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Review Article

A Compherensive Review of the Anti-Obesity Properties of Medicinal Plants

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Abstract

Obesity has emerged as a global health crisis, contributing to numerous comorbidities including diabetes, cardiovascular diseases, and chronic inflammation. Although lifestyle modifications are recommended to prevent obesity, the increase of obesity in last years has revealed the importance of drug research. While bariatric surgery remains an effective approach, the limited number of approved pharmacotherapies for obesity necessitates further investigation into novel therapeutic agents. Currently, there has been growing interest in exploring the potential of plant extracts in the treatment of obesity. Phytoextracts, derived from a variety of botanical sources, have attracted considerable attention due to their therapeutic properties and perceived lower risk profile compared to synthetic pharmaceuticals. These extracts often contain bioactive compounds such as polyphenols, flavonoids, and alkaloids, which have been extensively studied for their anti-obesity effects. Research indicates that certain plant extracts can modulate weight control by influencing metabolic processes, overall health, and lipid metabolism. The integration of herbal extracts into obesity treatment regimens offers a holistic approach to health, presenting a natural alternative to conventional medicine. Moreover, plant extracts often exhibit pleiotropic effects, targeting multiple pathways involved in the pathogenesis and progression of obesity. This multifaceted mechanism holds promise for enhancing clinical outcomes while minimizing the risks associated with monotherapy. However, despite the evident potential, further research is essential to elucidate the precise mechanisms of action, optimize dosage regimens, and evaluate the long-term safety and efficacy of these interventions.

Keywords: Obesity, pharmacological interventions, plant extracts, metabolic processes, weight management

1. Introduction

Obesity is recognized as a global health issue and a condition that can lead to serious health implications. Epidemiological, etiological, and therapeutic research conducted in this field contributes to a deeper understanding of obesity. Various strategies are employed in the treatment of obesity, including pharmacological methods, bariatric surgery, and lifestyle interventions. Pharmacological treatments for obesity include medications such as Orlistat, which reduces fat absorption, and Liraglutide, which suppresses appetite, both of which may support weight loss (1, 2).

Various investigations have demonstrated that obesity is a global health problem associated with serious health consequences and its rapid spread has been well-documented (3, 4). It remains the most prevalent metabolic disease worldwide, driven by lifestyle changes characterized by reduced physical activity and altered dietary patterns. The escalating trend of obesity, affecting both adults and children, is closely correlated with a surge in non-communicable diseases such as type 2 diabetes, dyslipidaemia, cardiovascular ailments, hypertension, and stroke (5). Obesity's pervasive impact extends to various bodily organs, notably central obesity's influence on respiratory disorders like obesity-hypoventilation syndrome and obstructive sleep apnoea syndrome. Efforts to comprehend obesity's roots and devise effective treatments are imperative, alongside the implementation of preventive measures to limit its rising prevalence. Failure to address this issue will exacerbate its global spread, posing significant public health and economic challenges (3, 5).

Obesity, a prevalent and complex condition in the developing world, significantly deteriorates health-related quality of life. Individuals grappling with obesity experience detrimental effects on both physical and psychosocial functioning due to weight-related issues. Besides adversely impacting morbidity and mortality rates, obesity compromises the ability of affected individuals to lead full and active lives. The negative impact of obesity intensifies with higher degrees of obesity, exacerbating public health concerns, particularly with the rising prevalence of associated diseases such as diabetes. Interventions aimed at reducing weight have demonstrated potential to improve health-related quality of life (6).

Obesity, acknowledged as a global epidemic fuelled by the consumption of energy-dense yet nutrient-poor foods and reduced physical activity, remains a significant medical challenge. Despite lifestyle modifications being a primary management approach, demand for more effective treatments like gastrointestinal surgery has increased, albeit their limited use and associated risks. Currently, only orlistat and sibutramine are approved medications for obesity, yet both exhibit limited efficacy and restrictive side effects (1,7). The withdrawal of sibutramine underscores the urgent need for safer and more efficient anti-obesity drugs. While promising, most developed medications have faced approval hurdles or market withdrawal due to adverse effects (8). Notably, orlistat stands as the sole long-term approved drug, highlighting the critical necessity for further effective medication options. Furthermore, studies should carefully evaluate the long-term safety and effectiveness of recently produced medications, taking into account the fact that low-calorie diets are frequently used in clinical trials, which may have an impact on real-world weight reduction results (7,8).

The rising prevalence of obesity worldwide has sparked significant interest from a multitude of disciplines, driving researchers to investigate and synthesize modern approaches to tackle this complex health issue. Obesity, characterized by excessive body fat accumulation, is associated with significant health risks and imposes multiple socioeconomic burdens on both the individual and society. Given these challenges, it is crucial to consolidate the current approaches and methodologies for effectively addressing the obesity epidemic. This discussion aims to synthesize and provide an overview of the varied perspectives and methodologies represented in contemporary obesity research and intervention. By doing so, it will facilitate the ongoing dialogue on effective strategies for obesity management and prevention.

2. Diagnosis of Obesity

A chronic illness brought on by excess body fat is obesity. Ideally, body fat percentage should be considered when defining obesity. However, establishing a population based on body fat measurement is very impossible due to the complex technology needed to assess body fat. Consequently, there isn't always a set amount for body fat (Fig 1). In practical terms, obesity is therefore determined by body mass index (BMI), which takes height and weight into account: Weight (kg) / height (m^2) = BMI. BMI has a beneficial effect on disease and is correlated with total body fat. Different definitions of obesity have been created by the World Health Organization (WHO) based on the link between obesity and mortality and BMI (3). In Fig 1, obesity indexes are given in the BMI chart prepared in the BioRender program (9).

Moreover, changes in the nature of work, with many jobs requiring less physical exertion, along with urbanization, which may limit opportunities for physical activity and exacerbate the problem. Environmental and social shifts associated with development frequently lead to alterations in dietary patterns and levels of physical exercise. Furthermore, the lack of government support in critical areas such as business, education, urban planning, transportation, agriculture, health, and food processing and distribution further compounds these challenges. It is important to recognize that obesity is a multifaceted issue with numerous contributing factors, many of which interact and reinforce one another (11).The factors of obesity are given in Table 1. People must reevaluate their lifestyles, establish healthy eating habits, and get regular exercise in order to prevent and manage obesity.

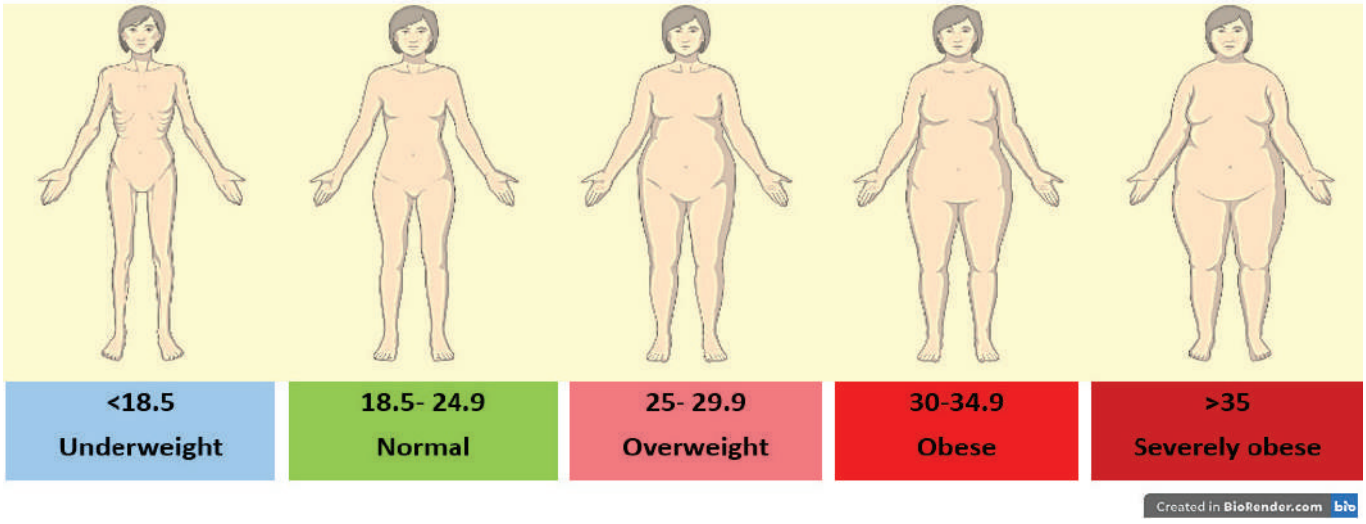


Figure 1. Body Mass Index (BMI) chart

Treatment for obesity should be seen as a way of life rather than as a quick "cure" for obesity. The American College of Physicians (ACP) advises that behavioural and lifestyle interventions, such as regular exercise, fitness, and good nutrition, be a part of fat and obesity control programs (10).

3. Pathophysiology of Obesity

The World Health Organization asserts that the fundamental cause of obesity and overweight is an imbalance between calorie intake and expenditure. This imbalance can be attributed to various factors worldwide, including increased consumption of sugar and energy-rich food, which often lead to reduced physical activity levels (WHO, 2021).

These variables differ from one person to another and frequently the factors above can combine (12). It may appear simple to attribute weight gain and obesity solely to long-term energy imbalance, however the pathophysiology of obesity is considerably more complex (Fig 2). Interactions among genetic, environmental, and psychological factors intricately shape food intake and energy expenditure. Although environmental and economic factors predominantly influence behavior and cannot be directly addressed at the molecular level, identifying genes and molecules associatedwith obesity reveals the underlying voluntary pathophysiological mechanisms at the molecular level (13).

Table 1. Determinants of obesity

Sedentary Lifestyle	• Insufficient physical activity can disrupt energy balance and lead to weight gain. Prolonged hours of sitting and irregular exercise habits contribute to obesity.
Psychological Factors	• Emotional eating, stress, depression, and other psychological conditions may drive some individuals to overeat, triggering weight gain.
Genetic Factors	• Obesity may arise as a result of genetic predisposition; individuals with a familial history of obesity are at an increased risk of developing the condition themselves.
Metabolic and Hormonal Disorders	• Metabolic and hormonal imbalances such as thyroid issues, insulin resistance, and hormonal fluctuations can affect weight control.
Alcohol Consumption	• Alcohol is associated with high-calorie beverages and excessive consumption can lead to weight gain.
Pregnancy	• Pregnancy can cause weight gain in women, and losing weight postpartum can be challenging.

4. Mechanism of Obesity and Endochronic Causes

The consumption of various energy-yielding macronutrients, including carbohydrates, proteins, and lipids, elicits undeniable metabolic effects contributing to the initiation of obesity (Fig 3). The relationship between macronutrient intake and the incidence of obesity involves complex physiological mechanisms regulating appetite, thermogenesis, and metabolic pathways, all intricately intertwined with an individual's genetic predisposition and the composition of their microbiota. The intricate interplay between an individual's genetic makeup and microbiota composition, coupled with their dietary behaviours and macronutrient intake, serves to elucidate individualized responses to shifting macronutrient compositions and dietary regimens. These nuanced revelations bear substantial potential in guiding the formulation of tailored precision nutrition interventions and individualized strategies aimed at mitigating the burden of obesity (14).

The convergence of central neurotransmitters and peripheral metabolic cues is widely acknowledged for its pivotal role in regulating energy balance, with profound implications for modulating feeding patterns and, consequently, impacting the development of obesity. Among the principal peripheral hormones implicated in the maintenance of energy equilibrium are leptin, ghrelin, insulin, peptide YY (PYY 3-36), and cholecystokinin (CCK) (15).

Globally, the escalating prevalence of obesity emerges as a pressing and imperative public health dilemma. While unhealthy dietary patterns and sedentary behaviours stand as primary instigators of the obesity epidemic, emerging research underscores the potential contribution of environmental chemicals to the etiologic of this condition. A substantial body of evidence indicates that certain endocrine-disrupting chemicals (EDCs) possess the ability to interfere with hormonally regulated metabolic pathways, particularly during critical developmental periods. Termed as "obesogens," these agents have the capacity to induce weight gain in susceptible individuals, even amidst efforts to restrict calorie consumption and enhance physical activity levels (16).

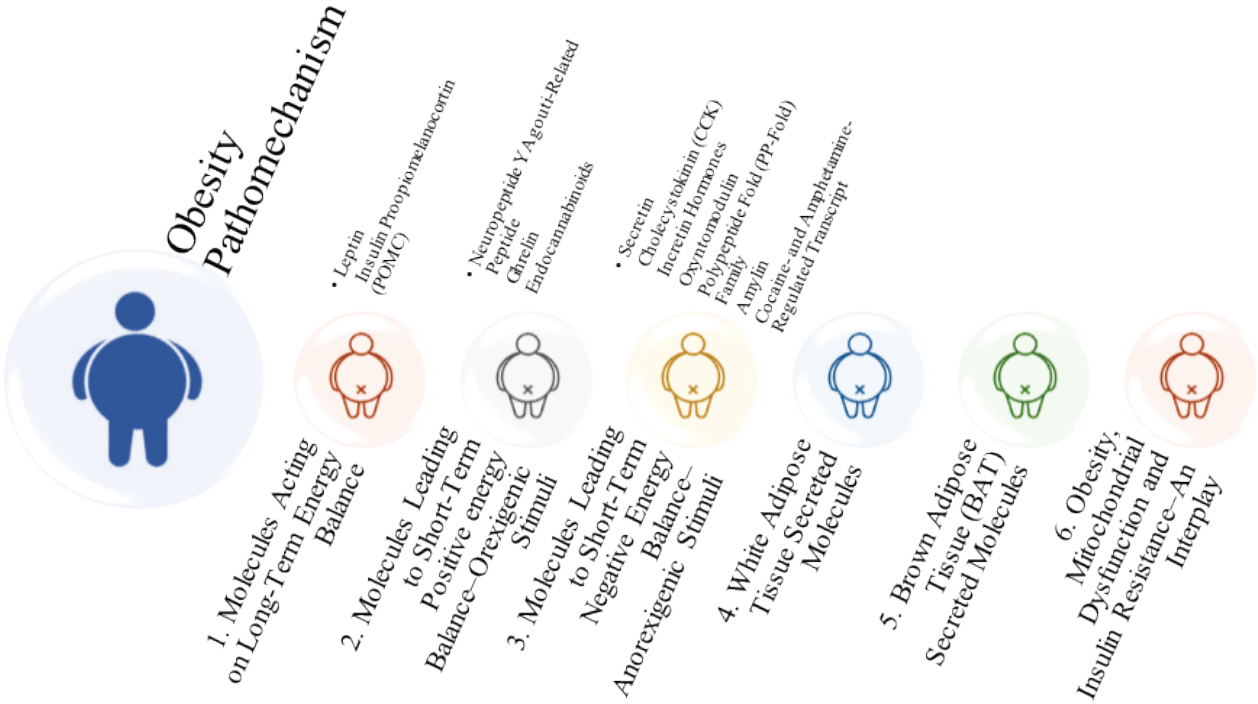


Figure 2. Obesity Pathomechanism (13)

4.1. Leptin

Leptin, a pivotal hormone in body weight regulation and adipose tissue management, exhibits a robust correlation with body fat composition in human, with serum leptin levels escalating proportionately in individuals with obesity (17). Prolonged activation of the leptin receptor can precipitate leptin resistance, either through hindering leptin signalling pathways or triggering hypothalamic proinflammatory responses due to heightened levels of saturated fatty acids, thereby instigating negative feedback mechanisms. Despite the multifaceted signalling initiated by the long form of the leptin receptor (Ob-Rb), involving over seven distinct pathways, the activity of suppressor of cytokine signaling-3 (SOCS-3) emerges as a plausible mechanism underlying leptin resistance observed in human obesity. Given the intricate integration of leptin-sensitive metabolic pathways with neuronal networks governing energy homeostasis, interventions aimed ameliorating leptin resistance demonstrate limited efficacy (18). Over the past two decades, genetic inquiries into both rare and common forms of obesity have unveiled two fundamental insights: firstly, the leptin-melanocortin pathway constitutes a pivotal circuitry in appetite regulation, and secondly, genes predominantly or exclusively expressed in the brain and central nervous system (CNS) assume a pivotal role in the ethology of obesity (19).

4.2. Adiponectin

In addition to its role in energy metabolism regulation, adipose tissue serves as a significant source of biologically active molecules known as "adipokines," which modulate diverse physiological processes. In the context of obesity, the aberrant production of adipokines stemming from excessive fat accumulation can precipitate the onset of obesity-associated ailments. Despite advancements in therapeutic strategies, extant treatments may encounter limitations, as the expansion of adipose tissue elicits oxidative stress and inflammatory cascades. Disrupted cytokine and adipokine secretion plays a critical role in the pathophysiology of issues such metabolic syndrome, cardiovascular diseases, respiratory disorders, diabetic retinopathy, and cancer. Pharmaceuticals such as pioglitazone and rosiglitazone have shown promise in the treatment of problems associated with obesity since their ability to effectively stimulate the expression of adiponectin (20).

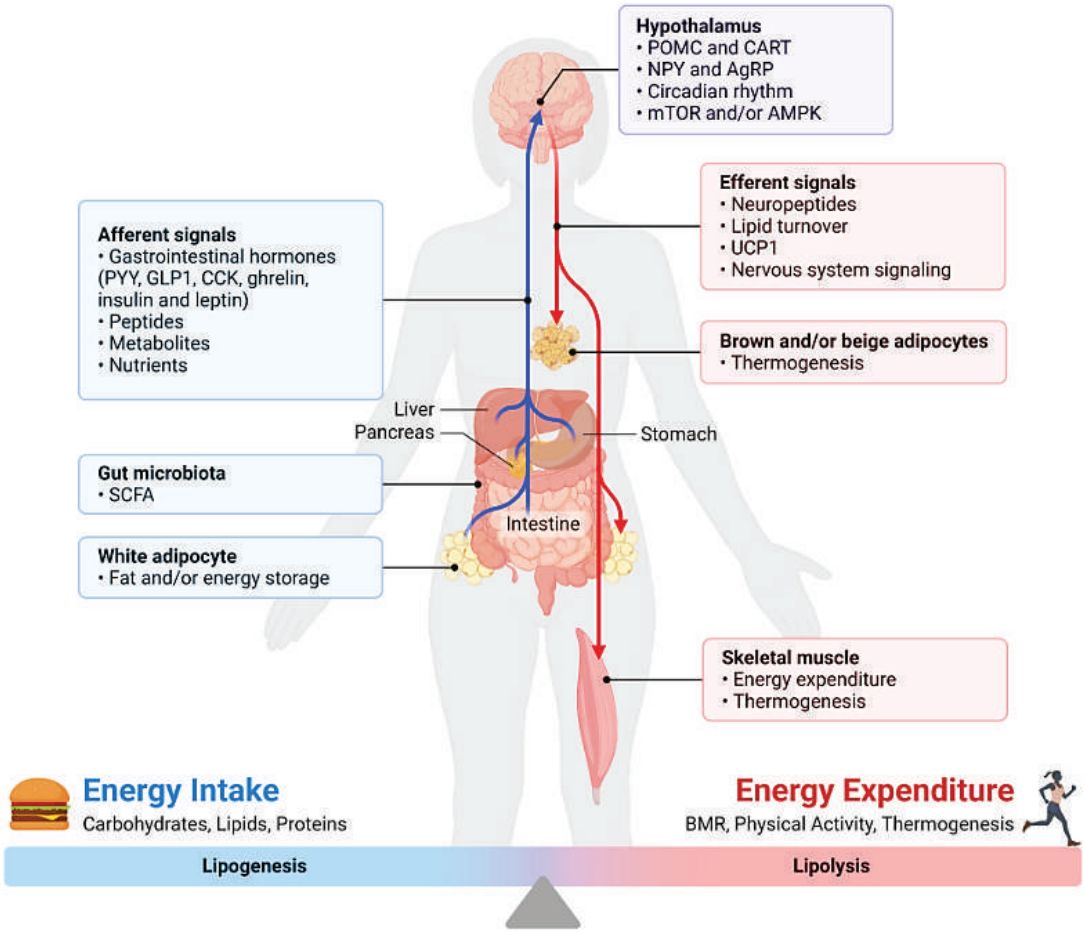


Figure 3. Key metabolic mechanisms of body weight regulation (14).

4.3. Vaspin

Vaspin, classified as a serine protease inhibitor and belonging to the serpin A12 family, was initially identified through research utilizing the Otsuka Long-Evans Tokushima fatty (OLETF) rat model, renowned for exhibiting symptoms of type 2 diabetes and obesity. Initially characterized as an adipokine predominantly secreted from visceral adipose tissue, subsequent investigations involving human subjects have corroborated this finding, highlighting a positive correlation between elevated circulating levels of vaspin and the presence of type 2 diabetes, obesity, and insulin resistance. The expression of vaspin is not limited to adipose tissue but extends to various organs within the human body, including stomach, liver, pancreas, and hypothalamus. Notably, despite being typically undetectable in visceral and subcutaneous adipose tissue among individuals with a lean body mass, vaspin mRNA expression increases with that mass. These findings underscore the potential significance of vaspin, particularly within visceral adipose tissue, elucidating its implications in the metabolic dysregulation observed in obesity (21).

4.4. Visfatin

Visfatin, a 52 kDa protein, is encoded by the PBEF/Visfatin gene situated on chromosome 7q22.2. This gene spans a length of 34.7 kb, encompassing 11 exons and 10 introns, primarily synthesized and secreted by immune cells, particularly macrophages. Visfatin's properties hint at its potential pivotal role in obesity and its related comorbidities. Its production can be attributed to adipocytes within visceral fat deposits or to macrophages dwelling in white adipose tissue, a process accentuated during obesity development. The theory emphasizing the substantial contribution of macrophages to Visfatin synthesis aligns with observed variations in Visfatin production across different adipose tissue types.

While mRNA expression levels in internal organs and subcutaneous fat tissue exhibit near uniformity in non-obese individuals, discrepancies surface among obese subjects. Specifically, visceral fat emerges as a prominent Visfatin source, whereas Visfatin production in subcutaneous tissue either diminishes or remains unaltered in the obese population (22).

5. Obesity and Health-Related Quality of Life

Overweight and obesity represent significant public health challenges due to their association with a wide array of severe medical conditions. These include various cancers such as breast, endometrial, ovarian, colorectal, oesophageal, kidney, pancreatic, and prostate cancers, among others. Additionally, conditions such as Type 2 diabetes, hypertension, stroke, Coronary Artery Disease, Congestive Heart Failure, asthma, chronic back pain, osteoarthritis, pulmonary embolism, gallbladder disease, and an elevated risk of disability are prevalent among individuals with overweight and obesity. The cumulative impact of these conditions results in over three million deaths annually. Moreover, early morbidity and mortality stemming from obesity and overweight during childhood and adolescence consistently correlate with an augmented risk of cardio-metabolic morbidity. The health repercussions of obesity and overweight pose a considerable burden, exerting a profound impact on future healthcare expenditures. A robust correlation exists between chronic medical conditions precipitated by obesity, diminished quality of life, and escalated healthcare and medication costs. Consequently, the healthcare expenses associated with health complications linked to obesity present a formidable challenge for individuals and healthcare systems alike. Thus, the implementation of effective measures to combat obesity and the cultivation of an enlightened society are imperative endeavours to ameliorate overall public health and address the dire consequences associated with this pervasive issue (23). At its very essence, obesity is a systemic condition that impacts much more than just physical appearance; rather, it extends to internal organs and metabolic activity.

Fig 4 represents the complex and multilayered effects of obesity on the human body. This graphic representation clarifies the interactive relation between obesity and some of the physiological systems and thereby provide insight into the profound ramification of excessive adiposity on general health. The figure also shows adverse effects on organ systems such as the respiratory system, with effects like obstructive sleep apnea; musculoskeletal system, with effects such as osteoarthritis; and the reproductive system, as seen with polycystic ovarian disease. Moreover, psychological and social consequences such as depression, stigma, and quality of life impairment are shown to highlight the holistic impact of this condition.

The World Health Organization has linked a number of serious non-communicable diseases to a higher BMI. Heart disease and stroke are indeed the leading causes of mortality among individuals with obesity, and elevated BMI serves as a significant risk factor for the development of these conditions. Similarly, high BMI is associated with diabetes, osteoarthritis, and other musculoskeletal conditions, as well as many types of cancer. The risk of developing these diseases escalates with higher BMI values. Furthermore, childhood obesity not only predisposes individuals to obesity later in life but also amplifies the risk of premature mortality and disability. Obese children may encounter a multitude of health complications, including respiratory difficulties, heightened susceptibility to fractures, hypertension, early indications of cardiovascular diseases, insulin resistance, and psychological ramifications (11).

Obesity is a serious health concern that goes beyond its individual status since it can increase the chance of developing a number of diseases by causing metabolic and cardiovascular problems. In this regard, it has been noted that obesity raises the chance of contracting a number of illnesses, including dyslipidemia, arterial hypertension, hyperuricemia, type 2 diabetes, and cardiovascular disorders (24).

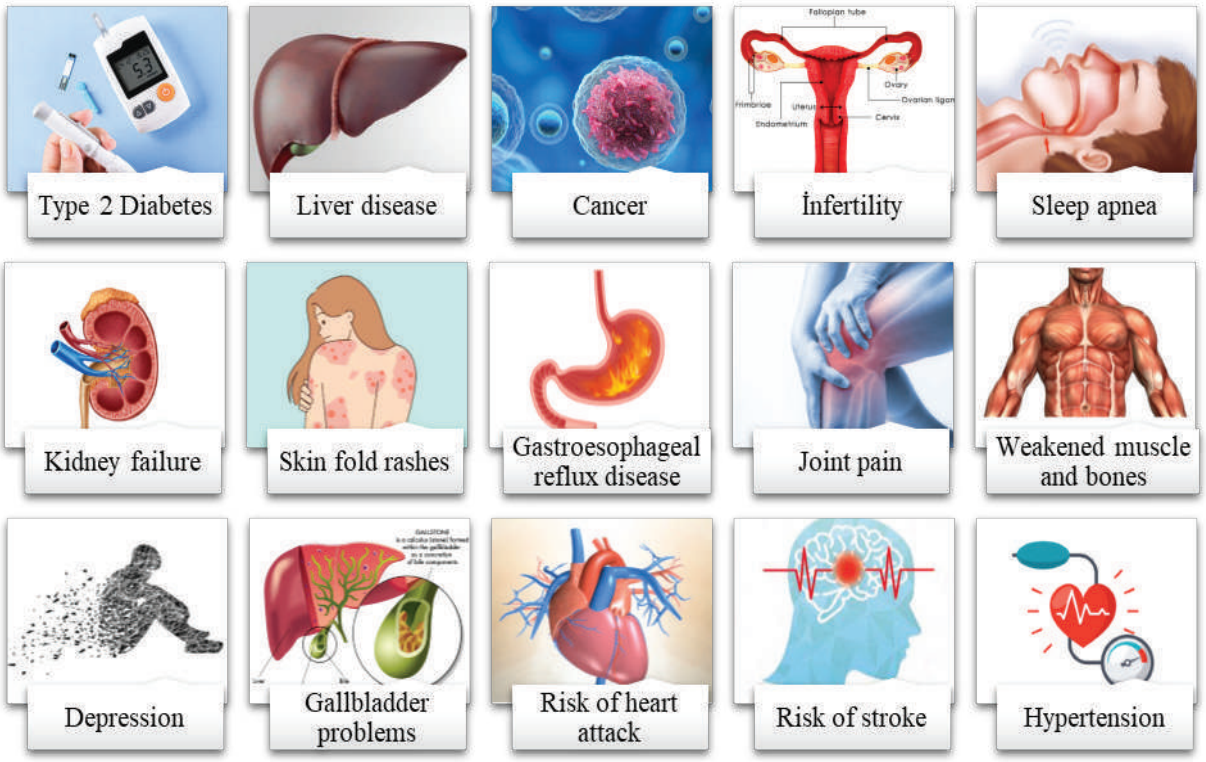


Figure 4. The effects of obesity on the body.

These diseases are among the most commonly triggered health issues associated with obesity. Additionally, obesity can have significant effects on the respiratory system and is strongly linked with respiratory tract diseases. Particularly, it is known to exacerbate respiratory problems such as hypoventilation syndrome and obstructive sleep apnea syndrome. Therefore, it should be emphasized that obesity has serious and diverse effects on overall health beyond being merely an aesthetic concern (3).

6. Treatment of Obesity

Obesity treatment involves many methods designed to address the complexity of this condition. Pharmacological intervention involves the use of medications to help control weight and is part of the treatment of obesity. These medications may work by decreasing appetite, absorption of nutrients, or increasing feelings of satisfaction. Another crucial component of treating obesity is bariatric surgery, which includes operations like sleeve gastrectomy and gastric bypass to change the digestive tract and lower stomach size, leading to weight loss. Lifestyle changes are the cornerstone of obesity management and often include diet, exercise and behavioural changes. These changes are designed to promote healthy eating patterns, increase physical activity levels, and create sustainable habits that encourage weight loss and proper weight maintenance. Additionally, behavioural interventions such as cognitive behavioural therapy or motivational interviewing can also be used to address psychological problems that lead to obesity. In summary, the treatment of obesity is multifaceted and includes medication, surgery, and lifestyle changes depending on the patient's needs and conditions.

6.1. Drug Treatments

A comprehensive multimodal approach to initiate and sustain effective obesity treatment is indispensable and also encompassing appropriate dietary modifications, consistent physical activity, and lifestyle adjustments are needed. Pharmacotherapy represents a viable option for individuals with accompanying comorbidities such as type 2 diabetes or those who are overweight. Anti-obesity drugs, classified based on their mechanisms of action, primarily fall into three categories: agents that enhance energy expenditure and thermogenesis, appetite suppressants, and inhibitors of fat absorption. Despite the numerous treatment

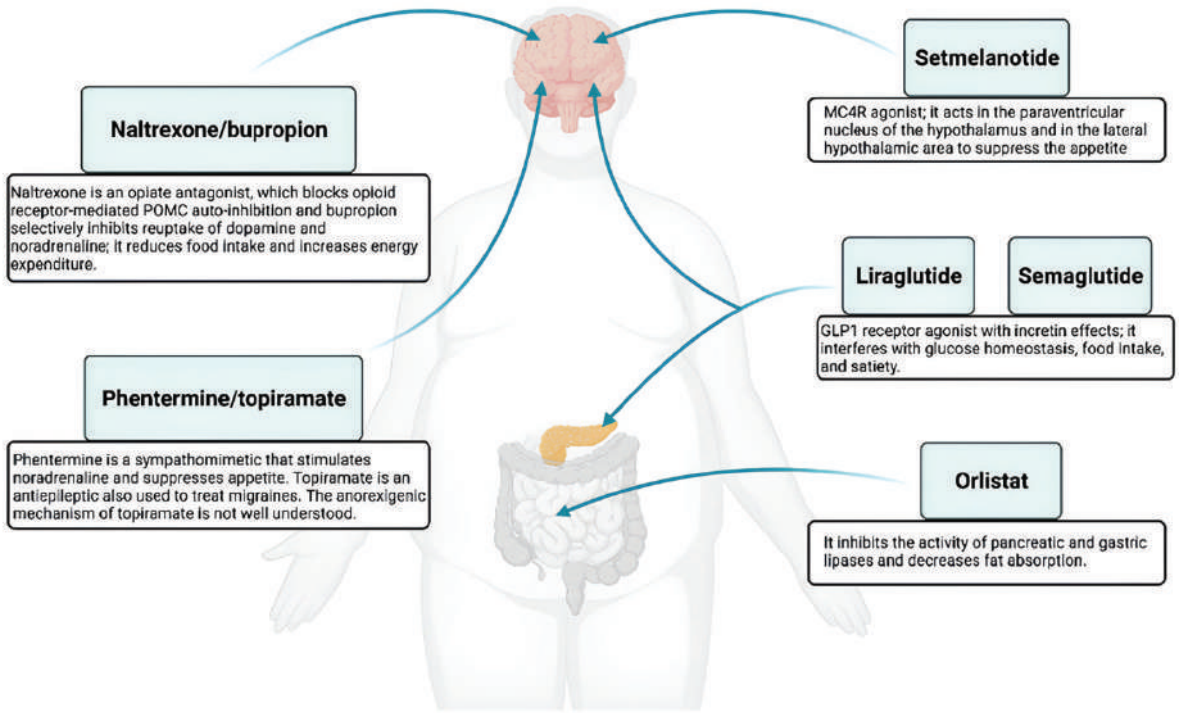


Figure 5. Summary of the mechanism of action for FDA/EMA approved anti-obesity drugs (13).

modalities employed over the years, the majority of anti-obesity therapies endorsed by the US Food and Drug Administration (USFDA) remain largely unavailable due to concerns regarding their safety and efficacy profiles (7). Among the pharmacological interventions recommended for managing obesity, Orlistat emerges as one of the most frequently prescribed medications. Orlistat facilitates weight loss by impeding fat absorption (1). Furthermore, promising outcomes have been observed with medications like liraglutide in combating obesity. It is known that Liraglutide helps weight reduction by suppressing appetite and enhancing feelings of fullness (2).

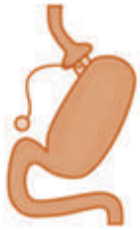
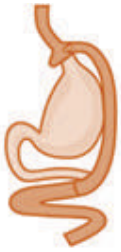
The United States Food and Drug Administration (FDA) has endorsed six major anti-obesity drugs, as depicted in Fig 5. However, the European Medicines Agency (EMA) has sanctioned only four medications for obesity management. This dissimilarity stems from the rejection of the phentermine/topiramate combination therapy by the EMA in 2013 and the ongoing assessment of semaglutide since January 2021 until the date of the current review (13).

6.2. Bariatric Surgery

Another avenue for against obesity is bariatric surgery, which has shown promise in alleviating obesity-related comorbidities (25). This invasive procedure involves altering the structure of the intestines or reducing stomach capacity to facilitate weight loss. However, it is crucial to carefully consider the pros and cons of different surgical techniques, as well as the suitability of each patient. Some bariatric surgery methods applied in the treatment of obesity are given in Table 2.

Bariatric surgery encompasses a range of surgical interventions aimed at promoting weight loss and managing health conditions associated with obesity. Candidates for these procedures typically include individuals with severe obesity, defined as a BMI of 40 or higher, or those with a BMI of 35 or higher who have obesity-related comorbidities, such as type 2 diabetes or hypertension, that may improve with weight reduction. While each bariatric surgery procedure aims to achieve weight loss and mitigate obesity-related health issues, they vary in their benefits, drawbacks, and potential risks. Therefore, selecting the most suitable treatment option requires careful consideration of patients' individual circumstances, medical histories, and lifestyles (26).

Table 2. Obesity bariatric surgery methods

Adjustable Gastric Banding (AGB)	Roux-en-Y Gastric Bypass (RYGB)	Vertical Sleeve Gastrectomy (VSG)	Biliopancreatic Diversion with Duodenal Switch (BPD/DS)
			
- A band is wrapped around the stomach to form a tiny pouch that can contain a tiny quantity of food. -The tightness of the band can be altered by adding or removing saline solution.	-A small gastric pouch is created by cutting the stomach. -Consumed foods are diverted away from the gastric body, duodenum, and proximal jejunum. - On the other hand, malabsorption of micronutrients such as calcium, iron, and vitamin B12 could happen.	-Approximately 80% of the stomach body is resected, creating a tubular-shaped stomach. -There is no need for a gastrointestinal-small intestine anastomosis. -This results in restriction on food intake, while speeding up gastric emptying.	-A sleeve gastrectomy is performed, followed by anastomosis between the bypassed intestine and the proximal duodenum. -This allows for partial malabsorption of nutrients. - Because of the high frequency of both short- and long-term problems, it is rarely practiced. (26)

6.3. Obesity Management Life Style Strategies

Lifestyle interventions are fundamental components of obesity management. Studies by Jensen et al. emphasize the efficacy of interventions such as nutritional counselling, structured physical activity programs, and behaviour modification strategies in tackling obesity. Embracing healthy dietary habits and maintaining regular physical activity are essential for achieving and sustaining weight loss. Additionally, the provision of psychosocial support and motivational guidance is vital for ensuring the success of obesity treatment journeys (27).

Nutritionists emphasize the detrimental impact of certain dietary habits, including reliance on diet culture, consumption of bakery products, processed foods (rich in refined carbohydrates), and excessive alcohol intake, all of which contribute to obesity development. Conversely, evidence suggests that incorporating breakfast and fruit consumption into one's diet can mitigate the risk of obesity, while evening snacking habits may exacerbate it. Attention is also drawn to the influential role of the school food environment and broader food landscapes, particularly in exposing school-age children to obesity risk factors.

Various scholarly investigations underscore the role of factors such as irregular physical activity or sedentary behaviour, excessive screen time, insufficient sleep duration or irregular sleep patterns, stress, obesogenic environments (linked to urbanization and industrialization), smoking, and frequent reliance on motorized transport as determinants of overweight and obesity. Prolonged screen exposure, for instance, may contribute to obesity development due to reduced glucose utilization by the brain during passive viewing. While scientific perspectives on the relationship between stress and obesity development vary, many researchers posit hormonal alterations as a potential contributing factor. Elevated cortisol levels, particularly in response to stress, can stimulate appetite and promote adipose tissue accumulation in the abdominal region, thereby exacerbating obesity

risk (28).

Obesity is a complex health problem that is influenced by many lifestyle factors, including dietary habits, sedentary behaviours, psychosocial factors, and environmental influences. Poor dietary choices, such as the consumption of energy-dense, nutrient-poor foods and sugar-sweetened beverages, together with a sedentary lifestyle characterized by low levels of physical activity, explain most of the weight gain and accumulation of adiposity. Psychosocial factors, including stress, emotional eating, and low-quality sleep, disrupt appetite regulation and promote disordered eating, further increasing the risk of obesity. In addition, cultural and environmental factors, such as obesogenic food environments and social disparities, foster the predisposition to gain weight of the individuals' lifestyle choices. Fig 6 shows the closely linked lifestyle factors and overweight and obesity, representing a combined illustration of the multiple interactions between different individual behaviours and weight status.

7. Medicinal Plants in the Treatment of Obesity

Notwithstanding the availability of numerous commercial drugs used in the treatment of obesity and diabetes treatment, many are limited in their suitability for a broad patient population since they may pose adverse effects. Therefore, using various medicinal plants and the phytochemicals they contain to treat diabetes and obesity may lead to the development of safer substitutes. These alternatives not only transiently lower blood sugar levels but also contribute to preventing hypertension and cardiovascular diseases. The significance of these plants lies in their potential to regulate the antioxidant system, insulin effects, and secretion. Identifying dietary components capable of regulating fat accumulation and blood sugar levels is paramount. Flavonoids, or bioflavonoids, derived from the Latin word "flavus," meaning yellow, represent the most prevalent polyphenolic compounds found in plants. These substances, which are found in fungi and plants as secondary metabolites, have a 15-carbon structure with two phenyl rings and one heterocyclic ring. More than five thousand naturally occurring flavonoid types have been found in different plants, and since their unique chemical makeup, each one offers a wide range of advantageous benefits (29).

In order to explore their potential in preventing obesity, numerous edible and medicinal plants have undergone thorough examination in studies employing diverse methodologies, including in vivo animal models and in vitro cellular assays. These investigations aim to uncover the underlying mechanisms related with the observed decrease in body weight (30).

Epidemiological and experimental studies have shown that selected edible and medicinal plants have anti-obesity activity (30-34). These results suggest that plant sources may have contribute effects on weight management and obesity prevention by modulating metabolic processes through their bioactive ingredients (35-37). Furthermore, clinical investigations exploring the potential effects of herbal ingredients on obesity enhance the scientific foundation of treatment and prevention strategies, creating new opportunities for clinical applications (30).

Earlier studies in literature related to the anti-obesogenic effects of such plants and the potential clinical application have been presented in Table 3. This review is based on findings from previous *in vitro* and *in vivo* studies. The table aims to provide a detailed overview of how these plants can contribute to the treatment of obesity by highlighting their mechanisms and evaluating their effectiveness based on scientific evidence.

Figure 6. Lifestyle factors associated with overweight and obesity.

Table 3. Anti-obesity potential of some medicinal plants.

Scientific Name (Plant)	Botanical Plant Images	Active Ingredient	Described Effect	References
Acacia mearnsii *de Wild.* **(Black wattle)**		Flavones (Flavan-3-ol catechins)	Upregulation of genes associated with energy expenditure in skeletal muscle and downregulation of fatty acid synthesis	(33)
Adiantum capilus veneris **(Maidenhair fern)**		Chlorogenic acid, Ellagic acid, Ferulic acid	Reducing weight gain Inhibiting pancreatic lipase activity	(34)
Allium cepa L. **(Onion)**		Quercetin	Significantly reducing lipids in 3T3-L1 cells Decreasing expression of lipogenesis-related genes, Restricting of lipid accumulation	(35)
Aloe barbadensis miller **(Aloe vera)**		Gallic acid, Quercetin	Reducing fat accumulation Activating fat lipolysis Improving oxidative stress	(36)
Brassica oleraea **(Red cabbage)**		Anthocyanidins	Inhibiting the activity of α-glucosidase and α-amylase	(37)
Camellia sinensis L. **(White tea)**		Flavones (Catechins) Alkaloids (Caffeine)	Serum triglyceride and non-esterified fatty acid levels	(38)
Capparis sicula ssp. sicula **(Capers)**		Rutin	Inhibiting pancreatic lipase activity	(39)
Carica papaya L. **(Papaya)**		Alkaloids, Saponins, Tannins, Anthraquinones, Anthocyanidins	Reducing triglycerides levels, LDL-C E VLDL-C	(40)
Centella asiatica **(Spadeleaf)**		Asiatic acid, Madecassic acid	Reducing in pancreatic lipase activity and α-amylase activity	(41)
Cinnamomum zeylanicum **(Cinnamon)**		Cinnamaldehyde	Reducing body weight gain Inhibiting the accumulative food intake Decreasing the secretion of ghrelin Reducing the gastric emptying	(42)

Cirsium setidens **(Gondre)**		Pectolinarin	Reduced intercellular lipid accumulation and lipid droplet sizes and numbers during adipogenesis	(10)
Coccinia grandis L. Voigt **(Scarlet gourd)**		Alkaloids, Cardenolide Glycosides, Phenols, Flavonoids	Reducing intracellular fat accumulation, as well as decreasing expression of PPARγ, C/EBPα, FAS, LPL, and GLUT4	(43)
Crataegus pubescens **(Tejocote)**		Gallic acid	Increasing excretion of faecal triacylglycerol Decreasing the accumulation of adipose tissue	(44)
Curcuma longa **(Turmeric)**		Curcumin	Changing into brown fat-like phenotype in white adipocytes, Cidea, Fgf21, Cited1 Increasing the brown adipocytes marker proteins, such as C/EBP-β, PGC-1α, PRDM16 and UCP1	(45)
Emblica officinalis **(Indian gooseberry)**		Terpenoids	Antidiabetic, hypolipidemic, and antioxidant effect, along with reducing serum AST and ALT levels	(46)
Garcinia cambogia **(Brindleberry)**		Hydroxycitric acid	Potentially inhibiting lipogenesis, including the inhibition of ATP-citrate lyase, and reducing triglyceride levels.	(47)
Garcinia cambogia Desr. **(Mala-bar tamarind)**		Hydroxycitric acid	Inhibiting the enzyme adenosine triphosphatase citrate lyase blocking lipogenesis	(48)
Hibiscus sabdarffa L. **(Roselle)**		Hibiscus acid, Anthocyanins, Flavonoids	Inhibiting activities of pancreatic lipase and α-amylase, cholesterol and triglycerides levels indicated reductions	(49, 50)
Hylocereus polyrhizus **(Red pitaya)**		Betacyanin's	Reducing body weight gain, modulating gut microbiota, downregulating the ratio of firmicutes and Bacteroidetes, increasing Akkermansia	(51)
Lactuca sativa L. **(Purple lettuce)**		Esculin, Chlorogenic acid	Decreasing body weight gain , reducing fat accumulation, Increasing energy consumption Regulating gut microbiota	(52)

Ligustrum robustum **(Kuding tea)**		Pomolic acid 19alpha-Hydroxyursolic acid	Inhibiting α-glucosidase	(35)
Litchi chinensis **(Lychee seed)**		Flavonoids	Inhibiting the differentiation of adipocytes, Downregulating PPAR-γ, C/EBP-α, -β, -δ and KLF9	(53)
Melissa officinalis L. **(Lemon balm)**		Hydroxycinnamic acid, Flavonoids	Blocking visceral obesity observed in female obese mice, Reducing increased fasting blood sugar, impaired glucose tolerance, and pancreatic dysfunction	(54)
Moringa oleifera **(Moringa)**		Isoquercitrin, Chrysin-7-Glucoside, Quercitrin	Decreasing TG accumulation in adipocytes	(51)
Morus bombycis Koidz. **(Mulberry)**		Alkaloids	Increasing lipolytic effects with decreased intracellular triglycerides and release of glycerol	(55)
Ocimum sanctum **(Tulsi plant)**		Ellagic acid, Epigallocatechin Gallate, Rutin	Increasing excretion of faecal triacylglycerol Decreasing the accumulation of adipose tissue	(44)
Orostachys japonicus **(*R*ock pine)**		Epicatechin Gallate, Quercetin, Kaempferol	Anti-adipogenic activity, inhibiting the major (PPARγ and C/EBPα) and minor (SREBP-1c, aP2 and leptin) adipogenic factors	(56)
Perilla frutescens **(Purple perilla)**		Rosmarinic acid	Decreasing body weight gain Upregulating ATGL and HSL in the adipose tissue and liver	(57)
Piper nigrum L. **(Black pepper)**		Alkaloids, Flavonoids, Tannins Saponins	Piperine suppresses the role of body weight, increases insulin and leptin sensitivity and ultimately balances obesity	(58, 59)
Portulaca oleracea L. **(Purslane)**		Crude	Decreasing total cholesterol (TC), triglycerides (TG), and low-density lipoprotein (LDL), increased high-density lipoprotein (HDL)	(60)
Rhizoma polygonati **(Huang jing)**		Kaempferol	Decreasing adipocyte differentiation markers, including PPARγ, SREBP-1c, Rxrβ, Lxrβ, Rorβ, Gpd1, Agpat2, and Dgat2, Increasing TNFα, Lsr, and Cel	(61)
Rubus coreanus **(Rubi Fructus)**		Ellagic acid	Reducing the weight of body and adipose tissue, Decreasing the expression of FAS, SREBP-1c, LXR, ACC, and LPL	(62)

Rumex pulcher L. **(Fiddle dock)**		Rutin, Luteolin, Apigenin	Preventing cardiovascular diseases by reducing oxidation of low-density lipoproteins	(63)
Salacia oblonga **(Ekanayaka)**		Polyphenols	Exhibiting antidiabetic, hypolipidemic, and antioxidant effects, along with reducing serum AST and ALT levels.	(46)
Salvia hispanica L. **(Chia)**		Quercetin, Chlorogenic acid, Caffeic acid	Chia oil increased glucose metabolism and it has revealed potential to protect against the development of obesity-related diseases.	(64, 65)
Solidago virgaurea **(Goldenrod)**		Keampferol-3-O-rutinoside, Chlorogenic acid, Protocatechuic acid	Reducing body weight gain adipose tissue size, WAT and liver fatty acid binding protein-4, PPAR-γ, C/EBP-α, FAS, SCD-1, SREBP-1c and CD36 inhibitory genes related to adipogenesis	(66)
Syzygium aromaticum **(Clove)**		Eugenol, β-caryophyllene	Reducing lipid accumulation Decreasing the weights of body and abdominal adipose tissue, Reducing the lipid deposition, Regulating TG, LDL-C	(67)
Terminalia paniculata **(Kindal)**		Polyphenols, Triterpenoids	Reducing body weight, inhibiting adipogenesis, Decreasing the expression of FAS, leptin, adiponectin, PPAR-γ, and SREBP-1c, Increasing the expression of AMPK-1α	(68)
Urtica dioica L. **(Stinging nettle)**		Caffeic acid, Chlorogenic acid, Malic acid, Rutin	It could potentially result in decreased weight gain induced by diet and improved insulin sensitivity.	(69, 70)
Vitis vinifera L. **(Grapes)**		Resveratrol, Flavonoids, Proanthocyanins, Stilbenoids	Containing tocotrienols, including significant amounts of α- and γ-tocotrienol (T3), which reduce the expression of mRNA protein (e.g., PPARγ and αP2), which are crucial for adipogenesis; reduces proinflammatory gene expression (IL-6 and IL-8)	(71)
Zingiber officinale **(Ginger)**		6-Gingerol	Improving obesity and inflammation downregulating the expression of microRNA-21/132 and activating AMPK in WAT	(72)

Among the plants used in obesity treatment, the primary plant metabolites that demonstrates anti-obesity effects through *in vivo* and/or *in vitro* biological tests explains their impact on delayed fat absorption, suppression of enzymatic activities, mediation of lipid levels, and enhancement of lipolytic effects. The plant species presented to the evaluation protocols for anti-obesity activity can be obtained from leaves, seeds, rhizomes, stems, flowers, fruits, and roots based on extraction solutions obtained through hot maceration, cold maceration, Soxhlet extraction, reflux, and accelerated extraction processes. Solvents used in extractive processes include water, ethanol, methanol, n-ethanol, hexane,

n-butanol, dimethyl carbonate, and ethyl acetate. Among the chemical substances responsible for pharmacological effects, phenolic compounds have been presented as primary secondary metabolites related with anti-obesity effects. The anti-obesity effects are attributed to secondary metabolites present and chemically characterized in samples used in biological protocols, including saponins, polyphenols, flavones, flavanols, tannins, and chalcones. Phenolic compounds (especially flavones, flavanols, flavanones, catechins, anthocyanins, isoflavones, and chalcones) and their functional derivatives offer various chemical structures and pharmacological activities, found in fruits, legumes, nuts, beverages, and drugs. These chemical compounds have emerged as significant anti-obesity agents, primarily attributed to their antioxidative properties, which aid in preventing oxidative damage in biological systems. Additionally, they demonstrate inhibitory effects on the proliferation of in vitro predispose cells, induction of apoptosis in adipocytes, mitigation of lipid accumulation, and inhibition of pancreatic lipase activity, consequently impeding the in vivo absorption of fatty acids (31, 32).

8. Conclusion

The findings obtained from the scientific literature concerning the role of medicinal plants in the identification and formulation of anti-obesity medications underscore the significance of this field of inquiry, highlighting the therapeutic promise of bioactive compounds found within these botanical sources. The main objective of these studies is to identify phytogenic compounds that can target the pathophysiological processes of obesity and have a positive effect on metabolic control and insulin sensitivity. For example, polyphenols with antioxidant properties have been shown to regulate adipocyte formation and lipid metabolism. Additionally, some plant responses have been reported to modulate the activity of neurotransmitters involved in appetite control and metabolic responses.

This review was conducted to expand the existing knowledge on medicinal plants used in treating obesity and to highlight the ethnopharmacological approaches implemented in this area. Our review aims to define the effectiveness, safety, and methods of action of traditional medicinal plants used worldwide in weight management. The results of the present study are far-reaching and relevant for the development of alternative or adjuvant therapeutic strategies in the management of obesity based on the use of medicinal plants. The amalgamation of evidence-based traditional knowledge with modern scientific research is expected to close the gap between traditional healing techniques and contemporary health systems and further enhance the scope of integrating plant-derived therapies into standard protocols used for obesity management.

In addition, the ethnopharmacological approach used in this review provides insight into the cultural relevance of medicinal plants and highlights the need for protection of traditional knowledge systems for the next generation. The documentation and scientific validation of traditional utilization of medicinal plants in the treatment of obesity are of great importance to biodiversity conservation and the maintenance of indigenous healing practices.

Overall, the findings of this review highlight the potential of medicinal plants in addressing the global obesity epidemic and support the need for further research. Traditional healers, researchers, and healthcare practitioners working in collaboration would be able to develop evidence-based botanical interventions for a better impact on healthcare and improve the life quality for those suffering from obesity worldwide.

However, more research is needed to evaluate the anti-obesity properties of medicinal plants and to understand their pharmacological properties. Comprehensive studies are needed on the pharmacokinetics, pharmacodynamics and safety of phytoactive compounds. In addition, increasing the number of clinical studies and using standard procedures will help to obtain strong evidence about the effectiveness and safety of herbal compounds in the treatment of obesity. Investigating anti-obesity drugs may represent an important area of research. However, multidisciplinary research and clinical studies are needed to evaluate this potential. A better understanding of the metabolic effects of bioactive compounds found in medicinal plants would help

develop new and effective approach in obesity treatments.

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Original Article

Dose Dependent Effects of Bisphenol A Exposure on Locomotor Activity, Acetylcholinesterase and Redox System Parameters in Zebrafish Embryos

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Abstract

Introduction: Endocrine disrupting chemicals (EDC) are either synthetic or natural compounds in the environment that can interfere with endocrine functions. Exposure to EDCs during development is a major concern, and the health consequences may be permanent or long-lasting. Bisphenol A (BPA) is known to be an EDC and prenatal BPA exposure has been related to differences in children's brain microstructure, leading to differences in children's behavioral symptoms. Moreover high BPA exposure during pregnancy is related to increased behavioral problems throughout childhood. In our study we aimed to evaluate the effects of BPA exposure in zebrafish embriyos focusing on locomotor activities and biochemical parameters.

Methods: Zebrafish embryos were exposed to 1µg/L and 10 µg/L BPA until 72 hpf. At the end of exposure period, locomotor activities were determined and acetylcholinesterase (AChE), glutathione S-transferase (GST) and superoxide dismutase (SOD) activities were determined using spectrophotometric methods.

Results: Concentration dependent changes were determined in GST and SOD activites indicating increased response to oxidative stress due to BPA toxicity. AChE acitivity alterations and locomotor activity changes pointed out the importance of concentration in the neurotoxic effects of BPA in zebrafish embryos.

Conclusion: The results of our study pointed out that new studies are needed to examine the effects of BPA, especially on cognitive and locomotor functions.

Keywords: Bisphenol A, locomotor activity, oxidative stress, acetylcholinesterase, zebrafish embryos

1. Introduction

Bisphenol A (BPA) is a chemical which is manufactured in large amounts for use principally in the production of polycarbonate plastics. It can be found in a variety of items, such as shatterproof glass, eyewear, water containers, and epoxy resins used to coat some metal food containers, bottle caps, and water supply piping. For the majority of people, eating habits are the main way they are exposed to BPA. Although additional potential exposure sources like air, dust, and water exist, the vast majority of daily human contact with BPA comes from food and drinks (1,2).

BPA is associated with harmful health effects in mammalian and non-mammalian systems, ecosystems, and in vitro models, according to a substantial body of evidence obtained from more than 300 published research. As a well-known endocrine disruptor BPA binds to estrogen receptors and has estrogenic effects (1). BPA is also referred as a popular model for illustrating the low dose and unconventional characteristics of hormones and endocrine disruptors that control or alter the endocrine system.

The cholinergic enzyme acetylcholinesterase (AChE) is mainly present in postsynaptic neuromuscular junctions, particularly in muscles and nerves. Acetylcholine (ACh), a neurotransmitter that occurs naturally, is instantly hydrolyzed into acetic acid and choline. AChE's main function is to halt synaptic transmission and messaging between neurons in order to stop ACh from spreading and activating adjacent receptors. AChE is inhibited by organophosphates. They play a significant role in the creation of insecticides and nerve agents (3). Cholinergic neurons in the brain regulate reception of stimuli, thinking, and awareness. Cholinergic neurons not only cover the forebrain, but also the brainstem and thalamus, including the reticular nucleus, which control cognition and attention. These cholinergic neurons have damaged projections in the setting of Alzheimer dementia as a degenerative illness, which correlate with the traditional signs of cognitive slowness and deterioration. Short-term memory loss, cerebrum atrophy, B-amyloid plaques, tangles, and tau protein deposits are all common symptoms of the condition (4).

In recent years BPA exposure has been shown to increase the risk of developing neurodevelopmental, neurodegenerative diseases including Alzheimer's and Parkinson's diseases (5). In line with this information, in our study, the effect of BPA exposure on locomotor activity and the possible relationship between this effect and AChE activity were investigated in zebrafish embryos. Antioxidant enzyme activities were also determined to determine the response to BPA toxicity.

2. Methods

2.1. Embryo exposure and determination of locomotor activity

Zebrafish embryos were exposed to BPA (5 µg/L and 10 µg/L) in well plates for 72 hours post-fertilization (hpf). The embryo medium eas used as blank control. For the biochemical analyses 50 embryos/pool and 3 biological replicates for each group was prepared. Exposure solutions were renewed with fresh solutions each day and the developmental parameters were investigated using a stereomicroscope (Zeiss Discovery V8, Germany). The locomotor activity of the zebrafish embryos at 72 hpf was evaluated described previously (6).

To accomplish this, a 60 mm Petri dish filled with embryo media was placed on top of the motility wheel, which is mounted on the microscope stage. The zebrafish embryo was then placed in the center of the motility wheel using an embryo poker tool, the length of time it takes for an embryo to swim that distance was recorded, and the average escape reaction was computed. Zebrafish embryos at 96 hpf have been immobilized at the conclusion of the exposure period by immersion in freezing water for 10 minutes, followed by five minutes of sodium hypochlorite, to assure death. No ethical permission was necessary for the techniques utilized because the zebrafish embryos used were no older than 5 days old, as indicated by the Council of Europe in 1986, Directive 86/609/EEC.

2.2. Determination of total protein

The concentrations of total proteins in the samples were assessed using Lowry's technique (7). In this procedure, proteins are first reduced by the Folin reagent before reacting with copper ions in an alkali media. At 500 nm, the absorbances are calculated. The values for each protein were computed and presented using the total protein levels.

2.3. Determination of Acetylcholinesterase activity

The supernatants were examined for acetylcholinesterase (AChE) activity using the method of Ellman (8). In this procedure, acetylcholinesterase produces thiocholine, which reacts with 5,5'-dithiobis (2-nitrobenzoic acid) to generate a yellow tint. The enzyme activity in the sample is determined by measuring the magnitude of the yellow product color at 412 nm.

2.4. Determination of Superoxide dismutase

The method used to assess superoxide dismutase (SOD) activity is based on SOD's capacity to enhance the effects of riboflavin-sensitized photo-oxidation of o-dianisidine. By shining a fluorescent lamp on the reaction mixture made up of riboflavin and O-dianisidine dihydrochloride, superoxide activity is created. Riboflavin sensitizes and SOD enhances the oxidation of O-dianisidine, and the enhancement is linearly correlated with SOD concentration. Using a spectrophotometer set at 460 nm, the absorbances at 0 and 8 minutes of illumination were measured, and the net absorbances were determined (9). The data were expressed as U/mg protein.

2.5. Determination of Glutathione-S-transferase

The evaluation of Glutathione-S-transferase activity was performed through the determination of the absorbance of the product obtained by the conjugation of GSH and 1-chloro-2,4-dinitro-benzene (CDNB) through spectrophotometric analysis at 340 nm (10).

3. Results

When compared with the control group, average speed and distance travelled decreased significantly in the 1 µg/L BPA exposed group (****p<0.0001). On the other hand, no significant change in average speed was observed in the 10 µg/L BPA exposed group while distance travelled decreased significantly in the same group (*p<0.05). Moreover, average speed and distance travelled levels of the 10 µg/L BPA exposed group was significantly higher than the 1 µg/L BPA exposed group (****p<0.0001) (Fig 1 and 2).

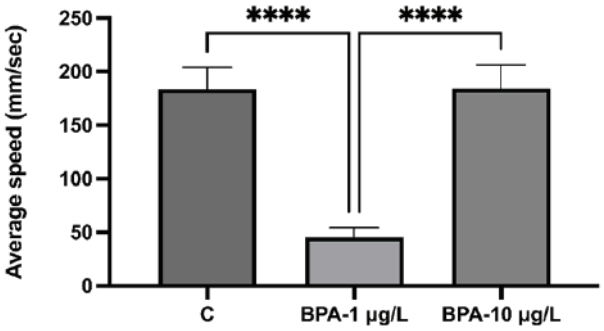

Figure 1. Average speed results of the embryos in in the control and BPA groups. *Data presented are mean ± SD. ****p<0.0001, SD: standard deviation.*

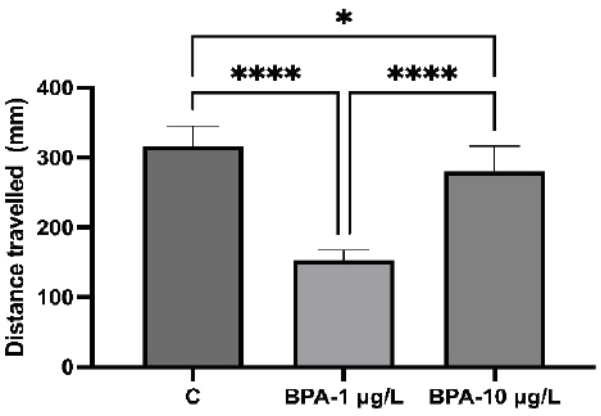

Figure 2. Total distance results of the embryos in in the control and BPA groups. *Data presented are mean ± SD. ****p<0.0001, * p<0.05. SD: standard deviation.*

AChE activity increased significantly both in the 1 µg/L and 10 µg/L BPA exposed groups (**p<0.01 and ****p<0.0001 respectively). AChE activity of the 10 µg/L BPA exposed group was significantly

higher than the 1 µg/L BPA exposed group (****p<0.0001) (Fig 3).

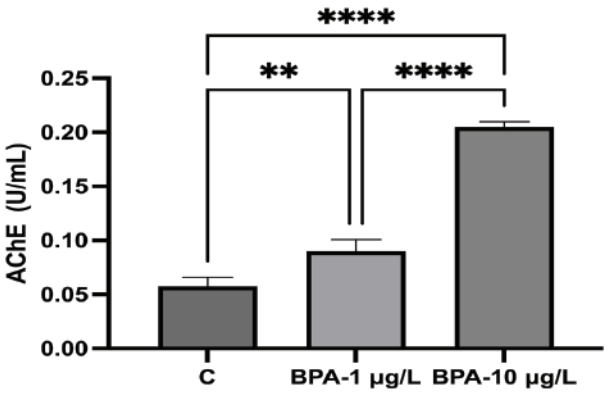

Figure 3. AChE activities of the embryos in the control and BPA groups. *Data are expressed as mean+SD from the three independent experiments. ****p<0.0001, ** p<0.01, SD: standard deviation.*

SOD activity decreased significantly both in the 1 µg/L and 10 µg/L BPA exposed groups (***p<0.001 and *p<0.05 respectively). SOD activity of the 10 µg/L BPA exposed group was significantly higher than the 1 µg/L BPA exposed group (*p<0.05) (Fig 4).

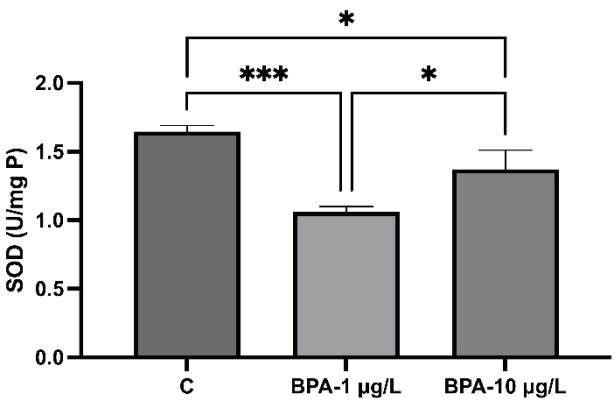

Figure 4. SOD activities of the embryos in the control and BPA groups. *Data are expressed as mean+SD from the three independent experiments. *** p<0.001, * p<0.05. SD: standard deviation.*

GST activity increased significantly both in the 1 µg/L and 10 µg/L BPA exposed groups (*p<0.05 and ***p<0.001 respectively). Moreover, GST activity of the 10 µg/L BPA exposed group was significantly higher than the 1 µg/L BPA exposed group (*p<0.05) (Fig 5).

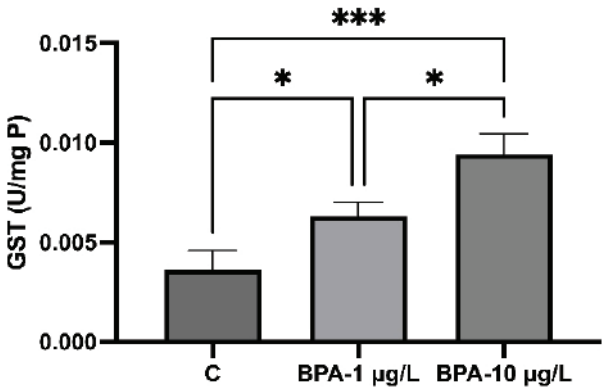

Figure 5. GST activities of the embryos in the control and BPA groups. *Data are expressed as mean+SD from the three independent experiments. *** p<0.001, * p<0.05. SD: standard deviation.*

4. Discussion

Results of our study showed that BPA caused dose dependent changes in locomotor activity in zebrafish embryos. Previous studies showed that BPA induced abnormalities in nonreproductive behaviors in rodents including locomotor activity, spontaneous motor activity, and aggressive behavior. Nojima et al., evaluated the effects of BPA on the spontaneous motor activity of adult male rats and reported that BPA induced hyperactivity in adult male rats (11).

In accordance with our results regarding the increased the locomotor activities, in male rats postnatal BPA exposure was found to increase the spontaneous motor activity. However, BPA exposure during perinatal and postnatal stages increased the locomotor activities in 1 month old female mice while it decreased it in the male mice of same age (12). According to the results of these studies BPA was shown to pass through blood −brain barrier and alter the functions of brain in adult rats. On the other hand, how intraperitoneally administered BPA affects brain and induce hyperactivity in adult is suggested to need further investigation (11).

In our study, AChE activity increased both in the 1 µL and 10 µL BPA groups,.The alterations in the AChE activities might be associated with the altered locomotor activity.

Xuereb et al., investigated the relation between AChE inhibition and the alterations in feeding and locomotor activity in male Gammarus fossarum exposed to chlorpyrifos which is an organophosphorous pesticide and carbamate pesticide methomyl (MT) for 96 hours (13). They reported reduced AChE activity in a concentration-dependent way and also altered behavioural parameters in both CPE and MT exposed groups. With regard to both the rate of feeding and locomotor behavior—both of which are recognized as important ecological responses—this study offers a basis for interpreting the biomarker AChE at the higher biological organisation stage.

In various laboratory models as well as in humans, oxidative stress and related indicators are linked to BPA toxicity. There is growing evidence that BPA organ toxicity is greatly influenced by the generation of reactive oxygen species (ROS) and/or a diminished ability of antioxidant defense, which changes the oxidative equilibrium in the mitochondria and throughout the cell (14). The effects of BPA on increasing oxidative stress in zebrafish and zebrafish embryos have been demonstrated in previous studies (15,16).

In our study SOD activity decreased in the 1 µg/L and 10 µg/L BPA groups. Increased activity of SOD may indicate the activation of the antioxidant defence mechanism in response to increased oxidative stress. On the other hand, GST activities increased significantly in the BPA exposed groups in a dose dependent pattern. Increased GST activity may also be regarded as a defence mechanism in case of increased oxidative stress due to BPA toxicity as GST plays a major role in the detoxification process. Similar to the results of our study, BPA has also been shown to induce oxidative stress which was decreased by antioxidant enzyme activities, showing the protective mechanism against oxidative stress. Moreover BPA is reported to be more toxic at the early stages of the embryonic development (17).

5. Conclusion

As a conclusion results of our study showed dose dependent alterations in the locomotor activities in BPA exposed zebrafish embryos. Increased antioxidant system parameters indicated the oxidant-antioxidant balance that was disturbed by BPA exposure. This finding supports the results of previous studies on BPA. We think that the changes observed in the form of stimulation of locomotor activity in our study may be associated with changes in AChE activity, which is closely related to cognitive functions. The results of our study pointed out that new studies are needed to examine the effects of BPA, especially on cognitive and locomotor functions.

Conflicts of interest: The authors report no conflicts of interest.

Ethics approval: As the zebrafish embryos used were no older than 5 days old, no ethical approval was required for the protocols applied as stated by the Council of Europe (1986), Directive 86/609/EEC.

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Original Article

Solubilization of Insoluble and Poorly-Water Soluble Drugs in Micellar Systems

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Abstract

Introduction: This study investigates the micellar solubilization of several insoluble and poorly soluble drugs—clopidogrel bisulfate, ganciclovir sodium, miconazole nitrate, brinzolamide, brimonidine tartarate, and dexamethasone—using sodium dodecyl sulfate (SDS), aerosol-OT (AOT), dodecyl trimethylammonium bromide (DTAB), and cetyltrimethylammonium bromide (CTAB) as surfactants.

Methods: The micellar solubilization experiments were conducted by preparing solutions of the drugs in the presence of SDS, AOT, DTAB, and CTAB micelles. Spectrophotometric measurements were performed at a constant temperature of 298 K to analyze the solubilization efficiency of each surfactant. Phase-solubilization graphs were plotted to visualize the relationship between drug solubility and surfactant concentration.

Results: The results indicated that hydrophobic interactions play a critical role in surfactant solubilization power. AOT was identified as the most effective surfactant among those tested. The solubility tendencies of the drugs in the presence of micelles were discussed based on the calculated K_M values and the spectral behavior of drug molecules.

Conclusion: Micellar solubilization offers a promising approach to characterize drugs with varying solubility profiles—ranging from slightly soluble to insoluble in water. Additionally, surfactant micelles serve as effective biomimetic models for membrane systems in pharmaceutical research. the findings from this study hold implications for drug formulation and design, particularly in addressing solubilization challenges and optimizing pharmaceutical dosage forms.

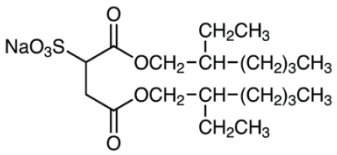
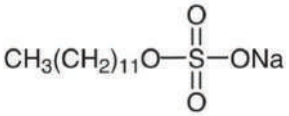
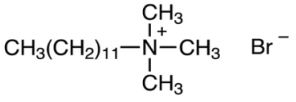
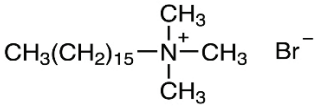
Keywords: Surfactant, poorly soluble drugs, critical micelle concentration, micelle, solubilization

1. Introduction

Solubility is of great importance in developing drug formulations and regulatory standards. Especially insoluble or poorly water-soluble drugs bring about problems in drug formulations at appropriate doses. The many pharmaceutical substances that have limited solubility in water present formidable problems to the acceptable dosage forms e.g. incomplete dissolution in body fluids. Therefore, solubility problems that make transport of drugs difficult are also present in many existing drugs. Solubility is a crucial chemical parameter in developing a drug, since of the current drugs in the industry are either insoluble or sparingly soluble in water. For this purpose, the dissolution method is usually performed to formulate. Among the most preferred methods are pH adjustment, cosolvents, solubilization with micelles (micellar solubilization), and complexation. One of the common methods in pharmaceutical applications practice to improve hydrophobic drugs' solubility is to use micelles since surfactants have many different structures and properties (1-7).

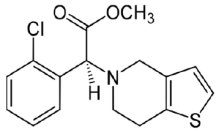
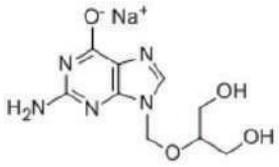
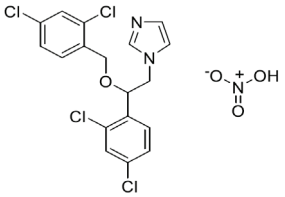
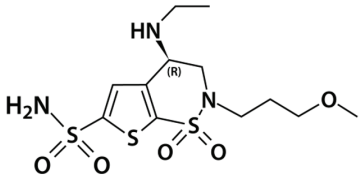
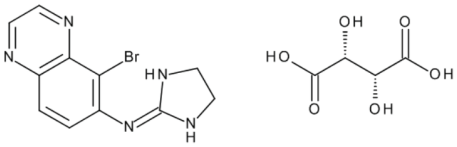
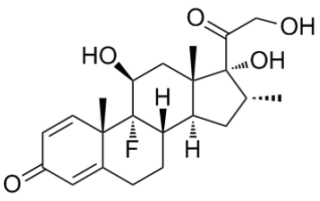
Molecules of surfactant composed of hydrophobic and hydrophilic parts associate in water to form colloidal aggregates called micelle if the concentration exceeds the critical micelle concentration (CMC). Owing to their unique structure and properties, surfactant micelles create a different microenvironment by binding organic ions and molecules in the solution with hydrophobic and/or electrostatic interaction and enhancing the solubility behaviour of molecules. From this point of view, surfactants and their role in pharmaceutical applications are paramount, especially concerning their ability to solubilize hydrophobic drugs. In recent years, there have been numerous studies on micellar solubilization of drugs to improve their

Table 1. CMC and molecular structures of surfactants

Surfactant	Molecular structure	CMC (mmol/L)
AOT $C_{20}H_{37}NaO_7S$		3.30
SDS $C_{12}H_{25}SO_4Na$		8.00
DTAB $C_{15}H_{34}BrN$		10.0
CTAB $C_{19}H_{42}BrN$		0.92

CMC: Critical micelle concentration.

Table 2. Solubility, IUPAC name and molecular structures of the drugs.

Drug	Solubility in water	IUPAC Name
Clopidogrel bisulfate (CBS)	practically insoluble	methyl (2S)-2-(2-chlorophenyl)-2-(6,7-dihydro-4H-thieno[3,2-c] pyridin-5-yl)acetate;sulfuric acid 
Ganciclovir sodium (GS)	soluble	sodium;2-amino-9-(1,3-dihydroxypropan-2-yloxymethyl)purin-6-olate 
Miconazole nitrate (MN)	limited soluble	1-[2-(2,4-dichlorophenyl)-2-[(2,4-dichlorophenyl)methoxy]ethyl] imidazole;nitric acid 
Brinzolamide (BRZ)	limited soluble	(4R)-4-(ethylamino)-2-(3-methoxypropyl)-1,1-dioxo-3,4-dihydrothieno[3,2-e]thiazine-6-sulfonamide 
Brimonidine tartarate (BRT)	practically insoluble	5-bromo-N-(4,5-dihydro-1H-imidazol-2-yl)quinoxalin-6-amine;(2R,3R)-2,3-dihydroxybutanedioic acid 
Dexamethasone (DEX)	practically insoluble	(8S,9R,10S,11S,13S,14S,16R,17R)-9-fluoro-11,17-dihydroxy-17-(2-hydroxyacetyl)-10,13,16-trimethyl-6,7,8,11,12,14,15,16-octahydrocyclopenta[a]phenanthren-3-one 

efficiency in solutions (8-12). Among the various experimental methods, spectrophotometry is the most preferred technique for the interaction of drugs with surfactants as well as micellar solubilization of hydrophobic drugs (8, 9-14). By considering all above points, the present study examines the solubilization of slightly soluble drugs using sodium dodecyl sulfate (SDS), aerosol-OT (AOT), dodecyltrimethylammonium bromide (DTAB) and cetyltrimethylammonium bromide (CTAB) micelles (Table 1). The properties and molecular structures of pharmaceutical compounds were given in Table 2. The obtained experimental data have been used to plot phase-solubility graphs of all drugs in aqueous solutions of surfactants according to the Higuchi-Connors method (15). Using the phase solubility graphs of drugs in different micellar media, the solubilizing capacity of micelles (K_M) was determined and the drug solubilization capacities of the studied surfactant micelles were compared.

2. Methods

2.1. Materials

CBS, GS, MN, BRZ, BRT, and DEX were supplied from World Medicine Pharmaceutical Company. SDS, AOT, DTAB and CTAB purchased from Sigma Co. All solutions were prepared using doubly distilled conductivity water. DMSO, methanol, and ethanol were of analytical grade. UV-visible spectrophotometer computer connected (Shimadzu 1700) was used to record the UV spectra of drugs in the absence and presence of surfactants.

2.2. Method

Phase-solubility experiments of CBS, GS, MN, BRZ, BRT, and DEX in aqueous solutions of AOT, SDS, DTAB and CTAB were conducted to the shake-flask method of Higuchi and Connors (1965) (15). Surfactant solutions in various quantities in water were prepared depending on their CMC i.e. at below the CMC (premicellar region) and well above the CMC (post micellar region) of surfactants. The concentrations of surfactants varied from 0.1 mM to 50 mM (from premicellar to micellar region). Then an excess of the drug was added in glass flasks and shaken up with surfactant at 298 K for 24 h to reach the equilibrium. After filtration, the total solubilized concentration of the drugs was analysed by UV absorbance spectroscopy. The calibration curve of drugs was constructed using UV-visible spectrophotometer absorption data. The drug concentrations dissolved in the presence of surfactants were determined using the calibration curves of the drugs constructed in appropriate solvents such as methanol, ethanol or DMSO. The detailed micellar solubilization experimental procedure has been previously reported. (8,9,15).

2.2.1. Determining the solubilizing capacity of micelles (K_M)

The solubility of a substance in the presence of a surface active agent can be explained by the two-phase model which assumes that micellization only occurs above the CMC and that the monomer concentration remains constant, independent of the total surfactant concentration, as described by the following equation (8,10,11,16,17).

$$S_m = K_M (C_S - C_{CMC}) + S_0$$

Here, CS is the surfactant concentration and CCMC is the critical micelle concentration of each surfactant. Sm and S0 are solubility of the drug in the presence and absence of surfactants, respectively. Determining the slope of the solubilization curve the solubilizing capacity of micelles (K_M) can be calculated and given in mmol/L (mM).

3. Results

3.1. Clopidogrel bisulfate (CBS)

CBS was practically insoluble in water. The maximum absorbance of CBS was recorded at 203 nm in methanol, based on the valid concentration range of Lambert-Beer Law. To compare the influence of micelles, the corresponding absorption spectra of CBS in the absence and the presence of DTAB, CTAB, SDS and AOT micelles are shown in Fig 1.

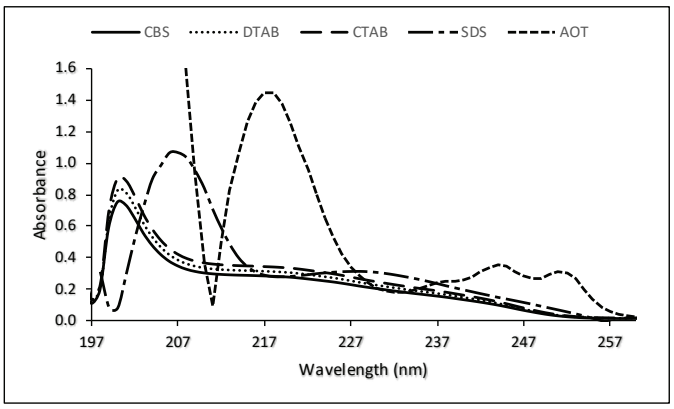


Figure 1. Absorption spectra of CBS in micellar solutions of DTAB, CTAB, SDS, AOT and in methanol.

As seen in Fig 1 the λmax of CBS shifted from 203 nm to 207 nm in the presence of SDS micelles while a significant red shift was observed from 203 nm to 219 nm in the presence of AOT micelles. In addition, as the surfactant concentration increased, an increase in the absorbance of CBS was observed. However, no significant shift was observed for CBS in the presence of DTAB and CTAB micelles. The solubility of CBS increased with the increase in AOT and SDS micelle concentration. The solubilization capacities (K_M) of AOT and SDS micelles were determined and are presented in Table 3. The variation in the solubility of CBS as a function of the micelle concentration of DTAB, CTAB, AOT, and SDS is shown in Fig. 2.

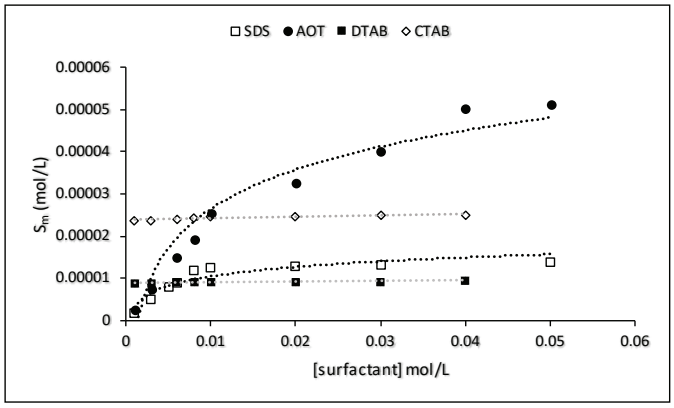


Figure 2. Phase–solubility plot of CBS in DTAB, CTAB, SDS and AOT micelles (298 K)

3.2. Brimonidine tartrate (BRT)

Brimonidine tartrate (BRT) was practically insoluble in water. The maximum absorbance of BRT was recorded at 247 nm in DMSO, based on the valid concentration range of Lambert-Beer Law. In order to compare the influence of micelles, the corresponding absorption spectra of BRT in the absence and the presence of DTAB, CTAB, SDS and AOT micelles are shown in Fig 3.

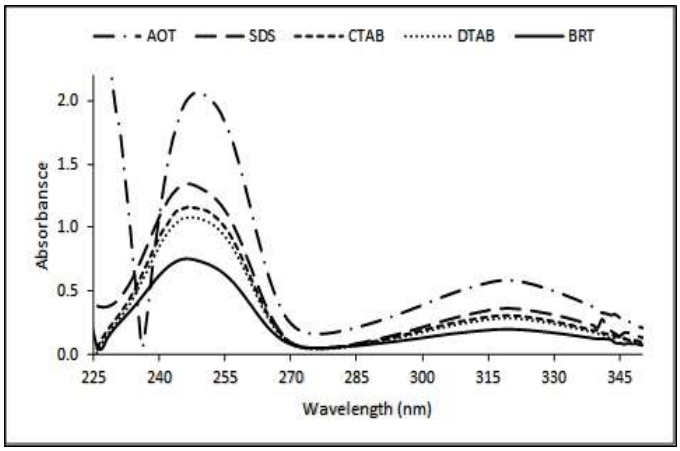


Figure 3. Absorption spectra of BRT in micellar solutions of DTAB, CTAB, SDS, AOT and in DMSO.

The λmax of BRT shifted slightly from 247 nm to 250 nm in the presence of all micelles. However, with AOT and SDS concentration increased and also an increase in the absorbance of BRT was observed. Since no significant change was observed in the absorbance of BRT in the presence of DTAB and CTAB micelles, K_M values could not be calculated. Solubility of BRT increased with the increase in AOT and SDS micelle concentration. The solubilization capacity (K_M) of SDS and AOT micelles were calculated and are given in Table 3. The variation of solubility of CBS as a function of micelles concentration of DTAB, CTAB AOT and SDS are shown in Fig 4.

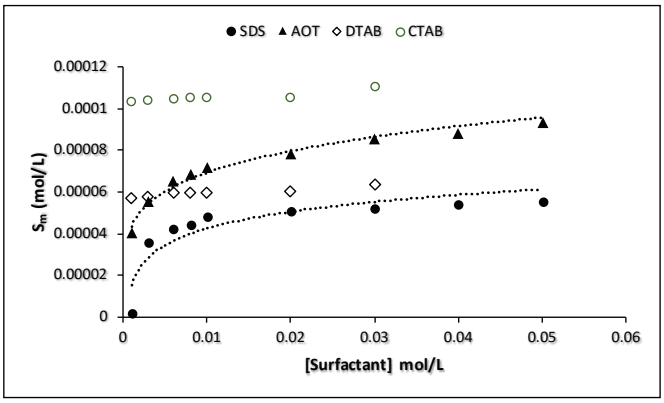


Figure 4. Phase–solubility plot of BRT in DTAB, CTAB, SDS and AOT micelles (298 K)

3.3. Dexamethasone (DEX)

Dexamethasone (DEX) was practically insoluble in water. The maximum absorbance of DEX was recorded at 242 nm in ethanol, based on the valid concentration range of Lambert-Beer Law. In

order to compare the influence of micelles, the corresponding absorption spectra of DEX in the absence and the presence of DTAB, CTAB, SDS and AOT micelles are shown in Fig 5.

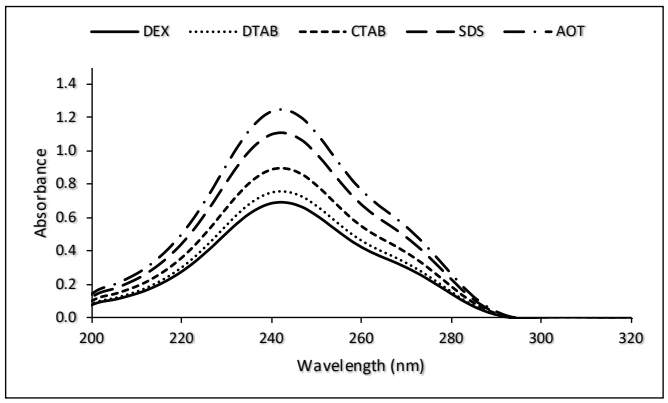


Figure 5. Absorption spectra of DEX in micellar solutions of DTAB, CTAB, SDS, AOT and in ethanol.

As seen in Fig 5 the λmax of DEX did not change in the presence of micelles but with the surfactant concentration increased and also an increase in the absorbance of DEX was observed. However, the solubility of DEX enhanced with the increase in DTAB, CTAB, SDS, and AOT micelle concentration. The solubilization capacity (K_M) of micelles were calculated and given in Table 3. The variation of solubility of DEX as a function of micelles concentration of DTAB, CTAB, AOT and SDS are shown in Fig 6.

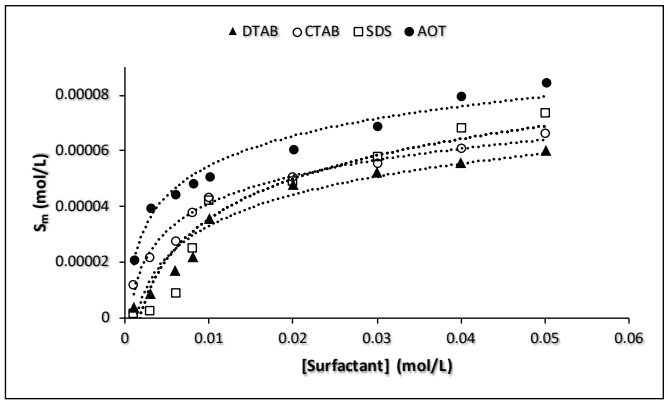


Figure 6. Phase–solubility plot of DEX in DTAB, CTAB, SDS and AOT micelles (298 K)

3.4. Miconazole nitrate (MN)

The solubility of miconazole nitrate (MN) in water was limited. MN exhibited two maximum absorbance at 231 and 238 nm in DMSO. Based on the valid concentration range of Lambert-Beer Law, the absorbance variation of MN was observed at 231 nm. To compare the influence of micelles, the corresponding absorption spectra of MN in the absence and the presence of DTAB, CTAB, SDS and AOT micelles were shown in Fig 7. As seen in Figure 7, no shift was observed in the presence of cationic DTAB and CTAB micelles while the presence of SDS and AOT affected the absorbance spectrum of MN with a significant red shift. Besides the λmax of MN at 238 nm disappeared.

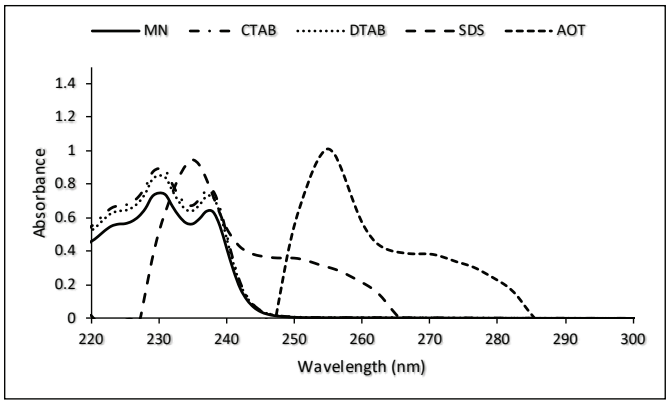


Figure 7. Absorption spectra of MN in micellar solutions of DTAB, CTAB, SDS, AOT and in DMSO.

The solubility of MN enhanced with the increase in DTAB, CTAB, SDS, and AOT micelle concentration. The solubilization capacity (K_M) of micelles were calculated and given in Table 3. The variation of solubility of MN as a function of micelles concentration of DTAB, CTAB AOT and SDS are shown in Fig 8.

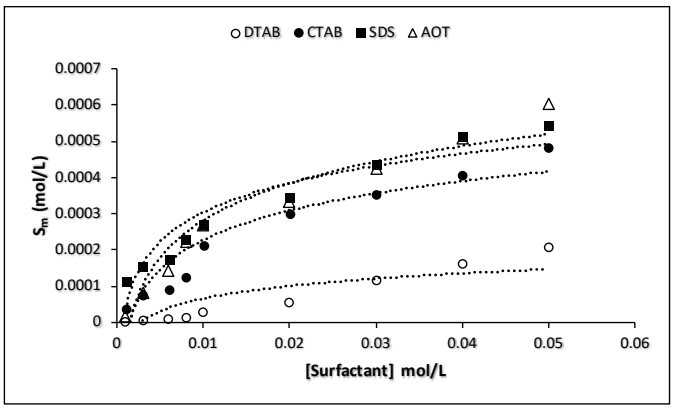


Figure 8. Phase–solubility plot of MN in DTAB, CTAB, SDS and AOT micelles (298 K)

3.5. Brinzolamide (BRZ)

The solubility of Brinzolamide (BRZ) in water was limited. The maximum absorbance of BRZ was recorded at 255 nm in ethanol, based on the valid concentration range of Lambert-Beer Law. In order to compare the influence of micelles, the corresponding absorption spectra of BRZ in the absence and the presence of DTAB, CTAB, SDS and AOT micelles are shown in Fig 9.

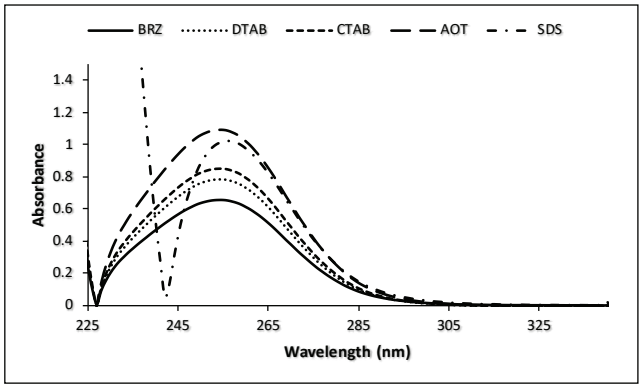


Figure 9. Absorption spectra of BRZ in micellar solutions of DTAB, CTAB, SDS, AOT and in ethanol.

As seen in Fig 9 the λmax of BRZ did not change in the presence of DTAB and CTAB micelles but with the surfactant concentration increased and also an increase in the absorbance of BRZ was observed. However, λmax of BRZ shifted from 255 to 257 nm in the presence of SDS and AOT micelles. The solubility of BRZ enhanced with the increase in DTAB, CTAB, SDS, and AOT micelle concentration. The solubilization capacity (K_M) of micelles were calculated and are given in Table 3. The variation of solubility of BRZ as a function of micelles concentration of DTAB, CTAB AOT and SDS are shown in Fig 10.

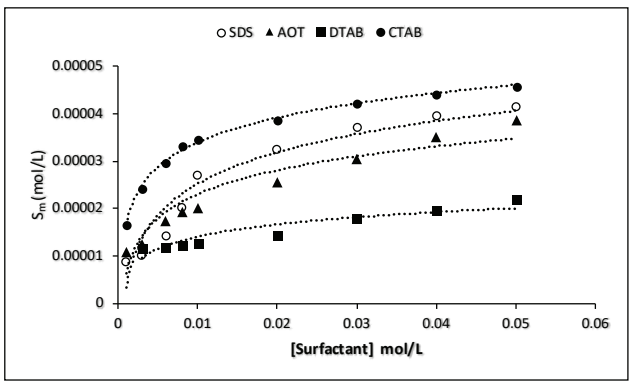


Figure 10. Phase–solubility plot of BRZ in DTAB, CTAB, SDS and AOT micelles (298 K)

3.6. Ganciclovir sodium (GS)

Ganciclovir sodium (GS) was freely soluble drug in water. The maximum absorbance of GS was recorded at 255 nm in water based on the valid concentration range of Lambert-Beer Law. In order to compare the influence of micelles, the corresponding absorption spectra of GS in the absence and the presence of DTAB, CTAB, SDS and AOT micelles were shown in Fig 11.

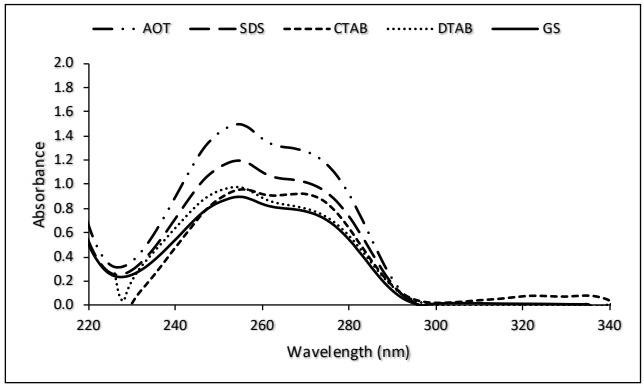


Figure 11. Absorption spectra of GS in micellar solutions of DTAB, CTAB, SDS, AOT and in water.

The λmax of GS did not change in the presence of DTAB, CTAB, SDS and AOT micelles but with the surfactant concentration increased an increase in the absorbance of GS was observed. The solubility of GS enhanced with the increase in DTAB, CTAB, SDS, and AOT micelle concentration. The solubilization capacity (K_M) of micelles were calculated and given in Table 3. The variation of solubility of GS as a function of micelles concentration of DTAB, CTAB AOT and SDS are shown in Fig 12.

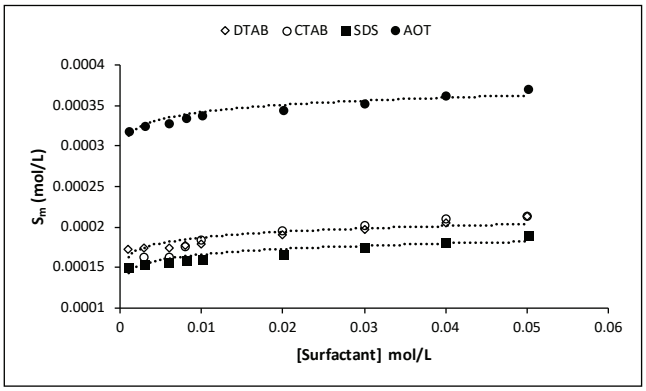


Figure 12. Phase–solubility plot of GS in DTAB, CTAB, SDS and AOT micelles (298 K)

4. Discussion

Each of the drugs used in this study had different chemical structures and properties. CBS, BRT and DEX were practically insoluble in water whereas MN and BRZ were sparingly soluble in water. Only GS was freely soluble in water among the drugs studied. It was observed that the solubility of drugs, in general, was almost constant until CMC. Then the significant increase observed after CMC indicated the solubilization of each drug molecule by the micelles. The solubilization capacities (K_M) of the selected micelles as models were calculated from the linear relationship between C_M and Sm which was valid for the postmicellar region and are given in Table 3. Fig 13 has illustrated C_M versus Sm as an example of determining the solubilization capacity of micelles.

Table 3. K_M (mmol/L) values of DTAB, CTAB, SDS and AOT for CBS, GS, MN, BRZ, BRT and DEX at 298 K. (Error limit in K_M ± 3%. The correlation coefficients are good in all cases (R^2 >0.9987).

CBS			
DTAB (mmol/L)	CTAB (mmol/L)	SDS (mmol/L)	AOT (mmol/L)
-	-	0.03	0.9
BRZ			
DTAB (mmol/L)	CTAB (mmol/L)	SDS (mmol/L)	AOT (mmol/L)
0.2	0.3	0.4	0.5
DEX			
DTAB (mmol/L)	CTAB (mmol/L)	SDS (mmol/L)	AOT (mmol/L)
0.4	0.6	0.8	0.9
MN			
DTAB (mmol/L)	CTAB (mmol/L)	SDS (mmol/L)	AOT (mmol/L)
5.1	6.4	7.2	8.5
BRT			
DTAB (mmol/L)	CTAB (mmol/L)	SDS (mmol/L)	AOT (mmol/L)
-	-	0.2	0.6
GS			
DTAB (mmol/L)	CTAB (mmol/L)	SDS (mmol/L)	AOT (mmol/L)
0.7	0.7	0.8	0.9

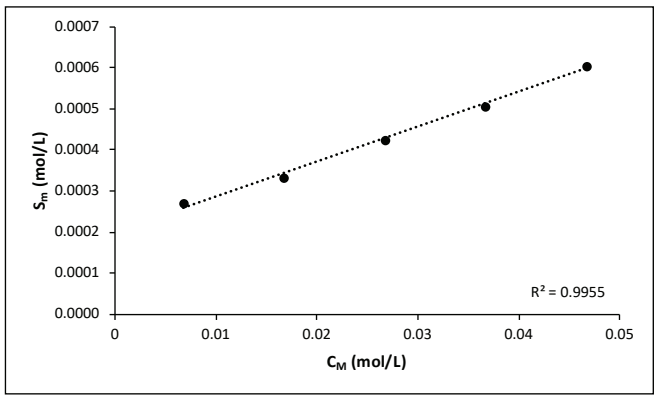


Figure 13. A representative plot determining the solubilization capacity of AOT micelles for MN.

As seen in Table 3, the solubilization capacities of the micelles contributed to the solubilization of drugs. On the other hand, the solubilization capacities of water-insoluble drugs CBS and BRT could not be calculated because no significant change was observed in their absorbance in the presence of cationic DTAB and CTAB. However, DEX, which is also insoluble in water, showed a tendency to dissolve in the presence of both types of surfactant micelles. This can be explained by hydrophobic and/or electrostatic interactions which constitute the basis of the interactions between drugs and surfactants. Surfactant micelles create a different microenvironment for substances by binding ions and molecules of drug to themselves through hydrophobic and/or electrostatic interaction. Therefore, the interaction of the drug with the micelle, their orientation varied in this microenvironment. While electrostatic attraction played a major role in the incorporation of drug ions into oppositely charged micelles, there was also a hydrophobic interaction depending on the structure of the drug. Mostly, the hydrophobic interaction assumes the role of driving force in solubilization, and in many cases, especially in environments where electrostatic repulsion was present, the hydrophobic interaction often predominated. All drugs used in the study were cationic (basic) except DEX which was strongly acidic). Accordingly, the low solubility tendency or no solubility tendency observed in the presence of DTAB and CTAB could be explained by the dominance of the hydrophobic interaction by the electrostatic repulsion forces between drug and micelles. For instance; since CBS and BRT had cationic character, the lack of solubilization tendency with DTAB and CTAB cationic micelles could be explained by the dominance of electrostatic repulsion over hydrophobic interaction. The solubilization tendency of DEX in the presence of DTAB and CTAB micelles might be expressed by the fact that DEX had an anionic character and therefore electrostatic attraction forces with DTAB and CTAB micelles played a role together with the hydrophobic interaction. However, the solubilization efficiency with anionic SDS and AOT micelles was a result of the dominant character of both electrostatic attraction and hydrophobic interaction. The strongest solubilization capacity observed in the presence of AOT could be explained by the fact that CMC of AOT (3.3 mmol/L) had more hydrophobic character than SDS (CMC: 8.0mM mol/L) (3).

In addition to monitoring variation of the absorbance change in spectrophotometric measurements used in solubilization and interaction studies, changes in the observed wavelength provided information about the degree of interaction. The shift of the wavelength at which a molecule exhibits maximum absorbance towards red (bathochromic effect) and shorter wavelengths in the presence of surfactant micelles was called blue shift (hypsochromic effect). There were changes in the absorption spectrum of the substance depending on the degree of interaction of the substance with the surfactant micelles, that was, where the substance is located in the micelle. A schematic representation of the micellar solubilization of a pharmaceutical compound was illustrated in Fig 14.

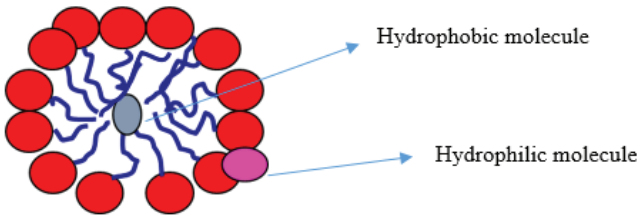


Figure 14. Possible location of a drug molecule in micelles

The further the wavelength of the substance shifts toward the red side in the presence of micelles, indicating that it moves toward the micelle core, i.e. the more hydrophobic region (8-10,18) This can be clearly seen when the absorption spectrum graphs of drugs in the presence of micelles are examined.

GS was the only water-soluble drug used in the study and has hydrophilic character. No significant shift in the wavelength of GS was observed in the presence of micelles. While the wavelength at which GS showed maximum absorbance did not change in the presence of DTAB and CTAB, it shifted from 255 to 256 and 257 nm in the presence of SDS and AOT, respectively. Whereas, MN, which had limited solubility in water, exhibits maximum absorbance at 231 and 238 nm. While no significant change was observed in the presence of CTAB and DTAB, it was observed that the wavelength shifted to 235 and 255 nm in the presence of SDS and AOT, respectively. This showed that the solubilization mechanism of MN was towards the micelle core in the presence of anionic micelles. The same situation applied to BRT, which had limited solubility in water. While no significant change was observed in the presence of DTAB and CTAB, the wavelength at 247 nm shifted to 249 nm in the presence of anionic micelles. The small shift in the wavelength of BRT observed in the presence of micelles indicates that the solubilization mechanism occurs at the micelle surface. When the solubilization results of water-insoluble CBS were examined, an increase in absorbance was observed in the presence of anionic and cationic micelles, while no significant shift was observed at the maximum absorbance wavelength of 201 nm. The solubilization results of water-insoluble CBS also showed, an increase in absorbance in the presence of anionic and cationic micelles, but no significant shift was observed at the maximum absorbance wavelength of 201 nm. This behaviour indicated that the solubilization mechanism of CBS into micelles occurs on the micelle surface. The same situation was applied to the solubilization mechanism of water-insoluble BRZ. While no change was observed in the presence of DTAB and CTAB, it was observed that the wavelength of BRZ at 255 nm, shifted to 257 nm in the presence of SDS and AOT micelles i.e. solubilization mechanism occurs on the micelle surface. Among the water-insoluble drugs, the most significant wavelength shift was observed in the case of DEX. The wavelength of DEX at 247 nm shifted to 253 nm in the presence of SDS and AOT micelles. This shift in the observed wavelength indicated that the solubilization mechanism occured from the micelle interface toward the micelle core.

Experimental data obtained from the presented study showed that as the hydrophobicity of surfactants increases the micellar solubilization of drugs is enhanced, especially for insoluble or poorly soluble drugs in water. The lower the CMC value of a surfactant, the more hydrophobic the micelles. The micellar solubilization efficiency followed the order for cationic micelles DTAB < CTAB and for anionic micelles SDS < AOT micelles. The most effective surfactant was also found to be AOT which has a two-branched hydrophobic tail that contributes to the highest micellar solubilization capacity. From this perspective, AOT had a low CMC value and the highest hydrophobic character to solubilize drug molecules with micelles. Therefore, it could be concluded that AOT played a very important role in increasing the solubility of drugs from a pharmacological point of view.

5. Conclusion

Micellar solubilization provided the potential to characterize slightly, sparingly, poorly, and insoluble drugs in water. Furthermore, surfactant micelles have been widely used as a biomimetic model for membrane systems in pharmaceutical research. The main purpose of this paper was to deal with the solubilization of different kinds of drugs by the various types of micelles. In our point of view, the results of this study can be used in drug formulation and design related to solubilization problems as well as assessing the pharmaceutical dosage forms.

Conflicts of interest: The authors have no conflicts of interest to declare.

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Original Article

The Cytotoxic and Genotoxic Potential of *Euphorbia macroclada* Boiss. Extract on Colon Cancer Cells

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Abstract

Introduction: Cancer continues to be the second most common cause of death globally. There is currently any proven treatment medication for cancer. Chemotherapy is one of the modern therapeutic approaches that not only kills cancer cells but also has major negative effects on healthy cells. 80% of people worldwide still get their basic medical care from traditional medications, according to data from the World Health Organization. Scientists have created new approaches to treating cancer by combining radiation, chemotherapy, and surgery with a variety of phytochemicals derived from different plant species. This is because plants and active pharmaceutical ingredients derived from plants have minimal to no side effects and high activity. The *Euphorbia macroclada*, found in different locations of Turkey, has long been utilized by the public for its traditional medicinal properties in treating a range of illnesses. Research conducted in current scientific databases has established that *Euphorbia macroclada* exhibits significant antioxidant activity.

Methods: This study aims to evaluate the potential antiproliferative effect of the dichloromethane extract of the *Euphorbia macroclada* on HT-29 and HCT-116 cancer cell lines using the *in vitro* MTT technique. Additionally, the possible genotoxic activity using the Comet assay on human colon cancer cells were also done.

Results: The IC_{50} concentration of *Euphorbia macroclada* was determined to be 133,835 µg/mL in HCT-116 cells and 111,215 µg/mL in HT-29 cells after 24 h. Concurrently with the MTT results, it was established that there was an increase in DNA damage in a dose dependent manner than those of control.

Conclusion: Following the collection of these data with the *Euphorbia macroclada*, it has been determined that dichloromethane extract of the *Euphorbia macroclada* has the potential to serve as a therapeutic agent for the treatment of colon cancer. On the other hand, in vivo and in vitro studies on this subject are needed.

Keywords: *Euphorbia macroclada*, colon cancer, cytotoxicity, genotoxicity

1. Introduction

Cancer is a highly detrimental illness that has a significant impact on the human population. The disease is defined by the persistent and uncontrolled growth of cells in the human body. There is a persistent need for novel medicines to address and mitigate this potentially fatal illness. Presently, the available treatments encompass chemotherapy, radiation, and pharmacologically synthesized medications. Existing techniques, such as chemotherapy, are restricted in their application because they have adverse effects on host tissues that are not the intended target. Consequently, experts have directed their attention on the utilization of alternative medicines and therapies in the battle against cancer. Currently, there is a trend towards using natural products that are believed to have fewer adverse effects than standard treatments like chemotherapy. As a result, there is a growing need for alternative medicines that contain naturally occurring substances with anticancer properties (1). Plants are essential elements of both traditional medicine used globally and current medication development research. This is because they are inexpensive, readily available, and have little side effects (2).

Euphorbia species, referred to as "euphorbia" in Turkish, have been utilized in traditional medicine in Turkey and various regions globally for the treatment of dermatological diseases, wounds, warts, gonorrhea, migraines, cancer, and intestinal parasites (3). There has been a growing interest in Euphorbia species due to their varied structures and therapeutic significance. These species possess a range of biological activities including cytotoxic, antitumor, antibacterial, anti-inflammatory (4), antiproliferative, antiviral, antidiarrheal, antimicrobial, and antipyretic-analgesic effects (5). Research indicates that numerous varieties of Euphorbia are used for medicinal purposes, specifically in the management of ailments such as respiratory infections, skin irritations, digestive disorders, inflammatory infections, body discomfort, microbiological infections, and snake or scorpion envenomation. (6-10).

Euphorbia species preparations are used in traditional therapy as skin medicine to alleviate various skin conditions such as itching, warts, eczema, hair loss, acne, dermatitis, boils, sunburn, calluses, rashes, irritation, and pustules. These preparations possess antiseptic, disinfecting, and emollient features (8).

Euphorbia macroclada (*E. macroclada*) is an important plant species commonly found in various regions of Central, Eastern and Southern Anatolia. Among these regions, the most common cities are Ağrı, Ankara, Antalya, Bitlis, Burdur, Çorum, Elazığ, Erzincan, Erzurum, Eskisehir, Gümüshane, Hakkari, Kars, Kayseri, Malatya, Maras, Mardin, Niğde, Osmaniye, Sanliurfa, Tunceli, Van and Yozgat (11). The fact that the plant spreads over such a wide area shows its ability to adapt to different environmental conditions. The use of the plant's latex in traditional medicine reflects its long-standing cultural and medicinal importance in the region. This latex is traditionally used to treat various digestive disorders such as constipation, ulcers, hemorrhoids, and various skin problems such as wounds, stretch marks, warts, eczema, and fungal infections, arthritis, scorpion stings, bee stings, malaria, and body parasites (12).

The current scientific literature study reveals that comprehensive investigation has been done on numerous species of Euphorbia, however only a few investigations have been carried out on *E. macroclada.* Kirbag et al. evaluated the potential antimicrobial activities of *E. macroclada, E. aleppica, E. szovitsii var*. and found that they had antibacterial and antifungal activity (13).

Mahmoudi et al. considering the paucity of studies on the antifungal effects of *E. macroclada latex*, aimed to evaluate the antifungal activity of *E. macroclada* latex in hospitalized patients. As a result of this study, it was determined that *E. macroclada* latex showed antifungal activity against some pathogenic Candida species. (14).

The literature also includes studies on the antioxidant properties of *E. macroclada*. A study was conducted to examine the antioxidant activity in several extracts of *E. macroclada*. The results

of the research revealed that all tested extracts of *E. macroclada* exhibited more antioxidant activity compared to BHT and α-tocopherol (15).

In another study the chemical composition and antioxidant properties of *E. macroclada* were examined.The results indicated that both the ethanolic and aqueous extracts of this plant exhibited significant antioxidant activity, suggesting that it could potentially be utilized in the prevention of certain diseases associated with oxidative stress (16).

A further study was conducted to analyze the chemical composition and antioxidant capabilities of *E. gaillardotii* and *E. macroclada*. The levels of rutin, hesperedin, and hyperoside in the extract of *E. macroclada* were significantly higher compared to the extract of *E. gaillardotii*. In contrast to the absence of rosmarinic acid in the *E. gaillardotii* extract, a significant amount of rosmarinic acid was detected in the *E. macroclada* extract. Therefore, the antioxidant activity of the extract from *E. macroclada* stems and leaves can be attributed to the presence of a significant quantity of e chlorogenic and rosmarinic acid, rutin and quercetin compounds known for its antioxidant properties (17).

Recent investigation has indicated that Euphorbia species possess various pharmacological activities, including *in vitro* anti-cancer effects, owing to their abundant synthesis of bioactive chemicals (18). The plant is believed to have cytotoxic properties through various mechanisms, including its impact on cell proliferation and differentiation, inhibition of apoptosis and metastasis, excessive formation of reactive oxygen species, and promotion of angiogenesis (19).

This study aimed to evaluate the antiproliferative properties of the dichloromethane extract of *E. macroclada* on colon cancer cell lines (HT-29 and HCT-116) using the MTT assay. Furthermore, our objective was to examine its genotoxic potential by employing the Comet assay on human colorectal cancer cells, marking the first instance of such investigation.

2. Methods

2.1 Data collection and identification of plant sample

The aerial parts of the *E. macroclada* were collected from its natural environment and crowded populations in the Tunceli province of Türkiye's eastern Anatolia region. The taxonomic description of the species was made using appropriate scientific sources. The plant dried at room temperature and in the shade. Additionally, a sample of it has been kept as voucher specimen (MARE 20618) at Herbarium of Marmara University Faculty of Pharmacy.

2.2 Preparation of crude extract

The aerial parts of the *E. macroclada* plant were dried at room temperature and ground into powder. Dichloromethane extract will be obtained by the maceration method of the aerial parts. After the maceration process, the liquid part were filtered through filter paper. The solvents were then filtered via filter paper and evaporated under low pressure in a rotary evaporator, with the obtained dried extracts stored in the refrigerator at +4 ºC.

2.3 Preparation of the extracts

The dried residues (10 mg) were dissolved in 100 µL of dimethyl sulfoxide (DMSO) and diluted to a final volume of 2 mL with distilled phosphate buffer saline (PBS) then filtered through 0.22 µm microbiological filters. The concentrations of 10, 20, 50, 100, and 200 µg/mL were achieved by further dilution.

2.4 Determination of antiproliferative activity

Human colorectal cancer cell lines, HT-29 (ATCC, HTB-38) and HCT-116 (ATCC, CCL-247), were used in this study to investigate the effects of dichloromethane extract of *E. macroclada*. HT-29 and HCT-116 cells were cultured in DMEM medium supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C and 5% CO_2 atmosphere. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphentyltetrazolium bromide) method was used to determine the cytotoxic effects on HT-29 and HCT-116 cancer cells (20).

The cells (1 × 10^4 cells per well) were seeded in 96-well plates and cultured for 24 h. After that, the cells were treated with different concentrations of dichloromethane extract of *E. macroclada*. The plates were incubated for 24 h. Then the medium was discharged from the 96-well plate and 10 µL of 3-(4,5 – dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide (MTT) was added per well. Additionally the plate kept for 4 h in 5% CO_2 humidified incubator at 37°C to allow reaction of yellow colored MTT reduced by mitochondrial dehydrogenases in viable cells to form pink to purple because of formazan. Excess MTT was taken off, and the resulting formazan crystals were dissolved in 100 µL of dimethyl sulfoxide (DMSO). The optical density (OD) was read at 570 nm using 630 nm as reference wavelength on a multiwell plate reader (Biotech Instruments, Winooksi, VT, USA). All the experiments were repeated twice, and each treatment was run in triplicate.

The percentage of cell viability was calculated the following formulae:

% Cell viability= Mean (OD) of treated cells / Mean (OD) of the untreated cells × 100

2.5 Determination of genotoxicity

The genotoxic effects of *E. macroclada* on HT-29 and HCT-116 cells were evaluated by using alkaline single cell gel electrophoresis assay (Comet Assay) according to Singh et al. (21) with slight modification. Comet Assay was conducted *in vitro* in control, positive control (hydrogen peroxide) and plant extract groups. To determine the genotoxic potential of *E. macroclada* cells were seeded into 6-well cell culture plates (approximately 5x10^5 cells per well) with cell culture medium and incubated at 37 ºC in 5% CO_2 for 24 h. for cell proliferation. After 24 h, three concentrations of *E. macroclada* (100, 200 and 400 µg/mL in 1% DMSO) were added to the cells and incubated for another 24 h at 37ºC, here DMSO (0.1 %) was used as control. A concentration of 50 µM H_2O_2, which is known to cause DNA damage, has been used as a positive control. After incubation, the cells were washed with PBS, harvested using trypsin/EDTA and collected for centrifugation at 400 x g for 5 min. at 4 ºC. The supernatant was discharged, and the cell density was adjusted to 1x10^6 cells/ml by using cold PBS. 10 µL cell suspension was mixed with 90 µL of 0.6% low melting agarose (LMA) and added to the slides precoated with 1% high melting agarose. After solidification of the agarose, the slides were immersed in lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100 and 10% DMSO, pH 10) for 1 h at 4°C. Upon removing the slides from the lysing solution, they were washed with cold PBS and placed in a horizontal electrophoresis tank side by side. DNA was allowed to unwind for 20 min. in freshly prepared alkaline electrophoresis buffer containing 300 mM NaOH and 10 mM Na_2EDTA (pH 13.0). Then, electrophoresis was run at 25 V, 300 mA for 20 min. at 4 °C under minimal illumination to prevent further DNA damage. The slides were washed three times with a neutralization buffer (0.4 M Tris, pH 7.5) for 5 min. at 4 ºC and then treated with ethanol for another 5 min. before staining. Dried microscope slides were stained with ethidium bromide (20 µg/mL in distilled H_2O; 50 µL/slide) covered with a coverslip and analyzed using a fluorescence microscope (Olympus BX51, Japan) at a 400 × magnification. Percentage of DNA in the tail (% DNA_T) were scored using Comet Image Analysis-BABSOFT, and A total of 100 cells in triplicate per group were used to calculate the DNA damage. The DNA percentage in tail was used as the primary measure of DNA damage according to Hartmann et al. (22).

2.6 Statistical analysis

All data were expressed as the mean and standard error of the mean, derived from the repetition of each test at least three times. Statistical analyses were conducted using the SPSS 20.0 software. Student t-test was used to assess IC_{50} values derived from MTT test results.

The Comet assay findings were assessed using one-way analysis of variance (ANOVA) with the One-Way ANOVA test. Post-hoc analyses, specifically comparing the differences between groups, were conducted using Fischer's least significant difference (LSD) test. Comet data were expressed as the mean ± standard deviation (SD) of the data. *p*-values of less than 0.05 were considered statistically significant.

3. Results

The antiproliferative effect of *E. macroclada* dichloromethane extract on human colon cancer cell lines HT-29 and HCT-116 was assessed using the MTT assay. Various concentrations of *E. macroclada* extract (10-20-50-100 and 200 µg/mL) were administered to both cell lines, and the viability percentages were determined after 24 hours. The results indicate a dose-dependent inhibition of cell proliferation in HT-29 cells treated with *E. macroclada* extract (Fig 1A). The Half Maximal Inhibitory Concentration (IC_{50}) value for HT-29 cells was calculated as 111.215 µg/mL. *E. macroclada* inhibited 79.5% of HT-29 cells at a concentration of 200 µg/mL.

The genotoxic potential of *E. macroclada* was assessed by exposing cancer cells to various dosages of *E. macroclada* (ranging from 100 to 400 µg/mL for HCT-116 cells and from 50 to 400 µg/mL for HT-29 cells) for 24 hours. The extent of DNA damage was measured using the Comet Assay. Nuclei exhibiting DNA damage displayed a comet-like structure characterized by a luminous head and a trailing tail, while nuclei with intact DNA exhibited a spherical shape devoid of a tail. Each figure depicted a characteristic comet tail formed by the observed cells. The data was collected from at least 100 cells on two slides in each experiment. Significant alterations in the percentage of tail DNA were observed between the control cells and cells

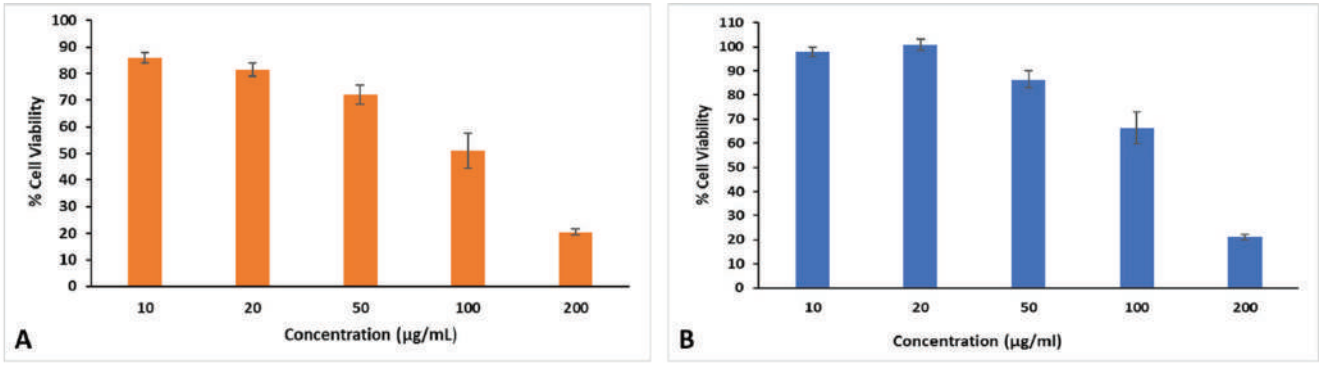

Figure 1. The antiproliferative effect of *E. macroclada* on HT-29 (A) and HCT-116 (B) cells.

In HCT-116 cells, viability showed a dose-dependent decline following exposure to *E. macroclada* extract, particularly at concentrations of 50 µg/mL and above (Fig 1B). At a concentration of 200 µg/mL, *E. macroclada* demonstrates an inhibition of 78.9% on HCT-116 cells, while the IC_{50} value for these cells was calculated as 133.835 µg/mL.

that were exposed to *E. macroclada,* as indicated in Table 1 and Table 2. As it is seen tables significant increase in the percentage of DNA damage and length of comet tail in a concentration-dependent manner in cancer cells exposed to *E. macroclada*. After 24 h. *E. macroclada* treatment, cancer cells showed different sizes, fragmentation and comet structures with increased

Table 1. The genotoxic effect of *E. macroclada* extracts on HCT-116 cells

	%DNA_T				
	Control	**Positive Control**	**100 µg/mL**	**200 µg/mL**	**400 µg/mL**
HCT-116	23.82 ± 0.56	52.64 ± 2.75***	32.16 ± 0.39$^{*,+++}$	45.44 ± 2.02$^{***,+}$	All Cells were Death

Data are represented as mean±standard deviation. Groups of data were compared with an analysis of variance (ANOVA) followed by Tukey's multiple comparison tests.$^{*}p$ <0.05, $^{**}p$<0.01 and $^{***}p$ <0.001 compared with the control group,^{+}p<0.05, ^{++}p<0.01 and ^{+++}p<0.001 compared with the positive control group.

% DNAT : Percentage of DNA in the tail

Table 2. The genotoxic effect of *E. macroclada* extracts on HT-29 cells

	%DNA_T					
	Control	**Positive Control**	**50 µg/mL**	**100 µg/mL**	**200 µg/mL**	**400 µg/mL**
HT-29	25.49 ± 0.87	64.72 ±1.79***	35.75 ± 4.29$^{***, +++}$	48.27 ± 0.11$^{***, +++}$	70.94 ± 1.10$^{***,+}$	All Cells were Death

Data are represented as mean±standard deviation. Groups of data were compared with an analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. $^{*}p$ <0.05, $^{**}p$<0.01 and $^{***}p$ <0.001 compared with the control group, ^{+}p<0.05, ^{+}p<0.01 and ^{+++}p<0.001 compared with the positive control group.
% DNA_T : Percentage of DNA in the tail

% tail intensity when compared to the normal cells. The results indicated that *E. macroclada* induced DNA damage in a concentration-dependent manner and there were significant changes in the tail % of DNA between the normal cells and cancer cells at all doses.

4. Discussion

Despite significant improvement in chemotherapeutic strategies for cancer treatment, current methods still fall short in effectively combating the disease. This statement is mainly due to the non-specific targeting of tumor cells by drugs, their adverse side effects, and the emergence of drug resistance through various mechanisms. Consequently, there is a desperate need to explore alternative therapeutic agents that can enhance the efficacy of existing chemotherapeutics. In this context, the potential application of compounds isolated from natural sources as chemotherapeutic or chemopreventive agents is increasingly recognized.

The genus Euphorbia, which includes over 5000 species, is valued as a therapeutic resource in traditional medicine. Numerous scientific articles have shown the utilization of Euphorbia species in the treatment of various ailments, including microbiological infections, malaria, cancer, ringworm, tuberculosis, as well as sexually transmitted diseases like syphilis and gonorrhea (8, 23, 24). In addition, the latex from Euphorbia plants has been utilized for the treatment of lice infestations and mange, which is seen in animals. Furthermore, it has been documented that this substance has been used in the therapeutic management of parasitic illnesses, such as measles. Moreover, the investigation discussed the utilization of Euphorbia species for the management of respiratory ailments such as asthma, cough, and pneumonia (8, 23, 25).

The Euphorbiaceae family is known for its wide variety of diterpenoids that possess therapeutic activities such as antiproliferative, anti-inflammatory, and immunomodulatory properties (8). Notably, some diterpenoids from *Euphorbia esula L.* have shown antitumor activity against both multidrug-resistant (MDR) and non-resistant human gastric cancer cells (26, 27). Recently, an extract derived from *Euphorbia esula L.* was reported to exhibit antiproliferative activity against various types of cancer such as lung, cervix, stomach, breast, and liver. Additionally, diterpenoids isolated from the ethanol extract of *Euphorbia helioscopia* have demonstrated cytotoxic effects in renal cancer cell lines (28, 29).

However, investigations on the effects of plant extracts from the *Euphorbia* genus on human colon cancer cell lines *in vitro* is still limited. In a study, the latex of *E. trigona Mill.* was tested on the HT-29 colon cancer cell line and found to be inactive (30). In Aliomrani and colleagues' study, it was reported that the dichloromethane extract of *E. turcomanica* exhibited cytotoxic effects on HT-29 cells, with an IC_{50} value of 115 µg/mL as determined through their investigation (31).

The literature review reveals that numerous studies have demonstrated that *E. macroclada* have high antioxidant activies. However, the antiproliferative effect of the plant has only been the subject of two studies. One of the investigations aimed to examine the harmful effects of dichloromethane, ethylacetate, and methanol extracts from *E. macroclada* Boiss and the plant's latex on the MDA-MB-468 breast cancer cell line using the MTT method. The findings of this research study showed that the dichloromethane and

ethylacetate extracts exhibited cytotoxic properties against the MDA-MB-468 cell line, however the methanol extract and latex did not display cytotoxicity at the evaluated quantities. According to the findings of the study, *E. macroclada* Boiss non-polar extracts had shown greater cytotoxic action (32).

In the second study, using the MTT method, Tas et al. (2018) investigated the effects of acetone extracts of *E. macroclada* Boiss flower stems and leaves on the human breast cancer cell line (MCF-7) and mouse fibroblast healthy cell line (L-929). The study has found that the cytotoxicity of the extracts changed based on the concentration and duration of exposure, and the viability of cells decrease as the concentration increased. The acetone extract derived from the leaves of the plant exhibited superior efficacy compared to other extracts at all measured time intervals. Furthermore, it was found that after 72 hours of incubation, the leaf extract of *E. macroclada Boiss* exhibited greater cytotoxic activity in MCF-7 cell lines when compared to the control (L-929) (33).

The findings from these two investigations provide evidence about the potential of *E. macroclada* to be utilized as an anticancer agent. There is currently no research in scientific literature indicating the cytotoxic effects of *E. macroclada* on colon cancer cell lines.

In our study, the IC_{50} concentration of *E. macroclada* was determined to be 111.215 µg/mL in HT-29 cells and 133.835 µg/mL in HCT-116 cells. These findings suggest that the dichloromethane extract of *E. macroclada* is more effective in a dose-dependent manner on HT-29 cells when compared to HCT-116 cells.

Additionally, the genotoxic effects of the dichloromethane extract of *E. macroclada* on colon cancer cell lines (HT-29 and HCT-116) significantly increased in a dose-dependent manner in correlation with the MTT results.

5. Conclusion

Based on our research, it can be concluded that the components of *E. macroclada* may have cytotoxic effects on colon cancer cells. Furthermore, the genotoxic effect of the plant extract has been demonstrated for the first time. However, further research is needed to purify and identify the active components in order to develop a new cytotoxic agent.

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Original Article

Cytotoxic activities of *Helichrysum arenarium* on ECV 304 and Ishikawa cells

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Abstract

Introduction: In this study, the cytotoxic activity of *Helichrysum arenarium* plant on various human cells was examined.

Methods: ECV 304 (human endothelial cell line) and Ishikawa cells (human endometrial adenocarcinoma cells) were used in order to find out the cytotoxicity results. Dimethyl sulfoxide (DMSO) extract of *Helichrysum arenarium* showed the most significant cytotoxicity effect on these cells and also lipid peroxidation (MDA) assay was performed. Also, the essential oils of immortelle extracts were analyzed using gas chromatography-mass spectrometry (GC-MS).

Results: Cell viability and MDA levels were decreased by the extract of *Helichrysum arenarium* both in Ishikawa cells and ECV304 cells at 500 µg/mL. 2-Palmitoylglycerol and palmitic acids were found as the major essential oils in DMSO extracts of *Helichrysum arenarium*.

Conclusion: *Helichrysum arenarium* may show an antioxidant activity *in vitro*.

Keywords: *Helichrysum arenarium,* cytotoxicity assay, MDA assay, GC-MS, Ishikawa cells

1. Introduction

Helichrysum arenarium (L.) Moench is an herbaceous perennial plant which belongs to the Asteraceae family (1,2). *Helichrysum arenarium* (L.) Moench has been used in folk and modern medicine since antiquity (3). Due to its diuretic, anti-infective, hepatoprotective, detoxifying, cholagogue, and choleretic effects it has been used in various treatments in folk medicine. These treatments include regulating gallbladder disorders, relieving stomach pain, improvingressings, relieving coughs, treating erythema, and diabetes mellitus (4-6). Today, scientific studies confirm the characteristics of Helichrysum arenarium since its antioxidant, antimicrobial, antifungal, and anti-inflammatory properties. Some of the most popular utilizations are treating digestive problems and infections, ,

improving respiratory conditionssupporting heart health, and the nervous system. It has been traditionally used in European folkloric medicine for many years as well as in modern times.

Several studies have been carried out over the past years to highlight some of the traditional uses of *Helichrysum arenarium* extract and other potential applications. Many studies aimed to determine how gold grass behaves as an antimicrobial, anti-inflammatory, and natural antioxidant agent (4,7-9). *Helichrysum arenarium* helps improve heart health due to its anti-inflammatory properties and also shows positive cardiovascular effects on hearth health (10). Previous studies have showed that *Helichrysum arenarium* contains special flavonoid antioxidant compounds that inhibit cancer growth and oxidative stress. Also, *Helichrysum arenarium* has anti-inflammatory properties and helps intestinal healing (11). Studies have shown that *Helichrysum arenarium* extract is effective against radiation-induced DNA damage, cell death, and mutation, as well as cancerous tumor growth. It also helps to stimulate the stomach secretions (12). In clinical trials, has been shown to contain flavonoids and fluoroglycinols, which can kill harmful bacteria, fungi, and viruses, and has been strong enough to reduce the risk of HIV contraction (13). On the other hand, *Helichrysum arenarium* is a diuretic and supports digestion through natural routes (14). Additionally, it is also used in the production of some creams (15).

In this study, the cytotoxic activities of the *Helichrysum arenarium* plant on ECV 304 and Ishikawa cells were examined using MTT assay. Malondialdehyde levels were also measured using TBARS assay. Gas chromatography mass spectrometry (GC-MS) was used for the analysis of the chemical composition of the *Helichrysum arenarium* extracts.

2. Methods

2.1. Preparation of plant extracts

Helichrysum arenarium were pulverized to weigh 10 mg. It was then extracted in DMSO (VWR, Cat No: 16I054006, France) at concentrations of 10 mg/mL.

2.2. Cell culture

In this study, ECV 304 and Ishikawa cell lines were studied. The cells were grown in a 37 °C incubator balanced with 5% CO_2. The cells were diluted to 10^5 cell/mL with DMEM (1x) medium (GIBCO) containing 10% of FBS, L-glutamine, and penicillin-streptomycin (16, 17). Both ECV and Ishikawa cells were in cell culture petri dishes for 24 h before treatment with the extracts. Various concentrations of the plant extract were applied into the cells for 24 h.

2.2.1. Cytotoxicity assay

To determine the cytotoxic effect of plant extracts on the cells, MTT (3- (4,5-dimethyl thiazole-2-yl) -2,5-diphenyl tetrazolium bromide) assay was used (18). Here 10 mg/mL of DMSO extracts was prepared as the stock solution, and diluted with serum medium (5 µg/mL, 10 µg/mL, 50 µg/mL, 500 µg/mL). The cells were diluted to 10^5 cells/mL. Then, 90 µL of cells were placed in each well of 96 well-plate. The cells were incubated for 24 hours in a 5% CO_2 incubator at 37 ºC. After 24 h, 10 microliters of plant extract were applied. Then, the MTT solution with a concentration of 5 mg/mL dissolved in sterile PBS was added. After 3 h incubation, 100 µL of 50% DMSO-50% isopropanol mixture was added and incubated for 45 minutes at room temperature. Finally, optical density was measured with 570 nm with a multiwell spectrophotometer (Thermo Scientific, USA). Cytotoxicity index (CI) was calculated to following formula;

CI % (Cytotoxicity index) = 1- OD treated wells / OD control wells X 100.

2.3. Lipid peroxidation (LPO)

Oxidizing agents can alter lipid structure forming lipid peroxides that result in the formation of malondialdehyde (MDA), which can be measured as Thiobarbituric Acid Reactive Substances (TBARS). 150 µL of standards and samples were added to each well, and 75 µL of TBA Reagent was added to each well. The optical density of each well was measured sing a microplate reader at 532 nm. Then microplate was incubated for 2-3 hours at 45-50 °C. The optical density of each well using a microplate reader was measured at 532 nm.

2.4. GC-MS analysis

1 mL of the extract sample was transferred into the test tube. 10 mL of hexane was added to the test tube and the mixture was mixed using a vortex for 1 minute. Then, 0.5 mL methanolic 2 N KOH was added and the mixture was mixed using a vortex for 1 minute and kept in the dark for 30 minutes. Then, 0.5 mL 0.1 N aqueous HCl was added and kept in the room temperature for 5 minutes for phase separation. Clear phase separation was injected into the GC system. Essential oils were analyzed by using a Shimadzu GC-2010 plus gas chromatography (Shimadzu Scientific Instruments, Columbia, MA, USA), equipped with an Rtx®-5MS column (30 m × 0.25 mm ID, 0.10 µm film thickness) (Restek, USA) where helium was used as carrier gas (average flow rate, 1.50 mL/min). The oven temperature program increased from 140 °C (5 min) to 240 °C at 4 °C/min and kept isothermal at 240ºC for 15 min. Injector temperatures were 250 °C and, mass spectrometer parameters were as follows: source and interface temperatures, 250 °C and 275 °C, respectively; electron Impact ionization mode, at 70 eV; acquisition mass range,40–400 m/z with a scan speed of 2000 amu/s and a scan-interval of 0.20 s. Data handling was supported by the software GC-MS solution, ver. 2.51 (Shimadzu).

The oil content of algal extracts was identified using the National Institute Standard and Technology (NIST) library. Components' relative percentages were calculated based on GC peak areas without using correction factors.

2.5. Statistical analysis

GraphPad Prism software was used to perform statistical analysis according to Dunnett's test.

3. Results and Discussion

The cell viability of the *Helichrysum arenarium* plant on ECV 304 was calculated as 81.8% at concentrations of 5 µg/mL and 56.35% at 500 µg/mL respectively. However, the cell viability levels in Ishikawa cell line were found as 83.06% at 5 µg/mL and 39.24% at 500 µg/mL. These results indicate that *Helichrysum arenarium* may show cytotoxic effect on Ishikawa cells. Mao et al. (19) reported that flavonoids obtained from *Helichrysum arenarium* may have potential for preventing AS formation (Fig 1).

In the study of Les et al. (20) which was done with the *Helichrysum stoechas (L.) Moench* plant, the basic phytochemical components of the plant were determined and evaluated in terms of antioxidant, antidiabetic, and neuroprotective activities of this plant. As a result of the phytochemical analysis of the plant extract prepared with methanol, ten different components were identified, some of which include two heterodimeric phloroglucinol, oryzanol, one homodimeric α-pyron, three phenolic acids, p-hydroxybenzoic, 5,7-dihydroxy -3,6,8-trimethoxyflavone, isoquerythrin. Bioassays have shown significant antioxidant and antiproliferative effects. The antiproliferative effects in HeLa cell line (line of human cells derived from cervical cancer) were assessed using MTT assay (mitochondrial viability test). It was found that at doses of 60 µg/ml and above applied to the cancer cell line HeLa cells, cell viability decreased approximately by 25%. In addition, no significant difference was observed at low concentration doses. In our study, Ishikawa cells were used as a cancer cell line, and cell proliferation was decreased gradually with increased concentrations of the extract.

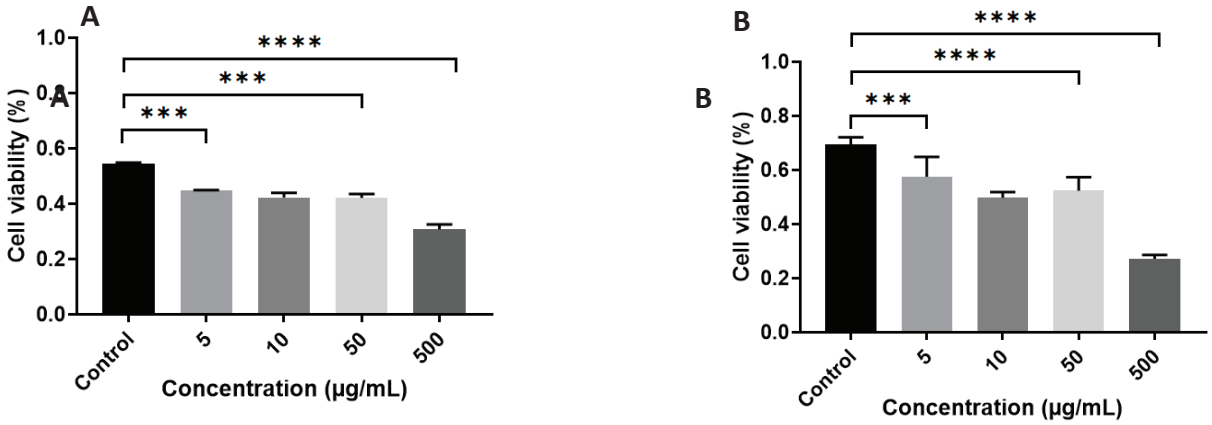

Figure 1. Cell viability results of ECV304 (A) and Ishikawa cells (B) in the presence of *Helichrysum arenarium* DMSO extracts (**** $p < 0.0001$).

TBARS assay was used to analyze the biological antioxidant level of *Helichrysum arenarium* (Fig 2). In the experiment, *Helichrysum arenarium* was applied to ECV304 cells (Fig. 2A) and Ishikawa cells (Fig.2B). According to the results, 50-250-500-2500 µg/mL doses of *Helichrysum arenarium* applied to cancer cell line caused a significant inhibition in MDA levels. At the dose of 5000 µg/mL, a similar result was obtained with the MDA level of the control group.

Elaguel et al. (21) applied the antioxidant *Lawsonia inermis*(henna) essential oil to the human lymphatic cancer cell line (Raji) and analyzed the biological antioxidant levels with the TBARS assay method. They reported that henna essential oil inhibited the MDA level significantly (80%).

There have been found very few studies in the literatüre related with the effects of antioxidant substances on MDA levels in human cell lines, However, there are some studies showing that plants with antioxidant properties can inhibit MDA levels other cancerous animal tissues (22, 23). Elaguel et al. (21) reported that when 50-5000 µg/mL doses of *Helichrysum arenarium* applied to ECV304 cells, MDA levels significantly decreased. In our opinion, the decrease in MDA level of the study above may be due to the inhibition of peroxidase enzyme activity and the decrease in lipid peroxidation.

In our study, 23 essential oil compositions were determined in the extract of *Helichrysum arenarium*. The most abundant compounds were 2-Palmitoylglycerol and palmitic acids. The detailed composition of the identified compounds is given in Table 1.

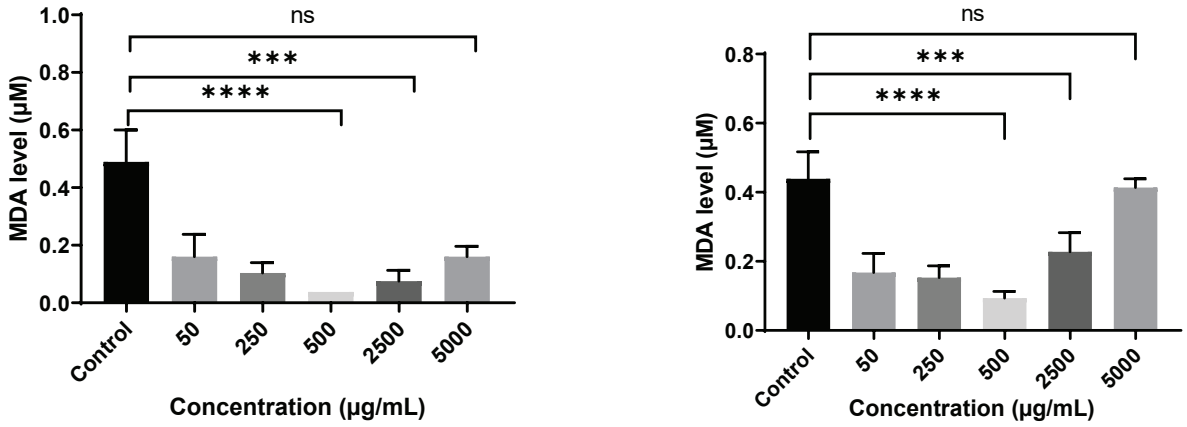

Figure 2. Changes of malondialdehyde levels in the presence of *Helichrysum arenarium* DMSO extracts in ECV304 cells (A) and Ishikawa (B) (**** $p < 0.0001$).

Table 1. Chemical composition analysis of *Helichrysum arenarium* extract detected by GC-MS.

	Compound	***Helichrysum arenarium*** (%)
1	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	18,79
2	Hexadecanoic acid (Palmitic acid)	16,75
3	Phenol	14,59
4	9-Octadecenoic acid (Oleic acid)	9,49
5	9,12-Octadecadienoic acid (Linoleic acid)	7,70
6	Octadecanoic acid (Stearic acid)	7,36
7	N ,N-Dimethylpalmitamide	4,94
8	Nonadecanoic acid, 18-oxo-	3,63
9	Tridecanal	2,55
10	Borane, diethylmethyl-	2,32
11	Tetradecanoic acid (Myristic acid)	2,29
12	Palmitic acid, 2-(tetradecyloxy)ethyl ester	1,81
13	1-Octanol, 2-butyl-	1,22
14	Tetratetracontane	1,05
15	Triacontane, 1-Bromo-	0,94
16	Dodecane	0,81
17	Tetratriacontane	0,81
18	1H-Purin-6-amine, [(2-fluorophenyl)methyl]-	0,73
19	Nonadecane	0,57
20	Hexadecane	0,54
21	Pentadecane	0,54
22	Docosane	0,28
23	Octadecane, 1-chloro-	0,27

Essential oils were reported very diverse in some studies (24, 25). It was reported that the major essential oil constituents were: decanoic acid (9.8%), dodecanoic acid (11.9%) and ester methyl palmitate (28.5%) (24), caprinic acid (19.8%) and methyl palmitate (28.5%) (26), limonene (11.4%), cyclosativene (11.9%), α-ylangene (13.9%), and diepi-α-cedrene (17.9%) (27), β-caryophyllene (9.0–25.6%), heneicosane (3.0–32.1%) and α-copaene (1.5–7.2%) (28), α-humulene (15%), 1,8-cineole (16%) and α-pinene (32%) (29) and 1,8-cineole (8.9%), δ-cadinene (9.0%) and β-caryophyllene (5.8–36.2%) (27).

The differences in essential oil constituents could be explained by diversities in their origin and the climate. Also, esterification was used to increase the volatility of fatty acids in the sample preparation of our study.

Previously, Judzentiene et al showed that essential oils of *Helichrysum arenarium contains p*almitic, myristic and lauric acids, *n*-nonanal, and trans-β-caryophyllene (≤6.5%) (30). In various studies it has been shown that phenolic compounds of *Helichrysum arenarium* had anti-atherosclerotic and antimicrobial activities (19,31). Additionally, Kucukoglu et al. suggested that Helichrysum extracts can inhibit

mammalian DNA topoisomerase I enzyme and these extracts may used to prepare anticancer medications in the future (32). Since the potential functions of substances found in herbal ingredients can be investigated by using computational methods (33), extracts of the *Helichrysum arenarium* can be find out by using computational methods and possible signaling mechanisms.

4. Conclusion

This study investigated the cytotoxic activities of *Helichrysum arenarium* on several types of human cells including ECV304 and Ishikawa cells. The results of our study have established the most significant extract dose. Furthermore, immortelle extracts' essential oils were examined by using gas chromatography-mass spectrometry. The *Helichrysum arenarium* extract reduced both cell viability and MDA levels in Ishikawa and ECV304 cells at 500 µg/mL. We have found that the main essential oils in the DMSO extracts of *Helichrysum arenarium* were palmitic acids and palmitoylglycerol. In conclusion, *Helichrysum arenarium* has exhibited antioxidant activity.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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