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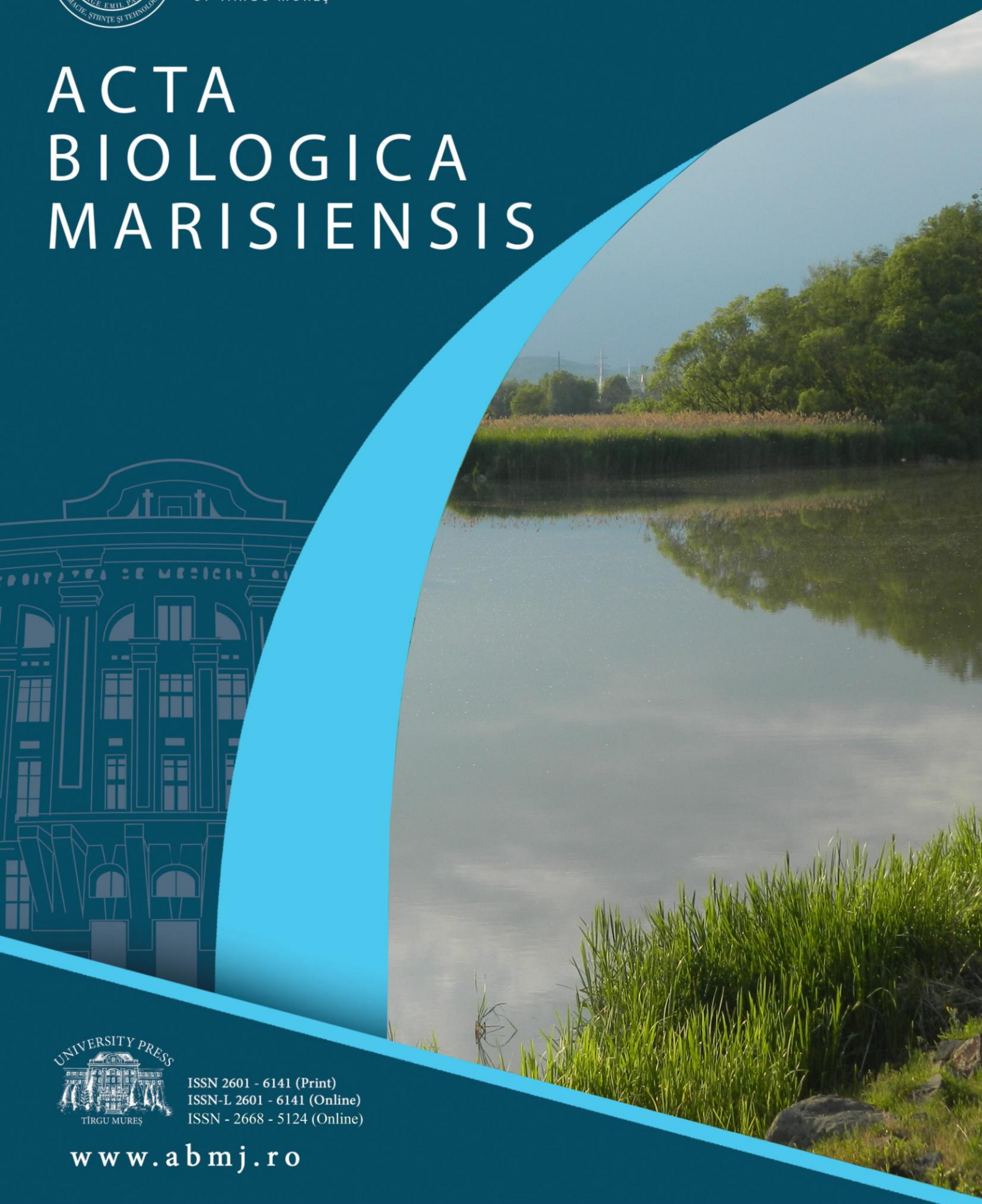

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CHEMICAL COMPOSITION OF VOLATILE ORGANIC COMPOUNDS OF AN EXTREMELY RARE AND ENDEMIC ALGERIAN APIACEAE SPECIES, *BUNIUM CRASSIFOLIUM* BATT.

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Abstract: *Bunium crassifolium* Batt. (*B. crassifolium*) (Apiaceae) is an extremely rare endemic species from the North East of Algeria. In this study, we extracted the volatile organic compounds (VOC) of *B. crassifolium* Batt. aerial parts using an Agilent G1888 network headspace sampler coupled with an Agilent 7890 GC system. The results revealed the presence of twenty-two (22) compounds, twenty (20) of which were identified as representing 97.48% of the total composition, the major components are: 44.67% of β-Cubebene, 8.82% of β-Caryophyllene, 7.04% of γ-Elemene, 4.70% of δ-Cadinene, 4.11% of γ-Cadinene, 3.77% of Ascaridole and 3.33% of β-Elemene, along with other constituents at a relatively low amount.

Keywords: *Bunium crassifolium*, Apiaceae, volatile organic compounds (VOC), GC-MS and GC-FID

1. Introduction

The genus of *Bunium* belongs to the family of Apiaceae, and represented about fifty species distributed in North Africa and Europe, to the central and southwest Asia. It contains medicinal and aromatic plants such as *B. persicum* (Hajhashemi et al., 2011) and *B. bulbocastanum* (Haroon et al., 2014). In Algeria, genus of *Bunium* contain seven species, four of which are endemic such as *B. crassifolium* Batt., *B. elatum* Batt., *B. chaberti* Batt., and *B. fontanessii* (Pers.) Maire (Quezel and Santa, 1963). *B. crassifolium* Batt. is an

endemic and rare extremely species distributed and growing in the North-East of Algeria. It is a perennial plant 30-60 cm in height. The leaves are pinnatisect with long linear divisions of 2-4 cm. Flowers in large umbels 7-10 cm. Fruits are blackish, nearly as wide as long, with very marked primary ribs, keeled on the back and sometimes a little winged (Pottier-Alapetite, 1979).

In Algeria, bulb of the genus *Bunium* were used by some people as a food in colonial period in the nineteenth century. During this period, the Atlas Mountains people use to dry the bulb of *B. incrassatum* in the sun in the form of powder to use it as flour mixed with barley and some wheat to consume it as bread or couscous and sometimes without any other flour (Souilah et al., 2021).

Volatile organic compounds studies previously on species of genus *Bunium* L. reported the presence of a broad range belonging to a lot of classes such as non-terpenoids and terpenoids, which are applied in different medical areas and pharmacological (Mohammadhosseini, 2017a; Mohammadhosseini et al., 2017b; Mohammadhosseini et al., 2021; Zhang et al., 2021).

Different species of the genus *Bunium* usually are potential sources of secondary metabolites and possess a pungent odor, mainly terpenoids, which constitute the volatile organic compounds presented in their secretory glands (Mohammadhosseini et al., 2021), from various organs such as fruit, flowers and leaves (Tholl et al., 2006). A large wide of reports are found in the scientific researchers with the quantitative and qualitative characterizations of several species of *Bunium* (Mohammadhosseini et al., 2021).

Recently, the interesting of scientific researchers in the chemical composition of essential oil of various plants (VOCs) has led the progress of a several systems for the extraction and analysis of volatiles compounds. The developed headspace sampler for Gas Chromatography–Mass Spectrometry (GC-MS) explorers more volatile compounds profile of fresh plants than classic methods by solvent extraction or steam distillation.

In our case, this method is most appropriate because we only have a small amount of a plant endemic and extremely rare (*B. crassifolium* Batt.). To the simplest of our knowledge, the chemical composition of organic volatile compounds (VOCs) of *B. crassifolium* species has not been reported before. For this reason, our study aimed to explore the VOCs for the first time to know their health benefits.

2. Materials and methods

Plant material

Samples of the aerial parts of *B. crassifolium* Batt. were collected during the flowering period in Sérraïdi (Annaba, Algeria), during the month of May (**Fig. 1**). The plant was identified by Dr. Tarek Hamel lecturer in the department of Plant Biology and Environment at University of Annaba (Algeria). A voucher specimen was deposited in the herbarium of the University of Constantine 1. Samples were shade dried, then they were cut into smaller pieces.

Analyses of volatile organic compounds

Volatile organic compound (VOCs) analyses of *B. crassifolium* aerial parts were performed using an Agilent G1888 network headspace sampler coupled with an Agilent7890A GC system coupled also with an Agilent 5975C inert triple-axis MS detector.

Fig. 1. *B. crassifolium* Batt. (Aerial part and bulb) (Original)

Results were evaluated as percentage (%) area of VOCs of *B. crassifolium*. The oven temperature program, using an Agilent DB-WAXETR column (60m×0.32mm, 0.25µm), was as follows: between 60 to 150°C at 5°C.min−1, 2min hold; between150 to 220°C at 3°C.min−1, 2min hold. The injection volume was 1µL and the carrier gas was helium at a flow rate of 1.5mL min−1. To perform the analyses, 2g aerial part samples of *B. crassifolium* were placed in 20 mL glass vials and sealed with aluminum seals and polytetrafluoroethylene septa (Koldas et al., 2015).

Components were identified by matching their mass spectra with those in the NIST and Wiley libraries.

3. Results and discussions

The composition and percentages of different compounds are summarized in **Figure 1** and **Tables 1 and 2**. The compounds are classed with their order of retention time. Twenty (20) compounds were identified, corresponding to a total of 97.48%: One (1) oxygenated monoterpene, fifteen (15) sesquiterpene hydrocarbons and four (4) oxygenated sesquiterpenes. Moreover, the results of all scientific literature studied the genus of *Bunium* reported that the number of VOCs were ranged between 9 and 48. Higher values have been recorded for *B. wolffi* Klyuikov from India with the presence of 48 components, *B. ferulaceum* Sm. from Algeria with 40 components and *Bunium* spp from Iran with 37 components. Lowest value has been detected for *Bunium* spp. from India with 9 components (Mohammadhosseini et al., 2021).

The components of essential oils showed several important roles of natural substances for health, such as resistance to diseases and against insects (Gershenzon & Dudareva, 2007), physiological function of growth, development and ecological function (Wink, 2003). They also possess antimicrobial, anti-oxigenic, anti-mycotic, antiviral, anti-parasitic and insecticidal properties (Bishop, 1995; Juglal et al., 2002; Lamiri et al., 2002; Moon et al., 2002; Michaelakis et al., 2007). Regarding phyto-pathogenic viruses, several substances of natural and synthetic origin have been assessed for their anti-phytoviral activity (Yordanova et al., 1996; Rusak et al., 1997; Othman & Shoman, 2004; Krcatović et al., 2008).

The results of VOCs were dominated by a large amount of sesquiterpenes with a 91.58% of the total of compounds identified (97.48%). Divided into 87.87% sesquiterpene hydrocarbons and 3.71% oxygenated sesquiterpenes. Bezic et al., (2011), demonstrated that sesquiterpene are potent inhibitors of Cucumber Mosaic Virus (CMV) infection and bio-substances in the control of plant virus diseases.

The results indicate that the aerial part of *B. crassifolium* is a potential source of producing volatile profiles of sesquiterpene hydrocarbons where the main compounds are: β -cubebene with 44.64%, β -Caryophyllene with 8.82%, β -Elemene with 7.04%, δ-Cadinene with 4.70%, γ-Cadinene with 4.11%, and β -Elemene with 3.33% and oxygenated sesquiterpenes where the majority compounds are: cis-sesquisabinene hydrate with 1.44% and aromadendrene oxide with 1.22%. We notice the conspicuous absence of monoterpene hydrocarbons, products that make up the major part of the essential oils of higher plants. Unfortunately, the only component identified monoterpene oxygenated was ascaridole with 3.77%. This compound with high toxicity that avoids the usage of oils containing it internally or externally as well, except as plants' infusions, given its very low water solubility.

The β -cubebene was recognizable as an induced chemical plant defensive and beneficial attract organisms. The variability of chemical compounds in plants as well as of biotypes or races in insects was considered as a response to selection pressure exercised by insects and flora on each other; flora synthesize new chemicals compound against insects and insects elaborate detoxification systems, preparing each to enter on new zone of adaptation (Murugesan et al., 2012).

The team of Hernandez-Leon (2020) showed that the β-caryophyllene, had a potent agonist selective of subtype 2 receptor of cannabinoid, for that was considered such as a neuropathic pain model in the central nervous system and against nociceptive activity (Segat et al., 2017). Any psychoactive responses are related with central nervous system effects of β-caryophyllene; for that it is reported as a neuroprotective compound (Machado et al., 2018). In addition, it was considered as an anti-inflammatory by oral administration (Klauke et al., 2014). β-elemene was considered as an antitumor drug extracted of *Curcuma wenyujin* by traditional Chinese medicine. The β-elemene exerts its effects by inhibiting and arresting cell proliferation, provoking cell apoptosis and boosting the immune system (Zhai et al., 2018).

Ascaridole has been reported as antifungal, sedative, anti-parasitic and antimalarial (Okuyama et al., 1993). Pollack et al. (1990), reported that ascaridole inhibit the development of some bacteria *in vitro* such as: *Trypanosoma cruziand*, *Plasmodium falciparum* and *Leishmania amazonensis*. Moreover, Valery et al. (2008) reported its exhibition against various tumor cell lines *in vitro* (CCRF-CEM, MDA-MB-231 and HL60).

Volatile organic compounds of *B. crassifolium* also contain a highly oxidative product carbamic acid synonym of butylated hydroxytoluene (BHT). The BHT was considered as a synthetic antioxidant used to prevent oxidative deterioration in fatty foods and fat (Addis, 1986), and applied also in many commercial foods and constitute a little part of many people diet (Hocman, 1988). BHT was reported as anti-carcinogens in several animal models (Wattenberg et al., 1980; Wattenberg, 1986; Williams, 1986; Hocman, 1988; Williams and Iatropoulos, 1996). On the other hand, BHT has carcinogenic effects in experimental animals (Ito et al., 1983; Williams, 1986; Hocman, 1988), especially in the liver of mice and rats (Verhagen et al., 1991; Clayson et al., 1993 and Papas, 1993).

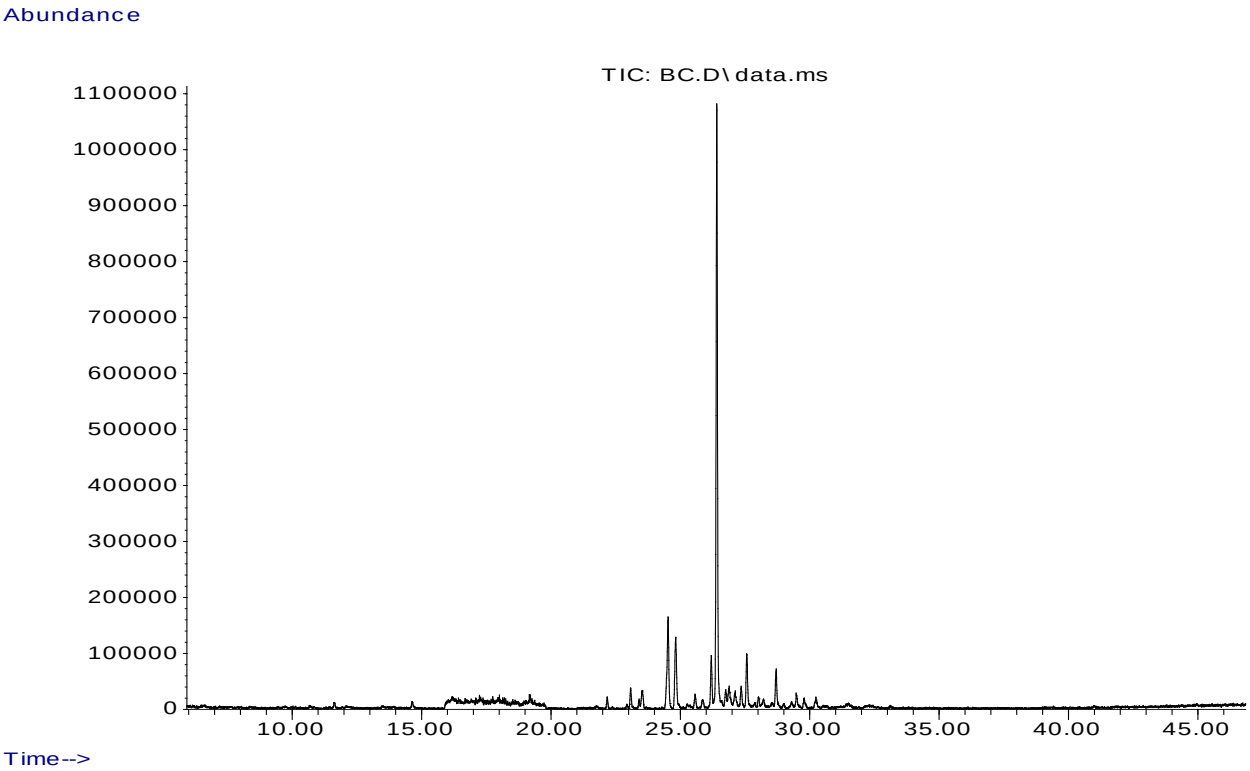

Fig. 1. The spectrum of GC-MS analysis of the oil of *B. crassifolium*

Table.1. Volatile organic compounds of *B. crassifolium* Batt. determined by HS-GC/MS

N°	RT(mn)	Formula	Class	Product	%
1	16.185	$C_{10}H_{16}O_2$	OMT	Ascaridole	3,77
2	17.283	-	N.I	-	1,68
3	22.174	-	N.I	-	0,82
4	23.081	$C_{15}H_{24}$	STH	α-Copaene	1,60
5	23.532	$C_{15}H_{24}$	STH	1,5-Cyclodecadiene, 1,5-dimethyl-8-(1-methylethenyl)	2,52
6	24.525	$C_{15}H_{24}$	STH	β –Caryophyllene	8,82
7	24.819	$C_{15}H_{24}$	STH	γ –Elemene	7,04
8	25.576	$C_{15}H_{24}$	STH	α-Bisabolene	1,23
9	25.865	$C_{15}H_{24}$	STH	Bicyclo[4.4.0]dec-1-ene, 2-isopropyl-5-methyl-9-methylene-	1,18
10	26.2	$C_{15}H_{24}$	STH	γ –Cadinene	4,11
11	26.408	$C_{15}H_{24}$	STH	β –Cubebene	44,67
12	26.754	$C_{15}H_{24}$	STH	β –ylangene	1,66
13	26.887	$C_{15}H_{24}$	STH	α-Muurolene	2,69
14	27.118	$C_{15}H_{24}O$	PC	2,6-bis (1,1-dimethylethyl)-4-methylphenol (BHT)	2,13
15	27.355	$C_{15}H_{24}$	STH	Germacrene D	2,00
16	27.568	$C_{15}H_{24}$	STH	δ –Cadinene	4,70
17	28.025	$C_{15}H_{24}$	STH	Valencene	0,98
18	28.215	$C_{15}H_{24}$	STH	Selina-3,7(11)-diene	1,34
19	28.7	$C_{15}H_{24}$	STH	β -Elemene	3,33
20	29.492	$C_{15}H_{26}O$	OST	cis-sesquisabinene hydrate	1,44
21	29.786	$C_{14}H_{20}O_2$	OST	3,4,4-Trimethyl-3-(3-oxo-but-1-enyl)-bicyclo[4.1.0]heptan-2-one	1,05
22	30.242	$C_{15}H_{24}O$	OST	Aromadendrene oxide	1,22

Table.2. Percentage of different classes of volatile organic compounds

Class	%	Number of compounds
Oxygenat monoterpene (OMT)	3.77	15
Sesquiterpene hydrocarbon (STH)	87.87	1
Oxygenat sesquiterpenes (OST)	3.71	3
Not idendied (NI)	2.5	2
Phenolic compound (PC)	2.13	1
Total	**99.98**	**22**

Conclusions

This study was performed to investigate the volatile organic compounds (VOCs) of *B. crassifolium* by the headspace sampler with gas chromatography-mass spectroscopy. The result reveals the presence of twenty-two (22) products of which twenty (20) were identified representing 97.48% of the total components. The major component identified were: 44.64% of β-cubebene, 8.82% of β-Caryophyllene, 7.04% of γ-Elemene, 4.70% of δ-Cadinene, 4.11% of γ-Cadinene, 3.77% of Ascaridole and 3.33% of β-Elemene, along with other constituents at relatively low amount. The composition of the (VOCs) is dominated by sesquiterpenes (91.58%). Among these sesquiterpenes, the ones without oxygen are mainly represented (87.87%). The aim of this study based at the research on chemical components useful in food preservation, insecticide and some biological activities *in vitro* and *in vivo*, such as anti-cancer. Finally, we recommended in the future the extracting of essential oil from various plant organs, including flowers, leaves and stems, to find out the chemicals and their concentrations to determine the more active plant organ.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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HORMONE IMBALANCE IN POLYCYSTIC OVARIAN SYNDROME

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Abstract: Polycystic Ovarian Syndrome (PCOS) is a disease that is characterized by an increased Gonadotropin-Releasing-Hormone (GnRH) pulsatile frequency, causing an altered LH/FSH ratio. More precisely an increased Luteinizing Hormone (LH) secretion compared to a decreased Follicle-Stimulating Hormone (FSH) secretion leads to the development of hyperandrogenism and to a low-level concentration of estrogens and therefore decreased negative estrogenic feedback in the control axis. The purpose of this review is to connect the physiological Hypothalamic-Pituitary-Ovarian (HPO) axis with said pathology and the ensuring discussion about the possible mechanisms of pathogenesis and guidelines for relieving associated symptoms.

Keywords: Polycystic Ovarian Syndrome, hyperandrogenism, hyperestrogenism, infertility, hirsutism.

Introduction

Polycystic Ovarian Syndrome (PCOS) is extremely frequent in the general population (4-20% of women of fertile age are affected), yet it is still not perceived as a major health problem although, in addition to anovulation, hyperandrogenism, and polycystic ovaries, PCOS can lead to insulin resistance, Luteinizing Hormone (LH) hypersecretion, dyslipidemia, hirsutism, type 2 diabetes and/or infertility (Deswal et al, 2020). Despite the prevalence, the ethiology of PCOS is poorly known, the disease is incurable, and the treatment is symptomatic. Due to the extremely varied symptomatology, several diagnostic criteria have been established over time: *National Institute of Health (NIH) criteria* (Nicolaides et al, 2020), *Rotterdam criteria* (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004) and *AE-PCOS Criteria* (Azziz et al, 2006).

The purpose of this review is to emphasize the most important mechanisms of estrogen secretion regulation in women, insisting on the biochemical mechanisms underlying the hormonal imbalance that occur in PCOS and some therapeutic possibilities for the treatment of the associated symptoms.

Biochemical aspects of estrogen secretion

The estrogen secretion underlies the Hypothalamic-Pituitary-Ovarian (HPO) axis. The HPO axis starts in the hypothalamus, where production of Gonadotropin-Releasing-Hormone (GnRH) takes place especially in the preoptic and anterior nuclei of the hypothalamus (Lee et al, 2021). The GnRH will use the hypothalamic hypophyseal portal system to reach the hypophysis, where it will act on gonadotroph cells. Depending on the frequency of release of GnRH, the women can either have FSH or LH release. High frequency of GnRH release will cause more LH release compared to FSH, while lower frequency of GnRH release will stimulate more FSH release compared to LH (Stamatiades and Kaiser, 2018).

The FSH and LH will then enter the bloodstream and act on the primordial follicles of the ovary. FSH is especially important as it causes not only proliferation of granular cells (follicular cells), but it also causes development and formation of thecal cells. This newly formed layer can be divided into theca interna and theca externa. Regulation of estrogen and progesterone production by the hypothalamic-pituitary axis is presented in **Figure 1**.

The theca interna is especially important because it causes formation of progesterone and other similar hormones, which can be used by the granular cells to produce estrogens. Another important effect of FSH is that it causes increased expression of estrogen receptors to which estrogen will bind and cause an increased expression of LH receptors. The combination of increased LH receptors, increased estrogen receptors and FSH causes increased proliferation of granular cells, which will cause more estrogen secretion. Once the estrogen reaches a specific threshold, it will cause a switch from negative feedback of estrogen to positive feedback of estrogen (Orisaka et al, 2021).

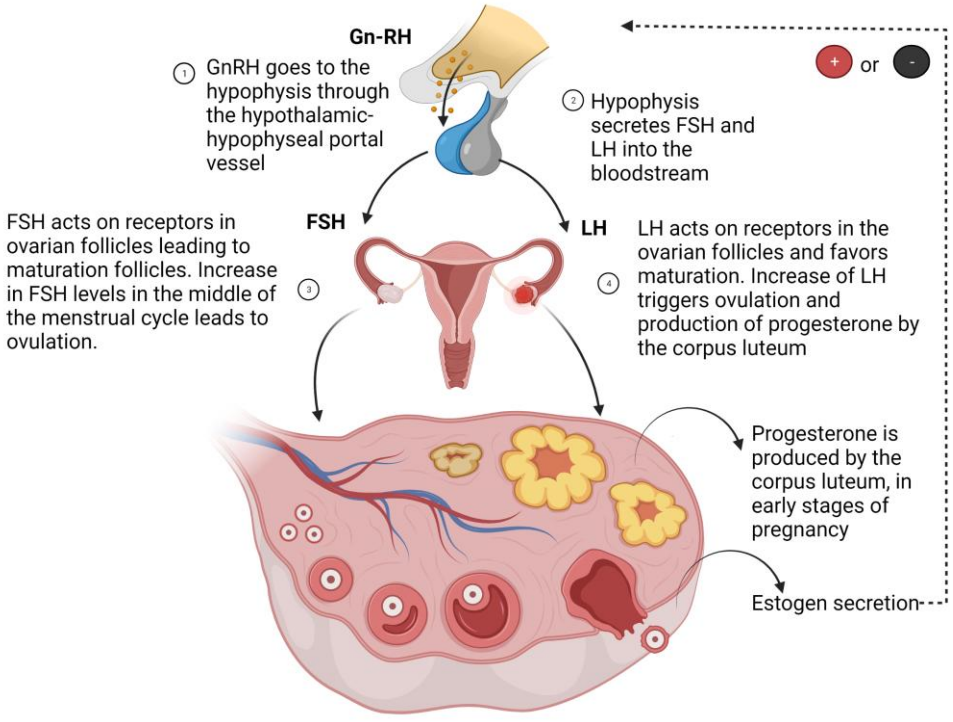

Fig. 1. Regulation of estrogen and progesterone production (FSH - Follicle-Stimulating Hormone, LH - Luteinizing Hormone, GnRH - Gonadotropin-releasing hormone) (da Costa CS et al., 2021)

In addition to the above-mentioned process, androgens are very important for the development of primordial follicles to primary follicles and from primary follicles to secondary follicles. They exert these effects through Insulin-like Growth Factor-1 (IGF-1) which is produced by androgens binding to the androgen receptor (AR). Androgens seem also to facilitate further development to the Graafian follicle by increasing sensitivity to FSH, via increased production of FSH receptors. (Sanchez-Garrido and Tena-Sempere, 2020).

Although a lot of primordial follicles will start to develop during the follicular phase, only one primordial follicle will become a mature Graaf follicle. Although it is not clear how this is happening, one of the theories is that the increase in estrogen will cause a reduced secretion of GnRH due to the negative feedback. The predominant follicle will be able to keep producing more estrogen with such a low level of GnRH but the other follicles, which were not as developed will not produce enough estrogen to survive, and therefore involute (Ding et al, 2021). Another hormone which inhibits follicular differentiation is the Anti-Müllarian Hormone (AMH). AMH is secreted in high amounts by tertiary and Graaf follicles, and will further inhibit the FSH and estrogen release, accentuating the abovementioned process (Rudnicka et al, 2021). It is important to mention that the release of this hormone is constant and does not vary throughout the ovarian cycle. It will vary however throughout the life of the female, being at its peak at around 25 years and minimal at menopause. AMH in fact seems to represent the available follicles in the ovary and is therefore often used to denote fertility. High levels of AMH however are not always representative for increased fertility, as they will be elevated in PCOS, even though the patients are infertile (Moolhuijsen and Visser, 2020).

Recent research has suggested that both negative and positive feedback act via an indirect pathway. Experiments on transgenic mice have shown that there are specific neuron groups residing in the hypothalamus, which via connections with GnRH neurons can cause both positive and negative feedback. The neuronal groups responsible for negative feedback in these mice are located in the arcuate and median eminences although the group of neurons behind negative feedback doesn't seem to have been fully discovered. The positive feedback neurons are located in the Anteroventral Periventricular nucleus (AVPV), median preoptic, periventricular preoptic and suprachiasmatic nucleus. The first three nuclei form the RP3V group while the suprachiasmatic nucleus is responsible for connecting the estrogen feedback to the circadian rhythm (Moenter et al, 2020). In addition, a connection between the circadian rhythm and the estrogen feedback in humans could not be established while in other species like sheep and primates, circadian rhythms don't seem to be relevant in the cyclical changes of estrogen feedback (Webster and Smarr, 2020).

There are two types of Estrogen Receptors (ER), Erα/β, one that acts through an E-dependent pathway and an Estrogen Response Elements (EREs)–independent pathway. The former pathway mentioned acts through activation of the ERE to alter the transcription of specific genes. The ERE-independent pathway exerts its effect by influencing protein function which in turn influences gene transcription at non-ERE sites (Chen et al, 2022). Recent data agree that the ERα that act through the ERE-independent pathway are responsible for partially causing the negative feedback, that means that although this type of receptor causes negative feedback, alone it is not able to cause the strength of the negative feedback noticed in the normal organisms. To

cause the normal strength of the negative feedback, it is noticed that the ERα that acts through both the ERE-dependent and ERE-independent pathways is needed in every healthy organism. The ERα that act through the ERE-dependent pathway are responsible for the positive feedback and in part for the negative feedback (Moenter et al, 2009).

In the last decade, a lot of steps forward in understanding the positive feedback of estrogen were done. After each menstruation, the GnRH released in a pulsatile manner causes release of FSH and LH. The FSH acts on follicular cells in the follicles of the ovary. Those types of cells are very important as they are responsible for the production of estrogens. With each GnRH release from the hypothalamus the number of follicular cells increases and so does the amount of estrogen. These estrogens will act on the ERα, causing the increased transcription of the progesterone receptor gene, thereby increasing the amount of progesterone receptors on the cell membranes of the gamma-aminobutyric acid (GABA) and glutamatergic (Glu) neurons. Estrogens also act on astrocytes which cause the release of Ca^{2+} ions from the smooth endoplasmic reticulum, thus starting progesterone synthesis. By binding to the progesterone receptors, the progesterone causes the release of GABA and Glu, causing the LH surge (He et al, 2017).

Although a lot of receptors are responsible for the synthesis of progesterone in the astrocytes, the main receptor is the ERα (Sinchak et al, 2020). During the negative feedback, GnRH neurons have a pulsatile secretion, secreting GnRH for a few minutes every 1h to 3h. The advantage of such a pulsatile secretion is that the gonadotroph cells in the adenohypophysis will not suffer a downregulation of the GnRH receptors and therefore lose responsiveness to GnRH. During the positive feedback we have a change in secretion from only a pulsatile secretion to a pulsatile secretion superimposed with a continuous secretion. The reason behind this change is an increase of estrogen over a specific threshold for several hours (Herbison, 2018).

A specific group of GnRH neurons seems to be involved in the change of secretion. This group is composed of a sub-population of GnRH neurons located in the rostral preoptic area around the organum vasculosum of the lamina terminalis. The reason behind their involvement is still unknown. A hypothesis is that this subgroup is involved in the formation of the positive feedback due to specific properties, whereas another hypothesis is that they just receive a different type of signal causing them to react differently. As a result, the properties of this subgroup are unknown. As an example, it is not known if this subgroup is only responsible for the surge or if they also exhibit pulsatile secretion and are therefore involved in negative feedback too (Constantin, 2017).

Hormone imbalance in PCOS

The most important underlying reason for the pathogenesis of PCOS is genetics. The women suffering from PCOS have an increased frequency of release for GnRH and hyperandrogenism (Witchel et al, 2019). Hyperandrogenism is an essential characteristic of PCOS, being caused in women by the increase in serum values of androstenedione and testosterone, and in the case of 50% of women by the increase in dehydroepiandrosterone (DHEA) (Manu et al, 2022).

A high GnRH release frequency will lead to an increased release of LH and a decreased release of FSH and, in some patients the cells responsible for producing LH react strongly to GnRH leading to an excess LH production (Escobar-Morreale, 2018). As high quantities

of LH and a low FSH are released, the ovarian follicles will not develop, leading to lack of estrogen production. The cells in the follicles responsible for estrogen production are the follicular cells and the thecal cells (Richards et al, 2018). The thecal cells produce androgens, which will be used to produce estrogens. Due to the low production of FSH however, there will be a lack of granulosa cells which will lead to hyperandrogenism. The hyperandrogenism will lead to the partial development of the follicles, but due to the lack of FSH, estrogens are not produced. Furthermore, due to high levels of 5α-reduced androgens, the aromatase activity is reduced further inhibiting estrogen production. Due to the lack of ovulation overall, there will be more follicles, which combined with the partial development of the follicles due to hyperandrogenism will lead to very high levels of AMH. The increased AMH levels will lead to further inhibition of FSH release (Fujibe et al 2019).

Some comorbidities that will exacerbate the symptoms of PCOS are obesity and insulin resistance. Obesity will also allow for the transformation of androgens to estrogens since adipose tissues also contain aromatase. Insulin resistance is defined as resistance of the insulin receptors to the hormone because of increased levels of free fatty acids, increased levels of adipokines with the exception of adiponectin and inflammation. Obesity will increase the levels of adipokines, thereby increasing resistance and increase the levels of free fatty acids. The increased levels of free fatty acids favor insulin resistance directly and indirectly; directly by inhibiting insulin signaling and indirectly by favoring inflammation. Inflammation will lead to increased levels of IL1 which will lead to insulin resistance (Moghetti and Tosi, 2021).

It should also be noted that hyperandrogenism itself can cause insulin resistance. In addition to that, elevated levels of insulin found in patients suffering from insulin resistance, can exacerbate the already present hyperandrogenism by stimulating thecal cells to produce even more androgen (Unluhizarci et al, 2021).

The high levels of androgens and the elevated insulin levels due to insulin resistance will lead to a decrease in the production of Sex Hormone Binding Globulin (SHBG) which, as a result, leads to increased levels of androgens in the blood, and thus will exacerbate the effects of androgens. Since the effects of androgens are enhanced, insulin resistance will be increased (Zhu et al, 2019). High androgen levels in obese women suffering from PCOS, will lead to the production of estrogens in the adipose tissue due to the presence of aromatase in the adipocytes. Basic mechanism of PCOS pathogenesis and pathophysiology are presented in **Figure 2**.

Another secondary effect of hyperandrogenism in PCOS is hirsutism, while the hyperestrogenism will be responsible for the hyperplasia of the endometrium. Insulin resistance and obesity are responsible for the cardiovascular problems and the high risk for developing diabetes encountered in these patients (Spritzer et al, 2022; Hill et al, 2021).

Main biochemical pathways in PCOS

The main biochemical pathway responsible for the PCOS pathogenesis involves estrogen production in the ovaries. This pathway, at the base of the two-cell theory, necessitates both theca and follicular cells. In both thecal and follicular cells, we have LH receptors and Low-Density Lipoproteins (LDL) receptors. LH secretion in women determines synthesis of steroid hormones from the ovaries (Coutinho and Kauffman, 2019).

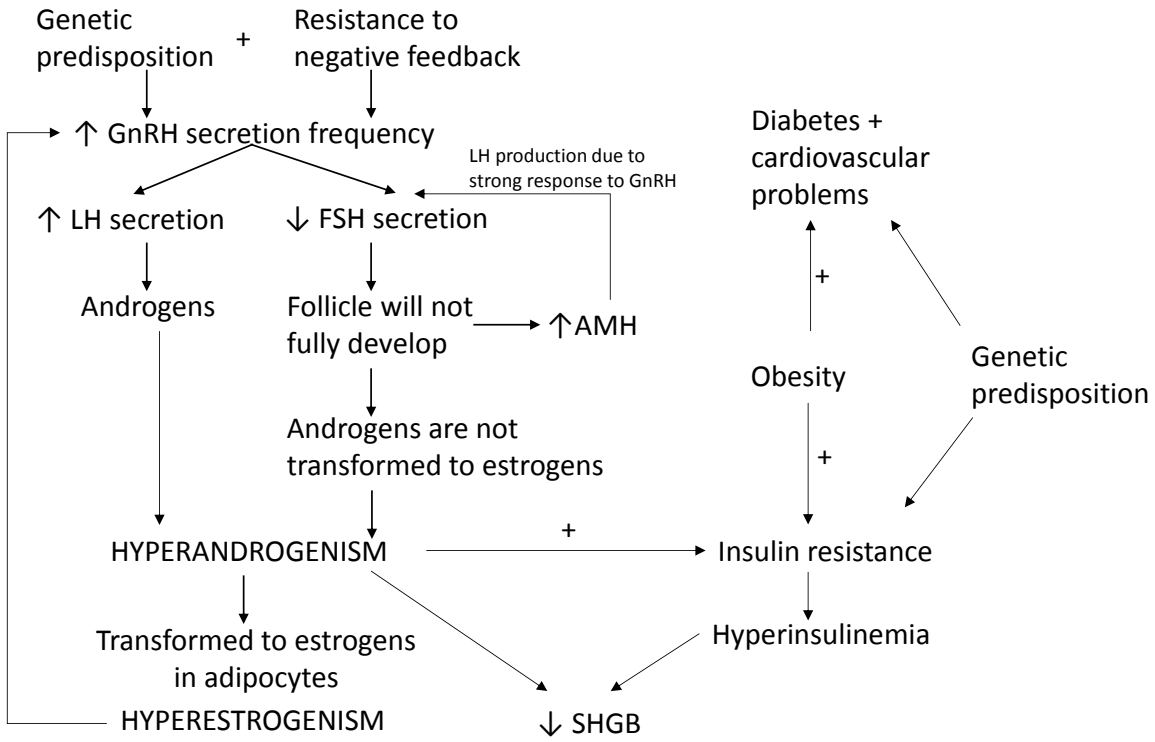

Fig. 2. Basic mechanism of PCOS pathogenesis and pathophysiology (FSH - Follicle-Stimulating Hormone, LH - Luteinizing Hormone, GnRH - Gonadotropin-releasing hormone, SHBG - Sex Hormone Binding Globulin, AMH - Anti-Müllarian Hormone)

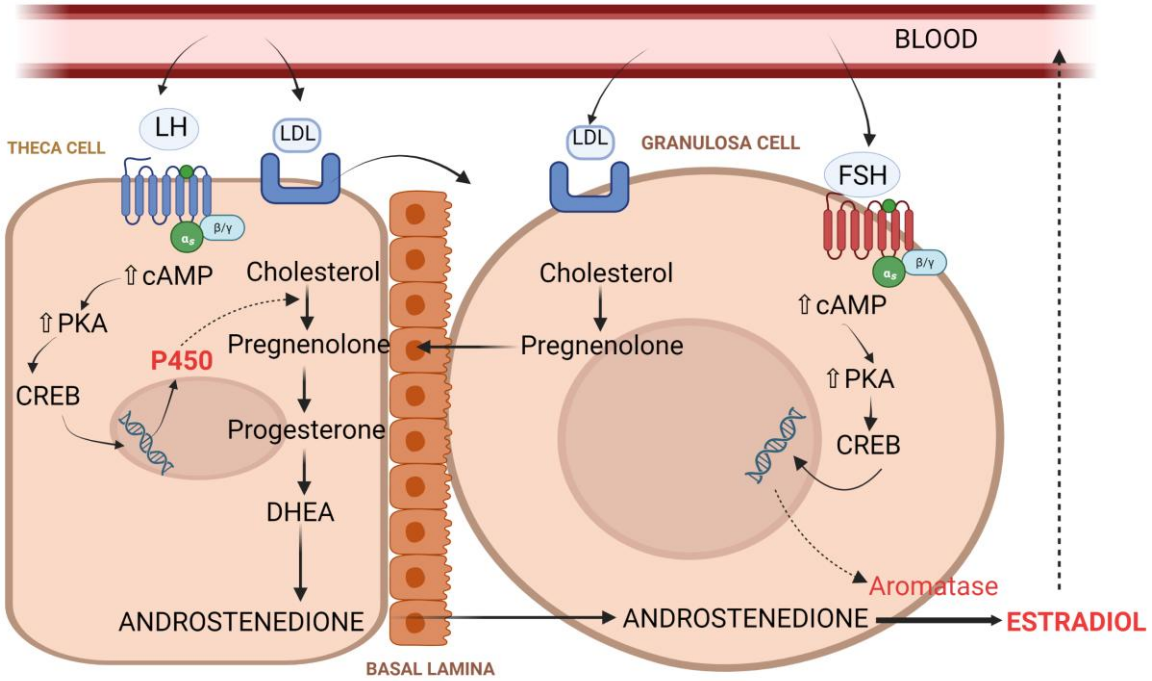

Fig. 3. Two cell theory of estrogen production (FSH - Follicle-Stimulating Hormone, LH - Luteinizing Hormone, LDL - Low Density Lipoprotein, cAMP - cyclic adenosine monophosphate, PKA - protein kinase A, CREB - cAMP response element-binding protein, P450 - Cytochromes P450, DHEA - dehydroepiandrosterone dehydroepiandrosterone) (adapted from Orisaka et al., 2021)

In the follicular cells LH leads to the production of progesterone. This newly formed progesterone will diffuse to the theca cells, where it will be added to the progesterone produced there. Progesterone will be transformed to androgens, which will diffuse to the follicular cells again, where the aromatase will transform it to estrogens. Here, by increasing the levels of cAMP, FSH activates protein kinase A (PKA) which will lead to the production of aromatase (Casarini and Crépieux, 2019). Two cell theory is presented schematically in **Figure 3**.

Depending on the concentration of androgens, these hormones will preferentially be transformed to estrogens or potent androgens thanks to the 5α- reductase. If the concentration is low, androgens will be transformed to estrogens thanks to the aromatase. If the concentration of androgens is increased, they will preferentially be transformed to potent androgens thanks to the 5α-reductase. To be noted that 5α-reduced androgens inhibit aromatase activity (Hammes and Levin, 2019).

Pharmacological alternatives to treat and relieve symptoms associated with PCOS

Considering that the symptoms of PCOS are varied (hirsutism, infertility, menstrual cycle irregularities, insulin resistance), treatment must be individualized according to the patient.

Thus, depending on the visible signs of PCOS, there are multiple pharmacological recommendations. For example, to limit hirsutism, the use of spironolactone and metformin is recommended (Onalan et al, 2005). For ovulation induction, the use of clomiphene and metformin (alone or in combination) is recommended, and depending on other comorbidities, rosiglitazone **(**Moll et al, 2006; Ganie et al, 2020). For the treatment of obesity-related insulin resistance and oligomenorrhea, the most recommended agent is also metformin (Patel, 2018). Since the disease seems to have genetic origins, the treatment itself is not curative but symptomatic.

Another pharmacological intervention to regulate the hormonal imbalance is represented by the use of oral contraceptives, if the patient can tolerate the side effects (nausea, headaches, thrombophlebitis, etc.) (Podfigurna et al, 2020).

Another aspect to consider in hyperandrogenism is that it is responsible for decreasing the levels of SHBG, whose level is low in the case of insulin resistance (Qu and Donnelly, 2020). Thus, by correcting the hyperinsulinemia, the level of SHBG returns to normal, managing to bind sex hormones, bringing back the free form of these hormones to normal concentrations.

A simpler representation of the agents used in PCOS is illustrated in **Table 1**.

Conclusions

PCOS is a complex pathology extremely frequently encountered among the female population, with multiple causes, leading to important hormonal changes and serious consequences upon the health and quality of life (diabetes, insulin resistance, hirsutism, infertility), whose treatment remains only symptomatic.

Considering the complex clinical picture, with multiple tissue modifications, but also the not to be neglected the psychological impact on the affected females, this article insists on understanding the mechanisms behind the various symptoms. One future goal is to understand the importance of individualized treatment and the early detection of risk groups.

Table 1. First and second-line agents for PCOS symptoms

First-line agents	Mechanism/Effect	Second-line agents	Mechanism/Effect	References
Clomiphene	SERM function, for infertility	Acarbose	reducing insulin resistance;	Moll et al., 2006
Metformin	Reduces insulin resistance and the manifestations related to hyperinsulinemia	Estroprogestative combinations	Reduce hirsutism	Onalan et. al, 2005
Thiazolidinediones	Similar to metformin	5-alpha-reductase inhibitors (finasteride)	Reduce hirsutism	Patel, 2018
Spironolactone	Anti-androgenic effect	Antiandrogens (flutamides)	Reduce hirsutism	Onalan et al., 2005
		Aromatase inhibitors (letrozole)	Infertility treatment	
		Anorexigenic agents (sibutramine)	Reduce hirsutism and insulin resistance	

Conflict of interest

The authors declare that there are no conflicts of interest related to this article.

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BREAKING DOWN TUMOR DRUG RESISTANCE: THE LINK BETWEEN CELL MEMBRANE CHANGES AND TREATMENT EFFICACY

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Abstract: There have been significant advances in our understanding of how changes in the fluidity and permeability of the cell membrane can affect drug resistance in cancer. Research has shown that cancer cells often have changes in the fluidity and permeability of their cell membrane that contribute to their resistance to drugs used to treat cancer. These changes may be due to changes in the composition and organization of the lipid bilayer that makes up the membrane, as well as changes in the expression or localization of proteins and other molecules embedded in the membrane. The lipid composition in the tumor cell membrane changes with drug resistance, which can affect the fluidity and permeability of the cell membrane. Reversal of drug resistance can be achieved by altering cell membrane fluidity and permeability. In recent years, there have been numerous studies aimed at understanding the mechanisms underlying these changes and identifying strategies to overcome drug resistance in cancer. This research has led to the development of new drugs and drug delivery systems that are designed to target specific changes in the cell membrane of cancer cells and improve the effectiveness of chemotherapy. Overall, the advances in our understanding of the role of cell membrane fluidity and permeability in drug resistance in cancer have led to the development of new approaches to treat cancer and improve patient outcomes and further research is needed to continue to improve the understanding of these mechanisms and to identify new strategies to overcome drug resistance in cancer. This article highlights the research status and detection methods of cell membrane fluidity and permeability affecting tumor drug resistance.

Keywords: tumor, drug resistance, cell membrane, fluidity, permeability, techniques

Introduction

Today, cancer poses a sombre threat to human health and life. It is projected that there will be more than a million new cancer cases and 609,820 deaths in the US alone by 2023 (Siegel et al., 2023). It is also estimated that the frequency of cancer increases at a rate of 3% to 5% each year. Radiotherapy and chemotherapy are important clinical treatments for cancer. Nevertheless as cancer cells are open to a variety of chemotherapy drugs over time, their sensitivity to these drugs progressively deteriorates, resulting in multidrug resistance. Multidrug resistance (MDR) refers to tumor cells becoming impervious not only to the chemotherapeutic drug, but also to other drugs with different structures and functions as a result of long-term exposure to that drug (Xiang et al., 2020). As a result of

chemotherapy, drug resistance can be distributed into two types: natural drug resistance (drug resistance that exists at the beginning of the treatment) and acquired drug resistance (induced by a chemotherapeutic drug during the treatment process) (Wu et al., 2016).

A major cause of chemotherapy failure is the emergence of multidrug resistance. The main mechanism of tumor drug resistance involves: 1) Abnormal expression of ABC transporter superfamily, such as P-glycoprotein (P-gp), multidrug resistance-associated proteins (MRPs), lung resistance proteins (LRPs) and mammary gland Drug efflux mechanism mediated by cancer resistance proteins (BCRPs); 2) Abnormal activity of topoisomerase II, reduced expression, reduced or lost targets of chemotherapeutic drugs, resulting in drug resistance (Sauna and Ambudkar, 2007; Huimin, 2005) Increased expression of glutathione-transferase, which catalyzes the combination of glutathione and chemotherapeutic drugs to form a complex, inactivating drug activity to produce drug resistance; 4) drug resistance mediated by enhanced DNA damage repair (Xiang et al.2020). Abnormal receptor expression leads to drug resistance mediated by abnormal apoptotic pathway (Zhou, 2016; Yang et al., 2015). As a result of the complexity and diversity of drug resistance, there are still many areas of unknowns despite the proposed mechanisms of tumor drug resistance mentioned above. One of the most widely studied drug resistance mechanisms is the overexpression of P-gp encoded by MDR1 (Xiang et al., 2020). In order to produce drug resistance, accumulation decreases. Drug-resistant tumor cells overexpressing P-gp have a significantly different membrane lipid composition, which affects its natural properties, such as fluidity, permeability, and phospholipid arrangement order (Baritaki et al., 2007). Researchers have discovered that chemotherapy drugs can be enhanced in sensitivity to tumor drug-resistant cells by altering the fluidity and permeability of tumor drug-resistant cell membranes. This may be due to changing the lipid composition or ratio of tumor drug-resistant cell membranes.

Structure and Composition of the Cell Membrane

The cell membrane, also known as the plasma membrane or cytoplasmic membrane, is a thin, flexible barrier that surrounds and encloses the contents of a cell. Cell membranes provide the structural basis for carrying out life activities. It is made up of a phospholipid bilayer, which is a double layer of phospholipid molecules that is arranged in a mosaic pattern (Singer and Nicolson, 1972). The phospholipid bilayer is composed of two layers of phospholipid molecules, with their hydrophobic tails facing each other and their hydrophilic heads facing outward. The structure of the cell membrane is fluid and dynamic, and the phospholipid molecules are constantly moving and interacting with each other. The fluidity of the cell membrane is determined by the composition and organization of the phospholipid bilayer, as well as by the presence of proteins and other molecules embedded in the membrane. A cell membrane is composed mainly of lipids and proteins, and lipid molecules can be categorized as phospholipids, cholesterol, and glycolipids.

A cell's internal environment cannot only be maintained, but it can also be accountable for the transmission of information and material between it and its external environment as well. The lipid bilayer model was first proposed by Gorter and Grendel, 1925 as a static and symmetrical model of the cell membrane. Over time, the understanding of the cell membrane has advanced to a highly

dynamic and asymmetrical structure. Phospholipids are a crucial component of the membrane and can be categorized into phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidylethanolamine (PE) (Xiang et al., 2020; Zalba and Hagen, 2017). PS, an essential component of the brain cell membrane, regulates various brain functions by influencing membrane fluidity and permeability. PI plays a vital role in metabolic regulation, signal transduction, and cell communication (Xiang et al., 2020; Zalba and Hagen, 2017). The composition and type of phospholipid molecules can differ between different cell membranes, regions on the membrane, and inner and outer phospholipid monolayers in bilayer lipids due to the role it plays in various physiological functions (Xiang et al., 2020). In the outer layer of the phospholipid monolayer, PC is particularly abundant, whereas PE and PS are predominant in the inner layer of the phospholipid monolayer (Alberts et al., 2002). At normal body temperature, cholesterol has a rigid structure because of its high melting point, which helps increase membrane stability, reduce water-soluble substance permeability, and contribute to the fluidity of cell membranes (Baritaki et al., 2007). In addition to completing most of its functions through lipids and proteins, the cell membrane also affects the normal division and proliferation of cells due to differences in composition (Hendrich and Michalak, 2003) . Overall, the structure and composition of the cell membrane are essential for the function of cells, as it controls the movement of molecules in and out of the cell and plays a key role in cell-cell communication and interaction.

Variances between Cancer Cells and Normal Cell Membranes

Cancer cells and normal cells have several differences in their cell membranes that can affect the effectiveness of drugs used to treat cancer and may contribute to tumor drug resistance. During the tumor development, phospholipid content of cell membranes can change. Merchant et al. (1991) used 31 P nuclear magnetic resonance spectroscopy to analyze phospholipids in human benign, malignant, and normal breast tissues. The results showed that compared with the phospholipid content of normal cell membranes, PC, PE, PI in malignant breast cancer cell membranes content increased. Compared with normal cells, the contents of sphingomyelin (SM), β-glycerophosphate and glycerol 3-phosphoserine in the colon cancer cell membrane increased. They measured phosphatidylcholine content in the membrane of malignant colon cancer cells at various differentiation stages by 31 P (P = 1215 MHz) NMR spectroscopy, and found that moderately differentiated colon cancer cells contain phosphatidylcholine in their membrane (Xiang et al., 2020, Merchant et al, 1995). Phosphatidylcholine content was significantly higher than that of poorly differentiated cells. According to Barker and Bowler, 1991, phospholipids do not fully reflect the differences between normal and tumor cell membranes. The membrane phospholipid composition content of liver cancer cells D23 and hepatic sarcoma cells MC7 did not differ significantly from normal rat liver cells (Xiang et al., 2020). However, the fluorescence polarization method proved that the cholesterol content in the cell membrane was significantly reduced. Compared with normal liver cells, the order of the membranes of the above two tumor cells was lower. By fluorescence polarization, Van-Blitterswijk et al. (1982) found that mouse

thymic leukemia cells had significantly higher membrane fluidity than normal thymocytes.

There was a significant increase in phospholipid ethanolamine, as well as an increase in unsaturated fatty acids, in tumor cell membranes compared to normal thymocyte membranes. Increasing fatty acid chains and decreased cholesterol and sphingomyelin are attributed to increased membrane disorder and decreased cholesterol/phospholipid ratio in tumor cell membranes (Xiang et al., 2020). The above studies have shown that the composition of tumor cell membranes is different in different types of tumor cells, and the degree of differentiation of tumors is different, and the proportion of membrane components will also be different.

Changes in the Membrane Composition of Tumor Drug-Resistant Cells

Tumor drug-resistant cells may have changes in the composition of their cell membrane that contribute to their resistance to drugs used to treat cancer. Changes in the composition of the cell membrane can affect the ability of drugs to interact with their target molecules or to enter the cell. Some of the changes in the membrane composition of tumor drug-resistant cells that may contribute to their drug resistance include: 1. Altered expression or localization of proteins: Tumor drug-resistant cells may have different levels or types of proteins embedded in the cell membrane, such as receptors, transporters, or enzymes, which can affect the uptake or efflux of drugs and other molecules. 2. Changes in the lipid composition: Tumor drug-resistant cells may have changes in the composition and organization of the lipid bilayer that makes up the cell membrane, which can affect the fluidity and permeability of the membrane. 3. Increased expression of membrane pumps: Tumor drug-resistant cells may have increased expression of proteins called ATP-binding cassette (ABC) transporters, which can pump drugs out of the cell and contribute to drug resistance (Xiang et al., 2020). During chemotherapy, drugs enter the cell through the membrane and reach their target by crossing the cell membrane. In tumors with drug-resistant cell membranes, drug resistance is facilitated by P-gp overexpression, which reduces drug accumulation intracellularly. The fluidity of the cell membrane is largely determined by the composition of the phospholipid bilayer. Changes in the saturation and length of fatty acid chains can alter the packing of phospholipids in the bilayer, affecting the fluidity of the membrane. For example, it has been shown that increasing the level of unsaturation of the fatty acid chains in the membrane can increase the fluidity of the membrane, resulting in decreased P-gp activity (Hendrich and Michalak, 2003). P-gp is more active in rigid membranes with low fluidity, and its activity decreases as the membrane becomes more fluid. One study showed that increasing the fluidity of the membrane by incorporating unsaturated fatty acids into the membrane decreased P-gp activity, while decreasing the fluidity of the membrane by incorporating saturated fatty acids increased P-gp activity (Drori et al., 1995). Another study demonstrated that cholesterol depletion from the membrane using cholesterol-sequestering agents reduced P-gp activity by disrupting its interaction with the membrane and altering the lateral mobility of P-gp within the membrane (Orlowski et al., 2007). In addition to changes in the lipid composition, alterations in the permeability of the membrane can also affect the activity of P-gp. One mechanism that describes the effect of membrane fluidity on P-gp activity is the role of lipid rafts. Lipid rafts are regions of the membrane that are enriched in cholesterol and sphingolipids and are thought to be important for the localization and

function of P-gp. Several studies have shown that P-gp is localized in lipid rafts, and that the fluidity of these rafts is important for P-gp activity (Siddiqui et al., 2007). Specifically, it has been proposed that the lateral mobility of P-gp within the membrane is influenced by the fluidity of the lipid rafts, and that this mobility is necessary for P-gp to interact with substrates and other components of the membrane. The P-gp expels its substrates into the extracellular medium, which incurs an energy cost for lipophilic species. Alternatively, it may operate as a drug flippase, moving its substrates from the inner to the outer leaflet of the membrane. This mechanism requires that drug molecules have a specific localization within each bilayer leaflet, rather than being randomly distributed. Evidence suggests that P-gp functions as an outwardly directed flippase for various fluorescent phospholipid and glycosphingolipid molecules in both intact cells and reconstituted proteoliposomes. Pgp-mediated drug efflux probably takes place by a flippase-like mechanism, as it requires ATP hydrolysis and is inhibited by known P-gp substrates, and inhibitory potency is highly correlated with their P-gp binding affinity (Sharom, 2014). The flippase model for P-gp function is well-supported by substantial evidence, which indicates that P-gp acts as an outwardly directed flippase for various fluorescent phospholipid and glycosphingolipid molecules in both intact cells and reconstituted proteoliposomes (Sharom, 2014). Cells that overexpress P-gp exhibit altered distribution and decreased accumulation of fluorescent phospholipid derivatives, along with increased outward transport of these analogs, which can be reversed by P-gp modulators. Studies have shown that purified P-gp can act as a broad-specificity, outwardly directed flippase for a variety of both short-chain and long-chain nitrobenzo-2-oxa-1,3-diazole (NBD)-labeled phospholipids and glycosphingolipids in proteoliposomes (Sharom, 2014). Pgp-mediated drug efflux is believed to occur through a flippase-like mechanism, as phospholipid and glycolipid flippase activity required ATP hydrolysis and was inhibited by known P-gp substrates, with inhibitory potency highly correlated with their P-gp binding affinity.

In the process of simulating the insertion of P-gp into the lipid bilayer in phospholipid liposomes, it was found that the fluidity of the membrane was enhanced; and the addition of cholesterol in the phospholipid liposomes could reduce the fluidity of the membrane and increase the ATPase activity of P-gp (Rothnie et al., 2001). By affecting the lipid composition of the cell membrane, the overexpression of P-gp may indirectly affect the biological characteristics of the cell membrane, which may explain the development of tumor drug resistance (Xiang et al., 2020). P-gp expression is commonly found in various types of cancer, including renal and colon carcinomas, melanomas, and central nervous system tumors. P-gp expression has been associated with drug resistance in these tumors, with levels often increasing after chemotherapy. P-gp is the primary cause of multi-drug resistance (MDR) in cancer cells, which is linked to reduced responses to chemotherapy and poor clinical outcomes (Sharom, 2014). Developing compounds that can inhibit Pgp-mediated MDR is a crucial goal in cancer therapy. Modulators, such as verapamil and cyclosporin A, have been identified as chemosensitizers that can reverse drug resistance in MDR cells in vitro. In comparison with mouse leukemia cells (P388), drug-resistant cells (P388/ADR) had reduced PC content and increased SM content, and electron spin resonance spectroscopy showed that the lipid structure of drug-resistant cells was more ordered (Xiang et al., 2020; Ramu et al., 1984; Ramu et al., 1983). Kok et al., 2000 found that in HT29

cells overexpressing MRP1, glucosylceramide and galactosylceramide were increased in the composition ratio of sphingolipids. Human leukemia lymphocytes and vinblastine-resistant cells were analyzed by May et al., 1988, and it was found that the membrane lipid components of drug-resistant cells had a 50% increase in cholesterol and 30% increase in phospholipids, respectively, and a 60% increase in lipoproteins (Xiang et al., 2020). Researchers have shown that phospholipid and cholesterol are embedded between phospholipid molecules in tumor drug-resistant cells, and the polar head of cholesterol is close to that of phospholipid molecules. As a result of the adjacent phospholipid hydrocarbon chains (Peetla et al., 2013), the order and rigidity of the cell membrane are significantly improved (Hendrich and Michalak, 2003, Eytan et al., 1996), resulting in a significant change in the cell membrane's fluidity and permeability, making chemotherapy drugs more difficult to enter the cell, leading to resistance to chemotherapy drugs.

Effect of Changes in Cell Membrane Fluidity on Tumor Drug Resistance

Changes in the fluidity of the cell membrane can affect the effectiveness of drugs used to treat cancer and may contribute to tumor drug resistance. Membrane fluidity refers to the extent to which the lipid bilayer of a cell membrane can move and change its shape. This property is important for various cellular processes such as signaling, transport, and fusion. The fluidity of the membrane is dependent on the composition of its lipids, including the length and saturation of the fatty acid tails, the presence of cholesterol, and the presence of various membrane proteins. Various researches had shed light on the dynamic nature of membrane fluidity and its importance in various cellular processes. For example, studies have shown that changes in the fluidity of the membrane can affect the activity of membrane proteins, such as ion channels and receptors, by altering their conformation and localization.In addition, new technologies such as super-resolution microscopy and single-molecule imaging have allowed researchers to directly observe and manipulate membrane fluidity at the nanoscale level. This has led to a better understanding of the mechanisms underlying the regulation of membrane fluidity, such as the roles of lipid rafts and membrane-cytoskeleton interactions. Cell membrane fluidity is determined by the composition and organization of the lipid bilayer that makes up the membrane (Baritaki et al., 2007), as well as by the presence of proteins and other molecules embedded in the membrane. There are several factors that influence membrane fluidity for cell membrane components, including cholesterol content, saturation levels, fatty acid chain length, lecithin/sphingomyelin ratios and interactions between lipids and proteins (Szlasa et al., 2020, Pallarestrujillo et al., 2000). Moreover, external physical factors, such as temperature, will affect it as well (Zalba and Hagen, 2017). It is the fluidity of the normal cell membrane that allows substances to exchange inside and outside the cell. If the properties of the plasma membrane change in tumor cells, the fluidity of the cell membrane will also change. One of the main mechanisms by which changes in cell membrane fluidity affect tumor drug resistance is through alterations in the expression of drug transporters, such as P-glycoprotein (P-gp) and multidrug resistance-associated protein (MRP). These transporters are membrane proteins that play a key role in the efflux of drugs from the cell. Studies have shown that increased expression of P-gp and MRP in tumor cells can lead to decreased intracellular drug accumulation and increased drug efflux, resulting in drug resistance (Sauna and

Ambudkar, 2007). The reduction of drug resistance of tumor drug-resistant cells is associated with changes in P-gp function and membrane fluidity (Callaghan et al., 1993). In addition to drug transporters, changes in cell membrane fluidity can also affect the uptake and efflux of drugs by altering the expression of ion channels and transporters. For example, changes in the expression of aquaporins, which are membrane proteins that regulate the movement of water and other small molecules across the cell membrane, can affect the uptake and efflux of drugs. A study by Frede et al., 2013 showed that the expression of aquaporin-1 (AQP1) was significantly higher in drug-resistant leukemia cells than in drug-sensitive cells, and that inhibition of AQP1 increased the intracellular accumulation of chemotherapy drugs and enhanced drug sensitivity. Cancer cells may alter the fluidity of their cell membrane in a way that affects the effectiveness of drugs used to treat the cancer. For example, cancer cells may have increased fluidity of the cell membrane, which can make it easier for drugs to interact with their target molecules or to enter the cell. On the other hand, cancer cells may also have decreased fluidity of the cell membrane, which can make it more difficult for drugs to enter the cell and may contribute to drug resistance.

Improved cell membrane fluidity is important for improving tumor drug resistance (Baritaki et al., 2007). In tumor-resistant cells, the increase or decrease in membrane fluidity can lead to increased drug uptake (Hendrich and Michalak, 2003) . Ginsenoside Rg3 has been shown to reduce membrane fluidity in human fibroblast carcinoma cells and inhibit the activity of P-gp, thereby reducing drug efflux and reversing drug resistance (Kwon et al., 2008). Tetrandrine reduced membrane fluidity in human breast cancer cells (MCF-7/ADR) doxorubicin-resistant to doxorubicin in a concentration-dependent manner (Fu et al., 2002). In contrast, the membrane fluidity of human leukemia drug-resistant cells (K563/MDR) was increased after microwave treatment, but also effectively reversed the drug resistance of K562/MDR cells (Xiang et al., 2020, Ziru et al., 2002). Although the mechanism by which changing cell membrane fluidity reverses tumor drug resistance is still ambiguous, it is clear that affecting cell membrane fluidity may be a new strategy for improving resistance to tumor drugs. Further, the phospholipid bilayer's structural disruption and changes in cell membrane fluidity may facilitate the entry of cells into apoptosis (Baritaki et al., 2007). The impact of early cell membrane fluidity on tumor cell growth, proliferation, and drug resistance has once again been demonstrated by studies showing that lipid compounds and drugs can enhance early cell membrane fluidity and promote cell apoptosis (Fujimoto et al., 1999). By investigating this relationship between cell membrane fluidity and tumor drug resistance, it is possible to get valuable insights that can potentially lead to innovative approaches for enhancing the efficacy of anticancer therapies.

Detection Method of Cell Membrane Fluidity

1. Fluorescence Polarization Method

Fluorescence polarization is a technique that measures the degree to which the fluorescence emitted by a sample is polarized, or oriented, in a particular direction. Fluorescence polarization is based on the fact that the fluorescence emitted by a sample will be more strongly polarized when the sample is excited by linearly polarized light, as compared to unpolarized light. The fluorescence polarization method involves measuring the intensity of the fluorescence emitted by a sample when it is excited by linearly polarized light, and comparing it to the intensity of the

fluorescence emitted when the sample is excited by unpolarized light. The difference in the intensity of the fluorescence signals is used to calculate the degree of polarization of the sample. One of the most commonly used fluorescent probes for measuring membrane fluidity is 1,6 diphenyl-1,3,5,-ethanetriene (DPH), embedded in the hydrocarbon chain region of the fatty acids in the cell membrane.

By using a fluorescence spectrophotometer to measure the fluorescence intensity of the fluorescent probe and the membrane lipid, fluorescence polarization is used to determine membrane fluidity (Xiang et al., 2020). There is an association between the degree of fluorescence polarization (P) and the degree of fluidity of the cell membrane (Yonggui et al., 2000), where the larger the P value, the weaker the fluidity, and vice versa (Guiqin et al., 2004). As per the findings of Kechun et al., 1981, the fluidity of lipids in the ascites cancer cell membrane is influenced by the microviscosity of the membrane. Conversely, when the microviscosity increases, the fluidity of the cell membrane weakens, as stated by Xiang et al. (2020). In another study conducted by Yuan et al. (2015), the fluidity of erythrocyte membranes was assessed using DPH fluorescence spectrophotometry. Fluorescence polarization is a sensitive method for detecting small changes in the orientation or conformation of molecules, and it is often used to study the interactions between molecules, such as the binding of ligands to receptors or the folding of proteins. It can also be used to study the mobility of molecules in cells or tissues, as the degree of polarization of the fluorescence signal can be affected by the motion of the molecules. This method is widely used because of its rapid detection, sensitivity, and repeatability.

2. Fluorescence Bleaching Recovery Technology

Fluorescencerecovery after photobleaching (FRAP) is a technique that is used to measure the brightness or intensity of a fluorescence signal over time. Fluorescence bleaching occurs when the intensity of the fluorescence signal decreases over time due to the loss of fluorescence from the dye or probe used to label the molecule of interest. Fluorescence bleaching recovery technology involves measuring the intensity of the fluorescence signal before and after bleaching, and using this information to calculate the rate of recovery of the fluorescence signal. In fluorescent recovery after photobleaching, fluorescent substances are labeled on cell membrane proteins or membrane lipids, and then laser light is used to bleach out some of the fluorescence. The fluorescence of the bleached part can be restored after laser irradiation is stopped if the membrane protein or cell membrane has fluidity. Based on the recovery speed, the diffusion rate of membrane proteins or membrane lipids can be calculated and the membrane fluidity can then be calculated (Axelrod et al., 1976; Xiang et al., 2020). In recent years, advances in microscopy and labeling techniques have made FRAP an increasingly powerful tool for studying membrane fluidity. For example, it is now possible to selectively label different types of lipids or proteins within the membrane, allowing researchers to study their mobility and interactions in more detail. Using specific probes to label the lipids of myocardial cell membranes, Zhe et al., 2017 determined the effect of bupivacaine and long-chain lipid emulsions on the fluidity of myocardial cell membranes by using laser confocal microscopy and fluorescence bleaching recovery. Fluorescence bleaching recovery technology can be used to study a variety of phenomena,

including the stability of fluorescent dyes, the dynamics of protein-ligand interactions, and the mobility of molecules in cells or tissues. In addition to studying membrane fluidity in intact cells or tissues, FRAP can also be used to study the properties of artificial lipid bilayers in vitro. This can be useful for understanding how different lipid compositions or modifications affect membrane properties, and for testing the efficacy of potential drugs or therapeutic agents. It can also be used to study the properties of fluorescent proteins, such as their fluorescence intensity and lifetime.

Fluorescence bleaching recovery technology can be used in conjunction with various fluorescence imaging techniques, such as fluorescence microscopy or fluorescence spectroscopy. It is a powerful tool for studying the properties and behaviors of fluorescently labeled molecules, and has a wide range of applications in the life sciences and other fields.

3. Electron Spin Resonance Spectroscopy

Electron spin resonance (ESR) spectroscopy is a technique that can be used to detect the fluidity of cell membranes. ESR spectroscopy detects the motion of unpaired electrons in a sample, which can be used to measure the fluidity of the membrane. The technique involves adding a spin probe, which is a molecule that contains an unpaired electron, to the membrane of interest. The spin probe molecules become embedded in the membrane, and their motion can be detected using ESR spectroscopy. The ESR spectrum of a spin probe in a fluid membrane is broad, while in a more rigid membrane, the spectrum is narrower. The amplitude of the ESR signal reflects the degree of molecular motion of the spin probe, which is in turn influenced by the fluidity of the membrane. By analyzing the ESR spectra of the spin probe in different membranes, it is possible to compare the fluidity of the membranes. One commonly used spin probe for ESR spectroscopy is 5-doxyl stearic acid (5-DSA). The spin probe is added to the membrane of interest, and the sample is then analyzed using an ESR spectrometer. The ESR spectrum is analyzed to determine the fluidity of the membrane. Several studies have used ESR spectroscopy to detect changes in cell membrane fluidity. For example, a study by Pawlikowska-Pawlęga et al., (2014) used ESR spectroscopy to investigate the effects of cholesterol on the fluidity of the plasma membrane in HeLa cells. The authors found that the presence of cholesterol decreased the fluidity of the membrane, as indicated by a narrower ESR spectrum. Another study by Spasojević, 2011 used ESR spectroscopy to investigate the effects of oxidative stress on the fluidity of the mitochondrial membrane. The authors found that exposure to hydrogen peroxide decreased the fluidity of the mitochondrial membrane, as indicated by a narrower ESR spectrum. In addition to its ability to provide information about the fluidity of the membrane, ESR spectroscopy can also provide information about the physical state of the membrane, such as its order or disorder. One of the key advantages of ESR spectroscopy is its ability to directly measure the motion of the lipids in the membrane, rather than relying on indirect measures such as fluorescence recovery after photobleaching (FRAP). This makes it a particularly valuable tool for studying the fluidity of membranes under a wide range of conditions, including those that may alter the behavior of fluorescent probes used in FRAP. ESR spectroscopy works by measuring the behavior of unpaired electrons in the membrane. These unpaired electrons are generated by exposing the membrane to a free radical probe that becomes trapped in the membrane. The unpaired electrons in the probe then interact with the

surrounding lipids, allowing their motion to be directly measured. One common approach in ESR spectroscopy is to use spin-labeled fatty acids, which are fatty acids that have been modified to include a stable free radical group. These spin-labeled fatty acids can be incorporated into the membrane and their motion can be monitored by ESR spectroscopy. The ESR spectra obtained from spin-labeled fatty acids can be analyzed to obtain information about the rotational motion and lateral diffusion of the lipids in the membrane, which can in turn be used to calculate the fluidity of the membrane. ESR spectroscopy has been used to study the fluidity of a wide range of biological membranes, including those from red blood cells, nerve cells, and cancer cells. For example, ESR spectroscopy has been used to investigate the effect of cholesterol on the fluidity of the plasma membrane in breast cancer cells (Zeisig et al., 2007). Another study used ESR spectroscopy to examine the effect of cell aging on the fluidity of the plasma membrane in human fibroblasts (Alonso et al., 2019). In all, ESR spectroscopy is a powerful tool for studying the fluidity of biological membranes, and has been used extensively to investigate the physical properties of cell membranes under a wide range of conditions.

4. Laurdan Two-Photon Microscopy

Laurdan is a membrane probe that exhibits a blue shift in its excitation and emission spectra when it is in a less polar environment and a red shift in a more polar environment. This property makes it a useful tool for detecting changes in membrane fluidity. Two-photon microscopy is a technique that allows for high-resolution imaging of samples using a low-energy, long-wavelength laser, which reduces photodamage to the sample. Laurdan two-photon microscopy uses this probe to measure the polarity of the local environment of the cell membrane. The probe is excited by a two-photon laser, and the emitted fluorescence is collected and used to calculate a generalized polarization (GP) value. GP values range from -1 to 1, with values closer to 1 indicating a more ordered, less fluid membrane and values closer to -1 indicating a more disordered, fluid membrane. Laurdan two-photon microscopy has been used to study membrane fluidity in a variety of biological systems, including cells, tissues, and even live animals. For example, a study by Balogh et al. (2011) used Laurdan two-photon microscopy to measure membrane fluidity in human fibroblast cells. They found that cells treated with a membrane fluidizer had a decreased GP value, indicating an increase in membrane fluidity. Another study by Yamamoto and Ando (2013) used Laurdan two-photon microscopy to investigate the effect of cholesterol depletion on the fluidity of giant unilamellar vesicles (GUVs). They found that the GP value decreased when cholesterol was removed from the membrane, indicating an increase in membrane fluidity. Another study by Jay and Hamilton (2017) used Laurdan two-photon microscopy to measure changes in membrane fluidity during the development of zebrafish embryos. The researchers found that the membrane fluidity of the yolk sac and somatic cells increased during early embryonic development, and that this increase was correlated with changes in the lipid composition of the membranes. Laurdan two-photon microscopy has also been used to investigate the effects of drugs and other agents on membrane fluidity. A study by Erazo-Oliveras et al. (2022) used Laurdan two-photon microscopy to investigate the effects of curcumin, a natural compound with anti-inflammatory and anti-cancer properties, on membrane fluidity in cancer cells. The researchers found that curcumin increased membrane fluidity in cancer cells, which was

correlated with increased cellular uptake of curcumin and increased cytotoxicity. Laurdan two-photon microscopy has several advantages over other techniques for measuring membrane fluidity. It is non-invasive and can be used in live cells and tissues. It is also relatively easy to perform and does not require any special sample preparation. However, it is important to note that the GP value is affected by other factors besides membrane fluidity, such as temperature and probe concentration, so careful controls and calibrations are necessary to ensure accurate results. Overall, Laurdan two-photon microscopy is a powerful tool for investigating membrane fluidity in a wide range of biological systems, from cells to whole organisms. Its sensitivity, specificity, and versatility make it a valuable tool for basic research, drug development, and clinical applications.

The Effect of Cell Membrane Permeability Changes on Tumor Drug Resistance

As a functional characteristic of the cell membrane, membrane permeability refers to how easily substances enter and leave the cell. Changes in the permeability of the cell membrane can affect the effectiveness of drugs used to treat cancer and may contribute to tumor drug resistance. For example, certain cancer cells may have increased permeability of the cell membrane, which can allow drugs to more easily enter the cell and interact with their target molecules. On the other hand, cancer cells may also develop decreased permeability of the cell membrane, which can make it more difficult for drugs to enter the cell and may contribute to drug resistance. It is not just the fact that the P-gp pumps out the drug from the cell that causes tumor drug resistance, but also the physical characteristics of the cell membrane, which reduce the diffusion rate of the drug and make it easier for it to enter the cell, may affect the drug when it enters the cell (Xiang et al., 2020). In cancer cells, the overexpression of P-gp can lead to resistance to chemotherapeutic drugs. This occurs because P-gp pumps these drugs out of the cancer cells, thereby reducing their effectiveness. P-gp achieves drug efflux by using energy from ATP hydrolysis to transport drugs from the cytoplasm of the cell to the extracellular space. The P-gp protein has a large transmembrane domain that forms a channel through which drugs can pass. The channel is lined with hydrophobic amino acid residues that interact with the hydrophobic portions of the drug molecules. Changes in the lipid composition of the membrane can alter the conformation and activity of P-gp, thereby affecting its ability to pump out drugs, while increasing the degree and speed of drug entry into the cell is of great importance for overcoming tumor drug resistance because the drug molecules within are reduced (Hendrich and Michalak, 2003).

Developing drugs that can alter the lipid composition or permeability of the membrane may increase the efficacy of existing drugs by reducing drug resistance. Alternatively, targeting P-gp itself may be more effective in cells with specific membrane properties. With a certain intensity of ultrasound, Jia et al., 2015 improved the permeability of the cell membrane, increased drug accumulation in MCF-7/ADR cells and reversed drug resistance in MCF-7/ADR cells. Both SM and glycolipids reduce membrane permeability in the outer leaflet of a cell membrane, but cholesterol is the decisive factor. By reducing cholesterol content, increasing membrane permeability, and reducing membrane rigidity, these compounds reduce tumor drug resistance. Other changes in cell membrane permeability may also affect the effectiveness of drugs used to treat cancer. For example, cancer cells may alter the expression or localization of proteins on the cell surface, such as transporters or receptors that play a role in the uptake or efflux

of drugs (Xiang et al., 2020). This can also contribute to drug resistance by affecting the ability of drugs to interact with their target molecules or to enter the cell. As phospholipid unsaturation increases, membrane permeability increases, and drug accumulates in the cell, the effect of reversing drug resistance increases (Hill and Zeidel, 2000). Apigenin enhanced cell membrane permeability in a dose-dependent manner and became an important factor in inducing apoptosis (Zhu et al., 2016). The PS component serves not only as an important component of phospholipids in this process, but also as a signal of early apoptosis when the cell membrane is damaged and the membrane permeability increases, PS is flipped from the inner leaflet of the cell membrane to the outer leaflet by flippase (Demchenko, 2012). A pro-apoptotic cationic host defense peptide containing lysine or arginine selectively binds to tumor cells through electrostatic interactions, causing apoptosis and reversing tumor drug resistance in human breast cancer paclitaxel-resistant cells (MCF-7/Taxol) (Xiang et al.2020, Dai et al., 2017). Several drugs targeting ion channels have been shown to be effective in overcoming drug resistance in cancer cells.

For example, a study by Spreckelmeyer et al. (2014) showed that the potassium channel blocker, tetraethylammonium (TEA), enhanced the efficacy of the chemotherapy drug, cisplatin, in cisplatin-resistant lung cancer cells by increasing cell membrane permeability and reducing the expression of drug transporters. By targeting membrane components and transporters, it is possible to enhance drug delivery and overcome resistance, while innovative drug delivery systems can exploit altered membrane permeability for improved therapeutic efficacy.

Detection method of cell membrane permeability

1. Fluorescence Labeling Method

Fluorescence labeling is a widely used technique in biology that has revolutionized the field of molecular imaging and cell biology. This technique offers a non-invasive way to visualize and study biological molecules, and is based on the fact that certain molecules can absorb light of a specific wavelength and emit it at a longer wavelength. The fluorescent dye or molecule is attached to the biological molecule of interest like protein, nucleic acid which then emits a fluorescence signal that can be detected, visualized and imaged using specialized microscopes and cameras. Fluorescence labeling can be used for a variety of purposes, including imaging, identifying specific molecules or cells, and studying the interactions between molecules. Flow cytometry is one of the most commonly used techniques for fluorescence labeling in biological research, and it allows for high-throughput screening of large numbers of cells or molecules. By using this technique, Ling et al. (2016) studied the effect of *Physcomitrella goldenscens* extract on K562 cells' membrane permeability using fluorescein diacetate/propidium iodide (FDA/PI) double staining. This method is based on the principle that fluorescein diacetate can enter live cells and be hydrolyzed by intracellular esterases, resulting in the production of fluorescein, which emits green fluorescence. Propidium iodide, on the other hand, can only enter dead cells with compromised membranes, and it intercalates into the DNA of the dead cells and emits red fluorescence. Guangyao et al., 2017 found that algae-dissolving active substances can destroy the integrity of algae cell membrane through acridine orange/ethidium bromide (AO/EB) double staining method. This method involves the use of acridine

orange, which can enter both live and dead cells, and stains the nuclei of cells with bright green fluorescence. Ethidium bromide, on the other hand, can only enter cells with compromised membranes, and it intercalates into the DNA of the dead cells and emits bright red fluorescence. While fluorescence labeling is a sensitive and easy-to-use technique, it can be affected by several factors during imaging. One of the main challenges in fluorescence imaging is the fast quenching speed of fluorescence, which can result in a loss of signal over time. Other factors that can affect fluorescence imaging include the imaging parameters, such as the excitation wavelength, exposure time, and magnification, as well as the properties of the fluorescent dye or molecule used for labeling.

2. SEM

Scanning electron microscopy (SEM) is a imaging technique that uses a focused beam of electrons to produce detailed, high-resolution images of the surface of a sample. SEM is often used to study the morphology, or shape, of cells and tissues, as well as their surface features and textures. According to Goldstein et al. (2018) SEM can provide high-resolution images with high depth of field, allowing for detailed observation of surface morphology, topography, and texture. The images generated by SEM can also provide information on chemical composition and crystallographic structure through the use of various detectors, such as energy-dispersive X-ray spectroscopy (EDS) and electron backscatter diffraction (EBSD). SEM can be used to detect changes in cell membrane permeability, as the permeability of the membrane can affect the appearance of the cell surface. The permeability of the cell membrane is responsible for controlling the movement of molecules in and out of the cell, and changes in this permeability can have a significant impact on the overall function of the cell. One of the key advantages of SEM is that it can be used to visualize changes in the size or shape of cells, as well as changes in the distribution of proteins or other molecules on the surface of the cells. These changes may be indicative of changes in the permeability of the cell membrane, and SEM can provide detailed images that allow researchers to study these changes in great detail. Surface irregularities are studied by bombarding them with atomic rays, which emit secondary electrons and other signals that are detected by detectors after they are received. The changes on the surface of the cell membrane were observed by SEM after ganglioside antibody was injected into mouse leukemia cells (Roquenavarro et al., 2008). SEM can clearly observe morphological changes on cell surfaces including microvilli, pseudopodia, and perforation, as well as changes in microvilli under different conditions (Xiang et al., 2020). It is worth noting that SEM is a surface imaging technique and cannot provide information about the interior of cells or tissues. Other techniques, such as transmission electron microscopy or fluorescence microscopy, may be needed to visualize changes in the interior of cells or tissues or to study other aspects of cell membrane permeability.

3. Electrophysiology

Another method to measure cell membrane permeability is electrophysiology, which involves the measurement of changes in the electrical properties of cells in response to changes in membrane permeability. Electrophysiology is a powerful tool that allows for the measurement of the electrical properties of cells in real-time. The technique is based on the fact that ion channels, which are transmembrane proteins that regulate the flow of ions across the cell membrane, play a critical role in regulating cell membrane permeability.

Changes in ion channel activity can alter the electrical properties of the cell membrane, which can be measured using electrophysiological techniques. This technique can provide real-time information about changes in membrane permeability and ion transport (Deng et al., 2004). Patch-clamp electrophysiology is a widely used technique to investigate ion channels in cell membranes, including the effects of drugs and toxins on membrane permeability (Zheng et al., 2004). One widely used electrophysiological technique for investigating ion channels is the patch-clamp technique, which was first developed by Erwin Neher and Bert Sakmann in the 1970s (Neher and Sakmann, 1976). The patch-clamp technique involves attaching a glass pipette to the surface of a cell and forming a high-resistance seal between the pipette and the cell membrane. This allows for the measurement of the electrical properties of a small patch of cell membrane, typically on the order of a few square micrometers. Patch-clamp electrophysiology can be used to investigate the effects of drugs and toxins on membrane permeability. For example, the technique has been used to study the effects of certain antibiotics on bacterial cell membranes (Kreir et al., 2008). Additionally, the patch-clamp technique has been used to study the effects of toxins on ion channels, such as the effects of tetrodotoxin on sodium channels (Brau et al., 1988). In addition to the patch-clamp technique, other electrophysiological techniques can be used to measure changes in membrane permeability, such as impedance spectroscopy (Matthews and Judy, 2003). Impedance spectroscopy measures changes in the electrical impedance of a cell or tissue sample in response to changes in membrane permeability, and has been used to study the effects of drugs and toxins on cell membranes (Crowell et al., 2020). These techniques have applications in drug discovery, toxicology, and basic research in cell biology.

4. Atomic force microscopy

Atomic force microscopy (AFM) is a high-resolution imaging technique that can be used to study biological samples, including cells and cell membranes. AFM is a powerful tool for studying membrane permeability because it can directly measure the mechanical properties of the cell membrane, including its stiffness and elasticity. In this way, AFM can provide valuable insights into the permeability of the cell membrane, which is critical for many cellular processes, including drug uptake and cell signaling. The basic principle of AFM is to use a small probe with a sharp tip to scan the surface of a sample in a raster pattern, similar to how a record player reads the grooves on a vinyl record. The tip is attached to a cantilever that deflects as it interacts with the sample, and the deflection is measured with a laser beam. By moving the sample and the probe relative to each other, a 3D image of the sample surface can be generated with nanometer-scale resolution. To study membrane permeability with AFM, the first step is to immobilize the cells on a substrate. This can be done by attaching the cells to a surface through a process called cell adhesion, which involves coating the substrate with molecules that the cells can bind to. Once the cells are immobilized, the AFM probe can be used to indent the cell membrane and measure its mechanical properties. There are several ways that AFM can be used to study membrane permeability. One approach is to measure the stiffness of the cell membrane using a technique called force spectroscopy. In force spectroscopy, the AFM probe is used to apply a small force to the cell membrane, and the resulting indentation of the membrane is measured. By analyzing the relationship between the applied force and the resulting

indentation, the stiffness of the membrane can be calculated. Changes in membrane stiffness can be an indicator of altered permeability, as increased permeability can lead to changes in the organization and composition of the membrane. Another approach is to use AFM to measure the force required to rupture the cell membrane, which is called membrane disruption force spectroscopy (MDFS). MDFS involves increasing the force applied to the cell membrane until it ruptures, and measuring the force required to do so. Changes in the rupture force can be an indicator of changes in membrane permeability. AFM can also be used to directly visualize the effects of membrane-permeabilizing agents on the cell membrane. For example, AFM has been used to study the effects of chemotherapeutic drugs on cancer cell membranes. One study used AFM to show that the drug doxorubicin caused changes in the stiffness and elasticity of the cell membrane, which could be indicative of increased membrane permeability (Agarwala et al., 2022). In another study, AFM was used to visualize the formation of nanoscale pores in the cell membrane caused by the drug cytochalasin B (Lamprecht et al., 2014). Overall, AFM is a powerful tool for studying membrane permeability in cancer cells.

5. Lactate Dehydrogenase Release Assay

A lactate dehydrogenase (LDH) release assay is a laboratory test that measures the amount of LDH in a sample. LDH is an enzyme that is involved in the anaerobic metabolism of glucose and other sugars because it does not require oxygen as a co-factor for its enzymatic activity, and is found in many tissues in the body, including the liver, heart, and skeletal muscles. Cell membrane damage is often detected by lactate dehydrogenase release (lactate dehydrogenase, LDH) and by the enhanced permeability of the membrane caused by the damaged membrane. The assay involves adding a sample containing LDH, such as cell culture supernatant or tissue homogenate, to a solution containing specific enzymes and substrates. The LDH in the sample catalyzes the conversion of the substrate to a product, and the amount of product produced is measured and used to calculate the amount of LDH in the sample (Xiang et al., 2020). When the cell membrane is damaged and its permeability is increased, LDH can be released outside the cell. LDH is an extremely stable cytoplasmic enzyme, which normally cannot pass through the membrane. As a result, lactic acid is converted into pyruvate, which reacts with tetrazole to produce red. A microplate reader can detect the formazan compound after dissolution, and the percentage of LDH activity in the total LDH can be calculated, which reflects the extent of damage to the membrane (Xiang et al., 2020). In a time-concentration-dependent manner, polypeptide BF-30 caused pancreatic cancer cells to release LDH under its action at different concentrations and times, suggesting that polypeptide BF-30 damages pancreatic cancer cell membranes in a time-concentration-dependent manner (Wang et al., 2017). Using the LDH release assay, Chang et al. (2011) also examined the effect of hepcidin 1-5 on human fibrosarcoma cells and human cervical cancer cells. This method has the advantages of being simple to use, highly sensitive, low in cost, and highly detectable. The LDH release assay can be used to evaluate the effectiveness of a treatment or to monitor the health of cells or tissues. It is often used in cancer research, as cancer cells are often more sensitive to certain treatments and may release more LDH in response to treatment. The LDH release assay can also be used to evaluate the effectiveness of a drug or to monitor the health of cells or tissues in other contexts, such as in drug development or toxicology studies. The LDH

release assay is relatively simple, quick, and cost-effective compared to other cytotoxicity assays such as the MTT assay and the trypan blue exclusion assay (Fotakis and Timbrel, 2006). However, the LDH release assay has some limitations. For example, the release of LDH is not always indicative of cell death, and some cells may release LDH without losing their viability. In addition, certain experimental conditions, such as pH, temperature, and the type of culture medium, can affect the accuracy and reproducibility of the assay.

6. Magnetic resonance imaging

Magnetic resonance imaging (MRI) is a non-invasive imaging technique that uses magnetic fields and radio waves to create images of the body. It has been widely used in medical diagnosis and research due to its high spatial and temporal resolution, and the ability to provide information on the structural and functional properties of tissues. Recently, MRI has been explored as a tool to evaluate membrane permeability in cancer cells. One of the most commonly used methods to assess membrane permeability using MRI is dynamic contrast-enhanced MRI (DCE-MRI). DCE-MRI involves the injection of a contrast agent, typically a gadolinium-based compound, into the bloodstream, and the subsequent measurement of the signal intensity in the tissue of interest as a function of time. The contrast agent leaks out of the blood vessels and accumulates in the extravascular space, leading to an increase in signal intensity. The rate of contrast agent uptake and washout can provide information on the membrane permeability and perfusion of the tissue.By analyzing the time course of contrast agent uptake and clearance from the tumor, researchers can estimate parameters such as the volume of the extravascular extracellular space (EES), the permeability-surface area product (PS) of the tumor vasculature, and the fractional plasma volume (fPV) of the tumor. DCE-MRI has been used to evaluate the membrane permeability of various types of cancer cells, including breast, prostate, and brain tumors. For example, in a study of breast cancer patients, DCE-MRI was able to distinguish between benign and malignant lesions based on differences in membrane permeability and perfusion (Choyke et al., 2003). Similarly, in a study of prostate cancer patients, DCE-MRI was able to predict the likelihood of tumor recurrence based on differences in membrane permeability and perfusion (Alonzi et al., 2007). Another method that has been used to assess membrane permeability using MRI is diffusion-weighted imaging (DWI). DWI measures the diffusion of water molecules in tissue, and can be used to evaluate changes in the extracellular space associated with membrane permeability. In cancer cells, increased membrane permeability can lead to an increase in the extracellular space, which can be detected using DWI.DWI has been used to evaluate the membrane permeability of a variety of cancers, including breast, prostate, and liver tumors. For example, in a study of breast cancer patients, DWI was able to differentiate between benign and malignant tumors based on differences in membrane permeability (Sinha et al., 2002). In short, MRI has been explored as a tool to evaluate membrane permeability in cancer cells which is quite useful in the diagnosis, prognosis, and monitoring of various types of cancer, and has the potential to improve patient outcomes.

7. TEER

Another commonly used methods to study cell membrane permeability is the measurement of transepithelial electrical resistance (TEER) using a voltmeter. TEER is a measure of the integrity of the cell monolayer like Caco-2, HT29, MDCK, A549 and is a

reflection of the tight junctions between the cells. It is an indirect measure of cell membrane permeability, as increased TEER indicates tighter junctions, which reduces the paracellular transport of drugs across the cell monolayer. The TEER method has been used extensively to study the effects of various drugs and compounds on cell membrane permeability in Caco-2 cells (Anderberg and Artursson, 1993). To measure TEER in Caco-2 cells, the cells are grown to confluency on a permeable membrane, such as a polycarbonate filter or a Transwell insert. The membrane separates two compartments, apical and basolateral, which represent the luminal and blood sides of the intestinal epithelium, respectively. The voltmeter is then used to measure the electrical resistance across the membrane, and the readings are recorded. The values are expressed as ohm*cm^2 to account for the surface area of the membrane. The measurement of TEER is a non-destructive method that can be used to monitor the integrity of the cell monolayer over time. A decrease in TEER value indicates an increase in paracellular transport, which could be due to the disruption of tight junctions between the cells. Conversely, an increase in TEER value indicates tighter junctions, which reduces paracellular transport.TEER measurement has several advantages over other methods of studying cell membrane permeability. It is non-invasive and does not require the use of exogenous markers or probes, which can interfere with the cellular processes being studied. Moreover, TEER measurement can be performed in real-time, allowing for the monitoring of drug effects on cell membrane permeability over time. Several studies have used TEER measurement to study the effects of various compounds on cell membrane permeability in Caco-2 cells. For example, it has been shown that the addition of polyphenols, such as quercetin and resveratrol, to the apical compartment of Caco-2 cells increased TEER values, indicating tighter junctions (JanssenDuijghuijsen et al., 2017). Similarly, it has been shown that the addition of the anti-inflammatory drug, dexamethasone, to the apical compartment of Caco-2 cells increased TEER values and reduced paracellular transport (Beck et al., 2007). One advantage of the TEER method is that it provides an indirect measure of cell membrane permeability, as increased TEER indicates tighter junctions between cells, which reduces the paracellular transport of drugs across the cell monolayer. This makes the TEER method a useful tool for evaluating the effects of various drugs and compounds on cell membrane permeability in Caco-2 cells (Anderberg and Artursson, 1993). Another advantage of the TEER method is that it is a non-destructive and non-invasive technique, which means that the cells can be used for further experiments after the TEER measurement. This makes the TEER method a useful tool for monitoring the integrity of cell monolayers over time, as well as for evaluating the effects of different culture conditions on cell membrane permeability. However, the TEER method also has some disadvantages that need to be considered. One disadvantage is that the TEER measurements are affected by a number of factors, such as temperature, pH, and culture medium composition, which can affect the tight junctions between cells and the overall integrity of the cell monolayer (Srinivasan et al., 2015). This means that it is important to standardize the experimental conditions when using the TEER method to ensure accurate and reproducible results. Another disadvantage of the TEER method is that it does not provide information on the permeability of specific molecules or drug compounds across the cell membrane. Instead, it provides an overall measure of cell membrane permeability, which can be affected by a number of factors. This means that it is

important to complement the TEER measurements with other assays, such as the microfluidic permeability assay (MPA), to obtain a more comprehensive understanding of cell membrane permeability and drug transport across cell membranes.

8. Microfluidic Permeability Assay

The microfluidic permeability assay (MPA) is a new method for studying drug permeability across cell membranes. This method uses microfluidic channels to create a continuous flow of drug molecules across a monolayer of cells like Caco-2, HT29, MDCK, A549 cells. The permeability of the drug molecules is measured by monitoring their concentration in the effluent stream over time. The MPA can be used to evaluate the permeability of different drugs and to study the effects of various factors, such as pH and temperature, on drug permeability. A study used the MPA to evaluate the permeability of different drugs across Caco-2 cell monolayers and found that the method provided accurate and reproducible results (Imura et al., 2009). To use the MPA to study membrane fluidity, Caco-2 cells are cultured on a porous membrane, such as a Transwell insert, in a microfluidic channel. A solution of fluorescently labeled molecules of different sizes and properties is introduced into the channel, and the fluorescence intensity in the effluent stream is monitored over time using a fluorescence microscope. The permeability of the fluorescent molecules across the cell monolayer is calculated based on their concentration in the effluent stream and the flow rate of the solution. The permeability of molecules with different properties, such as size, hydrophobicity, and charge, can be measured using the MPA. For example, a study by Ghaffarian and Muro (2013) used the MPA to evaluate the permeability of small molecules, such as glucose and mannitol, as well as larger molecules, such as albumin and dextran, across Caco-2 cell monolayers. The results showed that the permeability of these molecules varied depending on their size and charge, with the larger molecules exhibiting lower permeability across the cell monolayer. The MPA can also be used to evaluate the effects of factors that affect membrane fluidity on permeability. For example, a study by Tan et al. (2018) used the MPA to evaluate the effects of temperature on the permeability of fluorescently labeled molecules across Caco-2 cell monolayers. The results showed that increasing the temperature decreased the permeability of the fluorescent molecules across the cell monolayer, indicating a decrease in membrane fluidity. Another advantage of the MPA is its ability to evaluate the effects of drugs and other compounds on membrane fluidity and permeability. For example, a study by Babadi et al. (2020) used the MPA to evaluate the effects of the drug piperine on the permeability of fluorescently labeled molecules across Caco-2 cell monolayers. The results showed that piperine increased the permeability of the fluorescent molecules across the cell monolayer, indicating an increase in membrane fluidity. One advantage of the MPA method is that it provides a more precise and accurate measurement of drug permeability across the cell membrane compared to other methods, such as the TEER method. This is because the MPA method allows for continuous and controlled flow of drug molecules across the cell monolayer, which provides a more realistic and dynamic environment for drug transport studies. Another advantage of the MPA method is that it can be used to study the effects of various factors, such as pH and temperature, on drug permeability across cell membranes. This is important because these factors can significantly impact drug transport and absorption in vivo. The MPA method allows

for the evaluation of these effects in a controlled and reproducible manner.

Conclusion

To summarize this review, a new strategy to overcome tumor drug resistance based on changing the fluidity and permeability of cell membranes is expected to be developed. Lipids play a crucial role in the structure and function of cell membranes. The development of tumor drug resistance is closely related to changes in lipids within cell membranes. Increase in phospholipids and cholesterol make cell membranes more "hard," preventing drugs from entering. Changing the fluidity and permeability of the cell membrane essentially changes the arrangement order of the phospholipid bilayer of the cell membrane, the ratio of cholesterol/phospholipid, etc., and then affects the amount of drugs entering the cell, as well as the growth or apoptosis of the cell. For example, increasing the fluidity or permeability of the cell membrane may make it easier for drugs to interact with their target molecules or to enter the cell, which may increase the effectiveness of the drug and enhance the growth or survival of the cell. On the other hand, decreasing the fluidity or permeability of the cell membrane may make it more difficult for drugs to enter the cell and may contribute to drug resistance, as well as impair the growth or survival of the cell. However, the specific mechanism that affects the fluidity and permeability of the cell membrane, and the internal mechanism that affects tumor drug resistance need to be further studied. In a broader context, the fluidity and permeability of the cell membrane are important factors that can affect the effectiveness of drugs and the growth or apoptosis of cells, and understanding these factors can help to identify strategies to overcome drug resistance in cancer and other diseases and a further understanding of the specific mechanisms affecting cell membrane fluidity and permeability, as well as the internal mechanisms affecting tumor drug resistance, is needed.

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THE EFFECT OF ZINC ON GERMINATION IN SPECIES OF THE GENUS *ECHIUM* (FAMILY BORAGINACEAE)

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Abstract: The effect of zinc on seed germination in two species of the *Echium* genus (*Echium russicum* J.F. Gmelin and *Echium vulgare* L.) was investigated. Zinc was used as sulphate in three different concentrations: 100 mg⁄l; 200 mg⁄l și 300 mg⁄l; the treatment was applied to the seeds before germination, the exposure time being short (4 hours and 8 hours). Seed germination was monitored daily for a period of 14 days. The effect of treatment on seed germination in the test species was assessed by calculating four germination indicators: final germination percentage (FGP), germination index (GI), mean germination time (MGT) and coefficient of rate germination (CRG). The results show the following: the seeds of *Echium russicum* germinate faster than those of *Echium vulgare*; statistically insignificant changes in the indicators associated with germination, except for GI in *Echium vulgare* for the 8 hours exposure time; reduction of germination percentage (in both species) and increase in germination time (more evident in *Echium vulgare*) with increasing exposure time. The test species showed tolerance throughout the germination process to the zinc concentrations used.

Keywords: zinc, *Echium russicum*, *Echium vulgare*, germination indices

1. Introduction

Zinc belongs to group II-B of the periodic table of elements and is a transition metal.

Zinc is a microelement involved in many biochemical (enzyme activity, metabolism of some groups of organic compounds) and physiological processes (growth and development, photosynthesis, defense against diseases and pests, etc.) specific for plant life. The role of zinc for plants is described in literature by various authors (Rout and Das, 2003; Zamfirache, 2005; Sadeghzadeh, 2013; Wyszkowska et al., 2013).

According to Jain et al. (2010), zinc is the second most abundant transition metal in organisms after iron.

Although zinc is a micronutrient for plants, at high concentrations it can negatively influence biochemical and physiological processes (enzyme activity, cell division, germination, photosynthesis, plant growth and development) (Rout and Das, 2003; Jain et al., 2010). Toxicity limits vary according to species, ontogenetic phase, exposure time. According to Macniol and Beckett (1985), the

upper toxicity level for zinc is between 100 ppm and 500 ppm.

Anthropogenic sources of environmental pollution with zinc are represented by industrial activities (mining/processing of ores), industrial waste disposal, road transport, agricultural activities (excessive application of pesticides and fertilizers), etc. (Vasiliev et al., 2011; Wyszkowska et al., 2013).

In Romania, according to the legislation in force (Ordin nr. 756/1997) the maximum allowed limit for the total zinc content in the soil is 100 mg/ kg. The alert threshold is 300 mg/ kg for sensitive land and 700 mg/kg for less sensitive land. For the intervention threshold, the following values are established: 600 $mg \cdot kg^{-1}$ for sensitive lands and 1500 mg/kg for less sensitive lands.

Some species of the genus *Echium* such as *Echium russicum* and *Echium vulgare* have been studied for their physiological response to stress conditions caused by the presence of heavy metals or their phytoremediation potential (Izmaiłow and Biskup, 2003; Dresler et al., 2014; Dresler et al., 2017a; Dresler et al., 2017b.; Jakovljević et al., 2019; Murtić et al., 2021).

Echium russicum J.F. Gmelin, syn. *Echium rubrum* Jacq., non Forssk. (Gibbs, 1972), syn. *Pontechium maculatum* (L.) Böhle & Hilger (Hilger and Böhle, 2000), syn. *Echium maculatum* L. (Sârbu et al., 2013) is a biannual, heliophytic, subthermophilous, xerophilous, neutrophilous, oligo-mesotrophic, therophyte-hemiterophyte species. (Sârbu et al., 2013). The species has melliferous, decorative and medicinal importance (Grințescu, 1960; Chwil and Weryszko-Chmielewska, 2007***;*** Moazzami Farida et al., 2020). *Echium russicum* is a species of community interest, being considered a good indicator of the state of conservation of dry and semi-dry steppe and forest grasslands (Mănoiu and Brînzan, 2013). It is included in Annexes II and IV of the Habitats Directive 92/43/EEC on the conservation of natural habitats and of wild fauna and flora species, as well as in Annex I of Resolution 6 (1998) of the Berne Convention, revised in 2011 (https://eunis.eea.europa.eu/species/162097). According to Jakovljević et al. (2019) *Echium russicum* grows on both metalliferous and non-metalliferous soils and is considered a facultative metallophytic species. The results of the research carried out by the authors noted above with plants taken from sites with ultramafic soils in Serbia indicated the efficient absorption of zinc and copper from the soil and their translocation in the aboveground organs, a translocation which is considered a detoxification mechanism.

Echium vulgare L. is a hemiterophytic, biannual (or sometimes perennial), heliophilous, eurythermal, xero-mesophilic, oligo-mesotrophic, eurythermal species (Sârbu et al., 2013). It is a common species in Romania and has medicinal, melliferous, tanning importance (Grințescu, 1960; Eruygur et al., 2012). According to Dresler et al. (2014), *Echium vulgare* as a pseudometalophyte can grow on both uncontaminated and heavy metal polluted land; plant populations on heavy metal polluted land showed high tolerance to cadmium. Studies conducted in Poland on *E. vulgare* populations established spontaneously on lands with heavy metal waste from mining activity showed that plants growing on metalliferous lands produced smaller seeds with lower germination abilities (Dresler et al., 2017a). The root and stem of *Echium vulgare* accumulate heavy metals (Cu, Zn, Pb, Cd, Fe) and show a higher concentration of secondary metabolites (allantoin, shikonin derivatives), as a response to stress conditions caused by heavy metals in the growth environment. (Dresler et al., 2017b). Studies conducted in Bosnia and Herzegovina by Murtić et al. (2021) showed that *Echium vulgare* plants grown

spontaneously on the land located in the vicinity of a steel mill accumulated heavy metals (Cr, Cd, Zn, Pb, Ni) in the above-ground parts, but the ability to remove/stabilize heavy metals in the polluted soil was decreased.

The present work aims to investigate the influence of zinc treatment on the germination process in *Echium russicum* J.F. Gmelin and *Echium vulgare* L. species.

2. Materials and methods

Plant material

The plant material was represented by seeds belonging to two species of the *Echium* genus: *Echium vulgare* (EA020) and *Echium russicum* (EA017) purchased from Jelitto Staudensamen GmbH in Schwarmstedt, Germany (https://www.jelitto.com/).

Experimental procedures

Zinc was used in three different concentrations (100 mg/l, 200 mg/l and 300 mg/l), in the form of aqueous zinc sulphate solutions [$ZnSO_4.7\ H_2O$].

The seeds were immersed in zinc sulphate solutions (treatment variants) and respectively in distilled water (control), for a period of 4 hours and 8 hours, respectively.

The treated seeds were transferred for germination in sterile Petri dishes, on filter paper moistened with distilled water (4 ml each). For each experimental variant, four replications were used, each replication with 10 seeds. The Petri dishes were kept under laboratory conditions (with the naturally day/night photoperiod characteristic of March-April, year 2021 and an average temperature of approximately 21°C) for a period of 14 days. During this experimental period the germinated seeds were counted at intervals of 24 hours.

Studied parameters

The effect of the treatment on the germination of the test species was evaluated by calculating 4 germination indicators, according to the formulas described by Islam and Kato-Noguchi, (2014): final germination percentage (FGP), germination index (GI), mean germination time (MGT) and coefficient of rate germination (CRG).

Statistical analysis

All results noted in the tables are expressed as mean value ± standard error. The data obtained for each indicator studied were statistically analyzed using the one-way ANOVA test and the Tukey HSD test (to test the difference between mean values). Differences were considered significant when the *p*-value was ≤ 0.05.

3. Results and discussions

The final percentage of germination (FGP), for the treatment with exposure duration of 4 hours, registered a slight value increase for all the concentrations used, in the case of the species *E. vulgare* (by 9% - 13.63%) and for the concentrations of 200mg /l (by 15.78%) and 300mg/l (by 21.05%) in the species *E. russicum*. Also, FGP recorded a decrease of 25% in the case of the concentration of 100mg/l in the species *E.russicum* (**Table 1, Table 2**).

For the treatment with an exposure duration of 8 hours, in both test species, FGP recorded lower values than in the control in all treatment options: by 17.25% - 31.04% in *E. russicum* and by 14.29% - 32.15% in *E. vulgare* (**Table 3, Table 4**).

From a statistical point of view, the recorded changes are not significant, a situation valid for each exposure period and for each test species (**Table 1-4**).

Regarding FGP, the results obtained in the present study for the control in the case of the *E. russium* species are in agreement with those presented by Nowak et al. (2020).

The mean germination time (MGT) presented (with few exceptions) slightly higher values in the treatment variants compared to the control (by 0.43% to 23.52%) for each test species and each exposure period. MGT recorded statistically insignificant changes ($p \geq 0.05$) (**Tables 1-4**).

Depending on the experimental variant, the seeds of the two test species germinated as follows: in *E. russicum* - after a period of time between 5.9 and 7.72 days, and in *E. vulgare* after 8.53 – 10.03 days. This fact denotes a faster germination of the seeds of *E. russicum*, compared to those of *E. vulgare*. The obtained results confirm the data reported by Nowak et al. (2020) regarding the fact that *E. russicum* germinates quite quickly.

For the treatment with the exposure duration of 4 hours, the germination index (GI) registered statistically insignificant changes, compared to the control ($p \geq 0.05$). In both test species, a slight value increase was found (by 9% - 16% in *E. russicum* and respectively by 9% - 37% in *E. vulgare*), compared to the control, with only one exception (the concentration of 100 mg/l in *E. russicum)* (**Table 1, Table 2**).

In the case of the treatment with an exposure duration of 8 hours, in both test species, GI recorded slightly lower values in the treatment variants, compared to the control: by 21.06% - 41.06% in *E. vulgare* and 21% - 37.5% in *E. russicum* (**Table 3, Table 4**). The changes were statistically significant ($p \leq 0.05$) at the concentration of 100 mg/l in the case of *E.vulgare* species (statistical $F = 4.5345$; $p = 0.024$). By increasing the exposure period, the GI decreases in both species.

The coefficient of the germination rate (CRG) showed a slight value reduction in most treatment variants compared to the control, which was statistically insignificant, a situation valid for both treatment periods and both test species (**Table 1-4**).

The results obtained practically indicate that the seeds of the two species of the genus *Echium* studied have the ability to germinate quite well after a short period of exposure (4 hours and 8 hours, respectively) to different concentrations of zinc (100mg/l, 200mg/ l, 300mg/l).

For the two exposure periods, by comparatively analyzing the results obtained from the treatment variants with the control, the following aspects were found: a tendency towards a slight stimulation of germination in both species, although the germination time was longer than in the control, for the exposure period of 4 hours; a tendency for a slight delay in germination and a decrease in the germination index in both species for the 8-h exposure period, accompanied by an increase in germination time, more evident in *E. vulgare.* In the case of the *E. vulgare* species for the concentration of 100mg/l, statistically significant differences ($p \leq 0.05$) were found for some indicators analyzed at the 4-hour exposure time, compared to the 8-hour exposure time: FGP (F statistic = 7.7143; $p = 0.0321$) and GI (F statistic = 6.0586; $p = 0.049$). These results indicate that increasing the exposure time to certain concentrations can affect germination in the test species.

Studies have shown that the seminal integument and the other tissues around the embryo constitute the main barrier against heavy metals, and in the stages following imbibition the embryo becomes sensitive to heavy metals (Li, 2005).

In the species *E. russicum* and *E. vulgare* the term seed is used in a broad sense; in this case it is actually the fruit called nucula. Nucula is trigonous, pronounced coarse with a flat base, 2-3 mm long in *E. russicum* and

obliquely ovoid trigonous, coarse, with flat base 3-4 mm long in *E. vulgare* (Grințescu, 1960; Gibbs, 1972).

Germination tests performed by Izmaiłow and Biskup (2003) with *E. vulgare* seeds collected from two categories of plants (plants grown on heavy metal-polluted sites and control plants), using polluted soils as substrate showed good seed germination from the first category and the reduction of germination in the case of seeds from control plants. The authors suggest that the seeds of control plants are more sensitive to pollutants than the seeds from specimens grown on soils polluted with heavy metals (zinc, lead, cadmium). According to the authors, the fruit coat and seed coat are permeable to heavy metal ions.

The results obtained in the present study are in agreement with those reported by other authors regarding the effect of different concentrations of zinc on germination. In the specialized literature, the data reported on the influence of zinc (in the form of zinc sulphate) on germination are very different, depending on the species, variety, on the concentrations used, on the duration of the experiment (Ashagre et al., 2013; Menon et al., 2016; Stratu and Costică, 2018; Bezini et al., 2019).

Table 1: The effect of different concentrations of zinc on the indicators of the germination process in *Echium russicum* seeds - exposure time 4 hours.

Zn treatment (mg/l)	**FGP (%)**	**GI (seeds day^{-1})**	**MGT (day)**	**CRG**
0	47.5±10.35 (100)	0.85±0.2 (100)	6.25±0.68 (100)	16.77±2.34 (100)
100	37.5±7.5 (78. 94)	0.58±0.12 (62,23)	7.72±0.31 (123.52)	13.01±0.52 (77.57)
200	55±11.9 (115,78)	0.93±0.22 (109.41)	6.52±0.83 (104.32)	15.95±1.64 (95.11)
300	57.5±11.08 (121,05)	0.99±0.26 (116,47)	7.07±0.66 (113.12)	14.51±1.36 (86.52)

Results are means ± SE of the four replicates

Parentheses contain the percentage values of each parameter relatively to the control.

Table 2: The effect of different concentrations of zinc on the indicators of the germination process in *Echium vulgare* seeds - exposure time 4 hours.

Zn treatment (mg/l)	**PG (%)**	**GI (seeds day^{-1})**	**MGT (day)**	**CRG**
0	55±14.43 (100)	0.62±0.13 (100)	9.21±0.74 (100)	10.88±1.13 (100)
100	62.5±2.5 (113.63)	0.85±0.07 (137.09)	8.63±0.52 (93.70)	11.7±0.7 (107.53)
200	60±7.07 (109.09)	0.68±0.06 (109. 67)	10±0.26 (108.57)	10.01±0.26 (92)
300	62.5±7.5 (113.63)	0.72±0.1 (116.12)	9.25±0.48 (100.43)	10.88±0.51 (100)

Results are means ± SE of the four replicates

Parentheses contain the percentage values of each parameter relatively to the control.

Table 3: The effect of different concentrations of zinc on the indicators of the germination process in *Echium russicum* seeds - exposure time 8 hours.

Zn treatment (mg/l)	PG (%)	GI (seeds day^{-1})	MGT (day)	CRG
0	72.5±6.29 (100)	1.28±0.13 (100)	6.43±0.38 (100)	15.68±0.88 (100)
100	50±10.8 (68.96)	0.8±0.23 (62.5)	7.37±0.55 (114.61)	13.8±1.09 (88.01)
200	55±5 (75.86)	1.01±0.13 (78.90)	5.9±0.41 (91.75)	17.18±1.22 (109.56)
300	60±12.45 (82.75)	1.01±0.21 (78.90)	6.7±0.29 (104.19)	14.97±1.05 (95.47)

Results are means ± SE of the four replicates

Parentheses contain the percentage values of each parameter relatively to the control.

Table 4: The effect of different concentrations of zinc on the indicators of the germination process in *Echium vulgare* seeds - exposure time 8 hours.

Zn treatment (mg/l)	PG (%)	GI (seminte day^{-1})	MGT (day)	CRG
0	70±8.16 (100)	0.95 ±0.09 (100)	8.53±0.41 (100)	11.8±1.22 (100)
100	47.5±4.78 (67.85)	0.56*±0.05 (58.94)	9.3±0.11 (109.02)	10.74±0.25 (91.01)
200	60±5.77 (85.71)	0.75±0.06 (78.94)	9.34±0.65 (109.49)	10.87±1.55 (92.11)
300	55±2.88 (78.57)	0.62±0.07 (65.26)	10.03±0.63 (117.58)	10.09±1.30 (85.5)

Conclusion

Zinc in the concentrations used in the present study does not, in most cases, produce significant changes in the germination indicators analyzed in *E. russicum* and *E. vulgare* species, which suggests that the test species are tolerant to these concentrations.

The obtained results indicate that zinc, although a micronutrient, can adversely influence germination at certain concentrations and exposure times.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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BILBERRY ANTHOCYANINS - POSSIBLE APPLICATIONS IN SKINCARE PRODUCTS

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Abstract: *Vaccinium myrtillus* fruits are a significant source of anthocyanins and have been linked to a number of health advantages. Recent data, however, point towards the possible benefits of topical use for anthocyanins. The purpose of this study was to assess the antioxidant potential of two extracts obtained through maceration. Total anthocyanin content and HPLC-DAD qualitative analysis were completed prior to include the extract in a cream-type topical formulation. The extract with the highest anthocyanin content was incorporated in a basic O/W cream formulation and the antioxidant effect of the cream was evaluated using the DPPH radical scavenging assay. The extract's stability seven months later was also assessed. Our findings suggest that, the cream formulation offers antioxidant activity, but the activity declines by 27% when it is stored. Additionally, after seven months of storage, the formulation's color changed, probably as a result of the anthocyanins' instability.

Keywords: bilberry, antioxidant, natural cosmetics, anthocyanins, face cream formulation

1. Introduction

Vaccinium myrtillus L, or bilberry (**Fig. 1.**), belongs to the Ericaceae family, order Ericales. Fresh bilberry fruits (*Myrtilli fructus recens*), contain high concentrations of anthocyanins (Mikulic-Petkovsek et al., 2015). In addition to anthocyanins, bilberry fruits also contain 7-10% tannins (determined for dried fruits). Due to the tannin content, dried bilberry fruits (*Myrtilli fructus siccus*) are used as antiseptics and antidiarrheals in Romanian traditional medicine. The fruits also contain other polyphenolic compounds, glucids, pectins, and ascorbic acid (EMA-HMPC 2015; Gaspar et al., 2021). The physiological roles that anthocyanins play inside the plant are multiple: mediation of the response to oxidative stress through their antioxidant capacity, protection of plants from ultraviolet rays and other mechanical threats from the environment (Ma et al., 2021; Saigo et al., 2020).

The popular use of anthocyanins extracted from bilberry fruits is that of vasoprotective of capillaries, especially at the ocular level, but the extracts are also used in vascular complications induced by diabetes, as we have demonstrated in

our previous preclinical study, on diabetic Wistar rats (Ştefănescu (Braic) et al., 2018).

Anthocyanins also have a protective role in cardiovascular diseases, cancer, diabetes, etc, proved *in vitro* as well as *in vivo* in preclinical and clinical studies (Neamtu et al., 2020).

Recent years have seen consumers become more knowledgeable about the substances found in skincare products attributable to the Internet, and they frequently choose the purest, most "clean" versions with the fewest ingredients to run the lowest possible risk of skin sensitivity or allergy (Ahmed et al., 2020; Boon, 2020).

Flavonoid-containing fruits that prove photoprotective potential through various direct or indirect mechanisms have been called "*green sunscreens*" (Nunes et al., 2018). The fact that anthocyanins have antioxidant capabilities, that they can mediate the response to oxidative stress with the potential to prevent or delay the oxidation of lipids, proteins, and DNA, has led to a dizzying increase in the number of studies (Choi et al., 2016; Sarkar et al., 2014). These effects can protect the skin from the damage caused by environmental stressors such as UV radiation, pollution, and oxidative stress (Mattioli et al., 2020). Also, due to their multiple effects, anthocyanins can improve the appearance of the skin by reducing the appearance of fine lines, wrinkles, and age spots (Tsuda, 2012). However, studies focused on the effects of *Vaccinium myrtillus* anthocyanins on the skin are very limited. For this reason, the present work can contribute to the completion of knowledge regarding the behavior of the extract in a cosmetic product. Although the information that bilberries are among the best sources of anthocyanins with major health benefits is not current, research data in the dermato-cosmetic field is still scarce.

2. Materials and methods

Plant material

Vaccinium myrtillus fruits were collected in July 2020 from the spontaneous flora of Călimani Mountains, Mureș County, Romania. The fruits were immediately transferred to the laboratory. A sample of the herbal product was deposited at the Department of Pharmacognosy and Phytotherapy, Faculty of Pharmacy, George Emil Palade University of Medicine, Pharmacy, Science and Technology of Târgu Mureș (voucher specimen: FS-VM-19-20).

Chemicals and reagents

Cyanidin chloride and keracyanine chloride (Cyanidin-3-*O*-rutinoside chloride) were purchased from Carl Roth GmbH (Karlsruhe, Germany).

Fig. 1. *Vaccinium myrtillus*

Kuromanin chloride (Cyanidin 3-O-glucoside chloride) was purchased from PanReac (Barcelona, Spain), and delphinidin 3-O-rutinoside chloride, delphinidin chloride, 1,1-Diphenyl-2-picrylhydrazyl (DPPH•) were purchased from Sigma Aldrich Chemie GmbH (Steinheim, Germany). All solvents used in determinations were of HPLC grade. Purified water was obtained using a Milli-Q system from Millipore (Bedford, MA, USA).

Extract preparation

Two types of extracts were performed. The fresh fruits were macerated for 24 hours at room temperature with a mixture of either glycerin: ethanol (1:2) → BF1 extract or with glycerin: water (3:1) → BF2 extract.

Quantitative determination of anthocyanins

Total anthocyanin content was determined according to the method described in the European Pharmacopoeia 10th edition in the monography *Bilberry fruits, fresh*, with slight modifications. Briefly, 1 mL of extract was diluted (50x) with a 0.1% HCl solution. The absorbance was read at 528 nm and the concentration was calculated using the specific absorbance of cyanidin 3-O-glucoside at this wavelength. The results were expressed as cyanidin 3-O-glucoside equivalents (CGE) / 100 g fruits (*European Pharmacopoeia*, 2019).

Identification of individual anthocyanins through HPLC-DAD

Identification of anthocyanins was performed using a modified method from the European Pharmacopoeia 10th edition (*European Pharmacopoeia*, 2019). HPLC analysis was performed on a Merck HPLC system equipped with a quaternary pump Merck Hitachi L-7100, an L-7200 autosampler Merck Hitachi and a L-7360 column thermostat. Chromatographic separation of anthocyanins was performed on an Inertsil ODS-3, 3m, 150 x 4.6 mm (GL Sciences) column. The mobile phase consisted of a mixture of A - anhydrous formic acid: water (8.5:91.5, *V/V*) and B - anhydrous formic acid: acetonitrile: methanol: water (8.5: 22.5: 22.5: 41.5, *V/V/V/V*). The following multilinear gradient was applied: 95-75% A (0-14 min), 75-35% A (14-16 min), 35-0% A (16-17 min), and 0% A (16-20 min). The flow rate was set at 1.0 mL min^{-1}, the injection volume was 10 µL, and the detection wavelength was 535 nm. Stock solutions of 0.3 mg/mL were prepared for delphinidin chloride and of 0.5 mg/mL for cyanidin 3-*O*-rutinoside, delphinidin 3-*O*-rutinoside, cyanidin 3-*O*-glucoside, and cyanidin chloride. Prior to the injection, all samples were filtered through a 0.45 µm microporous cellulose syringe filter and transferred in HPLC vials. Identification of the peaks was based on the comparison of retention times of peaks in sample chromatogram and UV spectra with those of the standards.

DPPH· radical scavenging assay

Antioxidant activity was evaluated using a spectrophotometric method (Nisca et al., 2021). Briefly, 2.5 mL DPPH solution was mixed with different concentrations of the extract. The samples were mixed and allowed to stand at room temperature, in the dark, for 30 minutes. The absorbance was read at 517 nm. The inhibitory concentration (IC%) was calculated using the following formula: inhib%= [(A0-A1)/A0]*100. Ascorbic acid was used as the positive control. The concentration that inhibits 50% of the DPPH activity (IC_{50}) was calculated by plotting the inhibitory concentration versus the concentration on the *x*-axis (Laczkó-Zöld et al., 2018).

Cream base formulation

An oil in water (O/W) common cream base was prepared using *Prunus amygdalus* dulcis

oil, cetearyl olivate, sorbitan olivate, cetyl alcohol, ultrapure water, glycerin, benzyl alcohol, salicylic acid, and sorbic acid. The O/W formulation was prepared by the addition of the aqueous phase into the oily phase with continuous agitation. The proportion of the oily phase to aqueous phase was 27: 73. The bilberry glycero-alcoholic extract, in a concentration of 10% was added at the end. The antioxidant activity of the cream was determined using the DPPH radical scavenging assay imediately after preparation and after 7 months of storage at room temperature in air-tight containers.

Data analysis

Results were expressed as Mean ± Standard Deviation (SD) of three independent experiments for each determination. Data analysis was performed using GraphPad Prism® version 9. Student *t* test was used to compare the differences between two means. One-way ANOVA followed by posthoc Tukey test was used to compare the differences between samples. A value of *p* less than 0.05 was considered significant.

3. Results and discussion

The average anthocyanin content determined for the two extracts was higher in the glycero-alcoholic extract (**Fig. 2.**) with an average of 218.3 mg CGE/100 g herbal drug, compared with the macerate obtained with glycerol-water with an average of 187.6 mg CGE / 100 g. Considering these results, the glycero-alcoholic extract was further used in the cream formulation.

Vaccinium myrtillus is one of the best sources of anthocyanins, and until now there have been identified 15 different anthocyanins in the fruits. The identified anthocyanins are mainly glycosides of cyanidin, malvidin, peonidin, petunidin, or delphinidin (Lätti et al., 2008). Anthocyanins are a group of water-soluble pigments widely distributed in plants, with important therapeutic effects, such as antioxidant, anti-inflammatory, and anti-carcinogenic activities (Luca et al., 2020; Tena et al., 2020). The molecular structure of anthocyanins consists of a flavylium cation and one or more glycosyl groups attached to the phenolic hydroxyl groups (Hăncianu & Gîrd, 2020). The stability, bioavailability, and physiological activity of anthocyanins depend on various factors, such as pH, temperature, light, and enzymatic and chemical reactions (Dossett et al., 2011; He & Giusti, 2010). There is a significant variance in anthocyanin level and composition among bilberry populations, and is usually influenced by numerous factors, such as altitude, soil, precipitations and sun exposure (Lätti et al., 2008). It is thus very important that qualitative and quantitative determinations to be performed prior any preclinical and clinical studies.

Although there are many types of *Vaccinium* berries on the market, such as *Vaccinium corymbosum*, or *Vaccinium angustifolium*, the highest content of anthocyanins is found in *Vaccinium myrtillus* fruits, with 60-70% more than in the species mentioned above (Ștefănescu et al., 2017).

In the present study, a comparison with the anthocyanin standards mixture shown in **Figure 3A** revealed the presence of cyanidin 3-*O*-glucoside (Rt = 5.23 min) and delphinidin (Rt = 6.24) in the bilberry extract (**Fig. 3B**).

As it was expected delphinidin and cyanidin rutinoside were not present in the extract, because the anthocyanidins found in bilberries can be combined with three sugar moieties: glucose, galactose and arabinose. The presence of anthocyanins with rutinoside as a glycoside, is an indicator of adulteration of the sample with other berries (Govindaraghavan, 2014; Lätti et al., 2008).

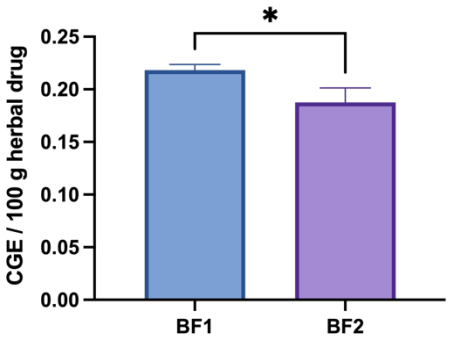

Fig. 2. Anthocyanin concentration in the extracts (*statistically significant difference at $p < 0.05$)

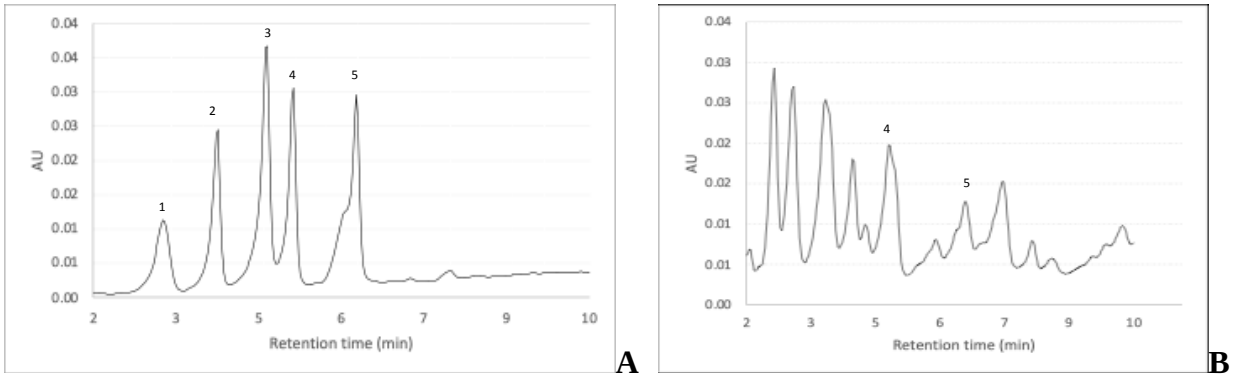

Fig. 3. Representative chromatograms of the standard mixture (**A**) and bilberry extract (**B**) 1 - cyanidin, 2 - delphinidin 3-O-rutinoside, 3 - cyanidin 3-O-rutinoside, 4 - cyanidin 3-O-glucoside, 5 - delphinidin

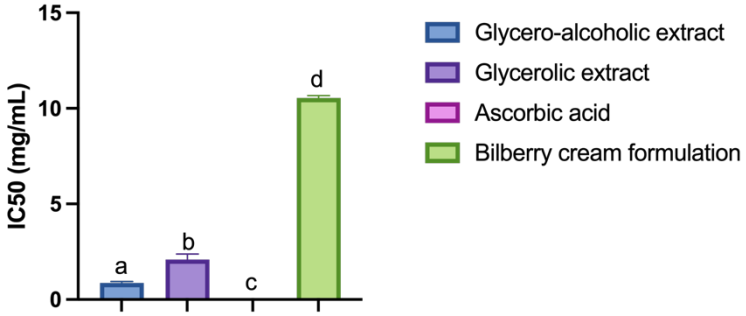

Fig. 4. Antioxidant activity with DPPH (* different letters above column, mean statistically significant differences at $p < 0.05$)

Regarding the antioxidant activity, our results have shown that all the tested samples had a good antioxidant potential. The highest antioxidant activity between the tested samples was observed for the glycero-alcoholic extract, with an IC_{50} of 0.87 ± 0.07 (**Fig. 4.**), while ascorbic acid solution had a more than 15 times lower IC_{50}.

The solvent used for maceration proved to be a significant factor for the antioxidant activity, probably correlated with the phytochemical profile.

The cream formulation, with 10% bilberry extract had a good antioxidant activity. However, statistically significant decline in antioxidant capacity is seen from 67.7% to 48.8% when comparing the percentage inhibition values of newly made cream versus the same cream after seven months. Data from other studies also suggest that formulations

with anthocyanins have stability issues, due to their chemical structure (Lee & Na, 2020).

Moreover, after seven months of storage under normal conditions, at room temperature, in a tightly closed container, protected from light, the preparation also underwent color changes from bright pink to a more faded pink. Because the color of anthocyanins is influenced by the pH, we have presumed that changes in pH occured during storage, but after the measurement of pH, we concluded that no changes appeard (pH = 5.6). No other changes of the formulation could be noticed, therefore the results confirm the instability of anthocyanins (Cai et al., 2022).

Abdellatif et al have also evaluated the stability of anthocyanins in a cream formulation and their results indicated that after 60 days, the formulation had a good stability and no changes appeared in the cream's colour (Abdellatif et al., 2021). The different results could be explain the by the longer storage time in our case. Different studies suggest an increased stability of anthocyanins in lyophilized form or formulated by microencapsulation (Gradinaru et al., 2003; Wang et al., 2017). Because more research is needed, until more stable extracts will be available, there is the possibility of offering consumers dual preparations, that can be extemporaneously prepared, in order to benefit from the effects of anthocyanin-rich extracts.

The limitations of the current study center around the methods for evaluating the antioxidant potential. Usually, different methods are used, due to different antioxidant mechanisms, but in this study only the DPPH method was used. This is due to the fact that the ABTS radical scavenging activity test produced clouding and opacification of the sample, rendering the method unsuitable for assessing the antioxidant potential of the cosmetic products.

Conclusions

The objective of the current study was to evaluate the antioxidant potential of a skincare product made with a bilberry fruit extract with a high anthocyanin concentration. In order to emphasize solely the anthocyanin-related qualities of the billberry fruit's glycerol-alcoholic extract, a simple cream base, while yet having moisturizing effects, was chosen. Although anthocyanin stability in cosmetics is limited, and more research is needed, anthocyanins have a longer shelf life in lyophilized form. This highlights the possiblity of producing dual cosmetic products that can be prepared extemporaneously, at the moment of use by the consumer.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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PHYTOCHEMICAL SCREENING AND *IN-VITRO* CYTOTOXIC ACTIVITIES OF METHANOLIC EXTRACTS OF *MEZONEURON BENTHAMIANUM* BAILL. AGAINST CANCER CELLS LINES

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Abstract: Despite the significant gains made in cancer therapy, cancer remains a major cause of global deaths due to rapid drug resistance. Therefore, urgent concerted efforts towards the discovery and development of newer and effective anticancer agents cannot be overemphasized. This study investigated *in vitro* cytotoxicity potential of methanol extracts of the root, stem, and leaves of *Mezoneuron benthamianum*. Leaf, stem and root samples were collected, authenticated, dried, separately pulverized and extracted in methanol. The methanol extracts were analysed for the presence of phytochemicals and cytotoxic potential evaluated by tetrazolium 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay on selected human cancer cells lines, HeLa (cervical cancer) and HEp-2 (epidermal carcinoma of the larynx), using mammalian Vero cells as a negative control. Data generated was subjected to descriptive statistics. The flavonoids in the plant was between 40 - 67.2% and was significantly higher ($p < 0.05$) compared to alkaloids and saponins. At concentrations of ≥ 50 µg/mL, the extracts exhibited 100% cytotoxicity on the cancer cells. The methanol root and leaf extracts with CC_{50} of 15.64 and 11.38 µg/mL were more cytotoxic on HeLa and HEp-2, respectively. In comparison to the stem and root extracts, the methanol leaf extract was selectively more toxic to cancer cell lines than Vero cells (CC_{50} = 28.89 µg/mL). Preliminary investigation reveals that *Mezoneuron benthamianum* contain bioactive compounds that possess promising anticancer potential that could be exploited.

Keywords: cancer cells, chemotherapy, *Mezoneuron benthamianum*, MTT

1. Introduction

Cancer is one of the leading causes of death in the world today. It is a chronic disease that occurs when cell growth gets out of control and divide uncontrollably to form lumps or masses of tissue called tumors. The tumor causes damage to healthy tissues *in vitro* that requires further investigation and exploitation (Avni *et al.*, 2008). Several plant derived

compounds such as vinca alkaloids, podophyllotoxin derivatives, taxanes, camptothecin, combretastatin, among others are used in various regimens as chemotherapy of various cancers (Anna and Krzysztof, 2018).

Although great advancements have been made in the development of anticancer drugs and the treatment, however, severe undesirable adverse effects associated with many of the chemotherapeutic regimens and resistance to anticancer drugs are common phenomena. The discovery and development of plant-derived effective and broad spectrum chemotherapeutics with less adverse effects will be valuable options in the cancer chemotherapy (Avni *et. al*, 2008), and (Sudhakar, 2009). Many plants have shown promising anti-cancer properties.

Mezoneuron benthamianum is a climbing shrub that belongs to the family leguminosae (Fabaceae). It contains hydroxystilbenes, piceatannol and *trans*-resveratrol, which have been reported to possess chemopreventive properties (Osamudiamen *et al*., 2020). This plant has been used in folk medicines to treat many diseases including general malaise, urethral discharge, inflammation, dropsy, swellings, oedema, cataract, wounds, skin infections, piles and ulcers (Burkill, 1985). In addition, plants belonging to the Fabaceae family have been reported to possess phytochemicals with anticancer activities and the phytochemical components of these plants inhibit carcinogenesis at various stages (Sharma *et al*. 2017). Previous reports also showed that the hexane extract of the root of *M. benthamianum* possesses phytochemicals that have *in vitro* cytotoxic activities against lung (NCI-H322), breast (T47D), prostate (PC-3) and lung (A549) cancer cell lines (Osamudiamen *et al*., 2017). However, cytotoxic activities of the leaves and stem bark of *M. Benthamianum* have not been investigated. This study was therefore designed to investigate the cytotoxic potential of the methanol extracts of the root, stem, and leaves of *Mezoneuron benthamianum* against HeLa (cervical cancer) and HEp-2 (epidermal carcinoma of the larynx).

2. Materials and methods

Collection of plant parts

The leaves, stem and roots of *Mezoneuron benthamianum* were collected from Afin-Iyanu area, Ologun-Eru, Ibadan. A mature part of the freshly collected plant with leaves, flowers and stem was authenticated at the herbarium unit of Department of Botany, University of Ibadan. The fresh leaves were spread in open air under a shade and away from direct sunlight, while the roots and stems were size reduced and air dried.

Extraction, qualitative and quantitative determination of phytochemicals in *M. benthamianum*

Dried leaves, roots, and stem of *M. benthamianum* were pulverized and extracted by cold maceration. The plant parts were weighed separately into separate glass containers and sufficient methanol was added and left to stand with stirring at intervals for 72 hours. The methanol extracts were filtered and concentrated under reduced pressure using rotary evaporator at 40^{o}C. The extracts were stored in labeled bottles at room temperature until further use. The presence or otherwise of phytochemical compounds in each extract was qualitatively determined using standard methods described by Oluremi *et al*. (2018).

Preparation and Maintenance of the Cell lines

Epidermoid carcinoma of the larynx (HEp-2), cervical cancer (HeLa) and African monkey kidney cells (Vero) used in this study were obtained from the WHO Polio Laboratory,

Department of Virology, University of Ibadan, Ibadan. Each cell line was cultured in separate T/45 tissue culture flasks using 10% (growth) medium and incubated in a 5% humidified CO_2 incubator at 37^{o}C until they were 80% confluent.

Determination of the cytotoxicity of *Mezoneuron benthamianum* extracts by MTT colorimetric assay

Sterile 96-well tissue culture microtiter plates were seeded with each cell lines. The plates were incubated in a 5% humidified CO2 incubator at 37^{o}C for 24 hours to attain 80-100% cell confluence. Spent media in the 96-wells were removed by aspiration and replenished with 100 µL of the different dilutions (0.001-100 µg/mL) of the methanol extracts in maintenance medium and the plates were further incubated. At the end, the plates were examined with an invertoscope for inhibition of cell growth. The medium was removed and 25 µL of a 5 mg/mL solution of MTT in phosphate-buffered saline was added to each well, the plates were incubated for 3 hours at 37^{o}C. Subsequently, 125 µL of DMSO was added into each well to solubilize the formazan crystals (Johan *et al*, 2011) and shaken using an incubator shaker (New Brunswick Scientific: Excella E24 incubator Shaker Series) at 120 rpm for 15 minutes. The optical density was determined at 570 nm wavelength with an ELISA multiple well plate reader (Multiskan FC). The experiment was performed in triplicates. Cell viability (%) and percentage cytotoxicity for the extracts on the cell lines was calculated using the following equations (1, 2) (Senthilraja and Kathiresan, 2015).

$$\text{Cell viability (\%)} = \left(\frac{\textit{Mean OD of treated}}{\textit{Mean OD of control}}\ x\ 100\right) \qquad (1)$$

$$\text{\% cytotoxicity} = \left[\frac{(\textit{Mean OD of control wells} - \textit{Mean OD of treated wells})}{\textit{Mean OD of control wells}}\right] x\ 100 \qquad (2)$$

Table 1. Percentage yield of methanol extracts

Plant Part Extracts	% Yield
Root	6.3
Stem Bark	2.5
leaves	3.6

Table 2. Qualitative Phytochemical Analysis

Phytochemicals	Leaves	Stem	Root
Steroids	-	+	+
Terpenoids	+	+	+
Saponins	+	++	+
Tannins	+	++	++
Flavonoids	+	++	++
Cardiac glycosides	-	-	-
Anthraquinone	+	+	+
Alkaloids	+	++	++

Key: - = absent, + = scanty, ++ = abundant

Data analysis

The data generated from the study were analysed using descriptive statistics and the cytotoxic concentration at 50% (CC_{50}) values determine using GraphPad prism.

3. Results

The result of the plant extraction is shown in **Table 1**. The root gave the highest extract yield with a value of 6.3% which is twofold that of the leaf (3.6%), and threefold the yield of the stem 2.5%. Phytochemicals such as steroids, terpenoids, saponins, tannins, flavonoids, cardiac glycosides, anthraquinones, and alkaloids were tested for in the three plant parts. The results showed that the three parts of *Mezoneuron benthamianum* contain six of the classes of phytochemicals as shown in **Table 2**. These phytochemicals were more abundant in the stem and the root extracts than in the leaves extract.

The quantitative analysis of certain phytochemicals in the different plant parts (**Fig. 1**.) shows that the root, stem and leaf had abundant quantities of flavonoids (67.2, 62.0 and 40.4%), the alkaloid content for root and stem was moderate (26.2 and 28.2%), while the leaf contained scanty amount (6.0%). Saponins were scanty in the root and leaf (2.6 and 5.8%), while stem had moderate quantity of 28.2 %.

Cytotoxicity effects

All the extracts caused 100% inhibition of growth of HEp-2 and HeLa cell line at concentrations of ≥50 µg/mL as shown in Figures 1-3. At a concentration range of 3.125 - 6.25 µg/mL methanol extract of *M. bethamianum* leaves and stem bark showed cytotoxic effects on HEp-2 cells, but not on HeLa cells (**Figures 2-3**).

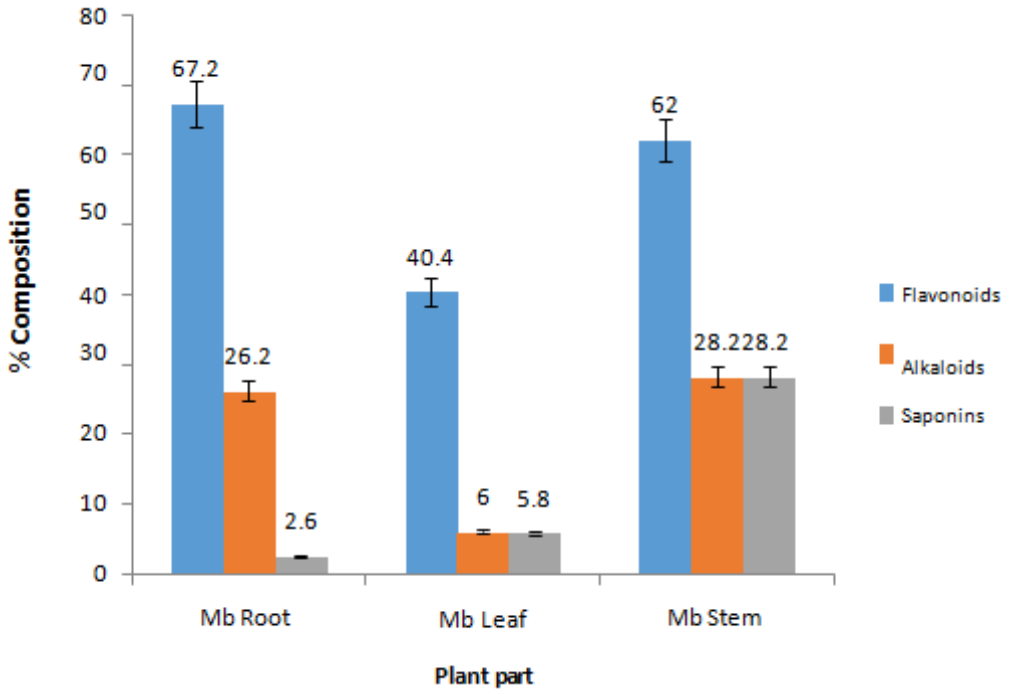

Fig 1. Quantitative Phytochemical constituents of the *M. benthamianum* parts
Abbreviations: Mb Leaf – *Mezoneuron benthamianum* leaf extract, **Mb Root** - *Mezoneuron benthamianum* root extract, **Mb Stem** - *Mezoneuron benthamianum* stem bark extract.

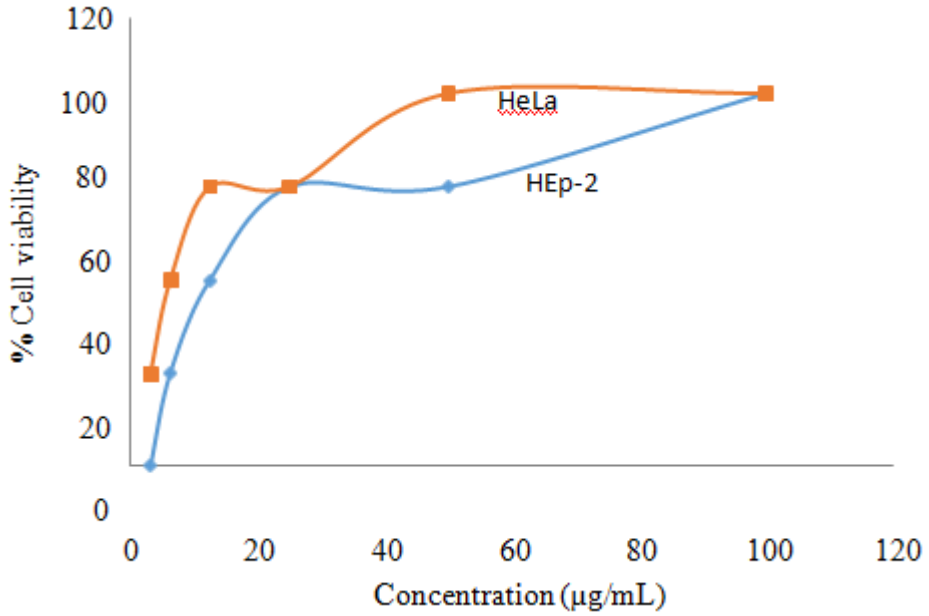

Fig. 2. Effects of root methanol extract of *M. benthamianum* viability of cancer cells

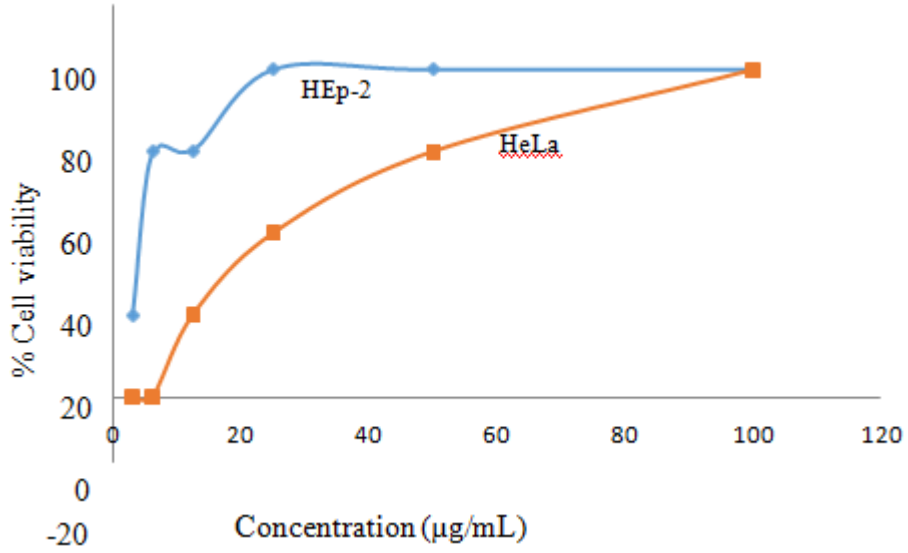

Fig. 3. Effects of leaf methanol extract of *M. benthamianum* viability of cancer cells

At 25 µg/mL, the cytotoxic effect of roots and leaves methanol extract on HEp-2 cells was 75% and 100%, respectively, and significantly ($p < 0.05$) higher compare to cytotoxic effect of stem bark.

The cytotoxic concentration (CC_{50}) of methanol extract of *Mezoneuron benthamianum* root on HeLa cells line was significantly ($p < 0.05$) lower compared to the CC_{50} of stem bark and leaves extracts (**Table 3**). The CC_{50} of methanol extract of *Mezoneuron benthamianum* leaves on HEp-2C cells line was significantly ($p < 0.05$) lower compared to the CC_{50} of stem bark and roots extracts. On Vero cells line, the methanol extract of *Mezoneuron benthamianum* leaves gave CC_{50} that was significantly ($p < 0.05$) higher compared to the CC_{50} of stem bark and roots extracts.

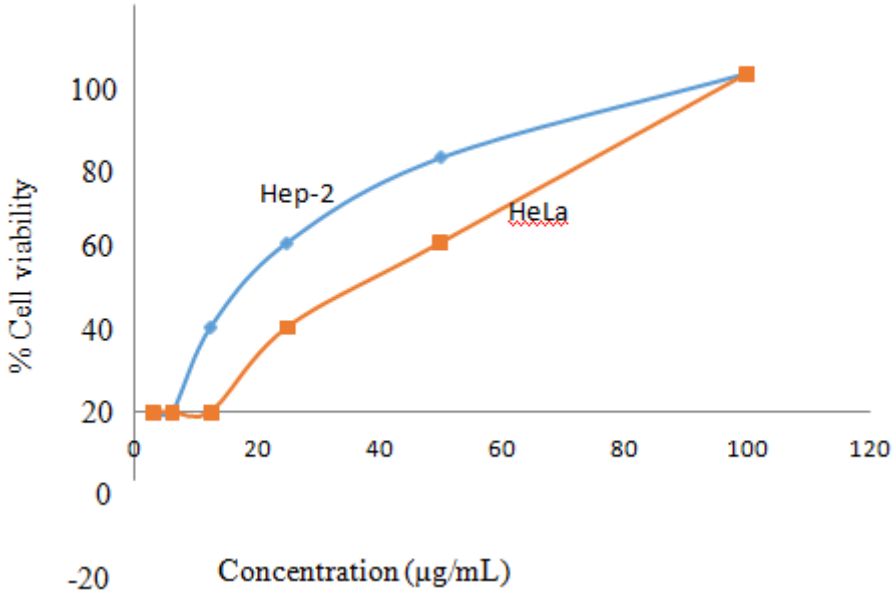

Fig. 4. Effects of stem bark methanol extract of *M. benthamianum* viability of cancer cells

Table 3. Cytotoxic concentration (CC_{50}) of methanol extracts on selected cells lines

Cell Lines	Extracts	CC50 (µg/mL)
HeLa	Mb Rt	15.64
	Mb Sb	34.20
	Mb Lf	23.06
HEp-2C	Mb Rt	21.23
	Mb Sb	23.26
	Mb Lf	11.38
Vero	Mb Rt	10.66
	Mb Sb	16.24
	Mb Lf	28.89

Abbreviations: Mb Lf= *Mezoneuron benthamianum* leaf; Mb Rt= *Mezoneuron benthamianum* root; Mb Sb= *Mezoneuron benthamianum* stem bark.

4. Discussion

Flavonoids, alkaloids, and saponins have been reported as phytochemicals that have cancer preventive activities against different types of cancer including estrogen-related cancers (Batra and Sharma, 2013; Isah, 2016; Shuli *et al*., 2010). Flavonoids are a group of polyphenolic compounds possessing low molecular weight that exhibit a common benzo-γ-pyrone structure. They are categorized into various subclasses including flavones, flavonols, flavanones, isoflavanones, isoflavanoids, anthocyanidins, and catechins (Hodnick *et al*., 1988; Cook and Samman, 1996). The average human diet contains a considerable amount of flavonoids and the major dietary sources are fruits (orange, grapefruit, apple, and strawberry), vegetables (onion, broccoli, green pepper and tomato), soybeans and different herbs. Flavonoids have been shown to possess chemopreventive properties by Batra and Sharma (2013) and they have been reported to possess broad

spectrum of biological activities such as antioxidant, antibacterial and antiviral activities (Friedman, 2007; Cazarolli, 2008). Alkaloids have also been reported to have antitumor, antiviral and antibacterial activities etc. (Maomao *et al*., 2015). Similarly, Isah (2016) reported that alkaloids had contributed immensely to the treatment of different types of cancers, while some saponins including ginsenosides and dioscin are reported to possess antitumor effects (Shuli *et al*., 2010).

Qualitative test carried out on methanol extract of the stem, was positive for the presence of steroids, but not methanol extracts the leaves and root. All the extracts tested negative for the phytochemical group, cardiac glycosides. Results of phytochemical constituents of methanol leaves extract of *Mezoneuron benthamianum* obtained in this studies agrees with the report of Osho (2013). These findings are consistent with that of Osho (2013) who also reported the presence of these six secondary metabolites in the leaves of *Mezoneuron benthamianum*, and only differs in that the stem had a trace amount of steroids while cardiac glycosides were absent in all the plant parts. The high quantity of flavonoids in the three parts of the plant with moderate quantity of alkaloids particularly in the root and stem might explain the significant cytotoxic activity of the plant extracts. Flavonoids, alkaloids, and saponins have been reported to possess cancer preventive properties, and activity against different types of cancer including estrogen-related cancers (Batra and Sharma, 2013; Isah, 2016; Shuli et al., 2010).

This study evaluated the cytotoxic potentials of the leaves, stems and roots methanol extracts of *Mezoneuron benthamianum* on some selected human cancer cell lines namely HeLa and HEp-2. In this study, the extracts exhibited concentration dependent cytotoxicity effects on the selected cancer cell lines with 100 µg/mL causing 100% inhibition of cells growth. To corroborate these findings, Osamudiamen *et al*. (2017) previously reported the cytotoxic activities of compounds isolated from the root extract of *Mezoneuron benthamianum*. They isolated two cassane diterpenoids, taepeenin A and nortaepeenin A, from the hexane extract of the roots of *Mezoneuron benthamianum*. Osamudiamen *et al*., (2017) evaluated these compounds for *in vitro* cytotoxic activities against four cancer cells lines which include lung (NCI-H322), breast (T47D), prostate (PC-3) and lung (A549). They found out that the two compounds were significantly cytotoxic to the selected cancer cells lines.

Attempt was also made to investigate selectivity of the extracts using Vero cells line. Vero cell lines simulate normal human cell line and are often used in anticancer studies to investigate the selectivity of experimental anticancer agents. Results from this study shows that methanol root extract of *M. benthamianum* was cytotoxic to both HeLa and Vero cell lines (CC_{50}= 10.66 µg/mL) which is an indication of non-selective toxicity, therefore making it unacceptable as a prospective anticancer agent. The leaf extract exhibited more superior cytotoxic activity and selective for HEp-2 cells (CC_{50}= 11.38 µg/mL), compared to methanol extracts of the root and stem plant parts. The leaf extract thus demonstrated low cytotoxic activity on Vero given rise to CC_{50} = 28.89 µg/mL. This result suggests that *Mezoneuron benthamianum* leaf extract could be an excellent prospective candidate for cancer chemotherapy. It further suggests that different parts of the plants contain variable phytochemicals that exhibited anticancer activities relative to the cancer cell lines.

Conclusions

Methanol extracts of *Mezoneuron benthamianum* is rich in bioactive constituents and can be explored as a source of highly safe and effective alternative cancer chemotherapy in addition to currently available anticancer drugs.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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