INTERNATIONAL STUDIES AND EVALUATIONS IN THE FIELD OF

HEALTH SCIENCES

December 2024

EDITORS

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SERÜVEN YAYINEVİ

Genel Yayın Yönetmeni / Editor in Chief • C. Cansın Selin Temana

Kapak & İç Tasarım / Cover & Interior Design • Serüven Yayınevi

Birinci Basım / First Edition • © Aralık 2024

ISBN • 978-625-5955-65-4

Serüven Yayınevi / Serüven Publishing
Türkiye Adres / Turkey Address: Kızılay Mah. Fevzi Çakmak 1. Sokak
Ümit Apt No: 22/A Çankaya/ANKARA
Telefon / Phone: 05437675765
web: www.seruvenyayinevi.com
e-mail: seruvenyayinevi@gmail.com

Baskı & Cilt / Printing & Volume
Sertifika / Certificate No: 47083

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CONTENTS

CHAPTER 1

ANTIOXIDANT ACTIVITY DETERMINATIONS BY COMPARATIVE PLANT SEEDS EXTRACTION METHODS

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1. INTRODUCTION

Medicinal plants have been traditionally used as natural healing medicines in folk medicine for many years (Wangchuk, 2018). Plants are an important source of drug formulations. In recent years, interest in medicinal plants and their benefits has increased. Bioactive compounds have numerous benefits, such as lowering blood pressure, preventing heart disease, providing a protective effect against cancer, or lowering blood sugar. Different parts of plants contain different bioactive compounds. The bioactive compounds found in medicinal plants and the antioxidant activity they contain have paved the way for these plants to be used as preservatives in the food and cosmetics industry. Volatile oils obtained from plant seeds are used for medical and industrial purposes. Seeds are one of the parts of the plant with the highest concentration of bioactive molecules (Gómez-Mejía et al., 2020).

The procedures and optimization of extraction methods are very important for the recovery of bioactive components from plant material. The quality of plant seed oil varies according to extraction methods. In recent years, new and effective extraction methods have been developed to expand the scope of conventional extraction methods. These extraction methods have advantages over conventional methods such as shorter extraction times, higher extraction yields, and lower solvent volumes. These new extraction methods are also known as green extraction methods (Khongthaw et al., 2021). Conventional methods such as soxhlet, percolation, maceration, boiling, filtration, and infusion are currently used, but better results are obtained using green methods such as supercritical fluid extraction, pressurized liquid extraction (or accelerated solvent extraction), ultrasonic-assisted extraction, enzyme-assisted extraction, and microwave-assisted extraction methods. Since these green extraction methods can be used at high temperatures and pressures, extraction times are significantly shorter, so these methods are preferred.

It is known that oils and their components obtained from plants by different extraction methods have a wide range of pharmacological and biological activities (Getahun et al., 2020). In this section, various methods studied in recent years for the extraction and isolation of bioactive substances from plant seeds are examined and the advantages and disadvantages of these methods are presented comprehensively.

2. COMPARISON OF PROPERTIES AND YIELDS OF BIOACTIVE COMPONENTS IN EXTRACTS OBTAINED FROM VARIOUS PLANT SEEDS BY CONVENTIONAL AND NEW EXTRACTION METHODS AND THEIR POTENTIAL APPLICATIONS

Extracts contain many plant metabolites, including phenolics, flavonoids, and fatty acids the types and numbers of which vary depending on the type

of seed and the method by which it is obtained. Flavonoids have the highest concentration in extracts obtained from plant seeds, followed by fatty acids. These plant metabolites have beneficial medicinal effects for human health.

In this section, we will briefly discuss the methods most commonly used by researchers for the extraction of plant seeds in the reviewed manuscripts.

Soxhlet extraction is mainly used for the extraction of organic compounds from solid samples. It is desired that the compounds are thermally stable at the boiling temperature of the solvent. The most important disadvantages of Soxhlet extraction compared to other solid sample preparation techniques are that it requires a long time and uses a large amount of organic solvent. Making large amounts of solvent harmless is not only expensive, but also a source of environmental problems (Büyüktuncel, 2012).

Hydro-distillation is a type of distillation. In the hydro-distillation extraction application, the plant sample and water are mixed, then the mixture is heated. The essential substances in the heated mixture are carried in the steam, then the steam is condensed and the substances are separated. It is a widely used extraction method to separate phytochemical compounds from the plant sample.

Pressurized liquid extraction is a technique developed for extraction of solid and semi-solid samples. The most critical factor affecting yield and selectivity in pressurized liquid extraction is the temperature applied during extraction. By keeping the sample to be extracted in a high-pressure environment, higher temperatures are used for conventional solvents, ensuring that they remain in liquid form. Its disadvantages are its high cost and matrix dependency.

Microwave-assisted extraction is a technique used to extract active ingredients from medicinal plants by using microwave energy to heat sample-containing solvents, thereby extracting analytes from the sample matrix into the solvent. Working at high temperature increases the solubility of the analyte of interest and reduces solvent viscosity, leading to more efficient sample preparation and reduced extraction times. Microwave-assisted extraction is selective, fast and provides more accurate and precise results than other methods. The disadvantage is that the selected solvents absorb microwave radiation (i.e. polar solvents).

Ultrasound-assisted extraction is one of the most effective methods used in the extraction of plant samples, with high efficiency, short extraction time, low solvent consumption, but high sensitivity (Maksoud et al., 2021; Dai and Mumper, 2010). This method is based on the mechanical effect of the acoustic cavity caused by ultrasound, which leads to increased surface contact between solvents and increased permeability of the cell walls of plant samples. Therefore,

under the influence of ultrasound, the physical and chemical properties of cell walls change, facilitating the release of compounds from plant cells into the solvent (Dai and Mumper, 2010; Azwanida, 2015). Its disadvantage is that it requires large amounts of solvent and filtration.

Supercritical fluid extraction is a technique used to extract components from a solid or liquid material by using a supercritical fluid (such as carbon dioxide) above or below its critical point. Supercritical fluid, which can be an element, substance or mixture; temperature above the critical temperature and pressure above the critical pressure can be applied. Supercritical fluids can be compressed, so their density can be changed. The higher the density of a supercritical fluid, the higher its dissolving ability. High selectivity is achieved by manipulating the dissolving power by changing the temperature and pressure. Disadvantages include high cost, dependence on matrix, difficulty in extracting more polar analytes since CO_2 is nonpolar, and difficulty in extracting wet or liquid samples and solutions.

Enzyme-assisted extraction is one of the new methods that rely on enzymes to break down the cell wall of the plant sample and facilitate the extraction of bioactive substances found in the plant. Commonly used enzymes for this method include cellulase, pectinase and protease.

The aim of the researchers in the presented manuscripts was to extract oils by changing the extraction conditions to obtain maximum efficiency from plant seeds and to determine the extraction method quality by analyzing the physicochemical properties, fatty acid composition, antioxidant activity and chemical composition of these essential oils.

Fennel seeds grown in Egypt and Pakistan were extracted by two methods. The extracts were analyzed by determining the total phenolic content and using different antioxidant methods. Total phenolic content was obtained for Egyptian fennel and Pakistani fennel by supercritical fluid extraction under 206 bar pressure. Antioxidant values were found to be higher in Pakistani fennel seeds than Egyptian fennel by supercritical fluid extraction method as FRAP, ABTS and ORAC. Among the extraction methods they compared, the researchers found that supercritical fluid extraction had higher essential oil yield, anti-inflammatory and antioxidant activities in the analysis of Pakistani fennel with ethanol solvent (Tanveer et al., 2024).

In this study, the amounts of kaempferol, apigenin, rutin, quercetin, silibinin and taxifolin were investigated in the extracts obtained by ultrasonic-assisted, supercritical fluid and soxhlet extraction of Date seed grown in the Balıkesir region. Total bioactive compound amounts were found to be with ultrasonic-assisted, soxhlet and supercritical fluid methods. As a result of ultrasonic-assisted extraction taxifolin, silibinin, kaempferol, quercetin and rutin values were obtained, which are 3.46 times more effective than soxhlet

and 16.29 times more effective than supercritical fluid. It has been reported that jujube seeds reduce the chronic diseases and risk of cancer and may shed light on subsequent studies that the plant seed can be used for pharmaceutical purposes (Nuralın, 2024).

Soxhlet, ultrasonic-assisted, and supercritical fluid extractions were compared to study the effects on yield and composition of tea seed oil grown in Iran. The oil obtained by supercritical fluid extraction is better in appearance than the oils obtained by other extraction methods. Palmitic, stearic, oleic, linoleic and gadoleic fatty acids were determined in the oil samples. Researchers reported that supercritical fluid extraction is a very useful method to extract tea seed oil without organic solvent (Rajaei et al., 2005).

Nigella sativa L. seed oil is rich in polyunsaturated fatty acids and bioactive components. The quality, phenolic compounds, antioxidant activity and antimicrobial activity of *Nigella sativa* L. seed oil obtained by three extraction methods were investigated. It has been reported that the oil obtained by supercritical fluid extraction has high DPPH and ABTS activities and strong antimicrobial activity against microorganisms *Staphylococcus aureus* and *Penicillium roqueforti* (Albakry et al., 2023).

It was aimed to recover oils from apple seeds using three different extraction methods. Researchers reached the best optimization parameters to obtain an apple seed oil with the highest yield and rich in fatty acid components by using response surface methodology. Similar yields were obtained when compared with the ultrasound-assisted extraction method (17.20±2.3%), supercritical fluid (19.3±2.1%) and soxhlet (19.1±1.5%). It was reported that the oil obtained with the ultrasound-assisted extraction method was rich in high amounts of unsaturated fatty acids and two important antioxidants (phloretin and foridzin) (Gasparini et al., 2023).

Ultrasound-assisted extraction was compared with conventional methods to extract phenolic compounds from date palm (*Phoenix dactylifera* L.) pulp and seed. Total phenolic content was found to be (18.53 mg GAE/g) in ultrasound-assisted date seed samples extracted with ethanol. Phenolic content of conventionally extracted seed samples was found to be between 1.30-14.46 mg GAE/g. Phenolic content was found to be the highest in the part extracted with ultrasound-assisted extraction method of date seed samples treated with methanol solvent. These have been reported as syringic acid, epicatechin gallate, epicatechin, p-hydroxybenzoic acid and coumaric acid. When researchers examined the extracts obtained from date samples, they reported that the highest yield was obtained with ultrasound-assisted extraction (Shi et al., 2023).

The oil yield and bioactive compounds of camellia seed (*Camellia oleifera* C. Abel) extracts prepared by supercritical fluid, aqueous, pressing and solvent

extraction were investigated. The highest oil yield (92.42%) was obtained by supercritical fluid extraction, and the contents of 89.34, 3173.23 and 6.20 mg/kg polyphenol, β-sitosterol and squalene were analyzed, respectively. When the quality of camellia seed oil obtained by different methods was examined, it was reported that the highest quality oil was obtained by the supercritical fluid extraction method (He et al., 2023).

The yield, properties and composition of pomegranate seed oil were investigated by soxhlet and microwave-assisted, ultrasound-assisted, subcritical extraction. Oil yield was obtained between 11.32% and 15.66% with soxhlet. High punicic acid content was found in microwave-assisted and subcritical extraction samples. Squalene, β-sitosterol and tocopherols were found in pomegranate seed oil. Three tocopherols (α, γ and δ) were detected in pomegranate seed oil, with γ-tocopherol being dominant. While relatively higher squalene and β-sitosterol contents were obtained with ultrasound-assisted extraction, the highest tocopherol content was reported in oils obtained with subcritical extraction (Liu et al., 2022).

Apricot kernel oil yield was compared using three extraction methods. The optimum extraction conditions fatty acid composition consisted of stearic acid and palmitic acid. The total content of cis-linolenic acid and cis-oleic acid was found to be 93%. The highest oil yield was achieved with soxhlet extraction. However, it has been reported that the green extraction method, ultrasound-assisted method, is advantageous in increasing oil extraction yield by reducing processing time, using less solvent and preserving the quality of the extracted oil (Hao et al., 2022).

Bioactive compounds were obtained from roasted date kernel using various solvent systems and extraction methods. Extraction efficiency, flavonoid compounds and antioxidant activity of the extracts were investigated. Phytochemicals and antioxidants in *Phoenix dactylifera* L. cv Kabkab were found using water, aqueous ethanol, aqueous acetone systems and ultrasound-assisted, microwave-assisted extraction and maceration and decoction-infusion extraction methods, which are combinations of these two methods. Fewer phytochemicals were extracted in the aqueous acetone solvent system compared to other systems. Although the extraction performance was high in maceration, other methods were shorter than maceration. Among the new methods, the highest and lowest performances were found with ultrasound-assisted and microwave-assisted extraction methods (Pourshoaib et al., 2022).

Three different extraction techniques were compared for the extraction of hemp seed oils: microwave and ultrasound-extraction techniques and mechanical cold pressing extraction technique. Oil yield was found as 41.0±2.1%(w/w) - 54.0±2.7%(w/w). The free radical scavenging activity of the oils was examined and it was reported that it showed 94% activity. The

combination of unsaturated fat and saturated fatty acids in hemp seed oil and its antioxidant value revealed that this oil can be used as vegetable oil (Mookerjee et al., 2022).

Ultrasound-assisted and conventional extraction methods were used for the extraction of *Nigella sativa* L. seeds. Total phenolic content and DPPH radical scavenging activity values of black cumin seeds were found. Optimum extraction conditions were determined with 59.1% ethanol solvent, 44.6°C temperature and 32.5 min extraction time to increase the efficiency of phenolics in *Nigella sativa* L. seeds by ultrasound-assisted extraction. Thus, total phenolic content and DPPH radical scavenging activity were found to increase by compared to conventional extraction. Epicatechin was found between 1.88-2.37 mg/g and rutin was found between 0.96-1.21 mg/g in *Nigella sativa* L. extracts. It was reported that ultrasound-assisted method could be a suitable extraction method for the extraction of phenolics from *Nigella sativa* L. seeds (Gueffai et al., 2022).

Flaxseed oil is known as a good source of α-linolenic acid. The quality of this oil was compared by examining the extracts obtained from solvent, hot pressing, cold pressing and aqueous enzymatic extraction methods. The contents of cold and hot-pressed linseed oils were found. The total cyclolinopeptide content was found to be approximately twice that of the linseed extract obtained by aqueous enzymatic and solvent extraction. Cold pressed linseed extracts were shown to have high γ-tocopherol content. The oil yield of cold pressing was lower than that of aqueous enzymatic extraction. The phytosterol content of aqueous enzymatic extraction was reported to be higher than that of pressed oils. As a result, it was reported that the importance of commercial processing of flaxseed oil extracted by different oil extraction methods was shown by this study (Zeng et al., 2022).

A volatile compound was obtained from *Tamarindus indica* (tamarind) seed by hydro-distillation using Clevenger apparatus and soxhlet extraction. Essential oil samples were extracted by hydro-distillation and soxhlet extraction, similar number of chemical compounds were found in the oil samples. The major essential oil components obtained from both extraction methods were calculated at varying concentrations. It has been reported that hydro-distillation using Clevenger apparatus is a better method than soxhlet extraction in obtaining essential oil from *Tamarindus indica* seed (Fagbemi et al., 2021).

Fenugreek seed (*Trigonella foenum graceum*) diosgenin was obtained by two green extraction methods, microwave and ultrasound-assisted. The concentration for acetone, ethanol, hexane and petroleum ether solvents was varied as 40, 60, 80 and 100% and the processing time was varied as 1.5, 3.0, 4.5 and 6.0 minutes and 30, 40, 50 and 60 minutes for microwave and

ultrasound-assisted extraction methods, respectively. In relation to better yield extract and diosgenin content, the yield and amount of fenugreek seed extract were found to be 7.83% and 35.50 mg/100 g with microwave-assisted extraction at 80% ethanol concentration in 6 and 60 minutes, respectively; and 21.48% and 40.37 mg/100 g with ultrasound-assisted extraction. In this study, it was reported that ultrasound-assisted extraction was more successful in obtaining diosgenin content from fenugreek seeds than the other method (Arya and Kumar, 2021).

Soxhlet extraction and microwave-assisted extraction and methods were compared to obtain oil from *Sapindus mukorossi* seed. The optimum condition of microwave-assisted extraction method was found as n-hexane and ethanol mixture as extraction solvent, 460 W, 8 mL/g solvent-material ratio, 72 extraction temperature and 42 minutes. All oils obtained showed similar fatty acid and triglyceride profiles and thermal behavior. However, the oil obtained by microwave-assisted extraction was found to have better quality than the oil obtained by soxhlet extraction in terms of low acid value and peroxide value. The results obtained from this study indicated that microwave-assisted extraction is an ecologically harmless method. (Hu et al., 2021).

Four extraction methods were applied to extract green coffee oil from Arabica coffee beans. The physicochemical properties, yield, and composition of green coffee oil were investigated and the extracted oils were compared. The yield of ultrasound/microwave assisted extraction was found to be 10.58±0.32% and that of pressurized liquid extraction was found to be 6.34±0.65%. The main fatty acids in green coffee oil were shown to be linoleic acid and palmitic acid, ranging from 40.67% to 43.77% and 36.57% to 38.71%, respectively (Dong et al., 2021).

Three methods were compared for the extraction of phytochemical compounds from fennel seeds: Thermosonication, soxhlet and percolation methods. The best conditions for extraction from fennel seeds by thermosonication were reported as 300 W and 60°C. With this method, the highest phenolic substance content of fennel extract and antioxidant activities determined by DPPH and ABTS methods were found. It was reported that the thermosonication method is a superior method to obtain phytochemicals from fennel seeds compared to percolation and soxhlet extractions, with 73% and 88% less energy consumption, respectively (Urango et al., 2021).

The researchers aimed to analyze the bioactive molecules in Apiaceae seeds *Carum carvi* L., *Pimpinella anisum* L., *Coriandrum sativum* L. and *Foeniculum vulgare* Mill. var. vulgare. For seed oil yield, seeds were extracted by soxhlet, shake-assisted and pressurized liquid extraction. Coriander seed oil yield was reported as 12.30%; fatty acid content was petroselinic acid, oleic acid and lauric acid. Cumin seed oil was found to contain caproic fatty acid.

It was reported that the yield of oils obtained from soxhlet and shake-assisted extraction methods was high, while total sterol and triterpene contents were higher in seed oils obtained as a result of pressurized liquid extraction (Balbino et al., 2021).

To study Baru (*Dipteryx alata*) seed oil, extracts were obtained by two supercritical fluid extraction methods, one supported by cold pressing and one unsupported. The oil yield, extraction kinetics, baru seed oil composition and production cost were evaluated in the two compared methods. Cold pressing supported supercritical fluid extraction yield was found to be higher than supercritical fluid extraction yield. The amounts of unsaturated fatty acids and other bioactive compounds were found to be quite high in the obtained oil. It was reported that the extraction of baru seed oil by supercritical fluid extraction supported by cold pressing resulted in higher yield and lower production cost compared to supercritical fluid extraction (Chañi-Paucar et al., 2021).

Mango seed kernel oil was extracted with ethanol and n-hexane solvents using soxhlet and microwave-assisted extraction methods. Extractions were performed at microwave power levels of 120 and 240 W for 5, 10 and 15 minutes. The yields in soxhlet extraction with ethanol and n-hexane were found to be 18.00±0.25% and 9.38±2.03%, respectively. In microwave-assisted extraction, 6.69±0.05% and 4.68±0.06% were obtained, respectively. In addition, microwave-assisted extraction with 120 W microwave power level was observed to be 15 minutes less compared to 8-hour Soxhlet extraction. The best solvent-feed ratio in the study was found to be 60/6 for all processes. It has been reported that better yield was obtained when ethanol was used as the solvent, and quality oil was obtained when n-hexane was used (Balacuit et al., 2021).

Soxhlet extraction and supercritical fluid methods were compared in obtaining oils from apple seeds. It was reported that both methods were rich in linoleic acid. The extract obtained from supercritical fluid extraction (63.76±4.96 g/100goil) was found to contain higher linoleic acid than that obtained from soxhlet extraction (49.03±3.85 g/100goil). The highest amount of phenolic compounds found in the extract was reported as phloridzin. Researchers reported that they could not analyze amygdalin, in the seed oil obtained by supercritical fluid extraction (Ferrentino et al., 2020).

Essential oils of *Lepidium sativum* L. (cress) seeds were obtained by Clevenger type apparatus, modified simple distillation, simultaneous distillation and soxhlet extraction methods. Benzaldehyde and 1-isocyano-2-methylbenzene were obtained in the highest amounts with Clevenger type apparatus. Benzyl isothiocyanate, benzaldehyde and 1-isocyano-2-methylbenzene were obtained in the highest amounts with simultaneous

distillation extraction. The highest amount of linoleic acid was obtained with modified simple distillation. Palmitic, linolenic, stearic, arachidic, linoleic, oleic, gondolaic and behenic acids were obtained by soxhlet extraction. The essential oils showed bactericidal activity against *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumoniae* bacteria. The IC 50 value with DPPH was found as 15.69±0.72 mg/mL and the IC 50 value with H_2O_2 was found as 19.18±0.60 mg/mL (Getahun et al., 2020).

In the study where eight tocols (α, β, δ, γ tocopherols and α, β, δ, γ tocotrienols) were investigated in *Capparis ovata* seed, pressurized liquid extraction and soxhlet methods were used. The amounts of δ-tocopherol, γ-tocopherol and α-tocopherol calculated by the methanolic pressurized liquid extraction method are higher than those obtained by soxhlet extraction. In the present study, δ-tocotrienol was found to be 32 times higher than α-tocopherol, and γ-tocopherol was found to be 20 times higher. All tocols were analyzed in about 10 minutes. The limits of quantification values ranged from 0.29×10^{-4} to 9.60×10^{-4} mgmL^{-1}, and recoveries between 95.84% and 113.83% were reported for all eight tocols (Bakir et al., 2020).

Studies on extraction methods, optimized conditions, phenolics, antioxidant analysis used in plant seeds are shown in the table below.

Table: Comparative analysis of extraction methods and conditions, bioactive constituents and antioxidant activities of plant seeds

Plant material	Extraction conditions	Antioxidant determination methods	Analytical Methods	Functional activity	Bioactive constituents	References
Fennel seeds	**soxhlet extraction:** ethanol, ethyl acetate, acetone **supercritical fluid extraction:** CO_2, 172, 206, 241 bar, 60°C, 4 h	Folin-Ciocalteu FRAP ORAC ABTS	Spectrophoto meter	antioxidant anti-inflammatory	anethole, fecone, limonene and estragole	(Tanveer al., 2024)
Fruit seeds of jujube	**ultrasound-assisted extraction:** 70.6% ethanol/water (v/v), 1:15 g/mL (w/v), 60 min, 35°C, ultrasonic power 100 W and frequency 40 kHz **soxhlet extraction:** 1:15 g/mL (w/v), 6 h, ethanol/water 50% (v/v), **supercritical fluid extraction:** 52.5°C, 27.1 MPa pressure, 113.4 min, 5 L/min CO_2 and 0.44 mL/min ethanol		HPLC	cancer and chronic diseases	Taxifolin, silibinin, rutin, quercetin, and apigenin	(Nuralin, 2024)
Tea seeds	**supercritical fluid extraction:** CO_2; 60, 70 and 80°C; 300, 350 and 400 atm; 20, 30 and 40 min.; 7.5% and 15% ethanol **soxhlet extraction:** 150 mL petroleum benzene; 50–70°C; 7.5 h **ultrasound-assisted extraction:** 70 mL petroleum benzene; 50–70°C; 30 min.		GC		palmitic, linoleik, oleic, stearic and gadoleic fatty acids	(Rajaei et al., 2005)
Black cumin seeds	**cold pressing:** with a household degreaser **solvent extraction**: 480 min, 55°C, 500 mL hexane **supercritical fluid extraction:** 90 min, 35°C, 40 g/min solvent (CO_2, 100%), 35 MPa	DPPH ABTS	HPLC-PDA FTIR	antioxidant and antimicrobial activity	unsaturated fatty acids, tocopherols, phytosterols, polyphenols	(Albakry et al., 2023)
Apple seeds	**ultrasound-assisted extraction:** 500 mL hexane, 30 min, 70 W, 30°C, 1:15 sample-solvent; **supercritical fluid extraction:** 40°C, CO_2, 1 L/h, 26 MPa, 140 min **soxhlet extraction:** 3 h, 150 mL hexane	Folin-Ciocalteu DPPH	Spectrophoto meter GC-FID	Antioxidant activity; total phenolic content, fatty acid composition	Phloretin, Phlorizin, Amygdalin	(Gasparini et al., 2023)
Phoenix dactylifera L. (*Medjool*) seeds	**ultrasound-assisted extraction:** 40 mL (70% ethanol, 70% methanol, water), amplitude of 40%, 5 min, 8000 rpm, 4°C **conventional extraction:** 30 mL (70% ethanol, 70% methanol, water), 16 h, 120 rpm, 10°C	Folin-Ciocalteu DPPH FRAP ABTS	LC-ESI-QTOF-MS/MS HPLC-PDA Spectrophoto meter		phenolic acid, flavonoid, polyphenol	(Shi et al., 2023)

Camellia oleifera C. Abel seeds	**supercritical fluid extraction:** 30 MPa, 40°C, 2.5 h. **aqueous extraction:** material-liquid, 1:4; 60 min, 85°C **pressing extraction:** with screw press **solvent extraction:** n-hexane material-liquid, 1:7, 4 h, 45°C		HPLC FTIR		Tocopherol Squalene Polyphenol β-sitosterol Fatty acid	(He et al., 2023)
Pomegranate seed	**soxhlet extraction:** n-hexane or petroleum ether, 40°C **shaking extraction:** (n-hexane or petroleum ether) 1:6 (w/v), 120 rpm 40°C, 2 h **microwave-assisted extraction:** (n-hexane or petroleum ether) 1:6 (w/v), 500 W, 40 kHz, 9 min **ultrasound-assisted extraction:** (n-hexane or petroleum ether) 1:6 (w/v), 5000 rpm, 10 min, 25°C **subcritical extraction:** 45°C, 40 min, 0.6 MPa		GC-FID HPLC-MS GC-MS HPLC-FLD		punisik asit, skualen, β-sitosterol, tokoferoller (α, γ, δ)	(Liu et al., 2022)
Apricot kernel	**pressing extraction:** with cold pressing, 20 min. **ultrasound-assisted extraction:** solid/liquid (1:7) petroleum ether, 80°C, 240 W, 50 min. **soxhlet extraction:** with petroleum ether, 3h		GC-MS FTIR NMR		stearic acid, palmitic acid, cis-linolenic acid, cis-oleic acid,	(Hao et al., 2022)
Kabkab date seed (*Phoenix dactylifera* L.)	**maceration extraction:** with water, aqueous ethanol or aqueous acetone mixture for 48 h, 25±2°C **decoction-infusion extraction:** with a mixture of water, aqueous ethanol or aqueous acetone at 70°C for 120 min. **microwave-assisted extraction:** with a mixture of water, aqueous ethanol or aqueous acetone for 5 minutes at 400 W **ultrasound-assisted extraction:** with water, aqueous ethanol or aqueous acetone mixture at 40 kHz, 500 W, 25°C, 30 min.	DPPH ABTS FRAP Folin-Ciocalteu	Spectrophotometer			(Pourshoaib et al., 2022)
Hemp seeds	**microwave-extraction:** 90 mL n-hexane (sample-solvent 1:10), 65°C-70°C, 10-15-20-30 min. **ultrasound-assisted extraction:** 250 mL n-hexane (1:10 seed-solvent), 0.5 s, 24 kHz **cold pressing extraction:** with screw press	DPPH	GC-MS ^{1}H NMR UV-VIS spectrophotometer Electro Paramagnetic Resonance (EPR) spectrophotometer		Linoleic acid, polyunsaturated fatty acid, γ-Sitosterol, stigmasterol, rodoksantin, karoten, metil kolat, β-Karoten, γ-Tokoferol	(Mookerjee et al, 2022)

Black cumin seeds (*Nigella sativa* L.)	**ultrasound-assisted extraction:** 200 mL of ethanol/water (30, 60, and 90%, v/v), 25, 50, 75°C, 15, 30, 45 min, 110 W, 40 kHz **conventional extraction:** 200 mL 50% (v/v) ethanol, 2 h, 50°C	DPPH Folin–Ciocalteu	HPLC-UV		epicatechin, rutin	(Gueffai et al., 2022)
Flax seeds (*Linum usitatissimum* L.)	**solvent extraction:** n-hexane (1:10 m/v), 2 h **the hot pressing:** 150°C, 15 min. **the cold pressing:** 60°C, 10 min. **the aqueous enzymatic extraction:** with water (1:5 m/v), 60°C alkaline protease (1.5 % w/w) pH of 9.00, 4 h, cellulose (1.5% w/w) pH of 5.00 4 h, 50°C		GC-FID HPLC-QTOF-MS		cyclolinopeptides, γ-tocopherol, phytosterols, squalene, phospholipid	(Zeng et al., 2022)
Tamarindus indica seeds	**soxhlet extraction:** petroleum ether (500 mL), 6 h, 30 and 60°C **hydro-distillation with the Clevenger:** 800 mL distilled water, 3 h		GC-MS		cis-vaccenic acid, 2-methyltetracosane, beta-sitosterol, 9,12-octadecadienoic acid (Z, Z)-, and n-hexadecanoic acid	(Fagbemi et al., 2021)
Fenugreek seed *(Trigonella foenum graceum)*	**microwave-assisted extraction:** 1:5 (w/v) acetone, ethanol, hexane and petroleum ether 1.5, 3.0, 4.5 and 6.0 min, 180 W **ultrasound-assisted extraction:** 1:5 (w/v) acetone, ethanol, hexane and petroleum ether 30, 40, 50 and 60 min, 30°C					(Arya and Kumar, 2021)
Sapindus mukorossi seeds	**microwave-assisted extraction:** 1 W-100 W, -40°C-500°C, 0–160 min, 2450 MHz **soxhlet extraction:** 360 min, 80°C, 200 mL n-hexane				oleic acid, eicosenoic acid, arachidic acid, linoleic acid	(Hu et al., 2021)
Arabica coffee beans (green coffee oil)	**ultrasound-assisted extraction:** 40 kHz, 50 W, ethanol 1:30 (g/mL), 35°C, 50 min **microwave-assisted extraction:** 100 mL ethanol, 60°C, 30 min, 200W **ultrasound/microwave-assisted extraction:** ethanol (solid:liquid ratio: 1:28 g/mL), 60°C, 10 min, 50 W	Folin-Ciocalteu DPPH ABTS FRAP	FTIR		cafestol, kahweol, α-tocopherol	(Dong et al., 2021)

	pressurized liquid extraction: 40 mL ethanol, 100 bar, 100°C, 30 min					
Fennel (*Foeniculum vulgare*) seeds	**thermosonication-assisted extraction:** 100, 200, 300, and 400 W; 40, 50, and 60°C; 15 min, 20 g ethanol **soxhlet extraction:** 60°C, 200 rpm, 90 g ethanol, 6 h **percolation:** 180 g ethanol, 6 h	Folin-Ciocalteu DPPH ABTS	spectrophotometer			(Urango et al., 2021)
Foeniculum vulgare Mill. var. vulgare, *Pimpinella anisum* L., Cumin (*Carum carvi* L.) and *Coriandrum sativum* L. seeds from Apiaceae family	**soxhlet extraction:** 100 mL hexane, 8 h **agitation assisted extraction:** 40 mL hexane, 30 min, room temperature, 1000 rpm **pressurized liquid extraction:** 25 and 100°C, 10 min, 10.34 MPa		GC-FID		Coriander seed: petroselinic and oleic, Caraway seed: caproic fatty acid, Anise seed: spinasterol and Δ7-stigmastenol	(Balbino et al., 2021)
Baru (*Dipteryx alata*) seeds	**supercritical fluid extraction:** 5 minutes, 150, 200, 250, 300 and 350 bar, 35 and 45°C **supercritical fluid extraction assisted by cold pressing:** cold pressing by applying a torque of 40 Nm		TLC GC-FID		Monounsaturated fatty acids, polyunsaturated fatty acids, saturated fatty acids	(Chañi-Paucar et al., 2021)
Mango seed kernel	**soxhlet extraction:** solvent (mL)/feed (g): 75/12, 75/10 and 60/6 mL absolute ethanol and 95% n-hexane **microwave-assisted extraction:** solvent (mL)/feed (g): 75/12, 75/10 and 60/6 mL absolute ethanol and 95% n-hexane; 5, 10 and 15 min., 120 and 240 watts		FTIR GC-MS		Oleic acid, Propanoic acid, Palmitic acid, Oxalic acid	(Balacuit et al., 2021)
Apple seeds	**supercritical fluid extraction:** 7.5 to 35 MPa, CO_2 flow rate 1 to 8 L/h, 25 to 90°C **soxhlet extraction:** 150 mL n-hexane, 6 h, boiling temperature	Folin–Ciocalteu DPPH FRAP	HPLC-MS		Arachidic acid, Linolenic acid, Linoleic acid	(Ferrentino et al., 2020)

3. CONCLUSION

In this section, articles examining the effectiveness of extraction methods, the amount, content and antioxidant capacity analyses of phenolic compounds in extracts obtained from plant seeds have been evaluated.

Sustainable environment and the possibility of performing cost-effective extraction methods with new technologies have directed researchers to green extraction methods. The most important reasons why researchers are turning to advanced extraction techniques are that these methods are suitable for automation, shortening extraction time, reducing organic solvent consumption, preventing pollution in analytical laboratories and reducing sample preparation costs.

Plant seeds are the most effective part of the plant from which herbal medicines are obtained. For this reason, obtaining extracts beneficial to human health has always attracted the attention of researchers. Researchers primarily prefer extraction from separation and purification methods to isolate and identify active substances from various parts of the plant, seeds. When deciding on the extraction method according to the plant sample, it is necessary to pay attention to the quality and efficiency of the bioactive substances and essential oils desired to be obtained. In order to choose the most effective extraction method, it is very important to evaluate the amounts calculated as a result of the analysis and compare them with the results of other methods to make the right decision.

In general, this section, which emphasizes that different extraction techniques (such as soxhlet extraction, hydro-distillation, pressurized liquid extraction, microwave-assisted extraction, ultrasound-assisted extraction, supercritical fluid extraction, enzyme-assisted extraction) are very important in determining the quality of extracts obtained from plant seeds, will benefit many researchers interested in this subject.

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CHAPTER 2

THE IMPORTANCE OF ZETA POTENTIAL IN BIOLOGICAL CELLS

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Introduction

As its simplest characterization, biological cells consist of a membrane and cytoplasm. The membrane exhibits distinct surface charges on its inner and outer layers. The ionic composition of the cytoplasm differs from that of the surrounding extracellular fluid, primarily as a result of the activity of membrane pumps. Additionally, charged macromolecules, including sialic acid, phospholipids, and integral proteins, collectively confer a net negative charge to the membrane, contributing to a surface charge that attracts counterions and repels coions (Weiss & Zeigel, 1971).

The membrane potential (*Vm*) is the difference in ion concentrations between the intracellular and extracellular compartments, which generates an electric potential across the membrane. In human cells, *Vm* is dynamic, fluctuating in response to cellular activities. The term 'potential' refers to this condition because it represents a form of stored energy, specifically potential energy. When opposing electrical charges, such as ions, are separated by a membrane, they have the potential to move toward each other, depending on the membrane's permeability. A membrane exhibiting this potential is considered polarized, characterized by a negative pole, where negative ions predominate, and a positive pole, where positive ions are more abundant. The magnitude of the potential difference between these poles is quantified in volts (V) or millivolts (mV), typically measured using a voltmeter. The polarity of a membrane's voltage reflects the charge on the inner surface of the polarized membrane. For example, a negative voltage, such as -60 mV, signifies a potential difference of 60 mV, indicating that the inner membrane is negatively charged relative to the outer surface. Conversely, a positive voltage indicates that the inner membrane is positively charged, while the outer membrane is negatively charged (Moini, Avgeropoulos, & Samsam, 2021). In excitable cells, particularly neurons, the regulation of *Vm* is crucial for facilitating long-distance electrical signaling. Neurons integrate excitatory and inhibitory synaptic inputs to generate all-or-none action potential spikes. Over the past decade, numerous fields of study have explored the role and significance of *Vm* in a variety of cell types beyond neurons (Adee, 2023). For instance, the functions of *Vm* have been demonstrated in fibroblasts in circadian rhythms, vascular smooth muscle cells and myofibroblasts in contractility, cochlear outer hair cells in hearing, pancreatic β cells in secretion, stem and cancer cells in proliferation, B and T cells in the cell cycle, melanocytes in cancer progression, corneal epithelial cells in migration and wound healing, and skin melanocytes in pigmentation (Aguilar-Bryan & Bryan, 1999; Amigorena, Choquet, Teillaud, Korn, & Fridman, 1990; Ashmore, 2008; Bellono, Kammel, Zimmerman, & Oancea, 2013; Blackiston et al., 2011; Chernet, Levin, & oncology, 2013; Chifflet, Hernández, & Grasso, 2005; Messenger & Warner, 1979; Neher & Sakmann, 1976; Nelson & Quayle, 1995; Noguchi, Wang, Pan,

& Welsh, 2012; Reid & Zhao, 2014; Wonderlin, Woodfork, & Strobl, 1995).

Conventionally, *Vm* has been evaluated using electrode-based methodologies, including whole-cell patch clamp and extracellular recording techniques. Although the patch clamp method remains the gold standard in electrophysiology due to its sub-millisecond temporal resolution and sub-millivolt sensitivity, it has several disadvantages, including significant invasiveness, inadequate spatial resolution, and limitations in the ability to simultaneously record from multiple neurons (Lazzari-Dean, Gest, & Miller, 2021). The advent of microelectrode probes, such as ultrathin complementary metal oxide semiconductor (CMOS) arrays, has substantially enhanced measurement throughput by at least three orders of magnitude (Shahrjerdi & Bedell, 2013). For example, the Neuropixels 2.0 probe features over 5,000 recording sites distributed along four narrow shanks, each approximately 1 cm in length. Nonetheless, like other extracellular recording methods, microelectrode arrays typically provide only spike timing information and do not measure sub-threshold voltage fluctuations (Steinmetz et al., 2021).

As described by the Goldman-Hodgkin-Katz (GHK) equation, electrophysiology focuses on potentials arising from differential ion concentrations-particularly of sodium, potassium, and chloride-between the intracellular and extracellular compartments (Goldman, 1943; Hodgkin & Katz, 1949). This approach largely overlooks the alterations in ion fluctuations resulting from charges on the cell membrane and also neglects the electric fields within the intracellular and extracellular areas, assuming that the electric field exists only within the membrane (Radványi et al., 2024). In contrast, fields such as electrochemistry and surface science frequently examine deviations in anion and cation concentrations from electroneutrality as one approaches a surface in an aqueous medium. These findings are derived from the works of Gouy, Chapman, Debye, Stern, and others, and are widely employed in the field of solution science to assess the stability of colloidal solutions. Specifically, they are used to evaluate whether colloidal solutions are stable or the likelihood of suspended particles aggregating into clusters (Lyklema & Leeuwen, 1991).

In discussing this scenario, an additional potential arises alongside the *Vm*, referred to as the zeta (ζ) potential. In the mid-18th century, electrokinetic phenomena, including streaming potential, electro-osmosis, and electrophoresis, were identified. In 1809, Ferdinand Friedrich Reuss documented the migration of colloidal particles in response to an applied voltage, a process now referred to as electrophoresis, as well as the flow of water through a clay plug when subjected to electrical voltage, known as electro-osmosis (Biscombe, 2017). Helmholtz was the first to describe the correlation between streaming potential and the electrical potential differences at the solid-liquid interface, utilizing the concept of the electrical double layer (EDL) model (Helmholtz, 1853). The term "zeta" (ζ) was introduced in 1904 by

Jean Baptiste (Předota, Machesky, & Wesolowski, 2016). Although there have been efforts to merge the principles of surface science with electrophysiology, these two fields have predominantly been treated as distinct entities. The Goldman-Hodgkin-Katz (GHK) equation describes the V_m as a result of ion diffusion across the membrane. In contrast, surface science has been employed to establish the ion concentrations on either side of the membrane, based on the surface potentials present on the inner and outer layers of the membrane. Moreover, it was suggested that V_m and ζ-potential are unaffected by each other. However, recent studies, such as that by Miyake et al. (Miyake & Kurihara, 1983) have found that the *Vm* of mouse neuroblastoma cells shows a significant relationship with ζ-potential, suggesting that the latter may influence the former. Similarly, Halder et al. investigated the impact of pharmacologically altering bacterial membrane permeability on ζ-potential and concluded that the observed changes could be due to modulation of *Vm*. However, it was previously assumed that changes in surface potential affected the *Vm*, rather than the reverse (Aiuchi, Kamo, Kurihara, & Kobatake, 1977).

Surface and Zeta (ζ) **Potentials**

The charge present on the surface of a particle influences the distribution and concentration of ions in the adjacent interfacial region, leading to the repulsion of like charges and the attraction of countercharges, as described by the Poisson-Boltzmann theory (Lyklema & Leeuwen, 1991).

$$c_i(0) = c_{0i} \exp\left[\frac{-z_i e \Psi_0}{kT}\right]$$

The equation shows how the ion concentration varies due to the electrostatic potential. Where the concentration $c_i(0)$ of ions *i* at a reference point, c_{0i} is the bulk concentration, z_i the valency, e is the electric charge, ψ_0 is the surface electrostatic potential due to surface charge, k is the Boltzmann constant, and T is the temperature.

An increased concentration of counterions forms an EDL around the particle. The charged surface modifies the charge distribution at the interface by attracting counterions from the bulk solution. This charge distribution at the interface generates a surface potential that varies with distance. Consequently, in the medium, a particle is surrounded by two distinct layers: the inner layer, where counterions are strongly bound, and the outer (diffuse) layer, where ions are less tightly associated.

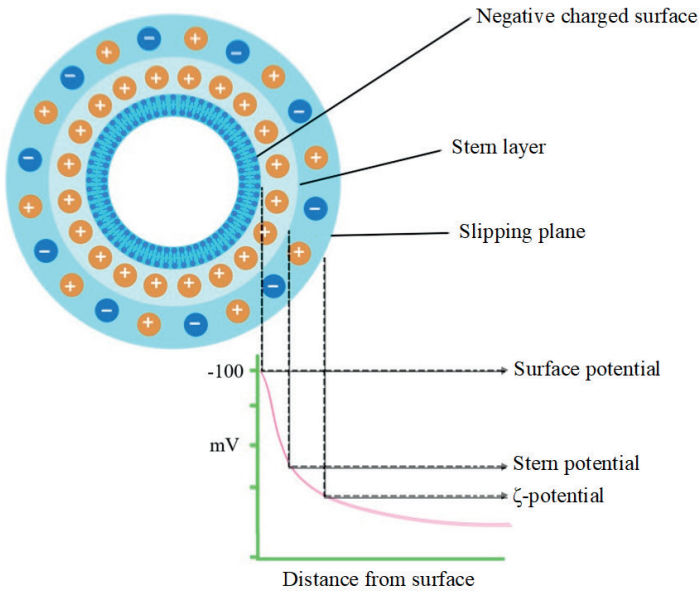

Figure 1. *Description of ζ-potential and different layers of charges around a cell in aqueous environment*

The inner layer is called the Stern layer. ψ_s is Stern potential at the end of this layer, which is influenced only by the surface potential and not by the ion concentration in the bulk medium (Stern, 1924). The outer layer is called the Shear plane, and it contributes to the stability of the particle while allowing the medium to flow freely. When a particle moves, such as due to Brownian motion or electrostatic interactions, the ions contained within this boundary move along with the particle; conversely, ions situated outside this boundary do not move in tandem with the particle. This boundary is referred to as the slipping plane or surface hydrodynamic shear. The electrical potential present at this boundary is termed the ζ-potential. The magnitude of the ζ-potential is a determinant of the stability of a colloidal system (Fatehah, Aziz, Stoll, & Biotechnology, 2014; Kamble et al., 2022). The ζ-potential represents the electric potential at the slipping plane relative to a point in the bulk medium, and it should not be conflated with the Stern potential or the surface potential, as these are defined at distinct locations on and around the particle (Figure 1). Ions situated within the Stern layer and the slipping plane are primarily transported along with the particle during transport processes, which leads to the common interpretation of the ζ-potential as the effective charge of the particle. Particles with similar charges exhibit a reduced tendency to agglomerate as their absolute ζ-potential increases. Generally, slurries with elevated particle

ζ-potentials are considered colloidally stable. The measurement of ζ-potential, which governs particle mobility, is a critical tool for understanding phenomena such as agglomeration, dispersion, coalescence, coagulation, and separation (Pate & Safier, 2022). Table 1. shows ζ- potential range and stability guidelines as a rule of thumb (33).

Table 1. *Zeta (ζ) potential range and stability guidelines (Nimesh, Chandra, & Gupta, 2017)*

Zeta (ζ) Potential (mV)	**Stability Behaviour of the Colloids**
0 to ±5	Rapid coagulation or flocculation
±10 to ±30	Incipient instability
±30 to ±40	Moderate stability
±40 to ±60	Good stability
>±60	Excellent stability

The potential within the medium beyond the Stern layer decays exponentially, approaching the Debye screening length. This length marks the threshold at which the influence of surface charge on ion concentration becomes negligible compared to the ion concentration in the bulk medium. The Debye screening length is defined by the following equation:

$$1/k = \sqrt{\left(\frac{\varepsilon RT}{2czF^2}\right)}$$

k is the Debye-Hückel parameter, which depends on the ionic strength of the solution and the charge of the ions, ε is dielectric constant, R is a distance from the surface, T is the absolute temperature, c the electrolyte concentration ($mol.m^{-3}$), z is the counterion valency, and F is Faraday's constant.

Gouy and Chapman proposed a model that describes the charge density near the surface. Stern later enhanced this model by incorporating the finite size of ions, which accounts for both electrostatic and van der Waals forces acting on the surface. This leads to the establishment of a fixed plane, known as the Stern plane (Svarovsky, 2000).

$$\psi(r) = \psi_{st} e^{-(kr)}$$

$\psi(r)$ is an electrical potential as a function of distance from the surface charge into an electrolyte, ψ_{st} is Stern potential, r is distance (Hodgkin & Katz, 1949).

Factors Affecting Zeta (ζ) Potential

The ζ- potential is influenced by primarly solvent parameters such as pH and the presence of ions.

1. **pH**

The ζ-potential is a key parameter that governs the stability of colloidal systems, including suspensions in biological environments such as cells (Morishita, Park, & SCIENCES, 2009). In suspensions with a negative ζ-potential, the addition of OH^- ions leads to an increase in the negative charge on the particles. In contrast, the addition of H^+ ions leads to the protonation of the surface, neutralizing the negative charge. At specific concentrations of H^+ or OH^-, the overall charge reaches neutrality, after which further acid addition induces a positive charge. It is well-established that the ζ-potential is negative at high pH and positive at low pH levels (Berg, Romoser, Banerjee, Zebda, & Sayes, 2009). The isoelectric point corresponds to a state of instability for the colloidal system. This point can be determined by varying the pH through the controlled addition of OH^- and H^+ ions. In this case, the isoelectric point occurs at pH 5.5, with colloidal stability observed at pH values below 4 (where a dominant positive charge is present) or above 7.5 (where a dominant negative charge is present) (Moayedi, Asadi, Moayedi, Huat, & Kazemian, 2011).

2. **The Presence of Ions**

Fluctuations in ζ- potential relative to the concentration of formulation components provide insights into product stability. Sometimes, the presence of known impurities or contaminants can modify the ζ-potential, thus playing a significant role in preventing flocculation. Controlled drug release is a critical factor in the administration and transport kinetics of pharmaceuticals, such as ezetimibe, classified as a Class II drug in the Biopharmaceutical Classification System (Borkhataria et al., 2020; Kasthuri, Rajendiran, & Biointerfaces, 2009).

Importance of Zeta (ζ) Potential in Different Cell Types

1. Red Blood Cells (RBCs)

Red blood cells (RBCs) possess unique properties, attributed to glycoproteins embedded within their fluid lipid bilayer (Goldhaber & Grasso-Correnti, 2002). Sialylated glycoproteins on the Red Blood Cell (RBC) membrane provide a negatively charged surface, establishing a ζ-potential

between cells. This repulsive charge mechanism helps inhibit interactions between RBCs and other cells, thereby preventing them from aggregating. A high ζ-potential is associated with increased blood stability, whereas a low ζ-potential promotes particle agglomeration, leading to the initiation of hemagglutination at reduced ζ-potential values. In general practice, packed red blood cells used for transfusion are stored for 35 to 42 days. However, during this cold storage period, RBCs undergo gradual negative changes, collectively referred to as 'storage lesions. The reversibility of these storage lesions presents a significant challenge in RBC storage and transfusion research. Regulating the ζ-potential through modulation of ATP and 2,3-bisphosphoglycerate concentrations is essential for restoring RBC functionality (Barshtein et al., 2023). Research on human RBCs has been conducted for applications in drug delivery and transfusion medicine. The nanoparticles used in these studies maintained a sustained high ζ-potential in blood medium. The results indicated that the functionalization of particles significantly affects their stability, preventing membrane rupture and hemolytic activity. Therefore, it is imperative for blood banks to maintain optimal storage conditions, as leukodepleted or agglomerated blood is not suitable for transfusion. Thus, monitoring the ζ-potential is crucial for assessing the stability of stored blood (Silva et al., 2012).

2. White Blood Cells (WBCs)

The ζ-potential of macrophages undergoes changes during the activation process. Specifically, activated macrophages transition from a ζ-potential of -21.8 mV in the resting state to −17.8 mV upon activation, with this change correlating with their phagocytic capacity (Chakraborty et al., 2020). Similarly, the *Vm* of control macrophages is −39.0 mV, whereas activated macrophages exhibit a potential of −23.1 mV. Moreover, the activation of T lymphocytes is modulated by *Vm*, which is influenced by potassium channels, with activated T cells depolarizing from −56 mV to −40 mV (Rader et al., 1996). Considering the importance of ζ-potential in influencing suspensoid interactions at the point of contact—relevant during phagocytosis and other immune responses—it is suggested that further investigation into T-cell ζ-potential is warranted.

3. Platelets

The electrical characteristics of platelets, particularly their transition from a resting to an activated state, have been extensively investigated. Both *Vm* and ζ-potential are known to be influential factors in the modulation of platelet adhesion. Palés et al. (Palés, López, & Gual, 1988) reported that platelets exhibit a resting voltage of −63.8 mV after venepuncture, which rapidly depolarizes to −35 mV when stored in iced water for one hour. This depolarization effect reversed upon rewarming to physiological temperature. Depolarized platelets exhibited increased aggregation in response to activating agents such as

adrenaline, collagen, or Adenosine diphosphate (ADP). Similarly, Krötz et al. (Krötz et al., 2004) found that hyperpolarization of platelets inhibited their adhesion to substrates. However, neither study proposed a mechanism to explain the relationship between *Vm* and platelet aggregation. Although fewer studies have measured ζ-potential in platelets, Coller (Coller, 1983) was the first to demonstrate that activated platelets alter their ζ-potential, thereby enhancing their capacity for adhesion and aggregation. The proposed mechanism for this alteration involves the reconfiguration of surface charge due to changes in the composition of specific phospholipids in the outer membrane layer.

Contribution of Zeta (ζ) Potential in Medical Applications

1. Drug Delivery

The rapid cellular uptake of pharmaceuticals is often associated with significant adverse effects and toxicity (Ernsting, Murakami, Roy, & Li, 2013). The ζ-potential plays a critical role in regulating the physicochemical interactions at the cellular level. A high positive charge can enhance the rate of uptake, potentially leading to harmful consequences for the cell and compromising the therapeutic efficacy of the drug. Consequently, it is essential to modify nanoparticles appropriately using functional polymers. The ζ-potential serves as a valuable metric for assessing the equilibrium between negative and positive charges on the drug, thereby facilitating an efficient uptake and release process (Bernfield et al., 1999).

2. Medical Treatment

Magnetic hyperthermia, a radiative treatment for cancer, uses heat to target and treat cancer cells while exposing the body to electromagnetic radiation. In this method, a magnetic substance is introduced to absorb radiation and convert it into heat, effectively treating the cancer-affected cells. Although iron oxide is an inexpensive and promising material for this application, its colloidal stability is crucial for ensuring effectiveness (Hildebrandt et al., 2002).

In conclusion, the ζ-potential plays a crucial role in the stability, functionality, and interactions of biological cells, as well as in various medical and pharmaceutical applications. Its influence extends across a broad spectrum, from modulating cell membrane interactions and the stability of colloidal systems to impacting processes like drug delivery and cancer treatment. The ability to manipulate and measure ζ-potential is vital for optimizing therapeutic strategies, such as ensuring efficient drug uptake, minimizing toxicity, and enhancing the stability of treatments like magnetic hyperthermia. Given its significance in cellular behavior, it is essential to continue exploring and leveraging ζ-potential to advance both biological research and clinical applications.

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CHAPTER 3

A NEW MORPHOMETRIC ANALYSIS METHOD IN NEUROSCIENCE: EVALUATION OF NEUROANATOMICAL REGIONS IN NEURODEGENERATIVE DISEASES USING THE QUINT WORKFLOW

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Neuroscience is an interdisciplinary field that studies the brain and the nervous system. It aims to understand the fundamental characteristics of neurons and neuronal circuits. Psychiatry, computational sciences, genetics, and many other interdisciplinary areas converge under the umbrella of neuroscience to study the development, anatomy, and functions of the nervous system. Over time, the scope of neuroscience has greatly expanded, from molecular and cellular studies of neurons to the neuroimaging of sensory, motor, and cognitive functions in the brain (Gillath, Karantzas, & Fraley, 2016). Thanks to these advancements, extensive research is being conducted in many areas, from the formation mechanisms to the diagnosis and treatment of neurodegenerative diseases, whose structure remains largely unknown. Particularly in the last century, promising developments related to many neurodegenerative diseases have emerged.

Neurodegenerative diseases are a mixed group of disorders characterized by progressive neuronal degeneration and loss in different subregions of the nervous system (Agnello et al., 2021; Cheng, Lin, & Lane, 2021). Although the exact pathogenesis of neurodegenerative diseases remains ambiguous, it is observed that epigenetic, genetic factors and environmental conditions contribute to the onset of these diseases. Currently, no effective treatment has been found to prevent, slow down, or stop the progression of these diseases. Therefore, molecular mechanisms that may be related to the pathogenesis of neurodegenerative diseases continue to be investigated with great seriousness. In recent years, the rapid aging of the population has also increased the risk of the onset of neurodegenerative diseases. Particularly, dementia is one of the most feared neurodegenerative diseases in this context (Rossor, Fox, Mummery, Schott, & Warren, 2010). Although dementia is not observed in every elderly individual, the number of people with this disease is rapidly increasing. The mechanisms that lead to the onset of dementia are numerous. Additionally, due to overlapping symptoms observed in individuals, the definition of different types of dementia is quite complex. This situation highlights the need for personalized diagnosis and treatment protocols for each patient. However, personalized diagnoses and treatments require in-depth knowledge about each type of dementia, along with a multidisciplinary approach (Marcos-Rabal et al., 2021). Among the most common types in the world are Alzheimer's disease, Parkinson's disease, and Amyotrophic Lateral Sclerosis (Marcos-Rabal et al., 2021; Wilbur, 2023).

In neurodegenerative disorders, different types of atrophy or increases in brain ventricular volume are typically observed in disease-specific groups, involving the cerebral cortex, basal ganglia, brainstem, cerebellum, and spinal cord. For example, in Alzheimer's Disease, a commonly observed form of dementia, atrophy in brain tissue, along with an increase in ventricular volume, has been reported, particularly in the hippocampus, entorhinal cortex, cingulate

gyrus, structures of the limbic system, and the parietal lobe (Fayed, Modrego, García-Martí, Sanz-Requena, & Marti-Bonmatí, 2017; Mak et al., 2016; Saka, Dogan, Topcuoglu, Senol, & Balkan, 2007). In hypokinetic diseases such as Parkinson's Disease, morphometric and molecular changes are primarily observed in the basal nuclei, including the putamen, globus pallidus, substantia nigra, subthalamic nucleus, and red nucleus (Sulkava, Haltia, Paetau, Wikström, & Palo, 1983). These neurodegenerations may also be accompanied by atrophy of the cerebellum and its pathways. In diseases affecting the spinal cord, such as Amyotrophic Lateral Sclerosis, lesions are observed in the posterior segments of the spinal cord (Cummings & Zoghbi, 2000).

The neuronal losses and morphometric changes observed in neurodegenerative diseases have shown researchers the need to focus more on biochemical and structural changes in the brain. Studies aimed at understanding neurodegeneration, using the human brain or experimental models, remain a prominent area of research today (Blixhavn et al., 2024; Zhang et al., 2023). When using stereological approaches or image-based cellular analyses, determining the anatomical regions' locations and quantifying cells are crucial for the validity of findings. However, since these classical methods require very detailed manual identification of anatomical regions, they have limitations. Therefore, neuroscientists have long used brain atlases of the human brain or experimental animals to conduct their analyses (Goerzen et al., 2020; Nie et al., 2013).

Rodent animal models are invaluable research tools for explaining the pathways and mechanisms involved in the development of neurodegenerative diseases (Gurdon et al., 2023; Javanmard et al., 2024). Stereotaxic atlases are frequently preferred for rodent brains, particularly (Paxinos & Franklin, 2019; Paxinos, Petrides, & Evrard, 2023; Paxinos & Watson, 2006). During the use of these atlases, specific anatomical landmark points are used to guide procedures, such as injections or tissue sectioning. The section templates, determined based on their distance from these landmarks, are presented in the atlas. Researchers accept their sections as being in alignment with these templates when conducting their evaluations. However, during tissue sectioning in laboratory conditions, axial shifts often occur. These shifts create issues when aligning the sections exactly to the template, leading to errors in measurements within the desired anatomical regions. To address this need, 3D atlases have been introduced in the literature (Johnson, Calabrese, Badea, Paxinos, & Watson, 2012; Kleven et al., 2023; Wang et al., 2020).

The principle behind 3D atlases is to identify the location of anatomical structures on histological and radiological sections, either taken randomly or based on landmarks. Neuroimaging data are analyzed through atlas-based workflows. Atlas coordinates serve as spatial references that can be read by machines. When combined with atlas terminology, these references facilitate

the automatic analysis of data registered in the atlas. Atlas-based analyses rely on the spatial registration of anatomical locations assigned to each pixel or voxel in histological section images or MRIs. These workflows generally consist of three steps: 2D/3D image data registration to an brain atlas, extraction of features, digitization or rendering of extracted features (Liu et al., 2023; Shi et al., 2023). While existing 2D atlases neglect the shift in the third axis, researchers using 3D atlases can accurately analyze anatomical structures in their sections by adjusting the correct angle on each axis. To overcome these limitations and analyze atlas registrations, a QUINT workflow has been developed for quantitative and qualitative analysis using 3D reference atlases based on rodent brain image series (Whilden, Chevée, An, & Brown, 2021; Yates et al., 2019).

The QUINT workflow is a software package designed to support the atlas-based measurement of labeled areas in serial histological images taken from mouse or rat brains. All software tools have graphical user interfaces and do not require coding skills. In addition to visualizing cells in the anatomical region from the 3D atlas, the QUINT workflow also measures object counts and object areas. Currently, the software package includes atlases such as the Allen Mouse Brain Atlas version 3 (2015 and 2017) (CCFv3), the Waxholm Space Sprague Dawley Rat Brain Atlas versions 2, 3, and 4. The QUINT workflow can be used for cell counting and distribution, as well as for the assessment of anatomical structures (Puchades, Csucs, Ledergerber, Leergaard, & Bjaalie, 2019; Zhang et al., 2023)

A. QUINT Workflow Organization Diagram and Process Steps

The QUINT workflow is an analysis method used for microscopic data of rodent brains. By utilizing a reference brain atlas based on the rodent species, it allows for brain-wide mapping and regional quantification. The QUINT workflow takes as input a series of microscopic brain section images from the rodent, and outputs the number of labeled objects, the area ratio per atlas region, and coordinates to visualize objects within the three-dimensional atlas space (Figure 1.).

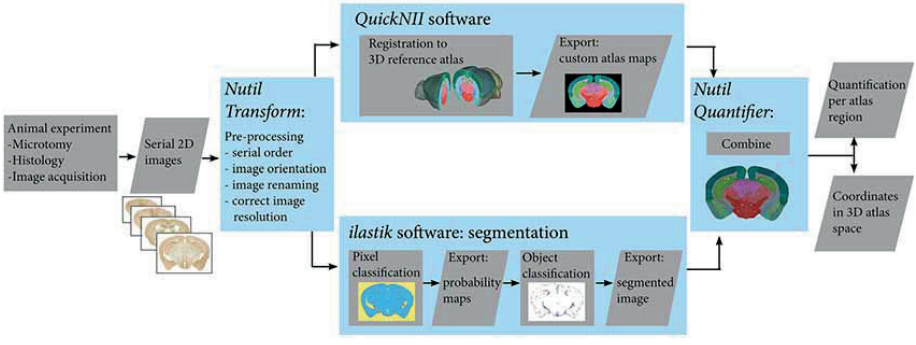

Figure 1. *Workflow Organization of QUINT (Yates et al., 2019)*

The process steps can be outlined as follows:

1) The image must be formatted to match the workflow using Nutil or another image analysis tool (Groeneboom, Yates, Puchades, & Bjaalie, 2020) (Figure 2.). QuickNII supports 24-bit .png (Portable Network Graphics) and .jpeg (Joint Photographic Experts Group) formats. Images up to 16 megapixels (e.g., 4000x4000 or 5000x3000 pixels) can be uploaded, but the optimal image area for QuickNII is approximately 1500x1000 pixels. For the workflow, each image file should be saved on the computer with the "_s00xx" suffix. This is due to the program scanning for the "_s" code.

2) Histological section images are registered to the reference templates according to the appropriate atlas using QuickNII, VisuAlign, or DeepSlice (Figure 2.). Axial registration is performed using QuickNII, followed by registration improvement using VisuAlign's planar methods. Additionally, the coronal rat brain sections are automatically registered using DeepSlice's axial registration step (Carey et al., 2022; Puchades et al., 2019). The data obtained from this process are saved in .json (JavaScript Object Notation) and .xml (Extensible Markup Language) formats (Figure 3.).

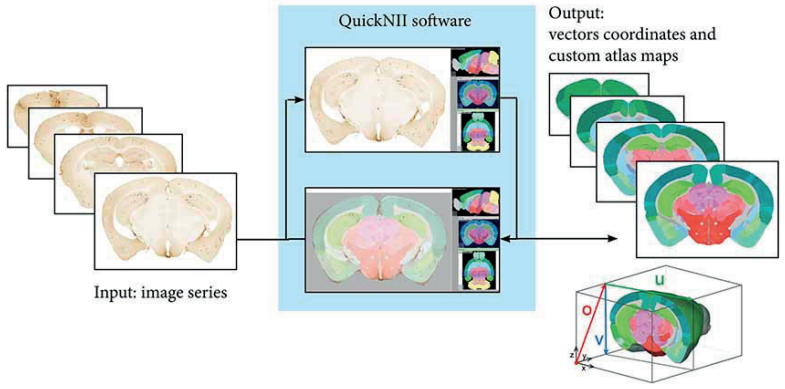

Figure 2. *Processing of histological serial brain section images in the QUINT workflow according to the 3D atlas template (Yates et al., 2019)*

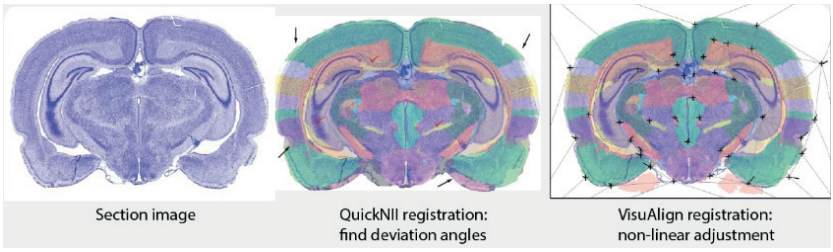

Figure 3. *Processing of microscopic brain section images using QUICKNII and VisuAlign applications (EBRAINS).*

3) Cell labeling is performed using ilastik or another image analysis tool (Berg et al., 2019) (Figure 4.). Ilastik is an intuitive and user-friendly tool designed for interactive image classification, segmentation and analysis. It can be used for automatic pixel classification, semi-automatic and fully automatic object tracking, as well as semi-automatic segmentation and object counting.

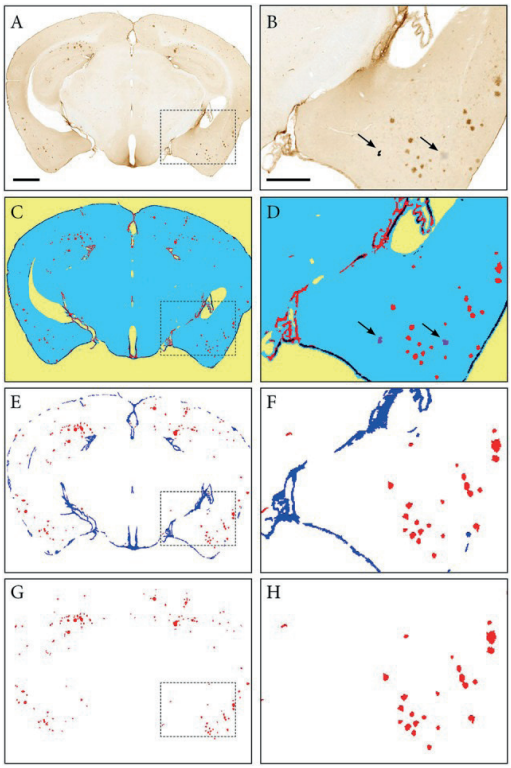

Figure 4. *In the image above from the study by Yates et al., A shows the histological section with neuron groups outlined in the square, while C, E, and G demonstrate the labeling of these neuron groups as objects within the ilastik program. B, D, F, and H display the zoomed-in versions of these processes (Yates et al., 2019).*

4) Quantitative analysis of reference atlas regions is performed using Nutil (Groeneboom et al., 2020).

5) Quality control (QC) is conducted using QCAlign. This step is optional and supports both the quality control of section images and the atlas registration. It also enables the exploration of the atlas hierarchy and the creation of a customized hierarchy level for quantitative determination.

6) The distribution of labeled cells in the 3D atlas visualization can be examined using the Meshview Atlas Viewer (Figure 5.) (Puchades et al., 2019).

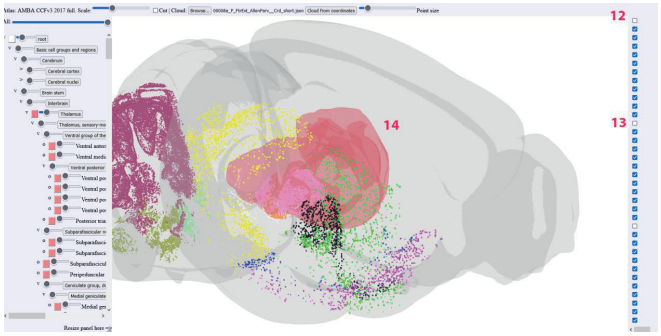

Figure 5. *Visualization of the distribution of labeled cells on neuroanatomical structures using the 3D Atlas in Meshview (EBRAINS, 2021).*

As a result of the conducted studies, it has been observed that the workflow is consistent with the results of stereology and similar applications, and validations have also been performed (Bjerke et al., 2022). With the QUINT Workflow, neuroanatomical structures can be analyzed morphometrically, and cell distributions in immunohistochemical or immunofluorescent sections of the examined region can also be analyzed. For example, in one study, tau pathology in the entorhinal cortex of wild-type mice was assessed using QUINT (Lubben et al., 2024). Bjerke at al., created a microscopic atlas of dopamine 1 and 2 receptors, DOPAMAP, using the QUINT workflow in both developing and adult mice (Bjerke et al., 2022).

In conclusion, for neuroscientists, the morphometric changes in neuroanatomical structures and cells in both human and rodent brains are of great importance. The structural assessment of neuroanatomical structures alone leads to incomplete cellular analysis and leaves hypotheses open-ended. In this context, the QUINT workflow, which allows both microanatomical and macroanatomical evaluations, is an extremely useful research method for neuroscientists working with experimental models. It is evident that this method will continue to maintain its validity in morphometric studies in the field of neuroscience, shedding light on clinical data.

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CHAPTER 4

THE EFFECT OF MUSIC IN PREGNANCY

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INTRODUCTION

Music therapy is a method that is on the agenda in health care in developed countries . It is a therapeutic practice that has been widely developed in the mid-century . Music was defined as a medical intervention in the time of Hippocrates. In the 19th century, Nightingale's statement about music therapy and Hippocrates' attempts often formed the basis of research today. In 1977, Clair defined the qualities of the music to be selected for therapeutic purposes as instrumental, slov tempo, accent-free, beat-free, and music without changing rhythms. The concept of "music therapy" was formed by the combination of the word "music" which means art and the word "therapy" which means taking the necessary measures for the care and treatment of patients.

According to the World Federation of Music Therapy, music therapy is defined as "the use of music and/or musical elements (sound, rhythm, melody and harmony) by a trained music therapist to develop and increase communication, relationship, learning, expression, mobilization, organization and other related therapeutic factors that a person or group needs to meet their physical, emotional, social and cognitive needs" (World Federation of Music Therapy, 2011).

Music therapy has been used in health care since time immemorial. It has been used by many cultures for therapeutic purposes in surgeries, during childbirth and in mental health. Therefore, music has a different place in almost many cultures. When we look at the literature, we see that music has been used in almost all branches of the health field. In the history of Islamic civilization, members of the Sufi school have dealt with music and have stated that Turkish music is effective especially in the treatment of psychological diseases. From past to present, Turkish maqams have been used in the treatment of various diseases, but there are no studies evaluating pregnant women and infants in the literature. Although pregnancy is a normal process, many women experience stress due to emotional, physical and social changes in this process, as well as anxiety due to the health of their babies and lifestyle changes after birth. Music reduces stress and anxiety in pregnant women and music listened to in the prenatal period affects the baby in the neonatal period. Hearing the sound that the fetus is used to hearing in the intrauterine period again after birth has a relaxing effect on the baby. It reduces infant stress, increases mother-infant attachment and causes positive physiological and behavioral changes.

Neurological and physical development is slower in babies of mothers who experience stress during pregnancy. The most important factor here is the high levels of cortisol, adrenaline and noradrenaline hormones released during stress. An increase in these hormones triggers maternal depression,

anxiety, anger and restlessness during the day. These hormones with high levels pass to the fetus through the placenta and negatively affect the neurological and physical development of the fetus. As a result, functional, behavioral and emotional disorders and deficiencies are observed in childhood. Music therapy has a therapeutic effect in reducing anxiety and stress in pregnant women. Music neutralizes negative emotions, increases the stress threshold, and helps the patient to reduce stress and relax . It also changes mood and stimulates autonomic response and imagination at the thalamic level. Feelings and emotions pass to the cerebral hemisphere. Affecting the brain communication network, music affects mood through the limbic system. Aesthetic pleasures are received by the right brain, causing the pituitary to release endorphins. Thus, the concentration of adrenocorticotropic hormone in the blood decreases. Music causes a change in the interaction between the thalamus and reticular activation system .

This results in changes in emotional state, changes in the muscular system, changes in autonomic functions such as blood pressure, heart and respiratory rate. It decreases heart rate, blood pressure, body temperature and respiratory rate. Music therapy is important not only in the treatment of diseases but also in the creation of protective behaviors in healthy individuals. People are more affected by the music of their own culture and can establish a healthier communication. Although there are many studies involving music therapy of different cultures in the prenatal and postnatal period, there are no scientific studies evaluating Turkish music, which has existed in our own culture in the past. Filling this gap in this field is important in terms of protecting the health of individuals as well as protecting our social and cultural values.

Music therapy, which is one of the non-pharmacologic methods used in pregnancy, is very important in terms of having no known side effects, being used whenever desired, being easy to learn, being cost effective and its effects can be observed immediately. The aim of this chapter is to examine the use of music therapy in pregnancy in line with the literature.

1. History of Music Therapy

Music, which has been the expression of many emotions throughout history, has been used for magical, religious, military and entertainment purposes as well as for therapeutic purposes. Healing the patient with different rhythms and lyrics has formed the basis of music therapy.

Music therapy has existed in the world for about 2500 years. Before Christ, Plato said that music affects the depths of the human soul, giving it tolerance and comfort. Celsus and Areteu reported that music relaxes spiritually and has a positive effect on mental health.

Confucius described the effect of music on people as 'with music, human relations improve, eyes brighten, ears sharpen, blood movement and circulation calm down' (Karamızrak, 2014). Music has been used in the treatment of physiological and psychological diseases in the healing centres of civilisations that existed after Christ. Researches mention a history of Turkish music that has been going on for 6000 years.

Many philosophers (Hippocrates, Plato, Aristotle, Pythagoras) have stated that the use of music in the field of health has positive contributions on individuals. Shamans also utilised the effects of music such as dance and drumming to cure diseases.

Shamans and local healers used music, drumming, singing and dancing to treat people. Hippocrates, known as the father of medicine, and philosophers such as Pythagoras, Plato and Aristotle have suggested the use of music in the field of health.

Music has been used in almost all civilisations and it has been seen that it has positive effects on health, especially on mental health .

2. Areas of Use of Music Therapy

Music started to be used in the Western world in the middle of the 20th century in the hospital environment. Considering the first purposes of use, it was seen that the sedative and painkiller effects of music were utilised. In the mid-twentieth century, researchers developed theories that the effects of music were neurologically based and its physiological effects were experimentally investigated. Music has been used in psychiatric diseases (mental retardation, autism, neurosis, substance addiction, etc.), oncology/terminal period patients, medical/surgical procedures that cause anxiety, delivery rooms/ operating theatres, intensive care units.

Today, music therapy is used in diseases such as dementia, Parkinson's, asthma, pain, anxiety , autism; palliative care, preoperative care and intensive care; oncology , paediatrics and women's health and obstetrics; heart patients , skin patients, haemodialysis patients and patients connected to mechanical ventilators. When the areas of treatment with music in gynaecology and obstetrics are examined; in vitro fertilisation (Aba et al., 2017), abortion (Wu et al., 2012), non-stress test (Küçükkelepçe, 2014), transvaginal ultrasonography (Shin & Kim, 2011) and during surgical operation procedures (Li & Dong, 2012; Wu et al, 2012); in terms of stress and maternal attachment factors during pregnancy (Chang et al., 2015); contribution to pain and stress management in labour (Sürücü et al., 2018; Dehcheshmed & Rafiei, 2015; Toker & Kömürcü, 2017); postnatal psychological health (Simavlı et al., 2014) and examination of neonatal parameters (Gonzales et al., 2017).

3. Effect of Music in Pregnancy

In an experimental randomised controlled study in which the effectiveness of music therapy during pregnancy was evaluated, pregnant women were made to listen to music consisting of lullabies, classical music, natural sounds and Chinese children's songs for two weeks and it was found that there was a significant decrease in the stress, anxiety and depression levels of pregnant women . It has been reported that music is very effective in reducing stress in high-risk pregnant women . In Korea, it is believed that relaxing music listened in the prenatal period provides stability in the physical and psychological state of the mother and creates a safe environment for the foetus. Music listened during the prenatal period also positively affects postnatal mother-infant attachment. In Korea, it is believed that relaxing music listened during the prenatal period provides stability in the physical and psychological state of the mother and creates a safe environment for the foetus. Music listened during the prenatal period also positively affects postnatal mother-infant attachment

Music listened to in the prenatal period affects the baby in the neonatal period. The right lobe is not sufficiently developed in the brain that does not receive any stimulation in the prenatal period and at birth. It is suggested that prenatal stimulation increases musical ability and language development. It is also reported in the literature that music therapy provides relaxation during pregnancy. (Chang et al., 2015; Dayyana et al., 2017; Persico et al., 2017). Chang et al (2015) evaluated the effect of music therapy on stress level and stress perception in pregnant women. In the study, music was played to the experimental group for fifteen days. As a result of the study, it was observed that the stress levels of pregnant women who were listened to music were lower.

Dayyana et al (2017) examined the effect of music therapy on anxiety and β-endorphin levels in pregnant women. As a result of the study, it was found that music therapy had a positive effect on anxiety and β-endorphin levels. In the study, it was concluded that music can be applied as an alternative treatment for women with anxiety. Persico et al (2017) looked at the effects of singing during pregnancy on stress and the baby. In the experimental group, it was observed that stress levels were less and mother-infant attachment was better in singing women.

Sleep problems are common due to hormonal changes that occur during pregnancy. Physical, psychological and hormonal changes caused by pregnancy lead to sleep disturbances. In studies examining the effect of music therapy on sleep problems during pregnancy, it has been reported that music therapy has a positive effect (Shobeiri et al., 2016; Liu et al., 2015). Oh et al. (2015) examined the effects of music therapy on maternal anxiety, blood pressure and pulse rate, fetal heart rate and NST duration. Music was found to reduce anxiety in women in the experimental group.

Liu et al (2015) examined the effect of music therapy on sleep quality in Taiwanese pregnant women and found that music had a positive effect and improved sleep quality in pregnant women. It was also found to reduce stress and anxiety in the same group. In another randomised controlled study, the effect of music therapy on stress and infant attachment in pregnant women was investigated. The study was conducted on 296 pregnant women. Women in the experimental group received routine antenatal care and music therapy. Women in the control group received only routine prenatal care. As a result of the study, women in the experimental group had lower levels of psychological stress, but there was no statistically significant difference in terms of maternal attachment (Chang, 2015). Akmeşe and Oran (2014) examined the effect of music therapy on pain and quality of life in women with low back pain. The study was conducted on 66 pregnant women. Pregnant women in the experimental group showed a significant improvement in their pain and an increase in their quality of life after music therapy.

Five studies including systematic review and meta-analysis studies examining the effect of music on stress and anxiety in pregnant women were evaluated. In the study, it was observed that music therapy significantly reduced anxiety, but did not have a significant effect on general stress and stress related to problems experienced during pregnancy. In these studies, it was observed that music therapy application was effective in reducing maternal anxiety, but these studies were found to be methodologically moderate and weak (Corbijn et al, 2017).

Anxiety levels of 154 women who were to undergo amniocentesis were investigated in three randomised controlled groups. The women were randomly divided into three groups as 'those who listened to relaxing music, those who sat and read magazines and those who stood in the waiting room'. The experimental group listened to music for 30 minutes twice a day in the morning and afternoon. Anxiety and cortisol levels of the women in the music group were found to be lower than the other groups. It was observed that women with younger gestational age had more anxiety than women in the other group and a greater decrease in cortisol and anxiety levels was observed in these women (Ventura, T., et al., 2012).

In another study conducted on 101 women hospitalised for abortion with a diagnosis of miscarriage, the effect of music therapy on pain was examined. It was found that music positively affected anxiety and satisfaction levels but had no effect on pain (Guerrero et al., 2012).

Wu et al (2012) examined the effect of music therapy on pain anxiety and satisfaction in patients undergoing surgical procedure for miscarriage. In the study, it was found that the satisfaction levels of the patients were high and anxiety levels were low.

Yang et al (2009) performed music therapy for 30 minutes for three days in their study on 120 women in China. Women in the control group also rested for 30 minutes for three days.

It was observed that anxiety decreased and physiological findings improved in the women in the experimental group who received music therapy.

CONCLUSION

Music therapy has been actively used in almost all societies in recent years and its effects have been reported to be positive. These applications, which have become increasingly important recently, are used effectively in different societies and areas. Today, it is seen that music-based applications are used in infertile individuals, prenatal, natal and postnatal periods and newborns. In the studies conducted, it is seen that music therapy is not only used in the treatment of certain diseases, but also as a preventive measure, appropriate types of music that can eliminate or reduce the stress factor that negatively affects healthy individuals can prevent discomfort that may occur. Music reduces stress and anxiety in pregnant women and has positive effects on the physiological conditions of infants. This situation also positively affects the harmony between mother and baby. For this reason, it is recommended that health professionals participate in music therapy certified training programmes and add music therapy to their practices. Suitable environmental conditions should be provided for health professionals to apply music therapy applications to pregnant women.

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CHAPTER 4

LARYNX ANATOMY

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The larynx is located at the entrance to the airways (1, 2). The main role is to prevent the lower respiratory tract from drawing food into the trachea during inspiration by acting as a sphincter (2). It is also a specialized organ responsible for vocalization (1). Phylogenetically, in humans, the larynx has reached its highest evolutionary development with the capacity to express speech, which is not found in invertebrates and fish (2). Its upper part opens into the laryngopharynx part of the pharynx, and below, it continues with the trachea (1). The larynx is approximately 4 to 5 cm long and wide, with a slightly shorter antero-posterior diameter. It is smaller in size in women compared to men, and is larger in adults. Larynx spans from roughly the C3 to C7 levels, and is held in place by muscles and ligaments. The epiglottis is connected to the hyoid bone and is its uppermost region (2).

It is adjacent to the hyoid bone on the upper side. Larynx is attached to the hyoid bone by the thyrohyoid membrane, thyrohyoid muscle and hyoepiglottic ligament. In the front, it is adjacent to the skin, superficial cervical fascia, platysma muscle and pretracheal layer of cervical fascia. On the anterolateral sides, it is adjacent to the anterior edge of the sternocleidomastoid, sternohyoid, sternothyroid, omohyoid and thyrohyoid muscles. It is also adjacent to the upper parts of the lateral lobes of the thyroid gland and the neurovascular bundle. Posteriorly, it is adjacent to the laryngopharynx (3).

Structure and Function

The larynx consists of cartilage, membranes, ligaments and muscles and is covered with mucous membrane (1, 3). Nine cartilages make up the skeleton. The unpaired cartilages are the epiglottis, thyroid, and cricoid. The paired cartilages comprise of the arytenoids, corniculates, and cuneiforms (2) (Fig. 1). There may also be paired cartilages, such as the triticeal and sesamoid cartilages, which are not always seen. The cartilages begin to ossify approximately at the age of 20. All larynx cartilages ossify, except the epiglottis, which is made of elastic cartilage, and the vocal process parts of the arytenoid cartilages (3).

The thyroid cartilage, the largest of the six cartilages, extends vertically and acts as a protective shield around the anterior part of the larynx (2). It consists of two laminae of hyaline cartilage that meet at the protruding V angle of the laryngeal prominence (Adam's apple) in the midline (1). Larynx has the appearance of a half-open book (2). The posterior border of each lamina extends upwards with the superior cornu and downwards with the inferior cornu (1). There are articular facets for the cricoid cartilage on the inner facing parts of the cornu inferior (3). On the outer surface of each lamina, there is a linea obliqua to which the sternothyroid, thyrohyoid and inferior pharyngeal constrictor muscles are attached (1). The internal surface of the laminae is smooth and covered with mucosa and externally limits the piriform recess.

The vocal ligament attaches to the small protrusion at the junction of the two laminae on the inner surface.

The cricoid cartilage is the strongest and thickest of the larynx cartilages. It resembles a signet ring, and is located under the thyroid cartilage (at the level of the C6 vertebra). It is located below the thyroid cartilage, at the level of the 6th cervical vertebra (C6) (2). It is composed of hyaline cartilage and is narrow anteriorly (about 5 mm) and long posteriorly (about 20 mm) (4). The tall posterior portion of the cricoid is known as the cricoid lamina and the anterior portion of the cricoid arch (5). On the back of the arch, there is an articular facet for the thyroid cartilage on both sides. The median cricothyroid ligament attaches to the cricoid arch anteriorly, and the lateral part of the conus elasticus attaches to the lateral sides. In the superior part of the lamina, there are articular surfaces that articulate with the arytenoid cartilages. The cricopharyngeal ligament, which is the starting tendon of the longitudinal muscle fibers of the oesophagus, is attached to the protrusion located behind. The cartilage is bonded to the 1st cartilage ring of the trachea via the cricotracheal ligament inferiorly (3).

The epiglottis is a leaf-shaped, elastic cartilage located behind the root of the tongue and covers the entrance to the larynx. (1). It is connected to the thyroid cartilage, and projects above the pharynx, allowing air to pass into the trachea, larynx and lungs. As the hyoid bone rises, it pulls the larynx upward during swallowing, preventing food or drink from entering the esophagus and food from entering the trachea (2). Its edges are attached to the arytenoid cartilages by means of the aryepiglottic fold. Its upper edge is free and the mucous membrane jumps forward from the epiglottis to the back surface of the tongue. Here, the median and lateral glossoepiglottic folds are located. On both sides of the glossoepiglottic fold are mucosal depressions called epiglottic vallecula (1).

Arytenoid cartilages are located on the back of the larynx, on the upper edge of the lamina of the cricoid cartilage. It resembles a three-sided pyramid in shape. Its apex is at the top and articulates with the corniculate cartilages. Its base is triangular. Its outer corner is called muscular process. The lateral cricoarytenoid, and the posterior cricoarytenoid muscle attach here. The front corner of the base is called the vocal process. The vocal ligament attaches here. The parts other than the vocal process are hyaline cartilage. The back surface of the cartilages in the arytenoid is concave. The transverse and oblique arytenoid muscles attach here. The inner surface is the surface of the two arytenoid cartilages facing each other and the interarytenoid notch is located between them. On the outer front surface, there are structures called arcuate crest and colliculus. In addition, there are pits called triangular fovea, where the vestibular ligament is attached, and oblong fovea, where the vocalis muscle is attached (3).

Corniculate cartilages or Santorini cartilages are two small elastic cones that articulate with the apex of the arytenoid cartilages. These cartilages located in the aryepiglottic fold, form the corniculate tubercle.

The *cuneiform cartilages* are also known as the cartilages of Wrisberg. Each of them is a piece of cartilage located in the aryepiglottic fold (1, 2). They are not directly connected to other cartilages, but serve to support the vocal cords and the lateral aspects of the epiglottis (2).

Triticeal cartilages may not always be present. When present, they are located behind the thyrohyoid membrane (inside the lateral thyrohyoid ligament).

Sesamoid cartilages can be seen close to the outer surfaces of the arytenoid cartilages or the vocal cords (3).

The laryngeal cartilages move by means of several joints between them. The cricothyroid joint connects the thyroid cartilage to the cricoid arch. The cricoarytenoid joints connect each arytenoid cartilage to the cricoid cartilage, and the arycorniculate joint connects the arytenoid cartilage to the Santorini cartilage (2).

The internal surface is generally lined with stratified columnar epithelium. Stratified squamous epithelium is only present at the vocal fold level. Since there are no blood vessels and submucosa layer here, this part is observed in a pearly white color. Other parts are seen as pink. The mucosa covering the arytenoid cartilages is particularly sensitive to irritation and plays a role in the closure of the larynx entrance through reflex (3).

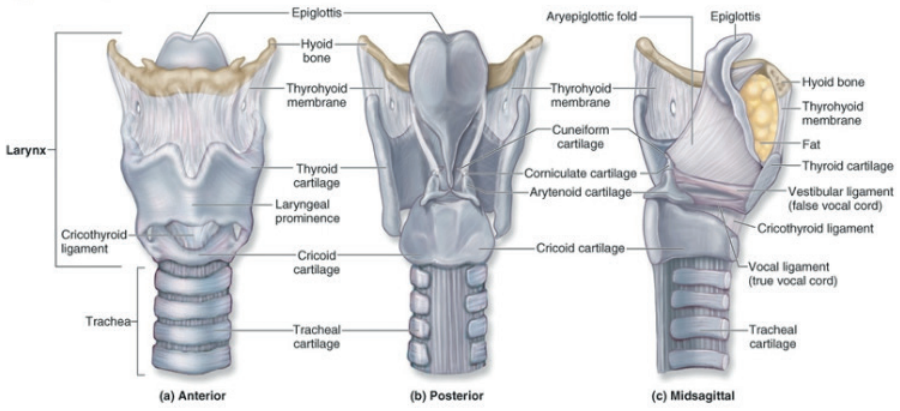

Fig. 1. *Larynx Cartilages (6)*

Membranes and Ligaments of the Larynx

The thyrohyoid membrane connects the superior horns of the thyroid cartilage with the greater horns of the hyoid bone. In the midline, this membrane thickens to form the median thyrohyoid ligament. The posterior edges thicken to form the lateral thyrohyoid ligament. The membrane is pierced on both sides by the internal branch of the superior laryngeal nerve (SLN), the superior laryngeal vein, and artery. The cricotracheal ligament connects the cricoid cartilage with the first ring of the trachea. The fibro-elastic membrane is under the mucosa covering the larynx. The upper part of this membrane is called the quadrangular membrane and is located between the epiglottis and arytenoid cartilage. At the same time, the lower edge of this membrane forms the vestibular ligaments. The lower section of the fibroelastic membrane is called the cricothyroid ligament (conus elasticus).

The anterior part of the cricothyroid ligament is thick and connects the lower border of the thyroid cartilage to the cricoid cartilage. The lateral part is thin and attaches below to the superior edge of the cricoid cartilage. The superior edge of the ligament, instead of attaching to the inferior edge of the thyroid cartilage, rises on its medial surface. The superior edge of the cricothyroid ligament thickens to form the vocal ligament, which is important on both sides. The anterior end of each vocal ligament is attached to the deep surface of the thyroid cartilage. Its posterior end attaches to the vocal process (arytenoid cartilage). The hyoepiglottic ligament connects the hyoid bone to the epiglottis. The thyroepiglottic ligament connects the inferior stem of the epiglottis to internal aspect of the thyroid cartilage.

Aditus Laryngis

The aditus laryngis is directed posteriorly and upwards towards the laryngeal part of the pharyngis. Aditus laryngis is bounded anteriorly by the upper edge of the epiglottis. It is bordered laterally and externally by the aryepiglottic fold, the mucous membrane that connects the epiglottis to the arytenoid cartilage, and posteriorly and inferiorly by the mucous membrane that lies between the arytenoid cartilages. The corniculate cartilage at the top of the arytenoid cartilage. The lesser cuneiform cartilage form a ridge at the upper margin of each aryepiglottic fold.

Laryngeal Cavity

The laryngeal cavity is the area between the aditus laryngis and the inferior margin of the cricoid cartilage. Cavity is divided into three parts. *(l)* Upper part; laryngeal vestibule *(2)* Middle part; intermediate laryngeal cavity *(3)* Lower part; infraglottic cavity.

The laryngeal vestibule extends from the entrance to the vestibular fold. The vestibular folds are two thick mucosal folds that cover the vestibular

ligaments. The laryngeal vestibule has anterior, posterior and lateral walls. The posterior surface of the epiglottis, covered with mucosa, forms the anterior wall. The posterior wall is formed by the arytenoid cartilages and the interarytenoid fold of mucosa covering the transverse arytenoid muscle. Below, the laryngeal vestibule is narrowed by the pink vestibular folds projecting medially. The rima vestibuli is the opening between the vestibular folds. The vestibular ligament, which lies inside each vestibular fold, is the thickened lower edge of the quadrangular membrane. This ligament extends from the thyroid cartilage to the edge of the arytenoid cartilage.

The middle section extends from the level of the vestibular fold to the level of the vocal folds. The middle part of the larynx is located between the vestibular folds and the vocal folds. The vocal cords contain the vocal ligaments and are white in color. Each vocal ligament is the thickened upper edge of the cricothyroid ligament. It extends from the thyroid cartilage anteriorly to the vocal process of the arytenoid cartilages posteriorly. The rima glottidis is an opening between the vocal folds anteriorly and the vocal processes of the arytenoid cartilages posteriorly.

There is a small opening on each side between the vocal and vestibular folds, called the laryngeal ventricle (sinus Morgagni). It is lined with mucosa. A small diverticulum called the laryngeal saccule passes upward from here, between the vestibular fold and thyroid cartilage (1). The laryngeal ventricles, the vocal cords and the glottis form the infraglottic cavity. The vocal cords are four folds of fibroelastic tissue, two above and two below. It is located anteriorly in the thyroid cartilage and posteriorly in the arytenoid cartilage. The upper vocal cords are thin and ribbon-shaped. They do not contain any muscular elements. The lower vocal cords are wider than the upper ones. They have bundles of muscles covering their entire length. The space between the upper vocal cords is larger than the space between the lower vocal cords. The lower vocal cords are the only ones that can approach each other, so they are considered true vocal cords. The upper vocal cords are called false vocal cords (2). The vocal cords consist of five layers: the thyroarytenoid muscle, squamous epithelium, deep, intermediate, and superficial lamina propria. The deep and intermediate lamina propria are grouped to form the vocal ligament. The superficial lamina propria provides a gelatinous surface during vocal cord vibration (7).

Embriology

The larynx develops from the endoderm and mesoderm during the 4th week of development. The muscles and cartilages develop from the third, fourth, and sixth pharyngeal arches, while the inner lining originates from the endoderm. In the fourth week of the developing foregut, the laryngotracheal groove appears. This groove deepens to form the esophagotracheal septum,

thus placing the esophagus dorsal to the septum and the rest of the respiratory tract anteriorly. The length of the groove continues to form the laryngotracheal diverticulum, which will eventually give rise to the larynx, trachea, and lungs. The laryngeal lumen initially disappears due to epithelial proliferation. The lumen recanalizes between weeks 7 and 10. The arches form the nerves, cartilages, and muscles of the larynx. These are:

Third branchial arch: Epiglottis, hyoid bone (greater horn), glossopharyngeal nerve

Fourth branchial arch: Thyroid and cuneiform cartilages, SLN, cricopharyngeal and cricothyroid muscles, epiglottis

Sixth branchial arch: Intrinsic muscles of the larynx, arytenoid, corniculate and cricoid cartilages, recurrent laryngeal nerve (RLN).

No part of the larynx is ossified at birth. Ossification of the thyroid cartilage occurs during adolescence. The cricoid cartilage does not ossify until the fourth decade of life (7).

Musculi Laryngis

There are intrinsic muscles responsible for sound production and extrinsic muscles that move the larynx. The extrinsic muscles are divided into two as elevators and depressors of the larynx. During swallowing, the larynx moves up and down. The hyoid bone is connected to the thyroid cartilage by the thyrohyoid membrane. Therefore, the larynx also participates in the movements of the hyoid bone. The elevator muscles that lift the larynx are the geniohyoid, mylohyoid, digastric and stylohyoid muscles. The palatoglossus, salpingopharyngeus and stylopharyngeus muscles that attach to the posterior margin of the thyroid cartilage lamina are also the muscles that lift the larynx. The sternohyoid, omohyoid and sternothyroid muscles are the depressors of the larynx. The movements of the trachea help these muscles. Intrinsic muscles are divided into those that control the entrance to the larynx, and those that move the vocal cords (1).

Intrinsic Muscles

Cricothyroid Muscle

It has two parts: pars recta and pars obliqua. Both parts originate from the cricoid cartilage. The pars recta located anteriorly, attaches to the anterior part of the lower margin of the thyroid cartilage above. The pars obliqua located at the back attaches to the anterior margin of the inferior horn above and the lower margin of the adjacent thyroid cartilage. The pars obliqua located on the posterior side is attached to the anterior margin of the inferior horn above and the lower margin of the adjacent thyroid cartilage. When the muscle contracts, if the thyroid cartilage is fixed by other muscles, it pulls the cricoid cartilage

upwards. However, the arytenoid cartilages that articulate with the cricoid cartilage are pulled backwards, resulting in the vocal cords being lengthened and higher-pitched phonation.

Posterior Cricoarytenoid Muscle

This muscle sits on the posterior aspect of the larynx and arises from the posterior part of the cricoid lamina. The muscle originating from here extends upwards and outwards and attaches to the muscular process (arytenoid cartilage). It pulls the muscular process inferiorly and posteriorly, when the posterior cricoarytenoid muscle contracts. As a result, the vocal process is pulled outward and slightly elevated. Along with this, all of the arytenoid cartilages also shift slightly outward. As a result of the vocal processes being pulled outwards, the rima glottidis widens. This muscle is the only muscle among the larynx muscles that expands the rima glottidis.

Lateral Cricoarytenoid Muscle

It originates from the upper edges of the lateral sides of the cricoid cartilage arch and the conus elasticus. It extends backwards and upwards, pulling the muscular process forwards and slightly downwards, causing internal rotation of the arytenoid cartilages. As a result of this movement, the rima glottidis narrows. It works as antagonist to the posterior cricoarytenoid muscle.

Thyroarytenoid Muscle

It has two parts: pars lateralis and pars vocalis. Pars lateralis originates from the angle of the thyroid cartilage. It extends posteriorly, covering the conus elasticus and laryngeal ventricle from the outside. Its insertion is the arytenoid cartilage. This part of the muscle narrows the pars intercartilaginea of the rima glottidis by pulling the arytenoid cartilage inward. Some bundles that branch off from the superior part of the muscle attach to the lateral margin of the epiglottis. These bundles, called pars thyroepiglottica, help to close the larynx entrance by pulling the epiglottis downwards.

Vocal Muscle

The inner part of the thyroarytenoid muscle is called the vocal muscle. The muscle originates from the thyroid angle under the vocal ligament, extends posteriorly within the vocal folds and ends at the oblong fovea, and partially at the vocal process. When contracted, the vocal cords stretch and thicken without shortening (8). Therefore, it causes the voice to deepen. When the medial part contracts, the voice becomes thinner (3).

Arytenoid Muscle

They lie between the two arytenoid cartilages. It has two parts: the oblique and transverse arytenoid muscles. The oblique arytenoid muscle originates from the muscular process of one side. It inserts at the apex of the cartilage

on the other side. Both muscles cross each other in the middle. As a result of their contractions, they bring the arytenoid cartilages closer together and narrow the posterior part of the rima glottidis (pars intercartilaginea). Some bundles of this muscle extend upwards and forwards within the aryepiglottic fold and attach to the epiglottis. These fibers are called pars aryepiglottica and when contracted, they help to close the larynx entrance by pulling the epiglottis down and back. The transverse arytenoid muscle is located deep to the oblique arytenoid muscle and performs the same function by bringing the arytenoid cartilages of both sides closer together. The internal surface, which lies between two cartilages, is covered with larynx mucosa and partially limits the interarytenoid notch from behind (8).

Extrinsic Muscles

Thyrohyoid Muscle

It originates from the oblique line of the thyroid cartilage. The muscle attaches to the body and greater horn of the hyoid bone as its insertion site. Innervation is provided by the C1 spinal nerve and the fibers that join the hypoglossal nerve. It pulls the hyoid bone downwards, and if the hyoid bone is fixed, it pulls the larynx upwards.

Sternothyroid Muscle

The sternothyroid muscle starts from the facies posterior of the manubrium sterni, under the origin of the sternohyoid muscle, and ends at the thyroid cartilage. It pulls the larynx downwards during swallowing and speaking. Branches of the ansa cervicalis innervate the muscle (3).

Inferior Pharyngeal Constrictor Muscle

The inferior pharyngeal constrictor muscles extend from the cricoid and thyroid cartilages to the pharyngeal raphe. The vagus nerve innervates the muscle via branches of the RLN and pharyngeal plexus. When contracted, they narrow the diameter of the pharynx to facilitate swallowing.

Stylopharyngeus Muscle

It arises from the medial side of the base of the styloid process (temporal bone). The insertion site is the thyroid cartilage. Its function is to lift up the pharynx and larynx. The glossopharyngeal nerve provides innervation (2).

Palatopharyngeus Muscle

Palatopharyngeus muscle originate from the palatine aponeurosis and the pterygoid processes and insert into the posterior margin of the thyroid cartilage (2, 3). Some of its fibers cross with the fibers of the opposite side on the midline. It is innervated by the pharyngeal plexus. It narrows the palatopharyngeal arch and elevates the pharynx forward and upward (3).

There are also muscles that are not located in the larynx but participate in the movement of the larynx. For example, the mylohyoid, digastric, geniohyoid or stylohyoid muscles elevate the larynx, while the sternohyoid and omohyoid muscles cause depression of the larynx.

Blood supply and Lymphatics

The superior and inferior laryngeal arteries provide blood supply to the larynx. The superior laryngeal artery is a branch of the superior thyroid artery that supplies blood to the supraglottic region, upper vocal cords, and epiglottis. The inferior laryngeal artery is also a branch of the superior thyroid artery and supplies blood to the lower vocal cords and subglottic area. The inferior laryngeal artery sometimes has a small branch of the superior thyroid artery that supplies the posterior cricoarytenoid and arytenoid muscles (2).

The venous blood of the larynx is poured into the superior laryngeal vein and the inferior laryngeal vein. The superior laryngeal vein empties into the internal jugular vein via the superior thyroid vein. The inferior laryngeal vein empties into the left brachiocephalic vein via the inferior thyroid vein (3).

The lymph vessels form two groups, above and below the vocal fold. The upper group follows the superior laryngeal artery and exits through the hole in the thyrohyoid membrane and opens into the deep lymph nodes of the neck near the carotid fork. Some of the lymph vessels in the lower group pierce the median cricothyroid ligament and open into the lymph nodes on the cricoid cartilage or the anterior side of the trachea (nodi lymphatici prelaryngeales and pretracheales). The remaining part opens into the deep lymph nodes of the neck along the inferior thyroid artery and into the supraclavicular lymph nodes. The lymph vessels originating from the vocal cords do not anastomose with the vessels of the opposite side. For this reason, cancer in the vocal cords does not metastasize to the opposite side. However, the lymph vessels of the mucosa on the back wall of the larynx anastomose in the submucosa.

Nerves

Innervation is provided by the SLN and the RLN, which are branches of the vagus nerve (Fig. 2). The SLN has two branches: external and internal. The cricothyroid muscle, one of the intrinsic muscles of the larynx, is innervated by the external branch of the SLN, and all other muscles are innervated by the accessory nerve coming from the ILN (terminal branch of the RLN). The part of the larynx mucosa above the rima glottidis is innervated by the internal branch of the SLN, and the part below it is innervated by the RLN.

The SLN originates from the lower half of the inferior ganglion of the vagus nerve at the upper part of the carotid triangle. It is divided into external and internal branches, which are terminal branches in the vagina carotica. The internal branch is thicker than the external branch and contains sensory

fibers. It passes to the piriform recess through the hole in the thyrohyoid membrane with the superior laryngeal artery. It receives sensation from the upper mucosa, including the upper surface of the vocal cords. The external branch is the thin branch of the SLN. It extends down from behind the sternohyoid muscle together with the superior thyroid artery. It initially lies on the inferior pharyngeal constrictor muscle. It then pierces this muscle and reaches the cricothyroid muscle. It innervates both the inferior pharyngeal constrictor muscle that it pierces and the cricothyroid muscle. The only muscle not innervated by the RLN is the cricothyroid muscle.

The RLN is the last branch of the vagus nerve containing somatomotor fibers. It rises up the tracheoesophageal groove. Here it is in close proximity with the inner surface of the thyroid gland. Along its way, it gives off branches to the pharynx, esophagus and trachea. The terminal branch, the inferior laryngeal nerve (ILN), innervates all the muscles of the larynx except the cricothyroid muscle. It receives sensation from the mucosa below, including the lower surface of the vocal cords. Taste buds, similar to those on the tongue, can be found in small numbers on the back surface of the epiglottis, in the aryepiglottic fold, and sometimes in other areas (8).

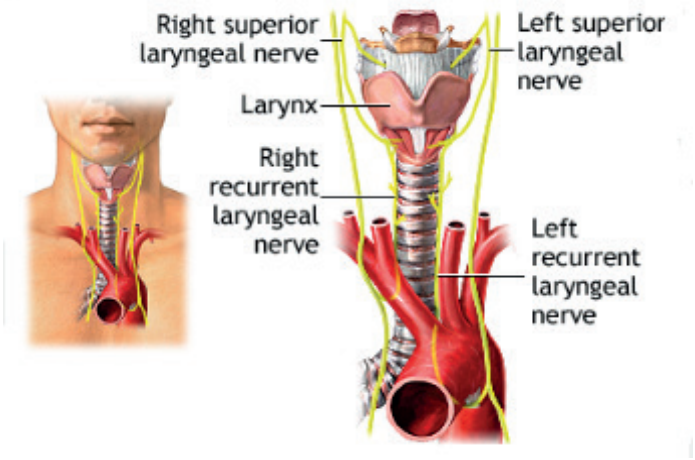

Fig. 2. *Larynx Nerves (9)*

Voice Formation

During the formation of the voice, in the first stage, the vocal cords approach each other (adduction). This movement is followed by the stretching of the vocal cords. The vocal cords are stretched by the work of the cricothyroid muscles and the bending of the thyroid cartilage. As the volume of the voice increases, the tension of the vocal cords increases. In the last stage, during expiration, the air coming from the lungs pushes the vocal cords from bottom

to top. When this pressure reaches a certain level, the rima glottidis opens and the voice is created. During whispering, the intermembranous part of the rima glottidis is closed while the intercartilaginous part is open (3).

Physiologic Variants

The larynx shows several differences according to gender. This is the primary cause of voice differences between men and women. The male larynx is often more prominent than the female larynx. This type of gender difference also exists in the thyroid, which is thicker in men and has a different angle of about 95 degrees in men and 115 degrees in women.

The innervation of the larynx can also vary from person to person. Therefore, the RLN has been widely studied and several variations have been described. The RLN divides into two or more branches, with the anterior branch entering the larynx anterior or posterior to the cricothyroid joint. Given that the left recurrent nerve loops around the aortic arch, its course may also vary due to aortic aneurysm or even interindividual aortic variation. Cases of nonrecurrent ILN have also been studied. The RLN was found to pass directly from the vagus nerve in the neck to the larynx and not to loop around the subclavian artery.

The relation between the inferior thyroid artery and the RLN has also been extensively studied. Usually the nerve ascends approximately 60% posterior to the artery. However, it has been found that it sometimes ascends approximately 32.5% anteriorly or approximately 6.5% between the branches of the artery. The location of the RLN is quite critical because of the risk of surgical injury.

Surgical Considerations

In life-threatening situations, a small incision in the cricothyroid membrane to open an airway is the most commonly used medical technique (cricothyrotomy). The most surgical risk of laryngeal surgery is iatrogenic injury to the RLN in thyroid surgery. Before and after thyroid surgery, the integrity of the RLN is examined by indirect laryngoscopy. Therefore, surgeons rely heavily on intraoperative landmarks to identify and avoid injury to the RLN. These landmarks include the inferior thyroid artery, the tubercle of Zuckerkandl and the ligament of Berry. But are not limited to these (2).

The ligament of Berry connects the thyroid to the first three rings of the tracheal cartilage. Studies have documented that the Berry ligament is the most reliable landmark. Because it goes superficial to the RLN in 78.2% of cases. The tracheoesophageal groove is also one of the most reliable landmarks for identifying the recurrent laryngeal nerve. It has been observed that the nerve is located in the tracheoesophageal groove in 63.7% of cases (7, 10).

Clinical Significance

When an emergency airway opening is required, an incision is made through the cricothyroid ligament during cricothyrotomy. Clinically, the cricoid cartilage serves as a palpable landmark to indicate the lowest part of the larynx where the trachea begins. This is vital in an emergency airway situation where the cricothyroid ligament is cut in the midline between the cricoid and thyroid cartilages (11).

Laryngitis

Laryngitis is an inflammation of the larynx. Chronic laryngitis, which lasts longer than 3 weeks, is more common than acute laryngitis. Cough, hoarseness, and pain are common symptoms. It is usually accompanied by fever, depending on the cause (12). Most viral upper respiratory tract infections cause acute laryngitis. Sometimes bacterial infections can lead to this situation. Fungal laryngitis is typically seen in 10% of cases and is underdiagnosed. Overuse of the vocal cords, such as singers and teachers, can cause laryngitis or laryngeal trauma. Among the causes of chronic laryngitis, allergies, smoking and reflux are frequently observed (2).

Vocal Fold Paralysis

RLN paralysis can lead to vocal fold paralysis. The etiology of RLN palsy includes a wide variety of diseases or disorders/causes (2, 13). It can be caused by diseases such as hydrocephalus, Goldenhar syndrome, and anatomic anomalies such as tracheoesophageal fistula. Although rare, it can be caused by viral infection. The most common cause is iatrogenic damage to the nerve. Lung, thyroid or esophageal tumors, systemic neurological diseases such as myasthenia gravis or multiple sclerosis can also cause vocal fold paralysis.

Laryngeal Cancer

Laryngeal cancer usually begins in the glottis and is a squamous cell carcinoma. Symptoms include hoarseness or voice change, cough, stridor, a lump in the neck, or difficulty swallowing (2).

Reinke's Edema (Polipoid Kordit)

The superficial lamina propria is also known as Reinke's Space. Its structure consists of loose connective tissue. Reinke's space is vulnerable to fluid accumulation due to inflammation and chronic irritation from smoking. Inflammation and irritation lead to polyp formation in the vocal cords, which causes difficulty with the natural vibration seen during phonation. Therefore, patients often present with a changed voice that is deeper. Women often sound more like men. The priority in treatment is to treat the underlying disease causing Reinke's edema, such as laryngopharyngeal reflux, hypothyroidism, vocal abuse, or smoking. If healing does not occur, surgical resection may be performed (7).

Neoplasms

Recurrent Respiratory Papillomatosis is a benign laryngeal neoplasm. The bimodal distribution of recurrent respiratory papillomatosis classifies the disease into juvenile type (before 12 years of age) and adult type (after 12 years of age). In children, infection is most commonly acquired through the vaginal canal via infected secretions from anogenital warts. Adults often come into contact with the infection through oral sex. Symptoms include stridor, dyspnea, and dysphonia. It can lead to respiratory tract involvement, causing pneumonia, atelectasis, and hemoptysis attacks mimicking tuberculosis. There is no complete cure for the disease yet. The best treatment is resection of as much of the exophytic papilloma as possible.

Cricoarytenoid Arthritis

Rheumatoid arthritis is an autoimmune disease with symmetric erosive arthritis and multisystem involvement. There is no definitive information yet about its etiology. It can cause destruction, deformity, and ankylosis in joints. The cricoarytenoid joints are true diarthrodial joints affected by rheumatoid arthritis (14). Symptoms of cricoarytenoid arthritis include hoarseness, pharyngeal fullness, and dyspnea during swallowing and speaking (11, 15). Normally, the joint has symmetric volume, density and orientation. In acute joint involvement, swelling of the vocal cords in fixed adduction can cause airway obstruction. In fact, the airway can become so narrow that serious respiratory distress or sudden death can develop with any infection (14).

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CHAPTER 6

EVALUATION OF OPERATIVE OUTCOMES AND THE RELATIONSHIP BETWEEN PROCEDURES AND PAIN DURING OFFICE-BASED HYSTEROSCOPY IN AN OUTPATIENT SETTING

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Introduction

The advancements in minimally invasive surgery have led to broader adoption and the ability to perform even complex procedures with minimal invasiveness [1]. The development of hysteroscopy has provided a minimally invasive approach to address common gynecological problems, such as abnormal uterine bleeding. Currently, the indications for hysteroscopic procedures are extensive and clearly defined. Hysteroscopy is not only utilized for visualizing the cervical canal and uterine cavity but is also widely accepted for the treatment of a variety of localized pathologies in these regions [2].

Hysteroscopy is considered the gold standard for evaluating the uterine cavity in cases of abnormal uterine bleeding, infertility, recurrent pregnancy loss, and suspected intrauterine space-occupying lesions [2]. This procedure can be performed in an office setting (outpatient hysteroscopy) or under anesthesia (inpatient hysteroscopy) as a day procedure. Outpatient hysteroscopy has been shown to be as accurate as inpatient hysteroscopy. However, compared to the traditional inpatient procedure, it offers several advantages, including reduced anesthetic risk, increased cost-effectiveness, and patient preference [3].

Pain is regarded as the most significant factor contributing to incomplete or unsuccessful office hysteroscopy procedures. The primary goal of hysteroscopy is to minimize patient discomfort by reducing pain levels while avoiding increased risks of complications or costs, thereby enhancing patient comfort. The development of small-diameter hysteroscopes has enabled hysteroscopy to be performed in outpatient settings [4]. Moreover, the introduction of an atraumatic insertion technique, known as the "no-touch" or vaginoscopic approach, has further reduced patient pain and discomfort [4].

During office hysteroscopy, various operative procedures can be performed, including the removal of missing intrauterine device strings, polypectomy, endometrial biopsy, and septum resection. Endometrial polyps are common pathologies of the female reproductive system, with a prevalence estimated to be around 8%. Among reproductive-aged women with abnormal uterine bleeding, the prevalence of endometrial polyps is thought to be between 20% and 40% [5].

Office hysteroscopy-based polypectomy is a safe and feasible procedure. However, patient compliance with the procedure is influenced by limitations related to the size of the polyps and the duration of the surgery. Factors such as menopausal status and previous vaginal deliveries are independent of the success of this procedure [6].

Despite these advancements, general anesthesia is still predominantly chosen for hysteroscopy-based operative procedures, and operative proce-

dures using mini-hysteroscopes are relatively limited. Outpatient operative hysteroscopy represents a safe, efficient, and cost-effective procedure with high levels of patient satisfaction. This approach should replace day-case hysteroscopy and be widely adopted in healthcare settings [7].

The aim of this study is to evaluate the outcomes of operative procedures performed during office hysteroscopy in patients who presented to the gynecology clinic for various reasons. Additionally, the study seeks to assess the relationship between operative procedures and pain, as well as to identify independent risk factors contributing to pain levels during these procedures.

2. Background

2.1. Uterine Anatomy and Histology

The uterus is a muscular organ located between the bladder and rectum, primarily responsible for creating a suitable environment for pregnancy, nourishing and protecting the fetus, assisting in childbirth, and contracting post-delivery to prevent excessive bleeding. In nulliparous women, the uterus measures approximately 4x6x8 cm, weighs 70–90 grams, and the distance from the ostium to the fundus is about 6.5 cm. Knowledge of these dimensions is critical in preventing perforation during intrauterine procedures. Anatomically, the uterus consists of two main parts: the corpus and the cervix. The corpus is further divided into the isthmus, cornu, and fundus, while the cervix includes the endocervical canal, internal os, and external os. The endocervical canal transitions into stratified squamous epithelium at the external os, forming a transitional zone that is particularly susceptible to dysplasia and malignancy [8].

The uterine wall consists of three layers: the serosal layer (perimetrium), the muscular layer (myometrium), and the mucosal layer (endometrium). The endometrium is composed of two sublayers—the basal layer and the functional layer—and is lined with glandular columnar epithelium. These structural and histological characteristics are essential in understanding uterine pathologies and their management.

Uterine cavity pathologies include structural abnormalities, such as polyps, atrophy, leiomyomas, and adenomyosis, as well as hyperplasia and carcinoma. Polyps are formed by localized hyperplastic growth of endometrial glands and stroma, while atrophy results from hypoestrogenic changes. Leiomyomas are the most common benign pelvic masses, and adenomyosis involves the infiltration of endometrial glands into the myometrium. Hyperplasia, characterized by glandular and stromal proliferation, may progress to malignancy. Endometrial carcinoma is the fourth most common cancer among women in developed countries, primarily affecting postmenopausal women and presenting with vaginal bleeding [9].

Endometrial polyps are a frequent cause of abnormal uterine bleeding in both premenopausal and postmenopausal women. They result from localized hyperplastic growth of endometrial glands and stroma, often forming a vascular core. Polyps can range in size from a few millimeters to several centimeters and may develop anywhere within the uterine cavity. Molecular mechanisms implicated in their development include excessive secretion of endometrial aromatase, monoclonal endometrial hyperplasia, somatic gene mutations (e.g., KRAS, PTEN, TP53), and hormonal receptor expression, particularly in postmenopausal women [10–14].

Risk factors for endometrial polyps include tamoxifen use, obesity, hormone replacement therapy, and genetic syndromes such as Lynch and Cowden syndromes. Tamoxifen, in particular, is associated with an increased risk of polyp formation and malignancy. Obesity has also been shown to correlate with higher rates of polyp development and related metabolic disturbances [15–20].

The most common clinical symptom of endometrial polyps is abnormal uterine bleeding, which is reported in up to 88% of patients. Polyps may also be asymptomatic and detected incidentally during imaging or routine examinations. Diagnostic approaches include a detailed medical history, pelvic examination, and imaging studies. Transvaginal ultrasonography (TVUS) is the first-line imaging modality due to its cost-effectiveness and reliability. In cases of inconclusive findings, saline infusion sonography (SIS) or diagnostic hysteroscopy may be employed. MRI is not routinely used for diagnosing polyps but may provide additional information in specific cases [24–28].

Management of endometrial polyps typically involves removal, with indications summarized in **Table 1**. Polypectomy is recommended for symptomatic polyps, recurrent polyps, or those associated with risk factors for hyperplasia or carcinoma. The procedure is particularly critical in patients with infertility, abnormal bleeding, or genetic predispositions [29].

Table 1: *Indications for Endometrial Polyp Removal [29].*

Indications for Endometrial Polyp Removal	**Comments**
Abnormal Uterine Bleeding	Associated with increased malignancy risk compared to asymptomatic cases.
Infertility	Often addressed through polypectomy despite limited evidence on its effect on fertility.
Multiple Polyps	Less likely to regress and often symptomatic.
Protruding Polyps	Typically symptomatic and easily removed in outpatient settings.

Recurrent Polyps	Removal is common practice, although data on management remain limited.
Risk Factors for Hyperplasia or Carcinoma	Includes obesity, tamoxifen use, early menarche, late menopause, and genetic syndromes (e.g., Lynch, Cowden).
Large Polyp Size	Some studies suggest polyps >1.5 cm may have a higher malignancy or hyperplasia risk.

2.2. Hysteroscopy

Hysteroscopy is a minimally invasive procedure that has revolutionized the diagnosis and treatment of various gynecological conditions, including abnormal uterine bleeding. Recent advancements, such as improved clinician training, the development of smaller-diameter hysteroscopes, and the increasing feasibility of office-based procedures, have contributed to its widespread adoption. The technique allows for direct visualization of the endometrial surface and cervical canal through an optical device while also enabling simultaneous operative interventions. It is now considered the gold standard for evaluating the endometrial cavity. Despite its advantages, hysteroscopy is not recommended as a first-line diagnostic tool due to its cost and invasive nature. Instead, alternative methods such as pelvic ultrasonography, saline infusion sonography (SIS), and endometrial sampling are preferred initially. However, hysteroscopy offers a distinct advantage by reducing the likelihood of missing focal pathologies compared to other diagnostic techniques [30,31].

2.2.1. Indications for Hysteroscopy

Hysteroscopy is indicated for a broad range of clinical scenarios, including the evaluation and management of:

- **Abnormal uterine bleeding:** This includes premenopausal and postmenopausal bleeding, where hysteroscopy can identify and treat structural abnormalities.
- **Endometrial thickening or polyps:** These conditions can be directly visualized and, when necessary, treated during the procedure.
- **Submucosal fibroids and some intramural fibroids:** Hysteroscopy provides an effective approach for diagnosing and treating these lesions.
- **Intrauterine adhesions (Asherman syndrome):** The procedure allows for both diagnosis and lysis of adhesions.
- **Congenital uterine anomalies (e.g., uterine septum):** Hysteroscopy is crucial for identifying and correcting müllerian anomalies.
- **Retained intrauterine devices or foreign objects:** Hysteroscopy offers a precise and minimally invasive method for removal.

· **Endometrial hyperplasia and malignancies:** Guided biopsies can be performed for diagnostic confirmation and post-treatment evaluation.

· **Cesarean section scar defects (isthmoceles):** The procedure enables accurate assessment and potential surgical correction.

· **Persistent bleeding after pregnancy termination:** Hysteroscopy helps identify retained products of conception or other abnormalities.

· **Endocervical lesions and chronic leukorrhea:** These conditions can be assessed and managed during hysteroscopy.

· **Preoperative planning and postoperative evaluation:** It is an indispensable tool for surgical planning and follow-up [32,33].

2.2.2. Contraindications for Hysteroscopy

Absolute contraindications include:

· Suspected or confirmed intrauterine pregnancy

· Known cervical or endometrial cancer

· Active pelvic infections, including genital herpes

· Pyometra

Relative contraindications include:

· Severe cervical stenosis

· Operator inexperience or lack of appropriate training

· Known advanced endometrial cancer [33,34].

Technique and Instrumentation

Hysteroscopy involves a combination of advanced instruments and techniques tailored to the patient's condition and procedural goals. The core components include a hysteroscope with a telescope, light source, distension medium, and camera system. The hysteroscope's size and design depend on whether it is used for diagnostic or operative purposes.

Telescopes: Telescopes are central to the hysteroscope and typically consist of three parts: the eye lens, body, and objective lens. A 4 mm lens is commonly used for its ability to provide clear and sharp images. Telescopes with 0° and 30° viewing angles are the most frequently utilized, offering either direct or oblique views of the uterine cavity.

Light Sources: Effective visualization during hysteroscopy requires robust illumination. Light systems include fiber-optic or liquid cables paired with generators such as xenon, halide, or tungsten. Xenon light sources are preferred for their ability to produce high-quality white light, while tungsten

generators, although cost-effective, produce lower-quality light.

2.2.3. Diagnostic and Operative Sheaths

· **Diagnostic sheaths** are small (4–5 mm) and allow for the transmission of distension media.

· **Operative sheaths** are larger (7–10 mm), enabling the introduction of instruments and distension media for surgical procedures. The latest models, with isolated channels, ensure continuous flow of media, improving visualization and surgical precision.

Flexible Hysteroscopes: Flexible hysteroscopes are designed to navigate the uterine cavity without requiring cervical dilation. They provide excellent maneuverability and patient comfort but are costlier and offer slightly lower image quality compared to rigid hysteroscopes [35–40].

2.2.4. Distension Media

The uterine cavity is a potential space that requires separation of its anterior and posterior walls for optimal visualization. This is achieved using distension media, which may be gaseous or liquid.

Gas Media: Carbon dioxide is the only gas used for uterine distension. It provides clear visualization and is rapidly absorbed, making it suitable for diagnostic hysteroscopy. However, improper use can result in complications such as gas embolism or shoulder pain [41,42].

Liquid Media: Liquid media are preferred for most hysteroscopic procedures. Low-viscosity fluids, such as isotonic saline and Ringer's lactate, are widely used due to their cost-effectiveness and safety profile. Non-electrolytic solutions like glycine and sorbitol are suitable for procedures involving electrosurgery. However, care must be taken to monitor fluid balance and avoid complications such as electrolyte imbalances, hypervolemia, or organ dysfunction [43,44].

2.2.5. Clinical Significance

Hysteroscopy offers unparalleled diagnostic and therapeutic capabilities in gynecology. Its ability to provide detailed visualization and targeted treatment makes it a cornerstone of modern gynecological practice. By minimizing invasiveness and enhancing accuracy, hysteroscopy continues to improve outcomes for both patients and clinicians [45].

2.3 Principles of Hysteroscopy

Hysteroscopy is performed using specialized instruments that can be sterilized with gas or soaked in a 2% glutaraldehyde solution for 30 minutes before use. Diagnostic office hysteroscopy typically requires only local anesthesia, while general anesthesia is preferred for longer and more complex

operative hysteroscopies, such as those performed in conjunction with laparoscopy. For operative procedures, it is advisable to schedule the procedure during the postmenstrual phase, when the endometrium is thin, or after preparing the endometrium with gonadotropin-releasing hormone (GnRH) analogs.

During the procedure, the telescope is inserted into a sterile sheath, and connections for the light source and distension medium are established. Adequate cervical dilation may be performed if necessary. The uterine cavity is distended using the selected medium to provide clear visualization. The cavity is systematically observed, and any pathological tissue is resected from the posterior to the anterior wall, ensuring the endometrial border is not breached. Hysteroscopy enables simultaneous diagnostic and therapeutic interventions, such as biopsy, polyp or myoma excision, and treatment of pathologies like uterine adhesions or septa. Operative procedures should not be performed without a clear view, as this increases the risk of complications like uterine perforation. If complications are suspected, laparoscopy may be performed to inspect the uterine serosa and confirm safety [45].

2.3.1. Complications

Complications from hysteroscopy are rare but can sometimes be life-threatening. The complication rate for diagnostic hysteroscopy is significantly lower than for operative procedures. Common complications include:

- Uterine perforation
- Injury to the bowel or urinary tract
- Cervical lacerations
- Excessive fluid absorption
- Embolism
- Hemorrhage
- Thermal injury from electrosurgical instruments
- Anesthesia-related complications
- Infections
- Tumor dissemination
- Complications related to distension media

While these complications are infrequent, careful procedural planning, appropriate instrument selection, and operator expertise are critical to minimizing risks [46,47].

2.4 Pain

Pain is a significant health concern, defined by the International Association for the Study of Pain (IASP) as an unpleasant sensory and emotional experience associated with actual or potential tissue damage. It may occur with or without an organic cause [48]. Pain assessment begins with asking the patient whether they are experiencing pain. If present, further details should be gathered, including the location, intensity, exacerbating and alleviating factors, and pain type. This information allows for a comprehensive understanding of the patient's condition.

Several tools are commonly used to evaluate pain, including:

· **Visual Analog Scale (VAS):** A widely used tool where patients rate their pain intensity along a 10 cm horizontal line, with one end representing "no pain" and the other "unbearable pain."

· **Verbal Descriptor Scale (VDS):** This categorizes pain into descriptive levels such as "none," "mild," "moderate," and "severe."

· **Verbal Numerical Scale (VNS):** Patients assign a numerical value to their pain, with 0 indicating no pain and 10 representing unbearable pain.

· **Wong-Baker Faces Scale:** Designed for patients with communication difficulties, this scale uses facial expressions to correlate with pain levels.

· **Analog Chromatic Continuous Scale (ACCS):** Similar to the VAS, this tool utilizes color gradients to indicate varying levels of pain intensity.

· **Dermatomal Pain Mapping:** This method involves marking pain intensity and location on a body diagram according to dermatomal regions.

Effective pain assessment relies on combining patient-reported experiences with these tools, enabling clinicians to tailor pain management strategies to individual needs.

Conclusions

Hysteroscopy has emerged as one of the most transformative tools in modern gynecology, offering both diagnostic and therapeutic advantages for a variety of uterine pathologies. Its minimally invasive nature, combined with its ability to provide real-time visualization and targeted interventions, has made it a cornerstone in the management of abnormal uterine bleeding, endometrial polyps, fibroids, and other intrauterine conditions. This dual functionality not only enhances diagnostic precision but also significantly reduces the need for multiple procedures, benefiting both patients and healthcare systems.

Diagnostic Significance

Hysteroscopy's ability to directly visualize the uterine cavity offers unparalleled diagnostic accuracy, setting it apart from other modalities such as ultrasonography, saline infusion sonography, and hysterosalpingography. These conventional imaging techniques, while useful as first-line diagnostic tools, are limited in their ability to detect focal lesions or provide definitive confirmation of findings. Hysteroscopy addresses these limitations by enabling the operator to directly observe and assess the endometrial and cervical regions. This is particularly important for identifying subtle or small pathologies such as polyps, adhesions, and submucosal fibroids, which might be overlooked by imaging studies alone.

Therapeutic Potential

Beyond its diagnostic utility, hysteroscopy provides an effective platform for minimally invasive surgical interventions. Procedures such as polypectomy, myomectomy, and adhesiolysis can be performed during the same session as diagnostic evaluation, reducing the need for multiple hospital visits and general anesthesia. This integration of diagnosis and treatment is especially valuable in cases where timely intervention is critical, such as in patients with abnormal uterine bleeding or infertility. Additionally, hysteroscopy allows for precise surgical correction of congenital anomalies, such as uterine septa, which can improve reproductive outcomes in affected patients.

Safety Profile and Patient Benefits

One of the key advantages of hysteroscopy is its favorable safety profile. When performed by skilled operators under appropriate conditions, the procedure is associated with a low incidence of complications. Diagnostic hysteroscopy, in particular, carries minimal risks, making it a highly attractive option for initial evaluation. Even in operative hysteroscopy, advancements in instrumentation and techniques, such as the use of smaller-diameter hysteroscopes and improved distension media, have further reduced the likelihood of complications. Patients also benefit from the procedure's minimally invasive nature, which typically results in shorter recovery times, lower pain levels, and minimal scarring compared to traditional surgical methods.

Challenges and Limitations

Despite its many advantages, hysteroscopy is not without challenges. The success of the procedure is heavily reliant on the expertise and experience of the operator. Inadequate training or improper technique can increase the risk of complications, such as uterine perforation, fluid overload, or thermal injuries. Therefore, structured training programs and continuous professional development are essential for ensuring optimal outcomes.

Another limitation is the cost and accessibility of hysteroscopic equipment and facilities, which may restrict its availability in resource-limited settings. This is particularly relevant in low- and middle-income countries, where access to advanced gynecological care remains a challenge. Efforts to develop cost-effective hysteroscopic systems and portable solutions could help address this disparity.

Complications and Risk Management

Although complications from hysteroscopy are rare, they can have serious consequences if not promptly addressed. The most common complications include uterine perforation, cervical lacerations, excessive fluid absorption, and thermal injuries. These risks underscore the importance of careful pre-procedural planning, including appropriate patient selection, the use of well-maintained equipment, and adherence to established safety protocols. For operative hysteroscopy, maintaining clear visualization throughout the procedure is critical to avoiding complications. In cases of suspected uterine perforation or other intraoperative complications, immediate laparoscopic evaluation may be necessary to ensure patient safety.

Future Directions and Innovations

The future of hysteroscopy is promising, with ongoing advancements aimed at improving both the safety and efficacy of the procedure. The development of flexible hysteroscopes with enhanced maneuverability and high-definition imaging capabilities is expected to further enhance diagnostic accuracy and procedural efficiency. Additionally, innovations in distension media, such as the use of safer and more effective solutions, are likely to reduce the risk of complications associated with fluid overload and electrolyte imbalances.

Another exciting frontier is the integration of artificial intelligence (AI) into hysteroscopic practice. AI-powered systems could assist in real-time image analysis, helping clinicians identify subtle abnormalities and standardize diagnostic criteria. This technology has the potential to reduce inter-operator variability and improve overall diagnostic accuracy, particularly in resource-constrained settings where expert operators may be limited.

Hysteroscopy has firmly established itself as an indispensable tool in gynecology, offering unparalleled diagnostic and therapeutic benefits. Its ability to combine accurate visualization with minimally invasive surgical interventions has revolutionized the management of uterine pathologies, significantly improving patient outcomes. However, realizing the full potential of hysteroscopy requires addressing existing challenges, such as ensuring adequate training for operators and improving the accessibility of equipment. Continued innovation and research into new technologies and techniques are essential to further expand the scope and impact of this transformative procedure.

As hysteroscopy continues to evolve, it is likely to play an increasingly prominent role in gynecological care, setting new standards for precision, safety, and patient satisfaction. By bridging the gap between diagnosis and treatment, hysteroscopy represents a true paradigm shift in the management of intrauterine conditions.

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CHAPTER 7

ANATOMY OF THE TEMPOROMANDIBULAR JOINT

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ANATOMY OF THE TEMPOROMANDIBULAR JOINT

A joint is the connection or junction between two or more bones. The temporomandibular joint (TMJ) is a joint located on either side of the head that allows the mandible to move during speech and chewing movements. It is of great interest to dentists, orthodontists, clinicians and radiologists (Alomar et al., 2007) .

The TMJ is the most protected joint in the body. Because it is necessary for survival, such as chewing, communication and reproduction (Hatcher, 2022). This structure may also be called mandibular articulation or craniomandibular articulation. The TMJ is one of the most significant and complicated joints in the human body. This joint performs its duties with the harmonious operation of the joint ligaments, muscles, tongue, teeth, pharynx, soft palate, peripheral nerves related to sensation and movement, all other systems in the head and neck, and even the mental structure (Durna, 2011). It is a compound joint that is a mixture of ginglymus and plana group joints and also morphologically varies from person to person and even the right and left joints in the same person (Dağ et al., 2011).

1. EMBRYOLOGY OF THE TMJ

The formation of joints in the body generally begins in the early stages of intrauterine life and is completed until the end of the embryonal period and they consists of a single blastema (undifferentiated cells that can regenerate). However, the development of the TMJ begins with the formation of the glenoid fossa in the 7th-8th weeks of the embryonic period. TMJ consists of two blastemas, not a single blastema like other joints (Velasco et al., 1999; Stocum and Roberts, 2018; Arslan and Altındağ, 2023).

During the formation of the TMJ, structures called arches (pharyngeal for humans) that play a role in the formation of external appearance features emerge in the 4th-5th weeks of development. The pharyngeal arches are the first pharyngeal arch (Meckel's cartilage), the second pharyngeal arch (Reichert's cartilage), the third pharyngeal arch (Thyrohyoid arch), the fourth and the sixth pharyngeal arches. The mandible and TMJ are formed by Meckel's cartilage (First Pharyngeal Arch) (Stocum and Roberts, 2018; Arslan and Altındağ, 2023).

In the development of the TMJ, the glenoid fossa is first completed, which occurs with the accumulation of mesenchymal cells in the 7th-8th weeks of intrauterine life. The disc and capsule then form in this region. Ossification of the glenoid fossa begins in the 10th-11th weeks. The development of the cortical layer and bone trabeculae in the fossa occurs much faster than in the condyle. Then, the glenoid fossa grows medially and anteriorly, starting from the zygomatic arch region. The articular eminence, which facilitates the

movement of the disc, also begins to develop during this period (Stocum and Roberts, 2018; Arslan and Altındağ, 2023).

The articular disc is first observed as a mesenchymal cell mass in the uterus at 7.5 weeks. The typical fibrocartilage structure of the disc begins to appear at 19 and 20 weeks. The joint capsule appears as thin bundles around the joint area at 9 and 11 weeks. At the end of the 17th week, the boundaries of the capsule begin to become clear. After the 26th week, the cellular and synovial differentiation of the capsule is completed. The condyle develops from mesenchymal cells lateral to the Meckel cartilage at 10-11 weeks and initially has cartilaginous structure. While the formation of the upper joint cavity begins at 12-13 weeks, the lower joint cavity takes its final shape and both joint spaces complete their formation after the 14th week. The critical period of TMJ morphogenesis occurs between the 7th and 11th weeks of development (Velasco et al., 1999; Stocum and Roberts, 2018; Arslan and Altındağ, 2023).

2. ANATOMY OF THE TMJ

The TMJ is the only movable joint among the bones that form the skull, connecting the mandible to the structures in the upper part of the head and playing an important role in functions such as chewing and speaking by regulating the movements of the mandible. It is also defined as the most complex joint of the body. TMJ has a very special structure in terms of connecting to both the mandible and the skull (Dere, 1994; Sancak and Cumhur, 2002; Arslan and Altındağ, 2023).

The TMJ is a diarthrodial (synovial) joint of the condylar joint type located between the mandibular fossa under the temporal bone and the mandibular condyle just in front of the external auditory canal (Sancak and Cumhur, 2002; Odabaş and Arslan, 2008; Hatcher, 2022). The articular surface is covered by a synovial membrane, and since this membrane secretes synovial fluid, the TMJ is also called a synovial joint (Bilgir, 2020). The upper articular surface is located in the temporal bone. The convex anterior part of this surface is called the articular tubercle, and the concave posterior part is called the mandibular fossa. The lower articular surface is located in the mandible and is called the mandibular condyle (Arıncı ve Elhan, 2001; Sancak and Cumhur, 2002; Dağ et al., 2011).

The TMJ is formed by the coming together of many anatomical structures such as ligaments, muscle groups, discs and capsules, as well as bones (Arslan and Altındağ, 2023). It is a highly specialized joint in many ways and is distinguished from other joints by the fact that the surfaces of the joint are covered with fibrous cartilage (fibrocartilage) containing varying degrees of cartilage cells, not hyaline (Alomar et al., 2007; Dağ et al., 2011).

The articular capsule resembles a funnel with its wide part at the top. The articular capsule is thin and loose above and narrow below. The joint capsule is made of loose connective tissue and its wide upper part is attached to the anterior articular surface of the articular tubercle at the front, the edges of the joint surface of the mandibular fossa at the back, leaving the petrotympanic fissure outside, and its narrow lower part is attached to the neck of the mandible at the bottom. This capsule is quite wide compared to the articular surfaces. Therefore, the mandibular condyle can easily slide forward without tearing the joint capsule (protraction) (Arıncı ve Elhan, 2001; Sancak and Cumhur, 2002). The back part of the capsule is longer and contains more elastic fibers compğared to the other parts. Therefore, articular capsule does not prevent the mandibular condyle from moving forward by lengthening during opening of the jaw. Thanks to this elasticity, it also helps the mandibular condyle to return to its place during closing of the jaw. The joint capsule is loose enough not to prevent the wide movement of the mandibular condyle, but it is solidly structured. The articular disc, located in the joint cavity, is attached to the part of the joint capsule close to the mandibular condyle with its surroundings. Therefore, the part of the joint capsule below the disc is short and tight, while the part above it is long and loose (Arıncı ve Elhan, 2001).

The articular disc is the most important anatomical structure of the TMJ (Alomar et al., 2007). Since the joint surfaces do not fit together, it is oval-shaped and made of biconcave dense fibrous cartilage and is located in the joint cavity. Thanks to this disc, the joint surfaces fit together and movement occurs (Arıncı ve Elhan, 2001; Sancak and Cumhur, 2002). The articular disc, also called the meniscus, is a structure consisting of dense fibrous connective tissue that does not contain blood vessels or nerve fibers (Durna, 2011). It is a fibrocartilage structure located in the joint capsule between the glenoid fossa and the mandibular condyle, separating the synovial space into two, and does not contain vessels or nerves. When the mouth is closed, it is located between the mandibular condyle and the mandibular fossa, and when the mouth is open, it is located between the mandibular condyle and the articular tubercle. The upper surface of the discus, which has a thicker peripheral part, is slightly concave in the front and clearly convex in the back to fit the joint surface in the temporal bone. Its lower surface, which is attached to the mandibular condyle like a hat, is concave (Alomar et al., 2007; Arslan and Altındağ, 2023). Unlike other synovial joints, the temporomandibular condyle and temporal bone do not fit over each other in the absence of the disc. The disc stabilizes the joint during rotation and translation (Bilgir, 2020).

The disc adheres to the mandible more tightly than the temporal bone. Therefore, in cases where the mandibular condyle dislocates forward, the disc also slides forward with the mandibular condyle and passes in front of the articular tubercle. The articular surfaces that form the jaw joint are covered

with fibrous cartilage. The articular disc adheres to the joint capsule that surrounds the articular surfaces (Sancak and Cumhur, 2002).

The upper beams of the tendon of the lateral pterygoid muscle attach to the anterior part of the discus articularis through the joint capsule. The remaining large part of the muscle attaches to the pterygoid fovea in the mandible. Therefore, when this muscle pulls the mandible forward, the disc is pulled forward along with the joint capsule. Short and strong fiber bundles connect the inner and outer sides of the articular disc to the lower-lateral parts of the mandibular condyle. Thus, it helps the disc to move together during the anterior-posterior movement of the mandibular condyle. The posterior part of the articular disc is divided into lower and upper parts by a vein network. The upper part is made of fibroelastic tissue and attaches to the temporale bone at the posterior border of the mandibular fossa. The joint surfaces are covered with fibrous cartilage. Most of it consists of collagen and some of it consists of cartilage (Arıncı ve Elhan, 2001).

Since the articular disc is attached to the articular capsule with its surroundings, it divides the joint cavity into two independent spaces, the lower and upper. However, sometimes the middle of the disc may be perforated. In such cases, both spaces are connected to each other (Arıncı ve Elhan, 2001; Sancak and Cumhur, 2002; Odabaş and Arslan, 2008). The lower and upper joint spaces are filled with synovial fluid and allow the synovial fluid to spread as a film layer on the joint surface, providing lubrication (Bilgir, 2020).

The disc is anatomically examined in the sagittal plane as three separate regions, central, anterior and posterior, according to its thickness:

1. Anterior band (pars meniscus): It is the thin end of the disc at the front and attaches to the fibers of the superior lateral pterygoid muscle and the capsule. The anterior part is thinner than the posterior part and is 2 mm thick.

2. Central (intermediate zone, pars gracilis): It is the thinnest part and is 1 mm thick.

3. Posterior band (pars posterior): It is the thickest part and attaches to the retrodiscal area (bilaminar zone) consisting of dense neural and vascular structures and is 3 mm thick (Alomar et al., 2007; Odabaş and Arslan, 2008; Bilgir, 2020; Arslan and Altındağ, 2023).

The position of the disc has long been described as the 12 o'clock position. In a clock dial that is assumed to be placed in the center of the condyle, if the hour hand is at the 12 o'clock position and the posterior band of the disc is at this limit, this situation is called the ideal disc position. However, studies have revealed that the posterior band positioned anywhere between the 11 and 12 o'clock positions is also frequently seen in healthy individuals at 11. Therefore, the disc located anywhere in this area is considered to be in a normal position (Durna, 2011).

The shock absorbing feature and resistance to deformation in the central region of the disc are higher than in the posterior and anterior regions. The function of the disc is to prevent the pressure on the joint from concentrating at one point and to spread it over a wide area (Bilgir, 2020). The main function of the articular disc is to reduce the load between the joint surfaces of the glenoid fossa and the condylar head by distributing it equally. If the articular disc is exposed to excessive pressure, collagen thickening is observed in these areas. The lateral surface of the disc is the area most exposed to pressure, therefore perforations are frequently seen in this area (Arslan and Altındağ, 2023).

The synovial membrane, a serous synovial membrane covers the joint surfaces in the joint cavities. The synovial membrane fills the gaps created by the harmony of the joint surfaces, thanks to its extensions towards the joint cavities (Durna, 2011). The synovial membrane is a thin and vascular-rich membrane that surrounds all intra-articular structures except the mandibular condyle, fossa, articular eminence, articular cartilage and disc. The synovial membrane is hard and rigid, but it has the ability to change shape to adapt to changes in position and intra-articular pressure (Arslan and Altındağ, 2023).

The articular disc divides the joint space into two separate spaces. Therefore, the synovial membrane is two, superior synovial membrane and inferior synovial membrane. The first of these covers the fibrous membrane that lies above the discus articularis, and the second covers the fibrous membrane underneath (Arıncı ve Elhan, 2001). In addition, the membrane secretes the joint fluid called synovia. Synovia is rich in mucin and has a color and fluidity similar to egg white (Durna, 2011). Synovial fluid provides the metabolic needs of nonvascular articular joint surfaces and lubrication of articular surfaces during function (Odabaş and Arslan, 2008).

The synovial fluid, which is responsible for meeting the nutritional and metabolic needs of the disc, is produced by the synovial membrane, which contains cells with phagocytosis and immunological properties. Synovial fluid is a transparent, viscous, light-colored fluid containing mucopolysaccharides. The hyaluronic acid found in the synovial fluid determines the fluidity level specific to the fluid. The volume of the synovial fluid in the joint space is regulated by the synovial membrane. Synovial fluid plays a role in reducing friction by lubricating the joint (Arslan and Altındağ, 2023).

Among the functions of synovial fluids;

1- It has a lubricating function to reduce friction between joint surfaces during function.

2- Since the articular surfaces of the joint and the disc are avascular, it acts as a mediator in the transport of metabolite needs of these tissues. There

is a separate rapid exchange between the capsule vessels, synovial fluid and joint tissues.

3- It has a cleaning function to remove waste materials from the joint cavity (Durna, 2011).

Although the TMJ has many things in common with other synovial joints, it has a special character. There are some features that make the TMJ different from other joints of the body. These features are;

• Although both joints act as separate functional units, since these two joints are connected to each other by the mandible, movement or functional changes in one also affect the other. Therefore, bilateral synchronization is required for the normal function of the TMJ.

• The articular surfaces are surrounded by fibroelastic tissue, not hyaline cartilage, and the condylar cartilage plays an significant role in the response to trauma to which the condyle is exposed. Fibrous connective tissue is more resistant to degenerative changes than hyaline cartilage.

• Another unique feature of the TMJ is that it is the only joint that contains the growth center activity in the joint capsule, which plays an significant role in the bone development of the mandible (Bilgir, 2020; Demirsoy and Akbulut, 2020).

2.1. Ligaments of TMJ

TMJ ligaments are collagenous connective tissue and play an significant role in preserving the joint structure, fixing the joint, and directing and limiting movement. They do not actively participate in joint functions, but act passively as inhibitory structures that limit joint movements together with the joint capsule. These ligaments, which do not stretch, protect the joint by lengthening against large and continuous forces and preventing the load from being transmitted to the joint. Abnormal pressure and tension can damage the ligaments (Durna, 2011; Arslan and Altındağ, 2023).

As in all joints, the ligaments of the masticatory system have three functions: stabilization, movement guidance and limitation of movement. From a functional perspective, their most important function is to limit movement (Bilgir, 2020).

TMJ ligaments are examined in two sections: functional and accessory ligaments. TMJ has three functional and two accessory ligaments (Odabaş and Arslan, 2008; Durna, 2011; Arslan and Altındağ, 2023).

2.1.1. Functional Ligaments

Capsular Ligament (Articular Capsule): Capsular ligament is a thin tissue cover surrounding the entire TMJ. This fibrous ligament is also called

the "articular capsule" by some researchers. It is reinforced laterally by the temporomandibular ligament, which restricts the posterior movement and distraction of the condyle. It attaches to the neck of the mandible below, to the pars squamosa of the temporal bone above, to the articular cartilage on the sides, to the petrotympanic fissure behind and finally to the anterior part of the articular tubercle in front. Since the front and back parts have a loose structure, movements can be made easily. The movement of the jaw is restricted by the temporomandibular ligament formed by the thickening of the back and side parts of the capsule. The capsular ligament prevents the lateral, medial and inferior forces that tend to separate and dislocate the joint surfaces and also surrounds the entire joint, ensuring that the synovial fluid remains within the joint (Durna, 2011; Bilgir, 2020; Arslan and Altındağ, 2023).

Collateral (Discal) Ligament: They attach to the lateral and medial parts of the condyle and disc. Collateral ligaments divide the joint mediolaterally into the lower and upper joint cavities. Their basic function is to prevent the condyle and disc from moving away from each other beyond a certain point. During the protraction of the condyle that accompanies the depression of the mandible, the outer horizontal band becomes tense. During this time, while the anterior rotational and gliding movements of the condyle occur, it limits its inferior distraction. The inner horizontal band becomes compressed during the retraction of the mandibular head and limits the posterior movement of the mandible. Discal ligaments are responsible for the hinge type movement between the mandibular condyle and the articular disc. They play a role in the harmonious movement of the disc during rotational movement. There are two collateral ligaments, lateral and medial (Bilgir, 2020; Arslan and Altındağ, 2023). The medial discal ligament connects the medial edge of the disc to the medial part of the condyle. The lateral discal ligament connects the lateral edge of the disc to the lateral pole of the condyle (Durna, 2011).

Temporomandibular Ligament (Lateral Ligament): The lateral part of the capsular ligament is strengthened by tight fibrils and is named after this ligament. This main ligament, located lateral to the capsule, cannot be easily distinguished by dissection, but can be easily distinguished by the orientation of the collagen fibrils (Durna, 2011). It resembles a triangle with its base up and its apex down. Above, it attaches to the outer-posterior part of the zygomatic process of the temporal bone and the outer-inferior edge of the articular tubercle. It narrows downward and towards the posterior and forms the top of the triangle. Below, it attaches to the posterior part of the lateral surface of the neck of the mandible. The fibrous capsule thickens on the outer side and forms this ligament. The fibers of this ligament extend downward and posteriorly in an oblique line. The outer surface of the laterale ligament is in close proximity to the parotid gland, while its inner surface is in close proximity to the articular capsule (Arıncı ve Elhan, 2001; Sancak and Cumhur, 2002). It consists of two

parts: oblique (outer) and horizontal (inner). The oblique part is responsible for restricting the forward movement of the condyle head and excessive opening of the mouth. The horizontal part prevents the backward movement of the articular disc and the condylar head, preventing traumas that may damage the retrodiscal tissues (Bilgir, 2020; Arslan and Altındağ, 2023).

There may also be a ligament called the medial ligament, which is thinner and weaker than the lateral ligament and sometimes cannot be noticed because it is fused with the joint capsule. It is closely related to the articular capsule, attached to the medial end of the petrotympanic fissure and the sphenoidal spine above, and to the inner side of the neck of the mandible below (Arıncı ve Elhan, 2001).

2.1.2. Accessory Ligaments

They are ligaments located a little farther from the joint but affect the movements of the joint. These are:

Sphenomandibular Ligament: It is the residue of Meckel's cartilage. It is a thin, flat, band-shaped structure located in the medial part of the TMJ and attaches to the sphenoidal spine, lateral to the foramen spinosum. It expands downward and attaches to the lingula of mandible on the inner surface of the mandibular ramus. The airuculotemporal nerve and the lateral pterygoid muscle are located in the upper-outer part of this ligament, and the medial pterygoid muscle is located in the inner part. In addition, maxillar artery and vein, inferior alveolar artery and vein, inferior alveolar nerve and a part of the parotid gland pass between this ligament and the neck of the mandible (Arıncı ve Elhan, 2001; Sancak and Cumhur, 2002; Alomar et al., 2007) . It has no direct effect on TMJ movements. Its function is to prevent damage to the veins and nerves exiting the mandibular canal during joint movements (Bilgir, 2020; Arslan and Altındağ, 2023).

Stylomandibular Ligament: It is a thickened section of the deep cervical facsia in the form of a band. It extends from the tip of the styloid process in the temporal bone to the lower part of the posterior edge of the mandibular ramus and to the mandibular angle. This ligament extends between the masseter muscle and the medial pterygoid muscle, as well as between the parotid gland and the submandibular gland. Some fibers of the styloglossus muscle also begin from the inner side of this ligament (Arıncı ve Elhan, 2001; Sancak and Cumhur, 2002). It is loose when the mouth is fully open or closed. It prevents excessive protrusive movement by stretching at maximum protrusion movement (Arslan and Altındağ, 2023). It even prevents excessive upward rotation of the mandible. This situation causes problems in patients with a significantly reduced vertical dimension (Bilgir, 2020). In some cases, due to calcification in the ligament, an image appears as if it were a continuation of the bone. In such a case, complaints similar to the symptoms of temporomandibular disorder can sometimes be seen. This disorder, which

is accompanied by symptoms such as a stinging sensation in the throat, limited neck movements, and pain in the eyes and ears, is known as "Eagle Syndrome". These symptoms must be distinguished from the symptoms of temporomandibular disorder (Durna, 2011).

The structure called pterygomandibular raphé, which is not as important as the previous two ligaments in terms of its effect on the movement of the TMJ, consists of tendinous fibers. It extends from the pterygoid hamulus of sphenoid bone above to the posterior edge of the mylohyoid line below (Arıncı ve Elhan, 2001).

2.2. Masticatory Muscles

Masticatory muscles are the muscles that play a role in the rotation and translation of the mandible. Masticatory muscles are divided into two groups: the muscles that open and close the jaw. Of these muscles, the masseter, temporal, and medial pterygoid muscles play a role in opening the jaw, while the lateral pterygoid muscle allows the jaw to close. All of these muscles are innervated by the mandibular branch of the trigeminal nerve, which is the Vth cranial nerve (Durna, 2011; Arslan and Altındağ, 2023).

In addition to these muscles, there are also muscles that assist in the movements of the mandible. These are the digastric, mylohyoid, geniohyoid, stylohyoid muscles in the suprahyoid group and the sternohyoid, omohyoid, sternothyroid and thyrohyoid muscles in the infrahyoid group. The assisting muscles are involved in processes such as swallowing. In addition to these, the buccinator muscle plays a role in chewing by ensuring that food enters between the occlusal surfaces of the teeth during the chewing movement (Arslan and Altındağ, 2023).

The bilateral and simultaneous contractions of the masticatory muscles and suprahyoid muscles allow the jaw to perform two types of movements. The first is the rotation movement of the condylar heads around the horizontal axis during mouth opening, and the second is the sliding movement of the articular disc and condyle together forward and downward. In the centric position, the thin middle part of the disc conforms to the convexity of the condylar head and articular tubercle, while during the sliding and rotation movement, it changes direction from the anterior-superior surface of the condylar head to its upper surface and then to its posterior-superior surface (Durna, 2011).

2.3. Arteries and Nerves of TMJ

Arteries: There is a rich vascular network around TMJ. The superficial temporal artery and the branches of the maxillar artery are the most important. In addition, there are deep auricular, anterior tympanic and ascending pharyngeal arteries. The condyle provides its own vascular support

from the bone marrow cavity via the inferior alveolar artery (Arıncı ve Elhan, 2001; Sancak and Cumhur, 2002; Durna, 2011).

Nerves: It is innervated by the auriculotemporal nerve and the masseteric nerve, which is a branch of the mandibular nerve (Arıncı ve Elhan, 2001; Sancak and Cumhur, 2002).

2.4. Biomechanics of TMJ

TMJ movements are anatomically shaped by muscles and limited by ligaments. In order to understand mandibular movements, it is very significant to understand that the right and left TMJ move as a single joint during function (Dağ et al., 2011; Arslan and Altındağ, 2023). TMJ can be called the entire joint as a ginglymoarthrodial joint due to the rotation movement seen in the lower part of the joint and the term "ginglimoid" meaning ginglymus (hinge joint) and the translation movement seen in the upper part and the term "arthrodia" meaning arthrodia (joint that allows sliding movement of surfaces) that allows forward and backward movement only in one plane (Alomar et al., 2007; Dağ et al., 2011; Demirsoy and Akbulut, 2020; Arslan and Altındağ, 2023).

The TMJ is a very complex joint system. The presence of a pair of joints associated with the same bone, and the ability to move separately at the same time, makes the functions of the masticatory system even more complex. The mandible can move downward (depression), upward (elevation), backward (retraction) and forward (protraction). In addition, the caput mandibulae rotates on the lower surface of the discus articularis. This movement is made around the longitudinal axis passing through the middle of the caput mandibulae (Sancak and Cumhur, 2002; Durna, 2011). The movements of the mandible, which are elevation, protrusion, retrusion, depression and laterotrusion, occur together with translation and rotation. Mandibular movement is a combination of rotation and translation movements. The TMJ can be thought of as two separate joints, one between the temporal bone and the articular disc, and the other between the head of the mandibular condyle and the articular disc. While rotational movements occur in the inferior section, translational movements occur in the superior section (Bilgir, 2020).

During the opening of the mandible, the movement is first seen in the lower joint. Then, the upper joint participates in the movement as the articular disc slides forward together with the head of the mandibular condyle. The lower joint continues its unique movement during the forward sliding of the articular disc. The articular disc moves forward until the fibroelastic lamella attached to the temporal bone in its posterior-upper part is stretched. When the jaw closes, this fibroelastic lamella pulls the articular disc back to its normal position. The disc moving towards the front, when it comes to the articular tubercle, the direction of movement is downward - forward. In this

position, the jaw becomes extremely opened. There is a loose fat-connective tissue between the articular capsule and the external auditory canal. When the articular capsule is pulled forward with the articular disc, this loose fat-connective tissue fills the cavity between the two formations. During the closing and opening of the mandible, the joints on both sides go through the same phases. During this movement, a specific axis cannot be shown. If the movement were only between the disc and the head of the mandibular condyle, we could talk about a transverse axis passing through the head of the mandibular condyle. However, the fact that the head of the mandibular condyle moves forward and backward with the articular disc complicates the movement of the joint. The opening-closing movement of both joints is directed by the stylomandibular ligament, sphenomandibular ligament, medial pterygoid muscle and masseter muscle. Therefore, considering the attachment points of these formations to the mandible, we can talk about a transverse axis that changes its place during the opening and closing phases of the mandible, close to the attachment points of the muscles and ligaments in the middle of the mandibular ramus (Arıncı ve Elhan, 2001).

In the normal position where the mandibular condyle is in the mandibular fossa, the lower and upper teeth do not touch each other and are in a very close position. In this position, the jaw can make slight anterior-posterior movements. If the jaw is pulled forward too much, the mandibular condyle comes over the articular tubercle located in the front. Thus, the mandible will be pushed down by the height of the articular tubercle, and the jaw will open a little more. However, in order to open the mandible completely, the infrahyoid muscles must also contract. In addition to these movements, our lower jaw can also move right and left as a grinding movement. In this movement, the joint of one side makes a sliding movement forward while the joint of the other side makes a rotation movement around the vertical axis. These movements continue by changing sides. During biting with incisors, some of the muscle force passes to the incisor teeth and some to the mandibular condyle. However, during mastication with molars, most of the muscle force comes to the teeth. Here the mandibular condyle only serves as a guide to regulate movement (Arıncı ve Elhan, 2001).

When the jaw is opened, the mandibular condyle slides forward with the articular disc until it reaches the lower level of the articular tubercle. As the forward sliding movement continues, the mandibular condyle rotates on the lower surface of the articular disc. This movement allows chewing and grinding movements (Sancak and Cumhur, 2002).

For effective temporomandibular joint movement, the muscles of the cervical region, the muscles and joint structures of the craniomandibular region, and the occlusal relationship between the teeth must be compatible. The basic osteokinetic movements of the mandible are depression, protrusion

and lateral movements. The 5 basic muscles that contribute to TMJ movements are;

1. The temporal muscle: Its function is to elevate the mandible, its anterior fibers pull the mandible upwards and its posterior fibers pull it backwards.

2. The masseter muscle: Primarily elevates the mandible. Its superficial fibers contribute to protrusion, while its deep fibers stabilize the condyle against the articular eminence.

3. The medial pterygoid muscle: When the fibers contract, the mandible elevates and the teeth come into contact. tI also allows the mandible to move forward..

4. The lateral pterygoid muscle: It has two parts. As a result of unilateral contraction of the inferior lateral pterygoid muscle, lateral movement of the mandible occurs in the opposite direction. The superior lateral pterygoid muscle draws the mandibular condyle and articular disc medially.

5. The digastric muscle: It pulls the mandible downward and backward. The TMJ has rotation (turning) and translation (sliding) movements. The rotation movement occurs between the mandibular condyle and the articular disc (inferior synovial cavity). The translation is between the temporal bone and the disc-condyle complex (superior synovial cavity) (Odabaş and Arslan, 2008).

The normal jaw opening is 35-50 mm. 25 mm of this movement is provided by rotation and 15 mm by translation. The resting position of the TMJ is the position where the mouth is slightly open, the lips are together, the teeth do not touch each other, and the first half of the tongue is on the hard palate (Odabaş and Arslan, 2008; Dağ et al., 2011; Gezer and Levendoğlu, 2016).

The term "stomatognathic system" is used for the TMJ, chewing muscles and related surrounding soft tissues. The structures that make up this system are as follows:

1. Muscles and Soft Tissues: The important muscles of the jaw consist of the temporal, masseter, lateral and medial pterygoid muscles that close the TMJ and the suprahyoid, infrahyoid and lateral pterygoid muscles that open the TMJ. The cervical region muscles to ensure the position of the head and stabilizing the neck in harmony during jaw joint movements are very important.

2. Joints: The TMJ includes dental alveoli and paravertebral joints and are important sources of pain. The TMJ is a synovial joint and contains an articular disc.

3. Bone Structures: The cranium, mandible, cervical and upper dorsal vertebrae, and sternum.

4. Teeth: Almost any dental pathology can naturally affect joint functions (Gezer and Levendoğlu, 2016).

3. DISORDERS OF THE TMJ

Temporomandibular disorders (TMD) are a heterogeneous group of neuromuscular and musculoskeletal disorders involving the TME complex and surrounding muscle and bone components (14). TMD affects 15% of adults and is the common name for a series of disorders that can be seen at any age, most commonly between the ages of 20-40, with common symptoms including pain in the jaw and surrounding tissues during jaw movements, limitation of jaw movements and/or sounds such as clicking and crepitation, and pain in the head, neck, ears, and teeth. The lifetime prevalence of TMD is between 3-15% in western societies (Gauer and Semidey, 2015; Gezer and Levendoğlu, 2016; Tanhan et al., 2019).

TMJ dysfunction is a condition that affects the stomatognathic system and related structures, such as the TMJ and masticatory muscles. TMJ dysfunction or disorders are one of the main causes of chronic pain in the orofacial region (Kaynak et al., 2019). Scientific research on TMJ dysfunction began in the 1950s. Early studies showed that occlusion affects masticatory muscle function. In 1956, Schwartz proposed the term "TMJ Pain-Dysfunction" to distinguish masticatory muscle disorders from organic disorders of the joint. Shore introduced the terms "TMJ Dysfunction" in 1959 and Voss introduced the terms "Pain-Dysfunction Syndrome" in 1964. Laskin proposed the use of the term "Myofascial Pain-Dysfunction" in his article in 1969, in which he stated that fatigue and muscle spasm resulting from chronic oral habits were responsible for the symptoms of pain-dysfunction syndrome. Ramfjord and Ash used the term "Functional TMJ Disorders" in 1971. McNeill, who thought that the symptoms could not be limited to TMJ, used the terms "Craniomandibular Disorder" in 1980 and Bell used the terms "Temporomandibular Disorders" in 1982 (Durna, 2011).

3.1. Etiology of TMJ Dysfunction

TMJ dysfunctions are complex and multifactorial. There is no clear etiology for temporomandibular joint disorders and many factors may contribute to this disorder (Sharma et al., 2011). However, it has been discovered that some factors cause or increase susceptibility to joint disorders. Biological, environmental, social factors and sensory and cognitive triggers are effective in the emergence of TMD, which is why their etiology is considered multifactorial (Durna, 2011; Gezer and Levendoğlu, 2016; Arslan and Altındağ, 2023). Factors that increase the risk of TMD are called "predisposing

factors", those that cause the onset of TMD are called "initiating factors", and factors that prevent recovery or increase the progression of TMD are called "perpetuating factors" (Sharma et al., 2011).

Etiological factors causing TMJ dysfunctions are classified into different groups. Occlusion is the first and probably the most discussed etiological factor of TMD (Sharma et al., 2011). In addition, pain, fibromyalgia, autoimmune diseases, sleep apnea and psychiatric diseases can also cause TMD (Durna, 2011; Gezer and Levendoğlu, 2016; Arslan and Altındağ, 2023).

Table 1. Classification of TMD

I. Disorders of the Masticatory Muscles	**II.TMD**	**III. Chronic Mandibular Hypomobility**	**IV. Developmental Disorders**
1. Protective co-contraction	1.Disorder in the condyle-disc complex a.Disc displacement b.Disc dislocation with reduction c.Disc dislocation without reduction	1. Ankylosis a. Fibrous b. Bony	1. Congenital and developmental bone disorders a. Agenesis b. Hypoplasia c. Hyperplasia d. Neoplasia
2. Local muscle pain			
3. Myofascial pain		2. Muscle contractures a. Myostatic b. Myofibrotic	
4. Myospasm	2.Structural incompatibility of the articular surfaces a.Shape changes i.in the disc ii.in the condyle iii.in the fossa b.Adhesions i.between the disc and the condyle ii.between the disc and the fossa c.Subluxation (hypermobility) d.Spontaneous dislocation	3.Coronoid impedance	2. Congenital and developmental muscle disorders a. Hypotrophy b. Hypertrophy c. Neoplasia
5. Myositis and others			
	3. Inflammatory diseases of the TMJ a. Synovitis/capsulitis b. Retrodiscitis c. Arthritis i. Osteoarthritis ii. Polyarthritis d. Inflammatory diseases of additional structures i. Temporal tendonitis ii. Inflammation of the stylomandibular ligament		

The role of occlusion in the development of TMD is controversial. It is now widely accepted that it contributes to TMD by initiating, perpetuating or predisposing them. Initiating factors lead to the onset of symptoms and

are primarily related to trauma or adverse effects on the masticatory system. Perpetuating factors include:

a. Behavioral factors (clenching, grinding, and abnormal head posture)

b. Social factors (may affect perception and learned response to pain)

c. Emotional factors (anxiety and depression)

d. Cognitive factors (negative thoughts and attitudes that may make the resolution of the disease more difficult) (Sharma et al., 2011).

Predisposing factors are psychological, pathophysiological or structural processes that alter the masticatory system to such an extent that they increase the risk of developing TMD (Sharma et al., 2011).

The etiology is multifactorial. These are:

1. Stress: Anxiety, stress and other emotional disorders can cause or increase TMD.

2. Occlusal causes: Malocclusion, hard bite, some occlusal interventions can be triggers. In addition, while some studies argue that orthodontic treatments increase TMD, in some studies, they have been shown to have no effect.

3. Parafunctional habits: Parafunctional habits such as gum chewing and bruxism cause microtrauma in the TMJ or hyperactivity in the masticatory muscles.

4. Other causes: Whiplash syndrome, genetics, inflammation caused by cytokines such as IL-1beta and TNF-alpha, hypermobility, female gender and hormonal factors also play an significant role in the etiology (Gezer and Levendoğlu, 2016).

3.2. Classification of TMD

TMD has been classified in many ways, but the classification developed by Bell in 1986, accepted by the American Dental Association (ADA) and modified by Okeson in 2019 is still valid today (Arslan and Altındağ, 2023). According to the classification made by the American Academy of Orofacial Pain and the International Headache Society, TMD is examined in four categories. These are; disorders of the masticatory muscles, TMD, chronic mandibular hypomobility, developmental disorders. These categories are also classified according to their clinical differences. This classification is shown in Table 1 (Odabaş and Arslan, 2008; Gezer and Levendoğlu, 2016; Arslan and Altındağ, 2023).

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CHAPTER 8

MOLECULAR DOCKING

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1. Introduction

Molecular docking is a computational technique that predicts the preferred orientation of one molecule (such as a drug or ligand) to a second molecule (typically a protein or nucleic acid) when bound to each other to form a stable complex. In medical biology and genetics, molecular docking is pivotal for understanding the interactions between small molecules and biological targets, aiding in drug discovery and the elucidation of biochemical pathways (Meng, Zhang, Mezei, & Cui, 2011).

2. History

The concept of molecular docking originated in the early 1980s with the advent of computational chemistry and the increasing availability of biological macromolecular structures. One of the pioneering efforts was the development of the DOCK program by Kuntz and colleagues in 1982, which used geometric algorithms to predict ligand binding sites on proteins (Kuntz, Blaney, Oatley, Langridge, & Ferrin, 1982).

Throughout the 1990s, advances in computational power and algorithms led to the refinement of docking techniques, incorporating scoring functions to estimate binding affinities and enhancing the accuracy of predictions. The integration of molecular docking into high-throughput virtual screening processes revolutionized drug discovery by allowing researchers to evaluate vast libraries of compounds efficiently (Kitchen, Decornez, Furr, & Bajorath, 2004).

In recent years, molecular docking has expanded beyond drug discovery to include the study of genetic variations affecting protein-ligand interactions, contributing to personalized medicine approaches. The continuous development of more sophisticated algorithms and the integration with other computational methods like molecular dynamics simulations have further enhanced the utility of molecular docking in medical biology and genetics (Pagadala, Syed, & Tuszynski, 2017).

3. Techniques in Molecular Docking

Molecular docking is a pivotal computational method in structural molecular biology and computer-assisted drug design, aiming to predict the optimal orientation of a ligand when bound to a protein to form a stable complex. Various techniques are employed in docking studies to simulate and understand these interactions. One fundamental approach is rigid docking, which assumes both the ligand and the receptor are rigid entities. While this simplifies computational requirements, it does not account for conformational changes that occur upon binding (Meng, Zhang, Mezei, & Cui, 2011).

To address this limitation, flexible docking techniques have been

developed. These methods allow for ligand flexibility, enabling the ligand to adopt multiple conformations while the receptor remains static. Some approaches also incorporate receptor flexibility, where certain side chains or regions of the receptor can move to accommodate the ligand. Induced fit docking further extends this concept by allowing both the ligand and the receptor to adjust their conformations for optimal binding, providing a more accurate simulation of biological interactions (Kitchen, Decornez, Furr, & Bajorath, 2004).

Advanced techniques such as consensus docking combine results from multiple docking programs to enhance prediction accuracy. Molecular dynamics-assisted docking incorporates molecular dynamics simulations to explore the conformational space of the ligand-receptor complex over time, capturing dynamic interactions that static models may miss. Additionally, quantum mechanics/molecular mechanics (QM/MM) docking utilizes quantum mechanics for the active site and molecular mechanics for the rest of the system, offering detailed insights into electronic interactions crucial for binding affinity and specificity (Pagadala, Syed, & Tuszynski, 2017).

4. Commonly Used Docking Software

A variety of software tools have been developed to perform docking studies, each employing different algorithms and scoring functions. AutoDock and AutoDock Vina are widely used open-source programs that utilize a Lamarckian genetic algorithm for conformational searching, handling ligand flexibility and some receptor flexibility (Morris et al., 2009). DOCK, one of the earliest docking programs, uses shape complementarity principles to predict binding modes, focusing on the geometric fit between the ligand and the receptor's active site (Kuntz, Blaney, Oatley, Langridge, & Ferrin, 1982).

Glide is a proprietary software known for its accuracy and speed, employing a hierarchical series of filters and an empirical scoring function to predict binding affinities (Friesner et al., 2004). GOLD uses a genetic algorithm for docking flexible ligands into protein binding sites, allowing for partial receptor flexibility to improve accuracy (Verdonk et al., 2003). FlexX employs an incremental construction algorithm for flexible ligand docking, efficiently handling ligand flexibility by building the ligand in the binding site step by step (Rarey, Kramer, Lengauer, & Klebe, 1996).

Other notable tools include Surflex-Dock, which uses a molecular similarity method to predict binding poses and incorporates a protomol-based approach for docking (Jain, 2003), and RosettaDock, part of the Rosetta suite designed for protein–protein docking, utilizing Monte Carlo methods and energy minimization techniques (Lyskov & Gray, 2008). Web-based services like SwissDock provide accessible docking tools without the need for software installation, based on the EADock DSS engine (Grosdidier,

Zoete, & Michielin, 2011). rDock is an open-source program suitable for high-throughput docking, supporting both proteins and nucleic acids, making it versatile for various applications (Ruiz-Carmona et al., 2014). The Molecular Operating Environment (MOE) is an integrated suite that includes docking, visualization, and simulation tools, offering multiple scoring functions and support for both ligand and receptor flexibility.

Different docking software packages have unique advantages and disadvantages. AutoDock and Vina are popular choices for their availability and versatility, whereas Glide and MOE provide advanced features and high accuracy but are cost-prohibitive for some users. GOLD offers unique optimization through genetic algorithms but may be computationally demanding. Table 1 summarizes the essential pros and cons of commonly used docking software for a quick comparison.

Table 1: Pros and Cons of Various Docking Software Tools

Docking Software	Pros	Cons
AutoDock	Open-source and free	Slow computational time for large ligands/proteins
	Flexible ligand and partial receptor flexibility	Limited accuracy for complex systems
	Extensive community support and documentation	
Vina	Faster than AutoDock, good for virtual screening	Limited manual parameter control compared to AutoDock
	Uses knowledge-based and empirical scoring for better predictions	Difficulty with unusual or complex ligands
	Ligand and partial receptor flexibility	
Glide	Advanced scoring function and algorithm	Expensive for academic use
	Integrated virtual screening and binding energy tools	Steeper learning curve
	Excellent graphical user interface	
GOLD	Uses genetic algorithm, enhancing exploration of binding poses	Computationally demanding
	Performs well in active sites with complex geometries	Requires a paid license
	Flexible parameter adjustments for different systems	

MOE	Integrated QSAR modeling and visualization	Requires a license, costly for some users
	High accuracy with sophisticated scoring functions	Requires substantial hardware for large docking studies
	Supports various ligand and protein flexibilities	
SwissDock	Free web-based tool for ease of use	Requires internet access and may have limited computational resources
	Automatically generates binding poses and free energy estimations	Limited control over docking parameters
	User-friendly and does not require specialized hardware	Cannot handle very large ligands or proteins efficiently
Surflex-Dock	Automated preparation of ligand and protein, simplifying the docking process	Commercial license needed
	Utilizes "protomol" concept for efficient ligand matching	Limited flexibility for receptor, which may reduce accuracy for certain targets
	Performs well for both pose prediction and binding energy calculation	
FlexX	Efficient fragment-based docking algorithm to build ligands incrementally	Prone to generating suboptimal binding poses if ligand has significant flexibility
	Fast docking, making it suitable for virtual screening	Relatively outdated compared to more recent docking tools with better scoring functions
	Flexible scoring functions	
rDock	Open-source, making it accessible	Limited user interface, requiring more command-line knowledge
	Useful for docking in both proteins and nucleic acids	Not as user-friendly compared to modern commercial docking tools
	Flexible parameter settings for custom docking	
DOCK	One of the first molecular docking programs, well-established	Outdated scoring functions compared to newer docking algorithms
	Good for fragment-based and rigid-body docking	Does not handle receptor flexibility as well as other modern programs
	Open-source and customizable for advanced users	Steeper learning curve and challenging setup for beginners

5. Key Components of Docking Software

Central to docking software are the search algorithms and scoring functions. Search algorithms determine how the conformational space is explored, with common methods including genetic algorithms, Monte Carlo simulations, incremental construction, and molecular dynamics simulations. Scoring functions evaluate and rank the predicted binding modes based on estimated binding affinities, which can be force-field-based, empirical, or

knowledge-based. These components are critical for accurately predicting how ligands interact with their target receptors and for identifying potential therapeutic compounds (Meng et al., 2011).

6. Application of Molecular Docking in Medical Biology and Medical Genetics

6.1. Importance of Molecular Docking in Medical Biology and Genetics

Molecular docking plays a crucial role in medical biology and genetics by providing insights into biomolecular interactions, which are fundamental to understanding disease mechanisms and discovering new drugs (Meng et al., 2011). Docking helps predict the binding affinity and specific interactions between ligands (e.g., drugs or inhibitors) and biological targets, such as proteins and nucleic acids. This is essential in identifying novel therapeutic agents, predicting genetic variations that affect binding efficacy, and designing personalized treatments based on patient-specific molecular profiles (Ferreira et al., 2015).

6.2. Current Research and Achievements

6.2.1. Drug Discovery and Repurposing: Molecular docking is widely used in virtual screening to discover new drugs or repurpose existing ones for new therapeutic indications. For instance, during the COVID-19 pandemic, docking studies were crucial in the rapid identification of small molecules capable of inhibiting SARS-CoV-2 proteins (Gordon et al., 2020). Docking helped determine potential binding interactions of known antiviral drugs, contributing to expedited clinical trials.

6.2.2. Genetic Disease Research: Docking can be used to study the effect of genetic mutations on drug binding. In cystic fibrosis research, for example, molecular docking has been applied to understand how different mutations in the CFTR gene alter drug binding efficacy. Such insights can guide the development of mutation-specific therapeutics (Bellacchio, 2023).

6.2.3. Targeting Oncogenic Mutations: In medical genetics, docking is used to assess the impact of mutations on proteins, particularly those involved in oncogenesis. For instance, inhibitors for mutant kinases such as EGFR and BRAF are docked to their respective targets to determine which mutations are responsive to specific targeted therapies. This helps guide treatment strategies for cancers, making them more targeted and efficient (Abdel-Mohsen et al., 2014).

6.2.4. Neurodegenerative Diseases: In diseases such as Alzheimer's, molecular docking has been employed to screen potential inhibitors for amyloid-beta aggregation or acetylcholinesterase inhibitors. Identifying drugs that can interact with these targets could slow the progression of neurodegenerative conditions (Yusufzai et al., 2018).

7. Future Perspective

Molecular docking has been extensively employed in virtual screening, where thousands of compounds are docked against a specific biological target to identify potential drug candidates. For example, docking has been pivotal in screening compound libraries for inhibitors of enzymes like kinases involved in cancer (Gagic et al., 2020). Docking also helps in understanding the mechanism of action of drugs by revealing the interactions at the molecular level. This is instrumental in rational drug design, where drug candidates are optimized to maximize their affinity and specificity for the target protein (Pagadala et al., 2017).

Molecular docking could be further integrated into personalized medicine by utilizing patient-specific genetic data to predict drug efficacy based on the binding interactions of drugs with their target proteins, which may be altered by genetic mutations. Docking could also contribute to designing molecules that selectively interact with nucleic acids. This has potential applications in gene therapy, where small molecules could be designed to bind to mutated DNA sequences or regulate gene expression at the RNA level. Expanding the use of molecular docking beyond small molecules to understand protein-protein interactions could reveal novel therapeutic targets for conditions currently considered undruggable.

8. Limitations of Docking Studies

While molecular docking is a powerful tool in drug discovery and genetics, it has following limitations: -Docking relies on scoring functions that approximate the free energy of binding. These scoring functions are often not accurate enough to predict binding affinities with high precision, especially when water molecules and solvation effects play a significant role in binding (Warren et al., 2006). -Most docking tools assume that the receptor structure is rigid, which limits the accuracy of docking predictions for proteins that undergo significant conformational changes upon ligand binding (Ciemny et al., 2018). -The computational power required for flexible docking and molecular dynamics simulations to refine docking poses is significant, which can be a limiting factor for screening large libraries or for proteins with multiple flexible domains.

9. Conclusion

Molecular docking has proven itself as an indispensable tool in medical biology and medical genetics, aiding in drug discovery, understanding genetic disease mechanisms, and optimizing targeted therapies. While it has limitations related to accuracy and computational demand, advances in scoring functions and incorporation of machine learning approaches promise to overcome some of these barriers. The integration of molecular docking into personalized medicine represents an exciting frontier for individualized treatment strategies.

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CHAPTER 9

ASSOCIATION BETWEEN MITOCHONDRIAL DYSFUNCTION AND DEPRESSION

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The relationship between mitochondrial dysfunction and depression has become a major focus of neuroscience research in recent years. Mitochondria are organelles at the center of cellular energy production and therefore play an important function in the regulation of many biological processes of the central nervous system (Lagouge and Larsson, 2013). Depression is defined as a disorder with a complex etiology, resulting from the interaction of biological, psychological and environmental factors (Malhi and Mann, 2018). Mitochondrial dysfunction is thought to have a substantial impact on the development of depression. This is associated with processes such as impairments in mitochondrial energy production, increased oxidative stress and disruption of cellular functions (Daniels et al., 2020). It has been suggested that mitochondrial dysfunction plays an important role in the pathophysiology of depression due to its critical role in brain energy metabolism and therefore may be a potential target for the development of new treatment strategies (Picard and McEwen, 2018). In this study, the relationship between mitochondrial dysfunction and depression was evaluated.

1.Depression

The DSM-5 was updated in 2013 to separate the diagnoses of depression from *"Mood Disorders"* and group them into a new category called *"Depressive Disorders"*. This category includes specific sub-diagnoses including disruptive mood dysregulation disorder, premenstrual dysphoric disorder, major depressive disorder, persistent depressive disorder, substance/drug induced depressive disorder and depressive disorder due to other medical conditions. According to the DSM-5, the diagnosis of depression requires symptoms lasting at least two weeks and the presence of at least five symptoms in addition to the main symptoms of depressed mood or decreased pleasure/desire. Symptoms should result in significant distress and impairment in functioning and should not be caused by other medical conditions or substances (Table 1). Based on Behavioral Risk Factor Surveillance System (BRFSS) 2020 annual report, approximately 18.5% of US adults receive a depression diagnosis at some point in their lives (Walensky et al., 2023). According to epidemiologic studies, the annual prevalence of depression was found to be 6% (Malhi and Mann, 2018). In Turkey, the annual prevalence of depression symptoms among individuals aged >15 years was similarly reported as 6.24% (Phiri et al., 2022). Although depression can occur at any age, it is the most common in young adulthood and middle age (Kessler et al., 2010; Platt et al., 2021). Women are approximately two times as likely to suffer from depression compared to men (Malhi and Mann, 2018; Platt et al., 2021). This difference can be explained by a number of factors including hormonal changes, social and cultural pressures and gender-specific stressors. Men are less likely to be diagnosed with depression; because, they are less likely to express symptoms or have different symptoms. Depression in men can often manifest itself with

symptoms such as anger and alcohol consumption (Albert, 2015; Malhi and Mann, 2018).

Table 1. DSM-5 Diagnostic criteria for depression

Diagnostic Criteria	**Explanation**
Symptom persistence	Significant symptoms must persist for at least two weeks.
Main required symptoms	Depressed mood and/or decreased desire or pleasure in almost any activity.
Other symptoms	Presence of symptoms such as weight change, sleep disturbance, psychomotor agitation or retardation, persistent lack of energy, feelings of worthlessness or guilt, concentration difficulties, indecisiveness, thoughts of death or suicide.
Number of main symptoms	The minimum number of symptoms required for diagnosis must be at least five. At least one of the main required symptoms must be present.
Degree of functional impairment	It must cause significant distress and impairment of functioning in social, work or other important areas
Source of symptom	Symptoms should not be caused by the physiological effects of any substance or by any other medical condition.

Depression is a serious mental health problem worldwide and is considered a biopsychosocial disorder. Depression affects approximately 300 million people globally (Zhu et al., 2023). Depression has been associated to serious health problems such as cardiovascular disease, diabetes, cancer and other psychiatric disorders (Khan et al., 2023). Although genetic and environmental factors are thought to play a role in etiology, a precise model or mechanism for the pathophysiology of depression has not yet been identified (Malhi and Mann, 2018).

1.2. Etiology of depression from a biology perspective

From a biological perspective, depressive disorders are predominantly characterized by four (the monoamine, stress, neurotrophic and cytokine) theories (Šalamon Arčan et al., 2022).

1.2.1. Monoamin theory

Emerging tools such as molecular neurobiology and functional brain imaging suggested that three major monoamine systems are involved including serotonin (5-hydroxytryptamine, 5HT), norepinephrine (NE) and dopamine (DA; Saveanu and Nemeroff, 2012). Decreases in neurotransmitter levels are thought to impair communication between neurons, leading to the emergence of symptoms of depression (Šalamon Arčan et al., 2022). Studies suggest that 5HT systems within the central nervous system (CNS) play a critical role in depression (Saveanu and Nemeroff, 2012). Postmortem studies illustrated a decreased serotonergic neuron activity in depressed patients. Postmortem and PET imaging studies showed decreases in serotonin transporter (SERT)

binding sites and 5HT receptor subtypes in the cerebellum and amygdala regions of drug-free depressed patients (Drevets et al.., 1999; Mann et al., 1996). The critical importance of 5HT circuits in depression is further illustrated by a study of patients treated with selective serotonin reuptake inhibitors (SSRIs) who went into remission (Charney, 1998). Research reveals important implications of neuronal circuits involving NE and DA on mood disorders and depression in particular. Moreover, dopamine pathways were observed to play a critical role in the depression pathophysiology with anhedonia symptoms in particular being linked to the activation of dopamine neurons. The suboptimal treatment responses provided by serotonin and norepinephrine reuptake inhibitors (SNRIs) may be explained by the insufficient effects of these drugs on dopamine circuits in the brain (Saveanu and Nemeroff, 2012). The limitations of the monoamine theory arise from a number of factors such as the large variations in the course of major depressive disorder (MDD), the fact that antidepressants are not effective in all patients and that their effects take weeks (Willner et al., 2013). It was suggested that other neurotransmitters may also play a role in the etiology of depression. It is emphasized that glutamate, one of the neurotransmitters, plays an important role in depression and plays a critical function in neurometabolic activity and neurotransmitter cycling. It is revealed that this may affect treatment methods (Sarawagi et al., 2021). It seems possible to use blood serum glutamate levels as a biomarker to assess treatment response, especially in patients with recurrent depressive disorder (Losenkov et al., 2018). These findings show that the multidimensional nature of depression and effective treatment methods require a deep understanding of the biological basis of the disease.

1.2.2. Diathesis–stress theory

The hypothalamic-pituitary-adrenal (HPA) axis is an essential neuroendocrine system that enables the body to adapt to environmental stresses and thereby plays a vital role in depression (Knorr et al., 2010; Šalamon Arčan et al., 2022). In a meta-analysis study, some HPA axis-related genes were associated with childhood trauma (Normann and Buttenschøn, 2020). The stress response is triggered by the production of corticotropin-releasing hormone (CRH) by the hypothalamus, which prompts the pituitary to release adrenocorticotropic hormone (ACTH). The release of ACTH into the bloodstream initiates the secretion of cortisol from the adrenal cortex. Cortisol regulates the stress response by binding to glucocorticoid receptors in the brain and also plays a negative feedback mechanism on the HPA axis. Disruption of this negative feedback is linked to depressive disorders (Šalamon Arčan et al., 2022; Shadrina et al., 2018).

1.2.3. Neurotrophic theory

Neuroplasticity is defined as the ability of neurons to grow and adapt,

and it is suggested that environmental stress-induced inflammation and HPA axis dysfunction may affect this process (Malhi and Mann, 2018). The regulation of neurogenesis is predominantly controlled by critical proteins, such as brain-derived neurotrophic factor (BDNF). Neurotrophic factors promote the growth, survival and differentiation of developing mature neurons. Serum BDNF levels are found to be lower in patients with depression (Chauhan et al., 2023; He et al., 2023; Rana et al., 2021). In addition, BDNF levels were inversely associated with symptoms such as severity of illness (Chauhan et al., 2023; He et al., 2023) and suicidal ideation (Khan et al., 2019). Furthermore, decreased BDNF expression has been linked to epigenetic changes in the BDNF gene (Wang et al., 2019). The relationship between depression and other neurotrophic factors were evaluated including glial cell line-derived neurotrophic factor (GDNF; He et al., 2023; Zhang et al., 2009), vascular endothelial growth factor (VEGF; Castillo et al., 2020) and nerve growth factor (NGF; Salsabil et al., 2023; Wiener et al., 2015). Neurotrophic factors appear to play a critical role in elucidating the pathophysiology and therapeutic approaches to depression. The precise neurotrophic basis however has not yet been defined and further research is required. (Sunay and Kurar, 2023).

1.2.4. Cytokine theory

Cytokines are small polypeptide regulatory mediator proteins synthesized by various cells in the body, especially white blood cells. These molecules play critical roles in the immune system, particularly in inflammatory processes and in the modulation of cell growth, differentiation and function. Most cytokines exert their effects mainly in the local microenvironment. However, some cytokines, such as IL-1, IL-6 and TNF-α as mediators of the acute phase response, can be released into the bloodstream and exert hormone-like functions in distant organs (Himmerich et al., 2019). According to the inflammation theory, cytokines play an important role in the pathophysiology of depression. Peripheral cytokines can act directly on neurons and glial cells by crossing the blood-brain barrier. Their effects may also be mediated via afferent pathways, including the vagus nerve (Miller and Raison, 2016). Recent studies have confirmed that depression cases without somatic comorbidity are associated with increases in proinflammatory cytokines, particularly TNF-α, IL-1 and IL-6. The findings supporting the link between these cytokines and depressive disorders are evaluated within the framework of the *"cytokine-induced depression theory"*, one of the theories of inflammation. A meta-analysis showed that MDD patients with lower circulating IL-8 levels at baseline responded better to antidepressant treatment. This finding suggests that IL-8 levels can be used as a potential marker to identify people who will fail to respond to current antidepressant treatments and to identify new treatment strategies (Liu et al., 2020).

1.3. Epigenetics and epigenetic mechanisms

DNA methylation is an epigenetic mechanism that regulates gene expression and thereby several key biological processes. Epigenetics is used to describe mechanisms that affect the function of the genome without altering the DNA sequence. These mechanisms include DNA methylation, histone modifications, chromatin rearrangement and genetic regulatory RNAs (Keverne and Binder, 2020; Kuehner et al., 2019). Disruptions in the functioning of these mechanisms cause different disorders for the organism (Kuehner et al., 2019).

Histone modifications including histone methylation and acetylation play a central role in epigenetic regulation, alter the chromatin structure making DNA functionally accessible and regulating gene expression. Chromatin rearrangement facilitates the access of transcription factors by reorganizing the compact structures of DNA. Non-coding RNAs (ncRNAs), another mechanism of epigenetic regulation, operate through a vast network of interactions in the regulation of gene expression. In this category, lncRNAs and miRNAs are critical in controlling gene expression at the post-transcriptional level. lncRNAs are associated with brain development and various neuropsychiatric disorders (Kapranov et al., 2007; Kwon et al., 2013).

In mammals, 5-methylcytosine (5mC) is formed by the transfer of the methyl group to the 5-position on cytosine by DNA methyltransferases (DNMT). A large proportion (70-80%) of CpG dinucleotide islands of the mammalian genome are modified with 5mC (Xie et al., 2023). DNA methylation plays a fundamental role in many biological processes, from regulation of gene expression to cellular differentiation, chromatin structure maintenance, X chromosome inactivation and genomic imprinting. DNA methylation contributes to the organization of chromatin and promotes the packaging of genetic material and thus the maintenance of genetic integrity. In female mammals, inactivation of an X chromosome through methylation allows gene dosage balancing (Carlberg, 2023). In the process of genomic imprinting, DNA methylation allows some genes to be expressed only maternally or paternally. In addition, DNA methylation has important roles in memory formation and storage.

1.4. DNA methylation and depression

DNA methylation of specific genes and global DNA methylation has been associated with depression. It has been suggested that chronic stress may be effective in the pathophysiology of MDD by increasing global DNA methylation (Byrne et al., 2013; Numata et al., 2015; Uddin et al., 2011). It has been reported that BDNF gene methylation level is closely related to the pathophysiology of depression and response to antidepressant treatment (Lieb et al., 2018; Tadić et al., 2014). Methylation of NR3C1 is linked with depression prevalence and

incidence, and social factors potentially mediating this association (Borçoi et al., 2020). Also, a possible association between SLC6A gene methylation status and depressive symptoms was suggested. It also provides important clues that this relationship may differ between genders and that there may be gender-related differences in the biological basis of depression. Studies have also revealed that the response to antidepressant treatment may differ depending on SLC6A4 methylation (Iga et al., 2016; Philibert et al., 2008; Zhao et al., 2013). It has been reported that the FKBP5 gene is also associated with depression and may cause structural changes in the emotion regulation regions of the brain through epigenetic mechanisms (Han et al., 2017; Klinger-König et al., 2019; Tozzi et al., 2018).

2. Structure and functions of mitochondria

Mitochondria have a double membrane structure and are organelles with their own genome (Archibald, 2015; Sagan, 1967). Structurally, they consist of four main parts: outer and inner membranes, interstitial space (peripheral region) and matrix. They form a dynamic network within the cell. Mitochondria function as *"power plants"* that control the energy production mechanisms of the cell. This function is fulfilled through protein complexes encoded by mitochondrial genome (Shaughnessy et al., 2015). The electron transport chain (ETC) converts metabolites into an electrochemical gradient, which fuels vital processes such as ATP production (Daniels et al., 2020). This gradient also supports the functions of mitochondria, such as ion and protein transport and the synthesis of steroid hormones (Hoffmann et al., 2018). At the end of the ATP production process, reactive oxygen species (ROS) are produced. ROS are formed when electrons leak from the ETC and react with oxygen (Allen et al., 2021). Apart from ATP production, mitochondria regulate cell death and survival by taking part in biological processes such as ROS metabolism and calcium homeostasis. These organelles are highly sensitive to environmental factors and are both targets and mediators of the stress response (Picard and McEwen, 2018). The dynamic structure of mitochondria is maintained by continuous fusion and fission processes. These processes are crucial for maintaining the structural integrity of mitochondria and for adaptation to stress conditions (Tripathi et al., 2021).

Human mitochondrial DNA (mtDNA) consists of 16,569 bp and contains 37 genes and regulatory elements responsible for the production of 13 polypeptides and 24 RNA components (22 tRNAs and 2 rRNAs), as well as the D-loop, a non-coding control region. There are several differences between mtDNA and nuclear DNA (nDNA). These differences include the number of stop codons and the circular structure of mtDNA. The mitochondrial genome can only be inherited from the mother (maternal inheritance). The small genome size, the absence of intronic regions and the dense arrangement of genes make mtDNA a vulnerable target for mutations. There are "hotspot

regions" areas in the mitochondrial genome, with high mutation susceptibility. These mutations become irreversible and accumulate in the mtDNA genome. For these reasons, mutations occur 25 times more frequently in mammalian mtDNA than in nDNA (Şoroğlu et al., 2021).

2.1. Mitoepigenetics and mitochondrial epigenetics

Although epigenetic modifications in mammalian mtDNA were first demonstrated in 1973, they have not received much attention (Nass, 1973; Vanyushin and Kirnos, 1974). In 2011, evidence for the presence of hydroxymethylation (5hmC) in mtDNA was shown (Dzitoyeva et al., 2012; Shock et al., 2011). Mitochondria have been shown to be the center of epigenetic mechanisms involving mitochondrial ncRNAs (Ro et al., 2013; Smalheiser et al., 2011). In this context, the field of mitochondrial epigenetics has an increasing attention. Different studies are being conducted to explain the functional significance of these epigenetic modifications and to elucidate the pathophysiology of different diseases.

The fact that mitochondria have their own DNA also affects the epigenetic mechanisms that take place in the nuclear DNA. In this context, the distinction between "*mitoepigenetics*" and "*mitochondrial epigenetics*" is important. The concept of mitoepigenetics refers to the bidirectional communication between mitochondria and the nucleus. Mitochondrial epigenetics includes epigenetic events within mitochondria and mitochondrial genome functions (Manev and Dzitoyeva, 2013; Manev et al., 2012). This interplay between nDNA and mtDNA is critical for cell homeostasis. Epigenetic mechanisms regulating gene expression in response to environmental stimuli stand out as the main regulators of this interaction (Castegna et al., 2015). Mitochondrial epigenetic changes include mtDNA methylation and mitochondrial RNA changes. mtDNA epigenetic changes regulate mitochondrial functions. They can also affect mtDNA replication and expression. In this respect, they play a critical role in different diseases (Ceylan et al., 2024).

2.2. Mitochondrial DNA methylation

It has been previously hypothesized that mitochondria, which play a critical role in the energy production and metabolic regulation of the cell, are not affected by epigenetic mechanisms due to the low number of CpG islands in their DNA and the fact that mtDNA is not thought to interact with histone proteins (Cardon et al., 1994; Manev et al., 2012). However, recent studies question this view. The structure of mtDNA has been shown to be shaped by various proteins found within mitochondria that form mitochondrial chromatin-like structures. As in nuclear epigenetics, mitochondrial epigenetic mechanisms may play a role in nucleotide modifications. In addition, as nDNA, the presence of 5mC and 5hmC has been detected in mtDNA and shown to be sensitive to modifications (Manev, 2014). It is not

yet known whether mtDNA methylation is reprogrammed during embryonic development. However, dynamic mtDNA methylation patterns associated with aging (Chen et al., 2012), environmental factors (Byun et al., 2013; Shock et al., 2011) and different diseases (Infantino et al., 2011; Shen et al., 2013) suggest that mtDNA methylation can be remodeled by a rapid methylation-demethylation conversion. This is supported by interesting findings that drugs such as valproic acid modulate mitochondrial epigenetics (Chen et al., 2012).

The presence of various DNMTs has been detected in mammalian mitochondria. These appear to play an important role in the addition of methyl groups to mtDNA, similar to nDNA. Among these enzymes, DNMT1, DNMT3A and DNMT3B have been identified. Among these enzymes, only a small fraction of DNMT1 mRNA is translated from an initial codon containing a mitochondrial transfer signal and produces a mitochondrial-specific copy called mtDNMT1. This protein has been documented to interact with D-loop regions, which are important in the control of the replication and transcription of mtDNA. Other DNMTs do not have mitochondrial targeting sequences. Since they are nuclear-coded, they must be transferred to mitochondria (Dzitoyeva et al., 2012; Hewitt et al., 2014; Shock et al., 2011). These three DNMTs can determine the 5mC pattern and levels in mtDNA. The presence and content of 5mC in mammalian mtDNA have been evaluated using different methods. It has been directly observed by using immunofluorescence methods in CNS (Chestnut et al., 2011). In addition, immunoprecipitation (Shock et al., 2011) and ELISA (Dzitoyeva et al., 2012) have been used to evaluate 5mC content and distribution. Bisulfite sequencing confirmed the presence of 5mC in the D-loop region in humans and mice and indicated that more concentrated in non-CpG nucleotides (Bellizzi et al., 2013). The fact that mtDNA methylation occurs in the D-loop region suggests that it plays a regulatory role in mtDNA replication (Manev, 2014). Bisulfite pyrosequencing also detected the presence of 5mC in the mitochondrial MT-TF and MT-RNR1 genes (Cardon et al., 1994), and these regions are sensitive to environmental influences and can function as biomarkers (Manev, 2014).

The functions of methylation in mtDNA have not yet been clearly defined. Methylation is thought to play important roles in regulating mitochondrial transcription (Bellizzi et al., 2013; Shock et al., 2011). It is thought that mtDNA may function as a detector of environmental stressors, as mtDNA methylation has been observed to be associated with a variety of environmental factors. In particular, certain mtDNA regions have been negatively or positively associated with various environmental influences (Stoccoro and Coppedè, 2021). There is evidence that epigenetic alterations of mtDNA may play a role in the biological functions of mitochondria. However, more research is needed to understand this extent how these changes regulate mtDNA gene expression and replication. It is emphasized that the well-known epigenetic arrangements

of the nuclear genome may differ in mitochondria, given that mtDNA differs significantly compared to the nuclear genome, and nucleosomal chromatin and CpG islands are absent (Jiang et al., 2004).

Several possible scenarios come to the fore to explain the origin of 5hmC in mtDNA. This formation may depend on the enzymatic activity of TET proteins and mechanisms that may occur from other sources. TET1 and TET2 proteins were found in mammalian mitochondria (Dzitoyeva et al., 2012), but TET1 did not affect mtDNA 5hmC levels in embryonic stem cells of knockout mice (Manev, 2014). This points to the possible role of TET proteins. 5hmC in mtDNA can also be caused by oxidative damage or the activity of mitochondrial enzymes. As a result of studies conducted with high-resolution mapping techniques such as Aba-seq, it has been determined that the density of 5hmC in the context of CH and CG in mtDNA is much higher than in nDNA (Sun et al., 2013). The mouse and human mitochondrial genomes encode a large number of small ncRNAs (mitosRNAs) derived from gene transcripts. The clusters of these mitosRNAs differ according to human tissue. For example, mitosRNAs has a similar profile in brain and heart tissues however can differentiate in gastrointestinal tissues. MitosRNAs are thought to have significant effects on mtDNA methylation status and mitochondrial gene expression (Manev, 2014).

Mitochondrial epigenetic studies showed that mtDNA methylation is a complex and dynamic process. While these regulations are similar to nuclear epigenetic mechanisms in some respects, they also contain unique differences. There are different technical challenges in the detection of mtDNA methylation. These challenges arise from the non-amplification of mtDNA methylation and the requirements for method sensitivity. The first approaches presented the main problems, yielding contradictory results. However, novel techniques, such as the bisulfate sequencing, provide single base resolution and allow for more detailed study of mtDNA methylation. Control experiments and alternative methods are critical in the accurate detection of mtDNA methylation. In the future, new techniques, such as nanopore sequencing, may contribute to a more precise assessment of the mtDNA methylation level (Stoccoro and Coppedè, 2021). Precise methodologies need to be developed for a better understanding of mtDNA methylation in the future. Thus, the relationship of mtDNA methylation with certain mitochondrial functions and diseases can be better understood.

2.3. Mitochondrial dysfunctions toward depression

One of the mechanisms recently identified in understanding the biological basis of depression is mitochondrial dysfunction. Mitochondria are seen as the power plants of the cell. The brain is the organ that needs the most energy due to its structure (Rich et al., 2019). A resting neuron is thought to consume

~4.7X10^6 ATPs per second (Zhu et al., 2012). In addition to energy production, mitochondria also control calcium levels during neurotransmission and regulate synaptic function (Mattson et al., 2008).

Mitochondrial dysfunction is defined as the dysregulation of processes such as apoptosis that may occur with a decrease in the energy capacity of mitochondria and disruption of calcium balance (Lagouge and Larsson, 2013). This situation may affect critical processes such as intracellular signal transduction, energy metabolism and cell survival. In addition, the production of reactive oxygen species produced in the oxidative phosphorylation process. The emergence of disruptions in this control mechanism can cause problems in cells. Thus, it may cause mitochondrial protein damage and, on a larger scale, disruption of brain processes (Khan et al., 2023).

Possible causes of mitochondrial dysfunction include genetic factors, environmental stress, infections and aging (Forlenza and Miller, 2006). In addition, genetic mutations and DNA polymorphisms can also cause mitochondrial dysfunction (Tripathi et al., 2021). An example of this situation is heteroplasmia. mtDNA, which is normally the same in a cell, can differentiate through mutations. Heteroplasmia thereby can cause various diseases (Manev, 2014). Chronic stress can also create an allostatic load on mitochondria, which can cause cells to lose their physiological function (Picard ve McEwen, 2018). Environmental toxins and infections can also increase oxidative stress by disrupting mitochondrial functions (Tripathi et al., 2021). In addition, mtDNA damage that increases with aging causes mitochondrial dysfunction (Lagouge and Larsson, 2013). Mitochondrial dysfunction plays a role in the pathogenesis of neuropsychiatric diseases such as Alzheimer's, Parkinson's and amyotrophic lateral sclerosis (Lagouge and Larsson, 2013; Rosebush et al., 2017). As a result, mitochondrial dysfunction may contribute to the development of psychiatric disorders by affecting synaptic functions and neurotransmitter release in the brain (Chandel, 2015).

Mitochondrial dysfunction has also been linked to psychiatric conditions such as anxiety, depression, schizophrenia and bipolar disorder (Rosebush et al., 2017). Chronic stress, which plays a critical role in the development of depression, can disrupt cellular functions that will disrupt mitochondrial energy production and oxidative balance (Picard et al., 2015). In addition, increased glucocorticoid levels due to hyperactivity of the HPA axis can lead to an increase in mitochondrial activity and oxidative stress. Studies have also demonstrated an increase in mtDNA damage and oxidative stress markers in depressed patients. Due to its role in the treatment of depression, alternative pharmacological treatments are being investigated to reduce mitochondrial dysfunction, regulate mitochondrial functions, and reduce oxidative stress are being investigated (Daniels et al., 2020). Studies conducted in this context indicate that antidepressants show neuroprotective effects by increasing

mitochondrial biogenesis and strengthening antioxidant capacities (Allen et al., 2018). In particular, agents with rapid antidepressant action, such as ketamine, can alleviate symptoms of depression by improving mitochondrial functions (Brymer et al., 2020). Some antidepressants, such as fluoxetine, can increase mitophagy, clear damaged mitochondria and reduce symptoms of depression (Shu et al., 2019).

Telomere length and mtDNA copy number characteristics of early life difficulties and psychopathologies were studied. These studies have shown that childhood traumas and psychopathologies lead to changes in parameters such as telomere length and mtDNA copy number, which are indicators of cellular aging. It has been observed that individuals who experience parental loss at an early age and abuse in childhood, especially with conditions such as MDD and anxiety disorders, have shorter telomeres. In addition, it has been found that the mtDNA copy number of these individuals is also higher (Tyrka et al., 2016). The association between mitochondrial dysfunction and MDD was examined in peripheral blood cells. The results suggested that patients exhibited an increased mtDNA copy number and reduced DNA methylation at the PGC1α promoter compared to the controls. Furthermore, mtDNA copy numbers were inversely correlated with age, psychomotor agitation and somatic symptoms in MDD (Chung et al., 2019). Other studies with similar results have found that circulating mtDNA levels are higher in MDD patients (Giménez-Palomo et al., 2021). In a study examining mtDNA modifications in bipolar disorder and MDD patients, mtDNA copy numbers were remarkable decreased as patients' symptoms improved at week 8. This draws attention to the relationship between remission and mtDNA copies (Ceylan et al., 2023). Different studies have also shown that mtDNA copy numbers increases during acute depressive episodes (Cai et al., 2015; Nicod et al., 2016; Tsujii et al., 2019). In a study conducted by Czarny et al. (2020), no significant difference was found between healthy individuals and MDD patients in terms of mtDNA copy number. However, it was determined that MDD patients had a lower mtDNA repair capacity. In a study examining the relationship between circulating mtDNA and inflammatory cytokines (GM-CSF, IL-2 and IL-4), a positive correlation was found between mtDNA levels and cytokines of MDD patients. In contrast to most previous studies, MDD patients were found to have lower mtDNA copy counts compared to healthy controls (Kageyama et al., 2018).

3. Conclusion

Although mtDNA methylation has become an interesting research topic in recent years, studies evaluating its relationship with depression are quite limited. Two studies examining the effects of mtDNA methylation on depression provide important contributions to our understanding of how epigenetic alteration is associated with MDD. The first study revealed that

the rate of DNA methylation in the PGC1α promoter region was significantly lower in MDD patients compared to the control group. It was observed that hypomethylation in the PGC1α promoter leads to increased PGC1α expression and thus increased mitochondrial biogenesis in the MDD group. However, these changes were not directly related to mtDNA copy number (Chung et al., 2019). In this study, moreover, no significant difference was found in methylation levels in the D-loop region. The second study found that mtDNA methylation levels in the D-loop region were significantly higher in with MDD compared to healthy controls. This finding suggests that depression may be associated with mitochondrial dysfunction and that hypermethylation in the D-loop region may play a role in the pathogenesis of MDD. In addition, the significant decrease observed in D-loop methylation levels during remission of depressive symptoms suggests that methylation increase during acute depression may be a reversible mechanism (Ceylan et al., 2023). Studies on mtDNA methylation and depression reveal potentially important findings in this field, and further research is required for a deeper understanding of the subject.

In conclusion, mitochondrial dysfunction appears to be an important mechanism of the pathogenesis of neuropsychiatric and psychiatric disorders. Therefore, it seems to be potential target in the treatment of depression. For this reason, the relationship between mitochondrial dysfunction and these diseases may contribute to the development of new treatment strategies.

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CHAPTER 10

PEGANUM HARMALA: A COMPREHENSIVE STUDY

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1. Introduction

Peganum harmala, commonly known as Syrian rue, is a pale green perennial flowering plant and a native of arid regions, with an extensive presence in the Mediterranean, Central Asia, Pakistan, and India. Here, it has been used as traditional folklore medicine by several tribes and cultures over the last many centuries. In Islam, it has great significance as well. The seeds of *P. harmala* have recently become an object of current scientific discourse because of their behavioral, neuropharmacological, and toxicological properties (Nedjimi2020; Gamoun, 2021; Basahi, 2023). This development was made possible with nifty and robust techniques coupled with the notable trends in psychopharmacological research (Mousavi and Moudi2020; Li et al., 2023; Abbas et al., 2021; Nadia et al.2022; El et al.2024; Niazi et al.2021; Abduraimov et al., 2023).

It is indeed an unclaimed fortune of multidimensional pharmacological activities ascribed to *P. harmala;* thus, it is becoming an exclusive area of research for scholars from various scientific domains (El et al.2024; Niazi et al.2021; Nadia et al.2022; Dhief et al. 2022). This comprehensive study thus aims to describe the botany, ethnopharmacological and religious importance, phytochemistry, mechanism of actions, toxicology, and evaluation of *P. harmala*. This, in consolidation, aspires to lay the foundation for reconsideration of *P. harmala* in the traditional and modern systems of medicine, besides the purging of controversies associated with the unintended toxicity that this species is known to cause today. *Peganum harmala L*., also known as Harmel or Syrian rue, is a well-known species belonging to the Nitrariaceae family worldwide. Peganum species have significant ethnomedicinal values for a variety of ailments. The most promising objective addressed is *P. harmala L*. subsp. harmala, which is a culinary medicine since primordial times, used by traditional healers as a cathartic and emetic and in religious activities. (Zhu et al., 2022; Arif et al.2022; Shahrajabian et al., 2021; Sharma et al.2022; Abbas et al.2021; Afzal et al.2021; Rashid et al., 2023)

1.1. Background and Significance

The plant, *Peganum harmala L*., has many folk names in different countries, such as Kure, wild rue, and Syrian rue, from Pakistan to Turkey. *P. harmala*, which falls into the order Nitrariaceae and family Peganaceae, is recognized as a subject of ethnomedicine in different cultures. The traditional use of the plant in North and West Africa, Turkey, and Iran has persisted for centuries. *Peganum harmala* is especially valued in Iran and Afghanistan, where it has been used as a psychoactive substance and for the treatment of various disorders for centuries (Abduraimov et al., 2023; Najem et al., 2020).

Numerous and contradictory reports from ancient to present times about psychoactivity, hallucination, photophobia, associated sedation and

stability, neuropharmacology, erythrocytic behavior, leather tanning, pesticide, nematocide, antibacterial, antifungal, antiprotozoal, antiviral, hypoglycemic, hypolipidemic, abortifacient, uterotonic, antihelminthic, male fertility, sedative, and neurological treatments are frequently reported. (Karamkhudoeva et al.2021; Almoneafy, 2020; Hussain et al., 2023; Benamara et al.2022)

Thus, investigations into this plant from many aspects of ethnopharmacology are of great importance, integrating the old with modern pharmacy and rational therapy. The extensive uses of Peganum as green and organically produced substances can increase employment and provide the opportunity to improve the socio-economic situation, creating a decreasing dependence on single valuable drugs relied upon with hard work (Sharma et al.2022). Toxicity, quality, and dosage concerns have to be addressed. In one approach, dose values have to be established by traditional practitioners, and the doses of alternative forms have to adapt to these doses. In another approach, the uptake of a toxic substance at a psychoactive dose without intoxication can be achieved only if the public is carefully taught in the correct application of hazardous organic products. The bottleneck of all of these is the standardization of plant extracts, particularly based on traditional methods. Ethnopharmacology is integrated with pharmacovigilance to recognize and develop new active remedies (Gökkaya et al.2023; Niazi et al.2021).

1.2. Research Objectives

The objectives of this comprehensive study on Peganum harmala are to cover the review of existing literature concerning mainly its toxic and addictive properties, as well as the existing and potential therapeutic uses. Furthermore, we aim to narrate the botanical description of P. harmala, list the main phytochemical compounds reported, and discuss its most relevant pharmacological properties (both for indoor administration and external use). P. harmala is a monoamine oxidase inhibitor, interacts with a number of targets in the central and peripheral nervous systems, and is also toxic. This study aims to give a comprehensive assessment of its normal dose, toxic levels, and proven toxicity through both a review of the most relevant literature about the molecules and the scientific evidence of harmful effects. Moreover, traditional and pharmaceutical uses will be disclosed and reported (Araba et al.2024).

The modern scientific validation of personalized uses of such substances is likely to be the future of this area of study. We expect to close the existing gap in the literature about this substance, discussing both the good and the evil of its administration. This can be crucial when considering the history of traditional use already in place in multiple cultures: its use as incense for psychoactive experience, its effectiveness as an anthelmintic, and psychoactive

properties. We have six main questions applied to P. harmala whole drug and all its parts, which are derived from field grey literature related to appreciating the beneficial and detrimental effects of P. harmala based on its chemical, pharmacological, and toxicological profile, as well as its traditional uses. These research objectives are derived based on current evidence gaps for P. harmala to foster future research. This gives a unique approach to appreciating the whole plant's beneficial and detrimental uses in addition to its extracts, which calls for consciousness during research (Rezzagui et al.2020).

2. Botanical Description

This herb is annual. Aerial stems ascend through 30 to 100 centimetres tall, rarely reaching up to 200 cm. The major taproot is fleshy and dark brown both outside and inside; branches are many, and primary roots are moreover branched, creating tiny fine rootlets. The roots are heavy. The aerial system can produce many lateral stems from the base; generally, the plants are found to be much branched at the base. Stems are conspicuously green and filled with a soft pith, tough but easy to break. The surface is rough, and irregular longitudinal grooves run below it. It is easy to recognize from the internal region or cross sections, as the pith is continuous without any cavity. The entire plant is smooth and glabrous, green, and characterized by the strong smell of the dried plant. This plant is sticky due to thick exudates, creating toughness with a friction sound when rubbed between the fingers (Shahrajabian et al., 2021).

The vegetative part, generally called shoots, branches emerge from the flexible to slightly hard growth tissue present within the stem. These branches usually appear with a smooth surface. The base of the stem is swollen, giving a club-shaped appearance or rooting in soil. The shoot extends out and produces many lateral, leaf-bearing leaves upon each internode. Leaves contain a short petiole and are more than 6 cm long, entire, glabrous, alternate, and recurved-pinnate, somewhat pungent, dark green above, glabrous, and light green beneath, sometimes darker with venial coloration, and smell aromatic when crushed. The leaves of the stem vary little from one another in size, style, and phyllotaxy. The arrangement of leaves in all cases is some distance apart, spacious, and regular. Leaves of the vegetative part emerge just below the inflorescence; a suprachoroidal apposition of lateral leaves of the main bracts is distinctly observed. Numerous quantities of leaves exist on the upper vegetative part, where leaves are distant, and leaves grow close on the lower portion. The Phyto form is therophyte, and its vegetation begins in early spring depending on the location. Flower buds appear from early to late May, and flowers start blooming by early June and last until early August. The seeds mature from early July to late August, depending on the climate zone (Niazi et al.2021).

2.1. Taxonomy and Nomenclature

Peganum harmala is a perennial herbaceous plant that belongs to the family Nitrariaceae. The plant family Nitrariaceae belongs to the order Sapindales, which is in the class Dicotyledoneae of the phylum or division Magnoliophyta. The botanical classification of *Peganum harmala L.* is as follows: - Kingdom: Plantae - Phylum: Magnoliophyta - Class: Magnoliopsida - Order: Sapindales - Family: Nitrariaceae - Genus: Peganum - Species: *Peganum harmala* the binomial name generally consists of the generic name first, which is followed by the specific name. The scientific name refers to the species under the genus Peganum, identifying a single species with that name. There are a few synonyms for *Peganum harmala* as well as a variety of common names. The synonyms illustrate various changes in the naming of the species over time and across different botanical traditions. The common names also reflect references to distinct cultural origins. Whether in science or in traditional knowledge, using the same name for a plant can save time and avoid confusion. Moreover, scientific names are essential for scientific communication. Biodiversity and taxonomy are important fields for understanding the history of species and evaluating the evolutionary potential of plants. The definitions of species and genus are categorical and often involve geographic regions and historical processes. Monophyletic relationships are revealed by the classification of families and are more likely to reflect the evolutionary history of the group. The study of Plantae must have a foundation in the science of taxonomy. The comprehensive study of Peganum harmala will have to include information about the researchers who collected the various species and any validation according to the nomenclature. Synonyms recovered for species from different sources have been published in different floras, which will be discussed below, and thus scientific value may be added to the medical knowledge (Rashid et al., 2023).

2.2. Geographical Distribution

Peganum harmala is native to North Africa, Southern Europe, and Western Asia, distributed over the belt at latitudes of 10–45°N. It mainly grows in arid and semi-arid areas, such as Pakistan, Iran, Northwest India, Spain, Greece, Syria, Tunisia, Algeria, Egypt, Iraq, Jordan, Israel, Saudi Arabia, Central Asia, and some areas of North and East China. *P. harmala* has become a new invasive plant in some regions outside this range through natural dispersal. Accordingly, P. harmala is mainly distributed in Western and Central Asia. *P. harmala* is distributed in extremes of temperatures and precipitation across different native environments in the world (Ahmed et al., 2021).

P. harmala is mainly distributed in the climatic types of Mediterranean, temperate, and warm, with cold and wet winters and hot and dry summers.

Under the conditions of an annual average temperature of 6–18 °C, it grows well. It is distributed mostly on well-drained alluvial, loessial, red, and brown soils with light to moderate salinity. The distribution zone of *P. harmala* grows in areas dominated by soil organic matter, soil texture, pH, and soil calcium concentration, and does not thrive in high-concentration sulfur, volatile salt, and chloride soil. The fruits and roots of P. harmala, in recent years, have been spread by human activities, animals, or elements, to be able to disperse and propagate, and its current distribution is not limited to the region of original distribution. The above distributions are included in the combined characteristics of dispersibility and suitable growth environments of hyperaccumulators. Understanding the specific distribution patterns of species is important in the development of plant exploitation as a research subject. It can provide a framework for the conservation management of medicinal plants and the protection of threatened or endangered plant species in the maintenance of their distribution. The results show that there are some selective pressures that have a significant impact on the adaptive process to the distribution and growth range of *P. harmala*, and some factors of selective pressure themselves change on the scale of distribution. Developing biocidal agents based on ethnic and regional distribution and irrigation of *P. harmala* will be effective. The effect of local human activities limits its natural expansion to the south. Climate change has also had an effect, which has been verified to promote its natural spread (Kharchoufa et al.2021; Zhu et al., 2022).

3. Chemical Composition

The chemical composition of *P. harmala* has been studied, and various bioactive compounds are identified in this plant. Some studies have mainly focused on alkaloids. Alkaloids are nitrogen-based compounds that act as bases in water. They are present in about 20% of plant species. Alkaloids occur in families and subfamilies of plant species and are often restricted to a single taxonomic group. Because of the alkaloids identified in *P. harmala*, the significant quantities of alkaloids are implicated in the biosynthesis, and the pharmacological properties of this plant are ascribed to the alkaloids. The first methods used to extract, identify, and/or quantify either all types of alkaloids or a specific type depended to a large extent on the method of extraction that was used during the analysis (Kharchoufa et al., 2021; Araba et al.2024). The various types of chemical constituents of *P. harmala* were elucidated by employing different techniques for extraction and analysis. The anti-inflammatory, antiulcerative, antimicrobial, antidiabetic, larvicidal, insecticidal, pesticidal, and antimolluscicidal effects of the alkaloids or other phytochemical bioactive compounds on the crude root bark and whole plant extract were investigated, and the total alkaloidal and phytochemical content present in the seeds of *P. harmala* and the medicinal plant was reported to act as nematicidal, biocidal, or bactericidal agents. Several synergistic interactions

occurred at a biological level among the alkaloids of P. harmala. The hepatoprotective effect of the alkaloids of *P. harmala* is due to the combination of α-pyrene and/or α-isoquinoline and β-carboline or α-carboline (Arif et al.2022). The method of storage and the type of location or environment in different regions also affect the type, quantity, and active constituents of the concerned plant. There are many bioactive chemical compounds present in the seeds of Peganum harmala, which are utilized for different purposes due to their wide potential pharmacological effects, but some alkaloids are reported for their toxic effects (Wang et al.2022; Kamran et al., 2021; Melloul et al., 2022; Najm and Sultan2022; Kaya & Akbas, 2023).

3.1. Alkaloids

Peganum harmala is a plant containing the psychoactive alkaloids harmine, harmaline, and tetrahydroharmine, as well as harmalol and harmalicine. The tryptophan-dependent β-carbolines, such as harmine and harmaline, and the corresponding 1-methyl substituted β-carboline harmalol are the main bioactive components. Numerous scientific investigations of the *P. harmala* plant extracts and isolated alkaloid mixtures have been conducted. The alkaloids were primarily assessed for their psychoactivity and serotonergic effects, followed by a wider evaluation of biological activities and therapeutic potential. The β-carbolines have been linked to diverse therapeutic effects, including antidepressant-like and antinociceptive effects, antioxidant, antidopaminergic, antipyretic, antimicrobial, anticancer, anti-acetylcholinesterase, antiestrogenic, antitubercular, and immunomodulatory effects, among others. There is an increasing awareness of the potential of the legal plant alkaloids in traditional medical paradigms, with the growing interest in the use of alternative and complementary medical practices. Harmine, harmaline, and tetrahydroharmine are the three main bioactive alkaloids present in the extract of the P. harmala seeds and various plant parts containing psychoactive properties. These compounds are widely reported to occur in the Peganum genus and other plant families. The biosynthesis of these alkaloids begins with the cyclization of the tryptophan derivative in the presence of prenyl-transferase. The basic β-carboline moiety consists of a double-ring structure that is fused by one benzene ring and one pyridine (Anstis et al.2023; Doskaliyev et al.2021). The large aromatic structure of these β-carbolines confers an adaptable chemical structure, which in turn leads to their interaction with diverse biomolecular targets present in the body. Although β-carbolines of various structures have unique pharmacological effects and toxicity, such as the ability to function as a receptor agonist or antagonist, their effect is typically via an allosteric attractive site. The β-carboline compounds noted for psychoactivity and minor toxicity belong to the two primary phytopharmaceutical *Peganum harmala* alkaloids, harmine and harmaline. Harmine and harmaline consist of free hydroxyl

in the activated and most stable form as the β-carboline form. These are the main bioactive compounds within the *Peganum harmala* plant and its extract (Sharma et al.2022; Sharifi-Rad et al.2021; Niazi et al.2021; Saeedeh et al.2022)

4. Traditional Uses

This section aims to shed light on the previous and current utilization of *P. harmala* in folk medicine and associated socio-cultural and spiritual practices, providing information on the ritualistic nature of *P. harmala* use among specific ethnic or religious communities. Such ethnobotanical studies have been deemed particularly potent along with a transdisciplinary examination of medicinal plants, as such ethnographic or indigenous knowledge may offer valuable information that can be utilized in scientifically valid research. Consideration of specific socio-cultural practices can also usefully highlight highly susceptible, high-risk, very remote, or geographically isolated communities that may approach medicinal treatment in non-traditional ways, particularly utilizing herbs (Fahmy et al.2021).

Peganum harmala L. is widely used in traditional healing systems throughout its range. In Afro-Asian countries, mostly in Iran, India, Turkey, and Arabia, the plant is prescribed to alleviate numerous diseases, particularly those associated with exorcism, envious light, and fire. In China, a survey suggested that a significant percentage of inhabitants use the seeds of the plant as medicine for treating various ailments. It is also commonly seen to be used as a ritual rather than a medicinal plant throughout certain communities in North Africa, Cuba, South America, Mexico, Jordan, Lebanon, India, and Pakistan (Brito-da-Costa et al.2020).

4.1. Historical and Cultural Significance

Peganum harmala has played important historical and cultural roles in different parts of the world. Peganum harmala is an old-world herb belonging to the family Zygophyllaceae. Its seeds and roots were identified from the ruins of ancient Persia, Mongolia, and Egypt, which had drug properties. It had both strong medicinal properties and religious significance for the ancient Brahmins. They would mix it with milk and a dash of sugar, drink it, and called it Soma. The plant was thought to be the 'progenitor' of the Avesta and the heliotrope. It was used as a desiccant and thirst quencher in the desert as well as an offering. The conquerors of the desert, the Aryan-speaking peoples, came to worship it, first as a deity and later, when the horse sacrifice became the predominant form of worship, to acknowledge that this plant made ritual experience possible. All the indices point to the conclusion that the powers of the Indian Soma and the Haoma mentioned in the Avesta came from *P. harmala*, though some other solanaceous species were also used in some areas for the same purpose (Calderoni et al.2021).

In addition to ritual uses, scattered accounts are available about some Muslim saints using it for revelatory purposes in the moving spirits. It is a matter of considerable importance to collect all the accounts, motives, and outcomes to detect some sort of consistency or uniformity in the results, and thus to define more clearly the psycho-pharmacological properties of the plant and the texts upon which they rest. Overall, *P. harmala* seems to have been a part of all mystical experiences throughout the Islamic world, particularly that which is Sufism. The traditional and serious scientific knowledge of human health and medicine is contained in folklore based on locality, customs, traditions, and religious beliefs, which people inherit from their ancestors. Indigenous knowledge of different communities is disappearing rapidly, particularly with rapid social, economic, cultural, and environmental change in both developed and developing countries. Due to the modernization and glamorization of drugs, several benefits and traditional knowledge of these plants have been lost. A few herbs still have cultural, spiritual, social, and religious importance. The detailed uses of *P. harmala* are given below (Semwal et al., 2021).

5. Pharmacological Properties

Since ancient times, humans have benefited from the therapeutic properties of natural resources. *Peganum harmala* has been widely used by humans since ancient times for the treatment of various ailments. There is a solid foundation for understanding the toxic and psychoactive effects of the biological compounds such as beta-carboline and its derivatives that *P. harmala* consists of. The harmala alkaloids in *P. harmala* have exhibited biological activities, such as ameliorating antimicrobial activities, and anti-inflammatory, antioxidant, anti-mutagenic, antibacterial, and cytotoxic activities (Sharma et al.2022; Abbas et al., 2021). The beta-carboline alkaloids that belong to this plant are psychoactive compounds. Interestingly, the bioactive effects of *P. harmala* can elucidate and support the potential applications mentioned in traditional medicine. Moreover, some of its compounds are currently known as top-priority critical compounds. On the other hand, there are two others different harmala alkaloids in *P. harmala* extract, which showed significant activity on the growth of human uterine cancer cells. Some clinical studies have been performed on the effects of the seeds of *P. harmala*. These observations clearly match with the traditional uses of *P. harmala* in cancer therapy (Sharifi-Rad et al.2021). This review represents a study on the pharmacological properties of secondary metabolites present in P. harmala. Besides, the possibilities for finding novel pharmaceutical agents for disease treatment based on the results that have already been obtained are addressed in the review. Overall, our short review paper classifies and answers a number of questions within the scope of P. harmala for interested researchers. In conclusion, more in vivo and in vitro studies with beta-carboline and harmala alkaloids against diseases are needed

to promote the use of bioactive compounds to treat diseases in humans or animals. (Zhu et al., 2022; Akhtar et al.2022; Gökkaya et al.2023; Rezzagui et al.2020; Mamadalieva et al.2022; Jalali et al.2021).

5.1. Antimicrobial Effects

5.1. Antimicrobial Effects

The potential for *Peganum harmala L.* to work against a broad spectrum of pathogens is investigated through in vitro research. Several studies have described the pharmacological effect of *P. harmala* on pathogenic and harmful microorganisms. Crude extracts, pure compounds, and ample crude extract fractions isolated from Peganum species have asserted productive antimicrobial activities; for bacteria, the MIC and MBC values are 8–3.46 μg/mL; for fungi, the MIC and MFC values are 0.625 μg/mL and 1.25–2.5 μg/mL, and for viruses, the TCID50 and EC50 values are 0.625 and 1.29 μg/mL, respectively (Khalid et al.2024; Hayat et al.2024; Rathore et al., 2023; Selem et al.2024). It has been demonstrated that harmine, harmaline, harmalol, and vasicine are the main active antimicrobial agents. Lately, several publications have examined compliance with standard characteristics and essential guidelines for preclinical and clinical research to treat infections and propose such a possibility in a timely manner (Choudhary et al.2021; Karakoti et al.2023; Sharma et al., 2023; García-Carrasco et al.2023; Barati and Chahardehi2023; Abdelmigid et al.2022).

P. harmala has demonstrated antimicrobial effects; it suppresses a large array of Gram-positive and Gram-negative bacteria and fungi. Researchers have suggested that the antibacterial activity is achievable by targeting DNA and protein synthesis and uncoupling the microorganism's electron chain, which dissipates energy (Filban et al.2022). Up to a certain extent, this may justify why, traditionally, *P. harmala* has been employed to treat a plethora of skin infections due to bacteria. Therefore, researchers recommend further exploration of the pharmaceutical potential of this plant. Studies based on the principles of ethnopharmacology suggest that some natural compounds, either from plants or chemically synthesized, protect against skin infections. Harmine, harmaline, harmol, harmalol, and vasicine are the main active components of biomedicines. Furthermore, the crude alcoholic extract of the seeds and other parts of the plant, harmalol, and their derivatives have broad-spectrum antimicrobial coverage against a series of both Gram-positive and Gram-negative bacteria, as well as other microorganisms; this could justify why *P. harmala* holds the therapeutic status of a parasiticide, acaricide, and insecticide. (Zhu et al., 2022; Khalid et al.2024).

5.2. Antioxidant Activity

Oxidative damage to cells and macromolecules, metabolic imbalances, or inflammatory processes contribute to the pathogenesis of many chronic and acute diseases and are involved in the natural process of aging. This deleterious process can be limited in two ways: by using natural antioxidants present in many foodstuffs and plants that can neutralize the adverse effects of free radical species or by the use of exogenous antioxidants. *Peganum harmala* contains several bioactive components that have shown potential antioxidant capacities (Mounira et al.2022; Khalid et al.2022; Kaya & Akbas, 2023). A plethora of results confirm that methanolic extracts have strong antioxidant properties, as indicated by a wide range of experimental models. These beneficial effects are closely related to the presence of bioactive components, primarily three principal indole alkaloids. Various assay methods are used to study the antioxidant activity of compounds, such as radical scavenging, ferric reducing antioxidant power, oxygen radical absorbance capacity, and lipid peroxidation assays. The basic principle of these techniques usually includes single electron transfer or hydrogen atom transfer, or a mixed mode of actions. However, a more potent set of antioxidant compounds is present due to the additive and/or synergistic interactions of phytochemicals in Peganum harmala. The present section reviews the studies that have explored bioactivities associated with Peganum harmala antioxidant capacities. The potential therapeutic range of antioxidants in relation to aging and disease prevention makes the topic truly significant (Melloul et al., 2022; Kemel et al.2024). To optimize antioxidant benefits, the proper method to prepare this plant is also discussed, as well as a comparison of P. harmala with other prevalent natural antioxidants. An efficacious amount of the ingestion doses is crucial in practical health applications. Each plant may develop both desirable and undesirable traits, and an evaluation of *P. harmala's* safety profile is warranted when the medicinal dosage is recommended to consumers based on the contents of its radical-scavenging compounds. Profiles should include evidence for the losses that can harm human health, but in general, *P. harmala* is viewed as safe and is approved for use in dietary ingredients by several authoritative organizations. Besides, certain research has identified the potential capacity of alkaloids and other bioactive components in the seeds of *Peganum harmala* to protect from inflammatory liver damage. (Abbas et al., 2021; Senhaji et al.2022; Djarmouni et al.2022; Ahmed et al.2021).

6. Toxicological Aspects

It is imperative to thoroughly understand the safety of a potential herb prior to its use in traditional medicine or as a food additive. Following the administration of an active alkaloid constituent of *P. harmala*, harmine, to rats and mice, the maximum tolerated single doses were found to be 250 and 125 mg/kg b.w., respectively. Fluctuating systolic, diastolic, and mean

blood pressure, as well as convulsions, were induced at these harmful doses in an earlier study of the whole extract. It was concluded that acute three-day intravenous dosage studies had not resulted in toxicity at 0.375 mg and 30 mg/kg in mouse and dog models, respectively, but a dose of 300 mg/kg resulted in negative side effects leading to loss of consciousness within 1 minute, requiring euthanasia (Gökkaya et al.2023; Miao et al.2020; Aryan, 2022). The clinical application of *P. harmala* Qur'anic and water extracts, and their alkaloids harmine and harmaline at their concentration, are discussed in this review related to toxicity. Doses of 200 mg of harmaline/harmine on an empty stomach to consciously locate the sacred underground mushroom suggest the lethal-to-resistant ratio is harmonious with that found in animal experiments with these hallucinogens. Consumption of 10-51 unroasted *P. harmala* seeds and three cooked seeds occasioned vomiting and intoxication, which was sufficiently potent to make all twelve patients delirious (Sharifi-Rad et al.2021; Semwal et al., 2021; Drioua et al.2023).

A potential cause for adverse effects by *P. harmala* might be its approximately equimolar concentration of harmaline and harmine, especially when taken in different amounts by different individuals or when prepared with more harmaline for idiosyncratic reasons, such as hallucinations, instead of harmine for pharmaceutical purposes (Rezzagui et al.2020; Gökkaya et al.2023; Doskaliyev et al.2021). Some discrepancies exist about this relationship, however. Although no chronic toxicity of *P. harmala* could be found in an extensive search of the literature, a deficiency of clinical research could compensate for any actual chronic toxicity differences. Administering a bolus of *P. harmala* at 366.5 mg/kg/day for 14 days significantly opposed reduced serum Na+ and Cl- target doses of *P. harmala* (183.3 and 91.7 mg/kg/day). The 91.7 mg/kg/day localized dose increased serum total protein, glucose, and liver and kidney enzymes, reduced kidney function, and diminished spleen weight. In previous work by this same team, *P. harmala's* acute and subchronic toxic effects also included gastrointestinal problems, muscular tremors and fasciculations, behavioral abnormalities, anorexia, weight loss, strabismus, and abnormal gait. Unlike the latest research, it was noticeable that other parameters, such as hematocrit concentrations, central nervous system damage, and liver and kidney effects, showed variations with the amount of the plant (Bettihi, 2023; Drioua et al.2023; Tekşen et al.2024; Irinmwinuwa et al.2023; Otimenyin, 2022; Bernardo & Valentão, 2024).

Food and Drug Administration guidelines for acceptable drug safety were met by past animal research on the acute and sub chronic toxicity of the crude alkaloid extracts of P. *harmala*. The studies classified this substance as a directly toxic agent for the kidney, lung, heart, liver, or muscles, and for the development of cancer, due to severe abnormalities in the laboratory results and behavioural irregularities that would reduce the animal's capacity to

survive everyday life (Rezzagui et al.2020; Bettihi, 2023; Elbah et al.2023). A minimum of two complementary long-term studies in quantitative-researched rodents, a general test on poultry and cattle, and a representative practical experiment for species-specific reactions are called for in the toxicity research pathway, while more animal tests and human clinical trials are still needed to prove that the plant is harmless and beneficial for the intended use. Regarding safety, the necessity for animal studies is not absolute; only if a substance is currently existing and can be compared with a small number of bases and other components does it start a preliminary individual human biological check in the early stages of natural or synthetic compound research. The value of running animal studies in current *P. harmala* toxicity studies is vital, though, because they provide data that can be compared to previous research. (Alhawiti, 2022; Irinmwinuwa et al.2023; Semwal et al., 2021).

6.1. Acute Toxicity

6.1.1. Overview Peganum harmala can be acutely toxic to humans.

A considerable amount of strong evidence shows that the ingestion of harmaline and harmine can cause violent reactions. The severity of these effects is mainly correlated with the dosage, in addition to other individual susceptibility factors. Acute human poisoning has been reported since the 19th century, and recent case studies have investigated human exposures to concentrated harmala (Rezzagui et al.2020; Nadia et al.2022). Neurological disturbances seem to be the main adverse effects in patients intoxicated with harmala. The maximum recommended dose is reported to be up to 85 mg/kg, although an analgesic effect is achieved with much lower doses (15–20 mg/kg). Clinical studies in humans have shown that lower doses of Peganum extract (2.0–2.5 mg/kg) can decrease motor activity and increase its reaction time. There are several reports of severe harmala poisoning cases in adults. In all these cases, the patients suffered neurological disturbances, including ataxia, myoclonus, dysarthria, choreoathetosis, and seizures. In a single fatal case due to *P. harmala* ingestion, the patient died from status epilepticus uncontrollable with antiepileptic drug therapy. Vomiting was also reported as a clinical effect but was reversible. There is no hard evidence concerning the adverse effects of higher single oral exposure in humans; thus, it is recommended that the high-dose mixture is not consumed in order to avoid any of the adverse medical sequelae of Peganum cocktails. (Abbas et al.2021; Gökkaya et al.2023; Shahrajabian et al., 2021; Sadaf et al.2021).

6.2. Chronic Toxicity

A series of health complaints and cases of hospitalization following the ingestion of *Peganum harmala*-containing snuffs have been reported from some of the countries surrounding the eastern Mediterranean, with indications that long-term (habitual) consumption may lead to a range of signs

and symptoms over time. There is also a likelihood that Peganum harmala-containing products are in use in other societies. The numerous alkaloids, collectively termed 'Harmaline,' are deemed the toxic components of the seeds. At this stage, we must be cautious about the degree of extrapolation from these findings to the Western world. Fundamental biological variations of metabolism and underlying genetically determined detoxification mechanisms clearly show considerable variability between individuals on genetic backgrounds. Clinical trials in the Middle East may also be less likely to properly adhere to the full conditions of informed consent, as health care in the region is still of the paternalistic rather than the autonomous or client-based type. Health outcomes from trials may therefore not be reliable indexes of safety. In order to adhere to this working, the expert committee for the Safety of Medicinal Products must emphasize the following: Studies of human chronic toxicity on the potential effects of habitual use of *Peganum harmala* should be urgently conducted in the countries where it is in prevailing use. (Manal et al.2021; Rachid, 2021; Semwal et al., 2021; Achour et al.2022; Elouardi et al.2022; Mamadalieva et al.2022; Filban et al.2022; Moaan et al., 2022; El et al.2024).

Although no data regarding long-term human clinical studies are available, the potential for harm from chronic ingestion of combination products containing multiple biologically active substances could be inferred from available studies for the alkaloids. *Peganum harmala*-containing snuff (nasal snuffs), some of which contain DMT, are used by some societies primarily for social, religious, and medicinal purposes, including treatment of many conditions. There is a school of thought by some traditional healers based upon anecdotal and empirical evidence that preceding the wastelands of lands and subsidies is of paramount importance if we are to ascertain the potential toxic effects of the woody perennial plant *Peganum harmala* and its derivative compounds that are used as abortifacients, snuff, fumigants, and pesticides, among others. If these studies are conducted, other countries importing derivative products or using non-toxic doses of the products should also be included to prove their safety. Indeed, this epidemiological information would be the first step in assessing their safety. It would also pave the way for the development of guidelines for the control of import and export of the species and their derivative products, including demand as a constituent of snuffs from *Peganum harmala* in food and feed. The potential for a full safe exposure assessment could also be performed (Ullah & Badshah, 2024; Shah et al.2023; Karous et al., 2021).

7. Clinical Applications

The increasing interest in herbals, coupled with the recent scientific attention given to the clinical use of these substances in traditional medicine, has opened new gateways for scientific breakthroughs. *P. harmala* is a

candidate among medicinal plants that offers an opportunity to develop traditional concepts of medical sciences (Sharma & Sharma., 2022; van, 2021; Duarte et al., 2022). Many researchers have convincingly endorsed its use in clinical settings. Clinical evidence shows the anti-anxiety, antidepressant, painkilling, and anti-Parkinson effects of *P. harmala* extracts. More than ten clinical trials in different countries, along with the current wealth of indigenous knowledge of Peganum, suggest that biological activities in psychiatric disorders, Parkinson's disease, or chronic hepatitis C infections may be treated with even safer, isolated, standardized therapeutic agents from Peganum. The new trend promises a bright future for Peganum. Future research in this area can focus on pharmacogenomics, determining the applicability of quality control measures for electronic prescribing software, and safety and efficacy monitoring in well-designed observational and prospective studies. Despite significant potential, efforts to promote the clinical use of plants used in traditional settings are challenged by regulatory constraints. Moreover, the disconnection between traditional concepts and modern science further hampers clinical use. Because herbal medicines are complex mixtures, when modern science is unable to study their nature, it cannot make a statement about these patients' progress. Treatment strategies and recommendations should be validated through clinical research based on clinical evidence (Narby & Pizuri, 2021; Burns & Phillipson, 2024; Alves et al., 2021).

7.1. Current Research and Clinical Trials

Current Research and Clinical Trials: A Randomized Controlled Trial is currently being conducted. The two centers involved in the maximal sample of 140 patients will be in Vienna and Vorarlberg. The aim is to investigate the safety of oral harmine administration in patients with treatment-resistant psychotic depression. This is part of a clinical project and is included in a proof-of-concept study on the mental health potential of mind-altering substances or treatment-resistant patients in general, enrolling around 30 patients (Ables et al.2024). Our aim is to identify potential indications and to further evaluate the safety of pharmacological interventions with *P. harmala* extracts or single ingredients by applying further state-of-the-art research methods. Results: The RCT is ongoing; first safety results are pending but are expected to be presented by mid-year 2023, which will be incorporated in the main paper. Inter-lab studies lead to good agreement of PET-tracer quantification, and a cognitive task-based functional imaging study showed a significant neural signature of an altered processing of salient stimuli by harmine, consistent with traditional claims. Additionally, *P. harmala* extracts might also have anti-infective properties (Meling et al.2024; Mueller, 2023; Rossi et al.2022; Rodrigues et al.2024; Tarpley et al.2021). A group of scientific researchers, together with scientists and practitioners from outside the academic spectrum, as well as representatives of regulatory agencies and the

wider interested public, has established the Safety and Metabolism of Peganum harmala consortium as a collaborative effort to translate the traditional uses of *P. harmala* into evidence-based medicine. The main activity of the collaborative is the undertaking of several national holistic studies to fully assess the plant and its effects. Based on the traditional use of *P. harmala*, the prospective assessment strategies aimed to capture detailed information on the circulatory and central nervous system effects, as well as the impact on mental health from acute and sub chronic poisoning with *P. harmala*. Although the data will only become available in late 2023, results received to date regarding the safety profile of P. harmala have inspired future research into species of the genus Harmal. The possibility to conduct trials with *P. harmala* as neoadjuvant drugs for metabolic or neurological diseases is being investigated, as well as preventive strategies (Aicher et al.2024; Dornbierer et al.2023; White et al.2024).

8. Regulatory Status

Peganum harmala is a sacred plant holding a significant place in the traditional practices of various cultures, ethnic communities, and societies since time immemorial (Karamkhudoeva et al., 2021; Samorini, 2021; Chaachouay and Zidane, 2022). However, the legal status of *Peganum harmala* is different in various countries. The medicinal use of *Peganum harmala* is also involved in various cultures. Some countries restrict its usage, cultivation, collection, and distribution. It is listed as a weed in some countries, and anybody can cultivate it without any legal involvement. The regulatory status of *Peganum harmala* is different in medicinal and other parts of the world, and it is the result of cultural aspects included in the laws and legislation of different countries. In traditional practices, *Peganum harmala* is safe, but safety and efficacy are still controversial in modern practices. Therefore, the countries that consider its use as a traditional use or allow its medicinal use do not apply such strict legislation on its usage. The traditional uses of various plants have also been used in the production of modern medicines (Hussain et al., 2023; DAĞDEVİREN, 2023; Hussain et al., 2023; Tanasi et al., 2023; Motyka, 2022; Saleem et al., 2024; Jafari, 2024).

Controversies on the Safety and Efficacy There is a trend in herbal medicine that has caused an urgent need for scientific evidence to support the efficacy of alternative medicines. Such alarming issues are causing countries to take necessary regulatory actions to ensure that all elements of manufacturing, importation, storage, and distribution of herbal medicines conform to the national medicine regulatory standards. Their benefits are therefore devoid of the efforts of research that would have been used to invent a drug with definite beneficial medical applications if their scientific support were known. There is therefore an issue of regulation and equipoise, apportioned to different organizations, including research sponsors, chemical companies, and

intellectual property regulators. Phase II/III are clinical studies for a single plant to decide the safety and benefits of a drug. Evaluation of the *Peganum harmala* plant may take less time than the evaluation of a single substance for its likely mixed clinical benefits and risks (Rezzagui et al.2020; Miao et al.2020; Abbas et al.2021; Saeedeh et al.2022; Khalid et al.2024; Sharma et al.2022; Asadzadeh et al.2021).

8.1. Legal Status in Different Countries

Peganum harmala used to be highly cultivated in Europe and Africa, but many countries classify this plant on the written list. Actually, harmal is still rare in many countries because the rules are getting stronger, causing new difficulties and discouragement; sometimes, cultural restrictions are a prohibition per se. Bulgaria banned it in 2014 (Ahmadianmoghadam et al., 2024). In Germany, the Federal Office for Consumer Protection and Food Safety prohibited the retail trade of this plant in November 2017. In Belgium, because of a European-level meeting concerning plants with diuretic effects that can cause doping false positives and thus cause problems for athletes, the president of an organization announced the invocation of new European regulations in order to stop the use of *P. harmala* teas by athletes. In France's DOM-TOM, it is still used. In Morocco, P. harmala is cited as currently traded for smoke production. In its original area, it was recently reported for smoking, but the individual who reported it stopped using it because of the bad taste (Semwal et al., 2021; Amiri and Fozouni, 2020); Jazayeri et al., 2022; Zhu et al., 2024).

However, in many countries, P. harmala still finds its use in traditional medicine to treat flu, stomach, or other diseases, or in ceremonies, or to induce spiritual purification for mental peace. In Tanzania, the Tuaregs use the incense to spiritualize guests and to curse them later with the same (Saleem et al., 2020). In Iran, al-Harmal was fumigated by the Zoroastrians to exorcize demons and divs, cleanse the eye, and ascribe supernatural qualities to objects used in Zoroastrian ceremonies. It is not a drug, but a plant with therapeutic use against various ailments: anti-tumor, antioxidant, anti-diabetes, etc. Thus, considering that exhaustive information cannot be summarized in a small table, the status of harmala in various countries and areas of the world based on different information, with detailed reasons, has been elaborated here (Shahrajabian et al., 2021; Afzal et al.2021; Sharma et al.2022; El-Zayat et al.2021; Abbas et al.2021).

9. Conclusion and Future Directions

P. harmala has a rich history in both social and religious practices that have been concurrent for thousands of years, particularly in the domains where this plant grows. Based on those historical references, the social and religious uses of *P. harmala* have been well documented. However, a comprehensive study on

modern pharmacological and toxicological perspectives of *P. harmala* has a rare existence in the scientific literature. Additionally, compared to the potent pharmacological properties of individual harmala alkaloids, only a limited number of experimental activities, as well as preclinical and a few clinical investigations, have been reported. Consequently, to justify the traditional belief and to exploit the hidden potential of Peganum alkaloids, especially the harmala alkaloids, large-population clinical studies are warranted. Moreover, to establish the role of adjuvant *P. harmala* use and its possible synergistic effects in anticancer, antidiabetics, and antimicrobials, the reported clinical trials need replication. It is highly accepted that further interdisciplinary research on *P. harmala's* potential and therapeutic applications should also be promoted to explore new clinical interventions for side effect management, a more acceptable regimen, and a strong plant-kept immune memory to enhance human health care. Furthermore, further clinical trials are needed to address the potential therapeutic benefits of standardized *P. harmala* preparations. The concentration-response relationships, beginning doses, doses of anticipated medical effect, and limited tolerated doses are still not fully investigated (Khalid et al., 2024; Jalali et al., 2021). In doing so, and to minimize potential hazards when used for its benefits, standardized *P. harmala* guidelines and regulations should be prepared. Additionally, to integrate traditional knowledge into medical circumstances, well-designed experimental and toxicological investigations are also warranted. The tolerance of the long-term side effects and the different doses of pure harmala alkaloids remain unclear. Furthermore, these reports may also aid in understanding the use of other toxic plant species. In conclusion, this review indicates that *P. harmala*, with its essential harmala alkaloids, possesses a number of valuable pharmacological uses, including antioxidant, anticancer, anti-inflammatory, antidepressant, inhibiting AChE, anti-AD, improving cognition, antiviral, anti-leishmania, immunomodulatory, antimicrobial, insecticidal, wound healing properties, analgesic, antipyretic, antidiarrheal, antispasmodic, antidyslipidemic, and blood glucose-lowering properties. In contrast, since adverse effects when using *P. harmala* may occasionally be life-threatening, caution must be exercised. The use of *P. harmala* and its isolated harmala alkaloids to handle a wide range of diseases warrants further investigation. (Sharma et al., 2022; Ahmed et al., 2021; Sadaf et al., 2022; Zhang et al., 2020; Jaradat et al., 2024; Akhtar et al., 2022).

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CHAPTER 11

Mentha piperita L.: OVERVIEW OF PHENOLIC COMPOSITION AND TRACE ELEMENT CONTENTS

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1. INTRODUCTION

Mentha piperita L., commonly known as mint in Turkish, is a perennial and aromatic plant from the Lamiaceae family. This plant has had an important place among medicinal and aromatic plants since ancient times (Lawrence, 2006). Today, the use of the plant in various fields such as food, cosmetics, pharmacy and aromatherapy is quite common. In alternative medicine, *Mentha piperita* L. is used in the treatment of ailments such as headache, digestive problems, respiratory diseases and muscle pain due to its anti-inflammatory and analgesic properties. Additionally, peppermint has significant antimicrobial and antiviral activities, strong antioxidant and antitumor effects, and some antiallergenic potential. In terms of medical use, it is considered to be antibacterial as it can prevent the growth of *Streptococcus* and *Lactobacillus* bacteria, so it is frequently used in toothpastes, mouthwashes, chewing gums, confectionery and cosmetic products. In folk medicine, peppermint oil is widely used as a carminative, antispasmodic and expectorant, as well as in aromatherapy and massage therapies (Mimica-Dukić ve Božin, 2008; McKay ve Blumberg, 2006). Phenolic compounds and essential oils have an important place among the chemical components of the peppermint plant. The plant contains essential oils including menthol, menthone, flavonoids, phenols, triperthene and tannin. Phenolic components of the leaves include rosmarinic acid and various flavonoids, mainly eriocitrin, luteolin and hesperidin. It is very rich in vitamins C and A. Additionally, it contains high amounts of potassium, protein, folic acid, calcium, zinc, iron and fibres (Berktaş ve Çam, 2020).

Phenolic structures greatly contribute to the antioxidant activity of the plant. Reactive oxygen species (ROS) include superoxide, singlet oxygen, hydrogen peroxide, and hydroxyl radical. Excessive production of ROS can lead to oxidative stress, which triggers damage to cell structures including lipids, proteins, and DNA. Antioxidants prevent cellular damage caused by free radicals, reducing oxidative stress.

Due to environmental factors, contamination of agricultural soil and therefore plants with pesticides and heavy metals is inevitable. Additionally, plants are an important means for transferring trace elements from soil to humans. Therefore, determining the macro and micro element content in plants is very important in the use of herbal infusions and in the evaluation of toxicity in terms of human health in the production of plants used as raw materials in the pharmaceutical industry (Seeniyasan et al., 2008; Kostić et al., 2011; WHO, 2007).

Scientific research on peppermint, which has not lost its popularity to this day due to its chemical components that justify its use in folk medicine since ancient times, continues today. In this review, scientific publications on the

phenolic composition and trace element contents of *Mentha piperita* L. from Google Scholar, Web of Science, Scopus, and PubMed databases are included.

2. PHENOLIC COMPOUNDS AND TRACE ELEMENT CONTENT OF *Mentha piperita* L. USED AS A MEDICINAL PLANT

2.1. Phenolic composition analysis of *Mentha piperita* L. with microelement effect

The ability to grow medicinal plants under harsh environmental conditions is important for sustainable agriculture. When obtaining pharmaceutical products, the concentrations of metals in the soil should not exceed the permitted limit values. Copper (Cu), zinc (Zn), manganese (Mn), iron (Fe), molybdenum (Mo) and boron (B) are the elements necessary for plant growth. Apart from boron, these elements are also heavy metals and their high concentrations are toxic to plants (Webber, 1981). For this reason, the analysis of these microelements added to the soil and plant leaves for plant growth has led researchers to studies on increasing the phenolic composition of plants. These studies conducted in the last year are shown in Table 1.

In the study of Mehdizadeh et al. *Mentha piperita* plants grown in hydroponic culture system were divided into four different groups. Control group (Zn and methyl jasmonate (MeJA) were not applied), only Zn applied group, only MeJA applied group and both Zn and MeJA applied group. Different concentrations of Zn and MeJA were applied to these groups by spraying on the leaves. The leaves collected from the plants after the applications were analyzed for phenolic compounds. The results showed that among all the phenolic contents detected in peppermint leaves, rosmarinic acid content was the highest (34.34 mg g DW-1 in 1 mM MeJA and 0.05 mg L-1 Zn application), followed by gallic acid, tannic acid, chlorogenic acid, caffeic acid, ferulic acid and p-coumaric acid (Mehdizadeh et al., 2024).

Adamczyk-Szabela and Wolf studied how adding copper or zinc to the soil in which peppermint, common nettle, basil and borage plants would be grown affected photosynthesis. The polyphenol content in common nettle and basil plants increased with the metals added to the soil, while there was no significant change in other plants. It was found that the plant extracts obtained from common nettle and basil had high antioxidant properties and it was reported that these plants could be good free radical scavengers. It has been reported that determining safe levels of metals added to soil to increase phenolic compounds in plants is very important to obtain pharmaceutical products (Adamczyk-Szabela and Wolf, 2024).

Asle-Mohammadi et al. aimed to reduce Cu toxicity in soil by adding Fe, Zn and Mn to peppermint leaves as micronutrients. They examined the positive effects of this application on peppermint growth and increasing

essential oil (EO) production. Menthofuran, menthol, menthone, menthyl acetate, pulegone and limonene were found among the EO components identified at all Cu toxicity levels. In the study, it was reported that the plant with Fe, Zn and Mn is a cost-effective and practical application to remediate Cu-contaminated soils and that EO production in peppermint can be increased with the application of 5 mg kg-1 Cu toxicity (Asle-Mohammadi et al., 2024).

To increase peppermint biomass and essential oil, a study was conducted using four fertilizer sources namely poultry manure, sheep manure, cattle manure and chemical fertilizers as well as different cuts namely first, second and third cutting. In a study conducted to preserve ecosystem sustainability by replacing chemical fertilizers in semi-arid plateaus, it was reported that the use of poultry manure and sheep manure can be recommended to increase the biomass of peppermint essential oil and improve its quality. Plants grown with poultry manure had the highest yield of peppermint essential oil in all three sections, and the components of the oil were menthol (35.2-58.3%), menthone (3.1-33.6%), menthyl acetate (1.1-9.4%), iso-menthon (0.41-8.8%) was found as (Fallah et al., 2024).

Mohammed et al. investigated the effects of different cadmium pollution levels in soil on the growth, chemical composition and metal accumulation in the leaves of *Mentha piperita* L. and *Mentha spicata* var. crispa plants. When 15 mg kg-1 cadmium was added to the soil in both types of plants (1.537% for peppermint and 1.340% for curly mint), the percentage of essential oil and the amount of essential oil per plant increased. But when twice as much cadmium was added to the soil, cadmium accumulation in the leaves of the plant increased compared to the control (0.165 mg kg-1). It has been reported that plants grown in cadmium-contaminated soils have high Cd concentrations in their structure and have adverse effects on health when consumed (Mohammed et al., 2024).

Pourhosseini et al. explained the benefits of vermicompost and biofertilizers in their studies to optimize peppermint cultivation and reduce environmental risks resulting from the use of chemical fertilizers. They stated that these increase the phenolic, flavonoid, anthocyanin and antioxidant properties of peppermint, further enriching the medicinal potential of peppermint extracts. They reported the importance of using organic fertilizers in peppermint cultivation, which is a medicinal plant for sustainable agriculture (Pourhosseini et al., 2024).

Bghbani-Arani and Poureisa reported in their studies that the application of organic fertilizers (especially pure vermicompost and azocompost) or their combination with chemical fertilizers in arid climates increased the organic carbon, macro (nitrogen and phosphorus) and micro (iron and zinc) nutrients

in the soil solution, thus increasing biological yield and essential oil content of the peppermint plant examined in the study (Bghbani-Arani and Poureisa, 2024).

A study was conducted to determine the effects of three macronutrient concentrations in hydroponic nutrient solution on three mint species (*Mentha spicata* L. var. viridis; *Mentha piperita* L.; *Mentha spicata* L. var. rubra) grown according to the New Cultivation System during three harvest periods. The study emphasized the importance of adjusting the optimum hydroponic nutrient solution ion concentration according to specific plant species and environmental conditions to increase the appropriate yield, product quality and shelf life of peppermint plants. (Hazrati et al., 2024).

In Egypt, the use of peppermint and eucalyptus essential oils instead of pesticides against the whitefly, the most damaging field pest in potato cultivation, has been investigated. The main components of these essential oils were determined and their coarse emulsion and nanoemulsion were prepared. The researchers examined the total soluble protein, total carbohydrate, total phenolic content and peroxidase activity to examine the effect of the emulsions of these essential oils. It has been reported that the essential oils contained in both types of emulsions of plants are an effective non-chemical alternative against the whitefly *Bemisia tabaci*, provided that they are applied according to weather conditions such as temperature, humidity and wind (Wahba et al., 2024).

Researchers have examined the growth of the medicinal plant *Mentha piperita* with fungi (*Glomus intraradices*) acting as a supporter in the soil where it grows, in terms of the biological activities of the plant. They even reported the benefits of growing in this way to increase the amount and quality of the plant against various pollutants (Pb, Cd) coming from the soil or the environment (Djerrad et al., 2024).

In the study, the effects of salt stress on plant nutrients and essential compounds were examined in *Mentha piperita* L. seedlings grown at different salt concentrations and periodically applied *Bacillus amyloliquefaciens*. *Bacillus amyloliquefaciens* was applied to *Mentha piperita* L. seedlings grown at various salt levels for six weeks. At the end of the study, when the seedlings were evaluated in terms of plant nutritional elements, *Bacillus amyloliquefaciens* showed a positive effect on Zn, Mn, Cu and Na values compared to the control, while it did not show any effect on B, Fe, K, P, Mg and Ca. Limonene was determined as the significant component in essential compounds in all groups (Üner and Turgut, 2024).

Shariatmadari et al. used a natural isolate of heterocystous cyanobacteria to stimulate biomass production and rosmarinic acid content in *Mentha piperita* L. They inoculated *Mentha piperita* L. explants into the medium

containing cyanobacterial lysate. They found that rosmarinic acid increased 2-3 times in their measurements after 50 days of culturing (Shariatmadari et al., 2024).

The study conducted in Pakistan aimed to highlight the importance of genetic characterization for conservation of Mentha species and the potential of DNA barcoding in overcoming the limitations of traditional taxonomic methods. Comparative analysis and correlation among different genotypes of Mentha are crucial for genetic improvement aimed at increasing the yield of secondary metabolites through development of new and improved genotypes (Naseem et al., 2024).

Table 1. *Evaluation of the effect of microelements added to the soil and plant leaves for the growth of Mentha piperita L. by phenolic composition analysis*

Plant material location	Extraction method	Antioxidant determination methods	Analytical methods	Bioactive constituents	Trace elements	References
Cultivated in the greenhouse of Ferdowsi University in Mashhad, Iran in 2021.	extraction with methanol	DPPH, FRAP, Phosphomolybdenum complex method	HPLC	rosmarinic acid, gallic acid, caffeic acid, ferulic acid, chlorogenic acid, p-coumaric acid and tannic acid	Zn	(Mehdizadeh et al., 2024)
Seeds of plants were purchased from the company P.H. Legutko, Poland.	extraction with boiling water	Folin-Ciocalteu	Spectrophotometer, High resolution continuum source atomic absorption spectrometer (HR CS AAS)	phenolic compounds	Mn, Fe, Cu, Zn	(Adamczyk-Szabela, and Wolf, 2024)
Cultivated in the research greenhouse of Urmia University, Iran in 2021.	hydro-distillation (Clevenger apparatus)		UV-VIS Spectrophotometer, AAS, GC-MS	Carotenoids, menthofuran, menthol, menthone, menthyl acetate, pulegone, limonene	Cu, Mn, Fe, Zn	(Asle-Mohammadi et al., 2024)
Collected in 2019 from Koohrang, Chaharmahal and Bakhtiari Province, Iran.	hydro-distillation (Clevenger apparatus)	DPPH	GC-FID, GC-MS, UV-spectrophotometer, Kjeldahl method, flame photometer, AAS	menthol, menthone, menthyl acetate, iso-menthon	N, P, K, Zn, Cu, Mn, Fe	(Fallah et al., 2024)
The plant was collected from the nursery area in Bagera, Iraq in 2023.	extraction with distilled water		Spectrophotometer, AAS	chlorophyll content, volatile oil	Cd	(Mohammed et al., 2024)
It was harvested from Varamin farm in Ali-Abad Village (Iran) in 2021-2022.	hydro-distillation (Clevenger apparatus), methanol	Folin-Ciocalteu, DPPH	GC-FID, GC-MS, spectrophotometer	phenols, flavonoids, and anthocyanins		(Pourhosseini et al., 2024)

The *M. piperita* seedlings were obtained from Iran's Forests and Ranges Organization (2017-2019).	hydro-distillation (Clevenger apparatus)		Kjeldahl method, flame photometry, AAS		N, P, K, Fe, Zn, Cu, Mn	(Bghbani-Arani and Poureisa, 2024)
Essential oils of plants were purchased from El-Gomhoria Chemical Company, Cairo, Egypt.	distilled water		GC-MS, spectrophotometer	levomenthol, p-menthone, menthyl acetate, L-menthone, eucalyptol	N, P, K	(Wahba et al., 2024)
M. piperita rhizomes were obtained from the National Agricultural Institute of Algeria.	hydro-distillation (Clevenger apparatus)		GC-FID, GC-MS	1,8-cineole, linalool, menthone, menthon, pulegone, menthyl acetate	Pb, Cd	(Djerrad et al., 2024)
Seedlings were purchased from in Antalya-Turkey.	solid phase microextraction		SPME/GC, ICP-OES	limonene	Zn, B, Mn, Fe, Cu, Na, K, P, Mg, Ca	(Üner and Turgut, 2024)
M. piperita explants were obtained at the Iran Institute of Medicinal Plants.	extraction with ethanol		HPLC-UV	rosmarinic acid		(Shariatmadari et al., 2024)
Plants were collected from various places in Pakistan.	hydro-distillation (Clevenger apparatus)	Folin-Ciocalteu DPPH	UV-VIS Spectrophotometer	total phenolic content, total flavonoid contents		(Naseem et al., 2024)

2.2. Pharmacological effect of *Mentha piperita* L.

Discovering the potential pharmacological effects of the plant's bioactive molecules makes the use of natural products as a pharmacological approach attractive. Medicinal plants are used in food, feed, pharmaceutical, cosmetic and other industries due to their antiviral, antitumor, anti-inflammatory and antioxidant properties (Ivanova et al., 2024). These studies conducted in the last year are shown in Table 2.

El Omari et al. investigated the therapeutic properties and medicinal potential of the essential oil of *Mentha piperita* L. in their study. They evaluated the antidiabetic effect of the plant by measuring the inhibition of two digestive enzymes (α-glucosidase and α-amylase), its dermatoprotective effect based on the inhibition of elastase and tyrosinase, and its neuroprotective activity by examining the inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). Peppermint has shown a rich potential in bioactive compounds and the main

components of its essential oil have been reported to be used in the development of drugs and natural therapeutic applications (El Omari et al., 2024).

Ogbuokiri et al. designed their study to investigate the protective role of peppermint leaf extract in the brain of Wistar rats exposed to lead. The results showed that peppermint extract had no effect on cerebellum histology. They also did not find satisfactory results regarding the effects of lead acetate on antioxidant enzymes and lipid peroxidation (Ogbuokiri et al., 2024).

Helal et al. examined histopathological changes in the brain and liver tissues of rats divided into eight groups to prove the healing effect of *Salvia hispanica* oil and *Mentha piperita* oil on neurological effects in Albino rats exposed to lead. It was reported as a result of their study that the synergistic effect of the mixture of peppermint oil and chia oil had a strong healing effect against lead poisoning (Helal et al., 2024).

A mixture of peppermint, celery, coriander, parsley and rosemary leaf flour was named as a phytogenic substance. The effect of this new substance used in the nutrition of weaned pigs on the growth performance and hemato-biochemical indicators of pigs was investigated. It was concluded that the use of phytogenic substances up to 15 g kg-1 can increase pig productivity at the weaning stage without causing a negative effect on the health status of the animals (John, 2024).

Melissa officinalis and *Mentha piperita* are plants with potential to improve aquaculture practices and protect fish production in agriculturally polluted water bodies. Changes in protein levels and micronucleus induction in major organs of freshwater fish *Channa punctatus* after exposure to chlorpyrifos were evaluated. The contributions of these plants in maintaining fish health in toxic environments were highlighted (Tiwari et al., 2024).

The effect of *Mentha piperita* or *Thymus vulgaris* on improving hematological changes due to chronic oxidized palm oil consumption in rats has been investigated. Red blood cells decreased and white blood cells increased in rats consuming oxidized palm oil. In rats fed with oxidized palm oil and *Mentha piperita* or *Thymus vulgaris* or extracts of both plants, a significant increase in red blood cells was observed while white blood cells decreased (Muhanna et al., 2024).

Abu Zahra et al. investigated the effects of *Mentha piperita* powder added to the feed as an additive to the Nile Tilapia, a predatory fish species living in the Nile River. They evaluated the growth performance, hematological, biochemical and immune parameters, intestinal histology and interleukin gene expression of the fish. They aimed to evaluate the protection against *Vibrio alginolyticus* infection in *Oreochromis niloticus* by the plant powder. As a result of their studies, they reported that *Mentha piperita* powder, a

medicinal plant, may have growth-promoting and immunostimulatory effects for sustainable aquaculture (Abu-Zahra et al., 2024).

In the study, the oil obtained from fresh powdered *Mentha piperita* leaves was evaluated for its insecticidal activity on *Callosobruchus maculatus* and *Sitophilus zeamais*. After exposure to essential oil vapor for 6-24 hours, mortality rates were recorded for both insect species between 68-94.5% and 65-90.2%, respectively. The insecticidal effect of peppermint oil was compared with the effect of chemical insecticides and it was reported that it can be used as an alternative insecticide (Olayemi et al., 2024).

In the study of Sing et al. the chemical components of the essential oil obtained from *Mentha piperita* were analyzed and identified by GC-MS. The essential oil obtained from *Mentha piperita* was reported to have good antiproliferative activity against the A549 cancer cell line (Singh et al., 2024).

Machewar et al. formulated a herbal microemulgel containing *Mentha piperita* extract. They evaluated the in vitro anti-inflammatory properties of this microemulgel. They reported in their study that herbal microemulgels enriched with *Mentha piperita* extract have promising anti-inflammatory effects (Machewar et al., 2024).

Anum et al. synthesized silver nanoparticles (AgNPs) using extracts from three medicinal plants (*Amaranthus viridis* L., *Mentha piperita* L. and *Ocimum basilicum* L.). They evaluated the antifungal activities of the AgNPs they synthesized against *Botrytis cinerea*. They reported that AgNPs showed significant antifungal properties and could be developed as environmentally friendly antifungal agent candidates (Anum et al., 2024).

The study aimed to synthesize methanol extracts of *Mentha piperita*, *Mentha spicata* and *Mentha longifolia* plants with silver nanoparticles. They used *Mentha piperita* silver nanoparticle loaded carbopol gel for wound healing in a diabetic rat model. *Mentha piperita* silver nanoparticles were reported to have the highest antioxidant activity, the best antiglycation potential and antimicrobial activity against *Bacillus subtilis*, *Micrococcus luteus* and *Escherichia coli* (Aftab et al., 2024).

The study aimed to investigate the acaricide effect of *Mentha piperita* essential oil. Since *Varroa destructor* parasite damages *Apis mellifera* (honey bee) farms and causes economic losses, the pharmacological properties of four plants were investigated against this parasite. The phytochemical composition and pharmacological activity of the essential oils of *Calamintha nepeta*, *Calamintha sylvatica*, *Lavandula austroapennina* and *Mentha piperita* plants were evaluated. It has been reported that essential oils can be applied to synthetic drugs against chemical residues in foods for the control of *Varroa destructor* parasite (Bava et al., 2024).

Table 2. *Evaluation of pharmacological effect of Mentha piperita L. by analysis of its bioactive molecules*

Plant material location	Extraction method	Antioxidant determination method	Analytical methods	Bioactive constituents	Trace elements	References
Harvested in Ouezzane Province, Morocco.	hydro-distillation (Clevenger apparatus)	FRAP, DPPH, ABTS	GC-MS/MS	pulegon, mintlactone, D-carvone, eucalyptol, thymol		(El Omari et al., 2024)
Fresh mint leaves harvested in Nigeria.	extraction with methanol, water, ethanole, hexane, petroleum ether			Ammodendrine, Cyanogenic glycosides, Spartein, Proanthocyanidin (phytochemicals), etc.		(Ogbuokiri et al., 2024)
Peppermint leaves were collected from the field (Egypt).	hydro-distillation (Clevenger apparatus)		GC-MS	enthol, menthone, menthofuran, menthyl acetate, camphene, D-limonene, eucalyptol, 1-Hexade canol,2-methyl, geranyl acetate, myrcene		(Helal et al., 2024)
M. piperita and other plants were harvested from Sumitra Research Institute gardens in Gujarat (India).	extraction with ether	Folin Ciocalteu	UV/VIS Spectrophotometer, AAS	Phenols, Flavonoids, Alkaloids	Ca, P, K, Mg, Mn, Zn, Fe, Na, Cu, Se, Cr, Ni	(John, 2024)
Dried leaves of the plants were obtained from the local market in Lucknow, India.	extraction with ethanol					(Tiwari et al., 2024)
M. piperita was purchased from a local traditional market in Jeddah, Saudi Arabia.	extraction with distilled water					(Muhanna et al., 2024)
Collected from natural habitat (Egypt).	The leaves were used in powder form					(Abu-Zahra et al., 2024)

Fresh leaves of *M. piperita* was harvested from the garden in Tudun Wada area of Kaduna State in Nigeria.	hydro-distillation (Clevenger apparatus)		GC-MS	menthol, menthone, menthofuran, 1, 8-cineole, limonene		(Olayemi et al., 2024)
Peppermint collected from natural environment was used.	hydro-distillation		GC-MS	D-carvone, L-limonene, squalene, cis-carveol, α-amorphene		(Singh et al., 2024)
M. piperita plant was procured from a nursery in Nagpur.	maceration with alcohol and extraction with ethanol using a Soxhlet apparatus		FTIR, UV Spectrophotometer	menthol, menthofuran, menthyl acetate, menthone, 1,8-cineole		(Machewar et al., 2024)
The plant materials were collected Punjab, Pakistan.	microwave-assisted extraction with methanol	DPPH	GC-MS, UV-VIS Spectrophotometer, FTIR			(Anum et al., 2024)
Collected from high altitude natural locations of Punjab, Pakistan.	extraction with methanol	DPPH	UV-VIS Spectrophotometer, FTIR, ESI-MS			(Aftab et al., 2024)
Aerial parts of *M. piperita* were collected from natural growing areas in Calabria, Southern Italy.	extraction by steam distillation process		GC-FID, GC-MS	Menthol, 1,8-sineol, linalool, menton, pulegon		(Bava et al., 2024)

2.3. Use of Mentha piperita L. essential oils as preservatives

The use of essential oils of plants instead of synthetic preservatives due to their antimicrobial and antioxidant properties can be a safer and more sustainable alternative for health. Essential oil nanoemulsion-loaded edible coatings offer a viable solution to the ever-increasing demand for a green alternative method to preserve nutrients. Preparing nanoparticles containing free essential oils of plants prevents microbial growth and oxidative degradation during storage, thus extending the shelf life of foods and preserving their contents, providing maximum health benefits (Sharma et al., 2024). These studies conducted in the last year are shown in Table 3.

The aim of the study is to use iron oxide nanoparticles together with magnetic solid phase extraction method of acetone extracts of green plants (*Mentha piperita, Urtica dioica*). As a result, it was found that iron oxide nanoparticles act as an adsorbent for the isolation of photosynthetic pigments (chlorophyll a, lutein) from the extracts of these green plants. Researchers suggested this method as a simple and inexpensive method for the isolation of plant pigments (Flieger et al., 2024).

In this study, free essential oil of *Mentha piperita* was used to increase the quality and shelf life of cherry tomatoes (*Solanum lycopersicum* cv. Santiago F1) during storage after harvest. The effect of solid lipid nanoparticles containing different concentrations of free essential oil of peppermint on cherry tomatoes was evaluated. It was shown that the combination of solid lipid nanoparticles and free essential oil of *Mentha piperita* extended the shelf life during storage and improved the quality characteristics of cherry tomatoes (Vakili-Ghartavol et al., 2024).

The study analyzed the oxidative stability of peppermint essential oil (PEO) loaded solid lipid nanoparticles (PEO-SLN) to extend the shelf life of trout fillets during cold storage. The effect of gelatin coating containing PEO-SLN was found to be the most effective method against chemical deterioration of trout fillets during 12 days of storage. In addition, researchers reported that FRAP and DPPH radical scavenging of PEO-SLN showed higher antioxidant activity than free PEO (Safaeian Laein et al., 2024).

Fuentes et al. found that *Mentha piperita* essential oil loaded with solid lipid nanoparticles exhibited high stability under environmental conditions and excellent antifungal activity to reduce the mycelial growth of *Botrytis cinerea*. The researchers aimed to reduce the losses caused by *Botrytis cinerea*, a phytopathogenic fungus responsible for gray mold disease affecting fruits and vegetables. They reported that it was possible to protect nutrients from this fungus using *Mentha piperita* essential oil loaded with solid lipid nanoparticles (Fuentes et al., 2024).

Table 3. *Evaluation of free essential oils of Mentha piperita L. as preservatives for foods*

Plant material location	Extraction method	Antioxidant determination method	Analytical methods	Bioactive constituents	Trace elements	References
The plant was harvested in the south-eastern region of Poland in August 2023.	acetone and ethanol with magnetic solid phase extraction		HPLC, UV-VIS Spectrophotometer, FT-IR/PAS	chlorophyll a, lutein		(Flieger et al., 2024)
M. piperita essential oil was purchased from Barij Medicinal Plants Research Center, Kashan, Iran.	extraction with acidic methanol	Folin-Ciocalteau, DPPH	Spectrophotometer	total phenolic content, polyphenol oxidase activity, antioxidant activity		(Vakili-Ghartavol et al., 2024)
M. piperita (peppermint essential oil PEO) was purchased from Nader agricultural industries, Mashhad, Iran.	extraction with methanol	FRAP, DPPH	Spectrophotometer	antioxidant activity		(Safaeian Laein et al., 2024)
Essential oil of *M. piperita* was purchased from Chile.						(Fuentes et al., 2024)

2.4. Phenolic composition of *Mentha piperita* L.

The essential oils of the peppermint plant also exhibit strong antimicrobial and antioxidant activities, increasing the pharmacological importance of the plant. Based on this, researchers have studied the antioxidant capacity of mint by applying different antioxidant activity determination methods to the extracts obtained with different solvents and different extraction methods for the phenolic composition and antioxidant activity of the plant. These studies conducted in the last year are shown in Table 4.

Sadowska et al. worked with *Mentha piperita*, lemon balm and lavender plants. They found that the aqueous extract of the *Mentha piperita* plant had the highest polyphenol content and antioxidant activity measured by the FRAP method. The highest antioxidant activity measured by the ABTS method was reported in the methanolic lemon balm extract. The highest amount of total phenolic compounds was determined in the water extract of *Mentha piperita*. It was reported that the water and acidified methanol extracts of the *Mentha piperita* plant contained high amounts of naringin, rutin, hesperidin and rosmarinic acid (Sadowska et al., 2024).

In their study, si Said et al. investigated the possible effects of the addition

of *Mentha piperita*, *Cinnamomum verum*, *Illicium verum*, *Zingiber officinale*, *Artemisia herba alba* and *Syzygium aromaticum* to green tea on the biological properties of tea. The plants added to green tea increased the phenolic content of the tea and increased its antioxidant capacity. They found that peppermint-flavored green tea showed the highest content of polyphenols and flavonoids. In addition, green tea combined with peppermint showed the second highest antioxidant activity (si Said et al., 2024).

Quinic acid and caffeic acid derivatives are important compounds for health and are found in medicinal plants. The concentrations of caffeic and quinic acid, especially caffeoylquinic acid derivatives, in water and water-ethanolic extracts obtained from plants *Mentha piperita* L., *Melissa officinalis*, *Ocimum basilicum* and *Ipomoea batatas* L. Lam were analyzed by HPLC method. The analyzed aqueous plant extracts exhibited significant polyphenolic compounds containing higher amounts of mono-, di- and tri-caffeoylquinic acids than ethanolic extracts, and showed significant phytochemical properties (Islam et al., 2024).

HPLC method with UV detection at 330 nm was developed for quantitative determination of rosmarinic acid content, an important phenylpropanoid in *Mentha piperita* L. leaves. Rosmarinic acid content in leaves was found to vary between 2.01±0.03% and 5.54±0.05% (Kurkin et al., 2024).

Up to 58 components, including 11 main components, were determined in the essential oil of *Mentha piperita* L. by GC-MS. It was found that the quantitative content of the essential oil is highly dependent on the climatic conditions in which the plant is grown, and the composition of volatile organic substances is highly variable (Alibegov et al., 2024).

In the study, the antioxidant, antifungal and antibacterial properties of the essential oils of *Mentha piperita* and *Ocimum basilicum* and their potential synergistic effects with various antibiotics were evaluated. It was shown that 1:1 mixtures of essential oils could be a good treatment option alone or as drug adjuvants due to their antibacterial and antioxidant properties (Türk et al., 2024).

The essential oil composition of *Mentha piperita* was investigated using methyl jasmonate for the essential genes Pulegone reductase, Menthofuran synthase and limonene synthase. Methyl jasmonate increased the activity of antioxidant enzymes and the reason why peppermint, a medicinal plant, is preferred in the treatment of many diseases was explained (Afkar and Karimzadeh, 2024).

The synergistic effect of mixing the sugarcane (*Saccharum officinarum* L.) - peppermint (*Mentha piperita* L.) mixture using sonication and microwave separately for different minutes on the resulting fruit juice mixture was

examined. It was reported that the synergy of sonication and microwave in improving the physicochemical and phytochemical quality of the sugarcane-peppermint mixture significantly increased the total phenolic, flavonoid content and antioxidant capacity compared to the unprocessed fruit juice mixture (Hussain et al., 2024).

In this study, the yield, physicochemical compound and biological properties of *Mentha piperita* L. essential oil obtained by hydro-distillation and supercritical fluid extraction methods were comparatively evaluated. Essential oils were found to be menthol, menthone and eucalyptus. The essential oil obtained by supercritical fluid extraction showed the highest antimicrobial activity against *Pasturella multocida* and due to its potent bioactive components, it was reported by researchers that it could be a potential candidate for developing nutra-pharmaceuticals (Abbas et al., 2024).

In the study, the chemical composition, physical parameters and antioxidant properties of the basic acids of *Mentha spicata*, *Mentha piperita* and *Mentha pulegium* were determined. It was suggested that the extracts obtained from these plants belonging to three geographical regions in Morocco could be used as an alternative to synthetic chemical products and could find applications in complementary medicine, pharmaceutical and food industries (Rayan et al., 2024).

Table 4. *Evaluation of phenolic composition of Mentha piperita L.*

Plant material location	Extraction method	Antioxidant determination method	Analytical methods	Bioactive constituents	Trace elements	References
Obtained from the in Kraków, Poland.	extraction with water (infusion), methanol, methanol acidified with formic acid	ABTS, FRAP, Folin-Ciocalteu	HPLC-DAD	naringin, rutin, hesperidin, rosmarinic acid		(Sadowska et al., 2024)
All plants were purchased from a local market (Bejaia, Algeria).	extraction with distilled water	Folin-Ciocalteau, TFC (total flavonoid content), DPPH, TAC (Total antioxidant capacity), FRAP	UV-VIS spectrophotometer, FTIR	polyphenol, flavonoid		(si Said et al., 2024)
Plants were purchased from a local nursery (USA).	extraction with water, water-ethanol	Folin-Ciocalteau, DPPH, ABTS	RP-HPLC	phenolic acid, caffeoylquinic acid, 3, 4, 5 tri-caffeoylquinic acid		(Islam et al., 2024)

Peppermint leaves grown in the Samara University Botanical Garden were used (Russia).	extraction with water-ethanol		HPLC-UV	rosmarinic acid		(Kurkin et al., 2024)
Plants grown in soils at altitudes between 1100 and 1650 m above sea level were used (Russia).	hydro-distillation (Clevenger apparatus)		GC-MS	limonene, eucalyptol, γ-Terpinene, menthone, isomenthone, menthofuran, menthol, terpinen-4-ol, piperitone, neomenthyl acetate, menthyl acetate		(Alibegov et al., 2024).
M. piperita essential oil was supplied from the commercial market (Turkey).		DPPH, CUPRAC	GC-MS	menthol, L- menthone, L-menthol, menthyl acetate		(Türk et al., 2024)
Plants grown in Tehran, Iran.	extraction with ethanol		GC	Menthol, pulegone, menthofuran, menthyl acetate, cineole, linalool, α-pinene, β-pinene		(Afkar and Karimzadeh, 2024)
M. piperita was collected in Pakistan as an indoor plant.	Ultrasound-assisted extraction, microwave digestion extraction	Folin-Ciocalteau DPPH	Spectrophotometer	total phenolic content, total flavonoid contents, total antioxidant capacity		(Hussain et al., 2024)
Leaves and stem of *M. piperita* plant were harvested from Soon Valley, Khushab District, Punjab, Pakistan in April 2020.	hydro-distillation (Clevenger apparatus), supercritical fluid extraction	DPPH	GC-FID / GC-MS	menthol, menthone, eucalyptus		(Abbas et al., 2024)
Plants were collected from three different geographical areas in Rabat-Sale-Kenitra region of Morocco.	hydro-distillation (Clevenger apparatus)	DPPH, ABTS, FRAP	GC-MS	linalool, D-carvone, 1,3,8-p-menthatriene		(Rayan et al., 2024)

2.5. Physicochemical properties and trace element content of Mentha piperita L.

The physicochemical properties and mineral content of *Mentha piperita*,

which is used both as herbal tea and in kitchens to benefit from its nutritional value and taste (aroma), were investigated. These studies conducted in the last year are shown in Table 5.

The determination of the physicochemical properties and mineral content (Ca, K, Fe, Na, Cu, Zn) of *Mentha piperita* used as herbal tea was studied by Indian researchers. The potential health effects of peppermint herbal tea were reported in the study (Afshiya, and Anil, 2024).

In their study, Turco et al. investigated the total polyphenol and mineral element contents in herbal infusions using two different brewing methods: traditional brewing and using a coffee machine. Among the plant materials studied, the peppermint plant was found to be the richest in terms of minerals and basic trace elements, while all plant infusions were reported to be safe to consume in terms of As, Cd, Pb and Hg (Turco et al., 2024).

In their study, Yalçın et al. examined the effect of carbonate addition on the basic element concentrations of peppermint tea and other types of tea (black, herbal and fruit). For this purpose, they analyzed the teas brewed by the infusion method with the addition of carbonate and the teas brewed after the microwave process, using ICP-OES. According to the results obtained after the microwave process, the major elements were found to be Ca, K, P, Mg and Mn. However, the concentration of the major elements in teas brewed with the infusion method with the addition of carbonate decreased, and brewing tea by adding carbonate was not recommended by the researchers (Yalçın et al., 2024).

Green vegetables (*Corchorusolitorius*, *Mentha piperita*, *Allium fistulosum*, *Ocimumbasilicum*, and *Murray koenigiican*) purchased from a garden in Nigeria were evaluated for health. Protein, vitamins, and minerals in green vegetables were analyzed. Compared to other vegetables, iron, zinc, and lead values were found to be highest in *Mentha piperita* (Nwachoko et al., 2024).

Guemidi et al. aimed to improve the functional properties of yoghurt by adding different doses of peppermint hydroethanolic extract to yoghurt. As a result, they revealed that the peppermint plant hydroethanolic extract has phenolic compounds (mostly rosmarinic acid) and a significant antioxidant potential, which significantly increases the antioxidant capacity of yoğurt (Guemidi et al., 2024).

Table 5. *Evaluation of physicochemical properties and trace element content of Mentha piperita L.*

Plant material location	Extraction method	Antioxidant determination method	Analytical methods	Bioactive constituents	Trace elements	References

Seeds purchased from Amazon were grown and used.	extraction with boil water	Folin-Ciocalteu	Titration with EDTA for Ca, Iron cell test kit from Spectroquant for iron, AAS for Na, ICP for K, spectrometry for Cu, and dissolving, filtering and adding ammonic sulphide for Zn	phenolic content	Ca, K, Fe Na, Cu, Zn	(Afshiya, and Anil, 2024)
Purchased in 2023 from a herbal shop in Messina (Sicily, Italy).	extraction with acetonitrile, distilled water	Folin-Ciocalteu	UV-VIS spectrophotometer ICP-MS	polyphenols	K, Mg, Ca, Na, Fe, Mn, Zn, Cu, Cr, Mo, Co, Se, Al, B, Ba, Ni, As, Pb, Cd, Hg	(Turco et al., 2024)
Peppermint tea were purchased from a local market in Istanbul, Turkey in 2016.	extraction with pure water (infusion), microwave digestion		ICP-OES		Ca, Co, Cu, Fe, K, P, Na, Mg, Mn, Se, Zn	(Yalçın et al., 2024)
Green vegetables were purchased at fruit garden market, Rivers State, Nigeria.	soxhlet extraction		AAS Spectrophotometer LC-UV	fat, protein, vitamin	Mn, Fe, Cu, Zn, Co, Pb, Cd, Cr, Ni, Se	(Nwachoko et al., 2024)
M. piperita L. was harvested in Ouargla, southeastern Algeria.	Hydro-ethanolic maceration	DPPH, ABTS	LC-MS/MS	rosmarinic acid		(Guemidi et al., 2024)

3. CONCLUSION

Peppermint, one of the medicinal plants known as a source of antioxidants since ancient times and used as food in folk medicine and cuisine, is the subject of this review. For this aim, scientific publications evaluating the antioxidant effect, phenolic compound content and interaction with the elements found in the environments where it grows of extracts obtained from the medicinal plant *Mentha piperita* L. using various solvents (distilled water, ethanol, methanol, acetone etc.) and different extraction methods (maceration, infusion, hydro-distillation, soxhlet, ultrasound-assisted, microwave digestion, solid phase extraction) were reviewed. When the studies on the biomass, nutritional content (macro and micro), yield, chemical composition and antioxidant capacity of the peppermint plant are examined, it is understood from the

number of experimental studies that the interest of researchers in this plant has not decreased. It has been observed that parameters such as the soil in which the plant is grown (metal toxicity), fertilizers added to the soil to increase the yield of the plant (chemical-organic), and weather conditions (arid-semiarid) guide the experimental studies of the researchers. Because determining the safe levels of metals added to the soil to increase the phenolic compounds of the peppermint plant is crucial to obtaining suitable pharmaceutical products. The main goal of the research has been to determine the phenolic components and antioxidant activity in the extract of this plant used in the food, feed, pharmaceutical and cosmetic industries, and to reveal the important phytochemical properties found in this medicinal plant, which may have potential therapeutic applications due to its health-supporting components. As a result, due to the pharmacological importance of *Mentha piperita* L., experiments on the analysis of its phenolic compounds, essential oils, antioxidant activity, macro and micro elements will continue to be the subject of research.

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CHAPTER 12

KEFIR AND GUT FUNCTION

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Introduction:

Kefir is a fermented drink which owes its popularity to people from ancient times and has been on the menu for thousands of years. It keeps on as a healthy food (Bekatorou, 2019; Terpou, 2020). Milk kefir – a fermented milk product that has been traditionally prepared by adding kefir grain to milk. The kefir grain is a complex matrix of both proteins and exopolysaccharides that serve as a storehouse of various microorganisms which are responsible for the fermentation process (Chin-Wen et al., 1999). Kefir usually contains yeasts, acetic acid bacteria, and lactic acid bacteria which have various microbiological compositions (Table 1). Additionally, some of these microorganisms possess probiotic properties (Guzel-Seydim et al., 2021; Spizzirri et al., 2023). Numerous studies investigating the beneficial effects of milk kefir on host health have shown good results (Bourrie et al., 2016; Slattery et al., 2019).It has also been indicated that milk kefir can be consumed by lactose intolerant patients (Hertzler & Clancy, 2003).during experimentation in rodent models has shown that specific milk kefirs or key ingredients can help in wound healing, reduce cholesterol levels, affect the gut-brain axis, possess anti-cancerous Nevertheless, although in some situations the mechanisms for their alleged ones were not stated, they can still be considered crucial (Bourrie et al., 2018, 2021; Rodrigues et al., 2005; van de Wouw et al., 2020).

Kefir is commercially made using freeze-dried direct vat set DVS cultures to directly inoculate milk for processing. It contains a high number of microorganisms in the correct proportions: 80% of the composition consists of *lactococci*, while *lactobacilli* make up 10-15% and yeast make up 5-10% (Wszolek et al., 2007). the formula of the microorganisms in DVS starters should emulate that of kefir grains including yeast strains that may not produce large excess of carbon dioxide (Stepaniak & Fetliński, 2002). Using DVS cultures during starter preparation in the plant helps to avoid phage contamination and ensures the right strain balance (Surono & Hosono, 2011).

Gut microbiota is a complex group of microbes that reside in the gastrointestinal systems of people. The gut microbiota contains the largest number of microorganisms and has the highest number of species than all other parts of the body in humans (Quigley, 2013). The gut microbiota playes a lot of significant roles in our body and this include supporting protection against pathogens by colonizing the mucosal surfaces as well as creation of different antimicrobial substances and better perform of the immune system (Mills et al., 2019). In addition, microbiota can convert dietary elements into bioactive food compounds. These bacteria could transform indigestible carbohydrates like cellulose, hemicelluloses, resistant starch as well as pectin and oligosaccharides into short chain fatty acids such as acetic, propionic, and butyric acids (Lin & Zhang, 2017; Thursby & Juge, 2017). Kefir contains a large number of probiotics which can regulate the balance of intestinal

flora and strengthen the host's immune system via short-chain fatty acids, polypeptides, lactic acid, and various other metabolites as well as the bacterial antigens themselves. The microbiota of Kefir can cause the mucosal immunity of the body, and Kefir can keep the balance of intestinal homeostasis (Chen et al., 2024).

Table 1 *Species found in the microbiota of kefir and its grains*

Microbial Group	Species	References
Lactobacilli	*Lactobacillus casei, Lactobacillus acidophilus, Lactobacillus kefiri, Lactobacillus plantarum, Lactobacillus helveticus, Lactobacillus fermentum, Lactobacillus reuteri*	(Bengoa et al., 2019; Plessas et al., 2016)
Lactococci	*Lactococcus lactis, Lactococcus cremoris*	(Bengoa et al., 2019; Plessas et al., 2016)
Streptococci	*Streptococcus thermophilus (less common in kefir), Streptococcus lactis (uncommon)*	(Ding Fan et al., 2022; Plessas et al., 2016)
Acetic Acid Bacteria	*Acetobacter lovaniensis, Acetobacter xylinum*	(Bengoa et al., 2019; Plessas et al., 2016)
Other Bacteria	*Bifidobacterium bifidum, Bifidobacterium longum, Enterococcus faecium (limited presence), Pediococcus pentosaceus, Leuconostoc mesenteroides*	(Ding Fan et al., 2022; Plessas et al., 2016)
Yeast	*Kluyveromyces marxianus, Candida kefyr, Saccharomyces cerevisiae, Saccharomyces unisporus, Torulospora delbrueckii, Pichia fermentans, Kazachstania spp.* (e.g., *Kazachstania turicensis, Kazachstania unispora, Kazachstania exigua)*	(Bengoa et al., 2019; Ding Fan et al., 2022; Plessas et al., 2016)

Nutritional Value of Kefir

Kefir's nutritional values are result of an abundant chemical composition that has minerals, sugars, carbohydrates, proteins, peptides, vitamins and fats in it. Beyond its chemical constituents, it is in the fermentation process that we are further enriched nutritionally owing to secondary bioactive compounds like vanillin, catechin, salicylic acid, and ferulic acid (Bensmira & Jiang, 2015). Moreover, kefir is a rich source of vitamins B1, B2, B5 and C, a list of minerals and essential amino acids that are crucial for the fitness enhancement, faster healing process and homeostasis (Table 2) (Figure 1) (Sarkar, 2007).

Vitamins and Minerals

Kefir, a sour fermented dairy drink full of beneficial bacteria, offers a significant vitamin profile to improve health (Chong et al., 2023). Traditionally, kefir was produced by fermenting cow's milk with microbes, but new research suggests that it can also be made using milk obtained from goats, camels, buffalo, and sheep (Farag et al., 2020; Guzel-Seydim et al., 2021). The kefir product made from the different milk of key cattle may slightly differ nutritionally. Kefir is a source of vitamins of the B1, B2, B5, C, and B5 groups, and all these, together with amino acids and minerals, improve healing, fitness, and homeostasis. On the part of milk that is utilized as well as the microbial flora added in kefir productionsal has a significant impact on the vitamin content. The vitamin B12 is produced by *Propionibacterium pituitosum* and *Propionibacterium peterssoni*, while the former is *Propionibacterium Shermanii* and *Freudenreichii subsp.* it helped make more vitamin B6 (Arslan, 2015; Sarkar, 2007). Kefir additionally possesses vitamin A, vitamin K, and carotene (Otles & Cagindi, 2003). Kefir contains high levels of macro-elements like calcium, magnesium, potassium, and sodium, which help the body utilize carbohydrates, fats, and proteins for cell growth, maintenance, and energy. Kefir also has trace elements like iron, zinc, and copper, important for cellular metabolism and blood creation (Bakircioglu et al., 2018).

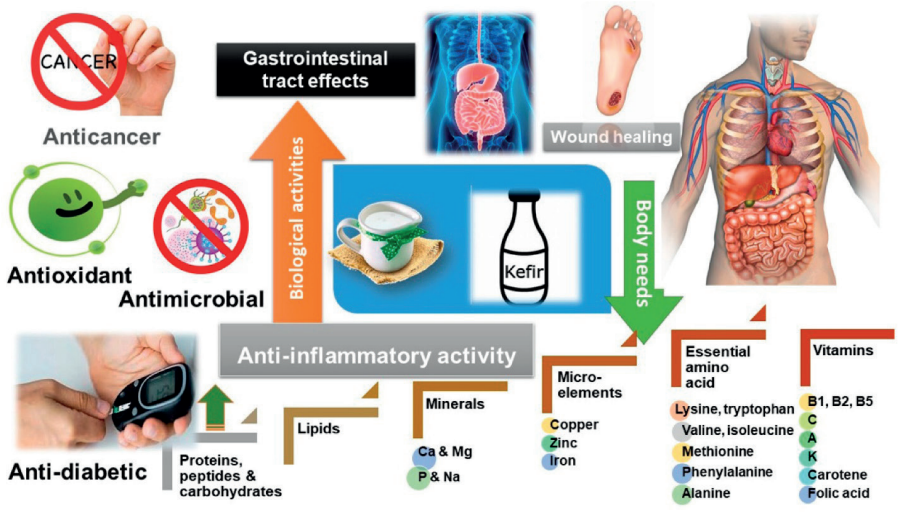

Figure 1 *The characteristics of kefir include its biological properties, nutritional value, as well as its macro- and micronutrient composition (Farag et al., 2020).*

Lactose

During fermentation, milk's lactose is broken down into acid, leading to a decrease in pH and a thickening of the consistency. Roughly 30% of the lactose

in milk is broken down by the enzyme β galactosidase, converting lactose to glucose and galactose. In addition, the bacteria found in kefir transform glucose into lactic acid(Egea et al., 2022). Because regular dairy milk contains a sugar known as lactose, numerous individuals are unable to properly digest it, leading to a condition called lactose intolerance (Deng et al., 2015). During the fermentation process of milk to make dairy products like kefir and kefir yogurt, the lactic acid bacteria turn the lactose sugar into lactic acid, reducing the lactose content in these foods compared to dairy milk. The fermented products also include enzymes which aid in further breaking down lactose. This is the reason why kefir is typically better digested by individuals with lactose intolerance when contrasted with regular unfermented milk. Hertzler and Clancy found that kefir enhances the digestion of lactose and increases its tolerance in adults with lactose maldigestion issues (Hertzler & Clancy, 2003). Kefir can be consumed right after removing the grains or can be stored in the refrigerator for future consumption. Throughout the cooling process, fermentation with alcohol causes the build-up of carbon dioxide, ethanol, and vitamin B complex (Farnworth, 2005; Santos JPV, 2008).This maturation process decreases the amount of lactose, making the product suitable for those with lactose intolerance and diabetes to consume (Farnworth, 2005).

Protein

Kefir contains a wide variety of amino acids viz; valine, isoleucine, methionine, serine, threonine, phenylalanine, tryptophan, alanine, and lysine that are essential for the functioning of the Central Nervous System (CNS). The kefir also has one of the most important protein that is effective in the absorption and digestion in the body since it has partially digested the protein of the casein (Simova et al., 2006). Kefir, a fermented dairy product, is rich in protein. It contains both casein and whey proteins, making it a complete protein source. The fermentation process enhances protein bioavailability, allowing for better absorption and utilization. Kefir's probiotic content also interacts with protein digestion, potentially benefiting gut health. Including kefir in your diet can contribute to meeting your protein requirements (Hertzler & Clancy, 2003; Kairey et al., 2023). Milk kefir is typically produced by fermenting milk with kefir grains, with a low-fat content (<10%), protein content of 2.7%, and lactic acid content of 0.6%. Milk is what determines this nutritional status (Prado et al., 2015).

Table 2 *Nutritional attributes of kefir*

Nutritional Attributes	Nutritional Components	Concentration	References
Macronutrients			
Carbohydrates	Total Carbohydrates	30-110 g/L	
	Sugars (including lactose)	20-60 g/L	
Protein	Total Protein	20-35 g/L	(Ding Fan et al., 2022; Prado et al., 2015)
Fat	Total Fat	6-30 g/L	
Micronutrients			
	Vitamin B1 (Thiamin)	20-40 mg/kg	(Brasiel et al., 2022; Prado et al., 2015)
	Vitamin B2 (Riboflavin)	40-120 mg/kg	
	Vitamin B12 (Cobalamin)	100-500 mcg/kg	(Brasiel et al., 2022; Ding Fan et al., 2022)
Vitamins	Vitamin K2 (Menaquinone)	Trace amounts	(Zhang et al., 2023)
	Macro-elements (%)		
	Calcium	0.4-1.2%	
	Magnesium	0.12-0.25%	
Minerals	Phosphorus	0.8-1.0%	
	Micro-elements (mg/kg)		
	Sodium	40-200	
	Potassium	800-1200	
	Iron	2-10	
	Zinc	20-40	(Ding Fan et al., 2022; Prado et al., 2015)
	Copper	1-2	
Minerals	Manganese	0.5-2	

Kefir and Health

Adding probiotic microorganisms to dairy products like milk, yoghurt, cheese, and buttermilk has been shown to be beneficial for producers in terms of texture and technological advancements, as well as offering health benefits through its bioactive components for the functional foods industry (Chand et al., 2021; Sharma et al., 2021). Kefir has been connected with promoting good health for a long time due to its ability to decrease and prevent the onset of diseases in individuals. Kefir's health benefits have made it a focus of research in recent decades due to its mixture of bacteria including lactic and acetic acid bacteria, yeast, all enclosed in kefiran, a polysaccharide matrix (Prado et al., 2015). Similar to milk, it also contains various bioactive peptides that

contribute to its antihypertensive, immune-modulating, antimicrobial, and antioxidative effects (Amorim et al., 2019; Guha et al., 2021). Nevertheless, it is reported that the microbiota in kefir not only boosts its health benefits, but the addition of certain metabolic products like organic acids, carbon dioxide, hydrogen peroxide, exopolysaccharides, and bacteriocins may provide it with an advantage compared to other fermented dairy products (Ismaiel et al., 2011). Kefir is recognized for its positive impacts on human health because of its two primary components: a solid bacterial fraction with probiotic properties and a soluble non-bacterial fraction with antimicrobial effects (Brasil et al., 2018). It has been investigated as a possible matrix for adding health-promoting bacteria and as an effective matrix for delivering probiotic organisms. Therefore, due to its ingredients and numerous health advantages, kefir is known as a "natural source of probiotics" (Nalbantoglu et al., 2014).

Change in The Gut Microbiome

The elements of gut microbiota, including archaea, fungi, bacteria, and other microorganisms (Dominguez-Bello et al., 2019), can exist in either a balanced state or an imbalanced state. In the initial scenario, referred to as eubiosis, the microbiota can handle slight alterations caused by factors like the surroundings, diet, or water intake, showing adaptability in preserving its equilibrium. Major changes like translocation or growth of specific bacterial groups, colonization by pathogenic bacteria, use of antibiotics, and changes in lifestyle can result in imbalance known as dysbiosis. The functionality of different organs like the brain, liver, pancreas, intestine, and heart is influenced by intestinal microbiota, regardless of balance (Iebba V et al., 2016; Weiss & Hennet, 2017). Moreover, the gut microbiota plays a role in the growth and maturation of organs and physiological functions (Iebba V et al., 2016), indicating that controlling the gut microbiota could be crucial for managing illnesses and promoting overall well-being (Peluzio, Dias, et al., 2021).

During a long-term research project on obese patients having bariatric surgery, weight loss and metabolic enhancements were achieved by changing the composition of the gut bacteria. Patients who had bariatric surgery showed differences in the *Firmicutes, Fusobacteria,* and *Verrucomicrobia phyla* compared to those with normal weight. Furthermore, *Akkermansia muciniphila* was found to be present following bariatric surgery. It is a species linked with lipid metabolism, which is a positive association, and not linked with inflammation in adipose tissue or high levels of glucose, insulin, leptin, and triglycerides in circulation. This indicates that its presence signifies an enhancement in the presence of markers for healthy metabolism (Palmisano et al., 2020).

During a study examining kefir's impact on mice's intestinal microbiota, researchers found that while total bacteria numbers remained unchanged, the

group consuming kefir experienced decreased *Enterobacteriaceae* and higher levels of *Lactobacillus* and *Lactococcus* over 3 weeks. Additionally, there was a decrease in Firmicutes and Proteobacteria and an increase in Bacteroidetes, Lactobacillus, and Lactococcus by the study's conclusion. Conversely, there was a notable rise in fecal yeast levels following the consumption of kefir (Kim et al., 2015).

The results show that kefir enhanced the gut microbiome of mice who ingested it, particularly by decreasing Enterobacteriaceae. This family is seen as harmful, often disrupted in conditions of behavioral and metabolic alterations—like eating high-fat, low-fiber diets, with aging, and during inflammation (Kim et al., 2015).highlighting that these dietary habits and inflammation are common in obesity (Bortolin et al., 2018; Cani et al., 2007; Fåk et al., 2015).

In humans, changes in intestinal microbiota also happen following kefir intake, as studied by (Bellikci-Koyu et al., 2019) in individuals with metabolic syndrome given kefir for 12 weeks. Following the treatment, there was a notable rise in Actinobacteria, along with alterations in the genera of the Bacteriodetes and Firmicutes phyla, among the kefir-consuming group (Bellikci-Koyu et al., 2019).

Kefir and Obesity

Obesity and being overweight, characterized by an excess of body fat leading to increased health risks, can also be influenced by the gut microbiome (Schetz et al., 2019). Kefir might aid in the fight against obesity by blocking enzymes that play a role in breaking down carbohydrates and lipids, resulting in a decrease in energy production (Tiss et al., 2020). In a study by Fathi et al., (2016) Women aged 25-45 with obesity received two kefir servings daily over 8 weeks. Results showed a significant reduction in serum levels and ratios of various lipoproteins, including total cholesterol (TC), low-density lipoprotein (LDL), non-high-density lipoprotein (non-HDL), and the ratios of TC/HDL and LDL/HDL. This intervention shows promise in enhancing lipid profiles and lowering cardiovascular disease risk in obese women. And in another study done by Seo et al., (2022) showed that supplementation with surface layer protein (SLP) and exopolysaccharides (EPS) from postbiotic lactic acid bacteria (PLAB) and prebiotic wine grape seed flour (GSF) effectively improved metabolic parameters and reduced obesity in mice fed a high-fat diet. The combined supplementation led to decreased body weight gain, adipose tissue weight, serum triglyceride levels, and insulin resistance, with the most significant effects seen in the group receiving all supplements. Changes in gut microbiota composition and adipocyte gene expression profiles were linked to these improvements, suggesting a potential role in preventing obesity and related diseases.

Kefir and Diabetes Mellitus

Chronic low-grade inflammation has been associated with the development of diabetes mellitus. When the balance of the intestinal microbiota is disrupted, it can increase intestinal permeability, allowing unwanted substances to enter the bloodstream and trigger an inflammatory response. This inflammation can lead to insulin resistance, which may ultimately progress to diabetes (Milani et al., 2017). In a study by Ostadrahimi et al., (2015) Adults aged 35 to 65 with type 2 diabetes consumed 600ml of probiotic kefir daily for eight weeks. The kefir contained *Lactobacillus casei, Lactobacillus acidophilus,* and *Bifidobacteria*. Results showed decreased HbA1C levels, improving long-term blood glucose control. Kefir with these probiotics may help manage diabetes as a dietary supplement. Another research conducted by Laela et al., (2021) explored the use of kefir and spirulina to combat the effects of diabetes in rats. Rats were divided into different treatment groups and their glucose levels and antioxidant activity were measured before and after a 28-day intervention. Rats that received kefir and spirulina showed lower glucose levels and higher antioxidant activity compared to control rats. The findings suggest that the combination of kefir and spirulina can offer nutritional and antioxidant benefits, helping to manage blood sugar levels and improve antioxidant status in diabetic rats.

Kefir and Liver disease

Toxins produced by gut bacteria and metabolic endotoxemia, caused by changes in intestinal permeability, contribute to mild, ongoing inflammation. This inflammation activates toll-like receptors and macrophages, which in turn results in liver and systemic inflammation, highlighting the link between gut microbiota and the onset of liver disease (Cani et al., 2012). A recent study done by Cui et al., (2024) investigated the effects of kefir supplementation on alcoholic liver disease (ALD), one of the leading causes of liver-related deaths. In a mouse model of Alcohol Liver Disease, C57BL/6J mice fed an alcohol diet were given oral kefir. Kefir improved liver health by lowering enzyme levels, decreasing inflammation, restoring the intestinal barrier, altering gut microbiota to increase beneficial bacteria, and adjusting bile acid profiles. These results suggest kefir may help alleviate alcohol-induced liver damage by promoting gut health. Another study by Santos et al., (2023) examined the impact of kefir supplementation on malnourished mice's metabolic outcomes and liver health. Mice were split into groups and put through a malnutrition and renutrition regimen. Those given kefir showed improved body weight, biochemical markers, and adipocyte size recovery. Gene analysis indicated a rise in antioxidant gene expression, hinting at reduced oxidative stress. Kefir supplementation aided in body weight gain, metabolism enhancement, and liver inflammation reduction.

Kefir and Cardiovascular Disease

Cardiovascular diseases are also linked to obesity's intestinal dysbiosis (Zhuang et al., 2019). In addition, changes in the intestinal microbiota can lead to the production of compounds such as trimethylamine N-oxide, which increase the risk of developing cardiovascular diseases (Peluzio, Martinez, et al., 2021). A clinical trial investigated by (Ghizi et al., 2021) the impact of kefir on individuals with metabolic syndrome. Forty-eight participants were split into a kefir group and a control group for 12 weeks. Results revealed that kefir improved health markers, such as blood pressure, glycemia, cholesterol levels, and reduced the risk of cardiovascular events over ten years. The study suggests that adding kefir to one's diet may benefit those with metabolic syndrome. Another study by Silva-Cutini et al., (2019) examined the effects of long-term kefir treatment on cardiac function and sympathetic signaling in spontaneously hypertensive rats (SHR). Kefir-treated SHR had lower mean arterial pressure and heart rate compared to untreated SHR. Kefir treatment also reduced cardiac hypertrophy and improved contractile protein expression in the left ventricle. Furthermore, it decreased tyrosine hydroxylase protein overexpression in central nervous system (CNS) regions involved in sympathetic regulation. These results indicate that kefir treatment lowers blood pressure by enhancing cardiac function and modulating sympathetic signaling in the CNS.

Kefir and Immunity

Development in babies depends on the establishment of intestinal microbiota during childhood, which plays a vital role in the immune system. Premature infants may possess underdeveloped immune, respiratory, and neurological systems, suggesting a potential link among them (Milani et al., 2017). The immune system collaborates with gut bacteria to uphold a harmonious, non-inflammatory condition. If the balance of gut bacteria is disturbed, it can cause the immune system to become too active, which may result in weakened immunity or autoimmune conditions such as type 1 diabetes (Weiss & Hennet, 2017). In study done by Tseng et al., (2023) three Lactobacillus plantarum strains from kefir were fed to white shrimp to assess their effects on immunity, gene expression, and disease resistance against *Vibrio alginolyticus*. Results indicated that certain strains, notably MRS18, improved immune responses, gene expression, and survival rates, suggesting potential benefits for aquaculture and human consumption. In other study done by Mansour et al., (2024) on carpet shell clams infected with *Vibrio alginolyticus*, those given a diet with 10% dried kefir showed a 100% survival rate after exposure to the pathogen. These clams also had higher weight and antioxidant responses, along with improved immune parameters such as phenoloxidase and lysozyme activity. Overall, the kefir-supplemented diet enhanced the clams' ability to fight off infection, suggesting that kefir could

be beneficial in boosting both antioxidant and immune responses in infected shellfish.

Future trends and perspectives on gut

For a long time, it has been thought that eating yogurt and other fermented dairy products offers multiple health advantages. Recent research on gut-related illnesses provides evidence supporting certain health benefits associated with these conditions (Adolfsson et al., 2004). Recent studies have highlighted the beneficial impact of kefir on gut health and its potential future applications. Kefir, a fermented milk product containing a symbiotic mix of bacteria and yeast, has been shown to influence the gut microbiota positively. Research indicates that kefir can enhance gut microbiome diversity, modulate immune responses, and improve gastrointestinal health by increasing beneficial bacterial populations and reducing pathogenic bacteria (Dahiya & Nigam, 2023; Gupta et al., 2024). For instance, a study demonstrated that kefir administration in critically ill adults was feasible and safe, showing potential improvements in gut microbiota composition and reduced inflammation (Gupta et al., 2024). Another study underscored kefir's ability to impact gut-brain communication, influencing behavior and immune responses in mice, which suggests broader therapeutic possibilities (van de Wouw et al., 2020). These findings support kefir's role as a valuable dietary supplement for maintaining and improving gut health, with future research likely to explore its application in personalized nutrition and medicine.

In the commercial market, there is a wide range of kefir products available, such as traditional fermented milk beverages, alternatives to dairy, and creative options like kefir cheese and powdered starter cultures. Emerging methods such as spray drying are being used to tackle challenges in commercial production, such as shelf life, storage, and packaging costs. Future possibilities include investigating new mediums for fermentation, like fruit juices and plant-based substitutes. Furthermore, adding probiotics, vitamins, and minerals to kefir products can improve their functional characteristics, meeting the needs of consumers' gut health and dietary variety. Further research and innovation will continue to reveal the potential of kefir as a functional food with various health advantages and culinary flexibility (Manjunatha et al., 2024).

Future studies on kefir should prioritize long-term clinical trials to validate its health benefits across diverse populations and varying health conditions. Researchers should investigate the specific mechanisms through which kefir modulates gut microbiota and its subsequent impact on metabolic and inflammatory pathways. Exploring optimal dosages, formulations, and the potential synergistic effects of kefir with other dietary components can enhance its therapeutic efficacy. Additionally, molecular studies focusing

on the interactions between kefir's microbiota and the host's immune and metabolic systems could provide deeper insights into its role in disease prevention and health promotion. Understanding these factors will be crucial for developing targeted nutritional interventions and personalized dietary recommendations.

Conclusion

kefir emerges as a potent natural source of probiotics with multifaceted health benefits. Its rich nutritional composition, diverse microbiota, and fermentation process contribute to its therapeutic properties, making it a promising dietary intervention for various health conditions. From its positive effects on gut microbiota modulation to its potential implications in managing obesity, diabetes mellitus, liver disease, cardiovascular disease, and immunity, kefir showcases its versatility in promoting overall health and well-being.

Studies have demonstrated kefir's ability to improve lipid profiles, blood glucose control, inflammatory markers, and gut microbiota composition. Moreover, its impact on conditions like obesity, diabetes mellitus, liver disease, and cardiovascular disease underscores its potential as a preventive and therapeutic agent. By influencing the gut microbiome, kefir contributes to better digestion, absorption of nutrients, and immune function, further enhancing its health-promoting properties.

As research continues to unveil the mechanisms underlying kefir's beneficial effects, it is poised to play a significant role in personalized nutrition and medicine. Future trends suggest expanding the exploration of kefir's applications, including its potential in gut-brain communication and its use in critically ill patients. Despite the growing body of research on fermented foods, there remains a necessity for additional clinical trials to comprehensively grasp their implications on diverse disorders. Subsequent investigations should prioritize extended intervention durations and the meticulous monitoring of gut microbiota to ascertain the enduring influence on human health.

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