT.

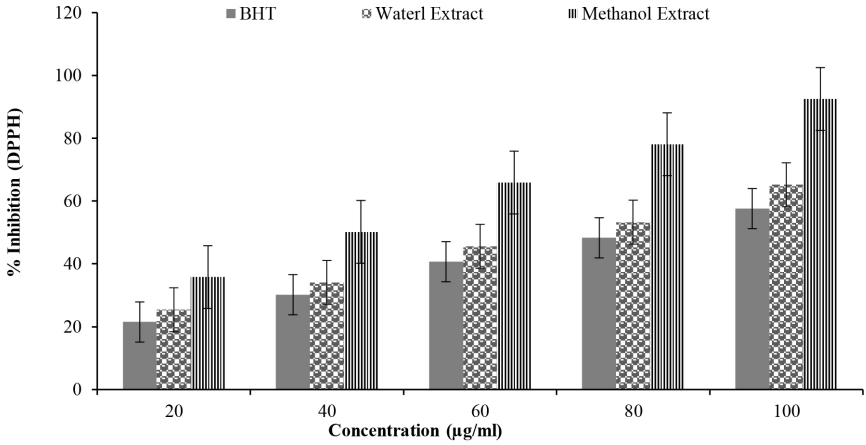

Figure 2. Percentage inhibition (DPPH) of aqueous and methanol extracts of *M. oleifera* dried leaves powder and BHT

The phytochemicals bear antioxidant properties due to the ability to prevent production free radicals, or scavenging free radicals in the body or chelating/reducing the content of transition metal[93,94]. An essential antioxidant method is considered to be the inhibition of the chain start phase by scavenging different reactive species like the free radicals[53,54,95,96].

ROS (reactive oxygen species) are continuously generated in animals due to some environmental factors encountered in daily life[97]. In such conditions, antioxidants are generated by the body cells to maintain the body's equilibrium with free radicals. Oxidative stress describes any imbalance brought on by a variety of diseases in the regular physiological system of the body. According to Karim *et al.*[98] at the harsh level, the oxidative stage transforms cellular damage into different chronic diseases. It has been found that the antioxidants have a good effect on these types of chronic diseases by preventing the initiation of any damage[99-101].

As per the findings of this study, the methanol extract of *Moringa* dried leaves powder displayed higher percentage of DPPH inhibition than the aqueous extract and BHT which are in accordance with previous investigations of Kumar *et al.*[102], Almaghrabi *et al.*[103] and Landazuri *et al.*[104].

The increased DPPH scavenging activity of methanol extracts of *Moringa* reported here might be associated with the presence of total phenolic and flavonoid contents. By providing hydrogen atoms to DPPH, these hydroxyl phenolic compounds can remove it from the environment. In order to evaluate the

antioxidant potentials of herbal extracts, the DPPH scavenging method is now frequently used[105-107] which is highly accurate, sensitive, and quick, depends on the transformation of unstable purple DPPH molecules into yellowish DPPH molecules in the presence of antioxidants[108,109].

The reducing power of *Moringa* dried leaves powder extract was assessed and compared with standard reference BHT on ferric to ferrous reduction in the presence of Fe (II) - stabilising ligand (Figure 3). The reducing power might be attributed to the hydrogen donating capacity, which is commonly related with the presence of reductants[110]. The extracts can convert $[Fe(CN)_6]_3$- to $[Fe(CN)_6]_4$-, which then interacts with Fe^{3+} to form $Fe_4[Fe(CN)_6]_3$ which is a Prussian blue coloured complex[111]. Our findings revealed that these extracts have a degree of hydrogen donation ability which varies with concentration.

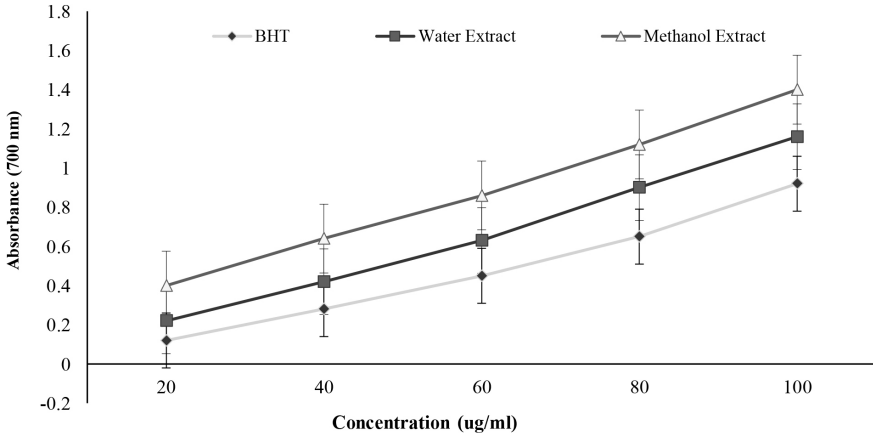

Figure 3. Reducing power of aqueous and methanol extracts of *M. oleifera* dried leaves powder and BHT

The reducing power increased with concentration and these capabilities were superior to those of standard synthetic antioxidant BHT. *Moringa* dried leaves powder extracted with methanol was found to be the most powerful reducing agent, followed by the aqueous extract and BHT. These findings form a clear relationship between the reduction efficacy and antioxidant capability of *Moringa* extracts with high phenolic content as the phenols with a greater number of hydrolysable groups (OH groups) connected to the ring are potent reducing agents (proton donors), resulting in the termination of free radical chain reactions[112].

Considering its myriad benefits, *Moringa* really does seem to be a Marvel plant. As such, this plant must be adapted as a high-quality, inexpensive memento from nature. In addition to its remarkable health benefits, this study found that the crude methanol and aqueous extracts of *M. oleifera* leaves possess essential nutrients, phytochemicals and natural antioxidants that could be significant for industrial and medicinal purposes. To have improved nutrition, the extracts of *Moringa* dried leaves powder should be used for better effects. To fully investigate and utilize the wonders of the *Moringa* tree, more robust research and product development strategies are required. The moment has come to investigate its route for food usage, standardize and commercialize technology for producing value-added and highly nutritious products.

CONFLICT OF INTEREST STATEMENT

Nothing to declare.

AUTHOR CONTRIBUTIONS

Design: Muhammad Khalid SAEED

Acquisition of data: Muhammad Khalid SAEED

Analysis of data: Muhammad Khalid SAEED

Drafting of the manuscript: Muhammad Khalid SAEED and Adil HUSSAIN

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